ADIPOKINE EXPRESSION IN MOUSE MODELS OF ENDOTOXAEMIA AND SEPSIS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of MD

BY

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ACKNOWLEDGEMENTS

I would like to thank my wife Ruth for her patience and support during the work that went into this thesis.

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INTRODUCTION

Sepsis kills. With an estimated annual mortality of 30 -50 per 100,000 population, although survival has slightly increased in the past 10 - 15 years (1) (2). It affects 18 million people worldwide every year and ranks in the top 10 causes of death with annual treatment costs in the US alone of over $16 billion (3).

SIRS	Two or more of: **Temperature** >38°C or <36°C **Tachycardia** >90 beats per minute **Respiratory rate** >20 breaths per minute or PaCO2 <4.3kPa **White Blood cell** count >12 x 10^9/L or <4 x 10^9/L or >10% immature (band) forms
Sepsis	SIRS due to infection
Severe Sepsis	Sepsis with evidence of organ hypoperfusion
Septic Shock	Severe sepsis with hypotension (systolic blood pressure < 90mmHg) despite adequate fluid resuscitation or the requirement for vasopressors / inotropes to maintain blood pressure

Table 1: Clinical definitions for the systemic inflammatory response syndrome (SIRS) and sepsis (4)

Sepsis, originally defined as putrification or decomposing of organic matter by micro-organisms (5), is the body's systemic response to infection. Consensus was reached with regards to clinically defining the condition in 1992 (table 1) (4) with the list of signs and symptoms increased in 2001 (6) and a simple haematological investigation has recently been shown to aid early diagnosis (7). However, these clinical criteria for the diagnosis of sepsis do not discriminate differences in underlying pathophysiology, thus hindering development and application of effective targeted therapies (8) (9) (10) (11).

The systemic inflammatory states of sepsis, septic shock and endotoxaemia affect most organs of the body including white adipose tissue with lipolytic activity and glucose metabolism particularly affected (12) (13). It is well established that glucose utilization is impaired and lipolysis altered in obesity, inflammation and sepsis (14) (15).

However, it has recently come to light that white adipose tissue (WAT), the body's main insulator and energy store, plays a mediatory role not confined to just glucose and fat metabolism and is a major endocrine secretory organ. WAT, by secreting protein mediators and signalling factors coined adipokines (16), contributes to inflammation related conditions in obesity such as the metabolic syndrome (17) (18).

Obesity is now a major health concern worldwide and has been described as an epidemic (19) (20). However, for obese ICU patients there appears to be a conundrum or even paradox with respect to outcomes. Obese patients were initially shown to have a poorer outcome from critical illness compared to non-obese patients (21-23) (24), but recent meta-analyses (25) (26) have suggested the reverse. But this is probably not the whole story, as a series of potential confounders may have affected these results (27)and indeed it may be the location of fat depots is a better predictor (28). Obese patients are still more difficult to manage due to difficulties and complications of ventilation and the high incidence of serious comorbidities such as ischaemic heart disease and diabetes.

As adipose tissue mass expands the concentration of a number of adipokines and proinflammatory markers expressed and secreted by adipocytes has been shown to rise. These include - cytokines (e.g. IL-6, IL-10), chemokines (e.g. IL-8, MCP-1, MIF) and acute-phase proteins (e.g. haptoglobin, serum amyloid A) as well as inflammation-

related adipokines such as leptin and nerve growth factor (NGF) (17) (18) (16) (29). These inflammatory responses may reflect hypoxia in the growing adipose tissue mass. Hypoxia is known to induce cytokine production in organs such as heart, kidneys and gut (30). Sepsis involves general microvascular dysfunction causing a decrease in oxygen delivery, producing tissue hypoxia and organ dysfunction (31). The recognition and appropriate response of cells to hypoxia is substantially mediated by the hypoxia inducible factor-1 (HIF-1) transcriptional system (32) (30, 33). Activation of HIF-1 during hypoxia, by stabilization of the HIF-1α subunit, triggers expression of glycolytic enzymes and membrane glucose transporters, such as GLUT-1 (32). HIF-1α was discovered more than 20 years ago when the expression of erythropoietin was being studied (34). It rapidly became clear that HIF-1α is a key regulator of many other systems and it is now apparent that certain cytokines and growth factors can make use of this 'hypoxic signaling system' under normoxia (35). Some of these responses are amplified in the presence of hypoxia (36). There appears to be distinct signalling pathways, induced by hypoxia or Toll-like receptor 4 mediated, for HIF-1α stabilisation leading to differential gene expression (37).

RESEARCH QUESTION

It was hypothesized that in endotoxaemia there is a major activation of the inflammatory response in adipose tissue, key inflammatory adipokines contributing to the metabolic dysregulation characteristic of endotoxaemia and sepsis. I used murine models of endotoxaemia and polymicrobial (surgical) sepsis to study the expression in both internal and subcutaneous adipose tissue of a series of inflammation-related adipokines. Evidence for the induction of adipose tissue hypoxia was sought through measurement of HIF-1α.

MODELS OF SEPSIS

Sepsis is a complex disease, affecting multiple organ systems, and frequently requires intensive care. The average age of patients is 60 - 65 years and it is more common in men (38) with higher mortality with increasing age (3). Severe sepsis has long term (>6 months) effects on both mental and physical health (39).

As well as treating the underlying infection with appropriate antibiotics and surgery etc, the patient often needs heamodynamic and respiratory support. This includes the use of sedative, analgesic, inotropic, steroid and other medication. Frequent observation and continuous invasive monitoring of vital signs are used to guide treatment.

KEY FEATURES OF A MODEL:

Standardisation - in order to minimise individual differences and genetic polymorphisms responsible for the spectrum of responses seen in human sepsis (40) and ensure all subjects experience identical conditions. Hence, animals should be of the same strain, age, weight and experience identical husbandry conditions and the intervention should be of a standard dose for each arm of the study.

The procedures performed should be easily reproducible and hence reduce any operator variability. Effects of the model need to be clearly measurable which could be by direct observation, invasive / non-invasive monitoring and laboratory tests. Ultimately, the model needs to be valid - i.e. produce a definite septic response due to polymicrobial contamination or endotoxaemia etc. The actual model should also be as simple and cost effective as possible and use the least number of subjects to answer the question.

MODELS USED:

There are mainly two widely used models:

1. Injection, either intraperitoneal or intravenous, of bacteria or bacterial contents (e.g. LPS). These are simple methods that can be used to produce **endotoxaemia** or sepsis due to specific organisms (41).

2. The release of faeces into the peritoneal cavity by caecal ligation and puncture (CLP) or ascending colon stent peritonitis. These are the preferred methods to model the **polymicrobrial** insult of **surgical / perioperative** sepsis (41) (42, 43).

AIMS:

To manage the complexity and demonstrate specific biological dysfunction in systems in order to gain a better understanding of the pathophyisology (Fig.1).

LIMITATIONS OF MODELS

Models by their nature are a controlled artificial environment with a set of specified procedures that in themselves can effect the outcome. Anaesthesia, which was a requirement of our licence, can effect onset and survival after LPS injection (44) and opiates, which were used in the CLP work, may alter the immune response (45). However, deep sedation incorporating opiates is used in the clinical management of patients with severe sepsis.

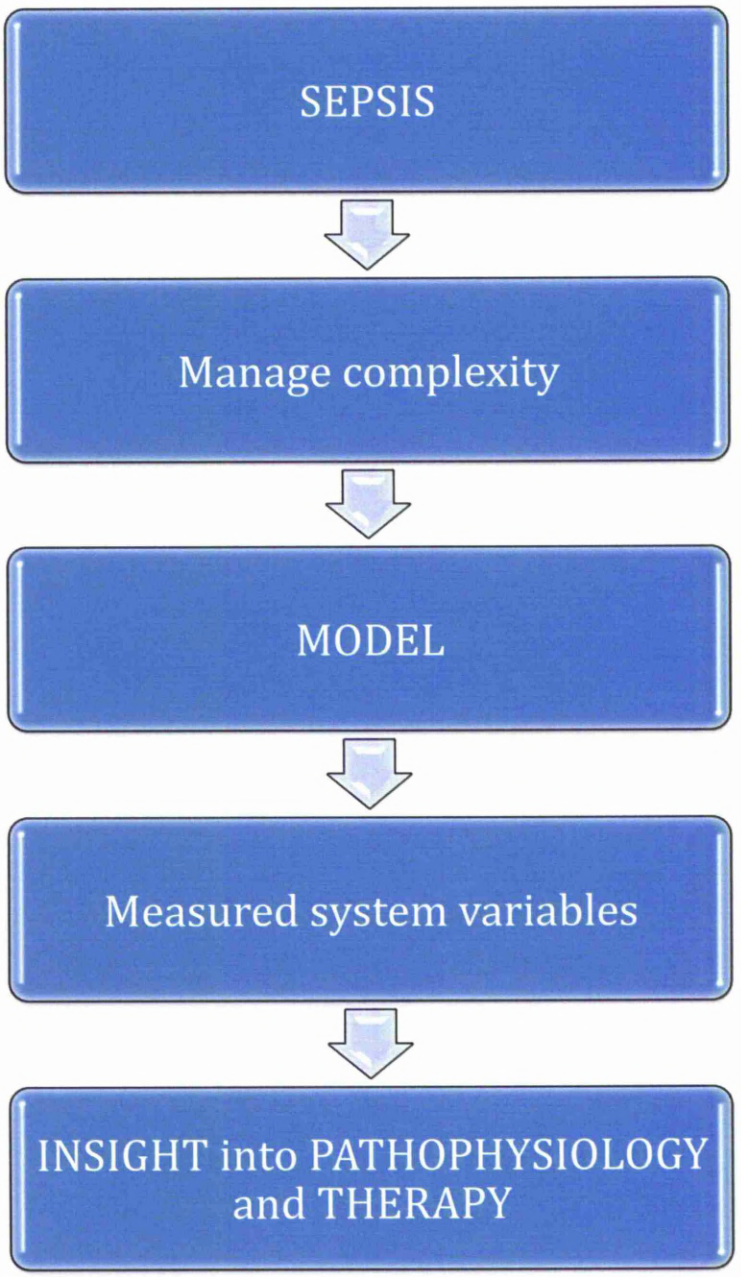


Fig 1: Aims of a model of sepsis

LEGAL REQUIREMENTS

IN ACCORDANCE WITH ANIMALS (SCIENTIFIC PROCEDURES) ACT 1986 THE SPECIFIC LICENCES UNDER WHICH WORK PERFORMED:

PROJECT LICENCE : PPL 40/2692

PERSONAL LICENCE : PIL 40/7646

In the first round of experiments both models have been implemented:

1. PRELIMINARY CLP (SURGICAL SEPSIS) AND LPS (ENDOTOXAEMIA) WORK

Caecal ligation and puncture produces a polymicrobial insult more akin to that seen in sepsis due to traumatic injury or surgery (42, 43).

METHOD

After obtaining a Home Office licence, we investigated thirty-two healthy, 8-10 week old, inbred, non-obese, male C57BL/6J mice (obtained from Charles River, UK) (body wt. 25-28 g). Eight animals served as controls; in eight animals, sepsis was induced by caecum ligature/puncture (CLP), in another eight animals by intra-peritoneal LPS injection (25 μg/g body wt) and eight animals were sham-operated. Twenty-four hours after induction of sepsis, the experimental animals were killed and RNA extracted from the epididymal fat pads (Fig.8). Real time PCR (see later experimental protocol) was used to quantify expression of IL-6, TNF_{α} , IL-18, MCP-1, nerve growth factor (NGF), hypoxia-induced factor (HIF-1α), adipsin, adiponectin and leptin, in comparison to controls.

CLP SURGICAL PROTOCOL

The mouse was weighed and anaesthetised (see separate protocol).

The mouse was positioned on a cork board using loops of micropore tape (only need to tape lower limbs) and a heating mattress placed under the board. The abdomen was shaved (hovering the shaved fur) and cleaned with aqueous, not alcoholic, iodine solution.

A midline abdominal incision was made using small scissors and opened in 2 layers - being careful not to damage bowel.

The caecum was exposed - cotton buds are useful to aid retraction and move bowel (the abdomen of sham operated animals would be closed at this point as described below).

Once mobilised the caecum was tightly ligated with a silk tie at the base.

The caecum was punctured twice with a 21G needle, distal to the ties, and squeezed to expel some of its contents.

The abdomen was closed in 2 layers with 4/0 Dexon - peritoneum and musculature with a continuous suture and skin with interrupted sutures.

The mouse was given a subcutaneous injection of 1ml warmed 0.9% Saline.

Analgesia was provided, anaesthesia reversed, and post op care provided as described in the separate protocol.

ANAESTHESIA PROTOCOL

The mouse was placed in the anaesthetic chamber and general anaesthesia induced with 1-2% isoflurane (Abbott, uk) in nitrous oxide / oxygen mixture (N2O/O2/ISO).

Once anaesthetised the mouse was removed from chamber and injected with:

- Ketamine 75mg/kg - 0.2ml of 1:10 dilution
- Medetomidine 1mg/kg - 0.3ml of 1:10 dilution (reduce to 0.2ml if weighs <25g)

Supplemental oxygen was provided while the injectable anaesthetic took effect.

REVERSAL

The mouse was administered an Antisedan injection - 0.1ml sc of a 1:10 dilution.

POST-OP ANALGESIA

Analgesia was provided by an injection of Buprenorphine - 0.15ml sc of 1:20 dilution

POST-OP CARE

The mouse was given a fluid bolus of 1ml 0.9% Saline sc to the scruff and placed on bubble wrap mattress in an incubator with 2L /min O2. The incubator temperature was monitored - aiming to maintain ~30-32 C (heating mattress may need turning of and on) and the mouse turned frequently - ~ 30 minutely intervals. It was transferred to an individual cage when became mobile. Further fluid boluses 0.5ml 0.9% Saline sc were administered as required (if lethargic). The mice were monitored regularly by a trained anaesthetist as per license requirements.

LPS PROTOCOL

24Hr LPS

The mouse was weighed.

The mouse was placed in the anaesthetic chamber and general anaesthesia induced with 1-2% isoflurane (Abbott, uk) in nitrous oxide / oxygen mixture (N2O/O2/ISO).

Once deeply anaesthetised 600 µg of LPS (Escherichia coli O 111:B4, Sigma-Aldrich) was injected ip (0.3ml) ~ 25µg / g

To compensate for fluid loses 1ml of Saline was administered sc to the scruff.

Analgesia was provided by giving Buprenorphine injection sc 0.15ml of 1:20 dilution.

Post procedure the mice were housed in separate clean cages and maintained in the same temperature controlled (24-28°C) conditions with free access to standard laboratory chow and water.

Repeat fluid boluses were administered as indicated.

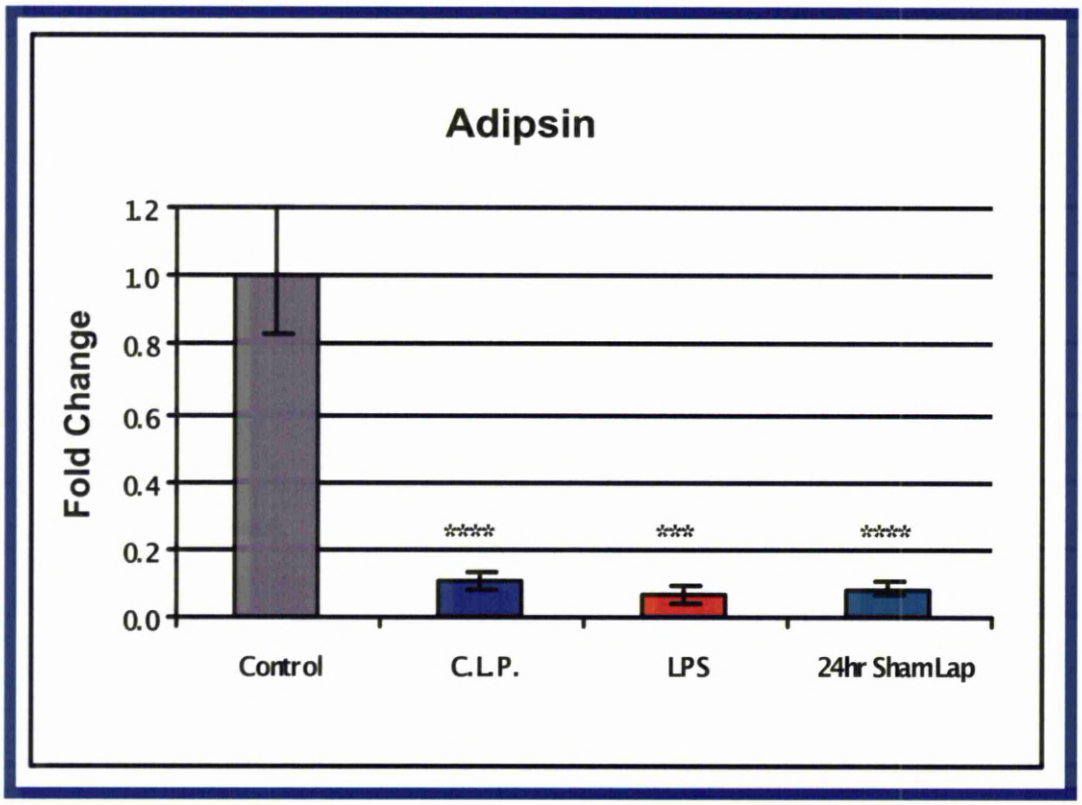
SAMPLE COLLECTION

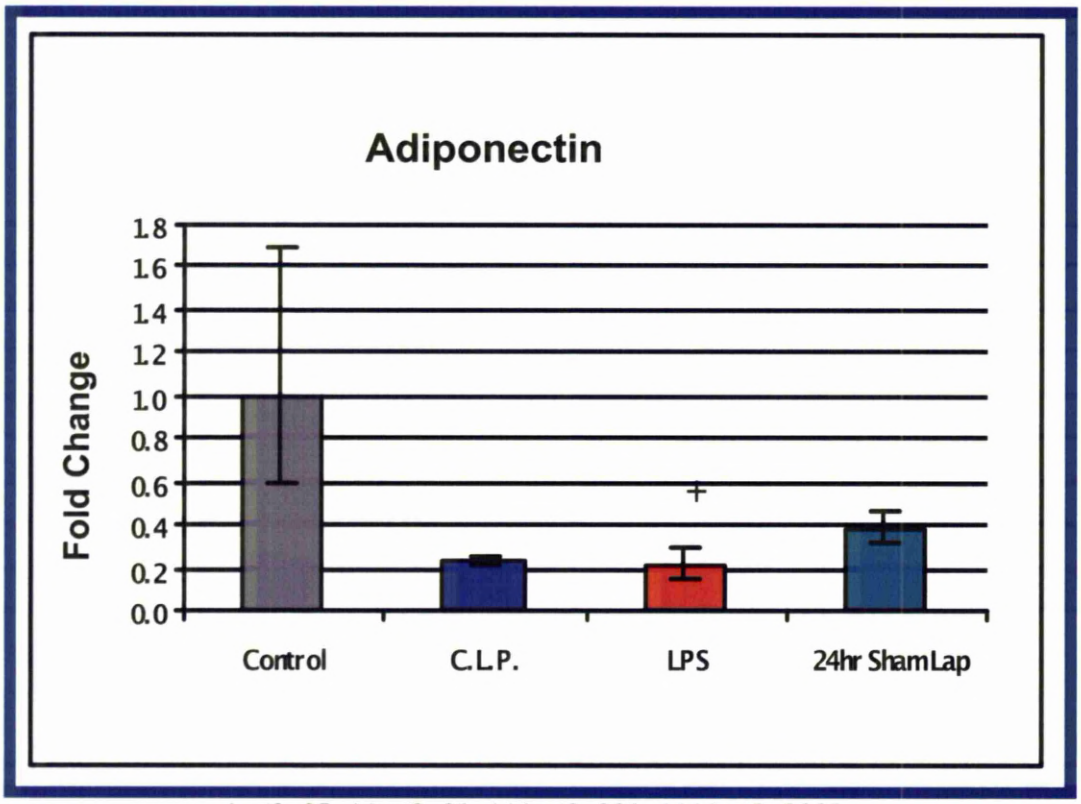
24 hours post procedure all animals were killed by the schedule 1 method of rising concentration of carbon dioxide.
The fat deposits were removed and immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

RESULTS

Twenty-four hours after induction of sepsis by LPS, real time PCR revealed substantial increases in the expression of IL-6 (500-fold), MCP-1 (55-fold), NGF (10-fold), TNF_{α} (5-fold) and HIF-1α (3-fold), while there were significant decreases in the expression of IL-18 (2.5-fold), adipsin (6-fold) and adiponectin (5-fold). The results in the CLP-group were qualitatively similar.

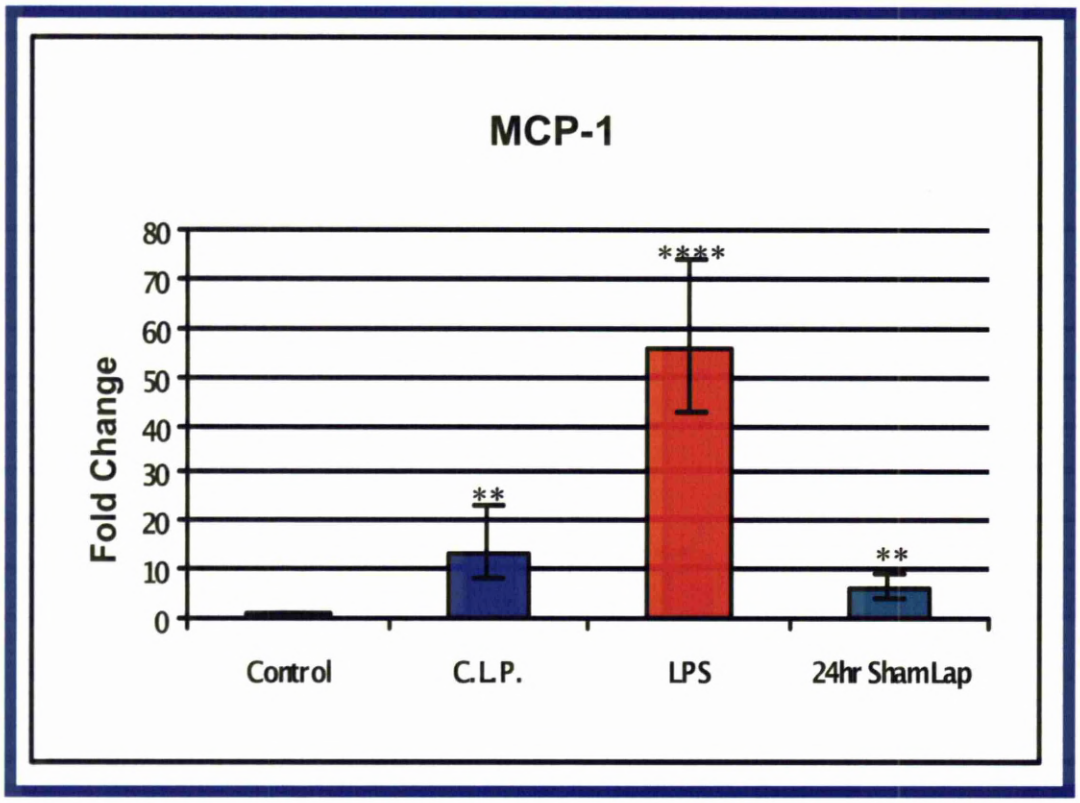
The work in these first set of experiments was presented as a poster (see appendix) at 2nd International Congress of the German Sepsis Society (GSS) in Weimar, Germany , September 7 - 10 , 2005.

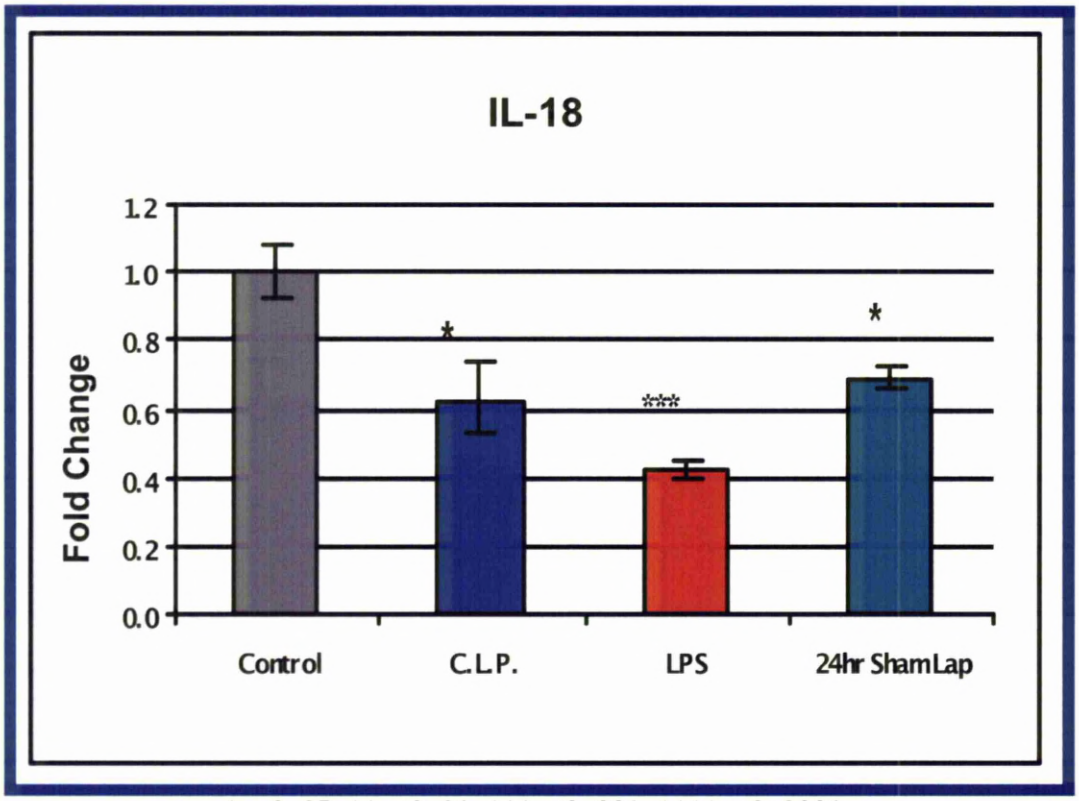




*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

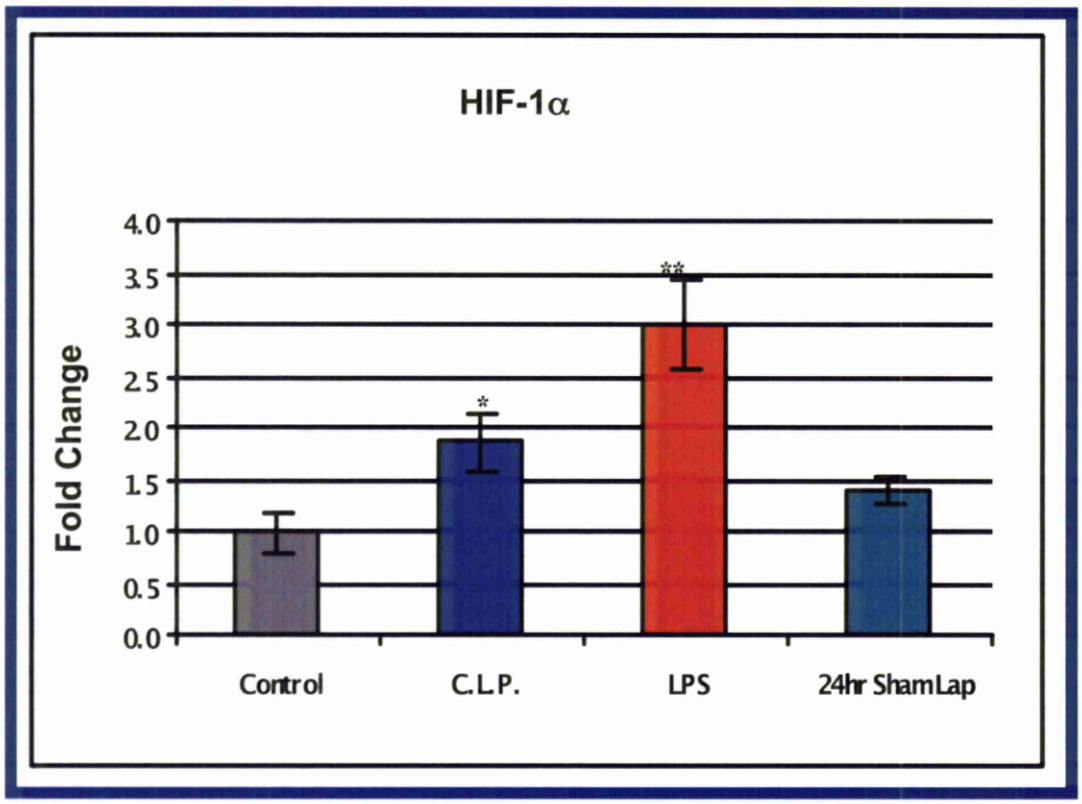
Fig. 2 Adipokine gene expression in sepsis and endotoxaemia. Twenty-four hours after induction of sepsis by CLP (blue bars) and endotoxaemia (red bars) or sham lapaparotomy (turquoise bars) surviving animals were killed by a rising concentration of CO2 and the epididymal fat depots removed. The level of each adipokine mRNA was measured by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Real time PCR revealed significant decreases in the expression adipsin and adiponectin which were qualitatively similar in both the CLP and LPS groups.
Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

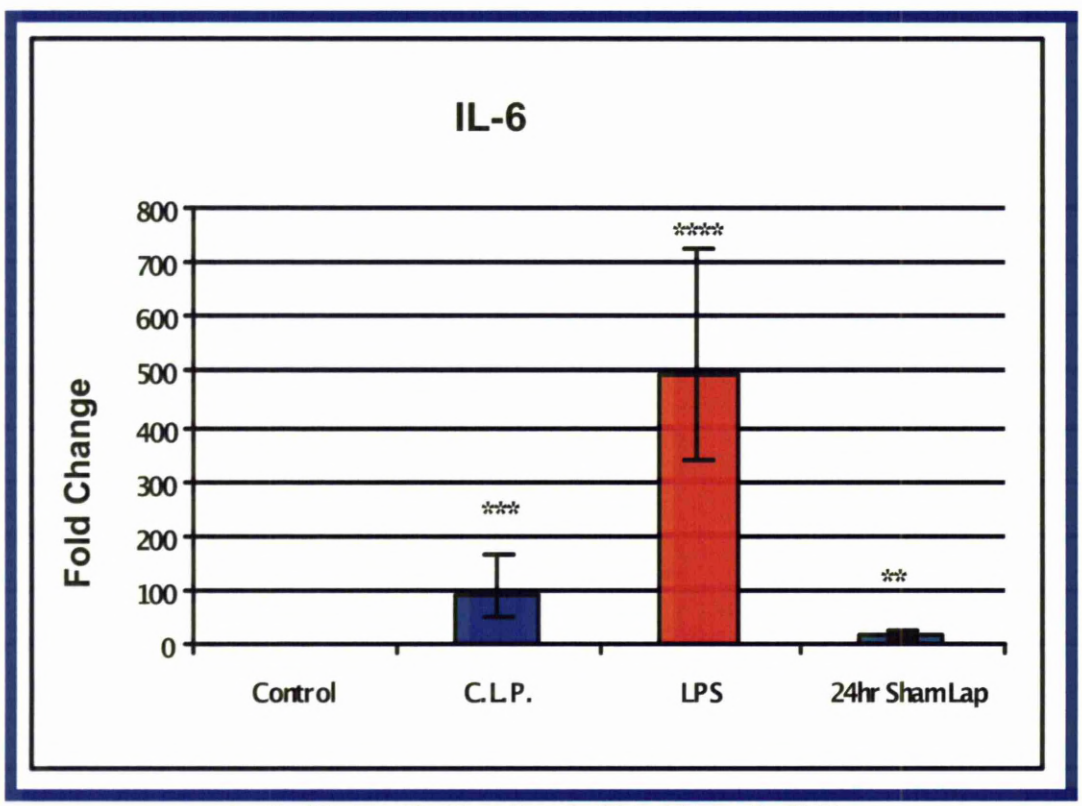




*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

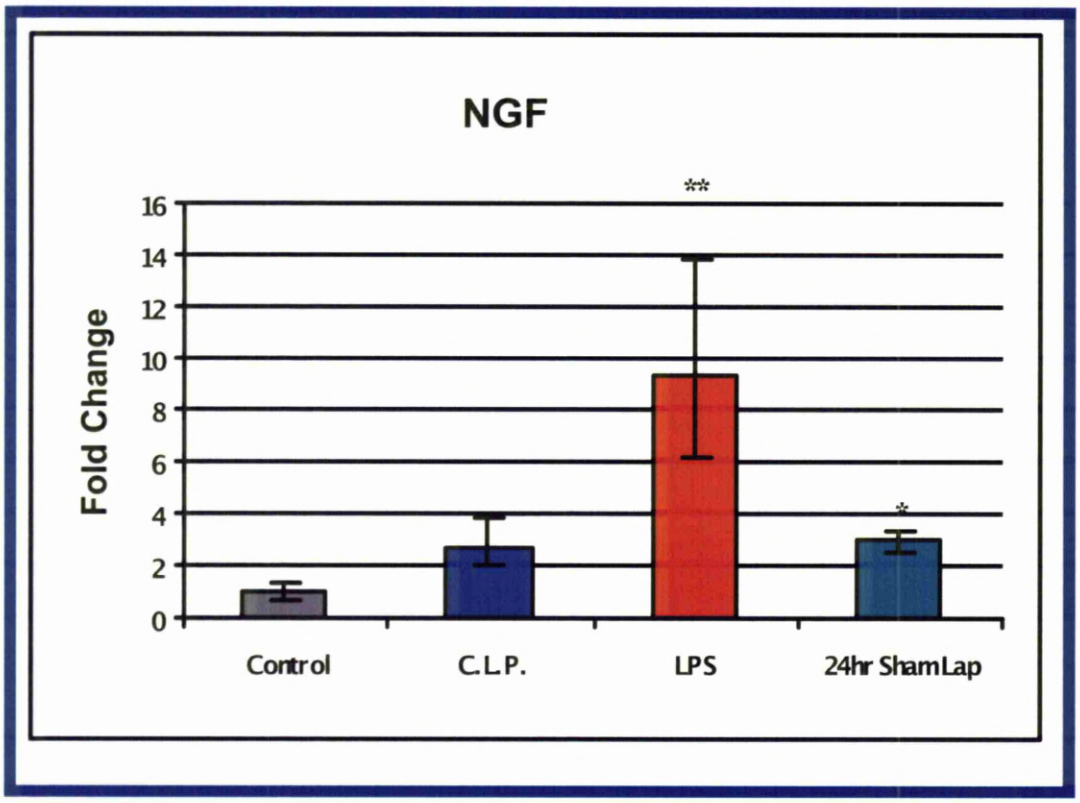
Fig. 3 T Adipokine gene expression in sepsis and endotoxaemia. Twenty-four hours after induction of sepsis by CLP (blue bars) and endotoxaemia (red bars) or sham lapaparotomy (turquoise bars) surviving animals were killed by a rising concentration of CO2 and the epididymal fat depots removed. The level of each adipokine mRNA was measured by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). In the LPS group real time PCR revealed a substantial increase in the expression of MCP-1 (55-fold, while there was a significant decrease in the expression of IL-18 (2.5-fold). The results in the CLP-group were qualitatively similar. Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

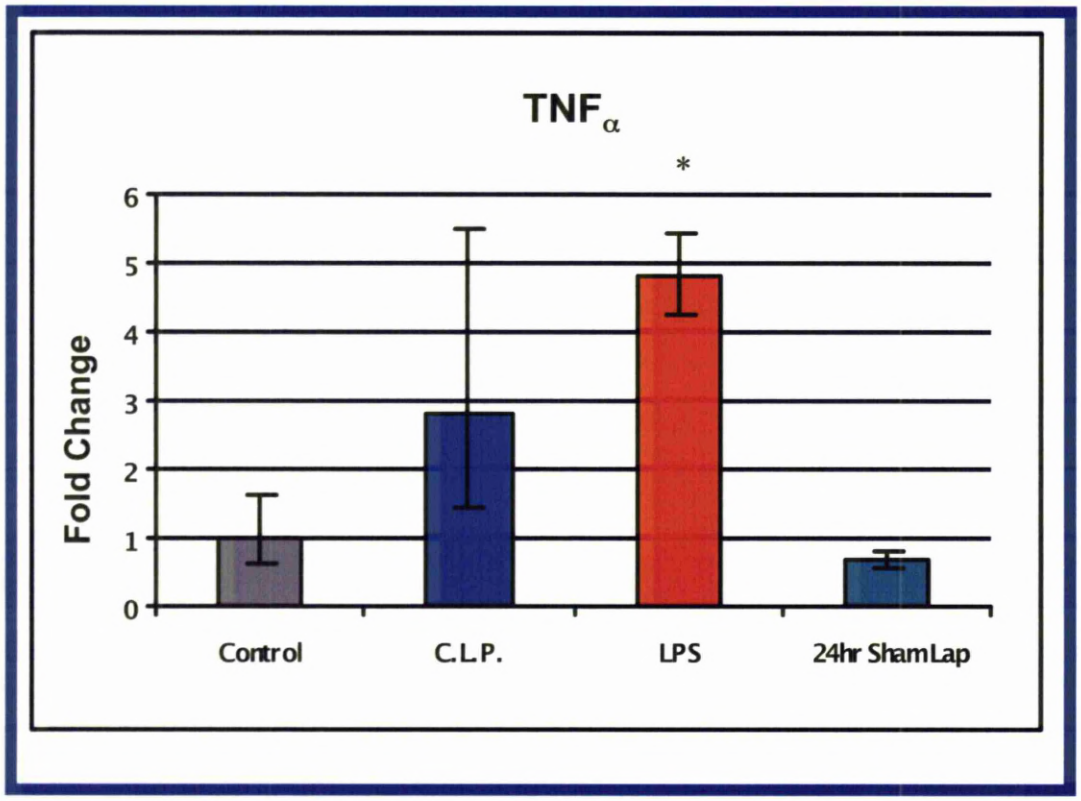




*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

Fig. 4 Adipokine gene expression in sepsis and endotoxaemia. Twenty-four hours after induction of sepsis by CLP (blue bars) and endotoxaemia (red bars) or sham lapaparotomy (turquoise bars) surviving animals were killed by a rising concentration of CO2 and the epididymal fat depots removed. The level of each adipokine mRNA was measured by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Real time PCR revealed substantial increases in the expression of IL-6 (500-fold), and HIF-1α (3-fold)in the LPS group with qualitatively similar results in the CLP-group. Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.





*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

Fig. 5 Adipokine gene expression in sepsis and endotoxaemia. Twenty-four hours after induction of sepsis by CLP (blue bars) and endotoxaemia (red bars) or sham lapaparotomy (turquoise bars) surviving animals were killed by a rising concentration of CO2 and the epididymal fat depots removed. The level of each adipokine mRNA was measured by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Real time PCR revealed substantial increases in the expression of NGF (10-fold) and TNF_{α} (5-fold)in the LPS group with results in the CLP-group being qualitatively similar. Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

2. REFINED LPS MODEL (ENDOTOXAEMIA)

Intraperitoneal injection of LPS was used which produces **endotoxaemia**. The efficacy had been proven from the preliminary work (see first set of experiments).

Further refinements were made to initial experimental model :

No opiates were used as they could effect inflammatory response (45).

The organs were harvested after cervical dislocation to minimise premortem hypoxia (compared to rising concentration of CO2).

As no laparotomy was performed there was no potential influence from the surgical trauma.

Confounding factors were reduced by using a single sex (male to exclude the oestrous cycle), strain of mice, and a single operator performing all procedures. Baseline and control groups were included to assess if the stress of the new environment for the mice had an effect. A mixture of procedures performed each day – as mice for different days could have been from different batches.

More fat depots were collected and analysed for more markers than in the previous set of experiments.

These experiments have led to a publication (46) in Pflugers Archiv : European journal of physiology which itself has been regularly cited - now (December 2012) by over 20 articles (see appendix for list and copy of paper).

LPS METHOD

Male 8 - 10 week old, inbred, C57BL/6J mice (obtained from Charles River, UK) were used in all experiments.

Mice were kept in the animal house for at least one week prior procedures, as per departmental protocol, to assure fitness.

The care of the mice and all experimental procedures were approved by the UK Home Office and were conducted in accordance with the appropriate Project License.

The mice were weighed prior to the procedure.

Anaesthesia was induced with 1-2% isoflurane (Abbott, uk) in nitrous oxide / oxygen mixture (N2O/O2) and maintained with the same agents.

Prior to injection the proposed site was cleaned with iodine solution.

LPS (Escherichia coli O 111:B4, Sigma-Aldrich) was injected intraperitoneally (ip) under general anaesthesia at a dose of 25 mg/kg.

Control animals were administered equivalent volumes of normal saline, ip.

All animals received 1 ml of normal saline subcutaneously (sc) to compensate for fluid losses.

Post procedure the mice were housed in separate clean cages and maintained in the same temperature controlled (24-28°C) conditions with free access to standard laboratory chow and water.

The animals were exposed to the same standard 12 hour day / night conditions.

The mice were monitored regularly by a trained anaesthetist as per license requirements.

All mice were killed at 4 or 24 hours after the injection of LPS by cervical dislocation.

The epididymal, subcutaneous and perirenal fat deposits were removed and immediately frozen in liquid nitrogen and stored at -80°C till further analysis.

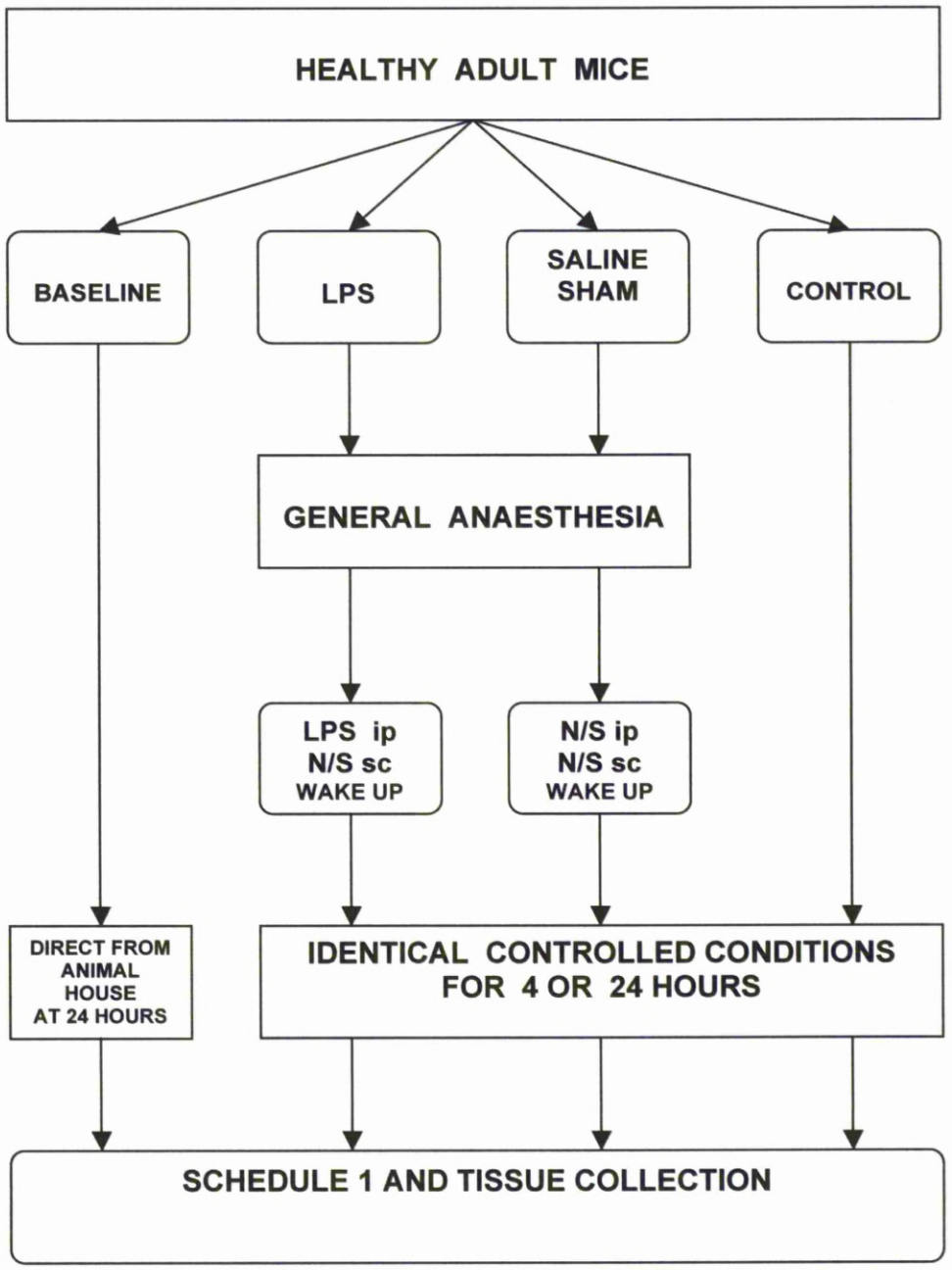


Fig.6: Flow diagram of procedures for the LPS model

LPS = Lipopolysacharide, ip = intraperitoneal, sc = subcutaneous, N/S = Normal (0.9%) saline

RNA EXTRACTION AND PCR

Total RNA was isolated from the adipose tissues using Trizol reagent (Invitrogen, UK).

Extracted RNA was quantified spectrophotometrically at 260 nm. Ratio of extinction at 260 and 280nm was between 1.7 and 1.9, and the quality was assessed by visualization of 18s and 28s ribosomal bands on an agarose gel.

1 µg of DNase I-treated RNA was reverse transcribed using a Reverse-iT™ 1ST Strand Synthesis Kit (Abgene, UK) in the presence of anchored oligo dT in a total volume of 20 µl. on a PCR Express thermal Cycler (Hybaid, Ashford, UK). Controls without reverse transcription were performed to exclude the possibility of DNA contamination.

Real-time polymerase chain reaction (PCR) reactions were carried out in a final volume of 12.5 µl consisting of 12.5-50 ng of reverse transcribed cDNA mixed with optimal concentrations of primers and probe and qPCR™ Core kit (Eurogentec, UK) in 96-well plates on a Mx3005P detector (Stratagene, USA). The primer and probe sets were designed using Primer Express software (Applied Biosystems) or Beacon Designer (Biosoft, USA) and synthesized commercially (Eurogentec). The sequence of primers and probes is shown in Table 2. Typically, the amplification started with 10 min at 95°C and then 40 cycles of the following: 15 s at 95°C and 1 min at 60°C. β-actin was used as an endogenous reference. Relative quantitation values were expressed using the 2-ΔΔCt method (see User bulletin #2, ABI Prism 7700, Applied Biosystems) as fold changes in the target gene normalized to the reference gene and related to the expression of the controls. The PCR efficiency in all runs was close to 100%, and all samples were analyzed in at least duplicate.

Gene	Sequence 5'-3'
Adiponectin	Forward GGCTCTGTGCTCCTCCATCT
	Reverse AGAGTCGTTGACGTTATCTGCATAG
	Probe CCCATACACCTGGAGCCAGACTTGGT
Adipsin	Forward ACGACCTCATTCTTTTTAAGCTATCC
	Reverse CCGGGTTCCACTTCTTTGTC
	Probe TCCCCACGTGAGACCCCTACCCTT
β-actin	Forward CAAGAAGGAAGGCTGGAAAAG
	Reverse ACGGCCAGGTCATCACTATTG
	Probe ACGAGCGGTTCCGATGCCCTG
F4/80	Forward AAGACTTGATACTCCAAAGTGAGC
	Reverse GAAGGAAGCATAACCAAGATCCC
	Probe CCCTGCACTGCTTGGCATTGCTGT
HIF-1α	Forward CAAGTCAGCAACGTGGAAGGT
	Reverse CTGAGGTTGGTTACTGTTGGTATCA
	Probe TTCACTGCACGGGCCATATTCATGTC
IL-6	Forward ACAACCACGGCCTTCCCTACTT
	Reverse CACGATTTCCCAGAGAACATGTG
	Probe TTCACAGAGGATACCACTCCCAACAGAACCT
IL-18	Forward GGCCTCTATTTGAAGATATGACTGATT
	Reverse CCTCTAGGCTGGCTATCTTTATACATACT
	Probe TGACTGTAGAGATAATGCACCCCGGACC
Leptin	Forward CATCTGCTGGCCTTCTCCAA
	Reverse ATCCAGGCTCTCTGGCTTCTG
	Probe AGCTGCTCCCTGCCTCAGACCAGTG
MAC-1	Forward GAATGGATTGTGCTATTTGTTCGG
	Reverse CGGAGCCATCAATCAAGAAGAC
	Probe TCCAACCTGCTGAGGCCGCCC
MCP-1	Forward GGCTCAGCCAGATGCAGTTAA
	Reverse CCTACTCATTGGGATCATCTTGCT
	Probe CCCCACTCACCTGCTGCTACTCATTCA
MT2	Forward CTTCTTGTCGCTTACACCGTT
	Reverse AGCAGCACTGTTCGTCACTTC
NGF	Forward GCCAAGGACGCAGCTTTCTAT
	Rev AGTGATCAGAGTGTAGAACAACATGGA
	Probe CTGGCCGCAGTGAGGTGC
TNFα	Forward CCCAGACCCTCACACTCAGATC
	Reverse GCCACTCCAGCTGCTCCTC
	Probe TAGCCCACGTCGTAGCAAACCACCAAG

Table 2. Primer and probe sequences used in real time PCR (amended) (46)

MEASUREMENT OF PROTEIN BY ELISA

The total amount of HIF-1α in adipose tissue homogenates was measured by enzyme-linked immunosorbent assay (ELISA) (R & D Systems, UK) as previously described. The adiponectin, IL-6 and MCP-1 content of adipose tissue homogenates was also measured using commercial ELISAs, according to the manufacturer's protocol. For adiponectin (R & D Systems), the stated minimal detectable amount was 3 pg/ml, and the interassay coefficient of variation was 6%. For Il-6 (RayBiotech, USA), the minimal detectable quantity was 6 pg/ml and the interassay coefficient of variation <12%, while for MCP-1 (RayBio- tech), the values were <3 pg/ml and <12%, respectively.

DATA PRESENTATION AND STATS

Data are expressed as the mean ± standard error of mean. The statistical significance of differences between treated and control groups was assessed with two-tailed Student's t test for independent samples. A p valve <0.05 was considered to be statistically significant.

RESULTS

Effect of LPS on adipokine expression 24 h after the induction of endotoxaemia

In order to induce severe endotoxaemia, a high dose of lipopolysaccharide was administered; the survival rate at 4 h was 100%, while the 24 h survival rate was 61% [14/23]. In the first experiments, the mice were treated with LPS for 24 h and the epididymal, perirenal and subcutaneous adipose tissue depots examined. Tissues were only analysed for those animals that survived. LPS administration had no significant effect on leptin mRNA level in the epididymal WAT; nor was there any change in metalliothein-2 mRNA (Fig.7). However, LPS induced a fivefold fall in adiponectin mRNA level, and there was a similar reduction in the level of both adipsin and IL-18 mRNA (Fig.7). In contrast to these genes, there were increases in mRNA level for IL-6, MCP-1, NGF and TNFα (Fig.7). The increase in NGF mRNA was small (twofold), but MCP-1 and TNFα mRNA levels increased by 24- and 18-fold, respectively, while IL-6 mRNA level increased as much as 250-fold.

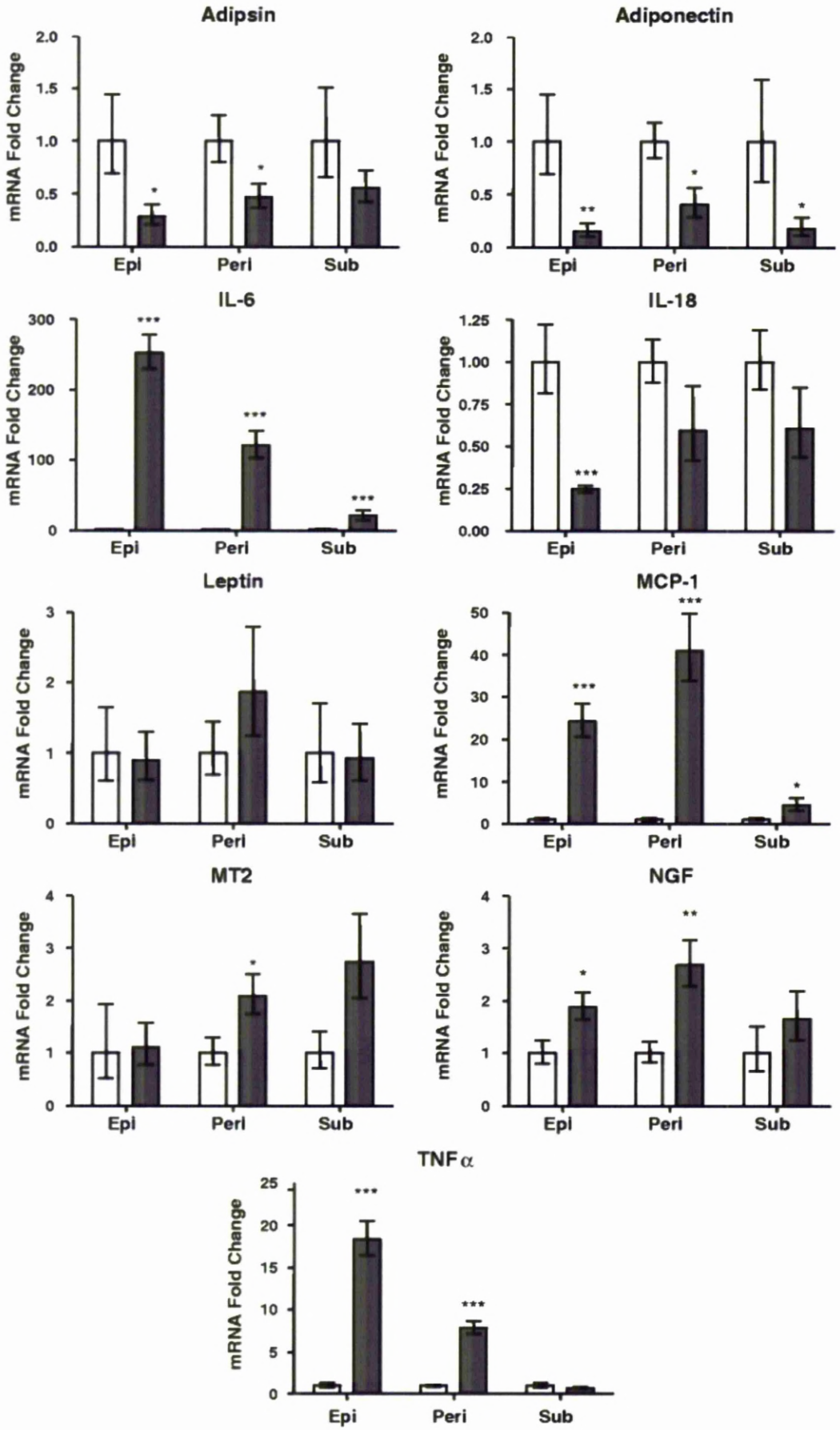

Adipsin
mRNA Fold Change
2.0
1.5
1.0
0.5
0.0
Epi
Peri
Sub
Adiponectin
IL-6
300
200
100
0
IL-18
1.25
1.00
0.75
0.50
0.25
0.00
Leptin
3
2
1
MCP-1
50
40
30
20
10
MT2
4
3
NGF
TNFα
25
20
15
10
5

Fig. 7 Adipokine gene expression in endotoxaemia in mice (46). Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 24 h later; control mice (*open bars*) received a saline injection. The level of each adipokine mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). *IL-6* Interleukin 6, *IL-18* interleukin 18, *MCP-1* monocyte chemoattractant protein 1, *MT2* metallothionein 2, *NGF* nerve growth factor, *TNFα* tumour necrosis factor α. Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

Adiponectin gene expression in epididymal fat pads previously published in M.Phil thesis by Honggwang Bao (47)

Examination of the perirenal adipose tissue depot gave broadly similar results, with reductions in adipsin and adiponectin mRNA levels (Fig.7). However, the changes were less substantial than with the epididymal depot, the decreases in IL-18 and adiponectin mRNA levels being statistically insignificant. Leptin was again unchanged following treatment with LPS. TNFα mRNA level increased by eightfold, MCP-1 by 40-fold and the largest increase (120-fold) was with IL-6 (Fig.7).

The results obtained with the subcutaneous adipose tissue were less dramatic than with either the epididymal or perirenal depots. Adiponectin mRNA level fell fivefold, but there were no significant changes with leptin, adipsin, IL-18, NGF, TNFα and MT-2 (Fig.7). The lack of change in TNFα mRNA is noteworthy given the increases observed in the other two fat depots. MCP-1 and IL-6 mRNA levels were, however, elevated in subcutaneous fat in response to LPS, the increases being four- and 20-fold, respectively (Fig.7).

Effect of LPS on adipokine expression 4 h after the induction of endotoxaemia

To assess the speed of the response to LPS, studies were next undertaken at 4 h after injection. Epididymal, perirenal and subcutaneous adipose tissue depots were again examined. In the epididymal depot at 4 h, there were no significant changes in adipsin, adiponectin, IL-18 and MT-2 mRNA levels (Fig.8). There were, however, small, but statistically significant, increases in leptin (fourfold) and NGF (3.8-fold) mRNAs. Substantial increases were observed for TNFα (20-fold) and particularly MCP-1 (250-fold) and IL-6 (500-fold) mRNA. A broadly similar pattern was observed in the perirenal depot, although in this case, there was no change in leptin

mRNA level. The largest changes were again with MCP-1 and IL-6 mRNA, the levels of which were increased by 35- and 78-fold, respectively (Fig.8). TNFα level increased 23-fold, and a modest increase (fourfold) was observed with NGF.

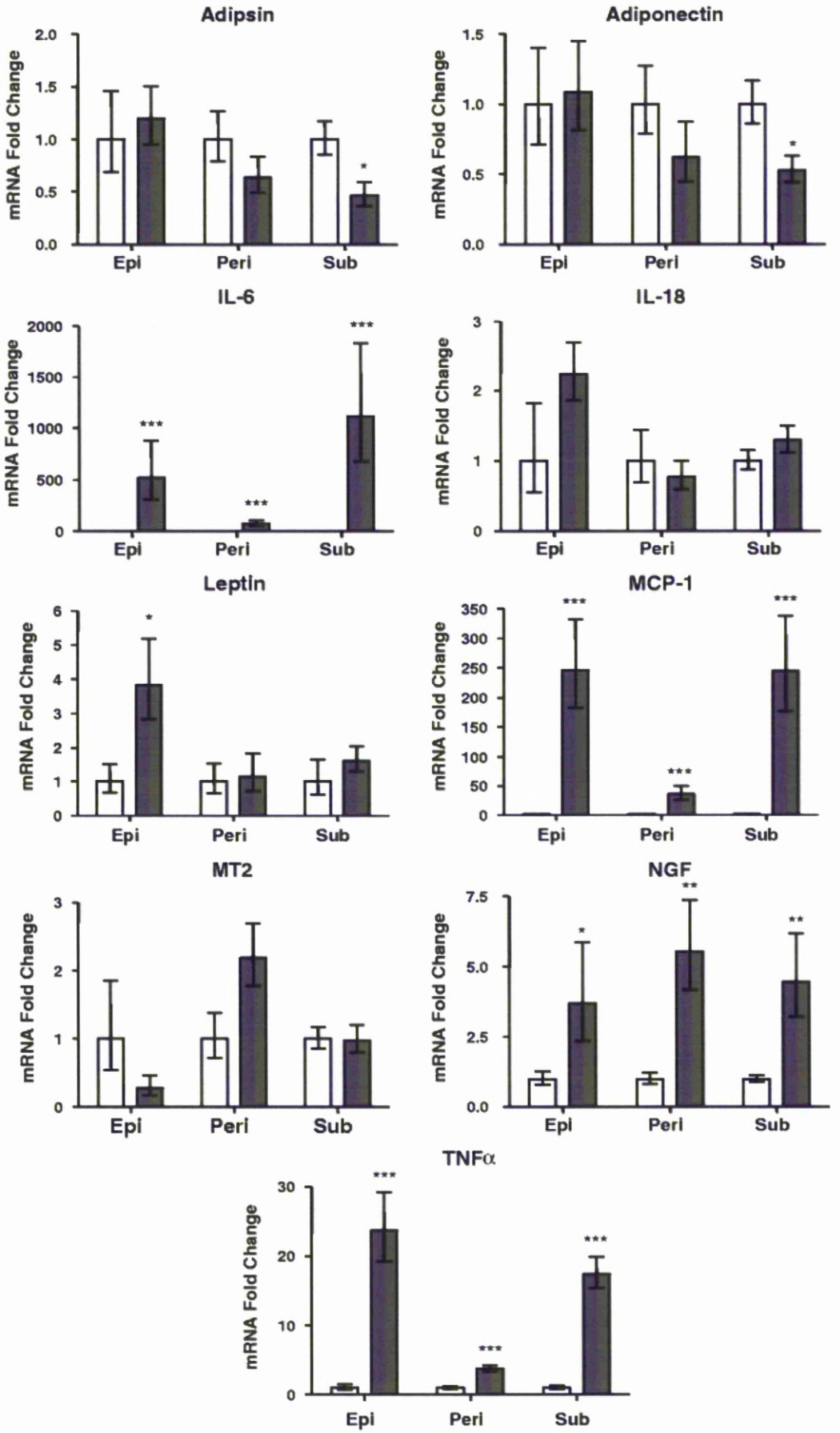

Adipsin
mRNA Fold Change
2.0
1.5
1.0
0.5
0.0
Epi
Peri
Sub
*
Adiponectin
mRNA Fold Change
1.5
1.0
0.5
0.0
Epi
Peri
Sub
*
IL-6
mRNA Fold Change
2000
1500
1000
500
0
***
***
***
Epi
Peri
Sub
IL-18
mRNA Fold Change
3
2
1
0
Epi
Peri
Sub
Leptin
mRNA Fold Change
6
5
4
3
2
1
0
*
Epi
Peri
Sub
MCP-1
mRNA Fold Change
350
300
250
200
150
100
50
0
***
***
***
Epi
Peri
Sub
MT2
mRNA Fold Change
3
2
1
0
Epi
Peri
Sub
NGF
mRNA Fold Change
7.5
5.0
2.5
0.0
*
**
**
Epi
Peri
Sub
TNFα
mRNA Fold Change
30
20
10
0
***
***
***
Epi
Peri
Sub

Fig. 8 Acute effects of endotoxaemia on adipokine gene expression in mice (46). Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 4 h later; control mice (*open bars*) received a saline injection. The level of each adipokine mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

Adiponectin and TNFα gene expression in epididymal fat pads previously published in M.Phil thesis by Honggwang Bao (47)

In the subcutaneous adipose tissue, there were no changes in mRNA levels for IL-18, leptin and MT-2 (Fig.8). There was, however, an approximately 50% reduction in mRNA levels for adipsin and adiponectin. NGF and TNFα mRNA levels increased moderately, by four- and 17-fold, respectively. Very major changes were observed with MCP-1, the mRNA level of which increased 250-fold, and with IL-6 where the increase was >1,000-fold (Fig.8).

White adipose tissue adipokine levels

The adipokine protein content was measured by ELISA in the epididymal and subcutaneous fat depots for the two inflammatory adipokines which exhibited the largest changes in mRNA level. IL-6, which showed the greatest elevation in mRNA, was increased in both the epididymal and subcutaneous fat at 4 and 24 h after the injection of LPS (Fig.9). The increase in the epididymal depot was between five- and eightfold, while in the subcutaneous tissue the rise was between three- and eightfold.

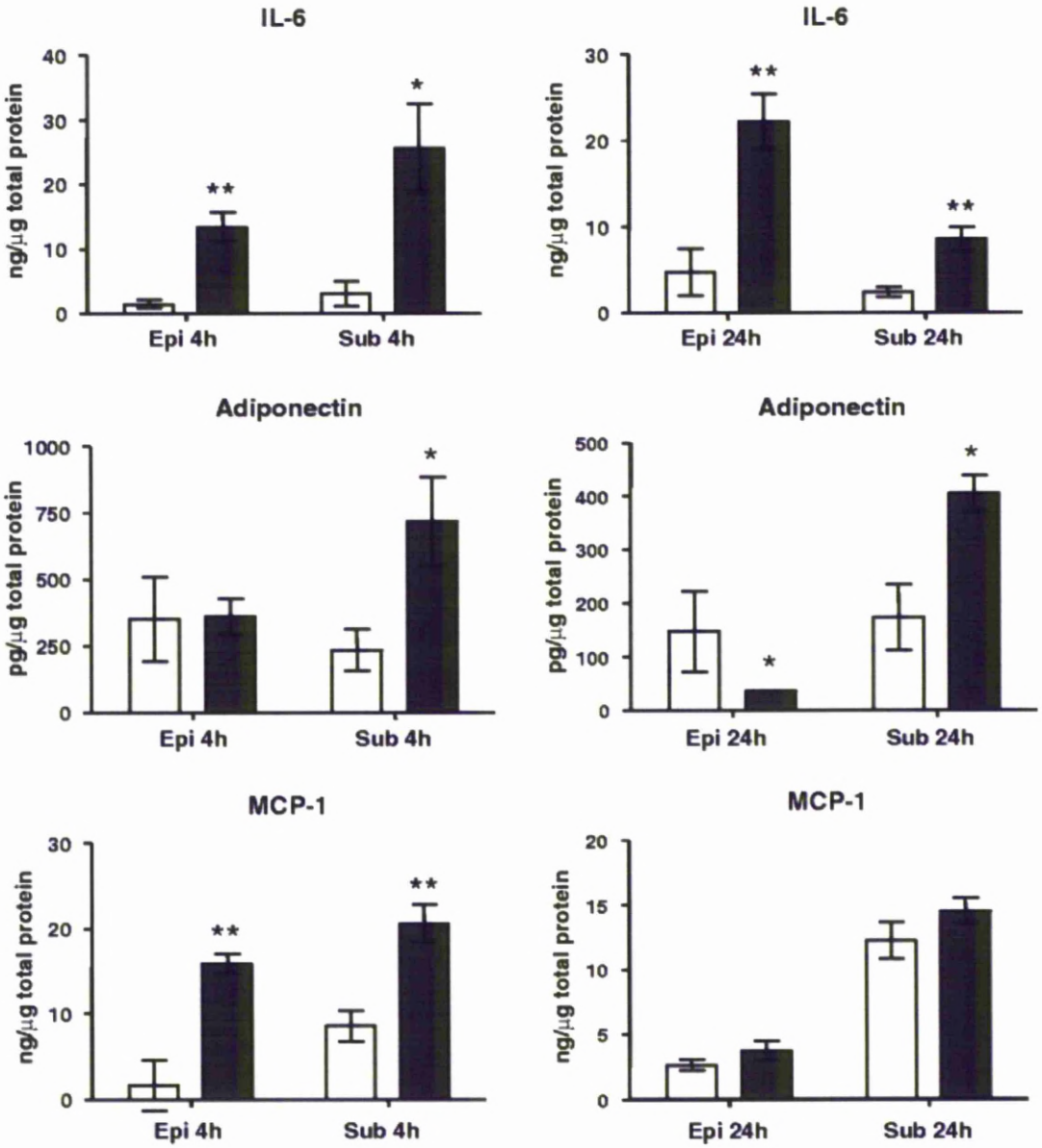


Fig. 9 Adipokine levels in adipose tissue in endotoxaemia in mice (46). Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. Adipokine proteins were measured in the epididymal (*Epi*) and subcutaneous (*Sub*) depots by ELISA. Results are given as mean values ± SE for three to six mice in each group. *$P < 0.05$, **$P < 0.01$ compared with controls.

In contrast to IL-6, there was no significant increase in MCP-1 protein in either tissue at 24 h. However at 4 h, the amount of MCP-1 was approximately eightfold greater in the epididymal fat and twofold greater in the subcutaneous depot (Fig. 9). For adiponectin, only in the epididymal depot after 24 h of treatment with LPS was there a significant fall in the protein, reflecting the changes in the mRNA level. However, paradoxically the adiponectin level in the subcutaneous depot was actually increased, rather than decreased, at both 4 and 24 h in the LPS-treated mice.

Macrophage marker expression

To assess whether the increases in the expression of key inflammatory adipokine genes, particularly MCP-1, reflected an infiltration of macrophages into adipose tissue, the mRNA level of two macrophage markers was examined. The markers were F4/80 and MAC-1, and these were examined in 4 and 24 h LPS-treated mice and in all three WAT depots. In no case was there a statistically significant increase in F4/80 and MAC-1 mRNA level (Fig.10). Indeed, in one case, the perirenal tissue treated for 24 h, the level of both mRNAs was significantly decreased. F4/80 mRNA level was also decreased in the epididymal depot at 24 h.

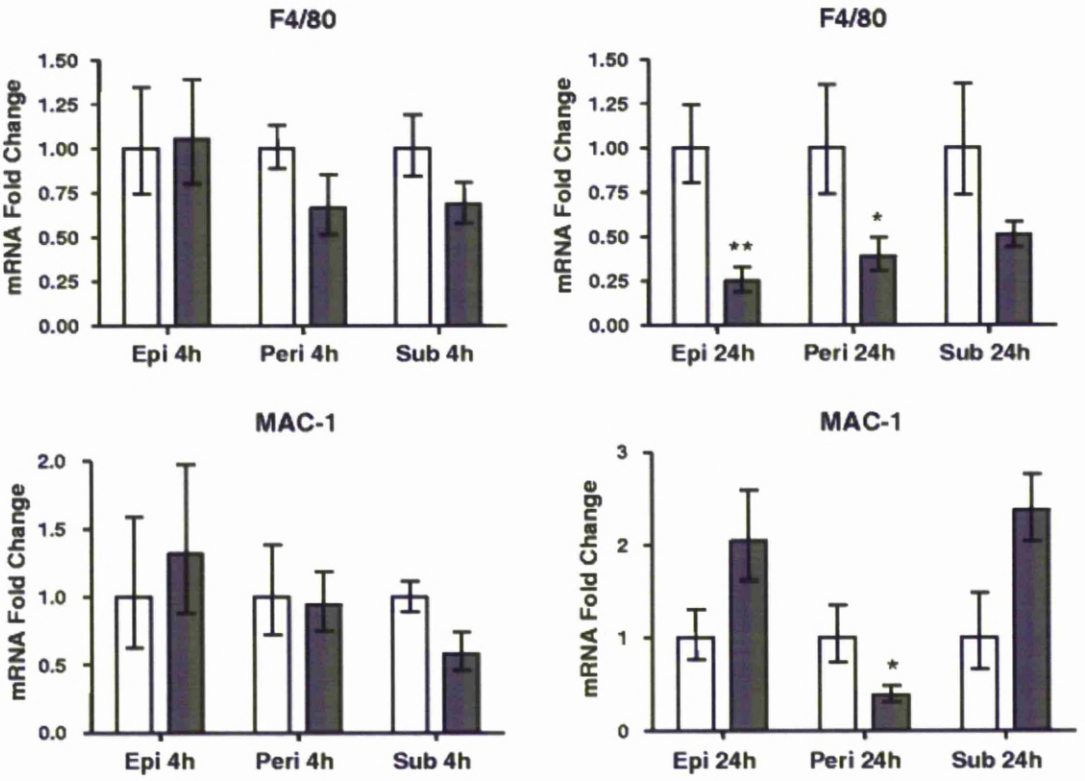


Fig. 10 Expression of macrophage markers in adipose tissue in endotoxaemia in mice (46). Mice were injected with LPS to induce endotoxaemia (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. The level of each macrophage marker mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$, $***P < 0.001$ compared with controls.

HIF-1α as an index of hypoxia

In the final investigation, the effect of LPS treatment on the key molecular index of cellular oxygen deficiency, the hypoxia-sensitive transcription factor HIF-1α, was examined. The expression and protein level of HIF-1α was determined in both the epididymal and subcutaneous adipose tissue depots. At 4 h after the treatment with LPS, there was a three to fourfold increase in HIF-1α mRNA in each depot, and importantly, this was accompanied by a two to threefold increase in HIF-1α protein (Fig.11). HIF-1α mRNA level was also twofold higher in both depots at 24 h, but at this later time point, there was no significant change in HIF-1α protein (Fig.11).

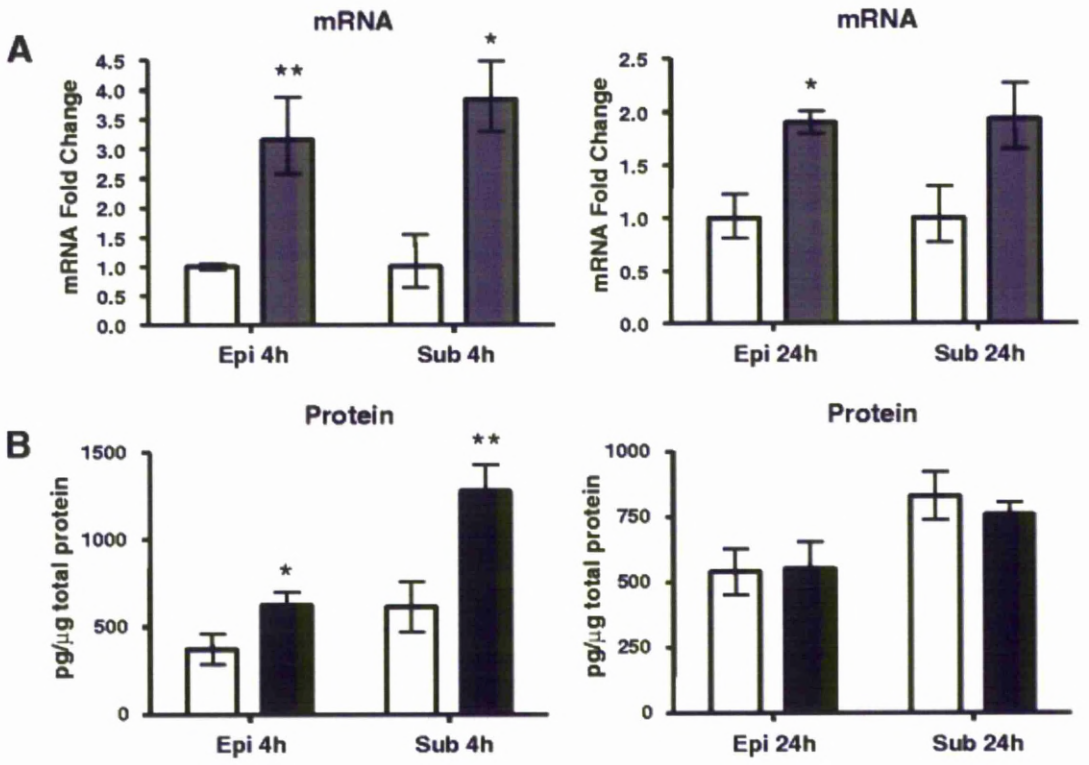


Fig. 11 HIF-1α gene expression and protein levels in adipose tissue in endotoxaemia in mice (46). Mice were injected with LPS to induce endotoxaemia (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. HIF-1α mRNA (**a**) and protein levels (**b**) were measured in the epididymal (*Epi*) and subcutaneous (*Sub*) depots by real-time PCR, the level being normalized to β-actin relative to the untreated control group (≡1), and by ELISA, respectively. Results are given as mean values ± SE for five to six mice in each group. $*P < 0.05$, $**P < 0.01$ compared with controls.

DISCUSSION

This study demonstrates that there are major changes in the expression of key adipokine genes in each of the main white adipose tissue depots in mice following the induction of endotoxaemia. The expression of several major inflammatory-related adipokines was examined, and by far the largest response was observed with IL-6 and MCP-1. Adipose tissue produces large amounts of IL-6 and is considered to be a substantial contributor to circulating levels of this cytokine (48, 49). Plasma IL-6 levels are increased in endotoxaemia as well as sepsis, and it seems likely that adipose tissue contributes directly to this (50-52), particularly since in this study the amount of IL-6 in the fat depots was increased alongside the increase in mRNA level. Nevertheless, it should be noted that the increase in protein was much less than the changes in mRNA level, although this could reflect rapid release of IL-6 from the tissue once synthesised.

The chemokine MCP-1 shows increased expression in obesity, like IL-6, and is linked to the recruitment of macrophages within tissues, including adipose tissue. Indeed, in obesity there is a major infiltration of macrophages into adipose tissue (53) (14). Since the mRNA level of two macrophage markers—F4/80 and MAC-1—did not increase in any of the three adipose tissue depots examined, either at 4 or 24 h after the administration of LPS, it is unlikely that there was an infiltration of immune cells during the duration of endotoxaemia in the present work. The increased MCP-1 expression suggests that macrophage recruitment into adipose tissue may well occur long-term; however, in contrast to IL-6, no sustained elevation in MCP-1 protein levels was evident in adipose tissue homogenates following LPS

treatment.

In addition to macrophage infiltration, MCP-1 has been associated with the development of insulin resistance (54). This is also the case with IL-6 and TNFα (55) (56-58). Thus, the major stimulation of the expression of these three factors in response to LPS suggests that adipose tissue may contribute to insulin insensitivity and the dysregulation of glucose homeostasis in infection. TNFα is a pleiotrophic pro-inflammatory cytokine, and its actions in adipose tissue include the stimulation of lipolysis and of apoptosis (59) (60). It also has a major effect on the expression of other inflammation-related adipokines including the strong upregulation of IL-6 and MCP-1 production (61, 62).

In marked contrast, adiponectin expression was downregulated in all three adipose tissue depots 24 h after the administration of LPS. There was also a significant reduction in adiponectin mRNA in the subcutaneous depot at 4 h. Adiponectin, which is a key adipocyte-derived hormone, has multiple physiological effects, and these include anti-inflammatory and insulin sensitising actions (63-66). Thus, a fall in adiponectin expression may add to the upregulation of IL-6, MCP-1 and TNFα expression in leading to a major pro-inflammatory response and insulin resistance in endotoxaemia. However, although adiponectin protein level decreased in the epididymal depot at 24 h (the absence of a change at 4 h may reflect the time delay between alterations in gene transcription and subsequent protein synthesis), in the case of the subcutaneous depot, there was a paradoxical increase in the amount of the hormone. We have no satisfactory explanation for this anomaly, although it may indicate that adiponectin is less readily released from the subcutaneous adipose tissue in endotoxaemia.

Furthermore, low adiponectin levels observed in obesity (67) as a state of low-grade chronic inflammation are associated with increased insulin resistance (63, 66) Low adiponectin levels on admission to the intensive care unit have also been identified as an independent predictor of survival (68). Stress-induced hyperglycaemia frequently occurs in infection and is associated with an adverse outcome (69) (70). Elevated TNFα levels, increased MCP-1 production as well as low adiponectin may act in concert to induce and to maintain hyperglycaemia in critical illness. Although this study does not reveal the extent to which adipose tissue might contribute quantitatively to alterations in circulating levels of cytokines and chemokines in endotoxaemia, overall, the results suggest a role for the tissue in the changes in glucose metabolism associated with infection. It is emphasised that since in the 24-h studies there was a distinct mortality rate (39%) and that only tissues from surviving animals were investigated, there could be a link between the gene expression profile and the protective survival response.

Expression of the complement-related factor, adipsin, also fell during endotoxaemia. Leptin, on the other hand, showed no changes in gene expression. This is in contrast with previous studies in experimental animals and in humans where an upregulation has been described (50, 71) (72). However, the previously reported changes in leptin mRNA were very small, particularly in comparison with the dramatic changes in IL-6 and MCP-1 mRNA level.

Small increases in NGF mRNA level were observed in the epididymal and perirenal depots but not in the subcutaneous tissue. This target-derived neurotrophin is also involved in the inflammatory response, its expression and secretion from both murine and human adipocytes being stimulated by TNFα (73) (61). The low molecular weight

cysteine-rich, metal-binding protein metallothionein showed little response to LPS except in the perirenal depot where there was a small increase at 24 h. Similarly, there was little change in the expression of IL-18; this pro-inflammatory cytokine plays an important role in innate and acquired immunity, and its expression in adipocytes (74, 75) has been shown to be strongly upregulated by TNFα (75).

Certain depot-specific responses to LPS were evident in this study. Of the three adipose tissue sites examined, the largest response was observed in the epididymal depot in the case of both IL-6 and TNFα (at 24 h). Indeed, the subcutaneous depot showed no increase in TNFα mRNA level at 24 h. In this context, it has been shown that subcutaneous adipose tissue is affected to a lesser degree by sepsis-induced malperfusion than the splanchnic area and does not contribute to lactate production during critical illness (15).

In addition to depot differences, there is the important question of which cells within adipose tissue may be responsible for alterations in adipokine/cytokine production in endotoxaemia. White fat consists of two major components, mature adipocytes and the stromal-vascular fraction. The stromal-vascular fraction includes macrophages, fibroblasts, vascular endothelial cells and preadipocytes. Adipocytes are able to express each of the inflammation-related signals examined in the present study (see (16) (29)). However, while the key cytokines and chemokines are also expressed by macrophages, adipocytes alone are responsible for adiponectin and leptin production.

It could be hypothesized that during endotoxaemia, macrophages infiltrate adipose tissue, causing further inflammatory damage and substantially contributing to mediator production. This would imply

that immigrating immune cells are mainly responsible for the changes observed. However, as noted earlier, the mRNA level of two specific macrophage markers did not increase in WAT and even decreased in the case of the perirenal depot at 24 h. This suggests that mature adipocytes may be the key site of the response to LPS. Indeed, adipocytes express the Toll-like receptor 4 through which LPS exerts its inflammatory effect (76) (77). Irrespective of the cell type involved, adipose tissue can be regarded as a key inflammatory organ, which may contribute to the systemic response observed during systemic infection by generating and releasing inflammatory mediators into the circulation. However, the possibility that resident macrophages are activated and may contribute to adipokine/cytokine production in the various adipose tissue depots in endotoxaemia, without increased immune cell infiltration, cannot be excluded. It would be useful to investigate the adipose tissue using immunohistochemisty techniques that are able to preserve the architecture of the tissue to help answer the question.

It has recently been proposed that the inflammation that occurs in adipose tissue in obesity may, at least in part, be a response to relative hypoxia in groups of adipocytes distant from the vasculature (16). Evidence in support of this hypothesis is emerging (78) (79) (80), and hypoxia has been shown to directly increase the expression and release of inflammation-related adipokines such as leptin, IL-6, MIF and VEGF in murine and human adipocytes, while adiponectin with its anti-inflammatory action is decreased (81) (78) (82) (83) (80). We were therefore interested in whether hypoxia might underlie the changes in adipokine expression in endotoxaemia and hence fat depots were harvested after cervical dislocation to minimise premortem hypoxia (compared to rising concentration of CO_2). Increases in the mRNA encoding the hypoxia-sensitive transcription factor, HIF-1α,

were evident following administration of LPS, and increased HIF-1α protein was found at 4 h. This suggests that at least in the early stages of endotoxaemia, hypoxia may occur in adipose tissue. The changes at the protein level were not sustained by 24 h, implying that any hypoxia may have been transitory. However, direct HIF-1α gene activation by LPS, in a Toll-like receptor 4 manner, has been shown in macrophages (84) (85). Further work is therefore warranted on hypoxia in adipose tissue in severe infection and whether it underlies the changes in adipokine expression or if it is by direct stimulation by LPS / bacterial components. It may be possible to investigate this using gene arrays to look for specific gene expression profiles (37) at different time intervals.

A high dose of LPS was employed to induce endotoxaemia, and it may also reflect sepsis. However, the LPS challenge may not adequately reproduce the complexity of human sepsis. In particular, investigators with an interest in peri-operative sepsis have increasingly used caecal ligation and puncture (CLP) as the model of choice. In the series of experiments with CLP in the same strain of mice, it was possible to reproduce the changes in IL-6 mRNA levels found in the epididymal fat depot after LPS challenge. Here IL-6 mRNA levels were 87-fold higher than in control mice and sixfold higher than in sham-operated mice at 24 h. Apparently, CLP leads to qualitatively similar changes in IL-6 expression as LPS, suggesting that white adipose tissue is activated in septic shock as in endotoxaemia.

Clinical sepsis is commonly polymicrobial, but experimentation involving bacterial products, such as LPS, may serve to characterize response patterns directed against one particular organism (42). LPS was therefore used as the model of choice to examine the potential

role of WAT during severe infection. The dose employed, although high, is within the range used in previous studies and elicited a severe, acute, systemic inflammatory response in mice with all the symptoms of sepsis and septic shock (86). Importantly, the substantial increase in IL-6 and MCP-1 gene expression in the subcutaneous adipose tissue at 4 h indicates a systemic response in WAT rather than an effect local to the i.p. site of injection. The mortality rate of 39% in the 24-h series of experiments reflects acute life-threatening gram-negative infection of a severity similar to that occurring in human sepsis (3). Further investigations using various models of sepsis are warranted to specify the role of WAT in infection caused by different organisms.

In conclusion, the results demonstrate that an LPS challenge sufficient to mimic endotoxaemia leads to major changes in the expression of key adipokine genes in different adipose tissue depots of mice. Thus, white adipocytes (and preadipocytes) may well contribute directly to the most prominent features of infection — systemic inflammation and metabolic dysregulation. If this could be corroborated in studies involving human patients and their various fat depots, it would allow a new interpretation of the correlation between body mass index and sepsis mortality as well as the development of novel approaches to treating insulin resistance in severe infection. It is evident from our results that not all fat is equal and it would be valuable to investigate the differences between depots in the human population with samples obtained at laparotomy in patients with sepsis or chronic inflammatory states such as pancreatitis. It may also be possible to look for hypoxic responses in these tissues using gene arrays. Quantification of the size of various fat depots in septic ITU patients from their CT images and their possible relation to outcome would also be interesting.

APPENDIX

LPS MOUSE EXPERIMENTS

MOUSE NUMBER	WEIGHT	EXPT LENGTH	PROCEDUE	SURVIVAL	OTHER
1	24g	24	LPS	Y	
2	25.4g	24	LPS	Y	
3	25.5g	24	LPS	Y	
4	24.6g	24	SALINE	Y	
5	24.6g	24	SALINE	Y	
6	25g	24	SALINE	Y	
7	22.8g	24	CONTROL	Y	
8	24.5g	24	CONTROL	Y	
9	25.8g	24	CONTROL	Y	
10		24	BASLINE	Y	
11		24	BASLINE	Y	
12		24	BASLINE	Y	
13	27.5g	24	LPS	Y	
14	23g	24	LPS	Y	
15	24.5g	24	LPS	N	DIED 11HOURS AFTER THE INJECTION
16	26.5g	24	SALINE	Y	
17	24g	24	SALINE	Y	
18	23g	24	SALINE	Y	
19	22g	24	CONTROL	Y	
20	24g	24	CONTROL	Y	
21	25g	24	CONTROL	Y	
22	26g	24	BASLINE	Y	
23	26.4g	24	BASLINE	Y	
24	23.4g	24	BASLINE	Y	
25	26.6g	24	LPS	Y	
26	23.9g	24	LPS	N	DIED 21HOURS AFTER THE INJECTION
27	23.5g	24	LPS	Y	
28	25.4g	24	LPS	N	DIED 19 HOURS AFTER THE INJECTION
29	24.5g	24	SALINE	Y	
30	26g	24	SALINE	Y	
31	24.9g	24	CONTROL	Y	
32	24g	24	CONTROL	Y	
33		24	BASLINE	Y	
34		24	BASLINE	Y	
35	27g	4	LPS	Y	
36	22.4g	4	LPS	Y	
37	25.4g	4	SALINE	Y	
38	24.9g	4	SALINE	Y	
39	26.7g	4	CONTROL	Y	
40	24.5g	4	CONTROL	Y	
41	23.5	4	LPS	Y	
42	26.5	4	LPS	Y	
43	21.5	4	SALINE	Y	
44	24	4	SALINE	Y	
45	22.5	4	CONTROL	Y	
46	24.5	4	CONTROL	Y	

47	25.5	4	LPS	Y	4 Hours later weight 25.9
48	24	4	LPS	Y	4 Hours later weight 23.3
49	23	4	SALINE	Y	4 Hours later weight 22.9
50	26	4	SALINE	Y	4 Hours later weight 26.2
51	25	4	CONTROL	Y	4 Hours later weight 24.4
52	24.5	4	CONTROL	Y	4 Hours later weight 23.4
		Schedule 1			DREW BLOOD BY CARDIAC PUNCTURE UNDER GA
		Schedule 1			DREW BLOOD BY CARDIAC PUNCTURE UNDER GA
53	24.5	24	LPS	N	DIED 20 HOURS AFTER INJECTION
54	26.7	24	LPS	Y	
55	24.8	24	LPS	Y	
56	24.8	24	LPS	N	DIED 17.5 HOURS AFTER INJECTION
57	26.4	24	LPS	Y	
58	24.5	24	LPS	N	DIED 18 HOURS AFTER INJECTION
59	28.7	24	SALINE	Y	Large cystic tumour in abdomen - SAMPLES NOT TAKEN
60	25	24	SALINE	Y	
61	25.1	24	SALINE	Y	
62	26	24	SALINE	Y	
63	26.4	24	LPS	N	DIED 14 HOURS AFTER INJECTION
64	26.4	24	LPS	Y	
65	25.3	24	LPS	Y	
66	24.1	24	LPS	Y	
67	26.4	24	LPS	N	DIED 19.5 HOURS AFTER INJECTION
68	26.9	24	LPS	Y	
69	26.5	24	LPS	N	DIED 17 HOURS AFTER INJECTION
70	25	24	SALINE	Y	
71	26.5	24	SALINE	Y	
72	25.1	24	SALINE	Y	

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A possible role of white adipose tissue in sepsis

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Background and Goal of Study: It has recently been established that white adipose tissue, besides its metabolic role, storing and releasing fatty acids, has a major role in secreting a variety of hormones and mediators which are referred to as adipokines.(1) In particular, it has been suggested that adipose tissue releases pro- inflammatory cytokines, e.g. IL-6 in response to hypoxia. Thus we felt tempted to investigate how adipokine expression relates to sepsis in an ani- mal model.
Methods: After obtaining a Home Office licence under the Animal Rights Act, we investigated sixteen healthy, non-obese male mice (body weight 25–28 g). Eight animals served as controls, in another eight animals, sepsis was induced by intra-peritoneal LPS injection. Twenty-four hours after induction of sepsis, the experimental animals were killed and RNA extracted from epididymal fat was investigated by real time PCR, in order to quantify expression of IL-6, IL-18, nerve growth factor (NGF), hypoxia-induced factor (HIF), adipsin and adiponectin in comparison to controls.
Results: Twenty-four hours after induction of sepsis, real time PCR revealed significant increases in the expression of IL-6 (500 fold), NGF (10 fold), TNF (5 fold) and HIF (3 fold), while there were significant decreases in the expres- sion of IL-18 (0.4 fold), adipsin (0.15 fold) and adiponectin (0.2 fold). **Conclusions:** These results show that white adipose tissue is an organ sys- tem which actually suffers from hypoxia during sepsis. It responds by a marked increase in the expression of pro-inflammatory cytokines. At the same time, the expression of anti-inflammatory cytokines as well as the expression of adipsin and adiponectin is reduced. Thus, we propose the hypothesis that white adipose tissue may be an important contributor to the pathophysiol- ogy of sepsis.
Reference:
1 Trayhurn P and Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *British Journal of Nutrition* 2004; 92: 347–355.

ADIPOKINE EXPRESSION IN A MURINE MODEL OF SEPSIS

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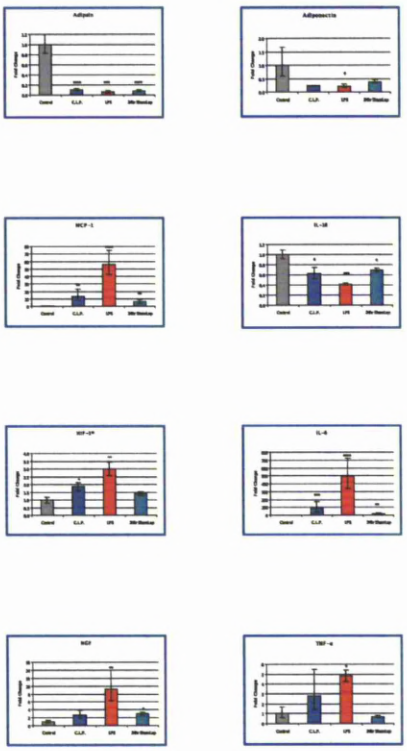

*p<0.05 **p<0.01 ***p<0.001 ****p<0.0001

INTRODUCTION

It has recently been established that white adipose tissue, besides its metabolic function in storing and releasing fatty acids, has a major role in secreting a variety of hormones and protein factors which are referred to as adipokines. (1) In particular, it has been suggested that adipose tissue releases pro-inflammatory cytokines, e.g. IL-6, in response to hypoxia. Thus we felt tempted to investigate whether adipokine expression relates to sepsis in an animal model.

METHODS

After obtaining a Home Office licence, we investigated thirty-two healthy, non-obese male mice (body wt. 25–28 g). Eight animals served as controls; in eight animals, sepsis was induced by intra-peritoneal LPS injection (25 µg/g body wt), in another eight animals by caecum ligature/puncture (CLP) and eight animals were sham-operated. Twenty-four hours after induction of sepsis, the experimental animals were killed and RNA extracted from the epididymal fat pads. Real time PCR was used to quantify expression of IL-6, TNF_{α}, IL-18, MCP-1, nerve growth factor (NGF), hypoxia-induced factor (HIF-1α), adipsin, adiponectin and leptin, in comparison to controls.

RESULTS

Twenty-four hours after induction of sepsis by LPS, real time PCR revealed substantial increases in the expression of IL-6 (500-fold), MCP-1 (55-fold), NGF (10-fold), TNF_{α} (5-fold) and HIF-1α (3-fold), while there were significant decreases in the expression of IL-18 (2.5-fold), adipsin (6-fold) and adiponectin (5-fold). The results in the CLP-group were qualitatively similar.

CONCLUSIONS

These results show that white adipose tissue is an organ system which responds to sepsis by a marked increase in the expression of pro-inflammatory cytokines. At the same time, the expression of anti-inflammatory cytokines as well as the expression of adipsin and adiponectin (anti-inflammatory) is reduced. Thus, we propose the hypothesis that white adipose tissue may be an important contributor to the pathophysiology of sepsis, and that this may relate, at least in part, to hypoxia within the tissue.

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INTEGRATIVE PHYSIOLOGY

Endotoxaemia leads to major increases in inflammatory adipokine gene expression in white adipose tissue of mice

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Abstract The proposition that white adipose tissue is involved in the inflammatory response and metabolic dysregulation of endotoxaemia has been examined. Mice were injected with lipopolysaccharide (LPS; 25 mg/kg) and epididymal, perirenal and subcutaneous adipose tissue removed 4 or 24 h later. The expression of genes encoding key inflammation-related adipokines was measured by real-time polymerase chain reaction. At 24 h after the administration of LPS, there was no change in leptin mRNA level, and adiponectin mRNA fell. However, major increases in TNFα, MCP-1 (up to 40-fold) and IL-6 (up to 250-fold) mRNA levels were evident; a substantial elevation in these mRNAs occurred by 4 h, and adipose tissue IL-6 protein also increased (three- to eightfold). At 24 h, the responses in the subcutaneous depot were much lower than in epididymal and perirenal adipose tissue, but at 4 h, the subcutaneous tissue showed major increases in IL-6, MCP-1 and TNFα gene expression. In contrast to the inflammatory adipokines, the mRNA level of two macrophage markers, F4/80 and MAC-1, was unaltered in adipose tissue during endotoxaemia. Expression of the hypoxia-sensitive transcription factor, HIF-1α, gene was increased at both 4 and 24 h, and HIF-1α protein was elevated at 4 h, suggesting that the tissue was hypoxic. It is concluded that white adipose tissue may play an important role in the production of inflammatory mediators in endotoxaemia.

Keywords Adipokines · Adipose tissue · Endotoxaemia · IL-6 · Inflammation · Sepsis

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Introduction

Sepsis, septic shock and endotoxaemia are systemic inflammatory states that affect most organs of the body, including white adipose tissue, and glucose metabolism and lipolytic capacity are particularly affected [21, 43]. It has been demonstrated that obese patients have a worse outcome from critical illness than non-obese individuals [33, 55]. To date, this has mainly been regarded as a result of difficulties in and complications of ventilation as well as an increase in the incidence of comorbidities such as diabetes and cardiac disease. While it is well established that, in obesity [54], as well as during endotoxaemia and sepsis, lipolysis is altered and glucose utilisation is impaired [25], it has recently become clear that the potential mediatory role of white adipose tissue (WAT) is not confined to glucose and fat metabolism. WAT has become recognized as a major endocrine and secretory organ which contributes to inflammation-related diseases in obesity, such as the metabolic syndrome, by the secretion of protein mediators and signalling factors called adipokines [16, 34, 46].

In obesity, the plasma concentration of a number of adipokines and proinflammatory markers expressed and

released by adipocytes has been shown to rise as adipose tissue mass expands. These include cytokines (e.g. IL-6, IL-10), chemokines (e.g. IL-8, MCP-1, MIF) and acute-phase proteins (e.g. haptoglobin, serum amyloid A) as well as inflammation-related adipokines such as leptin and nerve growth factor (NGF) [16, 34, 45, 46]. It has been suggested that these inflammatory changes may reflect hypoxia within the growing adipose tissue mass [46], and it is well recognised that in organs such as heart, kidneys and gut, hypoxia can induce the synthesis of cytokines and inflammatory mediators [40]. Sepsis is considered to be a state of generalised microvascular dysfunction with a consequent decrease in oxygen delivery, which is associated with tissue hypoxia and organ dysfunction [10]. The key intracellular pathway in hypoxia-induced metabolic changes involves activation of the transcription factor, HIF-1 [7, 39, 40]. Activation of HIF-1, through stabilisation of the HIF-1α subunit, during hypoxia triggers expression of glycolytic enzymes and membrane glucose transporters, such as GLUT-1 [7].

Previous studies have indicated that inflammation induced by lipopolysaccharide (LPS) and other agents leads to an increase in leptin expression and circulating levels in experimental animals [11, 12, 37], and increased circulating levels of the hormone have been observed in inflammatory conditions in humans [1, 4, 6, 44]. Evidence has also been presented for increases in IL-6 expression in humans during experimentally induced inflammation [1, 19]. In the present study, we hypothesized that in endotoxaemia there is a major activation of the inflammatory response in adipose tissue, key inflammatory adipokines contributing to the metabolic dysregulation characteristic of endotoxaemia and sepsis. A murine model of endotoxaemia was used to study the expression in both internal and subcutaneous adipose tissue of a series of inflammation-related adipokines. Evidence for the induction of adipose tissue hypoxia was sought through measurement of HIF-1α.

Materials and methods

Animals and tissue collection

All experiments were carried out on 8- to 10-week-old male C57BL/6J mice (Charles River, UK). The care of the mice and all experimental procedures were approved by the UK Home Office and were conducted in accordance with the appropriate Project License. LPS (*Escherichia coli* O 111: B4, Sigma-Aldrich) was injected intraperitoneally (ip) under general anaesthesia (2% isoflurane in N_2O/O_2) at a dose of 25 mg/kg. Control animals were administered equivalent volumes of normal saline, ip. All animals received 1 ml of normal saline subcutaneously (sc) to compensate for fluid losses. Mice were housed in separate cages post-procedures and maintained in the same temperature-controlled conditions with free access to standard laboratory chow and water. All mice were killed at 4 or 24 h after injection of LPS by cervical dislocation. A 24-h time point is commonly used in studies on LPS-induced endotoxaemia [12], providing a sufficient period for a severe response to be established, while 4 h [11, 37] was used to investigate acute effects. The epididymal, subcutaneous and perirenal fat depots were removed and immediately frozen in liquid nitrogen.

Real-time polymerase chain reaction

Total RNA was extracted from adipose tissues with Trizol reagent (Invitrogen, UK), and 1 μg of DNase I-treated RNA was reverse transcribed using a Reverse-iT™ 1ST Strand Synthesis Kit (Abgene, UK) in the presence of anchored oligo dT in a total volume of 20 μl.

Real-time polymerase chain reaction (PCR) reactions were carried out in a final volume of 12.5 μl consisting of 12.5–50 ng of reverse transcribed cDNA mixed with optimal concentrations of primers and probe and qPCR™ Core kit (Eurogentec, UK) in 96-well plates on a Mx3005P detector (Stratagene, USA). The primer and probe sets were designed using Primer Express software (Applied Biosystems) or Beacon Designer (Biosoft, USA) and synthesized commercially (Eurogentec). The sequence of primers and probes is shown in Table 1.

Typically, the amplification started with 10 min at 95°C and then 40 cycles of the following: 15 s at 95°C and 1 min at 60°C. β-actin was used as an endogenous reference. Relative quantitation values were expressed using the $2^{-\Delta\Delta Ct}$ method (see User bulletin #2, ABI Prism 7700, Applied Biosystems) as fold changes in the target gene normalized to the reference gene and related to the expression of the controls. The PCR efficiency in all runs was close to 100%, and all samples were analyzed in at least duplicate.

Measurement of protein by enzyme-linked immunosorbent assay

The total amount of HIF-1α in adipose tissue homogenates was measured by enzyme-linked immunosorbent assay (ELISA) (R & D Systems, UK) as previously described [51]. The adiponectin, IL-6 and MCP-1 content of adipose tissue homogenates was also measured using commercial ELISAs, according to the manufacturer's protocol. For adiponectin (R & D Systems), the stated minimal detectable amount was 3 pg/ml, and the interassay coefficient of variation was 6%. For Il-6 (RayBiotech, USA), the minimal

Table 1 Primer and probe sequences used in real time PCR

Gene	Sequence 5′–3′	
Adiponectin	Forward	ACGACCTCATTCTTTTTAAGCTATCC
	Reverse	TCCCCACGTGAGACCCCTACCCTT
	Probe	CCGGGTTCCACTTCTTTGTC
Adipsin	Forward	ACGACCTCATTCTTTTTAAGCTATCC
	Reverse	CCGGGTTCCACTTCTTTGTC
	Probe	TCCCCACGTGAGACCCCTACCCTT
β-actin	Forward	CAAGAAGGAAGGCTGGAAAAG
	Reverse	ACGGCCAGGTCATCACTATTG
	Probe	ACGAGCGGTTCCGATGCCCTG
F4/80	Forward	AAGACTTGATACTCCAAAGTGAGC
	Reverse	GAAGGAAGCATAACCAAGATCCC
	Probe	CCCTGCACTGCTTGGCATTGCTGT
HIF-1α	Forward	CAAGTCAGCAACGTGGAAGGT
	Reverse	CTGAGGTTGGTTACTGTTGGTATCA
	Probe	TTCACTGCACGGGCCATATTCATGTC
IL-6	Forward	CATCTGCTGGCCTTCTCCAA
	Reverse	CAGGCTCTCTGGCTTCTG
	Probe	AGCTGCTCCCTGCCTCAGACCAGTG
IL-18	Forward	GGCCTCTATTTGAAGATATGAC TGATT
	Reverse	CCTCTAGGCTGGCTATCTTTATA CATACT
	Probe	TGACTGTAGAGATAATGCACCCC GGACC
Leptin	Forward	CATCTGCTGGCCTTCTCCAA
	Reverse	ATCCAGGCTCTCTGGCTTCTG
	Probe	AGCTGCTCCCTGCCTCAGACCAGTG
MAC-1	Forward	GAATGGATTGTGCTATTTGTTCGG
	Reverse	CGGAGCCATCAATCAAGAAGAC
	Probe	TCCAACCTGCTGAGGCCGCCC
MCP-1	Forward	GGCTCAGCCAGATGCAGTTAA
	Reverse	CCTACTCATTGGGATCATCTTGCT
	Probe	CCCCACTCACCTGCTGCTACTCA TTCA
MT2	Forward	CTTCTTGTCGCTTACACCGTT
	Reverse	AGCAGCACTGTTCGTCACTTC
NGF	Forward	GCCAAGGACGCAGCTTTCTAT
	Rev	AGTGATCAGAGTGTAGAACAACA TGGA
	Probe	CTGGCCGCAGTGAGGTGC
TNFα	Forward	CCCAGACCCTCACACTCAGATC
	Reverse	GCCACTCCAGCTGCTCCTC
	Probe	TAGCCCACGTCGTAGCAAACCAC CAAG

detectable quantity was 6 pg/ml and the interassay coefficient of variation <12%, while for MCP-1 (RayBiotech), the values were <3 pg/ml and <12%, respectively.

Statistical analysis

The statistical significance of differences between treated and control groups was assessed with Student's *t* test for independent samples. $P<0.05$ was considered significant.

Results

Effect of LPS on adipokine expression 24 h after the induction of endotoxaemia

In order to induce severe endotoxaemia, a high dose of lipopolysaccharide was administered; the survival rate at 4 h was 100%, while the 24 h survival rate was 61%. In the first experiments, the mice were treated with LPS for 24 h and the epididymal, perirenal and subcutaneous adipose tissue depots examined. Tissues were only analysed for those animals that survived. LPS administration had no significant effect on leptin mRNA level in the epididymal WAT; nor was there any change in metalliothein-2 mRNA (Fig. 1). However, LPS induced a fivefold fall in adiponectin mRNA level, and there was a similar reduction in the level of both adipsin and IL-18 mRNA (Fig. 1). In contrast to these genes, there were increases in mRNA level for IL-6, MCP-1, NGF and TNFα (Fig. 1). The increase in NGF mRNA was small (twofold), but MCP-1 and TNFα mRNA levels increased by 24- and 18-fold, respectively, while IL-6 mRNA level increased as much as 250-fold.

Examination of the perirenal adipose tissue depot gave broadly similar results, with reductions in adipsin and adiponectin mRNA levels (Fig. 1). However, the changes were less substantial than with the epididymal depot, the decreases in IL-18 and adiponectin mRNA levels being statistically insignificant. Leptin was again unchanged following treatment with LPS. TNFα mRNA level increased by eightfold, MCP-1 by 40-fold and the largest increase (120-fold) was with IL-6 (Fig 1).

The results obtained with the subcutaneous adipose tissue were less dramatic than with either the epididymal or perirenal depots. Adiponectin mRNA level fell fivefold, but there were no significant changes with leptin, adipsin, IL-18, NGF, TNFα and MT-2 (Fig. 1). The lack of change in TNFα mRNA is noteworthy given the increases observed in the other two fat depots. MCP-1 and IL-6 mRNA levels were, however, elevated in subcutaneous fat in response to LPS, the increases being four- and 20-fold, respectively (Fig. 1).

Effect of LPS on adipokine expression 4 h after the induction of endotoxaemia

To assess the rapidity of the response to LPS, studies were next undertaken at 4 h after injection. Epididymal, perirenal and subcutaneous adipose tissue depots were again examined. In the epididymal depot at 4 h, there were no significant changes in adipsin, adiponectin, IL-18 and MT-2 mRNA levels (Fig. 2). There were, however, small, but statistically significant, increases in leptin (fourfold) and NGF (3.8-fold) mRNAs. Substantial increases were observed for TNFα (20-

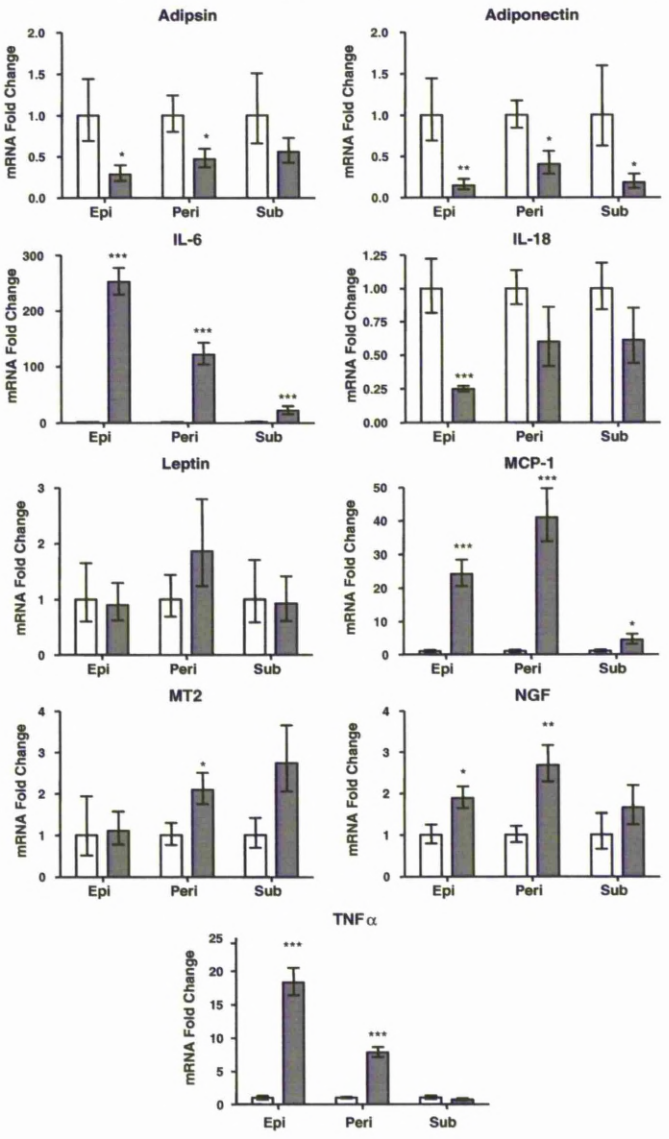

Fig. 1 Adipokine gene expression in endotoxaemia in mice. Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 24 h later; control mice (*open bars*) received a saline injection. The level of each adipokine mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). *IL-6* Interleukin 6, *IL-18* interleukin 18, *MCP-1* monocyte chemoattractant protein 1, *MT2* metallothionein 2, *NGF* nerve growth factor, *TNFα* tumour necrosis factor α. Results are given as mean values±SE for five to six mice in each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with controls

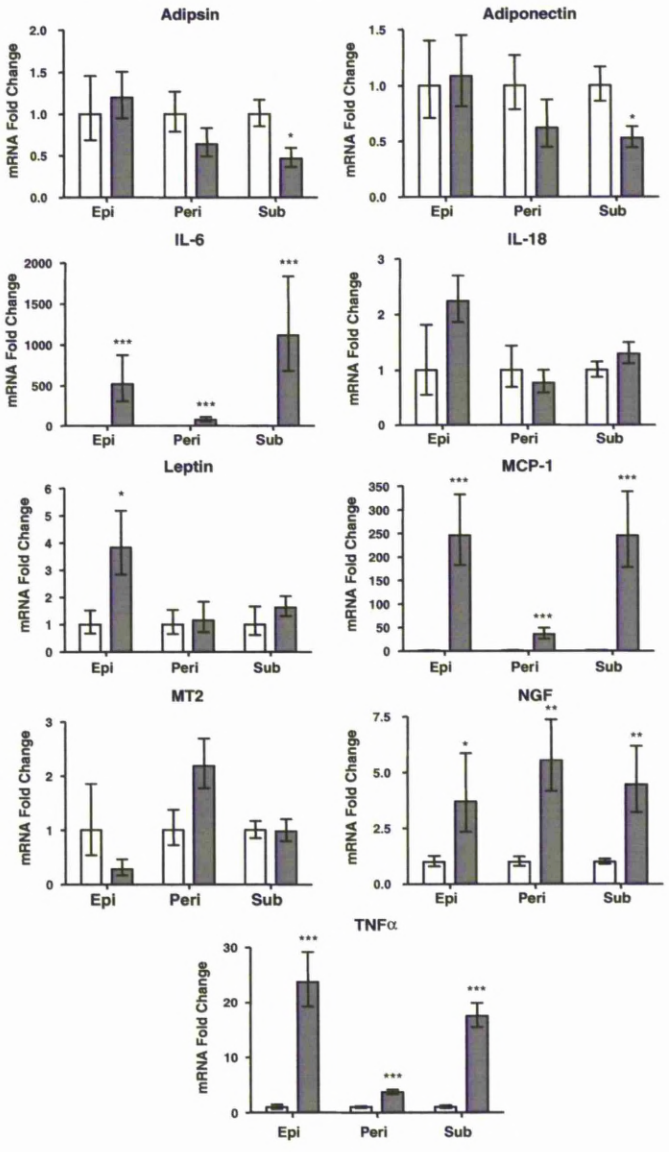

Fig. 2 Acute effects of endotoxaemia on adipokine gene expression in mice. Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 4 h later; control mice (*open bars*) received a saline injection. The level of each adipokine mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (≡1). Results are given as mean values±SE for five to six mice in each group. *$P<0.05$, **$P<0.01$, ***$P<0.001$ compared with controls

fold) and particularly MCP-1 (250-fold) and IL-6 (500-fold) mRNA. A broadly similar pattern was observed in the perirenal depot, although in this case, there was no change in leptin mRNA level. The largest changes were again with MCP-1 and IL-6 mRNA, the levels of which were increased by 35- and 78-fold, respectively (Fig. 2). TNFα level increased 23-fold, and a modest increase (fourfold) was observed with NGF.

In the subcutaneous adipose tissue, there were no changes in mRNA levels for IL-18, leptin and MT-2 (Fig. 2). There was, however, an approximately 50% reduction in mRNA levels for adipsin and adiponectin. NGF and TNFα mRNA levels increased moderately, by four- and 17-fold, respectively. Very major changes were observed with MCP-1, the mRNA level of which increased 250-fold, and with IL-6 where the increase was >1,000-fold (Fig. 2).

White adipose tissue adipokine levels

The adipokine protein content was measured by ELISA in the epididymal and subcutaneous fat depots for the two inflammatory adipokines which exhibited the largest changes in mRNA level. IL-6, which showed the greatest elevation in mRNA, was increased in both the epididymal and subcutaneous fat at 4 and 24 h after the injection of LPS (Fig. 3). The increase in the epididymal depot was between five- and eightfold, while in the subcutaneous tissue the rise was between three- and eightfold.

In contrast to IL-6, there was no significant increase in MCP-1 protein in either tissue at 24 h. However at 4 h, the amount of MCP-1 was approximately eightfold greater in the epididymal fat and twofold greater in the subcutaneous depot (Fig. 3). For adiponectin, only in the epididymal depot after 24 h of treatment with LPS was there a

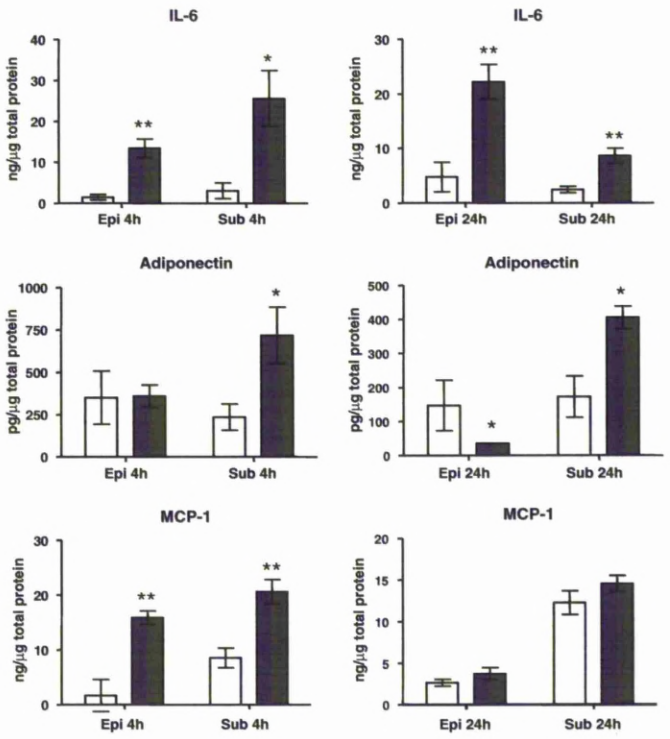

Fig. 3 Adipokine levels in adipose tissue in endotoxaemia in mice. Mice were injected with LPS to induce severe sepsis (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. Adipokine proteins were measured in the epididymal (*Epi*) and subcutaneous (*Sub*) depots by ELISA. Results are given as mean values±SE for three to six mice in each group. *$P<0.05$, **$P<0.01$ compared with controls

significant fall in the protein, reflecting the changes in the mRNA level. However, paradoxically the adiponectin level in the subcutaneous depot was actually increased, rather than decreased, at both 4 and 24 h in the LPS-treated mice.

Macrophage marker expression

To assess whether the increases in the expression of key inflammatory adipokine genes, particularly MCP-1, reflected an infiltration of macrophages into adipose tissue, the mRNA level of two macrophage markers was examined. The markers were F4/80 and MAC-1, and these were examined in 4 and 24 h LPS-treated mice and in all three WAT depots. In no case was there a statistically significant increase in F4/80 and MAC-1 mRNA level (Fig. 4). Indeed, in one case, the perirenal tissue treated for 24 h, the level of both mRNAs was significantly decreased. F4/80 mRNA level was also decreased in the epididymal depot at 24 h.

HIF-1α as an index of hypoxia

In the final investigation, the effect of LPS treatment on the key molecular index of cellular oxygen deficiency, the hypoxia-sensitive transcription factor HIF-1α, was examined. The expression and protein level of HIF-1α was determined in both the epididymal and subcutaneous adipose tissue depots. At 4 h after the treatment with LPS, there was a three- to fourfold increase in HIF-1α mRNA in each depot, and importantly, this was accompanied by a two- to threefold increase in HIF-1α protein (Fig. 5). HIF-1α mRNA level was also twofold higher in both depots at 24 h, but at this later time point, there was no significant change in HIF-1α protein (Fig. 5).

Discussion

The present study demonstrates that there are major changes in the expression of key adipokine genes in each of the main white adipose tissue depots in mice following the induction of endotoxaemia. The expression of several major inflammation-related adipokines was examined, and by far the largest response was observed with IL-6 and MCP-1. Adipose tissue produces large amounts of IL-6 and is considered to be a substantial contributor to circulating levels of this cytokine [9, 26]. Plasma IL-6 levels are increased in endotoxaemia as well as sepsis, and it seems likely that adipose tissue contributes directly to this [1, 13, 19], particularly since in the present study the amount of IL-6 in the fat depots was increased alongside the increase in mRNA level. Nevertheless, it should be noted that the increase in protein was much less than the changes in mRNA level, although this could reflect rapid release of IL-6 from the tissue once synthesised.

The chemokine MCP-1 shows increased expression in obesity, like IL-6, and is linked to the recruitment of macrophages within tissues, including adipose tissue. Indeed, in obesity there is a major infiltration of macro-

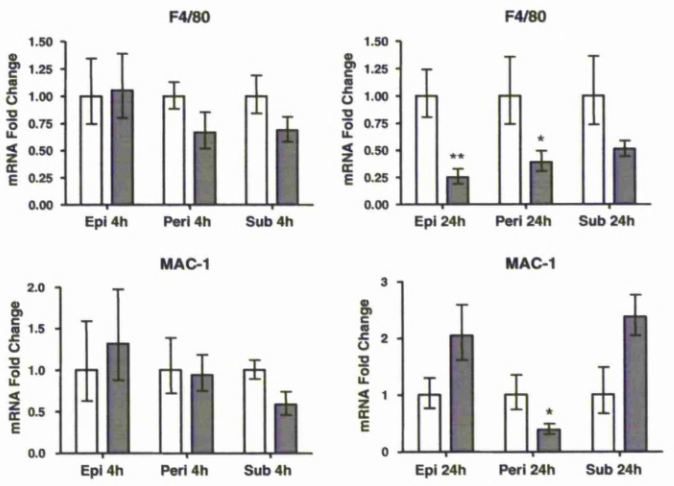

Fig. 4 Expression of macrophage markers in adipose tissue in endotoxaemia in mice. Mice were injected with LPS to induce endotoxaemia (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. The level of each macrophage marker mRNA was measured in epididymal (*Epi*), perirenal (*Peri*) and subcutaneous (*Sub*) depots by real-time PCR and normalized to β-actin relative to the untreated control group (=1). Results are given as mean values±SE for five to six mice in each group. $*P<0.05$, $**P<0.01$, $***P<0.001$ compared with controls

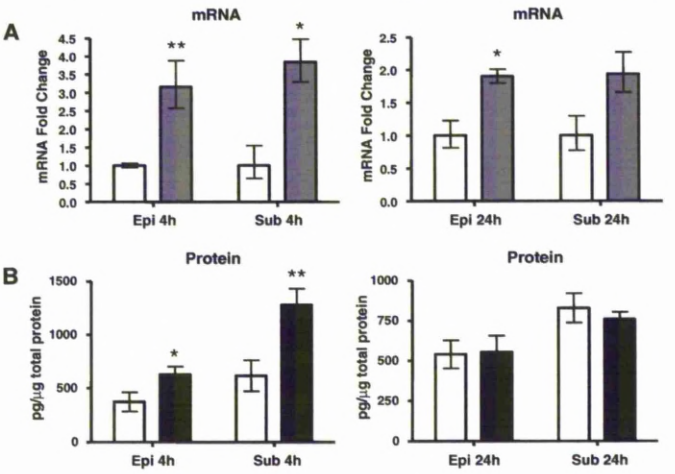

Fig. 5 HIF-1α gene expression and protein levels in adipose tissue in endotoxaemia in mice. Mice were injected with LPS to induce endotoxaemia (*closed bars*) and adipose tissue depots removed 4 or 24 h later; control mice (*open bars*) received a saline injection. HIF-1α mRNA (**a**) and protein levels (**b**) were measured in the epididymal (*Epi*) and subcutaneous (*Sub*) depots by real-time PCR, the level being normalized to β-actin relative to the untreated control group (≡1), and by ELISA, respectively. Results are given as mean values±SE for five to six mice in each group. *$P<0.05$, **$P<0.01$ compared with controls

phages into adipose tissue [52, 54]. Since the mRNA level of two macrophage markers—F4/80 and MAC-1—did not increase in any of the three adipose tissue depots examined, either at 4 or 24 h after the administration of LPS, it is unlikely that there was an infiltration of immune cells during the duration of endotoxaemia in the present work. The increased MCP-1 expression suggests that macrophage recruitment into adipose tissue may well occur long-term; however, in contrast to IL-6, no sustained elevation in MCP-1 protein levels was evident in adipose tissue homogenates following LPS treatment.

In addition to macrophage infiltration, MCP-1 has been associated with the development of insulin resistance [38]. This is also the case with IL-6 and TNFα [17, 20, 27, 35]. Thus, the major stimulation of the expression of these three factors in response to LPS suggests that adipose tissue may contribute to insulin insensitivity and the dysregulation of glucose homeostasis in infection. TNFα is a pleiotrophic pro-inflammatory cytokine, and its actions in adipose tissue include the stimulation of lipolysis and of apoptosis [31, 36]. It also has a major effect on the expression of other inflammation-related adipokines including the strong upregulation of IL-6 and MCP-1 production [49, 50].

In marked contrast, adiponectin expression was downregulated in all three adipose tissue depots 24 h after the administration of LPS. There was also a significant reduction in adiponectin mRNA in the subcutaneous depot at 4 h. Adiponectin, which is a key adipocyte-derived hormone, has multiple physiological effects, and these include anti-inflammatory and insulin sensitising actions [5, 28, 29, 56]. Thus, a fall in adiponectin expression may add to the upregulation of IL-6, MCP-1 and TNFα expression in leading to a major pro-inflammatory response and insulin resistance in endotoxaemia. However, although adiponectin protein level decreased in the epididymal depot at 24 h (the absence of a change at 4 h may reflect the time delay between alterations in gene transcription and subsequent protein synthesis), in the case of the subcutaneous depot, there was a paradoxical increase in the amount of the hormone. We have no satisfactory explanation for this anomaly, although it may indicate that adiponectin is less readily released from the subcutaneous adipose tissue in endotoxaemia.

Furthermore, low adiponectin levels observed in obesity [3] as a state of low-grade chronic inflammation are associated with increased insulin resistance [5, 56]. Stress-induced hyperglycaemia frequently occurs in infection and is associated with an adverse outcome [14, 48]. Elevated TNFα levels, increased MCP-1 production as well as low adiponectin may act in concert to induce and to maintain hyperglycaemia in critical illness. Although the present study does not reveal the extent to which adipose tissue might contribute quantitatively to alterations in circulating levels of cytokines and chemokines in endotoxaemia, overall, our results suggest a role for the tissue in the changes in glucose metabolism associated with infection. It is emphasised that since in the 24-h studies there was a distinct mortality rate and that only tissues from surviving animals were investigated, there could be a link between the gene expression profile and the protective survival response.

Expression of the complement-related factor, adipsin, also fell during endotoxaemia. Leptin, on the other hand,

showed no changes in gene expression. This is in contrast with previous studies in experimental animals and in humans where an upregulation has been described [1, 12, 37]. However, the previously reported changes in leptin mRNA were very small, particularly in comparison with the dramatic changes in IL-6 and MCP-1 mRNA level.

Small increases in NGF mRNA level were observed in the epididymal and perirenal depots but not in the subcutaneous tissue. This target-derived neurotrophin is also involved in the inflammatory response, its expression and secretion from both murine and human adipocytes being stimulated by TNFα [30, 49]. The low molecular weight cysteine-rich, metal-binding protein metallothionein showed little response to LPS except in the perirenal depot where there was a small increase at 24 h. Similarly, there was little change in the expression of IL-18; this pro-inflammatory cytokine plays an important role in innate and acquired immunity, and its expression in adipocytes [41, 53] has been shown to be strongly upregulated by TNFα [53].

Certain depot-specific responses to LPS were evident in the present study. Of the three adipose tissue sites examined, the largest response was observed in the epididymal depot in the case of both IL-6 and TNFα (at 24 h). Indeed, the subcutaneous depot showed no increase in TNFα mRNA level at 24 h. In this context, it has been shown that subcutaneous adipose tissue is affected to a lesser degree by sepsis-induced malperfusion than the splanchnic area and does not contribute to lactate production during critical illness [25].

In addition to depot differences, there is the important question of which cells within adipose tissue may be responsible for alterations in adipokine/cytokine production in endotoxaemia. White fat consists of two major components, mature adipocytes and the stromal-vascular fraction. The stromal–vascular fraction includes macrophages, fibroblasts, vascular endothelial cells and preadipocytes. Adipocytes are able to express each of the inflammation-related signals examined in the present study (see [46, 47]). However, while the key cytokines and chemokines are also expressed by macrophages, adipocytes alone are responsible for adiponectin and leptin production.

It could be hypothesized that during endotoxaemia, macrophages infiltrate adipose tissue, causing further inflammatory damage and substantially contributing to mediator production. This would imply that immigrating immune cells are mainly responsible for the changes observed. However, as noted above, the mRNA level of two specific macrophage markers did not increase in WAT and even decreased in the case of the perirenal depot at 24 h. This suggests that mature adipocytes may be the key site of the response to LPS. Indeed, adipocytes express the Toll-like receptor 4 through which LPS exerts its inflammatory effect [22, 42]. Irrespective of the cell type involved, adipose tissue can be regarded as a key inflammatory organ, which may contribute to the systemic response observed during systemic infection by generating and releasing inflammatory mediators into the circulation. However, the possibility that resident macrophages are activated and may contribute to adipokine/cytokine production in the various adipose tissue depots in endotoxaemia, without increased immune cell infiltration, cannot be excluded.

It has recently been proposed that the inflammation that occurs in adipose tissue in obesity may, at least in part, be a response to relative hypoxia in groups of adipocytes distant from the vasculature [46]. Evidence in support of this hypothesis is emerging [15, 32, 57], and hypoxia has been shown to directly increase the expression and release of inflammation-related adipokines such as leptin, IL-6, MIF and VEGF in murine and human adipocytes, while adiponectin with its anti-inflammatory action is decreased [8, 15, 23, 51, 57]. We were therefore interested in whether hypoxia might underlie the changes in adipokine expression in endotoxaemia. Increases in the mRNA encoding the hypoxia-sensitive transcription factor, HIF-1α, were evident following administration of LPS, and increased HIF-1α protein was found at 4 h. This suggests that at least in the early stages of endotoxaemia, hypoxia may occur in adipose tissue. The changes at the protein level were not sustained by 24 h, implying that any hypoxia may have been transitory. Further work is warranted on hypoxia in adipose tissue in severe infection and whether it underlies the changes in adipokine expression.

A high dose of LPS was employed to induce endotoxaemia, and it may also reflect sepsis. However, the LPS challenge may not adequately reproduce the complexity of human sepsis. In particular, investigators with an interest in peri-operative sepsis have increasingly used caecal ligation and puncture (CLP) as the model of choice. In a preliminary series of experiments with CLP in the same strain of mice, we were able to reproduce the changes in IL-6 mRNA levels found in the epididymal fat depot after LPS challenge. Here IL-6 mRNA levels were 87-fold higher than in control mice and sixfold higher than in sham-operated mice at 24 h (unpublished data). Apparently, CLP leads to qualitatively similar changes in IL-6 expression as LPS, suggesting that white adipose tissue is activated in septic shock as in endotoxaemia.

Clinical sepsis is commonly polymicrobial, but experimentation involving bacterial products, such as LPS, may serve to characterize response patterns directed against one particular organism [18]. We therefore used LPS as the model of choice to examine the potential role of WAT during severe infection. The dose employed, although high, is within the range used in previous studies and elicited a

severe, acute, systemic inflammatory response in mice with all the symptoms of sepsis and septic shock [24]. Importantly, the substantial increase in IL-6 and MCP-1 gene expression in the subcutaneous adipose tissue at 4 h indicates a systemic response in WAT rather than an effect local to the i.p. site of injection. The mortality rate of 39% in our 24-h series of experiments reflects acute life-threatening gram-negative infection of a severity similar to that occurring in human sepsis [2]. Further investigations using various models of sepsis are warranted to specify the role of WAT in infection caused by different organisms.

In conclusion, our results demonstrate that LPS challenge sufficient to mimic endotoxaemia leads to major changes in the expression of key adipokine genes in different adipose tissue depots of mice. Thus, white adipocytes (and preadipocytes) may well contribute directly to the most prominent features of infection—systemic inflammation and metabolic dysregulation. If this could be corroborated in studies involving human patients, it would allow a new interpretation of the positive correlation between body mass index and sepsis mortality as well as the development of novel approaches to treating insulin resistance in severe infection.

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INTEGRATIVE PHYSIOLOGY

Endotoxaemia leads to major increases in inflammatory adipokine gene expression in white adipose tissue of mice

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