Macrophage-derived factors impair insulin signalling pathway in human adipose cells: role of IL-1β

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By
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DOCTOR OF PHILOSOPHY DECLARATION

I hereby declare that the research reported in this thesis represents my own work at the Department of Obesity and Endocrinology, University of Liverpool. This work has not been previously submitted to the university or any other institution in application for admission to a degree or other qualification except where otherwise indicated as help which is appropriately acknowledged.

Mohamed F. Madi BSc, MSc

Liverpool, November 2015
DEDICATION

I dedicate this work to my dear parents and my beloved wife as well as my brothers and sisters. You have always been there as a source of encouragement and always supported me throughout my life. I hope I have made you proud.
LIST OF ABBREVIATIONS

aP2 Adipocyte Protein 2
ANOVA Analysis of variance between groups
AS160 Akt substrate of 160kDa
ATGL Adipose triglycerides lipase
ATP Adenosine triphosphate
BAT Brown adipose tissue
BCA Bicinchoninic Acid
BMI Body mass index
BSA Bovine serum albumin
CC/EBP C/EBP CCAAT/enhancer-binding protein
CLS Crown-like structure
CRP C-Reactive protein
CT Computerized tomography
CVD Cardiovascular disease
DMEM Dulbecco’s Modified Eagle’s Medium
ECM Extracellular matrix
ELISA Enzyme-linked immunosorbent assay
FCS Fetal calf serum
FFAs Free fatty acids
GAPDH Glyceraldehyde-3-phosphate dehydrogenase
GLUT Glucose transporter protein
GSK-3 Glycogen synthase kinase-3
HIF-1α Hypoxia-inducible factor-1α
HRP Horseradish peroxide
HSL Hormone-sensitive lipase
ICE Interleukin converting enzyme
ILs
Interleukins

IL-1β
Interleukin-1β

IL-6
Interleukin-6

IL-8
Interleukin-8

IR
Insulin receptor

IRS-1
Insulin receptor substrate-1

IL-1 Ra
Interleukin-1 receptor antagonist

JNK
c-Jun N-terminal kinase

LDH
Lactate dehydrogenase

LPL
Lipoprotein Lipase

MAPK
Mitogen-activated protein kinase

MC
Macrophage conditioned medium

MCP-1
Monocyte chemoattractant protein-1

MRI
Magnetic resonance imaging

NK
Natural killer

NAO
National audit office

NCMP
National child measurement programme

NF-κB
Nuclear factor-κB

PBMCs
Peripheral blood-derived monocytes

PBS
Phosphate-buffered saline

PCOS
Polycystic ovary syndrome

PDK1
Phosphoinositide dependent kinases 1

PGC-1α
Peroxisome proliferator-activated receptor gamma coactivator 1-α

PH
Pleckstrin homology

PIA-1
Plasminogen inhibitor activator

PI3K
Phosphatidylinositol 3-kinase

PIP2
Phosphatidylinositol 4,5-bisphosphate
<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
</tr>
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<tbody>
<tr>
<td>PIP3</td>
<td>Phosphatidylinositol 3, 4, 5-trisphosphate</td>
</tr>
<tr>
<td>PKA</td>
<td>Protein kinase A</td>
</tr>
<tr>
<td>PKB</td>
<td>Protein kinase B</td>
</tr>
<tr>
<td>PMA</td>
<td>Phorbol 12-myristate 13-acetate</td>
</tr>
<tr>
<td>PPAR-γ</td>
<td>Peroxisome proliferator-activated receptor-γ</td>
</tr>
<tr>
<td>RPMI-1640</td>
<td>Roswell Park Memorial Institute 1640</td>
</tr>
<tr>
<td>RT-qPCR</td>
<td>Real-time polymerase chain reaction</td>
</tr>
<tr>
<td>SD</td>
<td>Standard deviation</td>
</tr>
<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
</tr>
<tr>
<td>SDS-PAGE</td>
<td>Sodium dodecyl sulphate-Polyacrylamide gel electrophoresis</td>
</tr>
<tr>
<td>Shc</td>
<td>Src homology collagen</td>
</tr>
<tr>
<td>SH2</td>
<td>Src Homology 2</td>
</tr>
<tr>
<td>SOCS-3</td>
<td>Suppressor of cytokine signaling-3</td>
</tr>
<tr>
<td>SPARC</td>
<td>Secreted protein acidic and rich in cysteine</td>
</tr>
<tr>
<td>SREBP-1</td>
<td>Sterol regulatory element binding protein-1</td>
</tr>
<tr>
<td>SVF</td>
<td>Stromal-vascular fraction</td>
</tr>
<tr>
<td>TGs</td>
<td>Triglycerides</td>
</tr>
<tr>
<td>TMB</td>
<td>Tetramethylbenzidine</td>
</tr>
<tr>
<td>TNF-α</td>
<td>Tumor necrosis factor-α</td>
</tr>
<tr>
<td>TTBS</td>
<td>Tween 20-Tris-Buffered Saline</td>
</tr>
<tr>
<td>UCP-1</td>
<td>Un-coupling protein-1</td>
</tr>
<tr>
<td>UV</td>
<td>Ultraviolet</td>
</tr>
<tr>
<td>VEGF</td>
<td>Vascular endothelial growth factor</td>
</tr>
<tr>
<td>WAT</td>
<td>White adipose tissue</td>
</tr>
<tr>
<td>WC</td>
<td>Waist circumference</td>
</tr>
<tr>
<td>WHO</td>
<td>World health organization</td>
</tr>
<tr>
<td>WHP</td>
<td>Waist hip ratio</td>
</tr>
</tbody>
</table>
TABLE OF CONTENTS

LIST OF FIGURES .......................................................................................................................... XIII
LIST OF TABLES .............................................................................................................................. XV

ABSTRACT ...................................................................................................................................... XVI

1 INTRODUCTION ........................................................................................................................... 2

1.1 OBESITY .................................................................................................................................... 2

1.1.1 Prevalence of obesity .............................................................................................................. 2

1.1.2 Definition of obesity ............................................................................................................... 3

1.1.3 Assessment of obesity ........................................................................................................... 3

1.2 OBESITY ASSOCIATED METABOLIC DISORDERS ................................................................. 5

1.2.1 Obesity and type 2 diabetes ................................................................................................... 5

1.2.2 Insulin resistance and type 2 diabetes .................................................................................. 6

1.3 ADIPOSE TISSUE ORGAN ....................................................................................................... 7

1.3.1 Forms of Adipose Tissues: .................................................................................................... 7

1.3.2 Other cellular components of Adipose tissue: ....................................................................... 10

1.3.3 Development of adipocytes .................................................................................................. 12

1.4 METABOLISM OF ADIPOCYTES ............................................................................................ 14

1.4.1 Lipogenesis ......................................................................................................................... 15

1.4.2 Lipolysis ................................................................................................................................ 16

1.5 FUNCTION OF WAT .................................................................................................................. 16

1.6 THE INSULIN SIGNALLING PATHWAY IN ADIPOSE TISSUE .................................................. 19

1.6.1 Insulin action in adipose tissue ........................................................................................... 19

1.6.2 The insulin receptor and its role in insulin signalling ............................................................ 20

1.6.3 The insulin receptor substrates (IRs) .................................................................................... 21

1.6.4 The metabolic signalling pathway ......................................................................................... 22

1.6.5 The mitogenic signalling pathway ......................................................................................... 23

1.7 DYSFUNCTION OF ADIPOSE TISSUE IN OBESITY: ............................................................... 27

1.7.1 Adipose tissue storage dysfunction ...................................................................................... 27

1.7.2 The role of macrophages infiltration ..................................................................................... 27

1.7.3 The mechanisms of macrophage infiltration in adipose tissue in obesity ......................... 28

1.7.4 The consequence of macrophage infiltration: alteration of adipokine release and insulin sensitivity .................................................................................................................. 31

1.7.5 Interleukins (ILs) .................................................................................................................. 39

1.8 AIMS OF THE STUDY ................................................................................................................ 46

1.8.1 Hypothesis ........................................................................................................................... 46

1.8.2 Aims and objectives .............................................................................................................. 46

2 MATERIAL AND METHODS ........................................................................................................... 49

2.1 REAGENTS, MATERIALS AND EQUIPMENT ........................................................................ 49

2.1.1 Chemical reagents ............................................................................................................... 49

2.1.2 Kits ....................................................................................................................................... 51

2.1.3 Equipment and system .......................................................................................................... 51

2.1.4 Software ............................................................................................................................... 52

2.1.5 Supplier’s addresses and URLs ............................................................................................ 52

2.2 CELL CULTURE SYSTEM ......................................................................................................... 54
2.2.1 Cell culture basis ................................................................. 54
2.2.2 Method .............................................................................. 54

2.3 HUMAN PREADIPOCYTES CULTURE AND DIFFERENTIATION ....................................................... 55
2.3.1 Reagents and mediums ....................................................... 55
2.3.2 Human preadipocytes storage ........................................... 56
2.3.3 Resuspension of preadipocytes from cryopreservation .......... 56
2.3.4 Preadipocytes subculture .................................................. 57
2.3.5 Human preadipocytes differentiation ................................ 58

2.4 HUMAN THP-1 MONOCYTES CULTURE ...................................................................................... 58
2.4.1 THP-1 cell culture medium and equipment ....................... 58
2.4.2 Human THP-1 monocytes .................................................. 58
2.4.3 Human THP-1 monocytes generation and maintenance ...... 58
2.4.4 Human THP-1 monocytes differentiation and macrophage-conditioned medium preparation ............... 59
2.4.5 Human Peripheral blood mononuclear cells (PBMCs) culture ..................................................... 59

2.5 CELL TREATMENT .................................................................................. 60

2.6 CELL AND MEDIUM COLLECTION .................................................................................. 60
2.6.1 Cell culture medium collection ......................................... 60
2.6.2 Cell collection for Total RNA extraction ......................... 61
2.6.3 Collection of cell lysate ....................................................... 62

2.7 ANALYSIS OF LIPID ACCUMULATION IN ADIPOCYTES .......................................................... 63
2.7.1 Oil Red O Staining assay ...................................................... 63

2.8 DETERMINATION OF GLYCEROL RELEASE IN CELL CULTURE MEDIUM ........................................... 65
2.8.1 Reagent and equipment ....................................................... 65

2.9 TOTAL RNA ISOLATION ........................................................................ 65
2.9.1 Reagents and equipment ....................................................... 65
2.9.2 Method .............................................................................. 66
2.9.3 RNA quantification ............................................................. 66

2.10 REVERSE-TRANScription POLYMERASE CHAIN REACTION ....................................................... 67
2.10.1 The practical basis .............................................................. 67
2.10.2 Method .............................................................................. 68

2.11 REAL-TIME POLYMERASE CHAIN REACTION (RT-qPCR) ............................................................... 69
2.11.1 The practical basis .............................................................. 69
2.11.2 Taqman® system principles ............................................... 70
2.11.3 Preparation of 96 well plates for real-time PCR ................ 71

2.12 ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA) ................................................................... 75
2.12.1 The practical basis .............................................................. 75
2.12.2 Method .............................................................................. 76

2.13 WESTERN BLOTTING ........................................................................ 79
2.13.1 Western blotting principles ................................................ 79
2.13.2 Protein quantification by the Bicinchoninic acid (BCA) method ................................................................. 80
2.13.3 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) ........................................... 82
2.13.4 Electroblotting ................................................................. 84
2.13.5 Immunological detection of proteins ................................ 85

2.14 CELL VIABILITY ASSESSMENT .................................................................................. 88

2.15 STATISTICAL ANALYSIS ........................................................................ 88
3 CHARACTERIZATION OF INSULIN SIGNALLING MOLECULES IN HUMAN PREADIPOCYTES

3.1 INTRODUCTION.................................................................................................................90
3.2 AIM OF THE STUDY..............................................................................................................92
3.3 MATERIAL AND METHODS .................................................................................................93
  3.3.1 Culture of human preadipocytes and adipocytes .............................................................93
  3.3.2 Cell treatment..................................................................................................................93
  3.3.3 Western blotting ............................................................................................................93
  3.3.4 Statistical analysis.........................................................................................................94
3.4 RESULTS..........................................................................................................................95
  3.4.1 Basal and insulin-stimulated expression of the insulin signalling proteins in human primary preadipocytes and adipocytes .................................................................95
  3.4.2 Expression of glucose transporter 4 (GLUT-4) during preadipocytes differentiation 99
  3.4.3 Phosphorylation of the insulin signalling proteins p-IR, p-IRS-1(Ser612) and p-Akt (Ser473) in human preadipocytes in response to insulin ..........................................................101
  3.4.4 Phosphorylation of the insulin signalling proteins p-IR (Y1185), p-IRS-1(Ser612) and p-AKT (Ser473) in human adipocytes in response to insulin .........................................................105
3.5 DISCUSSION.....................................................................................................................109

4 IL-1B MODULATES INSULIN SIGNALLING PROTEINS AND MEDIATES MACROPHAGE-INDUCED PRODUCTION OF PRO-INFLAMMATORY FACTORS IN HUMAN PRE-ADIPOCYTES ..................................................................................114

4.1 INTRODUCTION.................................................................................................................114
4.2 AIM OF THE STUDY..............................................................................................................116
4.3 MATERIAL AND METHODS .................................................................................................116
  4.3.1 Culture of human preadipocytes.......................................................................................116
  4.3.2 Generation of THP-1 macrophages-conditioned medium ..............................................117
  4.3.3 Cell treatment.................................................................................................................117
  4.3.4 Western blotting ...........................................................................................................117
  4.3.5 Measurement of cytokine/chemokine release (ELISA).....................................................118
  4.3.6 Real-time PCR...............................................................................................................118
  4.3.7 Cell viability assay.........................................................................................................118
  4.3.8 Statistical analysis.........................................................................................................118
4.4 EFFECTS OF IL-1B ON EXPRESSION OF THE INSULIN SIGNALLING MOLECULES IN HUMAN PRIMARY PREADIPOCYTES ........................................................................119
  4.4.1 Effect of 4 hr treatment with IL-1β on insulin-stimulated phosphorylation of insulin signalling molecules in human preadipocytes ..............................................................119
  4.4.2 Effect of 24 hr treatment with IL-1β on insulin-stimulated phosphorylation of insulin signalling molecules in human preadipocytes ...................................................................124
  4.4.3 Influence of IL-1β on the effect of macrophage-derived factors on insulin signalling molecules in response to insulin in human preadipocytes ..................................................128
  4.4.4 Blocking IL-1β activity reverses the induction effect of macrophage-derived factors on mRNA levels of the pro-inflammatory factors in human preadipocytes ................131
  4.4.5 Blocking IL-1β activity reverses the effect of macrophage-derived factors on cytokine and chemokine release by human preadipocytes .........................................................133
  4.4.6 Cytotoxicity assessment.................................................................................................136
4.5 DISCUSSION.....................................................................................................................139
5 EFFECT OF LONG-TERM TREATMENT WITH IL-1B ON PREADIPOCYTE DIFFERENTIATION AND ADIPOCYTE FUNCTIONS........................................145

5.1 INTRODUCTION...........................................................................................................145
5.2 AIM OF THE STUDY....................................................................................................147
5.3 METHOD AND MATERIALS ..........................................................................................148
  5.3.1 Culture of human preadipocytes..............................................................................148
  5.3.2 Generation of THP-1 macrophages-conditioned medium......................................148
  5.3.3 Cell treatment.........................................................................................................148
  5.3.4 Measurement of lipid accumulation in adipocytes................................................149
  5.3.5 Measurement of glycerol release ..........................................................................149
  5.3.6 Real-time PCR.......................................................................................................149
  5.3.7 Cell viability assay..................................................................................................149
  5.3.8 Statistical analysis..................................................................................................150
5.4 LONG-TERM TREATMENT WITH IL-1B AFFECTS PREADIPOCYTE DIFFERENTIATION AND LIPID LEVELS DURING ADIPOCYTE DEVELOPMENT ........................................................................................................151
  5.4.1 Chronic treatment with IL-1β reduces lipid levels during the differentiation of primary human preadipocytes.................................................................151
  5.4.2 Long-term treatment with IL-1β downregulates mRNA levels for key adipogenic factors in human adipocytes..............................................................154
  5.4.3 Long-term treatment with IL-1β upregulates mRNA levels of the pro-inflammatory factors (IL-6, MCP-1, IL-8 and RANTES) in human adipocytes ............156
  5.4.4 Long-term treatment with IL-1β downregulates mRNA level of adiponectin in human adipocytes..........................................................158
  5.4.5 Long-term treatment with IL-1β upregulates mRNA level of leptin in human adipocytes 158
  5.4.6 MC medium and IL-1β stimulate lipolysis in human adipocytes .........................161
  5.4.7 IL-1β neutralizing antibody reverses MC medium-induced lipolysis in adipocytes163
  5.4.8 Blocking IL-1 receptor binding in human adipocytes partially reverses MC medium-induced lipolysis..........................................................163
5.5 DISCUSSION................................................................................................................166

6 IL-1B MEDIATES MACROPHAGE-INDUCED IMPAIRMENT OF INSULIN SIGNALLING IN HUMAN PRIMARY ADIPOCYTES........................................171

6.1 INTRODUCTION...........................................................................................................171
6.2 AIM OF THE STUDY....................................................................................................172
6.3 MATERIAL AND METHODS .......................................................................................173
  6.3.1 Culture of human adipocytes................................................................................173
  6.3.2 Cell treatment........................................................................................................173
  6.3.3 Western blotting....................................................................................................175
  6.3.4 Measurement of cytokine/chemokine and adiponectin release............................175
  6.3.5 Cell viability assay.................................................................................................175
  6.3.6 Statistical analysis..................................................................................................175
6.4 MACROPHAGE-DERIVED FACTORS INDUCE THE IMPAIRMENT IN THE INSULIN SIGNALLING PATHWAY IN HUMAN PRIMARY ADIPOCYTES .................................................................................................176
  6.4.1 Macrophage-derived factors inhibit the expression of insulin signalling molecules176
  6.4.2 Macrophage-derived factors inhibit insulin-stimulated phosphorylation of IR (Y1185) and Akt (Ser473)..............................................................................176
6.4.3 IL-1β induces impairment in the insulin signalling pathway in human primary adipocytes...........................................................................................................................................................................181
6.4.4 Blocking IL-1β activity reduces the effects of the MC medium on the insulin signalling pathway in human primary adipocytes ...........................................................................................................................................................................186
6.4.5 Blocking IL-1 receptor binding in human primary adipocytes .................................................................194
6.4.6 Inhibiting IL-1β production by macrophages protects insulin signalling pathway and reduced cytokines/chemokines release in human adipocytes ...........................................................................................................................................201

6.5 HUMAN PRIMARY MACROPHAGE-DERIVED FACTORS INHIBIT INSULIN SIGNALLING AND STIMULATE CYTOKINE/CHEMOKINES RELEASE BY HUMAN ADIPOCYTES AND THE EFFECT OF BLOCKING IL-1β ..................................................................................................................................................................................................................205

6.5.1 Human primary macrophage-derived factors inhibit the protein expression of IRS-1 and GLUT-4, and the effect of blocking IL-1β ...........................................................................................................................................................................................................................................205
6.5.2 Human primary macrophage-derived factors inhibit insulin-stimulated phosphorylation of Akt ....................................................205
6.5.3 Human primary macrophage-derived factors stimulate cytokine/chemokine release by human adipocytes and the effect of blocking IL-1β ...........................................................................................................................................................................................................................................205

6.6 CELL VIABILITY ASSESSMENT .........................................................................................................................................................................................................................................................................................211
6.7 DISCUSSION .................................................................................................................................................................................................................................................................................................................................213

7 GENERAL DISCUSSION AND FUTURE DIRECTIONS ..............................................................................218

7.1 INTRODUCTION .................................................................................................................................................................................................................................................................................................................................218
7.2 PREVIOUS WORK .................................................................................................................................................................................................................................................................................................................................218
7.3 CURRENT WORK .................................................................................................................................................................................................................................................................................................................................219

7.3.1 Characterization and expression of insulin signalling molecules in human preadipocytes 220
7.3.2 IL-1β modulates insulin signalling proteins and mediates inflammation-related protein in human preadipocytes ...............221
7.3.3 Effect of long-term treatment with IL-1β on preadipocytes differentiation and adipocytes functions .........................................................224
7.3.4 IL-1β mediates macrophage-induced impairment of insulin signalling in human primary adipocytes ...............................................................226
7.4 FUTURE DIRECTIONS .................................................................................................................................................................................................................................................................................................................................229
7.5 CONCLUDING REMARK.................................................................................................................................................................................................................................................................................................................................230

8 REFERENCES LIST .................................................................................................................................................................................................................................................................................................................................232

9 APPENDIX .................................................................................................................................................................................................................................................................................................................................276
LIST OF FIGURES

FIGURE 1-1: HISTOLOGICAL AND CELLULAR APPEARANCE OF WHITE ADIPOSE TISSUE (WAT) AND BROWN ADIPOSE TISSUE (BAT) ................................................................. 11
FIGURE 1-2: STRUCTURE AND COMPOSITION OF WHITE ADIPOSE TISSUE (WAT) ................... 13
FIGURE 1-3: WAT AS AN ENDOCRINE ORGAN ........................................................................ 18
FIGURE 1-4: THE METABOLIC INSULIN SIGNALLING PATHWAY .............................................. 25
FIGURE 1-5: THE MITOGENIC INSULIN SIGNALLING PATHWAY ................................................ 26
FIGURE 1-6: ADIPOSE TISSUE REMODELLING IN OBESITY .................................................... 32
FIGURE 1-7: SCHEMATIC ILLUSTRATION OF INFLAMMASOME ACTIVATION THAT LEADS TO IL-1B RELEASE ........................................................................................................ 44
FIGURE 2-1: OIL RED O STAINING TO DETERMINE NEUTRAL LIPID CONTENT IN MATURE ADIPOCYTES .................................................................................................................. 64
FIGURE 2-2: FIRST STRAND cDNA SYNTHESIS ......................................................................... 69
FIGURE 2-3: AMPLIFICATION PLOT; BASELINE-SUBTRACTED FLUORESCENCE AGAINST NUMBER OF PCR CYCLES ........................................................................................................ 72
FIGURE 2-4: SCHEMATIC ILLUSTRATION OF ELISA ................................................................... 79
FIGURE 2-5: STANDARD CURVE GENERATION FOR BICICHONIC ACID (BCA) METHOD .......... 82
FIGURE 3-1: BASAL EXPRESSIONS OF THE INSULIN SIGNALLING PROTEIN (IRS-1, PI3K-p85A AND GLUT-4) IN HUMAN PREADIPOCYTES AND ADIPOCYTES ....................... 97
FIGURE 3-2: TIME COURSE OF GLUCOSE TRANSPORTER 4 (GLUT-4) EXPRESSIONS DURING PREADIPOCYTE DIFFERENTIATION ........................................................................ 100
FIGURE 3-3: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF IR (Y1185) IN HUMAN PREADIPOCYTES ........................................................................................................... 102
FIGURE 3-4: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF IRS-1 (Ser612) IN HUMAN PREADIPOCYTES ............................................................................................. 103
FIGURE 3-5: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF AKT (Ser473) IN HUMAN PREADIPOCYTES ..................................................................................................... 104
FIGURE 3-6: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF IR (Y1185) IN MATURE HUMAN ADIPOCYTES ..................................................................................................... 106
FIGURE 3-7: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF IRS-1 (Ser612) IN MATURE HUMAN ADIPOCYTES ............................................................................................. 107
FIGURE 3-8: EXPRESSION OF INSULIN-STIMULATED PHOSPHORYLATION OF AKT (Ser473) IN MATURE HUMAN ADIPOCYTES ............................................................................................. 108
FIGURE 4-1: EFFECT OF 4 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF IR IN HUMAN PREADIPOCYTES ........................................................................... 121
FIGURE 4-2: EFFECT OF 4 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF IRS-1(Ser612) IN HUMAN PREADIPOCYTES .................................................. 122
FIGURE 4-3: EFFECT OF 4 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF AKT IN HUMAN PREADIPOCYTES .......................................................................... 123
FIGURE 4-4: EFFECT OF 24 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF IR IN HUMAN PREADIPOCYTES .......................................................................... 125
FIGURE 4-5: EFFECT OF 24 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF IRS-1(Ser612) IN HUMAN PREADIPOCYTES .................................................. 126
FIGURE 4-6: EFFECT OF 24 HR TREATMENT WITH IL-1B ON INSULIN-STIMULATED PHOSPHORYLATION OF AKT IN HUMAN PREADIPOCYTES .......................................................................... 127

XIII
Figure 4-7: Effect of IL-1B neutralising antibody on macrophage-induced p-IRS-1 (Ser612) .............................................................................................................................................. 129
Figure 4-8: Effect of IL-1B neutralising antibody on macrophage-modulated p-Akt (Ser473) .............................................................................................................................................. 130
Figure 4-9: Blocking IL-1B activity reverses the effect of macrophage-induced gene expression of cytokines/chemokines in human preadipocytes ...................................................................... 132
Figure 4-10: Blocking IL-1B activity reverses the effects of MC medium on cytokine/chemokines release in human preadipocytes ...................................................................................... 135
Figure 4-11: MC medium and IL-1B neutralizing antibody do not induce cytotoxicity in human preadipocytes ................................................................................................................... 137
Figure 4-12: Human recombinant IL-1B at different doses (2, 5, 10 ng/ml) do not induce cytotoxicity in human preadipocytes ...................................................................................................... 138
Figure 5-1: Effect of long-term treatment with IL-1B (1 ng and 5 ng/ml) on lipid levels in human mature adipocytes .................................................................................................................................. 152
Figure 5-2: Long-term treatment with IL-1B reduces mRNA levels of the key adipogenic factors in human adipocytes ........................................................................................................ 155
Figure 5-3: Long-term treatment with IL-1B increases mRNA levels of pro-inflammatory factors (IL-6, MCP-1, IL-8 and RANTES) in human adipocytes. 157
Figure 5-4: Long-term treatment with IL-1B downregulates mRNA levels of adiponectin in human adipocytes .................................................................................................................. 159
Figure 5-5: Long-term treatment with IL-1B upregulates mRNA level of leptin in human adipocytes ................................................................................................................................. 160
Figure 5-6: MC medium and IL-1B stimulate lipolysis in human adipocytes .......... 162
Figure 5-7: IL-1B neutralizing antibody reverses MC medium-induced lipolysis.... 164
Figure 5-8: Blocking IL-1 receptor binding in human adipocytes partially reverses MC medium-induced lipolysis .................................................................................................................. 165
Figure 6-1: Protein expression of the insulin signalling molecules (IRS-1, PI3K p85a and GLUT-4) in human adipocytes is reduced by MC medium ......................................................... 177
Figure 6-2: Insulin-stimulated phosphorylation of IR in human adipocytes is reduced by MC medium ............................................................................................................................................. 179
Figure 6-3: Insulin-stimulated phosphorylation of Akt (Ser473) in human adipocytes is reduced by MC medium .................................................................................................................. 180
Figure 6-4: Protein expression of the insulin signalling molecules (IRS-1, PI3K p85a and GLUT-4) in human adipocytes is reduced by IL-1B ........................................................................... 182
Figure 6-5: IL-1B inhibits insulin-stimulated phosphorylation of IR in human adipocytes ................................................................................................................................. 184
Figure 6-6: IL-1B inhibits insulin-stimulated phosphorylation of Akt in human adipocytes ................................................................................................................................. 185
Figure 6-7: Blocking IL-1B activity reverses the effects of MC medium on protein expression of the insulin signalling molecules in human adipocytes ................................................................... 187
Figure 6-8: Blocking IL-1B activity reverses the effects of MC medium on insulin-stimulated phosphorylation of Akt in human adipocytes ........................................................................... 190
Figure 6-9: Blocking IL-1B activity reverses the stimulatory effects of MC medium on cytokine/chemokine release by human adipocytes .............................................................................. 192
Figure 6-10: Blocking IL-1B activity reduced the inhibitory effect of MC medium on adiponectin release by human adipocytes .......................................................................................... 193
Figure 6-11: Blocking IL-1 receptor binding reverses the effects of MC medium on protein expression of insulin signalling molecules in human adipocytes ..... 196
Figure 6-12: Blocking IL-1 receptor binding reverses the stimulatory effects of MC medium on cytokine/chemokines release by human adipocytes..................199
Figure 6-13: Blocking IL-1 receptor binding reverses the inhibitory effect of MC medium on adiponectin release in human adipocytes.................................200
Figure 6-14: Inhibiting IL-1B production by macrophages reduces the effects of MC medium on protein expression of IRS-1, PI3K p85a and GLUT-4 in human adipocytes .........................................................202
Figure 6-15: Inhibiting IL-1B production by macrophages reduces the effects of MC medium on cytokine/chemokine release by human adipocytes ...............204
Figure 6-16: Human primary macrophage-derived factors inhibit IRS-1 protein expression and the effect of blocking IL-1B.........................................................207
Figure 6-17: Human primary macrophage-derived factors inhibit GLUT-4 protein expression and are reversed by the effect of blocking IL-1B .........................208
Figure 6-18: Human primary macrophage-derived factors inhibit insulin-stimulated phosphorylation of Akt at (Ser 473) in human adipocytes..............................209
Figure 6-19: Human primary macrophage-derived factors induce cytokine/chemokine release by human adipocytes and the effect of blocking IL-1B......................210
Figure 6-20: Effect of treatment with MC medium, IL-1B Ab, IL-1RA and caspase-1 inhibitor on the viability of human primary adipocytes ..................................212

List of Tables
Table 1-1: World Health Organization (WHO) classification of obesity .......... 4
Table 2-1: Primer sequence of human target genes ____________________________ 74
Table 2-2: Antibodies and standard concentrations used for ELISAs .......... 78
Table 2-3: Antibody dilution and condition used for western blots .............. 87
ABSTRACT

The prevalence of obesity and overweight has dramatically increased throughout the world and becomes a major health concern. In obesity, adipose tissue expansion is associated with increased macrophage infiltration and marked change in cytokine/chemokine production. Macrophage-derived factors significantly alter adipose cells function, inducing inflammatory responses, reducing insulin sensitivity and impairing differentiation capability which may contribute to obesity-related metabolic disorder such as insulin resistance and type 2 diabetes. Human adipose cells (preadipocytes and adipocytes) play an important role in adipose tissue remodelling but their role in the development of insulin resistance and type 2 diabetes remains to be established. Identification of the major factors that mediate the detrimental effects of macrophages on adipose cells may offer potential therapeutic targets. IL-1β, a pro-inflammatory cytokine, is suggested to be involved in the development of insulin resistance.

In this thesis, different models of cells in adipose tissue were used to establish the effect of the crosstalk between them on the function of adipose cells, including human primary preadipocytes, adipocytes, THP-1 macrophages (cell line) and peripheral blood-derived macrophages. More specifically, this work investigated the role of IL-1β in macrophage-adipose cells cross-talk which affects the insulin signalling pathway, inflammatory response and adipocyte development in adipose tissue.

The results presented in this thesis reveal that basal expression of insulin signalling proteins (IR, IRS-1 and PI3K-p85α) was present and detectable in both human adipose cells. IL-1β modulated the expression of insulin signalling proteins including IR (Y1185), IRS-1(Ser612) and Akt (Ser473) in human preadipocytes upon insulin stimulation. Furthermore, IL-1β mediated macrophage-induced modulation of the insulin signalling molecules in human preadipocytes. In mature adipocytes, IL-1β significantly modulated protein abundance of insulin signalling molecules, including IRS-1, PI3K-p85α and Glut4 and phosphorylation of IR and Akt.

In addition, IL-1β mediated macrophage-induced expression and release of the pro-inflammatory cytokine/chemokines (IL-6, IL-8, MCP-1 and RANTES) in both human adipose cells. Blocking IL-1β activity, its receptor binding and production can partially or totally restore the expression of insulin signalling proteins and insulin responsiveness in both adipose cells. IL-1β antagonism also protected against macrophage-stimulated release of the pro-inflammatory cytokines/chemokines and macrophage-inhibited release of adiponectin. Additionally, both IL-1β and MC medium reduced lipid accumulation by reducing expression of the adipogenic factors including C/EBPα, PPARγ and aP2 and stimulating lipolysis in human adipocytes.

In conclusion, the results of this thesis suggest that IL-1β mediates, at least in part, the effect of macrophages on insulin signalling and pro-inflammatory response in human adipose cells. Therefore, blocking IL-1β may serve as a beneficial target for reducing obesity-associated inflammation and insulin resistance in human adipose tissue.
CHAPTER 1:
Introduction
1 Introduction

1.1 Obesity

1.1.1 Prevalence of obesity

According to an expert technical consultation, which was organized in the mid 1990’s, the first ever World Health Organization (WHO) report was published about considering obesity with high importance and awareness. The prevalence of obesity and overweight has dramatically increased throughout the world and become a major health concern (WHO, 2000). Interestingly, obesity has significantly increased in developing economic countries, such as China, India, Brazil and South Africa, particularly in metropolitan areas where people are more exposed to high caloric foods with low physical activity (Lobstein, 2011).

Information from epidemiological studies from 199 countries using systemic analysis showed that approximately 1 billion adults were estimated to be overweight in the world in 2008, and half billion were obese (Finucane et al., 2011). In fact, obesity has also been found to affect both genders and all age groups, even the children (Kimm and Obarzanek, 2002), making it one of the most common chronic disorders in childhood, adolescent and adulthood. In England, for example, The National Child Measurement Programme (NCMP) provides the latest figures on children obesity, and this report showed that in 2013/14 more than a fifth of 4-5 year olds and third of 10-11 year olds were either overweight or obese (NCMP, 2015). Interestingly, it was estimated that the positive energy balance (over nutrition) increases mortality among people who suffered from obesity related complications more than people who will die due to food deficiency (malnutrition) (Mukhopadhyay et al., 2005).

Lean et al. stated that the prevalence of obesity has increased 20% in adult European citizen, 40-70% in the Gulf States and Polynesian islands, and the prevalence of obesity now exceeds 15% in most European countries (Lean et al., 2006). According to some national surveys such as a report revealed by the National Audit Office (NAO) in England ~ over half of women and two thirds of men were either overweight or obese (NAO, 2001). In the USA, more than 60% of people aged 20 and older (approximately 170 million) are overweight, 27% of adult American (about
70 million) are obese putting them at health risk (Hedley et al., 2004). Therefore, in the USA, the number of obesity-attributable deaths reached approximately 325,000 deaths each year, and cost the country annually billions of dollars to cover direct and indirect medical expense such as disability and lost productivity annually (Allison et al., 1999, Katzmarzyk and Janssen, 2004).

The prevalence of obesity varies between different countries, populations and further variations are found with age and gender. Moreover, a cluster of etiological factors involved in obesity development include both genetic and environmental factors that it is classified as a “multifactorial disorder”. Environmental factors require genetic tendency to induce the development of obesity. Alterations in endocrine functions, even though rare, are also an important cause of obesity. However, significant changes in economic, social, cultural, and environmental factors play key roles in the onset of obesity (Al-Malki et al., 2003).

1.1.2 Definition of obesity

The fundamental definition for both obesity and overweight is an abnormal or excessive fat (adipose tissue) accumulation that affects health (Marinou et al., 2010). Based on a simple calculation in which weight in kilograms divided by height square in meters (kg/m²) reflects the body mass index (BMI) for human body weight. BMI is considered as a potential indicator of obesity in humans by providing a comparable analysis of prevalence rates worldwide (James et al., 2001, Seidell and Flegal, 1997). According to BMI values human obesity status can be classified into several categories as in Table 1.1, where individuals with BMI ≥ 25 kg/m² and >30 are defined as overweight and obese, respectively. There are other methods such as waist hip ratio and imaging techniques which can be utilized to diagnose obesity by measuring body weight and fat distribution.

1.1.3 Assessment of obesity

Worldwide increase of obesity has resulted in the need to understand body fat distribution and its health consequences. Therefore, quantifying body composition in humans, particularly adipose tissue is essential to define how an excess of fat is being related to health risks (Davies and Cole, 1995). There are several techniques for diagnosis of obesity by measuring excess amount of accumulated adipose tissue and
identifying its location in whole and regional human body. These techniques include anthropometric and imaging techniques (Scafoglieri et al., 2014). Anthropometric techniques, such as BMI, skinfold thickness, waist circumference (WC) and waist hip ratio (WHP), have the advantages of being cheap, quick and easy. For this reason they are used in most clinical and epidemiological researches that studied obesity and its consequence. However, measures such as BMI also have limitations. BMI only measures total body weight without providing any data about the actual amount of body fat and where it is stored. Other anthropometric techniques (skinfold thickness, WC and WHP) are better to measure central obesity (visceral fat) than BMI (WHO, 2011, Scafoglieri et al., 2014, Chan et al., 2003). Imaging techniques including, but not limited, Computerized Tomography (CT) and Magnetic Resonance Imaging (MRI) are more accurate than anthropometric measures, providing separate measurements for specific fat depots (subcutaneous and visceral fat). However, these techniques are expensive and more likely complicated that need skilled and trained individuals to work on them. The use of imaging techniques substantially increases our understanding of the complex links between the body composition and various metabolic disorders and diseases (Lustgarten and Fielding, 2011, Ross, 2003, Smith et al., 2001).

Table 1-1: World Health Organization (WHO) classification of obesity

<table>
<thead>
<tr>
<th>Classification</th>
<th>BMI (kg/m²)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Underweight</td>
<td>&lt; 18.5</td>
</tr>
<tr>
<td>Normal weight</td>
<td>18.5 to 24.9</td>
</tr>
<tr>
<td>Overweight</td>
<td>≥ 25</td>
</tr>
<tr>
<td>Pre- obesity</td>
<td>25.0 to 29.9</td>
</tr>
<tr>
<td>Obesity class I</td>
<td>30.0 to 34.9</td>
</tr>
<tr>
<td>Obesity class II</td>
<td>35.0 to 39.9</td>
</tr>
<tr>
<td>Obesity class III</td>
<td>≥ 40.0</td>
</tr>
</tbody>
</table>

Table shows BMI values that classify human body weight and obesity status into several categories, adapted from WHO (2000).
1.2 Obesity associated metabolic disorders

1.2.1 Obesity and type 2 diabetes

Obesity is associated with a wide range of metabolic disorders, including hypertension, hyperlipidemia, atherosclerosis and type 2 diabetes (Kahn and Flier, 2000). The global prevalence of type 2 diabetes has increased during the last decades and has been confirmed by the World Health Organization as one of the major worldwide health problems (Tack et al., 2012). Type 2 diabetes is also known as non-insulin dependent diabetes mellitus (NIDDM) (WHO, 1999). Type 2 diabetes is estimated to increase from 171 million cases in 2000 to 377 million in 2030, and the highest proportion of type 2 increase will be seen in developing countries (Wild et al., 2004). The main reasons for this is due to increased incidence of obesity and a sedentary lifestyle with physical inactivity (Barnes, 2012). There are multiple factors contributing to the possibility of developing type 2 diabetes, such as having a family history of type 2 diabetes (Morris et al., 1989), glucose intolerance (Edelstein et al., 1997), insulin resistance, previous incidence of gestational diabetes mellitus (Kim et al., 2002), obesity (more specifically in visceral depots) (Wang et al., 2005b), physical inactivity (Kriska et al., 2003) and poor diet (particularly high fat diet) (Marshall and Bessesen, 2002).

The increase in the worldwide prevalence and severity of obesity is considered to be strongly responsible for the pandemic emergence of type 2 diabetes (Yaturu, 2011). It has become clear that lifestyle change, in most developed countries, towards physical inactivity and nutritional oversupply underlies the emergence of type 2 diabetes as a health problem in parallel with obesity (Diamond, 2003). Type 2 diabetes is strongly associated to obesity, particularly abdominal obesity (Wang et al., 2005b). Meta-analysis studies demonstrated that the association between these two conditions was observed in both genders those have higher BMI and waist circumference (Guh et al., 2009). Interestingly, not all obese people have type 2 diabetes. However, most patients with type 2 diabetes are found to be overweight or obese (Daousi et al., 2006). People can tackle and prevent overweight, obesity and type 2 diabetes by making change in their sedentary life style habits for example, increasing physical activity and reducing over consumption of high energy foods (Yaturu, 2011). The major origin for the association between these two health
problems is that obesity has the ability to induce inflammation (Lumeng and Saltiel, 2011a, Hotamisligil, 2006). Indeed, inflammation is considered as a crucial element of the aetiology of obesity-related insulin resistance and the development of type 2 diabetes (Xu et al., 2003, Cancello and Clement, 2006). Moreover, studies in obese mice and humans suggest that increased macrophage accumulation in adipose tissue during obesity might provide a causative link between inflammation and metabolic disorders such as insulin resistance and type 2 diabetes (Cancello and Clement, 2006, Weisberg et al., 2003, Xu et al., 2003).

1.2.2 Insulin resistance and type 2 diabetes

Insulin resistance manifests as an alteration in peripheral insulin target organs from insulin sensitive organs to insulin resistant organs (Vollenweider, 2003). Insulin resistance is a characteristic feature in type 2 diabetes and obesity plays an important pathophysiological role in both health conditions (Vollenweider, 2003). Insulin resistance is defined as a disorder in which a normal or elevated level of insulin provides insufficient biological responses (Cefalu, 2001), classically this definition usually refers to impaired sensitivity to insulin stimulated tissue glucose uptake (Reaven, 2004). Insulin resistance is characterized by decreased glucose uptake into peripheral tissue (skeletal muscle, adipose tissue and liver) leading to hyperinsulinemia, increased hepatic glucose production, and reduced insulin action to inhibit lipolysis in adipose tissue (Schenk et al., 2008). The combination between insulin resistance in peripheral tissues and low insulin secretion due to dysfunction in β cells in pancreas leads to development type 2 diabetes (Kasuga, 2006).

Insulin is the most efficient hormone that potently stimulates lipogenesis via increased glucose uptake in adipocytes by translocation of glucose transporter-4 (GLUT-4) from the intracellular compartment to the plasma membrane (Zimmermann et al., 2009). These effects occur after insulin binds to its receptor at the fat cell surface, consequently activating its tyrosine kinase activity (Saltiel and Kahn, 2001). Moreover, insulin also influences the expression of lipogenic enzymes including pyruvate dehydrogenase, fatty acid synthase and acetyl-CoA carboxylase (Assimacopoulos-Jeannet et al., 1995). This may be mediated by the transcription factor sterol regulatory element binding protein-1 (SREBP-1) which in turn mediates the effects of glucose on lipogenic gene expression (Kersten, 2001). In human
adipose tissue, increased expression of lipogenic enzymes are associated with improved insulin sensitivity (Roberts et al., 2009).

1.3 Adipose tissue organ

In 1551, the Swiss naturalist Conrad Gessner became the first person that identified adipose tissue in animals (Cannon and Nedergaard, 2008). Adipose tissues can be recognized as a significantly specialised loose connective tissue that contains energy in the form of fat, particularly triglycerides (TGs). Structurally, adipose tissue is classified into two forms: white adipose tissue (WAT) and brown adipose tissue (BAT), but each one has specific functions in the human body (Symonds, 2012, Cinti, 2001). WAT consists of two main depots, which are subcutaneous and visceral depots. The subcutaneous depot is composed from anterior and posterior which collectively forming 60-70% of adipose organ while remaining mass of adipose organ is visceral depot (Cinti, 2006). Visceral depots are rich with blood supply and are more innervated than subcutaneous depots (Wajchenberg, 2000). There are many other smaller fat depots in human body including epicardial and intermuscular which may provide particular functions related to their neighbouring tissues (Sacks and Fain, 2007, Cinti, 2001). On a cellular level, adipocytes are considered as the main cellular component, and they are larger than other cells in adipose tissue due to stored lipid droplets (Eto et al., 2009). In addition, there are a number of other cellular components located in adipose tissue, mainly in stromal-vascular fraction (SVF), including endothelial cells, smooth muscle cells, fibroblasts, blood cells, preadipocytes and immune cells (Collins et al., 2005).

1.3.1 Forms of Adipose Tissues:

1.3.1.1 Brown adipose tissues (BAT)

BAT is prominently different from WAT in several aspects such as anatomical distribution, histological appearance and physiological functions (Figure1.1) (Trayhurn, 1993, Cinti, 2001). However, both BAT and WAT are able to store energy as TGs in droplets through a process termed lipogenesis, and both also release stored energy as free fatty acids and glycerol by a process called lipolysis (Lowell and Flier, 1997). These metabolic functions are mediated by large number of genes, which are highly expressed in both depots of adipose tissues (Himms-Hagen J., 1989,
Lowe and Flier, 1997). BAT appears in the human body, particularly in the first decade of life. With ageing, BAT gradually disappears and is replaced with WAT (Heaton, 1972). BAT which is located in peripheral area such as the interior abdominal wall and inter scapular area, are the first to disappear (Heaton, 1972). On the other hand, BAT found in the body such as areas around the kidneys and aorta and in the neck and mediastinum, appears to be more preserved for longer time, roughly for eight decades later (Klingenspor and Fromme, 2012). With regard to BAT cellular components, brown adipocytes are smaller in size compared with white adipocytes, and it has a round nucleus located in the centre of each cell (Lowell and Flier, 1997). The triglycerides of the BAT are deposited in multiple lipid droplets which are referred to as 'multilocular' (Klingenspor and Fromme, 2012). In addition, there are large numbers of mitochondria in the cytoplasm of adipocytes, which are distinguished by existence of overcrowded cristae (Lowell and Flier, 1997). However, the most unique feature of brown adipocytes is their expression of uncoupling protein-1 (UCP1-) (Nicholls and Locke, 1984).

Regarding its role, BAT has a thermoregulatory role and is considered as the main player for heat production (non-shivering thermogenesis) (Trayhurn, 1993, Klingenspor and Fromme, 2012, Smith and Roberts, 1964). Thermogenesis is mainly regulated by two effects. Firstly, the release of noradrenalin from the sympathetic nerve system (sympathetic activity) that leads to an increase in both BAT hyperplasia and biogenesis of mitochondria (Trayhurn P., 1986). The second effect comes from UCP-1 which is located in the inner mitochondria membrane, which serves to uncouple mitochondrial respiration, by allowing proton translocation mechanism to take place in the mitochondria of BAT (Nicholls and Locke, 1984). The expression of UCP-1 is in response to either thermogenic stimulus, such as exposure to cold or thermogenic inhibitor, such as exposure to warm or hot weather (Himms-Hagen J., 1989). The importance of BAT in maintaining the energy homeostasis and in the modulation of body fat mass has been shown by a number of studies (Trayhurn, 1993, Cypess and Kahn, 2010). For example, transgenic mice (with reduced BAT) exhibited highly developed obesity with glucose intolerance and reduced insulin sensitivity (Hamann et al., 1995). Early study has shown that developing obesity in rodent models was partly due to impaired thermogenesis in BAT (Himms-Hagen, 1979).
1.3.1.2 White adipose tissues (WAT)

WAT is a major adipose depot in mammals. WAT consists of mostly mature adipocytes which make ~50% of the total cellular component of the tissue (Hausman et al., 2001) and constitutes approximately 90% of the tissue volume (Lee et al., 2012). The another fraction in WAT is stromal vascular fractions (SVF) which contains preadipocytes and other cell types such as macrophages, endothelial cells, smooth muscle cells, fibroblasts and immune cells (Figure 1.2) (Ouchi et al., 2011, Collins et al., 2005). In human, WAT is distributed in two main depots, the subcutaneous (under skin) and intra-abdominal (visceral) adipose tissues (Wajchenberg, 2000). White adipocytes are spherical with diameters varying between 20-200 µm (Lee et al., 2012). A single large lipid droplet fills most of the adipocyte’s intercellular space, thus the cytoplasm and nucleus are compressed in a thin area. Moreover, white adipocytes have a small numbers of mitochondria in comparison with brown adipocytes (Symonds, 2012). In addition, WAT is not highly innervated and vascularized as obvious appeared in BAT counterpart. Each white adipocyte can be connected to at least one capillary. The capillary acts as a main supplier to a vascular network of WAT (Symonds, 2012, Collins et al., 2005).

Based on an anatomical study, the WAT appearance starts approximately at 14 weeks of gestation (Kahn, 2012 ). Following this, the first WAT pads appear in the head and neck continued by abdominal pads; lastly WAT pad appear in the lower limbs (Kahn, 2012 ). Areas of WAT with abundant vascular supply appear to develop earlier than those with a low vascular supply (Langin et al., 2009). Classically, it is thought that the main function of WAT is to store a large amount of fat as energy in the form of TGs, which is important for the regulation of energy homeostasis (Korner et al., 2009, Symonds, 2012, Langin et al., 2009). However, WAT has recently been recognized to have an endocrine function since WAT secretes a variety of proteins, collectively named adipokines, which contribute to the regulation of energy homeostasis and insulin sensitivity (Kwon and Pessin, 2013).
1.3.2 Other cellular components of Adipose tissue:

1.3.2.1 Stromal vascular fraction (SVF)

The subcutaneous fat depot is an abundant source of SVF (Gimble et al., 2011). SVF is the second major cellular component in the adipose tissue. SVF contains a large pool of preadipocytes that was discovered by Poznanski et al. in 1971 following the enzymatic digestion of adipose tissue (Ng et al., 1971). These cells generally appear in a fibroblast-like morphology; thus they are indistinguishable from factual fibroblasts once they are grown in cell culture (Bourgeois et al., 1983). In addition, SVF contains fibroblasts, endothelial cells, vascular cells and immune cells including macrophages, dendritic cells, and T lymphocytes, all involved in a complex cross-talk with cells of adipocyte lineage (preadipocytes and adipocytes) (Patrick Jr, 2000, Ouchi et al., 2011). Interestingly, SVF also contains unidentified stem cells which are differentiated into a range of cell types including adipocytes, myocytes, chondrocytes and osteoblasts under optimized cell culture conditions (Bunnell et al., 2008, Zuk et al., 2002). The results of in vitro culture encouraged researchers to use SVF as source to isolate cells (Koh et al., 2011).
Figure 1-1: Histological and cellular appearance of white adipose tissue (WAT) and brown adipose tissue (BAT)

The multilocular brown adipocyte is characterized by numerous mitochondria as shown in the left half of the figure, while the unilocular white adipocyte has a small number of mitochondria as shown in the right half of the figure.

1.3.2.2 Brite cells (beige cells)

Brite cells are the novel brown adipocytes that appear among WAT, and are often called “brite or beige” cells (Giralt and Villarroya, 2013). These cells are closer to the cell lineage of white adipocytes, and are derived from precursor cells different from those in classical BAT (Petrovic et al., 2010). Brite cells are the result of a process called browning of WAT in which adipocytes are recruited at locations of WAT due to a thermogenic stimulus, such as prolonged cold exposure (Giralt and Villarroya, 2013). Brite cells can be distinguished from white adipocytes by the presence of structural gene markers for classic brown adipocyte including Peroxisome proliferator-activated receptor-γ (PPARγ), Peroxisome proliferator-activated receptor gamma coactivator 1-α (PGC-1α), Cidea and Elovl3 but lacking in both developmental genes and the myogenic signature that define classical brown adipocytes (Petrovic et al., 2009). Similar to white adipocytes, brite cells have an
extremely low basal expression of UCP-1; however, they can express high levels of UCP-1 as brown adipocyte upon stimulation by cold or pathways that elevate intracellular cyclic AMP (cAMP) (Wu et al., 2012).

1.3.3 Development of adipocytes

In WAT, adipocytes play a central role to regulate energy homeostasis in the whole body by storing food energy (Korner et al., 2009, Symonds, 2012, Langin et al., 2009). When energy intake is higher than energy expenditure, an increase in WAT growth occurs, which involves both an increase in the volume of existing adipocytes (hypertrophy) and an increase in the number of new adipocytes from precursor adipocytes (hyperplasia) (Gregoire et al., 1998). Adipocytes are genetically determined to not exceed a certain maximal size; thus adipose tissue has another alternative mechanism to regenerate adipocytes by *de novo* adipogenesis from adipocyte precursor cells, which is called hyperplasia (Spalding et al., 2008). Since Green and his colleagues established the first adipose cell culture system (3T3-L1 derived from murine), there are large number of studies using adipocyte precursors as a model for the study of cell differentiation (Green and Kehinde, 1975). Differentiation, also known as adipogenesis, is the transition process from undifferentiated cell state (fibroblast-like preadipocytes) into mature adipocytes (round lipid-filled fat cells) (Butterwith, 1994).

There are many cellular events that occur in the differentiation process such as growth arrest, clonal expansion, early differentiation, and terminal differentiation (Hausman et al., 2001). The main two phases of differentiation in the intermediates between multipotent mesenchymal precursors and mature adipocytes, include determination and terminal differentiation (Rosen and MacDougald, 2006a). In determination phase pluripotent stromal precursors commit themselves to adipocyte lineages which become preadipocytes but they lose their potential to convert to other cell types (Hansen et al., 1999). In terminal differentiation, the preadipocytes differentiate into mature adipocytes, accompanied by a change in morphology from fibroblast-like structures to the unilocular appearance of the mature fat cell. Terminal differentiation offers preadipocytes with mechanisms related to lipid transport and synthesis, insulin sensitivity, and the secretion of adipocyte-specific proteins (Hansen...
et al., 1999). These changes are coupled with alterations in the surrounding the ECM and cytoskeleton (Hansen et al., 1999).

**Figure 1-2: Structure and composition of white adipose tissue (WAT)**

This figure shows the cellular heterogeneity of WAT (mature adipocytes, preadipocytes, fibroblasts, blood vessel components and immune cells), adapted from Ouchi, Parker et al. (2011).
Differentiation has been found to be regulated by a number of environmental, genetic, hormonal, and pharmacological factors as well as positive energy balance (Sethi and Vidal-Puig, 2007). The above factors act modulates specific receptors to transduce external growth and differentiation signals through a cascade of intracellular events (Gregoire et al., 1998). These regulators have previously been demonstrated to affect differentiation in in vitro culture experiments using several cell models, such as preadipocyte cell lines derived from murine (3T3-L1 and 3T3-F442A), human primary preadipocytes, primary stromal-vascular fraction, embryonic fibroblasts and bone marrow stromal cells, all of which can be differentiated into adipocytes under optimised cell culture conditions (Butterworth, 1994, Gregoire et al., 1998).

Genetic transcription factors, including members of the PPAR families and the CCAAT/enhancer-binding protein (C/EBP), play a key role in adipogenesis and maintenance of adipocyte phenotypes (Tontonoz et al., 1995). PPAR\(\gamma\) is expressed in two isoforms, PPAR\(\gamma1\) and PPAR\(\gamma2\) (Fajas et al., 1997, Farmer, 2006). The expression of PPAR\(\gamma1\) is detectable in many tissues, whereas PPAR\(\gamma2\) is expressed exclusively in adipose tissue (Farmer, 2006). The function of PPAR\(\gamma\) is to induce expression of a group of target genes related to lipid and glucose metabolism, secretion of adipokines and mitochondrial biogenesis (De Pauw et al., 2009). The C/EBPs (\(\alpha\), \(\beta\) and \(\delta\)) are induced during adipocyte differentiation but the expression of the three members is different in mRNA levels (Cao et al., 1991). The early expression of two members of the C/EBP family including C/EBP\(\beta\) and C/EBP\(\delta\) stimulates the expression of C/EBP\(\alpha\) and PPAR\(\gamma\) during adipocyte differentiation (Cao et al., 1991). A study using C/EBP\(\alpha\)-deficient mice showed a reduced adipocyte ability to store lipid and reduced adipose mass (Wang et al., 1995). Moreover, both C/EPB\(\alpha\) and PPAR\(\gamma\) positively regulate each other and then activate adipocyte protein 2 (aP2) factor which is involved in lipid metabolism, leading to lipogenic capacity of the cells (Gregoire et al., 1998).

1.4 Metabolism of adipocytes

In mammals, the major physiological role of WAT is to act as a metabolic buffering system for storing energy as TGs and releasing energy in the form of glycerol and
FFAs when needed by other tissues (Frayn, 2002). This role is in particular regulated by two major metabolic processes known as lipogenesis (fat storing/synthesis) and lipolysis (fat breakdown/release) both of which regulate whole-body metabolism (Langin, 2006a).

1.4.1 Lipogenesis

Lipogenesis is the anabolic process for *de novo* synthesis of fat from glucose or other substrate (Lafontan, 2008). The process occurs through two physiological steps which are FAs synthesis followed by TGs synthesis in WAT particularly in adipocytes the postprandial period which then stored in cytoplasmic inclusions called lipid droplets (Martin and Parton, 2006). However, lipogenesis can mainly take place in the liver (Kersten, 2001). Fat synthesis is mediated by many different factors including nutritional, hormonal and transcriptions factors (Kersten, 2001). For example, consumption of high carbohydrate food leads to stimulated lipogenesis whereas consumption of polyunsaturated fatty acids inhabits lipogenesis (Jump et al., 1994). Fat can either be synthesized *de novo* from glucose or be recycled from the excess circulating FFAs; glucose also increases lipogenesis by stimulating the pancreas to release insulin.

In mammalian cells, lipid droplets contain a neutral lipid (TGs or cholesterol esters) which is enclosed by a phospholipid monolayer and associated proteins (Martin and Parton, 2006). One of these proteins is PAT-domain protein (Perilipin/APRP/TIP47) which is involved in stabilizing TGs' storage in adipocytes (Martin and Parton, 2006). There are also trafficking proteins such as Rab GTPase proteins which participate in the intracellular trafficking and storage of lipids in lipid droplets (Martin and Parton, 2006).

During lipogenesis adipocytes release lipoprotein lipase (LPL), which acts on the surface of endothelial cells in the WAT where it hydrolyses the lipoprotein bound TGs to FFAs (Abumrad et al., 1999). FFAs subsequently are taken up the adipocyte by both fatty acid transporter proteins (FATPs) (Abumrad et al., 1999) and by passive diffusion (Hamilton and Kamp, 1999). Inside the adipocyte, FAs are esterified into TGs in the endoplasmic reticulum (ER) and stored in the lipid droplet,
and become chaperoned by aP2 that functions to solubilize and transport FAs via the aqueous cellular environment (Thompson et al., 2009).

1.4.2 Lipolysis

Lipolysis is the catabolic process for hydrolysing stored TGs in adipocytes into FFAs and glycerol in times of energy deprivation, when energy is required by other tissues such as muscle, heart and liver (Langin, 2006b). Lipolysis is a highly regulated process which is stimulated by catecholamines and inhibited by insulin (Duncan et al., 2007). During lipolysis, catecholamine mediates stimulation of β-adrenergic receptors (β1, β2 and β3) on the cell surface of adipocyte, which in turn activates adenylate cyclase (Lafontan, 2008). Therefore, the intracellular cAMP level is increased leading to the activation of cAMP-dependent protein kinase A (PKA) (Duncan et al., 2007). PKA activates two protein targets including hormone-sensitive lipase (HSL) and perilipin and consequently, TGs are released from adipocytes by the action of the two main enzymes which are adipose triglycerides lipase (ATGL) and HSL (Duncan et al., 2007). PKA stimulates perilipin activation by phosphorylation of at least 5 different serine residues (Carmen and Víctor, 2006). Perilipin is required for translocation of HSL from the cytosol compartment to the lipid droplet in adipocytes; this translocation is essential to the admission of HSL into lipid droplet where HSL catalyzes the hydrolysis of TGs (Holm, 2003). For a long period, HSL was thought to be the only TGs lipase that could hydrolyse TGs but in 2004, the ATGL was discovered to be an additional TGs lipase with specific activity for TG hydrolysis (Zimmermann et al., 2004). ATGL acts as TGs hydrolyse in WAT and is responsible for the first step of the lipolytic activity and this activity is increased in the absence of HSL (Zimmermann et al., 2004). Lipolysis can be suppressed by insulin through the activation of its downstream kinase cascade (PI3K/Akt) leading to the reduction of cAMP levels and thus reduced PKA activity (Choi et al., 2010).

1.5 Function of WAT

The traditional view of the role of WAT was limited to lipid storage as a fuel reserve (Trayhurn and Beattie, 2001). In addition to fuel storage, WAT can act as a thermal insulator by helping to reduce heat loss through the skin such as in marine mammals
(seals and whales) (Trayhurn and Beattie, 2001). It also has a protective function by providing mechanical support (padding) and protection around some of the major organs (Trayhurn and Wood, 2004). Furthermore, WAT is involved in many cellular and molecular processes such as immune response, lipid metabolism, blood pressure control, haemostasis, appetite and energy balance, inflammation and insulin sensitivity (Trayhurn and Wood, 2004).

WAT is also as an active endocrine organ (Figure 1.3) (Mohamed-Ali et al., 1998). In 1987, Siiteri discovered that adipose tissue played an endocrine role especially once it produced steroid hormone (Siiteri, 1987). Subsequently in WAT, the discovery of leptin expression and release by adipocytes (Zhang et al., 1994) and other adipose-derived factors, including cytokines, chemokines, enzymes and hormones and growth factors, supports the endocrine role of WAT (Fruhbeck, 2008). In addition, FFAs which are considered as the major products are quantitatively released from WAT by lipolysis (Trayhurn, 2007). It is reported that raised circulating levels of FFA and TGs due to obesity contribute to insulin resistance and other metabolic disorders (Despres et al., 1990, Reaven, 1996, Boden, 2006). Therefore, the connection between obesity and type 2 diabetes has promoted research on the endocrine link between lipid and glucose homeostasis in adipose tissue (Kahn and Flier, 2000). Another major product that is directly produced from adipocytes termed adipokines are leptin, adiponectin, cytokines, chemokines and growth factors (Trayhurn and Wood, 2004). Currently, a large number of adipokines have been identified (Jung and Choi, 2014) that are produced by white adipocytes and released into circulation to act in an autocrine or paracrine manner in adipose tissue and also in endocrine manner in other insulin sensitive tissue (muscle and liver) to modulate energy metabolism, insulin sensitivity, immune response and inflammation (Gong et al., 2003, Trayhurn and Wood, 2004).

In WAT, the endocrine role is in response to altered physiologic, metabolic and pathologic states, particularly due to the expansion of adipose tissue which is characterized by low grade inflammation and increase in a wide range of pro-inflammatory factors probably due to an increase in the accumulation of macrophages during obesity (Hotamisligil, 2003, Weisberg et al., 2003). In addition, macrophages-derived factors have been found to potently stimulate the production of
pro-inflammatory cytokines and chemokines by human preadipocytes and adipocytes (Gao and Bing, 2011, Flower et al., 2003). These pro-inflammatory cytokines and chemokines are significantly increased in obesity and considered as potent inducers for insulin resistance and the development of type 2 diabetes (Pradhan et al., 2001, Festa et al., 2002, Trayhurn, 2007). Consequently, the increased pro-inflammatory factors including TNF-α, IL-1β, IL-6 and IL-8, C reactive proteins, CMP-1, plasminogen inhibitor activator (PIA-1) and acute phase proteins contributes in the obese state to a range of metabolic disorders such as insulin resistance and type 2 diabetes (Jung and Choi, 2014).

**Figure 1-3: WAT as an endocrine organ**

Figure shows that white adipocytes-secreted factors can play various roles in the whole body homeostasis.
1.6 The insulin signalling pathway in adipose tissue

It was well recognized that insulin signalling is initiated by a complex and highly integrated network that controls specialized cell functions (White, 1997, Avruch, 1998, Taniguchi et al., 2006). The main trigger for this signalling pathway starts once insulin is bound to its receptor (insulin receptor). There are two major signalling pathways which are known: the metabolic and mitogenic pathways. The metabolic pathway is activated through phosphatidylinositol 3-kinase (PI3K) with the downstream effector being the protein kinase B (PKB) or Akt pathway. The metabolic pathway is involved in most of the metabolic actions of insulin. The mitogenic pathway occurs throughout the Ras–mitogen-activated protein kinase (MAPK) pathway, which regulates the expression of some genes and collaborates with the PI3K/Akt pathway to control cell growth and differentiation (Saltiel and Kahn, 2001, Avruch, 1998).

1.6.1 Insulin action in adipose tissue

Since the discovery of insulin, considerable scientific studies have attempted to gain understanding of the mechanism of insulin action by focusing on the metabolic changes that occur during insulin stimulation (White, 1997). Insulin is an anabolic hormone, secreted from the pancreatic β-cells. At a cellular level, insulin has pleiotropic effects to control numerous metabolic processes (Saltiel and Pessin, 2002). Insulin is the most important hormone involved in whole body homeostasis, in particular glucose homeostasis, by increasing peripheral tissue glucose uptake and decreasing hepatic glucose production (Saltiel and Kahn, 2001, Gual and Tanti, 2005). Interestingly, glucose uptake only occurs in a small proportion in adipose tissue (10-15%) in comparison to skeletal muscle that takes up to 75% of whole circulating glucose (Saltiel and Kahn, 2001, DeFronzo et al., 1992). However, experimental study using murine model with a knockout of the insulin-sensitive glucose transporter protein-4 (GLUT-4) in adipose tissue whereas GLUT-4 expression was preserved in muscle, led to hyper-insulinaemia and impaired glucose tolerance, suggesting that decreased GLUT-4 expression in adipose tissue is important in inducing insulin resistance in muscle and liver (Abel et al., 2001). Other actions of insulin include promoting FFAs uptake into adipocytes (lipogenesis) and inhibiting lipolysis (Saltiel and Kahn, 2001). Additionally, insulin is also considered
as a key regulator for adipose tissue development and differentiation by inducing gene expression of several fat-specific transcription factors in preadipocytes, such as SREBP-1 and PPARγ during preadipocytes differentiation (Le Lay et al., 2002, Vidal-Puig et al., 1997).

1.6.2 The insulin receptor and its role in insulin signalling

The insulin receptor (IR) is abundant in all mammalian tissues but the highest expression has been observed in the major metabolic organs such as the liver and adipose tissue (adipocytes) (White, 1997). Skeletal muscle has a relatively low level of IR expression (Caro et al., 1987). In adipose tissue, the IR is localised in caveolae or flask-like invaginations of the plasma membrane (Karlsson et al., 2004, Gustavsson et al., 1999). The IR is a transmembrane glycoprotein which is composed of two extracellular α-subunits (130 kDa), and two intracellular β-subunits (96 kDa). These two parts of IR are linked to each other by disulphide bonds. The α-subunits are found outside of the cell and have insulin binding sites whereas β-subunits are located inside the cell and contain tyrosine kinase residues (Hubbard et al., 1993, Saltiel and Kahn, 2001, Cheatham and Kahn, 1995, Gual and Tanti, 2005). The IR is involved in several cellular functions including growth, differentiation, apoptosis and transformation (Lopaczynski, 1998). Insulin signalling is directly triggered and activated after insulin binds to the IR and it probably causes a conformational change by bringing the two α-subunits closer together; this allows Adenosine triphosphate (ATP) to bind to the intracellular β-subunit which in turn activates receptor leading to its auto-phosphorylation (Hubbard et al., 1993, Kido et al., 2001). Subsequently, auto-phosphorylation of IR leads to an increase in its tyrosine kinase activity by recruiting and phosphorylating intracellular docking proteins (IR’s substrate), including insulin receptor substrate-1 (IRS-1), insulin receptor substrate-2 (IRS-2), Src homology collagen (Shc) and adaptor protein with a pleckstrin homology domain (PH) and Src Homology 2 (SH2) domain (APS) (White, 1997, White and Kahn, 1994). As a result, these phosphorylated molecules are able to activate different signalling pathways such as metabolic and mitogenic signalling pathways (Gual and Tanti, 2005, Saltiel and Kahn, 2001). Both IR and IRS have been known as “critical nodes” due to their crucial role in the insulin signalling pathway (Taniguchi et al., 2006).
1.6.3 The insulin receptor substrates (IRSs)

The family of IRSs has nine protein members of which IRS-1 and IRS-2, in both the humans and mice genome, are widely expressed in insulin target tissue (White, 2002). Studies using IRS1 and IRS2 knockout mice have found that IRS1 may play a predominate role in insulin resistance in adipocytes and skeletal muscle whereas IRS-2 may be more important in hepatic cells (Rondinone et al., 1997, Kido et al., 2000). IRS-3 has only been expressed in mice and is largely restricted to adipose tissue but it might not be expressed in human tissue (White, 2002). Considering the role of IRS1-4 isomers, they are mainly expressed in thymus and hypothalamus and for completing insulin signal transduction (White, 1997). IRS-4 deficient mice have mild negative effects in growth and glucose homeostasis (Fantin et al., 2000).

The IRS family has low intrinsic catalytic activities but they have multiple interaction domains and phosphorylation motifs as part of their composition (White, 2002), characterized by the existence of N-terminal PH domain adjacent to a phosphotyrosine-binding (PTB) domain, followed by a C-terminal tail that contains several tyrosine and serine phosphorylation sites (Copps and White, 2012).

Regarding IRS-1, the N-terminal in PH domain helps to anchor the protein to the membrane close to the IR. Subsequently, PTB acts as a binding site for the phosphorylated tyrosine in the juxta membrane NPXY motif of the IR (Keller et al., 1993). IRS1 is known as a docking protein because C-terminal of the IRS proteins contain multiple tyrosine phosphorylation sites that link the activated IR to other signalling proteins (Sun et al., 1991). These signalling proteins contain SH2 domains such as p85α regulatory subunit of the PI3K, Grb2, and SHP-2, which propagate and regulate insulin signalling within the cell. Activation of IRS-1 through IR leads to generation of over 20 potential tyrosine phosphorylation binding sites for SH2 domain proteins (Myers and White, 1993, Sun et al., 1991). These binding sites include six in YMXM motifs, three in YXXM motifs, and with remaining sites found in several hydrophobic motifs (Myers and White, 1993). The PI3K p85 binds to phosphorylated IRS1 on YMXM motifs in SH2 domain (Backer et al., 1992). This binding site is very important for the metabolic signalling pathway which is responsible for growth and metabolism in mammalian cells (Cantley et al., 1991). The Grb2 also binds to IRS1 but on a YVNI motif and the binding site is also
essential for the metabolic signalling pathway (Lowenstein et al., 1992). Besides several tyrosine phosphorylation residues on IRS-1, there are 50 potential serine/threonine phosphorylation sites on IRS-1 such as Ser612 and Ser632 which are found near to the tyrosine residues that are involved in the binding of PI3K (Sesti et al., 2001, White, 2002). All tyrosine and serine residues are directly activated through IR by insulin (Gual and Tanti, 2005). Tyrosine phosphorylation residues of IRS-1 positively regulate and further propagate insulin signalling within the cells; however, serine/threonine phosphorylation of IRS may work mainly as important regulators with dual role either to reduce or enhance insulin signalling in response to feedbacks and crosstalk with other signalling pathways (Gual and Tanti, 2005). Previous animal studies have found that a group of serine phosphorylation residues at 629 and 1223 on IRS-1 have been shown to enhance phosphorylation of tyrosine residues in the insulin signalling pathway (Luo et al., 2007).

Previous studies have shown that phosphorylation of serine residues on IRS-1 also have negative effect on insulin signalling. For example, phosphorylation of Ser307 significantly inhibited the binding between IRS1 and the IR especially when IRS-1 associated with JNK (Aguirre et al., 2000), whereas Ser612 phosphorylation inhibited tyrosine residues close to the PI3K binding with IRS-1; PI3K activation is needed for insulin-stimulated glucose uptake (Gual and Tanti, 2005). In addition to insulin, there are wide ranges of agents that have the ability to stimulate serine phosphorylation on IRS-1. These agents include FFAs (Shulman, 2000), TNF-α (Kanety et al., 1995), amino acids (Tremblay and Marette, 2001), cellular stress and hyperinsulinemia (Schmitz-Peiffer and Whitehead, 2003).

1.6.4 The metabolic signalling pathway
Activation IR happens through phoshphating multiple tyrosine residues within the receptor β-subunits (Cheatham and Kahn, 1995). Subsequently, IR activation generates other docking sites of intracellular proteins including the IRS proteins (IRS-1 and IRS-2) which signal to both metabolic and mitogenic pathways (Laviola et al., 2006). The metabolic signalling starts after IRS proteins, particularly IRS-1, activate SH-2 domains with other several regulatory proteins which identify the specific downstream effectors (Sun et al., 1991). Among these, the major downstream effector seems to be PI3K which has a regulatory subunit (p85) and
catalytic subunit (p110). PI3K p85 is activated by binding to phosphorylated YMXM motifs in IRS-1 (Sun et al., 1993). PI3K p110 then catalyses the phosphorylation of phosphatidylinositol 4,5-bisphosphate (PIP2) into phosphatidylinositol 3, 4, 5-trisphosphate (PIP3). Subsequently, PIP3 regulates the activation of phosphoinositide dependent kinase (PDK), leading to the activation of Akt/PKB (Song et al., 2005).

Phosphorylation of Akt at both Ser473 and Thr308 is required to allow the kinase reach its full activity (Sarbassov et al., 2005), which in turn regulates cell growth, cell survival, differentiation and glucose uptake (Whiteman, Cho et al. 2002). Following Akt activation, phosphorylation was occurred at Ser9 leading to phosphorylate glycogen synthase kinase-3 (GSK-3), resulted in glycogen synthesis (Maedler et al., 2011). Akt has many substrates such as AS160 which is known to regulate GLUT-4 translocation in adipocytes (Thong et al., 2007). GLUT-4 is then translocated from the intra-cellular compartment to the plasma membrane of the cell mediating glucose uptake (Watson and Pessin, 2001) (Figure 1.4).

1.6.5 The mitogenic signalling pathway

In mammalian cells, mitogen-activated protein kinases (MAPKs) belong to a large family of serine/threonine kinases, including the ERK1/2, p38 and JNK. MAPKs are common contributors to the intracellular signal transduction that starts from the cell surface receptors and reaches to the nucleus (Lewis et al., 1998). The mitogenic signalling pathway or MAPK cascade is a highly integrated network, and has a central role in immune-induced inflammatory responses (Hommes et al., 2003). Moreover, the MAPK pathway is involved in many aspect of specialised cell functions including cell proliferation, cell differentiation and cell death (Kyriakis and Avruch, 2001) (Figure 1.5).

As part of the insulin signalling network in adipocytes, the mitogenic signalling pathway (MAPK cascade) is mainly initiated by insulin that is bound to its receptor and causes enhanced tyrosine kinase auto-phosphorylation, creating docking sites for proteins such as IRS and Shc proteins for signal transducers and adapters (Parpal et al., 2001, Stenkula et al., 2004). The adaptor molecules such as Grb2 was recruited to Shc and/or IRS-1 after bound to the guanine nucleotide exchange protein son of
sevenless (SOS) (Pawson and Scott, 1997, Li et al., 1993). The SOS catalyses GDP into GTP then binds to Ras; the activated Ras-GTP binds to its downstream effector which is protein kinase Raf (Jelinek et al., 1996). This then mediates the phosphorylation of the MAPK cascade and this signalling pathway is attached to mitogenic effects of insulin (Pelicci et al., 1992) leading to the phosphorylation of the extracellular signal regulated kinases (ERK1/2) (Avruch et al., 2001). ERK activation is linked to pro-survival signals in the cells (Chang and Karin, 2001). Moreover, the ERK pathway regulates gene transcription (Pellicci et al., 1992) and stimulates preadipocytes proliferation through growth factors (Boney et al., 2000).
Figure 1-4: The metabolic insulin signalling pathway

Figure shows that the insulin signals from the insulin receptor are transmitted through the insulin receptor substrate (IRS)-1. The phosphorylation of IRS-1 activates PI3kinase. The activation downstream effectors of PI3K, such as protein kinase B (PKB or Akt), leads to glucose transporter-4 (GLUT-4) translocation to the plasma membrane and subsequent glucose uptake.
Figure 1-5: The mitogenic insulin signalling pathway

Figure shows that the insulin signals from the insulin receptor are transmitted through the adaptor molecules such as Grb2 was recruited to Shc and/or IRS-1 after bound to the SOS. The SOS catalyses GDP into GTP then binds to Ras. The downstream effector which is protein kinase Raf which mediates the phosphorylation of the MAPK cascade leading to the phosphorylation of the extracellular signal regulated kinases (ERK1/2).
1.7 Dysfunction of adipose tissue in obesity:

1.7.1 Adipose tissue storage dysfunction
Although, adipose tissue only represents approximately 10% of glucose disposal from the whole body compared with muscle and liver which represent 60% and 30% of glucose uptake respectively (Smith, 2002), adipose tissue has been considered as an intrinsic inducer to initiate insulin resistance in muscle and hepatic tissue (Xu et al., 2003, Cancello and Clement, 2006). Functionally, increased and expanded adipose tissue mass, (either by hyperplasia or hypertrophy) results in increased storage capacity for lipids (Jo et al., 2009). However, in the obese state the lipid storage capacity of adipose tissue is reduced due to dysfunctional cells (hypertrophied adipocyte) (Le Lay et al., 2001). Thus, excess energy due to chronic nutritional overload may not be properly stored by the expansion of the adipose tissue, consequently inducing insulin resistance (Danforth, 2000). Furthermore, excess (triglycerides) TGs stored in muscle and liver tissues (as ectopic fat) contribute to the accumulation of various metabolic products which over time impair insulin action in both tissues, leading to insulin resistance by inhibiting insulin-stimulated glucose uptake and reducing glycogen synthesis (Lewis and Steiner, 1996, McGarry, 2002). Interestingly, ectopic lipid accumulation, particularly in the liver, is reduced with either improving lifestyle or drug-induced improvement of insulin sensitivity (Lettner and Roden, 2008).

1.7.2 The role of macrophages infiltration
As previously mentioned, adipose tissue expansion is a normal cellular process to help maintain healthy adipose tissue function, and this process relies on the capacity of preadipocytes to differentiate into new adipocytes (adipogenesis). However, in obesity either a decrease in preadipocyte number or their capacity to differentiate, in the presence of chronic positive energy balance due to energy intake exceeding energy expenditure, leads to expand adipose tissue by increasing adipocyte size (hypertrophied adipocytes) (Gesta et al., 2007).

In obese adipose tissue, hypertrophied adipocytes endure molecular and cellular changes which influences systemic metabolism (Greenberg and Obin, 2006). First, an increase circulating FFAs and glycerol from adipocytes in obese individuals is
observed compared to lean individuals (Horowitz and Klein, 2000). Moreover, elevated FFAs are positively linked to inducing insulin resistance in insulin target tissues (Shulman, 2000). Hypertrophied adipocyte is also associated with an increase in release pro-inflammatory adipokines such as leptin, TNF-α, IL-6 and IL-1β but a decrease in adiponectin release (Gutierrez et al., 2009). There are other alterations in companion with adipocyte hypertrophy such as rarefaction of vasculature and neural function and structure in adipose tissue (Coppack, 2005). For example, capillary diffusion capacity for glycerol is reduced in obesity (Coppack et al., 2005).

One fundamental cause for the increased release of FFAs is probably due to the impairment of insulin action such as to suppress lipolysis (Sorisky, 1999, Jensen et al., 1989). A second cause could be due to changes in perilipin expression which has protective role against natural enzymes that hydrolysis lipid droplet, even in obese individuals whose hypertrophied adipocyte, perilipin inhibiting lipases from hydrolysing triacylglycerol to facilitate the release of FFAs (Wang et al., 2003, Zhang et al., 2003). Consequently, this causes a cluster of metabolic disorders, including dyslipidaemia, increased hepatic glucose production and impaired insulin stimulated peripheral tissue glucose uptake (Despres et al., 1990, Reaven, 1996, Boden, 2006). One of the most crucial change which occurs in adipose tissue during obesity is due to mononuclear cells (such as monocytes and macrophages) infiltration. Previous studies in obese mice and humans have found that macrophage infiltration is increased in both species and macrophages infiltration is proportionally associated with BMI and to hypertrophic adipocytes (Weisberg et al., 2003, Wellen and Hotamisligil, 2003).

**1.7.3 The mechanisms of macrophage infiltration in adipose tissue in obesity**

Factors which induce infiltration and activation of macrophages in WAT are most likely multifactorial. Mechanical alterations of hypertrophied adipocytes may link with consequences, including altered adipokine secretion, local adipose hypoxia and adipocyte death together with alterations in paracrine, autocrine and endocrine signals which are considered as potential factors leading to macrophage infiltration (Bourlier and Bouloumié, 2009, Cancell and Clement, 2006).
1.7.3.1 Alterations of adipokine production

The size of the adipocyte is considered as a key factor of the alteration in producing several adipokines. Leptin, one of the major adipokines, is increased in obesity while it can also be produced by preadipocytes after stimulation with cytokines (Simons et al., 2007). Leptin has direct effects on endothelial cells through increasing of oxidative stress, activation of the NF-kB pathway, and increased expression of chemokines and adhesion molecules, which may induce macrophage infiltration in adipose tissue (Curat et al., 2004, Bouloumie et al., 1999). Furthermore, increased release of numerous chemoattractant chemokines such as MCP-1 and IL-8 from the expanded WAT markedly increased infiltration of monocytes which differentiated later into macrophages and eosinophils in WAT (Christiansen et al., 2005). Increased infiltration of macrophages and other immune cells in adipose tissue may constitute the major sources of adipose-derived pro-inflammatory factors, leading to a low grade of inflammation (Lumeng et al., 2007d, Weisberg et al., 2003).

1.7.3.2 Cell death

In addition, hypertrophied adipocyte in both obese mice and humans leads to increased cell death, a major contributor implicated in infiltration of macrophages in obese adipose tissue (Cinti et al., 2005). Cinti and Mitchell reported that over 90% of macrophages located in WAT of obese mice and humans formed crown-like structures (CLS) that envelopes and ingests the pool of dead or dying adipocytes and its cytotoxic residue lipid droplet (Cinti et al., 2005). The CLS of macrophages acts as scavenging to remove adipocyte debris due to necrotic cell death; this action is considered as a beneficial and essential process in physiology (Cinti et al., 2005). During necrosis adipocyte contents are released into the extracellular space which induces host inflammatory response (Rock and Kono, 2008). The clearance of dead adipocytes possibly enhances the activation of pro-inflammatory macrophages (Strissel et al., 2007). Therefore, adipocyte death has potentially important effects for the development of obesity-related inflammation.

1.7.3.3 Hypoxia

Local hypoxia (reduced tissue oxygenation) in obese adipose tissue may play a major role in macrophage infiltration (Bourlier and Bouloumié, 2009). Hypoxia causes
adipocyte death which has been suggested to increase macrophage infiltration into adipose tissue. This is supported by findings that macrophages are located around dead adipocytes formed CLS (Cinti et al., 2005). In obese adipose tissue of human, there was an increase in expression levels of hypoxia-inducible factor-1α (HIF-1α) (Hosogai et al., 2007), leading to induce fibrotic program which is characterized with a abundantly expressed secreted protein acidic and rich in cysteine (SPARC), profibrotic protein, which is linked to obesity induced insulin resistance (Kos and Wilding, 2010). However, expression levels of HIF-1α were decreased by weight loss (Cancello et al., 2005). In preadipocytes and adipocytes, leptin gene expression is stimulated by hypoxia and HIF-1 α thus leptin is considered as a hypoxia-sensitive gene (Grosfeld et al., 2002, Wang et al., 2008). Previous studies using several obese mouse models including Lep ob/ob, DIO and KKAy have demonstrated that hypoxia took places in obese adipose tissue (Hosogai et al., 2007, Ye et al., 2007). Hypoxia is also considered as a determinant factor that might connect obesity with altered adipokine production (Trayhurn et al., 2008). According to in vitro studies using different models of cultured adipocytes showed that expression levels of adiponectin were reduced by hypoxia whereas expression levels of pro-inflammatory factors, including, TNF-α, IL-6, IL-1, MCP-1 and PAI-1, were increased in parallel with hypoxia response genes (HIF-1α, glucose transporter 1, Vascular endothelial growth factor (VEGF)) (Wang et al., 2007, Hosogai et al., 2007). Alteration in adipokine production, in particular change in pro-inflammatory cytokine production under hypoxic conditions, is as a sign for responding to hypoxia (Lewis et al., 1999, Oda et al., 2006). Hypoxia has also been found to evoke cell necrosis in murine 3T3-L1 adipocytes (Ye, 2009). Collectively, hypoxia is involved in macrophage recruitment in obese rodents through several initiating factors including increased leptin production, adipocyte death and upregulation of pro-inflammatory genes. However, recent research has reported that obese insulin resistant subjects had significantly higher adipose tissue oxygen partial pressure (AT PO2) compared to lean insulin sensitive individuals (Goossens and Blaak, 2015). Further clinical studies are required to clarify whether hypoxia is present in adipose tissue in obese humans.
1.7.4 The consequence of macrophage infiltration: alteration of adipokine release and insulin sensitivity

The accumulation of macrophages in obese adipose tissue contributes to increased systemic concentrations of the pro-inflammatory cytokines/chemokines (Cancello and Clement, 2006). Interestingly, similarities in the expression of pro-inflammatory gene profiles between macrophages and mature adipocytes and their precursors have been reported, indicating that both preadipocytes and adipocytes could have some macrophage properties (Charrière et al., 2003, Cancello and Clement, 2006). In addition, adipokines including pro-inflammatory factors, growth factors and other proteins are very important in the regulation of lipid metabolism, glucose homeostasis, blood pressure, vascular haemostasis and angiogenesis (Trayhurn, 2007). The altered production of adipokines in obese adipose tissue, especially those with pro-inflammatory properties, is associated proportionally with the degree of obesity (Weisberg et al., 2003, Maury and Brichard, 2010). However, some adipokines with anti-inflammatory or insulin-sensitizing properties such as adiponectin are reduced in obese subjects (Arita, 2012). The dysregulation of adipokine production is implicated to induce a chronic low grade inflammation status in adipose tissue that which contributes to obesity associated complications such as triglyceridemia and insulin resistance (Weisberg et al., 2003, Constant et al., 2006, Constant et al., 2008). Studies in mouse and humans have shown that the macrophage crosstalk with adipocytes can lead to the alteration of adipocyte function, such as inhibition of preadipocyte differentiation (Constant et al., 2006, Gao et al., 2010), stimulating inflammatory responses (Lacasa et al., 2007) and reducing insulin sensitivity (Lumeng et al., 2007c). Therefore, identification of the key factors that mediate the effect of macrophages on adipose cells is important as it may provide potential therapeutic targets for obesity-related insulin resistance (Figure 1.6).
Figure 1-6: Adipose tissue remodelling in obesity

Edited_version

Figure shows unhealthy adipose expansion with massive enlargement of existing adipocytes (hypertrophied adipocytes), increased infiltration of macrophages and other immune cells. Hypertrophied adipocytes lead to structural and functional changes in adipose tissue including adipokine alteration, elevated FFAs release, limited angiogenesis, and subsequent hypoxia. All of which propagate an overall systemic inflammation associated with the development of obesity-related insulin resistance, adapted from Sun, K., et al. (2011).
1.7.4.1 Tumour Necrosis Factor α (TNF-α)

In adipose tissue, TNF-α is mostly produced by monocytes and macrophages and plays a central role in inflammatory and autoimmune diseases (Trayhurn and Wood, 2004). TNF-α is a 26-KDa transmembrane protein which is cleaved to produce a 17-kDa as the active form of TNF-α. In WAT, TNF-α expression was firstly recognized in rodents and found to be significantly increased in obese models; therefore, it was proposed as the first molecular link between obesity and insulin resistance (Hotamisligil et al., 1993). TNF-α has also been found to be secreted by human preadipocytes and adipocytes (Coppack, 2001). TNF-α is considered as a key regulator that plays an important role in the expression of a range of adipokines (Coppack, 2001), in particular pro-inflammatory factors such as interleukin 6, C reactive protein, neurotrophin, haptoglobin and nerve growth factor (Trayhurn and Wood, 2004). In WAT, both levels of gene expression and protein secretion of TNF-α are increased in obese individuals whereas TNF-α plasma levels decreased in same obese individuals upon weight loss (Kern et al., 1995, Dandona et al., 1998). Consistently, a community-based cohort study has found that the circulating levels of TNF-α were positively correlated with markers of insulin resistance (Hivert et al., 2008). A previous study using TNF-α deficient mice fed with a high fat diet showed that mice were protected from obesity-induced insulin resistance (Uysal et al., 1997).

Studies using adipocytes from murine and human adipose tissue showed that treatment with TNF-α inhibits insulin action by inhibiting insulin-stimulated phosphorylation of the IR, and subsequent inhibits phosphorylation of IRS-1 (Hotamisligil, 1999, Liu et al., 1998). In addition, an in vitro study demonstrated that TNF-α treatment induced phosphorylation of IRS-1 but at serine residues, which in turn inhibited IR activity and downstream signalling of PI3K activation in human adipocytes (Sykiotis and Papavassiliou, 2001). A growing body of evidence shows that role of TNF-α in inhibiting lipogenesis occurred through decreased FFA uptake and stimulation of lipolysis, impairing insulin signalling through induction of serine phosphorylation of IRS-1, inhibiting preadipocyte differentiation by suppressing expression of adipocyte-specific genes and stimulate an inflammatory response (Sethi and Hotamisligil, 1999). Lumeng et al found that TNF-α neutralizing antibodies partially reversed insulin resistance in 3T3-L1 adipocytes produced by MC medium collected from J774A.1 and RAW264 (Lumeng et al., 2007c).
Furthermore, thiazolidinediones (TZDs) which are used as oral hypoglycaemic agent, have inhibitory effect on gene expression of TNF-α and prevent TNF-α-induced insulin resistance (Quinn et al., 2008).

**1.7.4.2 Monocyte Chemoattractant Protein-1 (MCP-1)**

Adipose cells produce several chemoattractant proteins that facilitate monocyte infiltration from blood circulation into adipose tissue; monocytes then differentiate to macrophages (Rasouli and Kern, 2008). MCP-1, also named as chemokine (C-C motif) ligand 2 (CCL2) with molecular weight approximately 11-KDa. MCP-1 is expressed in several cell types including adipose cells, skeletal and smooth muscle cells and endothelial cells (Deshmane et al., 2009). MCP-1 plays an important role in the recruitment of immune cells (macrophages and lymphocytes), triggered through binding MCP-1 ligand to its target receptor on the cell surface that is termed C-C motif receptor 2 (CCR2) (Deshmane et al., 2009). Expression of MCP-1 increases with obesity in most fat tissue types, especially in visceral fat, and reduces after weight loss (Christiansen et al., 2005). Insulin, growth hormone, TNF-α and IL-6 induce MCP-1 expression in 3T3-L1 cells (Fasshauer et al., 2004). In humans, MCP-1 in adipose tissue is expressed predominantly in non-adipose cells (SVF) such as resident macrophages (Bourlier and Bouloumié, 2009). However, a recent an *in vitro* study by our group showed that expression and release of MCP-1 are low in human adipocytes but upon MC medium treatment MCP-1 levels are markedly increased (Gao et al., 2014).

Some *in vitro* and *in vivo* studies using obese and insulin resistant mouse models showed that increased expression and circulating levels of MCP-1 in adipose tissue is positively associated with obesity which may contribute to the development of insulin resistance and impair adipocyte differentiation (Di Gregorio et al., 2005, Sartipy and Loskutoff, 2003). In contrast, MCP-1 or its receptor (CCR2) knockout in mice reduces macrophage infiltration in adipose tissue (Tsou et al., 2007a, Kanda et al., 2006a). Overexpression of MCP-1 in WAT of mice increased infiltration of macrophages into adipose depot and reduced systemic insulin sensitivity assessed by insulin tolerance and hyperinsulinemic-euglycemic clamp tests (Kamei et al., 2006). In addition, MCP-1 overexpression in WAT affected insulin-stimulated tyrosine phosphorylation of IR and IRS proteins and decreased Akt phosphorylation in
muscle and liver, which in turn led to insulin resistance in both tissues (Kamei et al., 2006). Previous studies revealed that circulating levels of MCP-1 were increased in type 2 diabetes patients compared to non-diabetic individuals (Ezenwaka et al., 2009, Nomura et al., 2000). In addition, a case-cohort study based on human population found that raised circulating levels of MCP-1 were associated with the incidence of type 2 diabetes (Herder et al., 2006). Taken together, previous results suggest that MCP-1 could link obesity-related inflammation with the development of insulin resistance.

1.7.4.3 RANTES (CCL5)

RANTES, a member of the chemokine family and also known as (C-C motif) ligand 5 (CCL5), is a 8-KDa protein (Proudfoot et al., 1996). RANTES is released by a range of cells such as macrophages, eosinophils, platelets, fibroblasts, endothelium and epithelial cells (Marques et al., 2013). It is also classified as a chemotactic chemokine; an in vitro study has shown that RANTES induces recruitment of a range of different cells types including T cells, dendritic cells, eosinophils, NK cells, mast cells and basophils (Marques et al., 2013). RANTES binds with its receptors, on the cell surface, such as CCR1, CCR3 and CCR5 to mediate its biological effects (Murphy, 2002).

RANTES is a crucial pro-inflammatory factor in obesity-related adipose tissue inflammation (Matter and Handschin, 2007). A recent study has found that gene expression of RANTES in adipose tissue was increased in obese humans, and its secretion induced monocyte migration and macrophage survival in human adipose tissue (Keophiphath et al., 2010). Consistently, obese subjects with metabolic syndrome have higher mRNA levels of RANTES and CCR5 in visceral adipose tissue than in other depots (Wu et al., 2007). Moreover, obese mice with CCR5 knockout are protected from obesity-induced insulin resistance and this is related to reduced adipose tissue macrophage content and a M2 type-dominant polarization (Kitade et al., 2012a). It suggests that CCR5 plays a critical role in obesity-induced inflammation and insulin resistance.
1.7.4.4 Leptin

The mechanisms underlying the development of obesity and its metabolic disorders and complications have been studied intensively, particularly, after the discovery of leptin in 1994 (Zhang et al., 1994). Leptin is a mature hormone is a protein of the molecular weight 16 KDa encoded by (ob) gene. Due to the discovery of leptin, adipose tissue has been recognized as an endocrine organ (Zhang et al., 1994, Leopold et al., 1994). Leptin is considered as a major adipokine with multiple physiological roles such as regulating food intake, energy expenditure, body weight and neuro-endocrine functions (Otero et al., 2005, Howard et al., 2004). The hypothalamus is considered as the major site of leptin action (Kahn and Flier, 2000). Leptin action is triggered through binding to its receptors (obR) which are expressed in most tissues (Galic et al., 2010). Obesity is highly correlated with increased leptin production and increased plasma leptin concentrations (Flier, 2004, Considine et al., 1996), which may reflect leptin resistance (Ahima and Osei, 2008). Mice with leptin deficiency have obesity development such as in the phenotype of ob/ob mice (Zabolotny et al., 2002). Consistently, by giving recombinant leptin to morbid obese children suffering from a lack of leptin function, weight loss is successfully achieved (Shimabukuro et al., 1997).

White adipocytes in both human and rodents are considered as the main source of leptin from adipose tissue (Montague et al., 1998). However, leptin is also recognized to be produced at low levels in other places including BAT, muscles, stomach, placenta, and and fetal tissues (such as heart and bones) (Traughurn and Beattie, 2001). In addition, an in vitro human study using preadipocytes has reported that small levels of leptin was produced following stimulation with TNF-α and IL-1β (Simons et al., 2007, Simons et al., 2005). Leptin has a structure similar to helical cytokines like IL-2 and growth hormone 1, and is thought to have pro-inflammatory activities by increasing production of IL-6 and TNF-α from monocytes and stimulating the production of CC-chemokine ligands (Deng and Scherer, 2010, Santos-Alvarez et al., 1999, Kiguchi et al., 2009). Therefore, it was suggested that leptin production is altered during inflammation and infection period, which could be a part of the cytokine cascade which organizes (Simons et al., 2005) the host defense mechanisms and innate immune response (Faggioni et al., 2001, Pecoits-Filho et al., 2002).
Leptin was suggested to be a central player in glucose homeostasis as a previous study has shown that leptin appeared as a major insulin-sensitizing adipokine by reversing insulin resistance and diabetes mellitus in mice (Shimomura et al., 1999). In addition, studies on two groups of rodents (leptin deficient and obese with leptin resistance) demonstrated that both groups have severe insulin resistance which was rapidly improved by administration of leptin; thus leptin has insulin sensitizing effect on both groups (Farooqi et al., 2002). Further supporting evidence suggests that fatty acid oxidation is promoted by leptin and leptin can reduce ectopic fat accumulation in non-adipose tissues, as a result of increased insulin sensitivity (Muoiio et al., 1997). Leptin acts to activate adenosine monophosphate-activated protein kinase (AMPK) which is a key mediator of fatty acid oxidation (Minokoski et al., 2002). The effect of activation could be seen in certain skeletal muscles as a direct effect and also in the hypothalamus as an indirect effect (Minokoski et al., 2002). Furthermore, in the obesity state, resistance to leptin could promote ectopic lipid storage, which may in turn further impair insulin sensitivity (Ahima et al., 2006). However, several murine studies have showed that leptin impairs insulin action in different cell types such as adipocytes (Müller et al., 1997), skeletal muscle cells (Sweeney et al., 2001) and hepatocytes cells (Cohen et al., 1996). Leptin has ability to increase oxidative stress, activation of the NF-kB pathway, and increased expression of chemokines and adhesion molecules, which may induce macrophage infiltration in adipose tissue (Curat et al., 2004, Bouloumie et al., 1999). Macrophage-derived factors significantly alter adipose cells function, inducing inflammatory responses, reducing insulin sensitivity and impairing differentiation capability which may contribute to obesity-related metabolic disorder such as insulin resistance and type 2 diabetes (Bing, 2015).

1.7.4.5 Adiponectin
In the mid1990s adiponectin was originally discovered by four independent research groups through different experimental approaches (Hu et al., 1996, Scherer et al., 1995, Maeda et al., 1996, Nakano et al., 1996). Adiponectin is considered as the most abundant adipokine produced from adipocytes (Chandran et al., 2003). Adiponectin is a 30 KDa protein and its gene expression takes place from the middle stage of differentiation onwards (Scherer et al., 1995, Hu et al., 1996, Ouchi et al., 2011). The
circulating levels of adiponectin are present at high concentrations (3 to 30 µg/ml) in plasma (Pajvani et al., 2003, Ouchi et al., 2003a). Human adiponectin is recognized in different three forms as low molecular weight (trimer isoform), middle molecular weight and high molecular weight (hexamer isoform) (Kadowaki and Yamauchi, 2005). The high molecular weight isoform is reported as the most active form of adiponectin and considered as a better marker for the prediction of insulin resistance and metabolic syndrome than plasma total adiponectin level (Hara et al., 2006). Adiponectin is also detected in many different places such as the pituitary gland, brain and cerebrospinal fluid which is triggered by fasting (Steinberg and Kemp, 2007). Structurally, full-length of adiponectin is composed of a collagen-like domain (tail) and a globular domain head (Kadowaki and Yamauchi, 2005), that is similar to complement factor C1q (Wong et al., 2004).

An important difference between adiponectin and most adipokines is that adiponectin production and its plasma concentrations are decreased in extremely obese and insulin-resistant subjects (Hotta et al., 2001, Hu et al., 1996). Previous studies using obese/diabetic mice and human models have demonstrated that plasma adiponectin levels are significantly reduced (hypoadiponectinemia) (Arita, 2012, Hu et al., 1996, Yamauchi et al., 2001). In addition, circulating levels of adiponectin have also been found to be decreased in patients with metabolic disorders (Trujillo and Scherer, 2005) including type 2 diabetes, lipodystrophy, nonalcoholic hepatic steatosis (Deng and Scherer, 2010), cardiovascular diseases (Hotta et al., 2000, Kumada et al., 2003), hypertension (Ouchi et al., 2003b), and mutations in the adiponectin gene (Kondo et al., 2002). However, a number of studies in both mice and humans have confirmed that adiponectin expression and secretion are increased with weight loss and improved insulin sensitivity (Milan et al., 2002, Yang et al., 2001, Hulver et al., 2002). Adiponectin is essential for PPARγ agonists, such as TZD, to improve their anti-diabetic activities (Fasshauer and Paschke, 2003), mainly during exposure to a high fat diet (Nawrocki et al., 2006). Vice versa, PPARγ is found to be important to increase expression and plasma concentrations of adiponectin (Maeda et al., 2001). Thus, adiponectin has known to possess an anti-diabetic property that is proposed to treat obesity-related insulin resistance (Lindsay et al., 2002). Two case-control studies have demonstrated that individuals, who have high adiponectin circulating levels, are less likely to develop type 2 diabetes than individuals with low
adiponectin circulating levels (Lindsay et al., 2002, Spranger et al., 2003a, Yu et al., 2002b). Consistently, adiponectin has been shown to improve hepatic insulin action which suppressed hepatic glucose production and increased glucose utilization and fatty acids oxidation in adipose tissue through activation of AMPK (Berg et al., 2001, Rasmussen et al., 2006, Yamauchi et al., 2002).

Adiponectin is also commonly considered to have anti-inflammatory, pro-angiogenic and anti-apoptotic properties (Rajala and Scherer, 2003, Deng and Scherer, 2010, Landskroner-Eiger et al., 2009). An interesting observation of a study using murine pre-adipocytes (3T3-L1) was shown that adiponectin overexpression enhanced adipocyte differentiation and lipid accumulation (Fu et al., 2005). There are a range of therapeutic managements or interventions, such as weight loss, caloric restriction and TZDs treatment, which can improve adiponectin expression and secretion (Yu et al., 2002b, Bruun et al., 2003a, Combs et al., 2003). On the other hand, several studies have shown that TNFα treatment can inhibit gene expression and secretion of adiponectin in murine adipocytes (3T3-L1) and human primary adipocytes (Fasshauer et al., 2003, Kappes and Löffler, 1999). Furthermore, an in vitro study using murine adipocytes (3T3-L1) found that IL-6 treatment led to a reduction in adiponectin gene expression (50%) and secretion (75%) (Fasshauer et al., 2003). Thus, it was suggested that adiponectin expression and secretion are reduced due to pro-inflammatory cytokines which mainly increased in obesity.

1.7.5 Interleukins (ILs)

ILs are a cluster of polypeptides which are made up of a number of members with both pro and anti-inflammatory properties. ILs are cytokines that act on leukocytes and other tissue targets such as adipose tissue cells including preadipocytes, adipocytes and adipose tissue macrophages (Khadka, 2014). These cells also express and release a number of ILs such as IL-1 (IL-1α and IL-1β), IL-6 and IL-8 (Gao and Bing, 2011). Several ILs have been considered as potential effectors in the pathology of insulin resistance associated with type 2 diabetes (Fève and Bastard, 2009).
1.7.5.1 Interleukin-6 (IL-6)

IL-6 is a cytokine mostly associated with the immune system response (Torres-Leal et al., 2012). During the innate immune response IL-6 is mainly involved in the production of acute phase reactants such as C-reactive protein (CRP) from the liver (Steel and Whitehead, 1994). Unlike TNF-α, IL-6 has both pro-inflammatory and anti-inflammatory properties. This makes it play dual roles; at some level it provides a defence mechanism by promoting synthesis of anti-inflammatory cytokines which reduce inflammatory response where at other level such as in chronic inflammation it acts as pro-inflammatory cytokine (Kamimura et al., 2004). IL-6 is secreted in humans by a wide range of cells including monocytes, macrophages, endothelial cells, β pancreatic cells, osteoblasts, myocytes, adipocytes (Kamimura et al., 2004, Carey and Febbraio, 2004) as well as preadipocytes (Gao and Bing, 2011).

In humans, the expression and secretion of IL-6 was observed in adipocytes and preadipocytes (Sopasakis et al., 2004, Bastard et al., 2000, Vicennati et al., 2002, Krogh-Madsen et al., 2004). Substantial amounts of IL-6 are produced by WAT especially from the visceral depot where IL-6 may constitute 10-35% of circulating levels (Mohamed-Ali et al., 1997). However, non-adipose cells are considered as major source of IL-6 which may constitute about 60-90% of IL-6 secretion (Sopasakis et al., 2004). The circulating levels of IL-6 have been reported to be increased in obese individuals and also increased in individuals with type 2 diabetes (Vozarova et al., 2001b). Furthermore, evidence suggests that IL-6 directly plays a role in insulin resistance in 3T3-L1 adipocytes and primary mouse hepatocytes by altering insulin signalling through reduced gene and protein expression of IRS-1 and GLUT-4, and impaired insulin-induced glycogenesis in hepatocytes (Senn et al., 2002, Rotter et al., 2003). Another explanation for the role of IL-6 in insulin resistance is that several members of suppressor of cytokine signaling-3 (SOCS-3) in insulin target tissues are induced by IL-6 which in turn inhibits phosphorylation of insulin-stimulated IR, IRS-1 PI3K p85 and Akt (Senn et al., 2003). It appears from the literature that IL-6 is one of several pro-inflammatory cytokines implicated in insulin resistance and obesity.
1.7.5.2 Interleukin 8 (IL-8)

IL-8 is a member of the C-X-C motif chemokine family, a chemoattractant protein, which facilitates neutrophil and lymphocytes chemotaxis (Mackay, 2001) and also induces chemotaxis of macrophages (Waugh and Wilson, 2008). It is involved in many inflammatory diseases such as asthma, inflammatory bowel disease, sepsis and adult respiratory disease. IL-8 has commonly been recognized for its association with different inflammatory processes (Bagnolini et al., 1995, Roebuck, 1999). A previous in vitro study using human adipose tissue has found that IL-8 production was increased in dose dependent manner after stimulation with two pro-inflammatory cytokines treatment including IL-1β and TNF-α (Bruun et al., 2001). Therefore, IL-8 may be involved in obesity-related complications.

Similar to MCP-1, IL-8 has four conserved cysteine residues at N-terminus but its residues are separated by one amino acid (CXC chemokines) (Roebuck, 1999). IL-8 chemokine plays a key role in activation of multiple intracellular signalling pathways downstream through binding IL-8 ligand to its target G protein–coupled receptors on the cell surface that termed CXCR1and CXCR2 (Waugh and Wilson, 2008). IL-8 is secreted by a variety of cells including adipose cells (Gerhardt et al., 2001) and its circulating levels are increased in obesity (Rotter et al., 2003, Straczkowski et al., 2002). A research conducted to study the link between insulin sensitivity and plasma levels of IL-6, IL-8 and TNF-α, found that circulating levels of IL-8 and IL-6 were upregulated and associated with measures of insulin resistance in the abdominal depot of obese subjects. The circulating levels of these cytokines/chemokines were influenced by weight loss (Bruun et al., 2003b). Gene expression of IL-8 is upregulated in mammary adipose tissue of obese women, in parallel with increased macrophage infiltration (Sun et al., 2012). A previous study showed that IL-8 receptor (CXCR2) knockout in diet-induced obese mice prevents insulin resistance and macrophage recruitment into adipose tissue (Neels et al., 2009). Furthermore, an in vitro study using human adipocytes has found that IL-8 has an inhibitory effect on insulin action by reducing insulin-stimulated phosphorylation of Akt whereas it has a stimulatory effect on Akt mRNA expression (Kobashi et al., 2009).
1.7.5.3 Interleukin 1 family

IL-1 family is considered to be involved in several cellular activities such as regulating host defence and immune responses (Auron et al., 1984) and also implicated in the acute-phase response and is responsible for many alterations that are linked with the onset of a number of medical conditions (Di Iorio et al., 2003). The interleukin-1 family (IL-1) consists of 11 cytokines (Di Iorio et al., 2003). However, the two major cytokines are IL-1α and IL-1β (Dinarello, 2009). Structurally, IL-1α and IL-1β have similar biological properties with slight sequence homology and they are different in several regulation aspects such as localization, maturation, and secretion (Tack et al., 2012). Both major forms of IL-1 bind to common receptors, type I (signalling) and type II (decoy), that are present on a variety of target cells (Dinarello, 1998a, Mantovani et al., 2001, Fibbe and Willemze, 1991). Another member of the IL-1 family is an interleukin-1 receptor antagonist (IL-1 RA) which has similar sequence homology to IL-1β and binds to human IL-1 receptors without activating them and antagonizes both IL-1 subtypes by controlling their production and biological activity (Arend, 1990).

1.7.5.3.1 Interleukin 1β (IL-1β)

IL-1β is produced largely by macrophages (Sims and Smith, 2010), and also by several immune cells including dendritic cells, B lymphocytes and NK cells (Dinarello, 2009). Moreover, IL-1β can also be expressed and released by adipose cells (preadipocytes and adipocytes) (Xu et al., 2003, Weisberg et al., 2003) and its expression and release has been found to be enhanced in obesity (Fain, 2006). IL-1β plays an important role in mediating the inflammatory response (Arend, 2002). Furthermore, IL-1β has a role in development of systemic and local responses to injury, infection, and other immunological events and is the main cause of acute and chronic inflammation by inducing the production of cytokines and chemokines from epithelial, endothelial and immunocompetent cells (Dinarello, 1998b, Dinarello et al., 2010). For example, intravenous injection into humans with a small amount of IL-1β induces fever, thrombocytosis, leukocytosis, hypotension and the release of adrenal corticotropin hormone (Smith et al., 1993, Crown et al., 1993, Crown et al., 1991). IL-1β plays also a role in a range of cellular processes, including cell proliferation, differentiation and apoptosis (Arend, 2002) as well as a role in normal
homeostasis such as metabolism, hematopoiesis, regulation of blood pressure, renal and hepatic function and sleep (Dinarello, 1993).

Unlike IL-1α which is produced in an active form, IL-1β is firstly formed as pro–IL-1β (inactive form) which accumulates in the cytosol, and then it is cleaved and processed by the cysteine protease caspase-1 (also named interleukin converting enzyme (ICE) to produce the active form of IL-1β (p17). Caspase-1 has been known to be controlled by a multi-protein complex termed the nucleotide-binding oligomerization domain receptors (NLRP3) inflammasome (Figure 1.7) (Agostini et al., 2004). Once mature active form of IL-1β is released, it can increase its signals through binds to the IL-1 receptor I leading to a vicious cycle of inflammation, which in turn increase the activation and infiltration of macrophages into inflamed tissues, such as the islet and adipose tissue (Dinarello et al., 2010).

In the unhealthy state, IL-1β is expressed upon stimulation by stress-related pathways and the initial translation product is an inactive precursor. In vitro, stimulated human blood monocytes release less than 10% of the intracellular pool of IL-1β into the extracellular compartment (Schindler et al., 1990). Stienstra and et al reported elevated caspase-1 and increased levels of IL-1β from adipocytes isolated from mice that fed a high fat diet or obese ob/ob mice. However, adipose tissue from caspase-1-deficient mice is histologically distinct compared to fat from wild type mice; differentiation of preadipocytes isolated from caspase-1-deficient mice resulted in more metabolically active fat cells and are more insulin sensitive as compared to wild-type mice (Stienstra et al., 2010). Studies have shown that IL-1β upregulates the expression of inflammation–related genes in adipocytes and preadipocytes (Gao and Bing, 2011, Permana et al., 2006). Furthermore, a study showed that IL-1β induced a significant increase in pro-inflammatory cytokine (IL-6) in adipocytes and peripheral blood cells (Flower et al., 2003), consistently, a recent study conducted by our group showed that the release of a range of pro-inflammatory factors from human adipocytes including IL-6, MCP-1, IL-8 and RANTES were induced after treatment with IL-1β (Gao et al., 2014).

IL-1β is considered as a key pro-inflammatory cytokine which produced mainly by monocytes and macrophages (Agostini et al., 2004). Accordingly, an in vitro study
using murine and human adipocytes reported that long term treatment with IL-1\(\beta\) inhibited insulin stimulated phosphorylation of the p-IR \(\beta\) subunit, IRS-1 and Akt/protein kinase B. In addition, IL-1\(\beta\) changed adipocyte differentiation by reducing adipogenic factors such as C/EPB\(\alpha\) and PPAR\(\gamma\) (Lagathu et al., 2006). However, a recent study by Gagnon and other using an *in vitro* human cell models (THP-1 macrophage and human primary preadipocytes) reported that IL-1\(\beta\) was sufficient to inhibit preadipocytes adipogenesis but IL-1\(\beta\) is not required for the ability of macrophage CM to inhibit adipogenesis in human preadipocytes (Gagnon et al., 2013). Growing evidence based on mouse models suggests that IL-1\(\beta\) is essentially involved in the translation of obesity-associated inflammation into insulin resistance. However, little is known whether IL-1\(\beta\) mediates the effect of macrophages on insulin signalling in human preadipocytes and adipocytes.

**Figure 1-7: Schematic illustration of inflammasome activation that leads to IL-1\(\beta\) release**

Figure shows inflammasome activation is induced by a group of stimuli including (LPS, ceramides, glucose and uric acid). Inflammasome activation leads increased caspase-1-dependent cleavage of pro-IL-1\(\beta\) into mature IL-1\(\beta\), which is subsequently released from the cells, adapted from (Tack et al., 2012).
1.7.5.3.2 Targeting IL-1β in obesity and diabetes

Recent studies suggest that IL-1β may be a candidate in the development of insulin resistance and type 2 diabetes (Tack et al., 2012, Wen et al., 2011). Therefore, clarifying whether IL-1β mediate the effect of macrophages on the human adipose cells is important as it may provide potential therapeutic targets for obesity-related insulin resistance. Currently, most therapeutic agents used for inhibiting TNF-α activity are using neutralization approaches. However, there are several approaches for inhibiting interleukin IL-1β activities (Dinarello, 2004). Interestingly, the development of anti-IL-1β neutralizing antibody in humans is considered to be more beneficial as a therapeutic strategy than in IL-1α. This is because some studies using animal and human models demonstrated that IL-1β is released and found in pathological fluids while IL-1α remains an intracellular cytokine (Dinarello, 1996).

The IL-1RA (interleukin-1 receptor antagonist), also known as anakinra, is presently approved after used in clinical trials to treat diseases in human such as rheumatoid arthritis (Dinarello, 2004). At molecular level, IL-1RA binds to the IL-1 receptor I with high affinity and stops the binding of IL-1 to its receptor via a mechanism of competitive receptor antagonism (Dinarello, 2004). In studies using rodent models of obesity and diabetes (GK rats), blocking IL-1β by a neutralizing antibody or IL-1RA reduced hyperglycemia and tissue inflammation (Owyang et al., 2010, McGillicuddy et al., 2011, Ehses et al., 2009). Recently, several of studies conducted in patients with type 2 diabetes have shown that the IL-1β neutralizing antibody or IL-1RA (anakinra) have beneficial effects on glucose control and β-cell function, with a reduction in circulating pro-inflammatory markers (Larsen et al., 2007, Cavelti-Weder et al., 2012, Sloan-Lancaster et al., 2013).

As mentioned above, obese adipose tissue has been found to produce significant amounts of IL-1β via NLRP3 activation, which in turn is linked to inflammation and insulin resistance (Vandanmagsar et al., 2011, Stienstra et al., 2011). However, caspase-1-deficient mice have decreased body fat and improved insulin sensitivity (Stienstra et al., 2010). Therefore, reducing IL-1β production by inhibition of caspase-1 (ICE) has become a promising therapeutic strategy as well to reduce IL-1β role in many inflammatory diseases (Randle et al., 2001).
This thesis was conducted as one of the studies on the tissue specific effects of IL-1β inhibition in humans. IL-1β inhibition might decrease macrophage infiltration and inflammatory response in adipose tissue, thus improving adipose tissue function such as insulin sensitivity and differentiation of preadipocytes.

### 1.8 Aims of the study

**1.8.1 Hypothesis**

WAT is a heterogenous organ containing multiple depots and several cell types including preadipocytes, adipocytes and macrophages. These cells are able to produce and release a wide range of cytokines and other compounds. It has been shown that adipocytes are able to secrete adipokines. Therefore, adipose tissue is a key endocrine organ. Macrophages are considered to be the major source of pro-inflammatory cytokines in obese adipose tissue. IL-1β, a pro-inflammatory cytokine, is a major product of macrophages. Therefore, it is hypothesized that IL-1β could mediate the effects of macrophage-induced impairment of the insulin signalling pathway and induce inflammation in human adipose cells (preadipocytes and adipocytes).

**1.8.2 Aims and objectives**

Identification of the major factors that mediate detrimental effects of macrophages on adipocytes may offer potential therapeutic targets. IL-1β, a proinflammatory cytokine, is suggested to be involved in the development of insulin resistance. In this context, several *in vitro* human cell models were used, including primary preadipocytes, adipocytes, THP-1 macrophages (cell line) and peripheral blood mononuclear cells-derived macrophages (PBMCs), to establish the effect of cross talk between them on insulin signalling of adipocytes and their precursors (preadipocytes).

The specific objectives are listed below:

1. Characterize the key insulin signalling molecules in human primary preadipocytes in comparison with adipocytes;
2. Investigate the role of IL-1β in macrophage-preadipocyte crosstalk, and whether this crosstalk modulates the insulin signalling pathway in human preadipocytes;

3. Examine the role of IL-1β in modulating pro-inflammatory factors expression and release by preadipocytes;

4. Investigate the effect of long-term treatment with IL-1β on lipid storage functions and adipogenesis in human adipocytes;

5. Investigate whether IL-1β mediating macrophage-induced lipolysis in human adipocytes through blocking IL-1β activity;

6. Investigate the role of IL-1β in macrophage-adipocyte crosstalk, and whether this cross-talk modulates insulin signalling molecules and pro-inflammatory factors expression and release in human adipocytes;

7. Investigate whether inhibiting the effect of IL-1β by different approaches reduces the inflammatory response involved in adipose tissue dysfunction.

The results obtained after completing the experimental studies may provide some answers to above mentioned hypothesis. This may contribute to filling the gap in our knowledge and provide a better understanding of the role of the macrophage-derived factors, in particular IL-1β, in the development of insulin resistance and type 2 diabetes.
CHAPTER 2:
Material and Methods
## 2 Material and Methods

### 2.1 Reagents, materials and equipment

#### 2.1.1 Chemical reagents

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### 2.1.2 Kits

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### 2.1.3 Equipment and system

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### 2.1.4 Software

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<td>GraphPad Software</td>
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<td>Microplate Spectrophotometer</td>
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<td>Microsoft Excel 2007</td>
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<td>MxPro™ QPCR software</td>
<td>Stratagene</td>
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### 2.1.5 Supplier’s addresses and URLs

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<tr>
<td>Amersham</td>
<td>Amersham Pl, Little Chalfront, Buckinghamshire, United Kingdom, <a href="http://www.amershambiosciences.com">http://www.amershambiosciences.com</a></td>
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<tr>
<td>Bio-Rad</td>
<td>Bio-Rad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Hertfordshire HP2 7DX, United Kingdom, <a href="http://www.bio-rad.com">http://www.bio-rad.com</a></td>
</tr>
<tr>
<td>Cell Signaling</td>
<td>Cell Signaling Technology, New England Biolabs (UK) Ltd, 75-77 Knowl Piece, Wilbury Way, Hitchin, Hertfordshire SG4 0TY, United Kingdom, <a href="http://www.cellsignaling.com">http://www.cellsignaling.com</a></td>
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<tr>
<td>Enzo Life Sciences</td>
<td>Enzo Life Sciences (UK) LTD, Palatine House, Matford Court, Exeter EX2 8N, United Kingdom, <a href="http://www.enzolifesciences.com">http://www.enzolifesciences.com</a></td>
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<td>Eppendorf</td>
<td>Eppendorf UK Limited, Endurance House, Vision Park, Chivers Way, Histon, Cambridge CB24 9ZR, United Kingdom, <a href="http://www.eppendorf.co.uk">http://www.eppendorf.co.uk</a></td>
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<td>Eurogentec</td>
<td>Eurogentec Ltd, Old Headmasters House, Unit 1, Building 1, Forest Business Centre, Fawley Road, Fawley, Southampton, Hampshire SO45 1FJ, United Kingdom, <a href="http://www.fermentas.de">http://www.fermentas.de</a></td>
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<td>-------------------------------------------------------------------------------------------</td>
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<tr>
<td>Fermentas</td>
<td>European Head Office Germany. Fermentas GmbH, Opelstrasse 968789 St. Leon-Rot,Germany,</td>
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<tr>
<td>Fisher Scientific</td>
<td>Fisher Scientific UK Ltd. Bishop Meadow Road, Loughborough, Leicestershire, LE11 5RG, United Kingdom, <a href="http://www.fisher.co.uk">http://www.fisher.co.uk</a></td>
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<tr>
<td>Labnet International</td>
<td>Labnet International, PO Box 841, Woodbridge, NJ 07095, USA, <a href="http://www.labent.com">http://www.labent.com</a></td>
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<tr>
<td>Millipore</td>
<td>Millipore UK Ltd., Croxley Green Business Park, Watford, WD18 8YH, United Kingdom, <a href="http://www.millipore.com">http://www.millipore.com</a></td>
</tr>
<tr>
<td>Nikon</td>
<td>Nikon UK Limited. 380 Richmond Road, Kingston upon Thames, Surrey KT2 5PR, United Kingdom, <a href="http://www.europe-nikon.com">http://www.europe-nikon.com</a></td>
</tr>
<tr>
<td>PromoCell</td>
<td>PromoCell GmbH, Sickingenstraße 63/65, D-69126 Heidelberg, Germany, <a href="http://www.promocell.com">http://www.promocell.com</a></td>
</tr>
<tr>
<td>R &amp; D Systems</td>
<td>R &amp; D Systems Europe Ltd. 19 Barton Lane, Abingdon Science Park, Abingdon, OX14 3NB, United Kingdom, <a href="http://www.rndsystystems.com">http://www.rndsystystems.com</a></td>
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<td>SERVA</td>
<td>SERVA Electrophoresis GmbH, Carl-Benz-Str. 7 P.O.B.10 52 60, 69115 Heidelberg, Germany, <a href="http://www.serva.de">http://www.serva.de</a></td>
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<td>Sigma</td>
<td>Sigma-Aldrich Company Ltd. Fancy Road, Dorset BH12 4QH, United Kingdom, [<a href="http://www.sigmaaldrichie.com/united">http://www.sigmaaldrichie.com/united</a> kingdom.html](<a href="http://www.sigmaaldrichie.com/united">http://www.sigmaaldrichie.com/united</a> kingdom.html)</td>
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<td>Starlab</td>
<td>STARLAB (UK), Ltd. Unit 4 Tanners Drive, Blakenlands, Milton Keynes MK14 5NA, United Kingdom, <a href="http://www.starlab.de">http://www.starlab.de</a></td>
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<tr>
<td>Stratagene (Agilent)</td>
<td>Agilent Technologies, Inc. Life Sciences and Chemical Analysis, Group 5301, Stevens Creek Boulevard, Santa Clara, CA 95051-7201, USA</td>
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<td>Techno plastic products</td>
<td>TPPTechno Plastic AG, Zollstrasse 155, CH-8219, Trasadingen, Switzerland</td>
</tr>
<tr>
<td>UVP</td>
<td>Ultra-Violet Products Ltd., Unit 1, Trinity Hall Farm Estate, Nuffield Road, Cambridge CB4 1TG, United Kingdom, <a href="http://www.UVP.com">http://www.UVP.com</a></td>
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2.2 Cell Culture system

2.2.1 Cell culture basis

One of the most important and widely used methods to study cell biology and the effects of biologically active molecules on living organisms is cell culture. Cell culture systems provide excellent models that allow understanding of cellular and molecular basis of cells in both physiological and pathological conditions. Cell culture refers to the cell removal from human, animal or plant and their consequent expansion in an encouraging artificial environment (Aschner et al., 2011). Cells are contained mainly in a humidified atmosphere of 5% CO2/ 95% air at 37 ºC incubators unless specified otherwise to achieve successful cell cultivation. Moreover, cell culture can be performed using the cells which may be taken directly from the tissue as primary cells and disaggregated by mechanical or enzymatic methods before cultivation, or it can be achieved by cells derived from a cell line or cell strain that has already been established (Aschner et al., 2011).

2.2.2 Method

In this research project, there were three cell types used, namely primary human pre-adipocytes, human THP-1 monocytes (cell line) and peripheral blood mononuclear cell-derived macrophages (PBMCs). All were used as model systems to study the effects of the crosstalk between macrophages and adipose cells, and to demonstrate how adipose tissue dysfunction, such as the impairment of insulin signalling might occur during the development of obesity.

In culture of primary human pre-adipocytes and THP-1 cells, all appropriate bio-safety practices were followed to reduce and eliminate exposure to laboratory workers, and all required precautions were taken to avoid any contamination and infection into the candidate cells. Firstly, all glassware was well washed and autoclaved, and all plastic ware used was sterilized. Secondly, the UV-C lamp in the laminar flow hood or cell culture hood was used and surfaces were exposed to UV light for a minimum of 15 minutes before use. In addition, the cell culture hood was wiped by 1% Vircon solution, followed by 70% ethanol, to make sure a clean environment for cell culture was obtained. All items that were to be used in the hood were sprayed with 70% ethanol before entering the hood. This was to reduce the risk
of infection which may occur to cells. All medium components were mixed together by using single-use serological pipette (Starlab, Milton Keynes, UK) inside the hood and medium was filtered through a 0.22 µm filter membrane (TechnoPlastic Products, Trasadigen, Switzerland).

The culture growth medium and feeding medium were prepared, and they were kept at 4 ºC until use. They were then warmed in HB-1000 a hybridization incubator (Cambridge, UK) prior to use. Induction medium (Differentiation medium) (see below) was also prepared in the same way; however it had to be freshly prepared prior to the time of use and any extra medium was discarded afterwards.

2.3 Human preadipocytes culture and differentiation

2.3.1 Reagents and mediums

**Preadipocytes growth medium**

1x Preadipocytes Growth Medium (Promocell)
1x Preadipocytes supplementMix (Promocell)
100 ml pencillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericine mix

**Differentiation medium**

1x Dullbecco’s Modified Eagle’s Medium-Ham’s F12 (DMEM) (1:1, vol/vol)
Biotin 32 µM
Insulin 100 nM
Rosiglitazone 8 µM
Dexamethasone 1 µM
3-isobutyl-1-methylxanthine 200µM
L-Thyroxine 11 nM
100 U/ml penicillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericine mix

**Human primary adipocyte feeding medium (post differentiation)**

1x Dullbecco’s Modified Eagle’s Medium-Ham’s F12 (DMEM) (1:1, vol/vol)
Fetal calf serum 3%
Insulin 100 nM
Dexamethasone 1 µM
Biotin 32 µM
100 U/ml pencillin, 100 µg/ml streptomycin and 0.25 µg/ml amphotericine mix

2.3.1.1 Cell culture reagents
Phosphate- buffered saline (Autoclaved)
Trypsin 0.05% -EDTA 0.2% solution

2.3.1.2 Cell culture equipment
Autoclaved Pasture pipettes
Filter and filter collection bottle
150 cm² and 75 cm² cell culture flasks
Seriological pipette and sterile pipette tips
50 ml centrifuge tubes
Haemocytometer
6 /12 and 24 well plates

2.3.2 Human preadipocytes storage
For all experiments, PromoCell (Heidelberg, Germany) provided our lab with the human primary white preadipocytes that were originally derived from subcutaneous adipose tissue of a female Caucasian subject (age 44 years; BMI 21). Cryopreserved cells were stored in liquid nitrogen immediately after arrival, or were directly seeded. Cells could not be stored at - 80 ºC because this temperature was not sufficient for cell preservation and causes irreversible cell damage.

2.3.3 Resuspension of preadipocytes from cryopreservation
For suspending and culturing the human preadipocyte cells, Promocell’s protocol was followed in all cell culture experiments. An appropriate amount of culture growth medium was freshly prepared and warmed in 1000-HB a hybridization incubator (Cambridge, UK) to 37 ºC. Forty ml of growth medium was poured into a 150 cm² flask. A vial of preadipocytes was removed from a liquid nitrogen store and the vial lid was loosened to help release pressure then quickly transferred in a box of ice. The vial was then placed in a water bath at 37 ºC continuously swirled for 90 seconds to defrost cells. After defrosting, 1 ml of the warm culture growth medium
was added into the vial to ensure all cells were thawed completely, and mixed gently up and down using a pipette. The cell suspension was added to the 150 cm² flask contained growth medium and immediately incubated at 37 ºC with a humidified atmosphere of 5% CO2 /95% air for 24 hr, and the lid of flask was released a half turn. In the following, cells viability was directly checked under a phase contrast by Diaphot inverted microscope (Nikon, Surrey, UK) after which cells were removed from incubator. The culture medium was replaced with fresh growth medium and the cells were incubated for 3 to 4 days. Cells were observed during these days to see if the cells had become 70 to 90% confluent, but not reached 100% confluence to make sure cells will not undergo growth arrest.

2.3.4 Preadipocytes subculture

Once preadipocytes reached 70%-90% confluence, medium was discarded by aspirating it aseptically, after which attached cells were washed 2 times with autoclaved phosphate-buffered saline (PBS) by adding 10 ml each time from the non-layered side to avoid removing the cells. The PBS was removed and 6 ml of trypsin was added to the flask and placed in the incubator at 37°C for 3 to 5 minutes or placed at room temperature inside the laminar flow for detaching cells. The cells were inspected under a light microscope, and the flask gently shaken, to check if all cells had become completely detached. Once all the cells were fully detached, 10 ml of culture growth medium was added to the flask with swirling it to neutralize the trypsin solution and prevent toxic effects of trypsin that may cause cells death. The number of cells was counted using a haemocytometer. Flask suspension aliquot was used in both sides of haemocytometer by pipetting 10 µl of cell suspension to each side. The total cell number was used to determine the volume of cells' suspension which was needed to reach 40,000 cells per ml/well as the cell plating density. Remaining cell suspension was poured into a 30 ml centrifuge tube then spun at 250 x g for 5 minutes. The supernatant was aspirated aseptically from the tube and 5 ml of growth medium and Cryo-Stem Freezing Medium (Cryo-SFM) with ratio 4:1 were added to pellet of cells to obtain homogeneity. To store cells in liquid nitrogen with a new passage number put each 1 ml of cells suspension in separate vial. Alternatively, 1 vial of cells suspension was added to original flask (150 cm²) with adding 40 ml of growth medium to continue in experiment. The flask was then placed back into the incubator at 37 ºC and 5% CO2/95% air for 3 days. Once
preadipocytes reached proper confluence (70%-90%) the cells would be plated out and then placed back into an incubator at 37 °C and 5% CO2/95% air until cells had become 90% confluent. Depending on the experimental deign, preadipocytes could be used at this point for experiment or cells would be induced to differentiate into mature adipocytes.

2.3.5 Human preadipocytes differentiation
The growth medium was discarded from all wells, and preadipocytes were washed two times with sterile PBS that had been pre-warmed to 37 °C in 1000-HB a hybridization incubator (Cambridge, UK). PBS was then removed, and the induction medium was added to each well. This time point was defined as day 0 (zero). The plates were then incubated at 37 °C and 5% CO2/95% air for four days. Differentiation of the cells was checked using a light microscope under phase contrast, where small lipid droplets could be recognized within the cells. The Differentiation medium was then aspirated, and the feeding medium was replaced every three days with fresh feeding medium until day 12 post differentiation.

2.4 HumanTHP-1 monocytes culture
2.4.1 THP-1 cell culture medium and equipment
1x Roswell Park Memorial Institute 1640 medium (RPMI-1640)
10% Fetal calf serum
100 nM phorbol 12-myristate 13-acetate (PMA)
100 U/ml penicillin and 100 µg/ ml streptomycin mix
Pipette and sterile pipette tips
75 cm$^2$ culture flasks

2.4.2 Human THP-1 monocytes
The human THP-1 acute monocyctic leukemia cell line was purchased from Health Protection Agency Culture Collections (Porton Down, Salisbury UK). Liquid nitrogen storage was used to store all vials of cells until the day of use.

2.4.3 Human THP-1 monocytes generation and maintenance
To start a cell subculture experiment, a frozen vial of human THP-1 monocytes (1x10$^6$) was taken from the liquid nitrogen store and immediately defrosted in a water
bath at 37 °C or by holding in hand until completely thawed. One ml of pre-warmed THP-1 culture medium was added to the vial to facilitate defrosting and the cells were suspended gently being pipetted up and down a few times. The thawed aliquot was then poured into a 75 cm² flask containing 20 ml of THP-1 culture medium and then placed in an incubator at 37 °C in a humidified atmosphere of 5% CO₂/ 95% air for 24 hr with the flask’s lid released a half turn. After 24 hr of incubation, cells were poured into a 50 ml centrifuge tube and spun down at 200 x g for 5 minutes, and then the supernatant was discarded aseptically. The pellet of cells was mixed with 5 ml of fresh cell culture medium. Once again, the suspension of cells was decanted into 75 cm² flask, and 20 ml of THP-1 culture medium was added to the flask. Cells could be passaged every 3 to 4 days when cell density reached 10⁶/ml. For culturing, THP-1 cells (1X10⁶) were cultured in 24-well plates in Roswell Park Memorial Institute (RPMI-1640) medium with 10% FCS, 100 U/ml penicillin and 100µg/ml streptomycin at 37 °C.

2.4.4 Human THP-1 monocytes differentiation and macrophage-conditioned medium preparation
To prepare macrophage-conditioned (MC) medium, THP-1 monocytes were differentiated by the addition of PMA 100 nM (Sigma) for 48 hr. The culture medium was replaced with serum-free RPMI-1640 medium (without PMA) for 24 h. the MC medium was then collected and stored at -80 °C until needed.

2.4.5 Human Peripheral blood mononuclear cells (PBMCs) culture

2.4.5.1 PBMCs culture medium and equipment
1x Roswell Park Memorial Institute 1640 medium (No phenol red)
10% Fetal bovine serum
2 mM L-glutamine
20 Mm 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)
100 nM phorbol 12-myristate 13-acetate (PMA)
1 µg/ml Lipopolysaccharides (LPS; Sigma, UK)
1 mM Adenosine triphosphate (ATP; Sigma, UK)
Pipette and sterile pipette tips
Cell culture flasks (Corning, Amsterdam, Holland)
Cell culture plates (Corning, Amsterdam, Holland)

2.4.5.2 Generation of human primary macrophage-conditioned medium
Peripheral blood-derived macrophages were obtained from six healthy male and female donors BMI: (20-28). Blood sampling was approved by local and national ethics committees (UK National Research Ethics Service). PBMCs were differentiated into macrophages and macrophage-conditioned medium was collected, which was kindly provided by Dr Christopher Ford (Liverpool University, UK). All vials of medium were stored in -80 ºC until the day of use.

2.5 Cell treatment
According to the specific experimental design, the cells were treated with different agents and various concentrations as described in more details in each result chapter.

2.6 Cell and medium collection
All precautions needed were taken to avoid any contamination of the candidate cells and collected medium. Firstly, all glassware was well washed and autoclaved and all plastic ware used was as a brand new and sterile. Secondly, the UV-C lamp in the laminar flow hood was turned on to sterilise the surfaces for at least 15 minutes. Thirdly, the cell culture hood was wiped by 1% Vircon solution, and wiped by 70% ethanol as well, to make sure a clean environment for cell and medium collection was obtained. Fourthly, collection tubes were completely sprayed with 70% ethanol and opened inside a sterile cell culture hood. In aseptic way, cell lysate or extract and medium were transferred into RNase-free 2 ml centrifuge tubes or sterile 1.5 ml Eppendorf tubes (Eppendorf, UK).

2.6.1 Cell culture medium collection
2.6.1.1 The practical basis
Cell culture medium was collected prior to cell collection for measurement of secreted proteins as conditioned medium is a good source of secreted proteins by the cells. ELISA was used to measure secreted protein concentrations after a given treatment to study changes in the secretion pattern.
2.6.1.2 Medium collection method

**Equipment**

- Pipette and sterile pipette tips
- RNase-free 2ml centrifuge tubes
- Sterile 1.5 ml Eppendorf tubes
- Sterile centrifuge tubes

The steps were carried out as follows: once the cells were ready to study, Either the preadipocytes or adipocytes conditioned medium was collected into 1.5 ml Eppendorf or RNase-free 2 ml centrifuge tubes by taking just 1 ml from each well. The collected medium was stored at -80 °C until further use.

2.6.2 Cell collection for Total RNA extraction

2.6.2.1 The practical basis

A strong denaturing chemical compound used to denature protein is guanidinium thiocyanate, which is able to efficiently breakdown cells, denature protein and inactivate RNase. Guanidinium thiocyanate is a key reagent in the TRIzol® solution (Invitrogen, Paisley, UK). To extract total RNA from preadipocytes or adipocytes, guanidinium thiocyanate-phenol-chloroform extraction was used based on the method described by Chomczynski and his colleague (Chomczynski and Sacchi, 2006). Chloroform, as detergent, binds to protein and lipids of the cell membrane dissolving them as non-aqueous compounds. Thus, it disrupts cell membrane by breaking bonds that hold the membrane together. RNA and DNA are aqueous compounds. As for phenol, it is used for separating the RNA aqueous supernatant from other phases that contain DNA and non-aqueous compounds.

2.6.2.2 Reagent and equipment

- Cell culture in plates
- TRIzol® reagent (Contains phenol and guanidine isothiocyanate)
- RNase-free 2ml centrifuge tubes
- Pipette and sterile pipette tips
- Rocking platform
After the cell medium were collected or discarded, 500 µl of TRIzol® solution was added into each well of the plate which was then shaken by using a rocking platform for 5 minutes to detach cells. After cells have detached, the cells suspension was pipetted up and down to ensure that all cells were mechanically dislodged. Then from each well the mixture was collected into RNase tubes and stored at -80 °C until further investigation. The collected cells were used for RNA extraction (See section 2.9).

2.6.3 Collection of cell lysate

2.6.3.1 The practical basis

The cell collection was carried out for measuring biological molecules (intracellular proteins) through breakdown of candidate cells to allow isolation and extraction of the proteins which could be used for investigation. Lysis buffer was used to disrupt the cell membrane together with mechanical disruption by using pipetting for enhanced breakage. Most of the mammalian cells are generally fragile and can be disrupted by lysis buffer that contains sodium dodecyl sulphate detergent (SDS). SDS is considered as the main component of cell lysis buffer, where its function, in addition to lysing action, is to denature proteins into their primary structure. Protein extracts need to be collected as quickly as possible and stored at -80 °C until use to reduce degradation. Therefore, it is highly recommended to add protease and phosphatase inhibitor cocktails to lysis buffer. Protease inhibitor acts to inhibit endogenous proteases which are released during cell disruption and degrade other proteins (Deutscher, 1990). The phosphatase inhibitor acts to inhibit the action of exogenous and endogenous phosphatases (Deutscher, 1990). These phosphatases may remove phosphate group from proteins which are phosphorylated by protein kinases, most likely at tyrosine or serine residues, during cell signal transduction episodes that are involved in several cellular processes such as cell growth, proliferation and cell apoptosis (Alberts et al., 2015). Moreover, there are many methods for lysing cells to extract total protein carried out depending on the protein of interest.
2.6.3.2 Reagent and equipment

Cell lysis buffer
50 mM Tris-HCl, pH 6.7
5% glycerol
2% SDS
Protease inhibitor cocktail (1:100)
Phosphatase inhibitor cocktail (1:100)
Cold autoclaved PBS
Pipette and sterile pipette tips
RNase-free centrifuge tubes

2.6.3.3 Method
After the cell medium was aspirated, the monolayer of cells was washed two times with 1 ml of cold autoclaved PBS per well. 300 µl or 150 µl of lysis buffer was added into each well of a 6 well plate or 12 well plate respectively. Tapping the plate on the bench was needed to remove the attached cells from the bottom of the wells. The cells/lysis mixture was pipetted up and down to ensure that the maximum number of cells were collected and then transferred into RNase-free 2 ml centrifuge tubes and stored at -80 ºC to prevent protein degradation until further investigation.

2.7 Analysis of lipid accumulation in adipocytes

2.7.1 Oil Red O Staining assay

2.7.1.1 Reagent and equipment
Autoclaved PBS
Oil Red O stain
10% formaldehyde
Isopropanol
96-wells microplate
Shaker platform
Inverted microscope/D50 camera (Nikon,UK)
Benchmark™ Plus Microplate Spectrophotometer (Bio-Rad, UK)
Lysis buffer
2.7.1.2 Method
Post differentiation, mature adipocytes have neutral lipid droplets that were stained with Oil Red O as described previously (Ramirez-Zacarias et al., 1992) with some modifications. The cell culture medium was discarded from wells and monolayer cells were washed two times with PBS. Cells in each well of a 6 well plate were fixed in 0.5 ml 10% formaldehyde (BDH, UK) in PBS for 1 hr at room temperature. Freshly prepared Oil Red O solution (six ml 0.5% Oil Red O in isopropanol with 4 ml water mixed), then 375µl of Oil Red O solution was added into each wells of plates for 1 hour at room temperature. Cells subsequently were washed with PBS to remove excess Oil Red O stain, and cells were observed and images were taken by using a phase contrast microscope (Figure 2.1). The dye retained in cells was removed with 300 µl of isopropanol after that 200 µl of isopropanol from each well of plate was transferred into a new 96 well plate for measuring lipid content through the absorbance wave at 510 nm by a Benchmark™ Plus Microplate Spectrophotometer (Bio-rad, UK). Lipid levels were then correlated to total protein amount by using BCA protein assay (As described in section 2.13.2).

Figure 2-1: Oil Red O Staining to determine neutral lipid content in mature adipocytes

![a](image1) ![b](image2)

The figure shows the phase contrast photos of human preadipocytes post differentiation. (a) Differentiated adipocytes without staining as control; (b) Differentiated adipocytes stained with Oil Red O.
2.8 Determination of glycerol release in cell culture medium

Glycerol, release during lipolysis, was determined in adipocyte culture medium using a colorimetric method as described previously (Mracek et al., 2011).

2.8.1 Reagent and equipment

Glycerol standard solution (0.26 mg/ml) (Sigma, UK)
Reconstitution free glycerol reagent (Sigma, UK)
Double distilled water
96-wells microplate
Shaker platform
Benchmark™ Plus Microplate Spectrophotometer (Bio-rad, UK)

2.8.1.1 Method

Briefly, a 1:1 of reconstituted free glycerol and double distilled water was prepared and mixed well. Serial dilutions of glycerol standard solution were prepared as follows: 1 (blank), 1:5, 1:10, 1:20, 1:40, 1:80, 1:160. Medium (25 µl) or serial dilutions of glycerol standard solution were incubated with a free glycerol reagent (200 µl, Sigma) in a 96-well microplate at room temperature on a shaker platform to mix thoroughly for 15 min. The absorbance of the samples and standard were then measured using Benchmark™ Plus Microplate Spectrophotometer (Bio-rad, UK) at a wavelength of 540 nm. The concentrations of glycerol in samples were calculated by a glycerol standard curve constructed from the glycerol standard or alternatively by using the following formula:

\[
\frac{(A_{sample} - A_{blank})}{(A_{standard} - A_{blank})} \times \text{Concentration of standard}
\]

2.9 Total RNA isolation

2.9.1 Reagents and equipment

RNase AWAY®
TRIzol®/Cells mixture
Cholorform
Isoprpanol
75% pure ethanol
Refrigeration centrifuge
Sterile pipette tips and pipette
Ultra-pure water– molecular biology reagent, RNase and DNase-free (Sigma)

2.9.2 Method
To prevent any RNase and DNase contamination, all lab ware including glass, plastic ware, gloves and all surfaces around the RNA isolation work must be sprayed with RNase AWAY® as precaution. Cells were collected in TRIzol® as a mixture as mentioned in section 2.9.1. The TRIzol®/Cells mixture was placed in the box of ice and thawed at room temperature and homogenised by passing them through a 23 gauge needle three times, separate needles were used for each sample. 100 µl of chlorform reagent was added into each tube of the Trizol®/cells mixture and the tubes were vigorously shaken for 15 to 20 second after which the mixture was left to stand for 10 mins at room temperature. All mixture tubes were then centrifuged at 14000 rpm for 15 mins at 4 ºC. Three phases were created and seen in the tubes; a lower red colour containing protein, a white interphase containing DNA and colourless upper aqueous containing RNA. The colourless upper aqueous RNA layer was moved into a new RNase-free tube with extra care taken when pipetting to avoid any contamination from any of the other two layers. 250 µl of isopropanol was added to each aqueous tube which was then vortexed and left to stand at room temperature for 10 minutes. All tubes were then centrifuged once more at 4 ºC for 10 mins at 14000 rpm. Following this, the supernatant was aspirated away, making sure that pellets were not dislodged and still remained in the tubes. 100 µl of 75% ethanol was added into each tube, which were gently shaken up side down, after which tubes were centrifuged at 4 ºC for 10 minutes at 14000 rpm again. The supernatant was discarded by using a pipette (taking care not remove the RNA pellet ) and the pellets were allowed to air dry for 5 minutes before being dissolved in 12 µl of RNase-free water (Sigma); the RNA samples were then be stored at -80 ºC until required for RNA quantification.

2.9.3 RNA quantification
To measure RNA concentration and purity, RNA samples were first thawed at room temperature on ice and then the RNA samples were diluted 1:70 in RNase-free water. A spectrophotometer (Biophotometer Eppendorf, Cambridge, UK) was set to read
diluted RNA samples. 70 µl of RNase-free water was added into a 10 mm cuvette tube and then measured as a blank. The sample was prepared by adding 1 µl of RNA to 69 µl of the RNase-free water in a cuvette tube. The absorbance reading was taken at 260 nm (A_{260}) and 280 nm (A_{280}) and then the RNA concentration (µg/µl) was automatically calculated by the spectrophotometer. At 260 nm, the nucleic acids can reach to their maximum absorbance, and RNA has an extinct coefficient of approximately 44.19 at that wavelength. The concentration of RNA can be calculated by the spectrophotometer using the Beer-Lambert law:

\[
\text{Concentration of RNA µg/µl} = A_{260} \times 44 \times \text{Dilution factor}/1000
\]

This quantified the RNA purity to discover if there were any protein contaminations from the extraction. Therefore, if the value 260:280 ratio = 2 that would indicate a pure sample, where a value < 1.6 would show poor RNA quality with contamination which is probably from protein. RNA extracts were diluted to a concentration of 0.1 µg/µl by using this formula:

\[
\text{Concentration of RNA extract x 1 µl} = 0.1 \mu g/\mu l \times \text{total volume of dilution}
\]

2.10 Reverse-transcription polymerase chain reaction

2.10.1 The practical basis

This technique was used for generating single-strand DNA complementary to the mRNA sequences in total RNA samples. An RNA-dependent DNA polymerase enzyme (reverse transcriptase enzyme, RT) is not normally found in prokaryotic and eukaryotic cells, but is found in few types of viruses including retrovirus. In this reaction, reverse transcriptase enzyme derived from Monloney Murine Leukaemia Virus (MMLV) was used (Pelt-Verkuil et al., 2008). In addition, the iScript first strand cDNA Synthesis Kit (Bio-Rad, Hertfordshire, UK), including oligo (dT) and random hexamer primers, was used in the reaction mix. The poly (A) tail at the 3' end of the mRNA acts as a starting point for the reverse transcriptase to which a short complementary synthetic oligonucleotide (oligo dT primer) is hybridised to form a poly (dT/dA) hybrid. Random hexamers primers are synthesized completely randomly to provide a various range of cDNA sequences. Random hexamers primers were combined to allow priming all over the length of mRNA for uniform
representation of all RNA sequences ensures transcription of 5’ ends of long mRNA and allows for reverse transcription of RNAs without poly (A) tails (Figure 2.2). The cDNA synthesis on the mRNA template needs essential conditions and resources which are available and maintained in the kit buffer including all four deoxynucleotide triphosphates (dNTPs), MgCl₂ supplies Mg²⁺ as cofactors and a neutral pH.

2.10.2 Method

Reagent and equipment

5x cDNA synthesis kit buffer
iScript reverse transcriptase (Bio-Rad, UK)
0.1 µg/µl RNA sample
Thermal cycler (Eppendorf, UK)

After the total RNA was quantified, reverse transcription (RT) was performed to make DNA strands that are complementary to the mRNA. These strands are single stranded DNA (ssDNA) referred to as complementary DNA (cDNA).

Based on the manufacture’s protocol (Bio Rad), the total RNA was diluted 1:10 for use in RT-PCR. The RT procedure was performed in 0.5 ml sterile RNase-free tubes, with the total reaction volume of 10 µl. The Master mix was prepared in a RNase free tube using 2µl of 5XiScripit reaction (Bio-Rad, UK) mix, 0.5 µl of iScript reverse transcriptase, 2.5 µl of nuclease free water and the volume was made up to 10 µl by adding a 1:10 RNA template volume to give a concentration of 0.5 µg of RNA.

A master mix of 5xiScripit reaction mix and iScript reverse transcriptase was made up to carry out the required amount of reaction, plus two more reactions volume were needed in case any error happened in the pipetting. 5 µl of the master mix was then added to the 1.5 ml tubes containing water and RNA template, mixed by vortexing, and then centrifuged to spin the sample down. The RT reaction was carried out in a thermal cycler (PCR express). The tubes were incubated at 25 ºC for 5 minutes, followed by 42 ºC for 30 minutes, 85 ºC for 5 minutes and finally held at 4 ºC for 10 minutes to cool the tubes. Once the reaction was completed, cDNA was diluted 1:4.
by adding 30 µl of ultra-pure water into each PCR tube for use in quantitative PCR (qPCR) and standard PCR. The samples were stored at -20 ºC to prevent degradation until required for use.

**Figure 2-2: First strand cDNA synthesis**

The figure shows the traditional first strand cDNA synthesis by using oligo-(dT) primer and reverse transcriptase enzyme which hybrids to the mRNA sequences.

### 2.11 Real-time polymerase chain reaction (RT-qPCR)

#### 2.11.1 The practical basis

RT-qPCR is a reproducible and sensitive method for quantifying genes of interest (DNA and RNA target) in biological samples. It is considered to be a fundamental progress of the PCR technology that allows reliable detection and measurement of mRNA levels generated during each cycle of PCR process (Fraga et al., 2008). Its procedure follows the general principles of PCR and quantifies a targeted DNA molecule during the exponential phase based on amplification of DNA. In the exponential phase, the amount of PCR product approximately doubles in each cycle.
as the reaction proceeds until the reaction slows and enters a non-exponential phase (cycles 28–40) (Figure 2.3). Therefore, the exponential phase is considered to be a very efficiently phase that occurs early in the reaction process and gives reliable quantification of the starting DNA by correlating the measurement to the amount of specific starting DNA. Primarily, intercalating dyes such as SYBRGreen® was used by PCR product detection. After that qPCR systems were improved by the introduction of fluorogenic probes such as Taqman®. Taqman® system was used in this project (Eurogentec, UK).

2.11.2 Taqman® system principles
In this project, the Taqman® system was used to measure specific PCR product accumulation by using a pair of primers (sense and anti-sense) and a dual-labelled fluorogenic oligonucleotide probe (Fraga et al., 2008). The probe is a short oligonucleotide sequence which is labelled with two different fluorescent dyes. The reporter dye (fluorophore usually FAM) is covalently linked to the 5’ terminus end and a quenching dye (usually TAMRA) at the 3’ terminus end. A probe homologous to primers that is designed to anneal within cDNA regions by binding to specific area. The probe will be excited by energy emitted from the PCR cycler’s light source, and when the reporter and quencher are in close proximity, energy is transferred between the two fluorophores and emission from the reporter is quenched by the quencher by a process called FRET (Forster or fluorescence resonance energy transfer). As the reaction proceeds, the primes are extended toward the probe and synthesis the nascent strand by Taq polymerase that cleaves the probe, releasing the reporter from the oligonucleotide quencher. The activity of FRET is consequently reduced and the reporter emits fluorescence (518 nm or FAM) which increases during each cycle proportional to the rate of probe cleavage. As the reaction proceeds, the increased numbers of PCR products can be observed when the amount of fluorescence (ΔR) is increased. The Mx3005P qPCR software measures the fluorescent emission of reporter by using a reference dye (usually ROX), and a software algorithm is used to calculate and normalise the increase of reporter signal intensity over the extension phase (ΔR_N) to the reference dye (ROX). Normalization is essential to correct for difference in reaction mix volumes between samples. The amplification plot is examined at an early point in the log phase of product accumulation. This is done when fluorescence from a sample crosses the threshold,
and permits the fluorescence to be plotted on a graph against the cycle number (Figure 2.3).

2.11.3 Preparation of 96 well plates for real-time PCR

2.11.3.1 Taqman assay reagent and equipment

qPCR core kit consisting of:
10 x reaction buffer
50 mM magnesium chloride
5 mM dNTP mix
Forward and reverse primers
Taqman probe
RNase- free water
Strategne Mx3005P instrument (Thermal cycler)

2.11.3.2 Method

A master mix for each gene of interest without cDNA was made up to give a final volume of 11.5 µl per well, with a 2-well excess to allow for pipetting errors. 1 µl of the synthesised cDNA (equivalent to 12.5 ng of cDNA) was added into each well of a 96 well plate. Real-time amplification was performed in a final volume of 12.5 µl made up of cDNA, optimised concentration of primers, Taqman probe (FAM-TAMRA) and a master mix made from a qPCR core kit (Euroentech, Seraing, Belgium). Real-time PCR amplification was performed in duplicates using a Strategene Mx3005P instrument. A non-template control was incubated in each plate. The plate was spun at 500 x g for 1 minute to gather the contents to the bottom of the wells. The plate was then inserted into the heat block of the Mx3005P qPCR instrument with an optical pad placed on top and aligned with the well position. PCR cycling conditions were as follows: 95 ºC denaturation step for 10 minutes followed by 40 cycles (95 ºC denaturation step for 15 seconds and annealing-cum-extension step at 60 ºC) for 1 min. Data was collected automatically in real-time by MxPro software and analysed at the end of the run.
Figure 2-3: Amplification plot; Baseline-subtracted fluorescence against number of PCR cycles

The figure shows an amplification plot of cycle numbers on the X axis against fluorescence units (dRn) on the Y axis for each reaction. The blue line represents an amplification plot, while the exponential phase highlighted by the green arrow and the non-exponential phase is highlighted by the yellow arrow. The Ct (threshold cycle) value defined as the number of cycles required for the fluorescent signal to cross the threshold level.
2.11.3.3 Taqman assay reagents
Working solutions of primers and probes were reconstituted to produce a 100 μM stock.

Each 12.5 µl reaction contained the following:

<table>
<thead>
<tr>
<th>Component</th>
<th>Amount (µl) per well</th>
</tr>
</thead>
<tbody>
<tr>
<td>10x buffer</td>
<td>1.25</td>
</tr>
<tr>
<td>MgCl₂</td>
<td>1.25</td>
</tr>
<tr>
<td>dNTP</td>
<td>0.5</td>
</tr>
<tr>
<td>Forward Primer</td>
<td>0.038</td>
</tr>
<tr>
<td>Reverse Primer</td>
<td>0.038</td>
</tr>
<tr>
<td>Probe</td>
<td>0.063</td>
</tr>
<tr>
<td>Hot Goldstar Enzyme</td>
<td>0.0625</td>
</tr>
<tr>
<td>Ultra-pure water</td>
<td>8.3</td>
</tr>
<tr>
<td>Total master mix volume per well</td>
<td>11.5</td>
</tr>
</tbody>
</table>

2.11.3.4 Analysis of real-time PCR data
When data had been collected, the results were analysed by the Mx3005P software. Amplification plots were shown on log scale, and the threshold was manually adjusted for each plate to remove any background noise. The software automatically recalculated the Ct value for each well (Ct defined as the cycle number at which the fluorescence emitted from the well crossed the threshold). All samples were normalised to the value of the housekeeping gene (β-actin), the difference of Ct values between the control and treated samples were calculated and the relative fold changes obtained between the two groups. The formula used for calculation is as previously reported (Livak and Schmittgen, 2001):

\[
\Delta Ct = Ct (Target\ gene) - Ct (Reference\ gene)
\]

\[
\Delta \Delta Ct = \text{Mean}\ \Delta Ct (Treated\ sample) - \text{Mean}\ \Delta Ct (Control\ samples)
\]

\[
\text{Fold\ change} = 2^{-\Delta \Delta Ct}
\]
2.11.3.5 Primer and probe sequences

The sequences of primers and probes for human β-actin, adiponectin, Leptin, IL-6, IL-8, MCP-1, C/EBPα, aP2 and PPARγ are described in Table (2.1) as previously reported (Bing et al., 2006, Wang et al., 2005a, Bao et al., 2005). The primers and probes for RANTES (CCL5) and IL-8 were obtained commercially (Applied Biosystems, Applera; Hs99999048-m1 and Hs00174103-m1).

**Table 2-1: Primer sequence of human target genes**

<table>
<thead>
<tr>
<th>Transcript</th>
<th>Primer sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Human β-actin</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GGATGCAAGAGGAGAGTGACTG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CGATCCACCGGATCTTGG-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-CCCTGGCAACCCAGCAATG-TAMRA-3'</td>
</tr>
<tr>
<td><strong>Human Adiponectin</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CCAAAGAGGAGAGGAAGGCT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCCAGCAATGAGATGCAA-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-TTCCAGATGCCCCAGCAAC-TAMRA-3'</td>
</tr>
<tr>
<td><strong>Human Leptin</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CCAAAACCTCTCATCAAGACAATT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-AGTCCAAACCCTGTCTTCTG-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-TGACATTTTCACAGCGATCAGTCTCCT-3'</td>
</tr>
<tr>
<td><strong>Human IL-6</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GGTACATCCTCGACGGCATCT-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GTGCCTCTTTGCTGCTTTCAC-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAMTGTTACTCTTGTACATGTCTCCTTT CTCAGGGCTTAMRA-3'</td>
</tr>
<tr>
<td><strong>Human MCP-1</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-CATAGCAGCCACCTCTCATCC-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCTGCACCTGATCTTCTTATGG-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-FAM-ACCTGGACCTCTGGCACCCGATGTTAMRA-3'</td>
</tr>
<tr>
<td><strong>C/EBPα</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-AGAGCCGAGATAAAGCCAAACA-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-CGGTCATTGTCACTGGTCAACT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-AACGTTGGAGACCCAGGATGTGCT-3'</td>
</tr>
<tr>
<td><strong>PPARγ</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-AGTGGAGACCAGCGAG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-GCAGCAGTTGCTCTGGATGT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-TTGCTGAACGTGTAAGCCCATCGA-3'</td>
</tr>
<tr>
<td><strong>aP2</strong></td>
<td></td>
</tr>
<tr>
<td>Forward</td>
<td>5'-GAAGACAGCAACCACACCTAG-3'</td>
</tr>
<tr>
<td>Reverse</td>
<td>5'-TCGTGGAAGCTGCTTTCCTCT-3'</td>
</tr>
<tr>
<td>Probe</td>
<td>5'-ACGCATTCCACCCAGGTTATCGTCTCCT-3'</td>
</tr>
</tbody>
</table>
2.12 Enzyme-linked immunosorbent assay (ELISA)

2.12.1 The practical basis

Enzyme-linked immunosorbent assay (ELISA) is a biochemical assay used to detect the presence of proteins that are released from cells and measure their concentrations in the biological solution (usually in liquid or wet sample) (Engvall and Perlmann, 1971). ELISA is done by adding a specific antibody (Capture and detection) to the sample solution to attach to the antigen which can then be detected.

In this research, proteins released from primary human preadipocytes and adipocytes into the cell culture medium were measured after treating cells with many different treatments. The ELISA kits used in this research were all commercially available (R&D system), and were suited for the measurement of natural proteins produced by human cells. As mentioned in the ELISA procedure (Section 2.12.2.4), a microtiter plate was used and coated overnight with capture antibody (monoclonal) against the target protein. After the coating of the plate, unbound antibodies were washed and were blocked using reagent diluent or blocking buffer to block non-specific binding sites for 1 hr before adding cell culture medium into the plate for 2 hr. The wells were aspirated and then washed with washing buffer three times and incubated with the detection antibody (anti-human target protein antibody) for 2 hr to bind with the antigens. Washing steps were repeated before adding streptavidin–horseradish peroxide conjugate which binds to the biotin on the detection antibody for 20 minutes. Once again washing steps were repeated and then substrate solution consisting of equal volumes of hydrogen peroxide and tetramethylbenzidine (TMB) (usually called A and B substrate) was added for 20 minutes. The horseradish peroxide (HRP) enzyme oxidises TMB and providing a visible blue colour by catalyzing the electron transfer from TMB to the peroxide. Stop solution was added to stop colour development, which resulted in a yellow solution to appear. The absorbance is afterward measured at 450 nm with a wavelength correction set to 570 nm. Note, all wells were incubated at room temperature. IL-1β, IL-8, IL-6, MCP-1, RANTES (CCL5) and adiponectin, in cell culture medium were measured using DuoSet ELISA development kits purchased from R&D (Abingdon, UK) according to their protocols.
2.12.2 Method

2.12.2.1 Measurement of MCP-1, IL-6, IL-1β, RANTES (CCL5) and adiponectin

Reagents and equipment
Phosphate-buffered saline (PBS)
Reagent diluent (1% BSA in filtered PBS) as blocking buffer
Capture monoclonal antibody (Diluted to working concentration with PBS)
Detection monoclonal antibody (Diluted to working concentration with reagent diluent)
Recombinant human standard (Diluted in various 2-fold serial dilution for 7 points standard curve, excluding blank)
Wash buffer (0.05% Tween-20 in PBS)
Streptavidin-HRP
Substrate solution mixture (1:1 in volume)
Stop solution (2MSulphric acid)
96-well MaxiSorp® flat-bottom microplate
Benchmark™ Plus Microplate Spectrophotometer
Multi-channel pipette
Shaker plate form (Fisher Scientific, UK)

2.12.2.2 Measurement of IL-8

Reagents and equipment
Phosphate-buffered saline (PBS)
Tris-buffered Saline (20 mM Trizma base and 150 mM NaCl)
Blocking buffer (1% BSA in PBS with 0.05% sodium azide (NaN₃))
Reagent diluents (0.1% BSA and 0.05% Tween-20 in PBS in Tris-buffered Saline)
Capture monoclonal antibody (Diluted to working concentration with PBS)
Detection monoclonal antibody (Diluted to working concentration with reagent diluent)
Recombinant human standard (Diluted in various 2-fold serial dilution for 7 points standard curve, excluding blank)
Wash buffer (0.05% Tween-20 in PBS)
Streptavidin-HRP
Substrate solution mixture (1:1 in volume)
Stop solution (2MSulphric acid)
96-well MaxiSorp® flat-bottom microplate
Benchmark™ Plus Microplate Spectrophotometer
Multi-channel pipette

2.12.2.3 Plate preparation
1. The capture antibody was diluted with PBS to make a working solution (Table 2.2). A 96-well MaxiSorp® flat–bottom microplate was immediately coated with 100 µl of diluted antibody per well, sealed and incubated overnight.
2. The following day, each well was aspirated and washed 3 times with washing buffer by using a multi-channel pipette. To avoid excess wash buffer in the wells, plates were turned upside down and gently tapped against a clean paper towel.
3. The plate was blocked with 300 µl of reagent diluent for one hr at room temperature. Again, the aspiration and washing as mentioned in step 2 was repeated. The plate was then ready to use.

2.12.2.4 Assay procedure
1. 100 µl of sample or standard were added into wells after they were diluted in Reagent Diluent. The wells were covered with an adhesive film and left to incubate at room temperature for 2 hr.
2. Repeat the aspiration and wash as mentioned above, and 100 µl of detection antibody as added into wells after being diluted in reagent diluent. The wells were incubated for 2 hr and with a new adhesive film to cover them.
3. Repeat the aspiration and wash, after which 100 µl working solution of Streptavidin-HRP was added to each well and the plate was incubated for 20 minutes at room temperature.
4. The wells were aspirated and washed. 100 µl of substrate solution was then added to each well and incubated for at least 20 minutes in the dark until a blue colour developed. 50 µl of Stop solution was added to each well to stop colour development and the plate was gently tapped to ensure thorough mixing.
5. The optical density was determined using a microplate spectrophotometer at 450 nm with a reference wavelength at 570 nm to correct any optical imperfections if
found in the plate. The concentrations of proteins were calculated using the standard curve.

Samples treated with or without MC medium and several treatments according to the experimental design were diluted and some samples were used neat based on the pilot studies. The optical density of the samples were obtained using a Benchmark™ Plus microplate spectrophotometer (Bio-Rad). The Microplate Manger v5.2 software was used to collect and analyse the data. A standard curve was made and plotted to achieve a best-fit line and correlation coefficient of > 0.96 by applying the equation of the best-fit curve or line to calculate the concentration of protein.

Table 2-2: Antibodies and standard concentrations used for ELISAs

<table>
<thead>
<tr>
<th>Protein measured</th>
<th>Captured antibody working solution concentration</th>
<th>Detection antibody working solution concentration</th>
<th>Concentration of S1 (first point)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Human adiponectin</td>
<td>2 µg/ml</td>
<td>2 µg/ml</td>
<td>4000 pg/ml</td>
</tr>
<tr>
<td>Human IL-6</td>
<td>2 µg/ml</td>
<td>50 ng/ml</td>
<td>600 pg/ml</td>
</tr>
<tr>
<td>Human MCP</td>
<td>1 µg/ml</td>
<td>100 ng/ml</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>Human IL-8</td>
<td>4 mg/ml</td>
<td>4 mg/ml</td>
<td>2000 pg/ml</td>
</tr>
<tr>
<td>Human RANTES</td>
<td>1 mg/ml</td>
<td>20 ng/ml</td>
<td>1000 pg/ml</td>
</tr>
<tr>
<td>Human IL-1β</td>
<td>4 µg/ml</td>
<td>200 ng/ml</td>
<td>250 pg/ml</td>
</tr>
</tbody>
</table>
The figure shows briefly the procedure of the enzyme-linked immunosorbent assay (ELISA).

2.13 Western blotting

2.13.1 Western blotting principles

In 1979, the Western blotting method also known as protein immunoblot was introduced by Towbin and his colleagues (Towbin et al., 1979). It has become one of the most common techniques in the molecular biology field, which is used to separate and identify proteins’ expression within many different human cells including primary adipose cells. In this study, cell lysates were collected with lysis buffer that contained protease and phosphatase inhibitor cocktails (both from Sigma, UK). Tissue or cell lysate preparation is often carried out at cold temperatures to avoid protein denaturing and degradation as mentioned with more detail in section 2.6.3. After cell lysates were collected, the bicinchoninic acid (BCA) method was used to quantify protein concentrations. These values were used to calculate the volume of lysate with a protein concentration 30 µg/lane that will be loaded per lane.
of the acrylamide gel. Lysate was diluted 1:10 into a loading buffer. Loading buffer contains glycerol, SDS, β-mercaptoethanol and bromophenol blue dye. Glycerol helps samples sink easily into the gel’s wells. SDS offers the protein a negative charge and β-mercaptoethanol prevents the reformation of disulphide bonds. The bromophenol blue helps to visualise how far the protein separation through gel has progressed. The lysate was heated immediately after being diluted, to denature the higher protein structure; by denaturing the high structure it ensures that the negative charge of amino acids is not neutralized, enabling the protein to move during electrophorosis. Diluted lysates were then equally loaded into each well of sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) gel.

After the protein mixtures were separated, they were transferred to a nitrocellulose membrane. The transfer is done using an electric field oriented vertical to the surface of the gel, causing proteins to move out of the gel onto the membrane. The membrane was then probed using antibodies binding specifically to a protein of interest. To detect the protein of interest from total protein, an appropriate primary antibody was used. Firstly, the nitrocellulose membrane was blocked with bovine serum albumin solution (5% BSA) for 1 hr prior to addition of a primary antibody overnight at 4 °C. Secondly, a secondary antibody-HRP conjugate was then added to bind to the primary antibody for 1 hour. Finally, a luminol substrate was added to the secondary antibody which converted to a chemiluminescent substance due to the effect of HRP conjugate in the secondary antibody. The signal intensity was then detected with the camera of the ChemiDoc™ XRs+ molecular imager. Notably, Western blotting was used in this project to measure the relative expression of proteins and is considered as a semi-quantitative method.

2.13.2 Protein quantification by the Bicinchoninic acid (BCA) method

2.13.2.1 The principle of the BCA protein assay
This assay is used to measure total cellular proteins concentrations (from 0.2 to 1.0 mg/ml). It is completely reliant on the reduction process, where the reduction amount is proportional to the protein amount present in samples (Smith et al., 1985). By mixing bicinchoninic acid and copper with proteins this will provide Cu⁺-protein complex in alkaline environments. BCA forms a purple-coloured complex with Cu⁺
in alkaline conditions. This happens due to the reduction of the Cu$^{2+}$ to Cu$^+$. Consequently, the amount of Cu$^{2+}$ reduced is proportional to the amount of protein present in the solution. BCA assays are routinely performed at 37°C (Smith et al., 1985). It is always favourable to prepare the standard and the sample in the same buffer to reduce any interference effects.

2.13.2.2 Reagent and equipment

1 mg/ml bovine serum albumin (BSA-protein standard)
Bicinchoninic acid solution
Copper (II) sulphate solution
Double distilled water
96-well microplate
Orbital microplate shaker
Benchmark™ Plus Microplate Spectrophotometer

2.13.2.3 Method

Cell samples were diluted in lysis buffer that contained (25 mM Tris-HCl, pH 6.7, 10% Glycerol, 2% SDS) with freshly added protease inhibitor and phosphatase inhibitor cocktail to form cell lysates. A standard curve was plotted by using serial dilution of bovine serum albumin (BSA) with final concentrations of 0.2, 0.4, 0.6, 0.8, 1.0 mg/ml. Cell lysates were diluted 1:4 into wells by pipetting 7.5 µl of double distilled water to 2.5 µl of cell lysate. A 50:1 bicinchoninic acid to copper sulphate solution was made and 200 µl of this mixture was added to each well of diluted standard or samples by using a multichannel pipette. The plate was gently tapped to ensure thorough mixing, and then incubated for 30 minutes in a hybridization oven. The plate was read using a spectrophotometer at 570 nm. The concentrations of protein were determined using the standard curve generated (Figure.2.5) and multiplying it by a dilution factor of 4. Data was collected and schemed using the Microplate Manager V5.2 software. A standard curve was created relative to the obtained protein concentration of the samples that were determined. A standard curve was made and plotted to achieve a best-fit line and correlation coefficient of > 0.99 by applying the equation of the best-fit curve or line to calculate the concentration of protein, whereas the ideal correlation coefficient is equal 1. Samples
were then diluted 10 times with loading buffer and heated at 95 °C to denature proteins for 10 minutes. Samples can be stored at -80 °C or used directly in western blotting.

**Figure 2-5: Standard curve generation for Bicinchoninic acid (BCA) method**

The figure shows a standard curve for measuring protein concentration by using BSA as standard.

### 2.13.3 Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

#### 2.13.3.1 Reagent and equipment

PageRular protein marker (Fermentas)

**Loading buffer**

1 ml 0.5 M Tris-HCL, pH 6.8  
1.6 ml 10% SDS  
1 ml Glycerol  
0.4 ml β-mercaptoethanol  
0.01 g Bromophenol blue
**3x Gel Buffer**
18.16g Trizma base
10 ml 5M hydrochloric acid
0.75 ml 20% SDS
Double-distilled water (made up to 50 ml in total volume)

**Separating gel (10 ml) (10%)**
2 ml 48 % acrylamide/1.5 % bisacrylamide mixed with 36.4 ml double distilled water
3.3 ml 3x gel buffer
1 ml glycerol
50 µl ammonium persulphate
6 µl tetramethylethylenediamine (TEMED)

**Stacking gel (5 ml) (4%)**
0.4 ml 48% acrylamide/1.5% bisacrylamide mixed with 3.36 ml double distilled water
1.2 ml 3x gel buffer
40 µl ammonium persulphate
4 µl TEMD

**Cathode buffer (PH 8.25)**
12.11 g Trizma base (100 mM)
17.92 g Tricine (100 mM)
5 ml 20% SDS (0.1%)
Double distilled water (made up to 1000 ml of total volume)

**Anode buffer (PH 8.9)**
24.22 Trizma base
6 ml 1M hydrochloric acid
Double distilled water (made up to 2000 ml total volume)

**Gel electrophesis equipment**
Bio-Rad Mini-PROTEAN™ Tetra System
2.0 mm notched plates
2.0 mm plain glass plates
1.0 mm comb, 10 or 15 well
Casting base with silicone seals

2.13.3.2 Method
All gel electrophoresis components were cleaned with ethanol, dried and assembled according to the manufacturer’s instructions (Bio Rad). Once assembled and locked into position onto the casting base, distilled water was first poured between the glass plates to check for leak. The water was then poured and dried by using filter paper. Secondly, the separating gel was prepared and added between the glass plates by using a pipette and leaving a third of the space for the stacking gel. Then, 1x gel buffer or distilled water was added to the top of the gel to avoid gel from dehydrating while it sets. Once the gel had set, 1x gel buffer or distilled water was poured out. Thirdly, the stacking gel was prepared and pipetted into remaining third of space between glass plates. The combs were quickly inserted and the gel was stacked to set for 30 minutes. The combs were then removed from the gel’s top, and gels were removed from the casting base and placed into the gel tank. The tank was filled with Cathode buffer until the brim of the gel was completely submerged. 30 µg/lane of each sample was loaded slowly to each lane to avoid overflow and a PageRuler™ protein marker was added to the first lane. Anode buffer was then added to the tank to the labelled mark. The electrodes were run at 45v for 30 minutes to separate proteins, and increased to 85v until the blue dye migrated out of the gel. Finally, the electrophoresis system was disassembled and the gel was ready to be transferred.

2.13.4 Electroblotting

2.13.4.1 Reagents and equipment

Transfer buffer
3.03 g Tris-base (25 mM)
14.4 g Glycine (192 mM)
200 ml methanol (20%)
Double distilled water (made up to 1000 ml volume)

Electroblotting equipment
Compression cassettes
Fibre pads (foam pad)
Thick blotting paper (Filter paper)
Hybond ECL nitrocellulose membrane
Bio-Rad Mini-PROTEAN™ Tetra System

**Tris-base buffered saline (TBS) PH 7.2 -7.6**
2.42 g Tris-base
29. 24 g sodium chloride
0.1% Tween-20
Double distilled water (made up to 1000 ml volume)

In the electroblotting step, all components; nitrocellulose membrane and thick filter paper were soaked in distilled water for 5 minutes and then transfer buffer for 10 minutes. The blotting unit was assembled by placing components in the following order:
Cassettes were opened, from the bottom: foam pad- blotting paper- transfer membrane- gel- blotting paper-foam pad. It was necessary to ensure there were no bubbles between the components within cassettes. The cassettes were then placed into the adapter. The system was run at 100v for 80 minutes, with cold water or ice bag to keep the tank cool.

2.13.5 Immunological detection of proteins

2.13.5.1 Reagent and equipment
0.1% Ponceau S
Tris-base buffered saline (TBS) PH 7.2 -7.6
0.1% TTBS (TBS and 0.1% Tween-20)
Blocking solution (5% BSA in 0.1% TTBS)
Primary antibodies
Secondary antibodies
Luminol enhancer and stable peroxide buffer (Supersignal® West Pico ECL kit)
ChemiDoc™ XRS+ System (Bio-Rad)

2.13.5.2 Ponceau S staining
As soon as the transfer step was completed, the membrane was taken out and placed in a container and washed with 0.1% TTBS for 3 minutes. Detection procedure
began by submerging the membrane into Ponceau S stain for 5 minutes until the protein bands could be seen. The ponceau S was then discarded and the membrane was immediately washed with 0.1% TTBS to remove the red stain.

2.13.5.3 Signal detection

After the red stain was removed, the membrane was placed and incubated in a small container containing 5 ml of blocking solution at room temperature for 1 hour on a shaker. Primary antibody was added to the blocking solution to a ratio depending on the instructions supplied and optimization test. The container was incubated on a shaking platform overnight at 4 ºC. From experience, monoclonal antibodies were favoured than polyclonal ones, because they had less background on blot due to their high specificity. In the following day, the membrane was placed on a shaking platform and washed 4 times each for 10 minutes with fresh 0.1% TTBS. The membrane was submerged in blocking solution and the secondary antibody conjugated with HRP was added to a ratio depending on the instructions supplied and incubated for 1 hour at room temperature on a shaking platform. Repeat washing step again for 4 times in fresh 0.1% TTBS for 10 minutes each time. The membrane was finally washed in TBS (without Tween-20) to reduce the signal induced by Tween-20, before moving on to the development step. The enhanced chemiluminescence (ECL) solution was added in a 1:1 ratio, incubated for 5 minute and the membrane was taken out with forceps and placed in a transparent plastic folder; gently removing any bubbles that might be introduced into the folder. The membrane was scanned using the ChemiDoc™ XRS+ System and the accumulative signals were read over a period of 40 minutes. The antibody concentrations and conditions presented in Table 2.3 were used for each probe.

2.13.5.4 Determination of the optimal primary antibody dilution

Although the primary and secondary human antibodies concentrations were usually recommended by the manufacturers, it was also essential to define the optimal primary antibodies concentrations for detection which provides clean blots with low background. For this, a series of spot blots were carried out. Several dilutions of protein sample were applied directly onto the nitrocellulose membrane, then each blot of the protein sample being prepared for specific antibody dilution to be tested. The membranes were then blocked with 5% BSA and washed exactly as mentioned
above. A dilution of primary antibody was then prepared as described in Table 2.3 and incubated with the membrane overnight at 4°C.

2.13.5.5 Relative quantification of proteins
To determine the optical density of band images created by the molecular imager, densitometry was used for this purpose by the Image Lab software provided along with the ChemiDoc™ XRS+ System. The volume/density values were exported to Excel and the relative densities were obtained by the normalization of the optical density for GAPDH which was used as a housekeeping protein.

Table 2-3: Antibody dilution and condition used for western blots

<table>
<thead>
<tr>
<th>Primary Antibodies</th>
<th>Provider</th>
<th>Dilution</th>
<th>Molecular weight</th>
</tr>
</thead>
<tbody>
<tr>
<td>GAPDH</td>
<td>Abcam</td>
<td>1:2000</td>
<td>43 KDa</td>
</tr>
<tr>
<td>IRS-1</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>170 KDa</td>
</tr>
<tr>
<td>PI3K p85</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>60 KDa</td>
</tr>
<tr>
<td>GLUT-4</td>
<td>Millipore</td>
<td>1:2500</td>
<td>55 KDa</td>
</tr>
<tr>
<td>Total AKT</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>50 KDa</td>
</tr>
<tr>
<td>p-Akt (Ser473)</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>50 KDa</td>
</tr>
<tr>
<td>p-IR(Y-1185)</td>
<td>Abcam</td>
<td>1:1000</td>
<td>95 KDa</td>
</tr>
<tr>
<td>p-IRS-1(Ser-612)</td>
<td>Cell signalling</td>
<td>1:1000</td>
<td>170 KDa</td>
</tr>
</tbody>
</table>
2.14 Cell viability assessment

Cell viability was determined by using a colorimetric method for measuring cellular cytotoxicity from the release of lactate dehydrogenase (LDH) into cell culture medium. After treatment with THP-1 MC medium, IL-1β, IL-1RA, Caspas-1 inhibitor and IL-1β neutralizing antibody, the viability of preadipocytes and adipocytes were determined by using the LDH cytotoxicity assay (Thermo Scientific™ Pierce™, USA). Cell culture medium was collected for measuring LDH activity. LDH levels were measured using a spectrophotometer at 490 and 680 nm at room temperature. The LDH activity in the medium was determined once reducing of NADH was occurred as explained in the following reaction:

\[
\text{Lactate} + \text{NAD}^+ \rightarrow \text{Pyruvate} + \text{NADH}
\]

For calculation of cytotoxicity:

\[
\text{Cytotoxicity} \% = \frac{(\text{Experimental value} – \text{Low control})}{(\text{High control} – \text{Low control})} \times 100
\]

Low control was defined as the sample cell culture medium (not-treated cells) only while high control containing 1x LDH positive control solution. All samples were measured in triplicate.

2.15 Statistical analysis

All results are presented as mean ± SEM or mean ± SD and group size varied between experiments. Differences between two groups were analysed by Student’s unpaired t-test, while differences among more than two groups were assessed by one-way ANOVA coupled with Bonferroni’s t-test. Differences were considered as statistically significant when p< 0.05. Statistical tests were performed using Graphpad Prism version 5.03 (Graphpad software Inc., California, USA).
CHAPTER 3:
Characterization of insulin signalling molecules in human preadipocytes
3 Characterization of insulin signalling molecules in human preadipocytes

3.1 Introduction

Obesity, defined as an abnormal increase in WAT accumulation in the body, is a global health problem (Berry et al., 2014). Obesity is characterised by chronic low-grade inflammation, which contributes to the development of insulin resistance and type 2 diabetes (Lumeng and Saltiel, 2011a, Odegaard and Chawla, 2013). The development of obesity induces marked changes in WAT morphology and functions (Sun et al., 2011): WAT becomes inflamed with a significant increase in infiltration of macrophages and other immune cells (Lolmède et al., 2011, Weisberg et al., 2003). Macrophage-derived factors potently stimulate the production of pro-inflammatory cytokines and chemokines by human preadipocytes and adipocytes (Lacasa et al., 2007, Coppack, 2001).

Insulin as an anabolic hormone has crucial biological actions, mainly regulating glucose homeostasis by the stimulation of glucose uptake on insulin sensitive organs including adipose tissue, muscle and liver through the insulin signalling pathway (Gual and Tanti, 2005, Saltiel and Kahn, 2001). The insulin action is triggered by binding of insulin to the insulin receptor which induces auto-phosphorylation of intrinsic amino acids, including tyrosine and serine residues (Kido et al., 2001, Paz et al., 1999). The auto-phosphorylation leads to the activation of two major signalling pathways including the metabolic (PKB/Akt) and the mitogenic (MAPKs) cascades. In the metabolic cascade, insulin controls circulating glucose level through coordination of two processes: inhibition of glucose production by hepatic cells and stimulation of insulin target cells (muscle and adipose tissues) to uptake glucose (Cheatham and Kahn, 1995). To complete this metabolic function, phosphorylation of the IRS-1 and/or IRS-2 at tyrosine residues is considered as critical and required step to bind and activate PI3-kinase. The PI3-kinase has downstream effectors such as protein kinase B (PKB), also known as Akt. Akt mediates the metabolic actions of insulin through the phosphorylation of several substrates including several kinases and, leading to an increase in glucose uptake by inducing GLUT-4 translocation from intracellular vesicles to the plasma membrane (Taniguchi et al., 2006).
Interestingly, insulin sensitive organs have been shown to display insulin resistance particularly in the obese state (Hotamisligil, 2000). Although, adipose tissue only represents approximately 10% of glucose disposal from the whole body compared with muscle and liver which represent 60% and 30% of glucose uptake respectively (Smith, 2002), adipose tissue has been considered as an intrinsic inducer to initiate insulin resistance in muscle and hepatic tissue. This could be due to a decreased response to insulin in hypertrophied adipocytes, which may stimulate FFAs release into the blood circulation leading to systemic insulin resistance (Smith, 2002). This notion has been supported by the study of Krebs and his colleague Roden who found that insulin resistance is positively correlated with high circulating levels of FFAs which are released during the lipolysis process (Krebs and Roden, 2005).

Considering the importance of insulin action, firstly, it regulates cellular mechanisms in adipose cells by stimulating several operations, including glucose and FFAs uptake, increasing de novo fatty acid synthesis in adipocytes and inhibiting lipolysis (Saltiel and Kahn, 2001). Moreover, insulin modulates the development of adipose tissue by regulating the expression of a number of adipokines and adipogenic transcription factors including PPARγ and SREBP-1c (Vidal-Puig et al., 1997, Dif et al., 2006). Secondly, WAT is approximately composed of 50% adipocytes and 50% SVF cells which include preadipocytes, macrophages and other immune cells (Eto et al., 2009). Therefore, preadipocytes appear to be a major cellular component of the SVF. However, the characteristics of insulin signalling and the molecules involved in human preadipocytes are largely unknown.

A previous study using murine preadipocytes cell lines (3T3L-1 and 3T3-F442A) has shown that insulin signalling molecules including IR, IRS-1 and PI3K-p85α were increased during the differentiation of adipocytes but the auto-phosphorylation levels of IR and IRS-1 were decreased after chronic treatment with dexamethasone or insulin (Saad et al., 1994). Wortmannin has been shown to inhibit PI3K-p85α which in turn inhibits adipogenesis of 3T3L-1 (Tomiyama et al., 1995), activation of PKB is adequate to induce adipocyte differentiation (Magun et al., 1996). Wnt signaling is essential for healthy growth through promotes muscle and bone development and blocks the development of fat (Wright et al., 2007, Glass et al., 2005, Day et al.,
Interestingly, it has also reported that Wnt signalling interaction with insulin signalling in preadipocytes led to phosphorylation of key insulin signalling proteins through Wnt co-receptor LRP5 (Palsgaard et al., 2012).

Previous study on human preadipocytes has reported that insulin sensitization of preadipocytes by glucocorticoid, are produced in adipose tissue and contribute to metabolic disorders, may improve human preadipocyte differentiation and enhance components of the insulin signalling pathway (Tomlinson et al., 2010). Overall, it is still unclear whether insulin alone modulates the insulin signalling molecules in human preadipocytes. Therefore, this chapter will investigate and characterize the insulin signalling molecules and their response to insulin in human preadipocytes. The knowledge obtained would provide a basis for understanding the mechanisms that underlie how adipose tissue, as insulin target organ, contributes to insulin sensitivity or insulin resistance occurred in the physiological or pathological conditions.

3.2 Aim of the study

The aim of this study was to characterize the insulin signalling molecules, including IR (Y1185), IRS-1 (Ser612), PI3K p85α, GLUT-4 and Akt (Ser473), in human primary preadipocytes in comparison with adipocytes. By using in vitro human cell models, the specific objectives were set below:

- Basal expression of insulin signalling molecules in human primary preadipocytes;
- Expression of glucose transporter-4 (GLUT-4) during preadipocytes differentiation;
- Expression of insulin-stimulated phosphorylation of IR, IRS-1 and Akt in preadipocytes;
- Expression of insulin-stimulated phosphorylation of IR, IRS-1 and Akt in adipocytes.
3.3 Material and methods

3.3.1 Culture of human preadipocytes and adipocytes
Human white preadipocytes cell cultured based on the protocol from PromoCell (Heidelberg, Germany). Cells were cultured in preadipocyte growth medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Lonza, Tewkesbury, UK) at 37°C in a humidified atmosphere of 5% CO2-95% air. Preadipocytes were seeded at 40,000/cm² and divided into two groups. In the first group, preadipocytes were grown until confluence and then harvested as cell lysates for investigating the insulin signalling molecules. In the second group, preadipocytes were induced to differentiate (day 0) by incubation for 3 days in Dulbecco’s modified Eagle’s medium (DMEM)-Ham’s F-12 (1:1) medium containing 32 µM biotin, 1 µM dexamethasone, 200 µM 3-isobutyl-1-methylxanthine, 100 nM insulin, 11 nM L-thyroxine (all from Sigma, Poole, Dorset, UK), 8 µM rosiglitazone (GlaxoSmithKline, Uxbridge, UK), and 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B. After induction, cells were cultured in maintenance medium containing 3% fetal calf serum (FCS, Sigma), 100 nM insulin, 32 µM biotin, 1 µM dexamethasone and antibiotics until full differentiation into adipocytes. Adipocytes were then harvested as cell lysates for investigating the insulin signalling molecules.

3.3.2 Cell treatment
According to a time course experiment that was previously performed by our group, the optimal time point of maximum response to insulin was verified as at 5 min following the stimulation with insulin (data not shown). Therefore, preadipocytes and adipocytes were treated with 100 nM of insulin for 5 min to stimulate the autophosphorylation of insulin signalling molecules. Separate groups of preadipocytes and adipocytes were not treated with insulin and served as controls.

3.3.3 Western blotting
Protein concentrations in cell lysates were determined by the BCA assay as described in section 2.13.2. Protein samples (20µg/lane) were resolved by 10% Tricine-SDS polyacrylamide slab gels (Mini Protean Tetra, Bio-Rad, Hemel Hempstead, UK) and transferred onto a nitrocellulose membrane. For immunodetection, the membrane
was blocked for 1 h at room temperature with TBS containing 0.1% Tween-20 and 5% BSA and then incubated overnight at 4°C with the primary antibodies, including IRS-1 (diluted at 1:1000), PI3K p85α (diluted at 1:1000) and GLUT4 (diluted at 1:2500) in 5% BSA and 0.1% Tween-20. For insulin response study, primary antibodies phospho-insulin receptor (Y1185), phospho-IRS-1 (Ser612) and phospho-Akt (S473) were diluted at 1:1000. GAPDH (diluted at 1:20000). After four washes with 1xTTBS, the membranes were incubated with a HRP-conjugated secondary antibody (diluted at 1:2000). Signals were detected by enhanced chemiluminescence and scanned using Molecular Imager ChemiDoc XRS+ System (Bio-Rad). The size of the protein bands detected was estimated with PageRuler protein markers (Fermentas, York, UK). The membranes were further probed with GAPDH (1:20000 dilution) or total Akt (1:1000 dilution) as a loading control.

3.3.4 Statistical analysis
Data are expressed as means ± SEM. Differences between two groups were analysed by Student’s unpaired t-test. Differences were considered as statistically significant when p< 0.05.
3.4 Results

3.4.1 Basal and insulin-stimulated expression of the insulin signalling proteins in human primary preadipocytes and adipocytes

3.4.1.1 Basal expression of the insulin signalling proteins (IRS-1, PI3K-p85α and GLUT-4) in human primary preadipocytes and adipocytes

This experiment was to investigate whether preadipocytes would express any of the insulin signalling proteins at basal level in comparison with mature human adipocytes. One group of human primary preadipocytes was grown until confluence and then cells were harvested as cell lysates for investigating the basal expression of insulin signalling molecules, including total IRS-1, total PI3K-p85α and GLUT-4. The second group of preadipocytes was differentiated and maintained until became mature adipocytes (at day 12 post-differentiation), and then they were harvested as cell lysates for investigating the basal expression of insulin signalling proteins as positive controls.

3.4.1.1.1 Protein abundance of insulin receptor substrate-1 (IRS-1) in human preadipocytes and adipocytes

Basal IRS-1 protein expression was detected in preadipocytes and mature adipocytes. However, the basal level of IRS-1 expression was significantly higher (1.8-fold, p<0.05) in adipocyte compared with preadipocytes (Figure 3.1 a-b).

3.4.1.1.2 Protein abundance of phosphatidylinositol 3-kinase-p85α (PI3K-p85α) in human preadipocytes and adipocytes

Total PI3K-p85α protein expression in was also detected preadipocytes but it was significantly higher in adipocytes (1.4-fold, p<0.01) compared with preadipocytes (Figure 3.1 a, c).

3.4.1.1.3 Protein abundance of glucose transporter 4 (GLUT-4) in human preadipocytes and adipocytes

In preadipocytes, the expression of GLUT-4 protein was barely detectable. However, GLUT-4 expression was significantly increased (12-fold, p<0.001) at day 12 post...
differentiation (mature adipocytes) in comparison with preadipocytes (Figure 3.1 a, d).
Figure 3-1: Basal expressions of the insulin signalling protein (IRS-1, PI3K-p85α and GLUT-4) in human preadipocytes and adipocytes
Basal expression of insulin signalling proteins of IRS-1, PI3K-p85 and GLUT-4 in human preadipocytes were compared with mature human adipocytes. Both preadipocytes and adipocytes were unstimulated. (a) Western blotting was used for measuring protein expression in cell lysates, and GAPDH was used as a loading control. The relative protein expression of insulin signalling proteins including IRS-1 (b), PI3K-p85α (c) and GLUT-4 (d) were quantified and analysed by using densitometric analysis of signal intensity. Data are means ± SEM (n=3 per group). *p< 0.05, **p< 0.01, ***p<0.001
3.4.2 Expression of glucose transporter 4 (GLUT-4) during preadipocytes differentiation

GLUT-4 was largely expressed in mature adipocytes (Figure 3.1); its expression during the adipocyte development was then examined. Consistently, the level of GLUT-4 expression was barely detected at day 0 but interestingly, GLUT-4 expression was significantly increased from day 3 to day 12 post differentiation in human adipocytes (3.2-fold, p< 0.01) (Figure 3.2).
Figure 3-2: Time course of glucose transporter 4 (GLUT-4) expressions during preadipocyte differentiation

Expression of GLUT-4 protein during the development of human adipocytes. (a) Western blotting was used for measuring GLUT-4 protein expression in cell lysates, and GAPDH was used as a loading control. (b) The relative protein expression of GLUT-4 was quantified and analysed by using densitometric analysis of signal intensity. Data are means ± SEM (n=3 per group). *p< 0.05, **p< 0.01 between groups indicated; ND, none detected
3.4.3 Phosphorylation of the insulin signalling proteins p-IR, p-IRS-1(Ser612) and p-Akt (Ser473) in human preadipocytes in response to insulin

Insulin can trigger auto-phosphorylation events which act as signals in the cells (Taniguchi et al., 2006). This experiment was performed to investigate whether any of the key insulin signalling molecules expressed by preadipocytes are modulated after insulin stimulation. Western blotting was used to detect phosphorylated insulin receptor (p-IR-Y1185), phosphorylated insulin receptor substrate-1(p-IRS-1Ser612) and phosphorylated Akt (Ser473).

3.4.3.1 Protein abundance of insulin-stimulated phosphorylation of IR (Y1185) in human preadipocytes

In this experiment, it was observed that basal levels of p-IR were detectable and they were significantly increased (6-fold, p˂ 0.002) in preadipocytes which were stimulated with 100 nM insulin, compared with unstimulated preadipocytes (Figure 3.3).

3.4.3.2 Protein abundance of insulin-stimulated phosphorylation of IRS-1 (Ser612) in human preadipocytes

Protein level of the p-IRS-1(Ser612) in unstimulated preadipocytes was also detectable but at very low levels. In line with the result of insulin-stimulated p-IR, p-IRS-1(Ser612) was significantly increased (p< 0.001) in preadipocytes that were stimulated with 100 nM insulin (Figure 3.4).

3.4.3.3 Protein abundance of insulin-stimulated phosphorylation of Akt (Ser473) in human preadipocytes

The results have shown that basal protein level of p-Akt (Ser473) was not detectable in unstimulated preadipocytes (Figure 3.5). However, p-Akt (Ser473) was significantly increased (p< 0.001) in preadipocytes which were stimulated with 100 nM insulin.
Figure 3-3: Expression of insulin-stimulated phosphorylation of IR (Y1185) in human preadipocytes

Preadipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-IR and GAPDH was used as a loading control (b) The relative protein expression of p-IR was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). **p< 0.01
Figure 3-4: Expression of insulin-stimulated phosphorylation of IRS-1 (Ser612) in human preadipocytes

Preadipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-IRS-1(Ser612) which was normalised to the loading control GAPDH. (b) The relative protein expression of p-IRS-1(Ser612) was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). ***p< 0.001.
Figure 3-5: Expression of insulin-stimulated phosphorylation of Akt (Ser473) in human preadipocytes

Preadipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-Akt (Ser473) which was normalised to the loading control, GAPDH. (b) The relative protein expression of p-Akt (Ser473) was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n= 3 per group). ***p< 0.001, ND, none detected
3.4.4 Phosphorylation of the insulin signalling proteins p-IR (Y1185), p-IRS-1(Ser612) and p-AKT (Ser473) in human adipocytes in response to insulin

This experiment was performed in adipocytes to examine the phosphorylation of the key insulin signalling proteins, which served as controls to preadipocytes. Proteins including p-IR (Y1185), p-IRS-1(Ser612) and p-Akt (Ser473) were measured by using western blotting. Human primary preadipocytes were differentiated and maintained until they became mature adipocytes (at day 12 post-differentiation). The cells were then treated with or without 100 nM insulin. Cell lysates were collected for examining the expression of the phosphorylated insulin signalling proteins.

3.4.4.1 Protein abundance of insulin-stimulated phosphorylation of IR (Y1185) in human adipocytes

As shown by figure 3.6, phosphorylated IR (Y1185) was expressed at low levels in adipocytes; it was significantly increased (4.2-fold, p< 0.01) in response to insulin in comparison with unstimulated adipocytes.

3.4.4.2 Protein abundance of insulin-stimulated phosphorylation of IRS-1 (Ser612) in human adipocytes

A significant increase was observed in abundance level of phosphorylated IRS-1 (Ser612) (2.9-fold, p< 0.01) in insulin-stimulated adipocytes in comparison to unstimulated adipocytes (Figure 3.7).

3.4.4.3 Protein abundance of insulin-stimulated phosphorylation of Akt (Ser473) in human adipocytes

In agreement with the results of p-IR and p-IRS-1 (Ser612), phosphorylated Akt (Ser473) was expressed at low levels in adipocytes; its expression was significantly increased (1.7-fold, p< 0.05) in insulin-stimulated adipocytes in comparison to unstimulated adipocytes (Figure 3.8).
Figure 3-6: Expression of insulin-stimulated phosphorylation of IR (Y1185) in mature human adipocytes

Adipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-IR (Y1185) which was normalised to the loading control, total Akt. (b) The relative protein expression of p-IR was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group), **p<0.01
Figure 3-7: Expression of insulin-stimulated phosphorylation of IRS-1 (Ser612) in mature human adipocytes

Adipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-IRS-1(Ser612) which was normalised to the loading control GAPDH. (b) The relative protein expression of p-IRS-1 (Ser612) was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group), *p< 0.05
Figure 3-8: Expression of insulin-stimulated phosphorylation of Akt (Ser473) in mature human adipocytes

Adipocytes were treated with or without insulin (100nM) for 5 min. (a) The representative western blot is shown for p-Akt (Ser473) which was normalised to the loading control total Akt. (b) The relative protein expression of p-Akt (Ser473) was quantified by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group), **p< 0.01
3.5 Discussion

Adipose tissue is one of the main target tissues for insulin action and a major contributor to insulin resistance during obesity development (Smith, 2002b). There is clear evidence that preadipocytes and adipocytes, as the main adipose cells in the organ, secrete a range of bioactive proteins including hormones, pro-inflammatory factors and other signalling molecules that have a role in glucose and lipid homeostasis (Van Tienen et al., 2011). It is suggested that insulin modulates adipocyte development and differentiation (Le Lay et al., 2002, Vidal-Puig et al., 1997) but also acts as a key metabolic regulator (Saltiel and Kahn, 2001, Gual and Tanti, 2005). Moreover, preadipocytes are important in adipose tissue remodelling in obesity but their role in the development of insulin resistance is largely unknown. Therefore, the aim of the present study was to investigate whether insulin signalling molecules, particularly in the metabolic insulin cascade, are present and modulated by insulin in human preadipocytes.

To our knowledge the basal levels of insulin signalling proteins in human preadipocytes with a direct comparison with human mature adipocytes have not yet been fully investigated before. The primary objective of this study was to examine whether the key insulin signalling molecules (IRS-1, PI3K- p85α and GlUT-4) are present at the basal level in human preadipocytes. Results from this study showed that basal levels of insulin signalling proteins IRS-1 and PI3K-p85α were detected in human preadipocytes. Furthermore, these two insulin signalling molecules have significantly increased in mature adipocytes compared with preadipocytes (Figure3.1 a-c). These results are in agreement with previous studies in murine adipocytes (Saad et al., 1994). It suggests that the expression levels of insulin signalling molecules for both IRS-1 and PI3K-p85α in preadipocytes were increased during the course of the differentiation process, which may have importance in adipose tissue functions as well as development.

IRS-1 is a member of the insulin receptor substrate family of signalling molecules, and is considered as one of the main insulin receptor substrates in the insulin signalling pathway in muscle and fat cells (Schmitz-Peiffer and Whitehead, 2003). IRS-1 is a crucial mediator that plays a key role in insulin signalling particularly
once subsequently phosphorylated via activated IR (Sesti et al., 2001). IRS-1 function is positively regulated once phosphorylation occurs at its tyrosine residue which is required for insulin-stimulated responses. However, IRS-1 at serine/threonine residues has double roles which in turn leading to stimulate or inhibit the insulin action through its signalling pathway (Paz et al., 1997). Evidence has suggested that insulin signalling in an individual with insulin resistance and type 2 diabetes is often inhibited at the level of IRS-1 of tyrosine residues (Schmitz-Peiffer and Whitehead, 2003, Sesti et al., 2001).

PI3K is considered to be a downstream effector of IRS proteins (Cantley, 2002). After tyrosine phosphorylation, IRS proteins activate PI3K, where p85-α appears to be the subunit that links PI3-kinase activity in p110 to the tyrosine-phosphorylated proteins (Hara et al., 1994) leading to insulin-stimulated glucose transport and translocation through activation its downstream effector such as Akt (Cheatham and Kahn, 1995). Moreover, PI3K also plays an important role in insulin-reduced lipolysis in murine adipocytes (Okada et al., 1994). However, inhibition of PI3-kinase using a specific inhibitor such as wortmannin totally impairs insulin-stimulated glucose uptake in adipocytes (Okada et al., 1994). Collectively, it is suggested that increase in insulin signalling molecules including IRS-1 and PI3K is a prerequisite to promote the link of insulin signal transduction in adipose cells with glucose and lipid metabolism.

The GLUT-4 is the major glucose transporter in adipose tissue and one of the final molecules in the process by which insulin mediates cellular glucose uptake (Olson and Pessin, 1996). The present study also showed that GLUT-4 protein level was barely detectable in preadipocytes compared to mature adipocytes. A previous study that used crude membrane fractions of undifferentiated human preadipocytes showed that GLUT-4 protein expression was detectable in a very small amount while its expression was increased by 16.7-fold at 16 day post differentiation (Hauner et al., 1998). Interestingly, in preadipocytes there are repressor sequences in the 5'-flanking region of the GLUT-4 gene between positions -200 and -100 bp but not in adipocytes (Yokomori et al., 1999). Therefore, it has been suggested GLUT-4 protein is only abundantly expressed once preadipocytes differentiate into mature adipocytes. This study also performed a time course experiment to examine the levels of GLUT-4
protein expressions at several time points during preadipocytes differentiation. Results showed that GLUT-4 protein expression gradually increased from day 3 up to day 12 post differentiation (Figure 3.2). This is in agreement with previous studies using murine adipocytes (3T3L-1) in which several types of glucose transporter proteins are progressively expressed during the differentiation of adipocytes as demonstrated by kinetic studies (Weiland et al., 1990, Tordjman et al., 1989). Taken together, these results suggest that an increase in abundance of GLUT-4 expression in mature adipocytes could be responsible for the appearance of insulin responsiveness in adipose tissue to achieve metabolic function, such as insulin-stimulated glucose transport.

Another objective of the present study was to investigate whether the insulin signalling molecules, IR (Y1185), IRS-1 (Ser612) and Akt (Ser473) in human preadipocytes are modulated by insulin in comparison with mature adipocytes. The result showed that in both preadipocytes and mature adipocytes, exposure to insulin (100nM) led to the phosphorylation of IR (Y1185). Following insulin stimulation, there was a significant increase in the protein abundance of IR and this would be due to increased phosphorylation activity. This result is in agreement with the reports that once insulin binds to IR conformational changes are made (Kido et al., 2001, Sun et al., 1991), which in turn activates IR leading to its auto-phosphorylation (Hubbard et al., 1993).

Considering the regulation of IRS-1 function is crucial in insulin signalling, this study examined whether insulin affects the IRS-1 (Ser612), as one of the IRS’ inhibitory residues in insulin signalling, in preadipocytes compared with adipocytes. The result showed that the level of phosphorylated IRS-1(Ser612) was significantly increased in both cells following insulin stimulation. Previous studies have found that phosphorylation of IRS-1 at serine residues has an inhibitory effect on insulin signalling pathway that may contribute to insulin resistance (Tanti et al., 1994, Gual and Tanti, 2005, Paz et al., 1997).

Akt is known to serve as an important regulator of cell survival and proliferation (Song et al., 2005). The full activation of Akt needs phosphorylation on serine 473 of Akt by insulin or growth factors (Alessi et al., 1996). The present study also
examined whether Akt (Ser473) is modulated by insulin in preadipocytes in comparison with mature adipocytes. Result of the present study showed that insulin significantly stimulated the protein abundance of phosphorylated Akt (Ser473) in both adipose cells. These results suggested that treatment of adipose cells with insulin markedly induces the insulin signalling molecules that may have stimulatory or inhibitory roles on several biological processes such as proliferation, differentiation and development of adipose cells.

In summary, our data provides some evidence that the key insulin signalling molecules were detected in both human adipose cells (preadipocytes and adipocytes). Both IRS-1 and PI3K-p85α were expressed whereas Glut4 was barely detected in preadipocytes. The levels of insulin molecules (IRS-1, PI3K-p85α and GLUT-4) were significantly higher in differentiated adipocytes. Furthermore, insulin significantly stimulated the level of phosphorylated insulin signalling molecules, including IR (Y1185), IRS-1(Ser612) and Akt (Ser473), in human preadipocytes as well as mature adipocytes.
CHAPTER 4:

IL-1β modulates insulin signalling proteins and mediates macrophage-induced production of pro-inflammatory factors in human pre-adipocytes
4 IL-1β modulates insulin signalling proteins and mediates macrophage-induced production of pro-inflammatory factors in human pre-adipocytes

4.1 Introduction

In this chapter, it is investigated whether IL-1β plays a major role in mediating the effect of MC medium on insulin signalling pathways, and on cytokines/chemokines expression and release in human preadipocytes.

As mentioned in previous sections, obesity is now accepted to be a state of chronic low-grade inflammation (Hotamisligil, 2006, Rosen and Spiegelman, 2006b). Growing evidence from the studies in obese mice and human suggests that increased macrophage accumulation in adipose tissue during obesity might provide a causative link between inflammation and metabolic disorders such as insulin resistance and type 2 diabetes (Cancello and Clement, 2006, Weisberg et al., 2003, Xu et al., 2003). In addition, macrophages-derived factors have been found to potently stimulate the production of pro-inflammatory cytokines and chemokines by human preadipocytes and adipocytes (Flower et al., 2003, Gao and Bing, 2011).

WAT is made up of a number of different cell types including mature adipocytes, and the cells of the SVF which include fibroblast, preadipocytes, macrophages and other immune cells (T-Lymphocytes and neutrophils) (Lee et al., 2012, Fruhbeck, 2008). These cells produce cytokines/chemokines and growth and complement factors (Fruhbeck, 2008, Yang et al., 2006). The alteration in the release of pro-inflammatory factors by adipose cells has been associated with the metabolic disorders of obesity (Weisberg et al., 2003). Therefore, it is reasonable to expect that crosstalk will occur between these cells, possibly mediated by pro-inflammatory factors.

Several studies have provided evidence that some pro-inflammatory factors including TNF-α, IL-6, MCP-1 and IL-1β, are directly involved in obesity-associated pathologies, particularly insulin resistance and type 2 diabetes (Spranger et al., 2003c, Hotamisligil, 1999, Lagathu et al., 2003, Böni-Schnetzler and Donath, 2011, Sartipy and Loskutoff, 2003). An in vitro study using primary culture of human preadipocytes has shown that chemokines including IL-8, MIP-1 and MCP-1 were
highly produced, whereas their level of release was low in mature adipocytes (Gerhardt et al., 2001). This suggests that preadipocytes may have a central role in the recruitment of macrophages in comparison with mature adipocytes.

Identification of the key factors that mediate the effect of macrophages on human preadipocytes is challenging but it may provide better understanding for obesity-related insulin resistance in adipose tissue. IL-1β is considered as a key pro-inflammatory cytokine which produced mainly by monocytes and macrophages (Agostini et al., 2004). In adipose tissue, gene expression of IL-1β is upregulated in obese mice and humans. IL-1β is also released by human adipose tissue explants but by non-fat cells (Fain, 2006, Koenen et al., 2011) and the levels released are enhanced in obesity (Nov et al., 2010). Some studies suggested that adipocytes also release IL-1β with much less amount as compared to macrophages (Lagathu et al., 2006, Juge-Aubry et al., 2004, Wen et al., 2011, Tack et al., 2012). Previous studies also suggested a link between circulating IL-1β levels and metabolic disorders, such as insulin resistance and type 2 diabetes (Lagathu et al., 2006, Xie et al., 2010).

Recently, researchers have started to study the crosstalk between macrophages and preadipocytes by establishing in vitro models from human and rodents’ origin (Sorisky et al., 2013, Lacasa et al., 2007, Gagnon et al., 2013). Using the crosstalk models, some studies have demonstrated that macrophages-derived factors inhibit preadipocyte differentiation in human abdominal stromal as well as 3T3-L1 preadipocytes and stimulate inflammation in preadipocytes (Constant et al., 2006, Suganami et al., 2005). In addition, a recent study by our group revealed that macrophage-derived factors induce expression and release of matrix metalloproteins in human preadipocytes (MMP1 and MMP3) which are involved in the inflammatory response and adipose tissue remodelling in obesity (Gao and Bing, 2011). Furthermore, two studies suggest that pro-inflammatory factors from macrophages induce profound changes in human preadipocytes that may stimulate a fibrotic phenotype via modification of extracellular matrix (ECM) in adipose tissue (Lacasa et al., 2007, Keophiphath et al., 2009). Interestingly, it has been shown that macrophages-derived factors protect 3T3-L1 preadipocytes from apoptosis via activation the survival signalling that phosphorylates Akt and Erk molecules in the presence of ROS (Molgat et al., 2011). However, there is a lack of studies using
human primary preadipocytes as an in vitro model to demonstrate the effect of macrophages or IL-1β on insulin signalling pathway.

From the above, it has become clear that macrophages crosstalk with preadipocytes may influence preadipocytes differentiation, survival and proliferation. Insulin signalling is crucial and required for metabolic health (Fischer-Posovszky et al., 2012, Taniguchi et al., 2006). Beside the studies on direct effect of macrophages on preadipocytes, a number of cytokines/chemokines (TNF-α, IL-6, IL-1β and MCP-1) have been considered as candidates to impair insulin signalling in mature adipocytes. However, little is known whether macrophages-derived factors modulate insulin signalling pathways in human primary preadipocytes. Moreover, whether IL-1β mediates the effect of macrophages on insulin signalling in human preadipocytes has not been reported.

4.2 Aim of the study

The aims of this study were to investigate the role of IL-1β in macrophage-preadipocyte crosstalk, and how this crosstalk modulates the insulin signalling pathway in human preadipocytes. The specific objectives were set below:

- Effect of treatment with IL-β (2 ng/ml) for 4 and 24 hr on the insulin signalling pathway in preadipocytes;
- Effect of MC medium on expression of the insulin signalling molecules in preadipocytes;
- Effect of blocking IL-1β activity on MC medium-modulated expression of the insulin signalling molecules;
- Effect of blocking IL-1β activity on MC medium-induced gene expression of pro-inflammatory factors;
- Effect of blocking IL-1β activity on MC medium-induced release of pro-inflammatory factors including IL-6, MCP-1, IL-8 and RANTES.

4.3 Material and methods

4.3.1 Culture of human preadipocytes

Human white preadipocytes derived from subcutaneous adipose tissue of a female Caucasian subject (BMI 21kg/m²; age 44 yr) were obtained from PromoCell
Cells were cultured in preadipocyte growth medium supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin, and 0.25 µg/ml amphotericin B (Lonza, Tewkesbury, UK) at 37 °C in a humidified atmosphere of 5% CO2-95% air. Preadipocytes were seeded at 40,000/cm². Subsequently, preadipocytes were grown until confluence. At confluence, preadipocytes were harvested to use for the studies below.

4.3.2 Generation of THP-1 macrophages-conditioned medium

The human THP-1 monocytes were cultured in (RPMI-1640) medium with 10% FCS, 100 U/ml penicillin and 100µg/ml streptomycin and then placed in an incubator at 37 °C in humidified atmosphere of 5% CO2/ 95% air for 24 hr until cell density reached 10⁶/ml (see section 2.4.3). THP-1 monocytes were then differentiated by the addition of PMA 100 nM (Sigma) for 48 hr. The culture medium was replaced with serum-free RPMI-1640 medium (without PMA) for 24 h. the MC medium was then collected as previously mentioned in section 2.4.4.

4.3.3 Cell treatment

To assess whether IL-1β affects the insulin signalling pathway, human preadipocytes were treated with RPMI-1640 medium and with or without IL-1β (2 ng/ml) for 4 and 24 hr. Preadipocytes were also treated with 100 nM of insulin for 5 minutes to stimulate the autophosphorylation of insulin signalling molecules. Separate groups of preadipocytes were not treated with insulin and served as controls. To investigate whether IL-1β mediates the effects of MC medium, MC medium was pre-incubated with a human IL-1β neutralizing antibody (2 µg/ml) for 1 hr at 37 °C to block IL-1β activity; preadipocytes were then incubated with either RPMI-1640 medium (control), MC medium or MC medium neutralized by IL-1β antibody for 24 hr.

4.3.4 Western blotting

For assessing protein expression Western blotting method was used. For insulin response study, primary antibodies inducing phospho-insulin receptor (Y1185) (abcam, UK), phospho-IRS-1 (Ser612) and phospho-Akt (S473) (New England BioLabs Ltd, Hitchin, UK) were used and diluted at 1:1000. GAPDH (abcam, UK) diluted at 1:2000 or Akt (Cell Signalling) diluted at 1:1000 was used as a loading control. After 4 washes with 1xTTBS, anti-rabbit secondary antibody (New England BioLabs Ltd, Hitchin, UK) at 1:2000 dilution (or 1:20000 for GAPDH) were used.
Signals were detected by chemiluminescence (West Pico kit, Pierce, Loughborough, UK) and scanned using Molecular Imager ChemiDoc XRS+ System (Bio-Rad). The size of the protein bands detected was estimated with PageRuler protein markers (Fermentas, York, UK). The membrane was further probed with GAPDH (Abcam, Cambridge, UK) or total Akt (New England BioLabs Ltd).

4.3.5 Measurement of cytokine/chemokine release (ELISA)
The release levels of IL-1β by THP-1 macrophages and of IL-6, MCP-1, IL-8 and RANTES by preadipocytes were determined as the protein concentrations in cell culture medium, using ELISA kits (See section 2.12).

4.3.6 Real-time PCR
Total RNA was extracted from cells using Trizol as previously mentioned in section 2.9. RNA concentration was determined from the absorbance at 260 nm. First strand cDNA was reverse transcribed from 0.5 µg of total RNA using an iScript first strand synthesis kit in a final volume of 10 µl (Section 2.10). Real-time PCR amplification was performed in a final volume of 12.5 µl, containing cDNA (equivalent to 12.5 ng of RNA), optimized concentrations of primers, TaqMan probe (FAM-TAMRA) and a master mix made from qPCR core kit (Section 2.11). The sequences of primers and probes for human β-actin, IL-6, MCP-1 and IL-8 were as described previously (Table 2.1). Real-time PCR amplification was performed using a Stratagene Mx3005P instrument and the PCR cycling conditions were as follows: 95 ºC for 10 minutes followed by 40 cycles (95 ºC for 15 sec, 60 ºC) for 1 minute. The results were expressed as fold changes of Ct value relative to controls using the data analysis software from the manufacturer.

4.3.7 Cell viability assay
Cell viability was determined by using a colorimetric method for measuring cellular cytotoxicity from the release of lactate dehydrogenase (LDH) into cell culture medium (See section 2.14).

4.3.8 Statistical analysis
Data are expressed as means ± SEM or means ± SD. Differences between two groups were analysed by Student’s unpaired t-test; one-way ANOVA coupled with Bonferroni’s t-test was employed for comparison of multiple-groups. Differences were considered as statistically significant when p < 0.05.
4.4 Effects of IL-1β on expression of the insulin signalling molecules in human primary preadipocytes

4.4.1 Effect of 4 hr treatment with IL-1β on insulin-stimulated phosphorylation of insulin signalling molecules in human preadipocytes

This experiment was to investigate whether IL-1β, one of the major cytokines produced by macrophages, could affect insulin signalling molecules in human preadipocytes in response to insulin stimulation. It was first assessed that the effect of 4 hr treatment with IL-1β on response of insulin signalling molecules that includes p-IR, p-IRS-1 and p-Akt with or without insulin stimulation.

4.4.1.1 Effect of 4 hr treatment with IL-1β on protein expression of p-IR (Y1185)

As shown in figure 4.1, the basal protein abundance of phosphorylated IR was barely detectable in cells with or without IL-1β (2 ng/ml) treatment; there was no significant difference between the groups. However, there was a significant increase in insulin-stimulated p-IR abundance in untreated and IL-1β treated preadipocytes (p< 0.01 and p< 0.05) respectively in comparison with unstimulated cells. Interestingly, preadipocytes exposed to IL-1β resulted in a significant reduction in insulin-stimulated phosphorylation of IR at Y1185 (by 3.2-fold, p< 0.05) compared with corresponding controls (Figure 4.1).

4.4.1.2 Effect of 4 hr treatment with IL-1β on protein expression of p-IRS-1 (Ser612)

As shown in figure 4.2, low levels of basal protein abundance for p-IRS-1 (Ser612) were detected in both untreated and treated preadipocytes with IL-1β (2 ng/ml) treatment; there was no significant difference between the groups. In addition, there was a significant increase in insulin-stimulated p-IRS-1 in untreated and treated preadipocytes with IL-1β in comparison with unstimulated cells (p< 0.001 and p< 0.05). However, preadipocytes exposed to IL-1β resulted in a significant reduction in insulin stimulated phosphorylation of IRS-1 at ser612 (by 1.8-fold, p< 0.05) compared with corresponding controls (Figure 4.2).
4.4.1.3 Effect of 4 hr treatment with IL-1β on protein expression of p-Akt (Ser473)

As shown in figure 4.3, the basal protein abundance of Akt phosphorylation at serine 473 in both untreated and treated preadipocytes with IL-1β were undetectable. However, there was a significant increase in insulin-stimulated p-Akt abundance in untreated and treated preadipocytes with IL-1β in comparison with unstimulated cells (both p< 0.001). Another observation was that a slight reduction in p-Akt abundance level in preadipocytes after exposed to the effect of IL-1β, but this was not statistically significant (Figure 4.3).
Figure 4-1: Effect of 4 hr treatment with IL-1β on insulin-stimulated phosphorylation of IR in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 4 hr and then stimulated with or without 100 nM insulin. (a) The representative western blot is shown for p-IR, which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-IR was performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). *p< 0.05, **p< 0.01
Figure 4-2: Effect of 4 hr treatment with IL-1β on insulin-stimulated phosphorylation of IRS-1(Ser612) in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 4 hr and then stimulated with or without 100 nM insulin. (a) The representative western blot is shown for p-IRS-1(Ser612), which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-IR was performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). *p< 0.05, ***p< 0.001
Figure 4-3: Effect of 4 hr treatment with IL-1β on insulin-stimulated phosphorylation of Akt in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 4 and then stimulated with or without 100 nM insulin. (a) A representative western blot is shown for p-Akt, which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-IR was performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). ***p< 0.001. ND = non detectable. NS= Non significant.
4.4.2 Effect of 24 hr treatment with IL-1β on insulin-stimulated phosphorylation of insulin signalling molecules in human preadipocytes

Although 4 hr treatment with IL-1β affected the expression of insulin signalling molecules under insulin stimulation, it is unknown whether IL-1β has a longer time effect. The aim of this experiment was to study the effect of IL-1β treatment for 24 hr on insulin signalling molecules in response to insulin stimulation in human preadipocytes.

4.4.2.1 Effect of 24 hr treatment with IL-1β on protein expression of p-IR (Y1185)

There was a significant reduction in p-IR abundance level in preadipocytes treated with IL-1β for 24 hr (by 2-fold, p < 0.01) in comparison with untreated cells (Figure 4.4). In addition, there was a significant reduction in insulin-stimulated p-IR level in cells exposed to IL-1β in comparison to control (p < 0.05) (Figure 4.4).

4.4.2.2 Effect of 24 hr treatment with IL-1β on protein expression of p-IRS-1 (Ser612)

There was no difference in protein abundance of p-IRS-1 (Ser612) in untreated or IL-1β treated preadipocytes without stimulation with insulin (Figure 4.5). Furthermore, there was a significant increase in insulin–stimulated p-IRS-1 abundance level in both untreated or treated preadipocytes with IL-1β treatment (3.1-fold, p < 0.01 and 3.8-fold, p < 0.05) respectively, in comparison with unstimulated corresponding controls. However, this stimulatory effect was induced by insulin with slight effects caused by IL-1β.

4.4.2.3 Effect of 24 hr treatment with IL-1β on protein expression of p-Akt (Ser473)

Consistent with the results of 4 hr treatment with IL-1β, the basal abundance level of Akt phosphorylation at serine 473 was undetectable in both untreated and IL-1β treated preadipocytes (Figure 4.6). However, a significant increase in p-Akt abundance was observed in untreated and treated preadipocytes (p < 0.001 and p < 0.001) respectively, once stimulated with insulin in comparison with corresponding unstimulated cells. Interestingly, a 24 hr exposure showed a significant increase in insulin-stimulated p-Akt abundance (2.8-fold, p < 0.05) in preadipocytes treated with IL-1β compared to untreated cells (Figure 4.6).
Figure 4-4: Effect of 24 hr treatment with IL-1β on insulin-stimulated phosphorylation of IR in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 24 hr then stimulated with or without 100 nM insulin. (a) The representative western blot is shown for p-IR, which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-IR was performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). *p< 0.05, **p< 0.01
Figure 4-5: Effect of 24 hr treatment with IL-1β on insulin-stimulated phosphorylation of IRS-1(Ser612) in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 24 hr and then stimulated with or without 100 nM insulin. (a) The representative western blot is shown for p-IRS-1(Ser612), which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-IRS-1 was performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). *p< 0.05, **p< 0.01
Figure 4-6: Effect of 24 hr treatment with IL-1β on insulin-stimulated phosphorylation of Akt in human preadipocytes

Preadipocytes were either untreated or treated with 2 ng/ml IL-1β for 24 hr and then stimulated with or without 100 nM insulin. (a) The representative western blot is shown for p-Akt, which was normalised to the loading control, GAPDH. (b) The quantification and measurement for the protein bands from p-Akt were performed by using densitometric analysis of signal intensity. Data is shown as mean ± SEM (n=3 per group). ***p< 0.001 vs unstimulated cells, *p< 0.05 vs stimulated control cells. ND = none detectable levels
4.4.3 Influence of IL-1β on the effect of macrophage-derived factors on insulin signalling molecules in response to insulin in human preadipocytes

The presence of inflammatory mediators in MC medium may reduce and/or modulate insulin signalling proteins in adipose tissue (Lumeng et al., 2007c). To investigate whether IL-1β is a major factor which mediates the effects of MC medium, an IL-1β neutralising antibody was used to block IL-1β activity and the expression levels of insulin signalling molecules in human preadipocytes was determined by western blotting.

4.4.3.1 Effect of IL-1β neutralising antibody on MC medium-induced expression of p-IRS-1 (Ser612)

Upon insulin (100 nM) stimulation, significant increases in the phosphorylated level of IRS-1 (Ser612) in control (3.7 fold increase, p< 0.01), MC medium (2.9 fold increase, p< 0.05) and MC with IL-1β blocking antibody (2.1 fold increase, p<0.01) groups compared with unstimulated cell groups. Preadipocytes exposed to MC medium without insulin stimulation showed a significant increase (2.4-fold, p< 0.01) in protein expression of pIRS-1(Ser612) compared with the corresponding controls. However, MC medium-induced protein abundance of basal pIRS-1 was slightly reduced by IL-1β antibody but not statistically significant (Figure 4.7).

4.4.3.2 Effect of IL-1β neutralising antibody on MC medium-induced p-Akt (Ser473)

Insulin (100 nM) stimulation induced a significant increase in phosphorylation of Akt (Ser473) in control preadipocytes (6.4 fold increases, p< 0.01) compared with corresponding unstimulated group (Figure 4.8). However, MC medium increased the basal abundance of p-Akt (2.8-fold, p< 0.05) compared with control group but suppressed the induction of p-Akt in response to insulin (Figure 4.8). In contrast, IL-1β neutralising antibody reversed the inhibitory effect of MC medium on p-Akt as it significantly increased p-Akt abundance in response to insulin (Figure 4.8).
Figure 4-7: Effect of IL-1β neutralising antibody on macrophage-induced p-IRS-1 (Ser612)

Preadipocytes were treated with RPMI (control), MC medium or MC medium with 2 µg/ml IL-1β neutralising antibody and then stimulated with or without 100 nM insulin. The representative western blot is shown (a). The relative protein bands from p-IRS-1 (Ser612) were quantified and normalised to the loading control, total Akt (b). Data is shown as mean ± SD (n= 4 per group). *p< 0.05, **p< 0.01 and +p< 0.05 vs indicated group
Figure 4-8: Effect of IL-1β neutralising antibody on macrophage-modulated p-Akt (Ser473)

Preadipocytes were treated with RPMI (control), MC medium or MC medium with 2 µg/ml IL-1β neutralising antibody and then stimulated with or without 100 nM insulin. The representative western blot is shown with relevant molecular weight markers from the protein ladder (a). The relative protein bands from p-Akt (Ser473) was quantified and normalised to the loading control, GAPDH (b). Data is shown as mean ± SD (n= 4. Per group), **p< 0.01 and +p< 0.05 vs indicated group
4.4.4 Blocking IL-1β activity reverses the induction effect of macrophage-derived factors on mRNA levels of the pro-inflammatory factors in human preadipocytes

Several cytokines/chemokines have been shown to promote insulin resistance by influencing insulin signalling pathway (Tack et al., 2012). Cytokines/chemokines can also be produced by preadipocytes and potently induced by MC medium (O’Hara et al., 2012, Gao and Bing, 2011). The next experiments were carried out to verify the role of IL-1β in mediating the effects of MC medium on the gene expression of the cytokines/chemokines by preadipocytes. In this experiment, preadipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by the IL-1β antibody (2 μg/ml) for 24 hr. Gene expression of IL-6, MCP-1 and IL-8 was determined using real-time PCR.

4.4.4.1 IL-1β neutralisation reversed the effect of MC medium-induced gene expression of IL-6

As shown in figure 4.9 a, the gene expression of IL-6 by preadipocytes was barely detectable in the control group; MC medium potently increased the mRNA levels of IL-6 (85-fold increase, p< 0.001). However, this induction was blunted by IL-1β neutralization (p< 0.001) in preadipocytes (Figure 4.9 a).

4.4.4.2 IL-1β neutralisation blocked the effect of MC medium-induced gene expression of MCP-1

The gene expression of MCP-1 was detected in the control group of preadipocytes. MC medium strongly stimulated gene expression of MCP-1 (10-fold, p< 0.001) but this induction was totally reversed by IL-1β neutralization (p< 0.001) (Figure 4.9 b).

4.4.4.3 IL-1β neutralisation reversed the effect of MC medium-induced gene expression of IL-8

The gene expression of IL-8 was detectable in the control group of preadipocytes, and MC medium potently stimulated gene expression of IL-8 (17-fold increase, p< 0.001). However, this induction was partially blunted by IL-1β neutralization (p< 0.001) (Figure 4.9 c).
Figure 4-9: Blocking IL-1β activity reverses the effect of macrophage-induced gene expression of cytokines/chemokines in human preadipocytes

Inhibition of IL-1β activity reverses macrophage-induced gene expression of pro-inflammatory factors by human preadipocytes. Preadipocytes were incubated with either RPMI-1640 medium (control), MC medium or MC medium neutralized by IL-1β antibody (2 μg/ml) for 24 hr. The mRNA levels of IL-6 (a), MCP-1 (b) and IL-8 (c) were measured by real-time PCR. Data are means ± SEM (n=6 per group); *** p< 0.001 vs indicated groups
4.4.5 Blocking IL-1β activity reverses the effect of macrophage-derived factors on cytokine and chemokine release by human preadipocytes

In the present study, whether blocking IL-1β activity inhibits the effects of MC medium-induced release of cytokines/chemokines by preadipocytes was examined. The secretion levels of the cytokine/chemokines were determined using ELISA.

4.4.5.1 IL-1β neutralisation reverses the effect of MC medium-induced IL-6 release

As shown in figure 4.10 a, IL-6 was barely detectable in the medium of preadipocytes with a mean value of 8 pg/ml in the control group. Notably, MC medium potently stimulated the release of IL-6 (up to 11158 pg/ml) but this induction was largely blunted by IL-1β neutralisation (all p< 0.001). To distinguish the production of cytokine by preadipocytes from macrophages, the basal levels of IL-6 in THP-1 macrophage medium were shown as MC alone (72± 18 pg/ml) (Figure 4.10 a).

4.4.5.2 IL-1β neutralisation reverses the effect of MC medium-induced MCP-1 release

As shown by figure 4.10 b, the preadipocytes medium MCP-1 was detected with a mean value of 85 pg/ml in the control group of preadipocytes. MC medium also potently stimulated the release of MCP-1 (up to 3077 pg/ml) but this stimulation was markedly reduced by IL-1β neutralisation (all p< 0.001). To distinguish for the production of MCP-1 by preadipocytes from that by macrophages, the basal levels of MCP-1 in THP-1 macrophage medium were shown as MC alone (216 ± 56 pg/ml) (Figure 4.10 b).

4.4.5.3 IL-1β neutralisation reverses the effect of MC medium-induced IL-8 release

Further results have been observed to be in a similar pattern as IL-6 and MCP-1 release from preadipocytes. The release of IL-8 at basal level was detected with a mean value of 646 pg/ml in the control group of preadipocytes. MC medium strongly stimulated the release of IL-8 (up to 7081 pg/ml) but this induction was reversed by IL-1β neutralisation (all p< 0.001). The basal levels of IL-8 in THP-1 macrophage medium were shown as MC alone (762 ± 53 pg/ml) (Figure 4.10 c).
4.4.5.4 IL-1β neutralisation partially reverses the effect of MC medium-induced RANTES release

Lastly, the basal level of RANTES were determined and detected with a mean value of 88 pg/ml in the control group of preadipocytes. Consistent with the above data, MC medium strongly stimulated the release of RANTES (up to 3649 pg/ml) but this induction was significantly reduced by IL-1β neutralisation (all p< 0.001). The basal levels of RANTES in THP-1 macrophage medium were shown as MC alone (1014 ± 51 pg/ml) (Figure 4.10 d).
Figure 4-10: Blocking IL-1β activity reverses the effects of MC medium on cytokine/chemokines release in human preadipocytes

Inhibition of IL-1β activity reverses macrophage-induced cytokine/chemokine release by human preadipocytes. Preadipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (2 μg/ml), MC medium alone (without cells) for 24 hr. The releases of IL-6 (a), MCP-1 (b), IL-8 (c) and RANTES (d) by preadipocytes were measured as protein concentrations in cell culture medium by ELISA. Data are means ± SEM (n=6 per group); ***p< 0.001 vs indicated groups
4.4.6 Cytotoxicity assessment

It is not known whether the effects of both MC medium and recombinant human IL-1β on insulin signalling molecules in preadipocytes could be caused by cytotoxicity. To assess this possibility, cell viability was determined as LDH release by preadipocytes treated with MC medium, IL-1β and IL-1β neutralising antibody.

4.4.6.1 MC medium and an IL-1β neutralising antibody do not induce cytotoxicity

As observed in figure 4.11 there were no significant differences in LDH release by preadipocytes treated with MC or MC medium pre-treated with IL-1β neutralising antibody for 24 hr, compared with controls. However, there was a significant increase in LDH release (p˂ 0.001) in the LDH positive control group compared with all other groups. Therefore, there was no indication of increased cytotoxicity following the treatment with THP-1 MC medium or IL-1β neutralizing antibody.

4.4.6.2 Human recombinant IL-1β at several concentrations (2, 5, 10 ng/ml) does not induce cytotoxicity

As shown in figure 4.12, in the same manner with MC medium there were no significant differences in LDH release by preadipocytes treated with IL-1β at several concentrations (2, 5, 10 ng/ml) for 24 hr, compared with controls. However, there was a significant increase in the LDH release (p< 0.001) in LDH positive control group compared with all other groups. Therefore, there was no indication of increased cytotoxicity induced by IL-1β (Figure 4.12).
Figure 4-11: MC medium and IL-1β neutralizing antibody do not induce cytotoxicity in human preadipocytes

Human preadipocytes were either untreated (control) or treated with MC medium or MC medium pre-treated with IL-1β neutralizing antibody for 24 hr. Cell viability was assessed by measuring LDH release into the cell culture medium of preadipocytes. Data are means ± SEM (n=6 per group); ***p< 0.001 vs all other groups
Human preadipocytes were either untreated (control) or treated with IL-1β at different doses (2, 5, 10 ng/ml) for 24 hr. Cell viability was assessed by measuring LDH release into the cell culture medium of preadipocytes. Data are means ± SEM (n=6 per group); ***p< 0.001 vs all other groups.
4.5 Discussion

As mentioned in Chapter 1, both insulin resistance and type 2 diabetes are related to inflammation which is characterized by increasing a wide range of pro-inflammatory cytokines/chemokines probably due to an increase in the accumulation of macrophages during adipose tissue expansion in obesity (Hotamisligil, 2003, Weisberg et al., 2003). Preadipocytes at the non-differentiation state have capability to express pro-inflammatory factors that may contribute to the induction of inflammation (Poulain-Godefroy and Froguel, 2007). In the light of recent studies, some in vitro models have been used to study the crosstalk between macrophages and adipocytes and their precursors (preadipocytes). However, there is no study using human primary preadipocytes as an in vitro cell model to demonstrate the direct effect of macrophages-derived factors on the insulin signalling pathway. Furthermore, the key factors that mediate the effect of macrophages on the chemokines/cytokines expression and release by human primary preadipocytes which in turn may alter insulin signalling pathways are not well investigated.

In the present study, in vitro model of primary human preadipocytes and human recombinant IL-1β were used to investigate the acute and sustained effects of IL-1β at time points (4 and 24 hr) respectively on several insulin signalling molecules, including the key molecules which are involved in the metabolic pathway (PI3K-p85/Akt) in response to insulin stimulation. The activation of this pathway is also widely involved in conveying survival signals in response to a range of stimuli in many different cell types (Scheid et al., 1995, Yao and Cooper, 1995, Kennedy et al., 1997, Khwaja et al., 1997). Additionally, the present study also investigated whether IL-1β mediates the effects of MC medium on the insulin signalling molecules by using a neutralizing antibody to block IL-1β activity. Lastly, whether IL-1β mediates the effect of MC medium on the expression and release of pro-inflammatory factors by human preadipocytes was explored.

The activation and phosphorylation of the insulin signalling network is a prerequisite process for physiological insulin action in target tissues and cells. The first key protein in the insulin signalling pathway is the IR. IR is a tyrosine kinase that catalyses the phosphorylation of its substrates (Saltiel and Pessin, 2002). IRS-1 is one of the major IR substrates in insulin signalling pathway (Taniguchi et al., 2006). A previous study using human adipocytes from individuals with type 2 diabetes
showed that IRS-1 is the main docking protein for the activation of PI 3K in response to insulin (Rondinone et al., 1997).

The present study has demonstrated that in human preadipocytes, IL-1β strongly decreased the protein abundance of insulin-stimulated phosphorylation of insulin signalling molecules. The results showed that upon insulin stimulation, exposure of human preadipocytes to a human recombinant IL-1β (2 ng/ml for 4 and 24 hr) has led to significantly reduced insulin-stimulated phosphorylation of IR at tyrosine residues compared with untreated cells. This finding was in agreement with previous studies that observed IL-1β can reduce the ability of insulin to increase the tyrosine phosphorylation of IR (Lagathu et al., 2006). It is noted that both previous studies have used differentiating preadipocytes and differentiated adipocytes and high concentrations of mouse recombinant IL-1β (10 and 20 ng/ml). Taken together, it suggests that IL-1β could impair the initial and critical node of the insulin signalling pathway by acting on tyrosine phosphorylation of IR in adipose cells.

In insulin signalling pathways, IRS proteins are involved in regulate glucose uptake and, protein synthesis, lipogenesis and cell survival (Cheatham and Kahn, 1995). IRS-1 function is positively regulated once phosphorylation occurs at its tyrosine residues which are required for insulin-stimulated responses. However, upon insulin stimulation IRS-1 at serine/threonine kinases has dual roles which increase or inhibit the insulin action through its signalling pathway (Gual and Tanti, 2005). More specifically, activation of IRS-1 at inhibitory serine 612 is involved in the negative regulation of insulin signalling through IRS-1 by increasing its serine/threonine phosphorylation, which triggers dissociation with its downstream effector (PI3K) (Schmitz-Peiffer and Whitehead, 2003).

In the present study, treatment of preadipocytes with insulin significantly induced serine phosphorylation of IRS-1. This result was in agreement with a previous study that phosphorylation on several residues of serine/threonine kinases are also due to insulin stimulation (Mothe and Van Obberghen, 1996). Moreover, treatment of preadipocytes with IL-1β (2 ng/ml) for 4 hr in the presence of insulin has significantly increased p-IRS-1 (Ser612). However, the increase was less than controls (Figure 4.2). It has been shown that insulin; pro-inflammatory factors and activation of cellular stress pathways increased serine phosphorylation of IRS-1.
A previous study using Fao hepatoma cells has found that TNF-α induces serine phosphorylation of IRS-1 through activation of serine kinases. This increased serine phosphorylation interferes with tyrosine phosphorylation of IRS-1 and impairs insulin action through impairing the association of IRS-1 with PI3K (Kanety et al., 1995). In the current study, IL-1β treatment for 24 hr showed a slight increase in p-IRS-1 (Ser612) in preadipocytes but the strongest stimulatory effect was induced by insulin, suggesting that a possible effect of IL-1β on the induction of serine phosphorylation of IRS-1, which in turn inhibiting the insulin signalling transduction at tyrosine phosphorylation. Consistently, it has been shown that pro-inflammatory cytokine TNF-α has led to a decrease in tyrosine phosphorylation of IRS-1 on tyrosine residues through increased serine phosphorylation of IRS-1 (Kanety et al., 1995, Rui et al., 2001).

The activation of Akt was evaluated by investigating the phosphorylation of Ser473, one of two sites on Akt serine residues (Alessi et al., 1996). In the present study, preadipocytes exposed to IL-1β (2 ng/ml) for 4 hr showed an increase in insulin-stimulated phosphorylation of Akt but the level of increase was slightly lower compared with controls (Figure 4.3). On the other hand, IL-1β treatment for 24 hr led to a significant induction in the protein abundance of insulin-stimulated phosphorylation of Akt; the increase was higher compared with controls stimulated with insulin.

Previous studies using human and murine mature adipocytes have demonstrated that IL-1β has the inhibitory effect on insulin-stimulated phosphorylation of Akt (Lagathu et al., 2006). However, a previous study in human endothelial cells has shown a stimulatory effect of TNF-α or IL-1β on p-Akt in a time-dependent manner (Madge and Pober, 2000). Furthermore, previous studies have reported similar observations but in different cell types using a range of cytokines including IL-3, IL-4 and TNF-α (Hinton and Welham, 1999, Ozes et al., 1999), which showed that induced insulin-stimulated phosphorylation of Akt occurred due to the link between Akt activation and proliferation/survival function in response to these cytokines. Taken together, these results suggest that IL-1β could serve as a stimulus depending on cell type and/or specific function of the protein kinase such as Akt. Interestingly, it was observed that 4 hr exposure to IL-1β has led to decreases in insulin-stimulated
phosphorylation of IRS-1 and Akt, whereas a 24 hr exposure to IL-1β increased phosphorylation of IRS-1 and Akt. These results suggest that the stimulatory effect of IL-1β on phosphorylation of IRS-1 and Akt at serine residues is possibly in a time-dependent manner.

The above data led us to further investigate whether IL-1β is responsible for mediating the effect of MC medium on the insulin signalling molecules in human preadipocytes. The present study showed that there were significant increases in insulin-stimulated phosphorylation of IRS-1(Ser612) in all groups (Figure 4.7). Furthermore, the basal p-IRS-1 was induced by MC medium. This result is in agreement with some previous studies which showed that serine/threonine phosphorylation of IRS-1 is induced by a range of inducers such as okadaic acid on 3T3-L1 adipocytes (Tanti et al., 1994), PMA on 3T3-L1 adipocytes (Jiang et al., 2004), free fatty acids on muscle cells (Yu et al., 2002a), TNF-α on Fao hepatoma cells (Kanety et al., 1995) and amino acids on 3T3-L1 adipocytes (Gual and Tanti, 2005, Haruta et al., 2000). In the present study, blocking the activity of IL-1β using a neutralizing antibody did not alter the effect of MC medium on insulin-stimulated phosphorylation of IRS-1. The result suggests that other macrophage-derived factors in MC medium could contribute to the activation of p-IRS-1 at serine 612. Regarding, the basal p-Akt data, a recent study showed that the phosphorylation of Akt was significantly increased upon treatment with MC medium (derived from murine J774A macrophages), and that MC medium-induced murine preadipocytes survival through Akt/Erk pathway mediated via PDGFR (Molgat et al., 2011). Similarly, the current study observed that basal p-Akt was induced by MC medium in human preadipocytes (Figure 4.8). However, MC medium suppressed the effect of insulin on p-Akt, whereas, IL-1β neutralisation reversed inhibitory effect of MC medium on p-Akt expression in response to insulin (Figure 4.8). Therefore, it is suggested that IL-1β may contribute to some of the effect of MC medium on the activation of Akt in preadipocytes. It might be claimed that the modulating effects of IL-1β on the insulin signalling molecules in preadipocytes could be due to cytotoxicity. However, the current study has shown that there was no cytotoxicity after treatment with high doses of IL-1β as shown in figure 4.12.

The mechanisms of IL-1β in the modulation of macrophage-preadipocytes crosstalk on insulin signalling in adipose tissue are poorly understood. It is suggested that local
inflammation enhanced by macrophage infiltration in adipose tissue could be crucial leading to the impairment of insulin sensitivity (Lumeng & Saltiel, 2011). IL-1β has been particularly implicated in the inflammatory response (Gao and Bing, 2011) and the modulation of insulin sensitivity (Lagathu et al., 2006). Therefore, the second major aim of the present study was to examine the role of IL-1β in mediating macrophages-induced expression and release of pro-inflammatory factors by preadipocytes. The present study showed that MC medium potently induced gene expression and protein release of the pro-inflammatory factors including IL-6, MCP-1 and IL-8 in human preadipocytes. These results are in agreement with previous studies (Lacasa et al., 2007, Gao and Bing, 2011), suggesting that macrophages are strong inducers of an inflammatory state in preadipocytes which in turn could promote macrophages recruitment and maintenance in adipose tissue. Furthermore, inhibiting IL-1β activity in MC medium with a neutralizing antibody led to a significant reduction in gene expression of IL-6, MCP-1 and IL-8 (Figure 4.9). Consistent with the gene expression results, the release of pro-inflammatory factors including IL-6, MCP-1, IL-8 and RANTES by preadipocytes was markedly decreased by blocking IL-1β activity (Figure 4.10). Taken together; these results suggest that IL-1β is considered to be a key mediator for the pro-inflammatory activity of adipose tissue macrophages. The deleterious effect of macrophages on insulin signalling could be modulated by IL-1β through upregulation of the inflammatory response in preadipocytes.

In summary, this study demonstrates that IL-1β may modulate the protein expression of the insulin signalling molecules in human preadipocytes. IL-1β also mediates macrophage-induced modulation of the insulin signalling molecules in human preadipocytes. Blocking IL-1β activity partially reverses the effect of MC medium on protein expression of p-Akt in human preadipocytes. IL-1β blockade also protects against macrophage-stimulated expression and release of the pro-inflammatory cytokines/chemokines including IL-6, RENTS, MCP-1 and IL-8 by preadipocytes. These results suggest that inhibition of IL-1β may reduce macrophage accumulation-associated inflammation and dysregulation of insulin signalling molecules in human adipose tissue.
CHAPTER 5:

Effect of long-term treatment with IL-1β on preadipocyte differentiation and adipocyte functions
5 Effect of long-term treatment with IL-1β on preadipocyte differentiation and adipocyte functions

5.1 Introduction

Based on the results from Chapter 3 and 4, it is suggested that macrophage derived factors, in particular IL-1β, may play a detrimental role on human adipose cell development such as preadipocytes differentiation (adipogenesis), consequently leading to adipose tissue dysfunction. Adipose tissue has many functions; the traditional function, which is similar in all mammals, is storing energy in adipocytes in the form of TGs and this is important for the regulation of energy homeostasis (Korner et al., 2009, Symonds, 2012, Langin et al., 2009).

As mentioned earlier, adipose tissue releases energy, through cellular processes namely lipolysis, in the forms of glycerol and FFAs, when needed by other organs during exercise or fasting periods (physiological conditions) (Cinti, 2007, Trayhurn and Beattie, 2001, Langin et al., 2009). However, adipose tissue released FFAs are elevated in the circulation especially in the obese state which in turn induces adipose tissue remodelling (Sun et al., 2011, Lee et al., 2010, Boden, 2008). Increased FFAs release in obesity is probably due to the impairment of insulin action such as the suppression of lipolysis (Sorisky, 1999, Jensen et al., 1989). Consequently, this causes a cluster of metabolic disorders, including dyslipidaemia, increased hepatic glucose production and impaired peripheral glucose uptake (Despres et al., 1990, Reaven, 1996, Boden, 2006).

In addition to the traditional role, adipose tissue has also been discovered as an endocrine organ producing various bioactive proteins, known as adipokines that have both actions at local (autocrine/paracrine) and systemic (endocrine) levels (Kershaw and Flier, 2004, Mohamed-Ali et al., 1998). Moreover, these adipokines are involved in complex pathways to maintain metabolic and vascular functions, and many of which are linked to immunity and the inflammatory response (Kershaw and Flier, 2004). However, the overexpression and secretions of inflammation related-adipokines in adipose tissue, such as TNF-α, IL-1, IL-6 and MCP-1, have been found to link with a number of metabolic disorders including inflammation and insulin resistance (Tilg and Moschen, 2006).
In obesity, WAT expands through two processes including hypertrophy and hyperplasia. Hypertrophy, an increase in the volume of existing adipocytes prevails in obesity (Lacasa et al., 2007, Hirsch et al., 1989). When adipocytes become terminal cells, they have a lipid buffering capacity of \(~3\ \mu\text{g lipid/cell}\) (Danforth, 2000). This capacity may vary dramatically based on the physiological or pathological conditions. In the obese state this capacity is reduced due to dysfunctional cells (hypertrophied adipocyte) (Le Lay et al., 2001). Thus, excess energy may not be properly stored by the expansion of the adipose tissue (Danforth, 2000). Several studies have shown that a deficit in adipogenesis due to hypertrophied adipocytes is characterized by reduced lipid storage capacity. This in turn leads to the release of FAs into the circulation and favours accumulation of lipids in the liver and muscle with detrimental effects on insulin action in these tissues (Danforth, 2000, Weyer et al., 2000, Heilbronn et al., 2004). Furthermore, studies on obese animals and humans have shown that enlarged adipocytes are less responsive to insulin compared to lean adipocytes, developing insulin resistance (Friedman, 2000, Molina et al., 1989).

Hyperplasia (an increase in the number of new adipocytes) occurs by the proliferation and then differentiation of preadipocytes into adipocytes (Sun et al., 2011). Differentiation of preadipocytes is directed by serial expression of a range of important transcription factors including members of the C/EBP and the PPAR families (Tontonoz et al., 1995). C/EPB\(\alpha\) and PPAR\(\gamma\) are important during proliferation-differentiation conversion and terminal differentiation; aP2 is an adipocyte marker and its relative abundance reflects the terminal differentiation of the adipose cells (Yang et al., 2004). Moreover, both C/EPB\(\alpha\) and PPAR\(\gamma\) transcription factors positively regulate each other and then activate aP2 (Gregoire et al., 1998). Adiponectin and leptin are also considered as mature adipocyte markers (Yang et al., 2004).

During adipose tissue expansion there is an increase in infiltration of macrophages and other immune cells; these cells may constitute the major sources of adipose-derived pro-inflammatory cytokines/chemokines (Lumeng et al., 2007c, Weisberg et al., 2003). In addition to the pro-inflammatory role of macrophages and other immune cells in adipose tissue, preadipocytes and adipocytes can produce a large range of cytokine/chemokine such as TNF-\(\alpha\), IL-6, IL-8, MCP-1 and RANTES;
moreover, the production of these pro-inflammatory factors by preadipocytes and adipocytes can be markedly induced by macrophage-derived factors (Coppack, 2001, Weisberg et al., 2003, Gao and Bing, 2011). It appears that adipose cells and macrophages communicate each other through paracrine crosstalk or direct cellular processes (Bourlier and Bouloumié, 2009). Most previous studies have suggested that increased macrophage infiltration in adipose tissue in obesity could have an anti-adipogenic action (Xu et al., 2003, Sorisky et al., 2013). Recently, two in vitro studies using murine and human preadipocytes showed that MC medium strongly inhibited adipogenesis in both models by suppressing lipid accumulation as well as inhibiting fatty acid synthase, PPARγ, CEBPα, leptin and adiponectin (Lacasa et al., 2007). Adiponectin is produced primarily by adipocytes (Scherer et al., 1995), and has many functions such as anti-diabetic and anti-inflammatory properties (Trujillo and Scherer, 2005). However, pro-inflammatory cytokines including TNF-α, IL-6, IL-1β, IFN-γ, or TGF-β have been found to suppress in vitro preadipocytes differentiation into adipocytes, and also to increase lipolytic activity in adipocytes (Gregoire et al., 1998, Coppack, 2001).

IL-1β has been shown to play an important role in mediating the inflammatory response as well as being involved in a range of cellular processes, including cell proliferation, differentiation and apoptosis (Arend, 2002). Previous studies mostly using murine adipocytes reported that IL-1β inhibits adipogenesis (Lagathu et al., 2006, Xie et al., 2010). However, the precise molecular mechanisms of the anti-adipogenic action of IL-1β in human preadipocytes differentiation are still unclear. More importantly, whether IL-1β mediates macrophages-induced alteration of lipid storage in human adipocyte remain to be established.

5.2 Aim of the study
This chapter was therefore aimed to investigate the role of IL-1β in mediating macrophage-induced adipose tissue remodelling. By using in vitro human cell models, the specific objectives were set below:

- The effect of long-term treatment with IL-1β on the lipid storage functions of adipose tissue by influencing lipid accumulation in human adipocytes;
- The effect of long-term treatment with IL-1β on expression of adipogenic factors that are involved in the development of human mature adipocytes, and
also on expression of pro-inflammatory factors that are involved in adipose tissue remodelling and insulin resistance such as IL-6, MCP-1, IL-8 and RANTES;

- The effect of long-term treatment with IL-1β on expression of the key adipokines;
- The effect of IL-1β blockade on the effect of MC medium-induced lipolysis in human adipocytes.

5.3 Method and materials

5.3.1 Culture of human preadipocytes
Human primary preadipocytes were grown and induced to undergo adipogenesis (as described in section 2.3).

5.3.2 Generation of THP-1 macrophages-conditioned medium
The human THP-1 monocytes were cultured in RPMI-1640 medium as mentioned in details in section 2.4.3. Macrophage-conditioned (MC) medium was then prepared and harvested as previously mentioned in section 2.4.4.

5.3.3 Cell treatment
To investigate whether the long-term treatment of IL-1β inhibits human preadipocytes differentiation and impairs lipid storage function of adipose tissue by influencing lipolysis and lipid accumulation in human adipocytes, human preadipocytes were used and then treated with RPMI-1640 medium (control), IL-1β (1 ng/ml) or (5 ng/ml) from the induction day (Day 0) to day 12 post induction. To investigate whether IL-1β mediates macrophage-induced lipolysis in human adipocytes, the following experiments were carried out: firstly, MC medium was pre-incubated with a human IL-1β neutralizing antibody (2 μg/ml) for 1 hr at 37 °C to block IL-1β activity; adipocytes were then incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody or mouse IgG (as a negative control) for 24 hr. Secondly, differentiated adipocytes were pre-treated with a recombinant human IL-1β receptor antagonist (IL-1RA; Sigma) at 1 μg/ml for 2 hr and then incubated with MC medium in the presence or absence of IL-1RA or IL-1RA alone for 24 hr.
5.3.4 Measurement of lipid accumulation in adipocytes
Lipid content in adipocytes was measured using Oil Red O stain as detailed in section 2.7.1.2. The dye retained in cells was removed with isopropanol and pipetted into a 96 well-plate. After measuring the absorbance by a Benchmark™ Plus Microplate Spectrophotometer (Bio-rad, UK) at 550 nm, lipid levels were associated to total protein amount by BCA protein assay as described in section 2.13.2.

5.3.5 Measurement of glycerol release
Lipolysis was determined as the levels of glycerol in adipocyte culture medium using a colorimetric method as described in section 2.13.2. The absorbance of the samples and standard were then measured using a spectrophotometer (Bio-Rad) at a wavelength of 540 nm. The concentration of glycerol was calculated by a glycerol standard curve.

5.3.6 Real-time PCR
Total RNA was extracted from cells using Trizol as previously mentioned in section 2.9. RNA concentration was determined from the absorbance at 260 nm. First strand cDNA was reverse transcribed from 0.5 µg of total RNA using an iScript first strand synthesis kit in a final volume of 10 µl (Section 2.10). Real-time PCR amplification was performed in a final volume of 12.5 µl, containing cDNA (equivalent to 12.5 ng of RNA), optimized concentrations of primers, TaqMan probe (FAM-TAMRA) and a master mix made from qPCR core kit (Section 2.11). The sequences of primers and probes for human β-actin (control), inflammation factors (IL-6, MCP-1, IL-8 and RANTES), adipogenic factors (C/EPBα, PPARγ and aP2), and hormonal factors (adiponectin and leptin) were as described previously (Table 2.1). Real-time PCR applications were performed using a Stratagene Mx3005P instrument and the PCR cycling conditions were as follows: 95 °C for 10 minutes followed by 40 cycles (95 °C for 15 sec, 60 °C) for 1 minutes. The results were expressed as fold changes of Ct value relative to controls using the data analysis software from the manufacturer (section 2.11).

5.3.7 Cell viability assay
Cell viability was determined by using a colorimetric method for measuring cellular cytotoxicity from the release of lactate dehydrogenase (LDH) into cell culture medium as previously detailed in section 2.14.
5.3.8 Statistical analysis

Data are expressed as means ± SEM or means ± SD. Differences between two groups were analysed by Student’s unpaired t-test; one-way ANOVA coupled with Bonferroni’s t-test was employed for comparison of multi-groups. Differences were considered as statistically significant when p< 0.05.
5.4 Long-term treatment with IL-1β affects preadipocyte differentiation and lipid levels during adipocyte development

This study aimed to investigate whether long-term treatment of IL-1β impairs human preadipocyte differentiation and alters lipid accumulation during adipocyte development. Human primary preadipocytes were induced to undergo differentiation, and then treated with or without IL-1β (control) (from day 0 to day 12). Cells were collected, and lipid content was morphologically and quantitatively determined.

5.4.1 Chronic treatment with IL-1β reduces lipid levels during the differentiation of primary human preadipocytes

As shown by figure 5.1 a-b, treatment with IL-1β (1 and 5 ng/ml) led to reduced lipid levels in differentiated adipocytes (Day 12) as evaluated morphologically by microscopy and Oil red O staining. Although treatment with IL-1β at both doses (1 and 5 ng/ml) reduced lipid levels during adipocytes development the significant reduction was seen at 5 ng/ml (37 %, p< 0.01) compared with controls. These results showed that inhibitory effects were in a dose dependent manner (Figure 5.1 a-b).
Figure 5-1: Effect of long-term treatment with IL-1β (1ng and 5ng/ml) on lipid levels in human mature adipocytes

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<tr>
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<th>No staining</th>
<th>Oil red O staining</th>
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<td>Control</td>
<td><img src="image1.png" alt="Control" /></td>
<td><img src="image2.png" alt="Control" /></td>
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<td>IL-1β (1 ng/ml)</td>
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Human preadipocytes were induced to undergo differentiation. Mature adipocyte samples were collected at day 12 post induction after long treatment with IL-1β (12 days) at two concentrations (1ng/ml and 5ng/ml). The effect of IL-1β on cell morphology, IL-1β inhibits differentiation of human preadipocytes, as assessed by morphology: using Oil Red O staining of neutral lipids (a) at day 12, cultures were photo-graphed and images shown are representative of two experiments. IL-1β induces lipid reduction in a dose-dependent manner. Lipid levels were determined at 510 nm and linked to total protein amount by BCA protein assay (b). Results are expressed as means ± SEM (n=3 per group). **p< 0.01 vs control
5.4.2 Long-term treatment with IL-1β downregulates mRNA levels for key adipogenic factors in human adipocytes

To study whether long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) inhibits gene expression of key adipogenic factors including C/EPBα, PPARγ and aP2, preadipocytes were treated from the onset of induction (day 0) until cells became lipid-laden mature adipocytes (day 12 post induction) as seen in Figure 5.1.a. Total RNA was extracted from cells (day 12) and mRNA levels were determined using real time PCR.

As shown by Figure 5.2. Long-term treatment with IL-1β at all concentrations significantly downregulated mRNA levels of C/EPBα (81%, 81 % and 82 %, all p< 0.001) compared with controls. In Figure 5.2.b, PPARγ mRNA levels were significantly decreased by 1ng/ml IL-1β (38%, p< 0.05), 5 ng/ml IL-1β (37%, p<0.01) and 20 ng/ml IL-1β (40 %, p< 0.01). In addition, the expression profile of aP2 was also significantly downregulated by 5 and 20 ng/ml IL-1β (40%, p< 0.05 and 43%, p< 0.01) compared with controls (Figure 5.2.c). However, the inhibitory effect of the IL-1β was not dose-dependent for the three genes studied. The greatest reduction was observed in C/EPBα gene compared with other adipogenic factors.
Figure 5-2: Long-term treatment with IL-1β reduces mRNA levels of the key adipogenic factors in human adipocytes

Mature adipocytes were collected at day 12 post induction after long-term treatment with IL-1β at various concentrations (1ng/ml, 5 ng/ml and 20 ng/ml). IL-1β treatment decreased (a) C/EBPα, (b) PPARγ and (c) aP2 mRNA levels in human adipocytes compared with controls. Gene expression levels were measured by real-time PCR and normalised to β-actin. Data are expressed as mean ± SEM (n=6 per group). * p< 0.05, **p< 0.01, ***p< 0.001 vs controls
5.4.3 Long-term treatment with IL-1β upregulates mRNA levels of the pro-inflammatory factors (IL-6, MCP-1, IL-8 and RANTES) in human adipocytes

Since pro-inflammatory factors are expressed and secreted by several types of cells in adipose tissue, this study examined whether long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) during differentiation of preadipocytes induces gene expression of the key pro-inflammatory factors including IL-6, MCP-1, IL-8 and RANTES in adipocytes. In this experiment, cells were treated from the onset of induction (day 0) until cells became lipid-laden mature adipocytes (day 12) and mRNA levels were measured in adipocytes at day 12 post-induction.

As shown in figure 5.3.a-d, in contrast with the effect of IL-1β on adipogenic factors, long-term treatment with IL-1β at all concentrations induced a significant upregulation in gene expression of the pro-inflammatory factors. IL-6 mRNA levels were significantly increased by 1 ng/ml IL-1β (186-fold, p< 0.001), 5 ng/ml IL-1β (273-fold, p< 0.001) and 20 ng/ml IL-1β (256-fold, p< 0.001); MCP-1 mRNA levels were significantly increased by 1 ng/ml IL-1β (23-fold, p< 0.001), 5 ng/ml IL-1β (23-fold, p< 0.001) and 20 ng/ml IL-1β (25-fold, p< 0.001); IL-8 mRNA levels were also significantly increased (243-fold, p<0.001), (414-fold, p< 0.001) and (372-fold, p< 0.001) and RANTES (722-fold, p< 0.001), (1469-fold, p< 0.001) and (1260-fold, p< 0.001) compared with controls. Interestingly, the stimulatory effect of IL-1β for both IL-8 and RANTES was dose-dependent and this appeared obvious at 1 and 5 ng/ml concentrations (P< 0.05 and p< 0.01) respectively. The greatest upregulation was observed in RANTES gene expression compared with other pro-inflammatory factors (Figure 5.3).
Figure 5-3: Long-term treatment with IL-1β increases mRNA levels of pro-inflammatory factors (IL-6, MCP-1, IL-8 and RANTES) in human adipocytes

Adipocytes were collected at day 12 post inductions after long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml). IL-1β treatment increased mRNA of the key pro-inflammatory factors (a) IL-6, (b) MCP-1, (c) IL-8 and (d) RANTES (CCL5) in human adipocytes. Gene expression levels were measured by real-time PCR and normalised to β-actin. Data are expressed as mean ± SEM n=6 per group***p< 0.001 vs controls; +p< 0.05 and ++p< 0.01 vs 1 ng/ml of IL-1β
5.4.4 Long-term treatment with IL-1β downregulates mRNA level of adiponectin in human adipocytes

This experiment investigated whether long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) affects gene expression level of adiponectin in adipocytes. Figure 5.4 has shown that long-term treatment with IL-1β at all three concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) significantly decreased mRNA levels of adiponectin (38%, p< 0.05, 37%, p< 0.01 and 40%, p< 0.01) when compared with controls. However, the inhibitory effect was not dose-dependent (Figure 5.4).

5.4.5 Long-term treatment with IL-1β upregulates mRNA level of leptin in human adipocytes

The present experiment investigated the long-term effect of IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) on gene expression level of leptin in adipocytes. As shown by figure 5.5, in contrast to adiponectin result, long-term treatment with IL-1β at all concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml) significantly upregulates mRNA levels of leptin (8-fold, 13-fold and 9-fold, all p< 0.001) when compared with controls.
Figure 5-4: Long-term treatment with IL-1β downregulates mRNA levels of adiponectin in human adipocytes

Adipocytes were collected at day 12 post induction after long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml). IL-1β treatment decreased adiponectin mRNA level in human adipocytes. Gene expression levels were measured by real-time PCR and normalised to β-actin. Data are expressed as mean ± SEM n=6 per group. * p< 0.05, **p < 0.01 vs controls
Figure 5-5: Long-term treatment with IL-1β upregulates mRNA level of leptin in human adipocytes

Adipocytes were collected at day 12 post inductions after long-term treatment with IL-1β at various concentrations (1 ng/ml, 5 ng/ml and 20 ng/ml). IL-1β treatment increased leptin mRNA level in human adipocytes. Gene expression levels were measured by real-time PCR and normalised to β-actin. Data are expressed as mean ± SEM n=6 per group. **p< 0.001 vs controls
5.4.6 MC medium and IL-1β stimulate lipolysis in human adipocytes

To determine whether macrophage-derived factors or IL-1β exposure alters lipid mobilization in differentiated human adipocytes, cells at day 12 post-induction were treated either with TPH-1 MC medium (25%) or with IL-1β (2ng/ml) for 24 hr. Lipolysis was determined as glycerol release from adipocytes into cell culture medium.

It was shown that both MC medium and IL-1β significantly increased glycerol release from differentiated adipocytes into culture medium (>2-fold, p< 0.001) (Figure 5.6.a) and (<1.5-fold, p< 0.001) (Figure 5.6. b) respectively.
Figure 5-6: MC medium and IL-1β stimulate lipolysis in human adipocytes

Adipocytes were treated with either (a) RPMI-1640 medium (control) or THP-1 MC medium for 24 hr or (b) RPMI-1640 medium (control) or RPMI-1640 medium with IL-1β (2 ng/ml) for 24 hr. Lipolysis was determined as glycerol release into the culture medium in (a) and (b). Data are means ± SEM (n=6 per group); **p< 0.01, ***p< 0.001 vs indicated groups
5.4.7 IL-1β neutralizing antibody reverses MC medium-induced lipolysis in adipocytes

As macrophage-derived factors and IL-1β enhance adipocyte lipolysis, we next investigated whether IL-1β mediates macrophages-induced lipolysis in human adipocytes. MC medium was pre-incubated with a human IL-1β neutralizing antibody (2μg/ml) for 1 hr to block IL-1β activity. Differentiated adipocytes (day 12) were then incubated with either RPMI-1640 medium (control), MC medium or MC medium neutralized by IL-1β antibody or mouse IgG for 24 hr. The results showed that adipocytes treated with MC medium for 24 hr significantly increased glycerol release (>2-fold, p< 0.001) compared with controls; however, MC medium-stimulated lipolysis was completely abolished by blocking IL-1β activity with a neutralizing antibody (p< 0.001) (Figure 5.7). However, treatment with IgG (negative control) had no effect on MC medium-induced lipolysis in adipocytes (Figure 5.7).

5.4.8 Blocking IL-1 receptor binding in human adipocytes partially reverses MC medium-induced lipolysis.

The second experimental approach was to block IL-1β receptor binding in adipocytes. Differentiated adipocytes were pre-treated with an IL-1β receptor antagonist (IL-1RA) for 2 hr and then incubated with MC medium in the presence or absence of IL-1RA for 24 hr. As shown in Figure 5.8, adipocytes treated with MC medium significantly increased lipolysis (6.5-fold, p< 0.001) compared with controls. Consistent with the results of the IL-1β neutralizing antibody, MC medium-stimulated lipolysis was partially reversed by blocking IL-1β receptor with an IL-1Ra (p< 0.001). Adipocytes treated with IL-1RA alone (negative control) did not show any difference in glycerol release compared with controls (Figure 5.8).
Adipocytes treated with MC medium for 24 hr showed a marked increase in glycerol release compared with controls. This upregulation was abolished by the pre-treatment of MC medium with an IL-1β neutralizing antibody (2 μg/ml). Data are mean ± SEM (n= 6 per group).***p< 0.001 vs control; +++p< 0.001 vs MC medium group and MC+IgG group

Figure 5-7: IL-1β neutralizing antibody reverses MC medium-induced lipolysis
Figure 5-8: Blocking IL-1 receptor binding in human adipocytes partially reverses MC medium-induced lipolysis

Treatment with MC medium significantly increased lipolysis in adipocytes compared with controls. This up-regulation was abolished by the pre-treatment of adipocytes with a recombinant IL-1RA (1 µg/ml) for 2 hr to block IL-1 receptor binding in adipocytes; adipocytes were then incubated with MC medium in the presence or absence of IL-1RA for 24 hr. Data are mean ± SEM, n= 6 per group. *** p< 0.001 vs control; +++ p< 0.001 vs MC medium group
5.5 Discussion

As reported in previous studies, certain pro-inflammatory cytokines known to be mainly produced by macrophages including, TNF-α and IL-16, may have inhibitory effects on adipogenesis. TNFα has been documented earlier as a strong cytokine that inhibits 3T3-L1 and human preadipocyte differentiation (Torti et al., 1989, Petruschke and Hauner, 1993, Ruan et al., 2002) and induces lipolysis in human adipocytes (Rydén et al., 2004). IL-6 may have paracrine effect which inhibits differentiation of preadipocytes obtained from subcutaneous adipose tissue (Sopasakis et al., 2004). Recently, IL-1β has also been reported as a key anti-adipogenic factor (Lagathu et al., 2006). However, there were conflicting observations about the effect of IL-1β and it is unclear whether IL-1β mediates the effect of macrophage-derived factors on adipogenesis (Simons et al., 2007, Gagnon et al., 2013).

The present study investigated the long-term effect of IL-1β on adipocyte development, including lipid accumulation and the expression of the key adipogenic factors. The study also examined the long-term effect of IL-1β on the expression of the pro-inflammatory cytokines/chemokines and the key adipokines by adipocytes. Finally, whether IL-β acts to mediate the effect of MC medium on lipid mobilization was explored.

The results have shown that exposure of human preadipocytes to IL-1β (1 and 5 ng/ml) during the 12-day period of differentiation impaired adipogenesis. The inhibition with a 5 ng/ml of IL-1β occurred with a significant reduction in the cellular lipid content in human adipocytes. Consistently, cell morphology exhibited a significant inhibition of lipid accumulation in differentiated adipocytes after incubated with IL-1β (Figure 5.1). These results are consistent with a previous study which found that 6-10 days of treatment of murine adipocytes with IL-1β (10 or 20 ng/ml) decreased cellular lipid content (Lagathu et al., 2006). The same study also shown that mature human adipocytes (day 22 post-induction) treated with IL-1β for 7 days had reduced cellular lipid content. These results suggest that IL-1β exposure inhibits the adipogenic process and impairs lipid storage function of adipocytes.

The present study also examined the long-term effect of IL-1β at several concentrations (1, 5 and 20 ng/ml) on the gene expression of the key adipogenic
factors. The results from this study showed that prolonged IL-1β exposure during preadipocytes differentiation has inhibitory effects on the expression of the key adipogenic genes. IL-1β has significantly downregulated mRNA levels of C/EBPα, PPARγ and aP2, suggesting that IL-1β has an inhibitory effect on preadipocyte differentiation.

In contrast to the inhibitory effects of IL-1β on adipogenic factors, the present study showed that IL-1β had a stimulatory effect on pro-inflammatory factors. Prolonged IL-1β treatment significantly upregulated gene expression of pro-inflammatory factors, including IL-6, IL-8, MCP-1 and RANTES in adipocytes. Previous studies have suggested that IL-1β may play a role in the increased production of MCP-1 in adipocytes (Bruun et al., 2005, Fain and Madan, 2005). MCP-1 is a chemokine produced by a variety of adipose cells, including macrophages and human adipocytes (Gerhardt et al., 2001, Gao et al., 2012) and is responsible to recruit macrophages into tissues (Christiansen et al., 2005). A recent study from our group has shown that 24 hr treatment with IL-1β also stimulates IL-6, IL-8 and RANTES production in adipocytes (Gao et al., 2014). Collectively, it is suggested that IL-1β could have a role in inducing pro-inflammatory responses that may be responsible for adipose tissue dysfunction in obesity (Arvin et al., 1996, Di Iorio et al., 2003). Taken together, these results suggest that IL-1β could be a major factor that has linked an adipogenic deficit and an increase in the production of pro-inflammatory factors in obese adipose tissue (Heilbronn et al., 2004, Le Lay et al., 2001).

Macrophages, adipocytes and preadipocytes within adipose tissue have been shown to produce various pro-inflammatory protein factors including but not limited to TNF-α, IL-6, IL-1β, MPC-1 and RANTES (Gao and Bing, 2011, Coppack, 2001, Weisberg et al., 2003, Strissel et al., 2007). Other adipokines such as adiponectin and leptin are mainly produced by adipocytes (Yadav et al., 2013); leptin can also be produced by preadipocytes after stimulation with cytokines (Simons et al., 2007). Both adiponectin and leptin are considered as adipocyte markers in terminal adipocyte differentiation (Yang et al., 2004). Both are important in the regulation of energy homeostasis and insulin action (Havel, 2002). Previous studies have shown that macrophage-secreted factors influence adipokine expression by adipocytes, such as increasing the expression of a wide range of pro-inflammatory genes (O’Hara et
al., 2009) and stimulating leptin expression (Hirasaka et al., 2007) while decreasing adiponectin (Constant et al., 2006). In addition, several in vitro studies demonstrated that adiponectin expression and secretion levels were reduced by pro-inflammatory factors such as TNF-α (Kappes and Löffler, 1999, Bruun et al., 2003a, Degawa-Yamauchi et al., 2005, Wang et al., 2005a) and IL-6 (Fasshauer et al., 2003, Bruun et al., 2003a). However, there are few studies on the long-term effect of IL-1β on the expression of adiponectin and leptin. Therefore, the present study examined the effect of long-term treatment with IL-1β at several concentrations (1, 5 and 20 ng/ml) on gene expression of adiponectin and leptin in differentiated adipocytes.

Results in the current study have shown that prolonged exposure to IL-1β had an inhibitory effect on adiponectin mRNA levels. However, the inhibitory effect of IL-1β was not in a dose dependent manner. Adiponectin has been considered as a positive regulator of insulin sensitivity through regulating glucose and lipid metabolism in insulin target tissues (Berg et al., 2002). Adiponectin also possesses anti-inflammatory and antidiabetic properties (Ouchi et al., 2001). Considering the antagonistic actions between the role of adiponectin and IL-1β (Lagathu et al., 2006, Lihn et al., 2004, Simons et al., 2007), reduced adiponectin expression by IL-1β may enhance inflammation and insulin resistance in obesity.

In contrast to adiponectin, the expression of leptin is increased with the increase in body weight (Yadav et al., 2013). In addition to inhibiting food intake and increasing energy expenditure (Mantzoros, 1999), leptin is involved in the inflammatory response (Bernotiene et al., 2006). Leptin is generally considered as a pro-inflammatory factor (cytokine) due to the similarity in protein structure between leptin and its receptors with some cytokines such as IL-6 (Dessolin et al., 1997). In this study, our results showed that prolonged treatment with IL-1β had stimulatory effect on leptin gene expression, suggesting that IL-1β may act together with leptin to stimulate inflammation in obesity. In agreement with the effect of IL-1β on adipogenesis, previous in vitro and in vivo studies demonstrated that leptin increased lipolysis and inhibited adipocyte lipogenesis (Harris, 2014). It could be suggested that IL-1β and leptin may work synergistically to mediate the inhibition of adipogenesis and stimulation of lipolysis.
The present study observed that MC medium reduces the lipid storage function of human adipocytes through increased lipolysis. Interestingly, IL-1β at 2 ng/ml concentration was shown to have a similar potency as the MC medium to induce lipolysis in differentiated adipocytes (Figure 5.6 a-b). However, it is not known whether IL-1β acts as a key factor in mediating the effect of MC medium. In the present study, our data showed that the induction of lipolysis by MC medium was completely attenuated by IL-1β neutralization that blocks IL-1β activities. Therefore, the current study provides evidence for the role of IL-1β in mediating macrophage-induced lipolysis. This is further supported by the observation from the current study that the effect of MC medium was reduced by an IL-1RA which blocks IL-1 receptor binding in adipocytes (Figure 5.8).

In summary, this study has focused on the effect of IL-1β, as a major cytokine produced by macrophages, on adipogenesis and function of human adipocytes. Prolonged exposure to IL-1β reduced lipid accumulation and the expression of the adipogenic factors by human adipocytes. This is associated with an increase in the expression of pro-inflammatory factors (IL-6, IL-8, MCP-1, RANTES and leptin) and a downregulation of adiponectin. Furthermore, this study has used two IL-1β blocking approaches to confirm that IL-1β could be a key player that mediates macrophage-induced reduction in lipid storage ability of human adipocytes.
CHAPTER 6:
IL-1β mediates macrophage-induced impairment of insulin signalling in human primary adipocytes
6 IL-1\(\beta\) mediates macrophage-induced impairment of insulin signalling in human primary adipocytes

6.1 Introduction

Based on the results from Chapters 3, 4 and 5, it is suggested that macrophage-derived factors particularly IL-1\(\beta\) may induce impairment of insulin signalling by affecting the key insulin signalling molecules in human primary adipocytes. In addition to skeletal muscle and liver, adipose tissue develops insulin resistance in obesity (Hotamisligil, 2000). The reduced insulin sensitivity in adipocytes may increase FFAs release into the circulation inducing hepatic and muscle insulin resistance (Smith, 2002a). Therefore, obese adipose tissue plays a crucial role in initiating systemic insulin resistance and the development of type 2 diabetes (Xu et al., 2003, Cancello and Clement, 2006).

In obesity, adipose tissue is remodelled and the infiltration of macrophages is increased (Suganami and Ogawa, 2010). These cells may constitute the major sources of adipose-derived pro-inflammatory cytokine/chemokines (Cinti et al., 2005, Lumeng et al., 2007b, Fain, 2010, O'Rourke et al., 2009). Macrophage infiltration in excess fat has been reported in both mice and humans, and is implicated in the development of insulin resistance (Bourlier and Bouloumié, 2009). It is estimated that over 20-30 million macrophages accumulate per kilogram of excess fat in obese human individuals (Lumeng and Saltiel, 2011b).

Studies in murine and human have shown that macrophages crosstalk with adipocytes and can lead to the alteration of adipocyte function, such as inhibiting of preadipocyte differentiation (Constant et al., 2006, Gao et al., 2010), stimulating inflammatory responses (Lacasa et al., 2007) and reducing insulin sensitivity (Lumeng et al., 2007c). Therefore, identification of the key factors that mediate the effect of macrophages on adipocytes is important as it may provide potential therapeutic targets for obesity-related insulin resistance. IL-1\(\beta\), a key pro-inflammatory cytokine, is produced mainly by monocytes and macrophages, which is then activated through caspase-1 via the NLRP3 inflammasome complex (Agostini et al., 2004). Recent studies suggest that IL-1\(\beta\) may be a candidate in the development
of insulin resistance and type 2 diabetes (Tack et al., 2012a, Wen et al., 2011). An increase in circulating levels of IL-1β together with IL-6 has been shown to increase the risk of type 2 diabetes (Spranger et al., 2003b). Studies in obese mice and diabetic rats showed that IL-1β inhibition reduces hyperglycemia and tissue inflammation (Owyang et al., 2010, McGillicuddy et al., 2011, Ehses et al., 2009, Sauter et al., 2008).

In the insulin signalling pathway, IR is a core node protein activated by insulin and other polypeptide growth factors (Taniguchi et al., 2006). Once insulin is bound to the IR auto-phosphorylation occurs and the IR auto-phosphorylation leads to subsequent phosphorylation of its substrates including IRS-1 (Cheatham and Kahn, 1995). IRS-1, as major substrate of the insulin receptor, is required to activate PI3K in response to insulin, which promotes the phosphorylation of protein kinase B (also known as Akt) and subsequent glucose uptake (Taniguchi et al., 2006, Schmitz - Peiffer and Whitehead, 2003). It has been reported that gene expression of IL-1β in adipose tissue is upregulated in obese mice and humans (Juge-Aubry et al., 2004, Lagathu et al., 2006). IL-1β is also released by human adipose tissue explants mainly by non-fat cells (Fain, 2006, Koenen et al., 2011b), and the release is increased in obesity (Nov et al., 2010). Previous studies mostly using murine 3T3-L1 adipocytes have shown that IL-1β at a very high dose (20 ng/ml) reduced IRS-1 protein expression and GLUT-4 mRNA levels, and prolonged treatment abolished insulin-stimulated phosphorylation of IRS-1 and Akt (Lagathu et al., 2006, Xie et al., 2010). However, the effect of macrophage-adipocyte crosstalk on insulin signalling in human adipose tissue is largely unknown. Furthermore, whether IL-1β mediates macrophage-induced insulin resistance in human and rodent adipocytes has not been reported.

6.2 Aim of the study
The aim of this study was therefore to investigate the role of IL-1β in the macrophage-adipocyte crosstalk which affects insulin signalling in human adipocytes. By using in vitro human cell models including primary adipocytes, THP-1 macrophages (cell line) and primary macrophages, the specific objectives were set below:
• To investigate whether THP-1 macrophage-derived factors, using MC medium, induce the impairment of insulin signalling pathways in human primary adipocytes;

• To examine whether IL-1β induces impairment of the insulin signalling pathway in human primary adipocytes;

• To examine whether blocking IL-1β activity reverses the effects of MC medium on the insulin signalling pathway, and on cytokine/chemokine and adiponectin release in human primary adipocytes;

• To examine whether blocking the IL-1 receptor binding in human adipocytes reverses the effect of MC medium on the insulin signalling pathway, and also on cytokine/chemokine and adiponectin release in adipocytes;

• To examine whether inhibiting IL-1β production by macrophages reverses the effects of MC medium on expression of insulin signalling molecules, and also on cytokine/chemokine release in adipocytes.

• To investigate whether human primary macrophage-derived factors, using MC medium, impair the insulin signalling pathway and induce pro-inflammatory factors release in human primary adipocytes.

6.3 Material and methods

6.3.1 Culture of human adipocytes

Human primary preadipocytes were grown and induced to undergo differentiation until cells became lipid-laden mature adipocytes (day 12 post induction) (as described in section 2.3).

6.3.2 Cell treatment

According to the specific experimental design, the cells were treated with different agents as detailed below.

6.3.2.1 Effect of THP-1 macrophage conditioned medium

To assess the effect of macrophage-derived factors on insulin signalling, differentiated adipocytes were incubated with RPMI-1640 medium (control) or THP-1 MC medium for 24 hr as previously described in Chapter 2.
6.3.2.2 Effects of Human recombinant IL-1β
To assess the effect of IL-1β on insulin signalling, differentiated adipocytes were treated with RPMI-1640 medium or IL-1β (2 ng/ml) for 24 hr.

6.3.2.3 Effect of IL-1β inhibition
To investigate whether IL-1β mediates the effects of MC medium on insulin signalling, three of the following experiments were carried out. Firstly, MC medium was pre-incubated with a human IL-1β neutralizing antibody (2 µg/ml) for 1 h at 37 °C to inactivate IL-1β activity. Differentiated adipocytes were then incubated with either RPMI 1640 medium (control), MC medium or MC medium neutralized by IL-1β antibody or mouse IgG for 24 hr. Secondly, to block the IL-1β receptor in adipocytes, differentiated adipocytes were pre-treated with human recombinant IL-1β receptor antagonist (IL-1RA) at 1 µg/ml for 2 h and then incubated with MC medium in the presence or absence of IL-1RA for 24 hr. Finally, to inhibit IL-1β production by macrophages, THP-1 cells were incubated with RPMI 1640-medium (serum free) as controls or the 50 µM caspase1 inhibitor (Ac-YVAD-CMK) in RPMI 1640 medium (serum free) for 48 hr, with fresh medium replenished at 24 hr; the medium was collected from macrophages without treatment (MC medium) or treated with caspase 1 inhibitor (MC medium + caspase 1 inhibitor). Differentiated adipocytes were then incubated with either RPMI-1640 medium (control), MC medium or the MC medium + caspase 1 inhibitor for 24 hr.

6.3.2.4 Effects of primary macrophage conditioned medium
To further examine whether IL-1β mediates the effect of primary macrophages on adipocyte insulin signaling and the inflammatory response, MC medium generated from human PBMC-derived macrophages was used (kindly provided by Dr. Chris Ford). Differentiated human adipocytes were incubated with either RPMI 1640 medium (control), MC medium, MC medium neutralized by an IL-1β antibody, MC medium neutralized by an IL-1β antibody and a TNFα antibody, mouse IgG or MC medium with an IL-1RA for 24 hr. At the end of each experiment, cells and the culture media were collected and stored at -80°C until analysis.
6.3.3 Western blotting

For assessing protein expression Western blotting method was used. For the insulin response study, primary antibodies for IRS-1, PI3Kp85α (both from Cell Signalling), Phospho-insulin receptor (Y1185) (Abcam, UK) and phospho-Akt (S473) (New England BioLabs Ltd, Hitchin, UK) were used and diluted at 1:1000. GLUT-4 (Millipor, UK) was used and diluted at 1:2000. GAPDH (Abcam, UK) diluted at 1:2000 or Akt (Cell Signalling) diluted at 1:1000 were used as a loading controls. After 4 washes with 1xTTBS, anti-rabbit secondary antibody (New England BioLabs Ltd, Hitchin, UK) at 1:2000 dilution (or 1:20000 for GAPDH) were used. Signals were detected by chemiluminescence (West Pico kit, Pierce, Loughborough, UK) and scanned using the Molecular Imager ChemiDoc XRS+ System (Bio-Rad). The size of the protein bands detected was estimated with PageRuler protein markers (Fermentas, York, UK). The membrane was further probed with GAPDH (Abcam, Cambridge, UK) or total Akt.

6.3.4 Measurement of cytokine/chemokine and adiponectin release

The secretion levels of IL-1β by THP-1 macrophages or PBMC-derived macrophages and of IL-6, MCP-1, IL-8, RANTES and adiponectin by adipocytes were determined as the protein concentrations in cell culture medium, using ELISA kits (R&D Systems, Abingdon, UK).

6.3.5 Cell viability assay

Cell viability was determined by using a colorimetric method for measuring cellular cytotoxicity from the release of lactate dehydrogenase (LDH) into cell culture medium (See section 2.14).

6.3.6 Statistical analysis

Data are expressed as means ± SEM or means ± SD. Differences between two groups were analyzed by Student’s unpaired t-test; one-way ANOVA coupled with Bonferroni’s t-test was employed for comparison of multiple-groups. Differences were considered as statistically significant when p < 0.05.
6.4 Macrophage-derived factors induce the impairment in the insulin signalling pathway in human primary adipocytes

6.4.1 Macrophage-derived factors inhibit the expression of insulin signalling molecules

To study the effects of macrophage-derived factors on the insulin signalling pathway in human adipocytes, human primary adipocytes (at day 12 post-differentiation) were incubated with RPMI medium or MC medium for 24 hr and the effect of macrophage-derived factors on protein expression of IRS-1, PI3K p85 and GLUT-4 as the key insulin signalling molecules was examined. Results have shown that treatment with MC medium for 24 hr led to a significant reduction in protein abundance of all the proteins; IRS-1 protein was reduced by 40% (p< 0.01), PI3K p85 was reduced by 28% (p< 0.05) and GLUT-4 was reduced by 51% (p< 0.01) in human primary adipocytes (Figure 6.1 a-e).

6.4.2 Macrophage-derived factors inhibit insulin-stimulated phosphorylation of IR (Y1185) and Akt (Ser473)

In this study, human adipocytes were incubated with RPMI medium (control) or MC medium for 24 hr before stimulation with 100nM insulin. In control groups, insulin stimulated phosphorylation of IR level was significantly increased (by 3.5-fold, p<0.001) in insulin-treated adipocytes compared with unstimulated adipocytes (Figure 6.2 a-b). In contrast, adipocytes exposed to MC medium, had a reduction in insulin-stimulated phosphorylation of IR (by 2-fold, p< 0.001) compared with the corresponding controls (Figure 6.2 a-b). The level of serine phosphorylation of Akt was also examined and results showed that the p-Akt level was also increased but not statistically significant in response to insulin compared with basal levels in controls. However, adipocytes exposed to MC medium resulted in a significant reduction in phosphorylation of Akt at ser473 (p< 0.05) compared with the corresponding controls (Figure 6.3 a-b).
Figure 6-1: Protein expression of the insulin signalling molecules (IRS-1, PI3K p85α and GLUT-4) in human adipocytes is reduced by MC medium.
MC medium reduces protein expression of insulin signalling molecules in human adipocytes. Adipocytes (at day 12 post-differentiation) were treated with RPMI-1640 medium (control) or THP-1 MC medium for 24 hr. Cell lysates were collected and analysed by western blotting and densitometry, using antibodies to IRS-1 and PI3K p85 (a, b, c) and GLUT-4 (d, e). Data are means ± SEM (n=3 or 4 per group). *p< 0.05, **p< 0.01 vs controls.
Figure 6-2: Insulin-stimulated phosphorylation of IR in human adipocytes is reduced by MC medium

For measuring basal and insulin-stimulated phosphorylation of IR, adipocytes were incubated with RPMI-1640 or MC medium for 24 hr before stimulation with 100 nM insulin; IR phosphorylation was analysed by western blotting and densitometry. Total Akt and GAPDH were used as loading controls (a, b). Data are means ± SEM (n=3 per group). ***p< 0.001 vs indicated group.
Figure 6-3: Insulin-stimulated phosphorylation of Akt (Ser473) in human adipocytes is reduced by MC medium

For measuring basal and insulin-stimulated Akt phosphorylation, adipocytes were incubated with RPMI-1640 or MC medium for 24 hr before stimulation with 100nM insulin; Akt phosphorylation at Ser473 (p-Akt) was analysed by western blotting and densitometry. Total Akt and GAPDH were used as loading controls (a, b). Data are means ± SEM (n=3 per group). *p< 0.05 vs controls.
6.4.3 IL-1β induces impairment in the insulin signalling pathway in human primary adipocytes

6.4.3.1 IL-1β inhibits the protein expression of insulin signalling molecules
To determine the effects of IL-1β on the expression of insulin signalling molecules in human adipocytes, human primary adipocytes (at day 12 post-differentiation) were incubated with or without 2 ng/ml of IL-1β for 24 hr and the effect of IL-1β on protein expression of IRS-1, PI3K p85 and GLUT-4 in adipocytes was examined. Consistent with the results of MC medium treatment which potently reduced the insulin signaling molecules, IL-1β significantly reduced protein abundance of IRS-1 (by 20%), PI3K p85 (by 28%) and GLUT-4 (by 21%) (All p< 0.01) (Figure 6.4 a-e).

6.4.3.2 IL-1β inhibits insulin-stimulated phosphorylation of IR and Akt
Because IL-1β inhibits insulin signalling molecules, it becomes important to study the effect of IL-1β on proximal insulin signaling steps involved in this process. Adipocytes were treated as described above and then stimulated with or without insulin (100 nM/ml) for 5 minutes. IL-1β significantly decreased insulin-stimulated phosphorylation of IR at Y1185 (by 60%, p< 0.05) (Figure 6.5 a-b) and insulin-stimulated phosphorylation of Akt at ser473 (by 50%, p< 0.001) (Figure 6.6 a-b) compared with the corresponding controls.
Figure 6-4: Protein expression of the insulin signalling molecules (IRS-1, PI3K p85α and GLUT-4) in human adipocytes is reduced by IL-1β
IL-1β impairs the insulin signaling pathway in human adipocytes. Differentiated adipocytes were cultured in the presence of IL-1β (2 ng/ml) or RPMI-1640 (control) for 24 hr. Protein expression of IRS-1 and PI3K p85α (a-c), and GLUT-4 (d,e) were determined in cell lysates by western blotting and densitometry. Data are expressed as means ± SEM (n= 5 or 6 per group). **p< 0.01 vs controls
Figure 6-5: IL-1β inhibits insulin-stimulated phosphorylation of IR in human adipocytes

Adipocytes were treated with IL-1β (2 ng/ml) or RPMI-1640 (control) for 24 hr followed by stimulation with 100 nM insulin; IR phosphorylation was analysed by western blotting and densitometry. Total Akt and GAPDH were used as loading controls (a, b). Data are expressed as means ± SEM (n= 3 per group). *p< 0.05, ***p< 0.001 vs indicated groups
Figure 6-6: IL-1β inhibits insulin-stimulated phosphorylation of Akt in human adipocytes

Adipocytes were treated with IL-1β (2 ng/ml) or RPMI-1640 (control) for 24 hr followed by stimulation with 100 nM insulin; Akt phosphorylation at Ser473 (p-Akt) was analysed by western blotting and densitometry. Total Akt and GAPDH were used as loading controls (a, b). Data are expressed as means ± SEM (n=3 per group). *p< 0.05, ***p< 0.001 vs indicated groups.
6.4.4 Blocking IL-1β activity reduces the effects of the MC medium on the insulin signalling pathway in human primary adipocytes

6.4.4.1 Blocking IL-1β activity reverses the effects of MC medium on protein expression of insulin signalling molecules.

A recent pilot study of our lab has shown that blocking IL-1β activity reduced effects of MC medium on expression of genes involved in insulin signalling pathway (data not shown), this study was aimed to examine whether it is effective at the protein levels. The results showed that MC medium significantly reduced protein abundance of IRS-1 (46%, \( p< 0.01 \)), PI3K p85α (31%, \( p < 0.01 \)) and GLUT-4 (53%, \( p < 0.05 \)) in adipocytes (Figure 6.7 a-f); however, blocking IL-1β activity with a neutralizing antibody abolished the effect induced by MC medium on IRS-1 ( \( p < 0.001 \)) and GLUT-4 (\( p < 0.05 \)) (Figure 6.7 a-f). Treatment with IgG (as a negative control) did not alter the effect of MC medium.

6.4.4.2 Blocking IL-1β activity reverses the effects of MC medium on insulin-induced phosphorylation of Akt.

It was shown that MC medium also led to a reduction in insulin-stimulated phosphorylation of Akt at ser473 compared with controls (>3-fold, \( p < 0.001 \)) but this effect was blunted by IL-1β neutralization (\( p < 0.05 \)) (Figure 6.8 a-b). Treatment with IgG (as a negative control) did not affect the action of MC medium (Figure 6.8 a-b).
Figure 6-7: Blocking IL-1β activity reverses the effects of MC medium on protein expression of the insulin signalling molecules in human adipocytes.
Inhibition of IL-1β activity abolishes the inhibitory effect of macrophages on protein expression of IRS-1, PI3K p85α and GLUT-4 by human adipocytes. Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (2 μg/ml) or mouse IgG (2 μg/ml, as negative control) for 24 hr. Cell lysates were analysed by western blotting and densitometry, using antibodies to IRS-1 (a, b), PI3K p85α (c, d) and GLUT-4 (e, f). Data are means ± SEM (n=3 per group). *p< 0.05, **p< 0.01, ***p< 0.001 vs indicated groups.
Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (2 μg/ml) or mouse IgG (2 μg/ml, as negative control) for 24 hr followed by stimulation with or without insulin (100 nmol/ml) for 5 min; Akt phosphorylation at Ser473 (p-Akt) was analysed by western blotting and densitometry. Total Akt and GAPDH were used as loading controls (a, b). Representative blots are shown; data are means ± SEM (n=3 per group). ***p< 0.001 vs indicated groups
6.4.4.3 Blocking IL-1β activity reverses the stimulatory effect of MC medium on cytokine/chemokine release by human adipocytes.

In addition, we evaluated whether blocking IL-1β activity inhibits the effect of MC medium on the release of pro-inflammatory factors which are known to impair insulin signalling in adipocytes.

As shown in Figure 6.9 a-d, basal secretion of cytokine and chemokines by adipocytes was barely detectable (IL-6, IL-8 and RANTES) or very low (MCP-1 with a mean value of 140 pg/ml). MC medium potently stimulated the release of IL-6 (up to 19623 pg/ml), MCP-1 (42776 pg/ml), IL-8 (88806 pg/ml) and RANTES (5618 pg/ml) but this induction was blunted by IL-1β neutralization (all p< 0.001). Treatment with IgG did not affect the action of MC medium in comparison with IL-1β neutralization groups for all IL-6, IL-8 and RANTES and MCP-1 releases (p< 0.001). To distinguish the production of pro-inflammatory factors by adipocytes and macrophages, the basal levels of the cytokines in the THP-1 macrophage medium were shown as ‘MC alone’ (mean ± SEM): IL-6 (42 ± 2 pg/ml), MCP-1 (127±40 pg/ml), IL-8 (31623 ± 1937 pg/ml) and RANTES (1902 ± 237pg/ml) (Figure 6.9 a-d).

6.4.4.4 Blocking IL-1β activity reduces the inhibitory effect of MC medium on adiponectin release by human adipocytes

This study also evaluated whether blocking IL-1β activity inhibits the effect of MC medium on the release of adiponectin (adipokine). As shown in Figure 6.10, basal secretion of adiponectin was significantly higher with a mean value of 22343 pg/ml in control group. MC medium potently inhibited the release of adiponectin with a mean value of 6448 pg/ml but this inhibition was reduced by IL-1β neutralization (p< 0.001). Treatment with an IgG did not affect the effect of MC medium.
Figure 6-9: Blocking IL-1β activity reverses the stimulatory effects of MC medium on cytokine/chemokine release by human adipocytes

Inhibition of IL-1β activity reverses macrophage-induced cytokine release by human adipocytes. Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (2 μg/ml), mouse IgG (2 μg/ml, as negative control) or MC medium alone (without cells) for 24 hr. The release of IL-6 (a), MCP-1 (b), IL-8 (c) and RANTES (d) by adipocytes was measured as protein concentrations in cell culture medium by ELISAs. Data are means ± SEM (n=6 per group). ***p< 0.001 vs indicated groups
Inhibition of IL-1β activity partially reverses macrophage-reduced adiponectin release by human adipocytes. Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (2 μg/ml), mouse IgG (2 μg/ml, as negative control) for 24 hr. The release of adiponectin by adipocytes was measured as protein concentrations in cell culture medium by ELISA. Data are means ± SEM (n=6 per group). *p< 0.05, ***p< 0.001 vs indicated groups
6.4.5 Blocking IL-1 receptor binding in human primary adipocytes

6.4.5.1 Blocking IL-1 receptor binding in human adipocytes reverses the inhibitory effect of MC medium on expression of insulin signalling molecules

To further examine whether IL-1β has a key role in macrophage-induced impairment of insulin signalling, a recombinant IL-1RA was used to block IL-1 receptor binding in adipocytes. Adipocytes treated with MC medium significantly reduced protein expressions of IRS-1 (p< 0.001), PI3K p85α (p<0.05) and GLUT-4 (p<0.001) compared with controls. However, blocking IL-1 receptor binding by the IL-1RA blunted the inhibitory effect of MC medium on IRS-1 (p< 0.01), PI3K p85α (p< 0.05) and GLUT-4 (p< 0.01) in adipocytes (Figure 6.11 a-f).

6.4.5.2 Blocking IL-1 receptor binding reverses the stimulatory effect of MC medium on cytokine/chemokine release by human adipocytes

This study also evaluated whether blocking the IL-1 receptor binding inhibits the effect of MC medium on the release of pro-inflammatory factors which are known to impair insulin signaling by adipocytes. Consistent with data shown in section 6.4.4.3, basal secretion of cytokines/chemokines was barely detectable (IL-6 and IL-8) or very low (MCP-1 with a mean value of 275 pg/ml) by adipocytes; MC medium potently stimulated the release of IL-6 (up to 10934 pg/ml), IL-8 (46572 pg/ml) and MCP-1 (26282 pg/ml). This induction was largely reversed by blocking IL-1β receptor binding as there were marked decreases in protein release of IL-6 (97%), MCP-1 (93%) and IL-8 (90%) by human adipocytes (all p< 0.001; Figure 6.12 a-c).

6.4.5.3 Blocking IL-1 receptor binding reverses the inhibitory effects of MC medium on adiponectin release by human adipocytes

This study next evaluated whether blocking the IL-1 receptor binding inhibits the effects of MC medium on the release of adiponectin which is known to enhance insulin signaling by adipocytes. Consistent with data shown in section 6.4.4.4, basal secretion of adiponectin was markedly high with a mean value of 17638 pg/ml in control adipocytes; MC medium potently inhibited the release of adiponectin with a
mean value 6162 pg/ml. This inhibition was largely reversed by blocking IL-1β receptor binding as there was a marked increase in protein release of adiponectin (56%, p< 0.001) (Figure 6.13).
Figure 6-11: Blocking IL-1 receptor binding reverses the effects of MC medium on protein expression of insulin signalling molecules in human adipocytes
Blocking IL-1 receptor with IL-1 receptor antagonist reverses the effects of MC medium on protein expression of IRS-1, PI3K p85α and GLUT-4 in human adipocytes. Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium or MC medium with a recombinant IL-1 receptor antagonist (IL-1RA, 1 µg/ml) for 24 hr. Cell lysates were analysed by western blotting and densitometry, using antibodies to IRS1 (a, b), PI3K p85α (c, d) and GLUT-4 (e, f). Data are expressed as means ± SEM (n=3 per group). **p< 0.01, ***p< 0.001 vs indicated groups
Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium or MC medium with a recombinant IL-1 receptor antagonist (IL-1RA, 1 µg/ml) for 24 hr. The release of IL-6 (a), MCP-1 (b), and IL-8 (c) by adipocytes was measured as protein concentrations in cell culture medium by ELISA. Data are means ± SEM (n=6 per group). ***p< 0.001 vs indicated groups
Figure 6-13: Blocking IL-1 receptor binding reverses the inhibitory effect of MC medium on adiponectin release in human adipocytes

Differentiated adipocytes were incubated with either RPMI-1640 medium (control), MC medium or MC medium with a recombinant IL-1 receptor antagonist (IL-1RA, 1 µg/ml) for 24 hr. The release of adiponectin by adipocytes was measured as protein concentrations in cell culture medium by ELISA. Data are means ± SEM (n=6 per group). ***p< 0.001 vs indicated groups
6.4.6 Inhibiting IL-1β production by macrophages protects insulin signalling pathway and reduced cytokines/chemokines release in human adipocytes.

6.4.6.1 Inhibiting IL-1β production by macrophages reduces the inhibitory effects of MC medium on the protein expression of insulin signalling molecules

To further examine the role of IL-1β in mediating the effects of MC medium on insulin signalling pathway, IL-1β release by THP-1 macrophages was suppressed with an inhibitor of caspase 1 (IL-1β converting enzyme). Caspase-1 inhibitor (50 µM) significantly reduced IL-1β production by THP-1 macrophages by 84% (317 ± 56 vs 1936 ±190 pg/ml, p< 0.001). Adipocytes were then incubated with RPMI medium (control) or the medium from THP-1 macrophages treated with or without the caspase-1 inhibitor for 24 hr. As shown in Figure 6.12 a-d, MC medium treatment significantly decreased protein expression of IRS-1, PI3K p85α and GLUT-4 in adipocytes (all p< 0.001); however, this inhibition was partially (for IRS-1 and PI3K p85α) or totally (GLUT-4) abolished in adipocytes exposed to MC medium derived from THP-1 macrophages treated with the caspase-1 inhibitor (Figure 6.14 a- d).

6.4.6.2 Inhibiting IL-1β production by macrophages reduces the stimulatory effects of MC medium on the cytokine/chemokines release by human adipocytes

The effect of blocking IL-1β production by macrophages on the release of pro-inflammatory factors was also examined in this study. The MC medium with inhibited IL-1β production reduced protein release of IL-6 (29%, p< 0.05), IL-8 (86%, p< 0.001), MCP-1 (19%, p< 0.05) and RANTES (66%, p< 0.001) by adipocytes compared with the corresponding group treated with MC medium (Figure 6. 15 a-d).
Figure 6-14: Inhibiting IL-1β production by macrophages reduces the effects of MC medium on protein expression of IRS-1, PI3K p85α and GLUT-4 in human adipocytes.

(a) Blot analysis showing the expression levels of IRS-1, PI3Kp85α, GLUT4 and GAPDH in control, MC, and MC + Caspase 1 inhibitor conditions.

(b) Bar graph demonstrating the fold change in IRS1/GAPDH expression levels compared to control.
Inhibition of IL-1β production by THP-1 macrophages reduces the effect of MC medium on protein expression of IRS-1, PI3K p85α and GLUT-4 by adipocytes. THP-1 macrophages were incubated with RPMI-1640 medium (control) or the caspase 1 inhibitor (50 µM) for 48 hr (with freshly changed medium at 24 hr) and the culture medium was collected. Differentiated adipocytes were then treated with either RPMI-1640 medium (control), MC medium or MC medium in the presence of caspase 1 inhibitor for 24 hr. Cell lysates were analysed by western blotting and densitometry, using antibodies to IRS-1, PI3K p85α and GLUT-4 (a, b, c, d). Data are expressed as means ± SEM (n=6 per group). *p< 0.05, **p< 0.01, ***p< 0.001 vs indicated groups.
Inhibition of IL-1β production by THP-1 macrophages using a caspase 1 inhibitor reduces the effect of MC medium on cytokine/chemokine release including IL-6 (a), MCP-1 (b), IL-8 (c) and RANTES (d) by adipocytes. The levels of cytokine/chemokines were measured by ELISA. Data are expressed as means ± SEM (n=6 per group). *p< 0.05, ***p< 0.001 vs indicated groups.
6.5 Human primary macrophage-derived factors inhibit insulin signalling and stimulate cytokine/chemokines release by human adipocytes and the effect of blocking IL-1β

6.5.1 Human primary macrophage-derived factors inhibit the protein expression of IRS-1 and GLUT-4, and the effect of blocking IL-1β

To further examine whether IL-1β mediates the effect of primary macrophages on adipocyte insulin signalling and the inflammatory response, MC medium generated from human PBMC-derived macrophages were used. MC medium significantly reduced protein expression of IRS-1 (Figure 6.16 a-b) and GLUT-4 (Figure 6.17 a-b) in adipocytes (all p< 0.01). This inhibition was reversed by IL-1β neutralization (both p<0.05) or by both IL-1β and TNFα neutralization (p< 0.05 for IRS-1 and p< 0.01 for GLUT-4) or an IL-1β RA (all p< 0.01).

6.5.2 Human primary macrophage-derived factors inhibit insulin-stimulated phosphorylation of Akt

In line with the result from the THP-1 MC medium that reduced phosphorylation of Akt, human primary MC medium also led to a reduction in insulin-stimulated phosphorylation of Akt at ser473 compared with controls (>2-fold, p< 0.001) (Figure 6.18 a-b).

6.5.3 Human primary macrophage-derived factors stimulate cytokine/chemokine release by human adipocytes and the effect of blocking IL-1β.

As shown in Figure 6.19 a-d, protein release of IL-6, MCP-1, IL8 and RANTES from adipocytes was significantly induced by MC medium compared with the control group. This substantial release of pro-inflammatory factors was significantly reduced by blocking IL-1β activity in MC medium by IL-1β neutralization (90%, 55%, 65% and 26%, all p<0.001). This up-regulation was also partially reversed by both IL-1β and TNFα neutralization (92%, 67%, 63% and 21%) or by blocking the IL-1 receptor binding with an IL-RA (95%, 89%, 67% and 32%), respectively (all p< 0.001). Consistent with the effect of TPH-1 MC medium, human primary macrophage MC medium showed a similar induction effect on adipocytes to release...
pro-inflammatory proteins. Moreover, the effect of primary macrophage-derived factors was also inhibited by blocking IL-1β activity and IL-1 receptor binding.
Figure 6-16: Human primary macrophage-derived factors inhibit IRS-1 protein expression and the effect of blocking IL-1β

Differentiated human adipocytes were treated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (15 μg/ml), MC medium neutralized by IL-1β antibody (7.5 μg/ml) and TNF-α (7.5 μg/ml), mouse IgG (15 μg/ml) or an IL-1β receptor antagonist (IL-1RA, 1 μg/ml) for 24 hr. Cell lysates were analysed by western blotting (a) and densitometry (b), using antibodies to IRS-1; GAPDH was used as a loading control. Representative blots are shown; data are means ± SEM (n=3 per group). **p< 0.01 vs controls; + p< 0.05, ++ p< 0.01 vs MC group
Figure 6-17: Human primary macrophage-derived factors inhibit GLUT-4 protein expression and are reversed by the effect of blocking IL-1β

Differentiated human adipocytes were treated with either RPMI-1640 medium (control), MC medium, MC medium neutralized by IL-1β antibody (15 μg/ml), MC medium neutralized by IL-1β antibody (7.5 μg/ml) and TNF-α (7.5 μg/ml), mouse IgG (15μg/ml) or an IL-1β receptor antagonist (IL-1RA, 1 μg/ml) for 24 hr. Cell lysates were analysed by western blotting (a) and densitometry (b), using antibodies to GLUT-4; GAPDH was used as a loading control. Representative blots are shown; data are means ± SEM (n=3 per group).*p< 0.05 vs controls; + p< 0.05, ++ p< 0.01 vs MC group
Figure 6-18: Human primary macrophage-derived factors inhibit insulin-stimulated phosphorylation of Akt at (Ser 473) in human adipocytes

Differentiated human adipocytes were treated with RPMI-1640 or MC medium for 24 hr before stimulated with 100 nM insulin; Akt phosphorylation at Ser473 (pAkt) (a, b) was analysed by western blotting (a) and densitometry (b); GAPDH was used as loading control. Representative blots are shown; data are means ± SEM (n=3 per group). **p< 0.01, ***p< 0.001 vs indicated group
Figure 6-19: Human primary macrophage-derived factors induce cytokine/chemokine release by human adipocytes and the effect of blocking IL-1β

Blocking of IL-1β reduces the effect of MC medium on protein release of IL-6 (a), MCP-1 (b) and IL-8 (c) and RANTES (d) by adipocytes. The levels of cytokine/chemokine were measured by ELISA. Data are expressed as means ± SD (n=6 per group); +++p<0.001 vs control group; ***p< 0.001 vs MC group
6.6 Cell viability assessment

To assess the possibility that the reduction of insulin signaling molecules by several treatments, including THP-1 MC medium, human IL-1β neutralizing antibody (IL-1β Ab, 2 µg/ml), interleukin-1 receptor antagonist (IL-1RA, 1 µg/ml) and caspase-1 inhibitor 50 µM, is due to cytotoxicity, cell viability was determined as LDH release by adipocytes treated with these agents. There were no significant differences in LDH release by adipocytes treated with THP-1 MC, IL-1β Ab, IL-1RA and caspase1 inhibitor for 24 hr compared with controls (Figure 6.20 a-c).
Figure 6-20: Effect of treatment with MC medium, IL-1β Ab, IL-1RA and caspase-1 inhibitor on the viability of human primary adipocytes

Cell viability was assessed by measuring LDH release into the cell culture medium of adipocytes treated with the MC medium (a-c), IL-1β Ab (a), IL-1RA (b) and caspase-1 inhibitor (c) for 24 hr. Data are means ± SD (n=6 per group). ***p< 0.001 vs all the other groups
6.7 Discussion

The macrophage-adipocyte crosstalk in obese adipose tissue affects adipose tissue biology (in both structure and function) but the molecular mechanisms and the key mediators, particularly in human adipose tissue, are poorly understood. This study used \textit{in vitro} models of human macrophages (derived from a monocytic cell line and also PBMC) and primary fat cells to examine the influence of macrophage-derived factors on the insulin signalling pathway and the role of IL-1β in human adipocytes. In this study, MC medium significantly reduced protein abundance of insulin signalling molecules, including IRS1, PI3K p85α, GLUT-4 and insulin-stimulated phosphorylation of IR and Akt. These results are in agreement with a previous study in murine 3T3-L1 adipocytes (Lumeng et al., 2007a), suggesting that macrophage-derived factors can impair insulin signalling in human adipocytes.

Although major factors that mediate the detrimental effect of macrophages on adipocytes remain to be identified, IL-1β has been implicated as a key regulator in the translation of obesity-associated inflammation into insulin resistance in rodent models (Jager et al., 2007, Owyang et al., 2010, McGillicuddy et al., 2011, Ehses et al., 2009). The current study has demonstrated that in human adipocytes IL-1β potently inhibited insulin signal transduction. Similar to the effect of MC medium, IL-1β (2 ng/ml) induced a reduction in protein expression of IRS-1, PI3K p85α, GLUT-4 and insulin-stimulated phosphorylation of Akt. These data raise a question whether IL-1β is responsible for the inhibitory effect of MC medium on the insulin signaling pathway in human adipocytes. This study observed that blocking the actions of IL-1β can in part reverse the effects of MC medium on insulin signalling in human adipocytes. IL-1β depletion with a neutralization antibody abolished the inhibitory effect of MC medium on protein abundance of insulin signalling molecules (IRS-1, PI3K p85α and GLUT-4) and insulin-stimulated phosphorylation of IR and Akt, suggesting that IL-1β blockade could restore insulin signal transduction in human adipocytes. IL-1β therefore could be a major contributor to macrophage-induced inhibition on the insulin signaling pathway.

To further study the role of IL-1β in mediating the effect of macrophages, in this study an IL-1RA was used to prevent IL-1β binding to its receptor in adipocytes. As
a result, protein expression of IRS-1, PI3K p85α and GLUT-4 suppressed by MC medium was restored. In addition, it suggested that IL-1β plays a major role in the macrophage-adipocyte crosstalk associated impairment of insulin signalling in adipose tissue. A recent study has shown that IL-1β production in macrophages (is caspase 1-dependent) isolated from human adipose tissue (Stienstra et al., 2010b). Caspase1 has also been reported to regulate IL-1β production in mouse adipose tissue (Vandanmagsar et al., 2011). To further examine the role of IL-1β in mediating the effect of MC medium, the current study assessed the effects of IL-1β inhibition with a caspase-1 inhibitor on insulin signalling in adipocytes. In this study, the reduction in protein expression of IRS-1, PI3K p85α and GLUT-4 by MC medium was partially or totally reversed when IL-1β production by macrophages was inhibited. These results further support the importance of IL-1β in mediating macrophage-induced inhibition of insulin signalling in human adipocytes.

The mechanisms of IL-1β in the modulation of macrophage-adipocyte crosstalk on insulin signalling in adipose tissue are not fully understood. It is suggested that local inflammation exemplified by macrophage accumulation in adipose tissue could induce the impairment of insulin sensitivity (Lumeng and Saltiel, 2011a). The present study showed that MC medium potently stimulated cytokine/chemokines release while inhibition adiponectin release by human adipocytes. This dysregulation was largely reversed by inhibiting IL-1β activity in MC medium, which led to a significant reduction in protein release of IL-6, MCP-1, IL-8 and RENTS by adipocytes (Fig. 6.9). The current study also demonstrated that blocking IL-1 receptor binding in adipocytes or IL-1β production by macrophages can reduce macrophage-induced release of these cytokine/chemokines. In line with the data of using THP-1 macrophages, conditioned medium from PBMC-derived macrophages significantly induced release of IL-6, MCP-1, IL-8 and RANTES by adipocytes but this can be largely reversed by IL-1β neutralization or blocking of IL-1 receptors (Fig.6.17). These results suggest IL-1β as a key mediator for the pro-inflammatory activity of adipose tissue macrophages. IL-6 is suggested to play a role in insulin resistance as its circulating levels are positively related to adiposity (Vozarova et al., 2001, Spranger et al., 2003b) and IL-6 is overexpressed in fat cells from insulin resistant subjects (Rotter et al., 2003a). In vitro, IL-6 inhibits gene transcription of IRS-1, GLUT-4 and PPARγ and reduces insulin-stimulated glucose uptake in 3T3-L1
adipocytes (Rotter et al., 2003). Recent studies suggest that RANTES (or CCL5) is another key player in obesity-related adipose tissue inflammation (Matter and Handschin, 2007); gene expression of RANTES in adipose tissue is increased in obese subjects which led to increased monocyte migration and macrophage survival in human adipose tissue (Keophiphath et al., 2010). Furthermore, obese mice with deletion of RANTES receptor are protected from insulin resistance and this is related to reduced adipose tissue macrophage content and a M2 type-dominant polarization (Kitade et al., 2012b). MCP-1 (or CCL2) also has a chemotactic activity for monocytes (Kanda et al., 2006), as MCP-1 or its receptor (CCR2) knockout in mice reduces macrophage infiltration in adipose tissue (Kanda et al., 2006, Tsou et al., 2007).

IL-8 a neutrophil chemotactic factor also induces chemotaxis in macrophages (Waugh and Wilson, 2008) and gene expression of IL-8 is upregulated in mammary adipose tissue of obese women, in parallel with increased macrophage infiltration (Sun et al., 2012). A previous study showed that IL-8 receptor (CXCR2) knockout prevents macrophage recruitment in adipose tissue and insulin resistance in diet-induced obese mice (Neels et al., 2009). Taken together, these results suggest that mediating the deleterious effect of macrophages on insulin signalling by IL-1β could be through upregulating the inflammatory response in adipocytes, especially the production of pro-inflammatory cytokine/chemokines. In addition to the endocrine and autocrine effects of these cytokines, adipocyte-derived factors may affect macrophage function in a paracrine manner. Our group has reported recently that conditioned medium from human preadipocytes and adipocytes can increase THP-1 monocytes migration and this is probably due to chemoattractants, such as MCP-1, secreted by preadipocytes and adipocytes (Gao et al., 2013, Ding et al., 2013). Thus, the enhanced macrophages-adipocyte crosstalk in adipose tissue could stimulate inflammation and reduce insulin sensitivity of adipose tissue in obesity.

In addition, the present study showed that blocking the activity of IL-1β or blocking IL-1 receptor binding can reverse the effect of MC medium on release of adiponectin which is considered as an insulin-sensitizing adipokine (Wellen and Hotamisligil, 2003) that is involved in glucose and lipid metabolism in skeletal myocytes, hepatocytes and adipocytes (Berg et al., 2002). Adiponectin and its agonists have
been suggested to have a role in the prevention of obesity related insulin resistance, diabetes and cardiovascular diseases (Lee and Kwak, 2014). Taken together, it is suggested that IL-1β as a major macrophage-derived factor that impairs insulin signaling pathway at least in part by reducing adiponectin release by adipocytes.

Although IL-1β is a macrophage-derived mediator in inducing insulin resistance in human adipocytes, the contribution of other macrophage-derived factors cannot be excluded. The present study and previous work have shown high levels of IL-6, TNFα, MCP-1, RANTES and IL-8 together with other factors present in the MC medium (Gao et al., 2010). As some of the effects of blocking IL-1β in this study were partial, the other mediators could contribute. TNFα can induce insulin resistance in rodents and decrease the expression of insulin receptor, IRS-1 and GLUT-4 in 3T3-L1 adipocytes (Hotamisligil et al., 1993, Uysal et al., 1997, Stephens et al., 1997). TNFα neutralizing antibody has been shown partially reverse glucose uptake inhibited by MC medium in 3T3-L1 adipocytes (Lumeng et al., 2007a). Blocking both TNFα and IL-1β additively restore MC medium-suppressed Akt phosphorylation in murine liver cells (Wen et al., 2011). A recent study from our lab has demonstrated that simultaneous neutralization of TNFα and IL-1β additively inhibited MC medium-induced gene expression of MCP-1 and IL-6 by human preadipocytes (Gao et al., 2013). However, in the current study, a combined effect of the two cytokines on insulin signalling molecules and cytokine/chemokine release by adipocytes was not apparent that in addition to TNF-α, other factors in MC medium may play a role.

In summary, this study demonstrates that IL-1β is a key factor in mediating macrophage-induced impairment of the insulin signalling pathway in human adipocytes. Blocking IL-1β activity, its receptor binding and production can partially or totally restore insulin signalling and responsiveness in human adipocytes. IL-1β antagonism also either protects against macrophage-stimulated release of the pro-inflammatory cytokines/chemokines, including IL-6, TNFα, MCP-1, RANTES and IL-8 or protects against macrophage-inhibited release of adiponectin. These results suggest that IL-1β may serve as a target for reducing obesity-associated insulin resistance in human adipose tissue.
CHAPTER 7:
General discussion and future directions
7 General discussion and future directions

7.1 Introduction
In obesity, there is correlative relationship between excess adipose tissue and a state of low-grade chronic inflammation and metabolic disorders (Odegaard and Chawla, 2013, Lumeng and Saltiel, 2011a). Obesity is highly associated with insulin resistance which is the major risk factor for the development of type 2 diabetes (King, 2007, Bogardus et al., 1984). Expansion of adipose tissue during obesity induces marked changes in the morphology and functional properties of WAT such as inducing an inflammatory response, suppressing adipogenesis and desensitizing insulin action (Sun et al., 2011, Bing, 2015). Consequently, WAT becomes an inflamed tissue with a significant rise in infiltration of macrophages and other immune cells which together alter the secretion of adipokines (Lolmède et al., 2011, Weisberg et al., 2003). In addition, both adipocytes and preadipocytes are able to release protein factors, and macrophage-derived factors can potently stimulate the production of pro-inflammatory cytokines and chemokines in human adipose cells (Coppack, 2001, Lacasa et al., 2007). IL-1β, a pro-inflammatory mediator, is a major cytokine from macrophages, and it has been implicated in the development of insulin resistance during obesity (Bing, 2015, Böni-Schnetzler and Donath, 2011, Fève and Bastard, 2009). The studies in this thesis were thus established to investigate the role of IL-1β in mediating macrophage-induced impairment of the insulin signalling pathway and adipogenesis in human adipocytes, and whether blocking the activity of IL-1β, its receptor binding or production improves insulin signalling in human adipose cells.

7.2 Previous work
In addition to skeletal muscle and liver, adipose tissue has also been found to develop insulin resistance in obesity (Hotamisligil, 2000). The reduced insulin sensitivity in adipocytes may increase FFAs release into the circulation inducing profound metabolic changes in adipose tissue (Sun et al., 2011, Lee et al., 2010, Boden, 2008). Previous studies in obese mice and humans suggests that increased macrophage accumulation in adipose tissue during obesity might provide a causative link between inflammation and metabolic disorders such as insulin resistance and type 2 diabetes (Cancello and Clement, 2006, Weisberg et al., 2003, Xu et al., 2003). Furthermore,
two studies have been conducted by using murine adipose tissue cells to study the effects of macrophages that disrupt insulin action through downregulation of the expression of the insulin signalling molecules (Lumeng et al., 2007c, Zhou et al., 2008). More specifically, macrophages of the pro-inflammatory phenotype have been found to be linked with insulin resistance in both humans and mice (Lumeng et al., 2007a, Fujisaka et al., 2009). The first documentation showing a close link between obesity and insulin resistance was published by Hotamisligil and et al who stated that TNF-α was the first macrophage-derived factor involved in the mediation and development of insulin resistance (Hotamisligil et al., 1993, Hotamisligil and Spiegelman, 1994). Pro-inflammatory factors including TNF-α, IL-6, MCP-1 and IL-8 are directly involved in obesity-associated pathologies, particularly inflammation, insulin resistance and type 2 diabetes (Hotamisligil, 1999, Lagathu et al., 2003, Sartipy and Loskutoff, 2003, Spranger et al., 2003c, Neels et al., 2009). Growing evidence suggests that IL-1β is critically involved in the translation of obesity-associated inflammation into insulin resistance in rodent and human models (Lagathu et al., 2006, Böni-Schnetzler and Donath, 2011, Bing, 2015). However, there is a lack of studies using human primary adipocytes and preadipocytes as in vitro models to demonstrate whether IL-1β mediates the effect of macrophages on the insulin signalling pathway.

Another research direction has been focused on the effect of macrophage-derived factors on adipocyte development particularly adipocyte differentiation. The precise mechanisms of the anti-adipogenic action of macrophages mediated by IL-1β in human adipose cells are still unclear. In addition, previous studies have shown that adipose cells play their own roles in the inflammatory state (Chung et al., 2006, Gao et al., 2010). Further studies have shown that macrophage products potently induce the production of pro-inflammatory cytokines and chemokines by human preadipocytes and adipocytes (O’Hara et al., 2009, Gao et al., 2014, Gao et al., 2012). However, the role of IL-1β in mediating the effect of macrophages that alter adipose tissue development (differentiation) and lipid storage function is largely unknown.

7.3 Current work
This project was aimed to study the effect of the crosstalk between macrophages and adipocytes/preadipocytes on adipose tissue biology and function based on the general
hypothesis that there is extensive cross-talk between the major cell types within adipose tissue. More specifically, the current thesis investigated the effects of macrophage-derived factors on the insulin signalling pathway and differentiation of human preadipocytes to adipocytes, and identified cytokine IL-1β as the potential mediator of the effects of macrophages.

Chapters 3, 4, 5 and 6 showed experimental studies which were carried out in order to demonstrate the role of IL-1β in mediating macrophages-induced impairments of the insulin signalling pathway and development of the adipose tissue cells in humans. Therefore, human primary adipose cells (preadipocytes and adipocytes) and human macrophages were used in *in vitro* models to study the interaction between each other.

### 7.3.1 Characterization and expression of insulin signalling molecules in human preadipocytes

Although there are few studies carried out investigating the insulin signalling pathway and its molecules in human adipose tissue (Laviola et al., 2006, Summers et al., 2000), to our knowledge, little is known about the characteristics of the molecules involved in the insulin signalling pathway in human preadipocytes. For this reason, the study presented in Chapter 3 was focused on characterizing the insulin signalling molecules in human primary preadipocytes in comparison with adipocytes. The first objective was to examine whether the key insulin signalling molecules (IRS-1, (PI3K- p85α) and GLUT-4) are present at the basal level in human preadipocytes. Results showed that basal levels of insulin signalling proteins IRS-1 and PI3K-p85α were detected in human preadipocytes. Furthermore, these two insulin signalling molecules were significantly increased in mature adipocytes compared with preadipocytes. These results are in agreement with previous studies in murine adipocytes (Saad et al., 1994). It suggests that the expression levels of insulin signalling molecules for both IRS-1 and PI3K-p85α in preadipocytes were increased during the course of differentiation process, which may have importance in adipose tissue functions as well as development.

Expression of the glucose transporter-4 (GLUT-4) was also investigated in human preadipocytes and results showed that GLUT-4 protein was barely detectable in preadipocytes compared to mature adipocytes. A previous study that used crude
membrane fractions of undifferentiated human preadipocytes showed similar results (Hauner et al., 1998). In the following experiment, a time course study was conducted to examine the levels of GLUT-4 protein expression at several time points during preadipocytes differentiation. Results showed that GLUT-4 protein expression gradually increased from day 3 up to day 12 post differentiation (Figure 3.2). This is in agreement with previous studies using murine adipocytes (3T3L-1) in which GLUT-4 is progressively expressed during the differentiation of adipocytes as demonstrated by kinetic studies (Weiland et al., 1990, Tordjman et al., 1989). Therefore, it suggested GLUT-4 protein is only abundantly expressed once preadipocytes differentiate into mature adipocytes.

The second objective of Chapter 3 was to examine whether the insulin signalling molecules, including IR (Y1185), IRS-1 (Ser612) and Akt (Ser473), in human preadipocytes are modulated by insulin in comparison to mature adipocytes. The result showed that in both preadipocytes and mature adipocytes, exposure to insulin (100 nM) led to the phosphorylation of IR (Y1185), IRS-1 (Ser612) and Akt (Ser473). Following insulin stimulation, there were significant increases in protein abundance of all signalling proteins and this was due to increased phosphorylation activity. These results suggested that insulin markedly induces the insulin signalling molecules in adipose tissue that may have stimulatory or inhibitory roles on several biological processes such as proliferation, differentiation and development of adipose cells.

7.3.2 IL-1β modulates insulin signalling proteins and mediates inflammation-related protein in human preadipocytes

Little is known whether macrophages-derived factors modulate insulin signalling pathways in human primary preadipocytes. Therefore, the main aims of Chapter 4 were to investigate whether IL-1β, one of the major cytokines produced by macrophages, could affect insulin signalling molecules in human preadipocytes in response to insulin stimulation, and affect the expression and release of pro-inflammatory factors in human preadipocytes. To address the first objective in Chapter 4, in vitro model of primary human preadipocytes and human recombinant IL-1β were used to investigate the acute and chronic effect of IL-1β at two different time points (4 and 24 hr) respectively on several insulin signalling molecules,
including the key molecules involved in the metabolic pathway (PI3K-p85/Akt) in response to insulin stimulation.

The finding has demonstrated that in human preadipocytes, IL-1β strongly decreased the protein abundance of insulin-stimulated phosphorylation of insulin signalling molecules. The results showed that upon insulin stimulation, exposure of human preadipocytes to human recombinant IL-1β (2 ng/ml for 4 and 24 hr) led to significantly reduced insulin-stimulated phosphorylation of IR at tyrosine residues compared with untreated cells. This finding was in agreement with previous studies which observed the IL-1β at higher doses (10 and 20 ng/ml) can reduce the ability of insulin to increase the tyrosine phosphorylation of IR (Lagathu et al., 2006). In contrast to p-IR, treatment of preadipocytes with IL-1β (2 ng/ml) in the presence of insulin has significantly increased p-IRS-1 (Ser612) and p-Akt although the increases in both signalling proteins were less than controls (Figures 4.2 and 4.3). Additionally, IL-1β treatment for 24 hr showed a slight increase in p-IRS-1 (Ser612) in preadipocytes but the strongest stimulatory effect was induced by insulin, suggesting that a possible effect of IL-1β on the induction of serine phosphorylation of IRS-1, which in turn inhibiting the insulin signalling transduction at tyrosine phosphorylation.

Interestingly, there were conflicting observations about the effect of IL-1β on p-Akt with previous studies using human and murine mature adipocytes in which it was demonstrated that IL-1β has inhibitory effects on insulin-stimulated phosphorylation of Akt (Lagathu et al., 2006). However, other studies have reported similar results as in our study using different cell types and a range of cytokines including IL-3, IL-4, TNF-α (Hinton and Welham, 1999, Ozes et al., 1999), which showed that induced p-Akt occurred due to the link between Akt activation and proliferation/survival function in response to these cytokines. A recent study showed that macrophages-derived factors protect 3T3-L1 preadipocytes from apoptosis via activation the survival signalling that phosphorylates Akt and Erk molecules in the presence of ROS (Molgat et al., 2011). Taken together, these results suggest that IL-1β could serve as a stimulus agent depending on cell type and/or specific function of the protein such as Akt.
Further experiments described in Chapter 4 investigating whether IL-1β mediating the effect of MC medium on the insulin signalling molecules in human preadipocytes. The results showed that there were significant increases in p-IRS-1(Ser612) in all groups after insulin stimulation (Figure 4.7). Furthermore, p-IRS-1 was induced by MC medium. This result is in agreement with some previous studies which showed that serine/threonine phosphorylation of IRS-1 is induced by a range of factors as previously mentioned in section (4.7). In the present study, blocking the activity of IL-1β with a neutralizing antibody did not alter the effect of MC medium on insulin-stimulated phosphorylation of IRS-1. The result suggests that other macrophage-derived factors in MC medium could contribute to the activation of p-IRS-1 at serine 612.

Similarly, the current study observed that basal p-Akt was induced by MC medium in human preadipocytes (Figure 4.8). However, MC medium suppressed the effect of insulin on p-Akt, whereas IL-1β neutralisation reversed the inhibitory effect of MC medium on p-Akt expression in response to insulin (Figure 4.8). Therefore, it is suggested that IL-1β mediates the effect of MC medium on the activation of Akt in preadipocytes.

The mechanisms of IL-1β in the modulation of the macrophage-preadipocytes crosstalk on insulin signalling in adipose tissue from above data are not clear enough. It is suggested that local inflammation enhanced by macrophage infiltration in adipose tissue could be crucial in leading to the impairment of insulin sensitivity (Lumeng and Saltiel, 2011a). IL-1β has been particularly implicated in the inflammatory response (Lacasa et al., 2007, Gao and Bing, 2011) and the modulation of insulin sensitivity (Lagathu et al., 2006). Therefore, the second major objective of Chapter 4 was to examine the role of IL-1β in mediating macrophages-induced expression and release of pro-inflammatory factors by human preadipocytes. Results showed that MC medium potently induced gene expression and protein release of the pro-inflammatory factors including IL-6, MCP-1 and IL-8 in human preadipocytes. These results are in agreement with the previous studies (Lacasa et al., 2007, Gao and Bing, 2011), suggesting that macrophages are strong inducers of an inflammatory state in preadipocytes which in turn could promote macrophages recruitment and maintenance in adipose tissue. Furthermore, inhibiting IL-1β activity in MC medium with a neutralizing antibody led to a significant reduction in gene
expression and protein release of IL-6, MCP-1 and IL-8 by preadipocytes (Figures 4.9 and 4.10). Taken together, these results suggest that IL-1β is considered to be a key mediator for the pro-inflammatory activity of adipose tissue macrophages. The deleterious effect of macrophages on insulin signalling could be modulated by IL-1β through upregulation of the inflammatory response in preadipocytes.

7.3.3 Effect of long-term treatment with IL-1β on preadipocytes differentiation and adipocytes functions

Results obtained from Chapter 4, led us to hypothesize that inflammation-related proteins in preadipocytes enhanced by macrophages-derived factors particularly IL-1β could contribute to the impairment of preadipocytes differentiation (adipogenesis) and altering lipid storage function in human adipocytes. It has been shown in Chapter 5 that IL-1β has adverse effects in adipocyte development and lipid levels deposition in adipocytes. These results are in agreement with previous studies using murine adipose cell lines, where pro-inflammatory cytokines IL-1β and TNFα strongly suppress adipogenesis through activation of the nuclear factor kB (NF-kB) pathway (Petruschke and Hauner, 1993, Ruan et al., 2002).

The results in Chapter 5 showed that exposure of human preadipocytes to IL-1β (1 and 5 ng/ml) during the 12-day period of differentiation impaired adipogenesis. The inhibition with a 5 ng/ml of IL-1β occurred with a significant reduction in the cellular lipid content in human adipocytes. Consistently, cell morphology exhibited a significant inhibition of lipid accumulation in differentiated adipocytes after incubated with a higher dose IL-1β (5 ng/ml) (Figure 5.1). These results are consistent with a previous study that examined long-term treatment with IL-1β on murine and human adipocytes and found that IL-1β reduced cellular lipid content (Lagathu et al., 2006). Furthermore, the results also showed that prolonged IL-1β exposure during preadipocytes differentiation has inhibitory effects on the expression of the key adipogenic genes. IL-1β significantly downregulated mRNA levels of C/EBPα, PPARγ and aP2, suggesting that IL-1β has an inhibitory effect on preadipocyte differentiation by inhibiting the adipogenic process and impairs lipid storage function of adipocytes. Obtained results are also in line with previous work by our lab group where both MC medium and IL-1β suppressed expression of genes involved in adipogenesis (C/EBPα, SREBP1 and ACLY) and stimulated lipolysis in adipocytes (Gao et al., 2014).
In contrast to the inhibitory effects of IL-1β on adipogenic factors, the data in Chapter 5 showed that IL-1β had stimulatory effect on pro-inflammatory factors. Prolonged IL-1β treatment significantly upregulated gene expression of pro-inflammatory factors, including IL-6, IL-8, MCP-1 and RANTES in adipocytes. In addition, a recent study from our group has shown that 24 hr treatment with IL-1β also stimulates IL-6, IL-8 and RANTES production in adipocytes (Gao et al., 2014). Collectively, it is suggested that IL-1β could have a role in inducing pro-inflammatory responses that may be responsible for adipose tissue dysfunction in obesity (Arvin et al., 1996, Di Iorio et al., 2003).

Other adipokines such as adiponectin and leptin are mainly produced by adipocytes (Yadav et al., 2013). Therefore, the present study examined the effect of long-term treatment with IL-1β at several concentrations (1, 5 and 20 ng/ml) on gene expression of adiponectin and leptin in differentiated adipocytes. The results in current study showed that prolonged exposure to IL-1β had inhibitory effect on adiponectin mRNA levels. Considering the antagonistic actions between the role of adiponectin and IL-1β (Lagathu et al., 2006, Lihn et al., 2004, Simons et al., 2007), reduced adiponectin expression by IL-1β may enhance inflammation and insulin resistance in obesity. In contrast to adiponectin, the expression of leptin is increased with the increase in body weight (Yadav et al., 2013), and it is known as a metabolic hormone affecting a range of processes such as insulin secretion, glucose transport and lipolysis (Trayhurn et al., 1999). The results in Chapter 5 have showed that prolonged treatment with IL-1β had stimulatory effect on leptin gene expression, suggesting that IL-1β may act together with leptin to stimulate inflammation in obesity. In agreement with the effect of IL-1β on adipogenesis, previous in vitro and in vivo studies demonstrated that leptin increased lipolysis and inhibited adipocyte lipogenesis (Harris, 2014). Therefore, it is suggested that IL-1β and leptin may provide a synergistic effect that could be responsible for the inhibition of adipogenesis and stimulation of lipolysis.

The effects of MC medium and IL-1β on lipid storage function of human adipocytes through increased lipolysis were investigated in Chapter 5. Interestingly, IL-1β at 2 ng/ml concentration was shown to have a similar potency as the MC medium to induce lipolysis in differentiated adipocytes (Figure 5.6 a-b). However, it is not known whether IL-1β acts as a key factor in mediating the effect of MC medium.
Our data showed that the induction of lipolysis by MC medium was completely attenuated by IL-1β neutralization that blocks IL-1β activities. Therefore, the current study provides evidence for the role of IL-1β in mediating macrophages-induced lipolysis. This is further supported by the observation from the current study that the effect of MC medium was reduced by an IL-1RA which blocks IL-1 receptor binding in adipocytes (Figure 5.8).

7.3.4 IL-1β mediates macrophage-induced impairment of insulin signalling in human primary adipocytes

Based on the results from Chapters 3, 4 and 5 and previous studies (Smith, 2002, Heilbronn et al., 2004, Krebs and Roden, 2005, Suganami et al., 2011), we next hypothesised that macrophage-derived factors particularly IL-1β may induce impairment of insulin signalling by affecting the key insulin signalling molecules in human primary adipocytes. In vitro human cell models including primary adipocytes, THP-1 macrophages (cell line) and primary macrophages were used to address this question.

In this study, MC medium significantly reduced protein abundance of the key insulin signalling molecules at basal (IRS1, PI3K p85α and GLUT-4) and phosphorylated levels (p-IR and p-Akt). These results are in agreement with a previous study in murine 3T3-L1 adipocytes (Lumeng et al., 2007c), suggesting that macrophage-derived factors can impair insulin signalling in human adipocytes. In addition, similarly to the MC medium effect, our results demonstrated that IL-1β (2 ng/ml) potently inhibits insulin signalling molecules (IRS1, PI3K p85α, GLUT-4, p-IR and p-Akt) in human adipocytes.

Interestingly, the current study used three different approaches to reverse the effect of IL-1β in mediating MC medium. The first approach was by using an IL-1β neutralizing antibody to block IL-1β activity. IL-1β depletion with a neutralization antibody abolished the inhibitory effect of MC medium on protein abundance of insulin signalling molecules. The second approach was by using an IL-1RA to prevent IL-1β binding to its receptor in adipocytes. As a result, protein expression of IRS-1, PI3K p85α and GLUT-4 suppressed by MC medium was restored by an IL-1RA action. A recent study has shown that IL-1β production is caspase 1-dependent in macrophages isolated from human adipose tissue (Stienstra et al., 2010). The third
approach was by using a caspase-1 inhibitor to inhibit IL-1β production from macrophages. Results showed that the reduction in protein expression of IRS-1, PI3K p85α and GLUT-4 by MC medium was partially or totally reversed when IL-1β production by macrophages was inhibited, suggesting that IL-1β blockade could restore insulin signal transduction in human adipocytes. IL-1β therefore could be a major contributor to macrophage-induced inhibition of the insulin signalling pathway.

The study in Chapter 6 also showed that MC medium from both THP-1 and PBMC-derived macrophages potently stimulated cytokine/chemokine release (IL-6, RANETS, MCP-1 and IL-8) by adipocytes. This finding is in agreement with our results in Chapter 4 and a previous study showed that MC medium stimulated pro-inflammatory factors release by preadipocytes (Lacasa et al., 2007). It is suggested that increased cytokine/chemokine release by adipose cells could induce local inflammation enhanced by macrophages accumulation leading to the impairment of insulin sensitivity in adipose tissue.

To further study the role of IL-1β in mediating the effect of macrophages, the current study has used the similar IL-1β blockade approaches to reverse the effect of IL-1β in mediating MC medium-induced cytokine/chemokine release by adipocytes. In contrast to the stimulatory effects of IL-1β on pro-inflammatory factors, the present study showed that IL-1β had inhibitory effect on adiponectin release. This dysregulation was largely reversed by inhibiting IL-1β activity in MC medium, which led to a significant increase in protein release of adiponectin (Figure 6.10). Interestingly, the similarity of the effect of IL-1β (1, 5 and 20 ng/ml) on differentiating cells in Chapter 5 and the effect of MC medium on differentiated cells in Chapter 6 was observed. Taken together, it suggested that IL-1β as a major macrophage-derived factor that impairs insulin signalling pathway may at least in part by reducing adiponectin release by adipocytes.

These, results obtained are in the line with work by another researcher in our laboratory who using the PCR array analysis, found that IL-1β downregulated expression of genes involved in insulin signalling, insulin sensitivity, glucose metabolism and lipid metabolism, including IRS-1, IRS-2, PPARα, PPARγ, PGC-1β, GLUT4, GPD1, GSK-3β, ACLY and CEBPα. When IL-1β activity was inhibited
with a neutralizing antibody, the expression of 22 of 27 genes being downregulated by MC medium was partially restored, including all genes that were mentioned above (Gao et al., 2015).

Collectively, these data suggest that IL-1β is appeared as a major macrophage-derived mediator in inducing insulin resistance in human adipocytes. Therefore, IL-1β may serve as a target for reducing obesity-associated insulin resistance in human adipose tissue. However, the contribution of other macrophage-derived factors cannot be excluded.

**The main findings in this thesis are summarized as follows:**

1. Regarding insulin signalling, basal expression of insulin signalling proteins (IR, IRS-1 and PI3K-p85α) was present and detectable in both human adipose cells (preadipocytes and adipocytes). However, the basal expression of GLUT-4 was barely detected in preadipocytes. Insulin as metabolic regulator has significantly stimulated the abundance of levels of phosphorylated insulin signalling molecules, including IR (Y1185), IRS-1(Ser612) and Akt (Ser473), in both human adipose cells.

2. IL-1β was able to modulate the expression of insulin signalling protein including IR (Y1185), IRS-1(Ser612) and Akt (Ser473) in human preadipocytes upon insulin stimulation. Furthermore, IL-1β also mediated macrophage-induced modulation of the insulin signalling molecules in human preadipocytes.

3. IL-1β also mediated the macrophage-induced expression and release of the pro-inflammatory cytokine/chemokines which contribute to local inflammation environment that could reduce the expression of insulin signalling proteins. However, IL-1β blockade has been found to protect against the effect of macrophage-derived factors on preadipocytes.

4. Regarding adipose cell development, prolonged exposure of cells to IL-1β, from the induction of the differentiation state until full differentiated adipocytes state, has shown an inhibitory effect on lipid storage ability (accumulation) during adipocyte development through to the differentiation process.

5. Prolonged exposure to IL-1β also has inhibited the expression of the key adipogenic genes which were significantly downregulated in mature adipocytes. At
the full stage of differentiation, prolonged exposure to IL-1β has also reduced the gene expression of adiponectin which is known as a mature adipocyte marker. In contrast, the long-term treatment which IL-1β has stimulatory effects on the expression of pro-inflammatory genes including IL-6, IL-8, MCP-1, RANTES and leptin in human adipocytes.

6. IL-1β blockade (using IL-1β neutralizing antibody and IL-1R antagonist) has confirmed that IL-1β could be a key player that mediates macrophage-induced reduction in lipid storage ability of human adipocytes.

7. In human primary adipocytes, IL-1β is a key factor in mediating macrophage-induced impairment of the insulin signalling pathway. Blocking IL-β activity, IL-1 receptor for binding and IL-β production using three different approaches (including IL-1β neutralizing antibody, IL-1R antagonist and caspase-1 inhibitor) either partially or totally protected against macrophage-induced insulin resistance by impairing the expression of insulin signalling proteins and reducing the release of adiponectin from adipocytes.

8. IL-1β blockade protected against macrophages-initiated pro-inflammatory state by inducing the release of the pro-inflammatory cytokine/chemokines from adipocytes.

7.4 Future directions

Obesity alters adipose tissue function (metabolic and endocrine) and leads to a pro-inflammatory state with immune responses that include infiltration of adipose tissue with macrophages. These macrophages are believed to alter insulin sensitivity in adipose cells that contributes to obesity-associated complications such as inflammation and insulin resistance leading to the development of type 2 diabetes (Weisberg et al., 2003).

It has been mentioned in Chapter 1 that the mitogenic cascade is also involved in the insulin signalling pathway. The mitogen-activated protein kinase (MAPK) pathway such as c-Jun N-terminal kinases (JNK) and extracellular signal-regulated kinases (ERK) provides another route in insulin-mediated regulation of adipocyte transcription factors and adipose tissue development. Therefore, there could be an opportunity to explore other mediators of the insulin signalling pathway and the role
of IL-1β on the insulin molecules involved in mitogenic cascade in human adipose cells.

As reported in previous studies, preadipocytes play a role as macrophage-like cells by acquiring high phagocytic activity and releasing several pro-inflammatory factors involved in the inflammatory response. This conversion appears in proliferating preadipocytes and disappears when preadipocytes differentiate into adipocytes (Charrière et al., 2003, Cousin et al., 1999). To increase our understanding about adipose tissue dysfunction and how it affects the insulin signalling pathway in adipose cells, further experiments will be needed to investigate whether human preadipocytes (using preadipocytes conditioned medium) as a new approach, show possible cross-talk between preadipocytes and adipocytes. This interaction between preadipocytes and adipocytes hypothesize that preadipocytes might be induced by adipose-derived factors which could impair the key insulin signalling molecules in the metabolic cascade such as IR, IRS-1, PI3K p85α and GLUT-4 in human adipocytes.

Another future direction is that freshly isolated adipose cells, as alternative approaches which may more challenging, taken from lean, obese and type 2 diabetes subjects would provide more information about the expression of genes involved in insulin signalling, insulin sensitivity, glucose metabolism and lipid metabolism in three different samples from human subjects by using PCR array analysis. In addition, there could be chance also to investigate the abundance of proteins involved in insulin signalling pathway by using western blotting methods.

7.5 Concluding remark

Studies undertaken for this project have demonstrated that IL-1β is a key pro-inflammatory factor in mediating macrophage-modulated insulin resistance in human adipose cells. IL-1β also mediated macrophage-induced expression and release of pro-inflammatory cytokine/chemokines in adipose cells. Identification of IL-1β as a key factor that mediates detrimental effect of macrophages on human adipose cells is important for better understanding of obesity-related adipose tissue dysfunction and for developing potential targets for treatment.
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Appendix: 9

Publication associated with this thesis
9 Appendix

LIST OF PUBLICATIONS

Part of the results in this thesis has been presented and published in:

A. Published articles


B. Published abstracts and posters

1. Madi, M, Gao, D, Trayhurn, P and Bing, C (2012). Interleukin-1β mediates macrophage-induced lipolysis and inhibits lipid accumulation in human adipocytes. 39th Adipose Tissue Discussion Group (ATDG, 2012), Path, UK.

2. Madi, M, Gao, D, Trayhurn, P and Bing, C (2012). Interleukin-1β mediates macrophage-induced lipolysis and inhibits lipid accumulation in human adipocytes, European Congress on Obesity (ECO, 2013), Liverpool, UK.


