Mechanisms determining severity in acute pancreatitis

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

Euan William McLaughlin
September 2012

This thesis is dedicated
to my loving wife, Jane.

Abstract

Background:

Acute pancreatitis (AP) remains a significant cause of human morbidity and mortality, and for which therapy remains limited in its ability to prevent the development of severe disease. Urgent work is needed to elucidate the disease process at the cellular level to identify suitable targets for therapy. Extensive research as been undertaken in animal models, which have helped clarify the underlying complex cellular mechanisms involved, yet much still remains unclear. In particular the use of the hyperstimulation-induced animal model, particularly with cholecystokinin (or its analogue caerulein), has been controversial in recent years with detractors claiming there is no clear relevance to human disease. Work with human tissue is required to validate the findings of such animal models of acute pancreatitis. Cell death pathways are suggested to play a pivotal role in determining disease severity, with apoptosis favouring milder outcomes, and necrosis favouring increasing severity. However in recent years it has been suggested that this view may be simplistic. The Bcl-2 family of proteins have been found to be important regulators of cell death, but there have been no published work investigating their action in *in vivo* pancreatitis. Caffeine has been suggested as a potential therapeutic at it inhibits the abnormal Ca^{2+} signals associated with pancreatitis *in vitro*. However it's actions have not been tested *in vivo*.

Methods:

Experiments using confocal microscopy to test the responses of two subtypes of cholecystokinin (CCK-8 and CCK-58), in both isolated murine and human isolated pancreatic acinar cells, found both subtypes elicited very similar responses in both species. These responses were linked to metabolic activity (as measured by increase in mitochondrial NADH) and secretion (measured by loss of quinacrine from zymogen granules). These results validate continued use of the hyperstimulation model of acute pancreatitis.

Results:

Caerulein-induced AP in Bak, Bax and Bcl-2 knockout (KO) mice revealed surprising results. Bcl-2 KO had little effect on AP, Bak KO led to less severe disease, but no change in apoptosis. Bax KO led to more severe disease and early necrosis (first 8hrs), but subsequently increased apoptosis at later time points (12-24 hrs). This suggests the current understanding of Bcl-2 family proteins is incomplete.

Conclusions:

Caffeine was found to have protective effects in caerulein murine AP when administered at a concentration of 10 mg/kg, and more so at 50 m g/kg but this dose produced severe toxic side-effects. Further work is needed to optimise the correct dose and test caffeine metabolites and/or analogues.

Acknowledgements

I would like to thank my supervisor Professor Robert Sutton for his advice, guidance and, above all, patience during my studies. His knowledge and passion for this subject have been invaluable and inspirational. I would like to thank Professor Ole Petersen, Professor John Neoptolemos and my co-supervisor Professor Alexei Tepikin for permission to work within their laboratories and for access to their expert advice.

I would like to thank all my previous co-workers from the departments of surgery and physiology who have helped support me throughout. In particular John Murphy, Dave Criddle, Dave Booth, Rishi Mukherjee, Svetlana Voronina, Mischa Chvanov and Mark Houghton. A special mention goes to my friend Wei Huang, who I took under my wing when he first arrived in our laboratory, but whose help I heavily relied upon in the latter years of my thesis.

I am grateful to the Royal College of Surgeons for awarding a Fellowship to support me through the final year of study, and for funding from the Royal Liverpool University Hospital. I also wish to acknowledge the time and support given by colleagues from other departments, including Prof. M

Pritchard and Prof. A. Watson (Division of Gastroenterology), Dr. B. Azadeh Prof. S Coupland and Dr H. Kalirai (Department of IHC, Pathology). Their gracious co-operation has been of immense importance.

Finally I thank my family for their love and support throughout my studies. To Jane, Dougal and Elizabeth.

Table of Contents

Abstract iii

Acknowledgements iv

List of Tables 5

List of Figures 6

List of Abbreviations 9

Chapter 1 – Introduction

1.1 The burden of human acute pancreatitis 12
1.1.1 Aetiology of human disease 14
1.1.2 Does aetiology influence severity? 19
1.1.3 Severity scoring systems 20
1.1.4 Improvements in management of acute pancreatitis 24

1.2 Experimental models of acute pancreatitis 26
1.2.1 Animal models of acute pancreatitis 26
1.2.2 Secretagogue-induced models 30
1.2.3 Other non-invasive experimental models 33
1.2.4 Invasive experimental models 36
1.2.5 Duct obstruction-induced model 37
1.2.6 Duct perfusion-induced model 39

1.3 Apoptosis and necrosis in acute pancreatitis 42
1.3.1 Morphology in apoptosis and necrosis 43
1.3.2 Caspase cascade 44
1.3.3 Bcl-2 family proteins 47
1.3.4 Role of the mitochondria in pancreatic acinar cell death 49
1.3.5 Role of calcium signalling and mitochondrial function 52
1.3.6 Mitochondrial membrane permeabilisation 54
1.3.7 Evidence that apoptosis is protective in acute pancreatitis 57

1.4 Pancreatic acinar cells and calcium signalling 58
1.4.1 Pancreatic exocrine function 58

1.4.2 *The pancreatic acinar cell* **61**
1.4.3 *Normal Ca^{2+} signalling in pancreatic acinar cells* **64**
1.4.4 *Toxic Ca^{2+} signalling in pancreatic acinar cells* **70**
1.4.5 *Premature trypsinogen activation and enzyme co-localisation* **72**
1.4.6 *CCK stimulation of pancreatic acinar cells* **80**
1.4.7 *The role of cytokines in acute pancreatitis* **81**
1.4.8 *Reactive oxygen species and cell stress* **84**

1.5 Caffeine **85**

1.6 Summary **88**

1.7 Aims and objectives **90**

Chapter 2 - Material and Methods

2.1 Materials
2.1.1 *Equipment* **93**
2.1.2 *Confocal microscopy* **94**
2.1.3 *Consumables* **96**
2.1.4 *Chemicals and reagents* **96**
2.1.5 *Animals* **98**

2.2 Methods for isolated murine and human PACs experiments
2.2.1 *Isolation of murine pancreatic acinar cells* **100**
2.2.2 *Isolation of human pancreatic acinar cells* **101**
2.2.3 *Loading of fluorescent dyes and measurement of secretion* **103**

2.3 Methods for experimental *in vivo* murine pancreatitis models
2.3.1 *Induction of experimental pancreatitis in vivo* **106**
2.3.2 *Removal of pancreas* **108**
2.3.3 *Measurement of oedema* **108**
2.3.4 *Analysis of serum amylase* **109**
2.3.5 *Preparation of histological slides* **110**
2.3.6 *Histological scoring of severity of pancreatitis* **110**
2.3.7 *Immunohistochemistry for caspase-3 stain* **113**
2.3.8 *Use of caffeine in experimental models* **116**
2.3.9 *Statistical analysis* **116**

RESULTS

Chapter 3 Direct activation of Ca^{2+} signalling and zymogen secretion from isolated murine and human pancreatic acinar cells in response to stimulation by cholecystokinin-8 and cholecystokinin-58

3.1 Introduction 119

3.2 Materials and Methods
3.2.1 *Isolation of murine and human PACs* 123
3.2.2 *Solution and chemicals* 125

3.3 Isolated murine PACs responses to CCK-8 and CCK-58
3.3.1 *Effects of CCK-8 and CCK-58 on $[Ca^{2+}]_i$ and mitochondrial stimulus-metabolic coupling in isolated murine pancreatic acinar cells* 127
3.3.2 *Effects of CCK-8 and CCK-58 on secretion in isolated murine PACs* 131

3.4 Isolated human PACs responses to CCK-8 and CCK-58
3.4.1 *Effects of CCK-8 and CCK-58 on $[Ca^{2+}]_i$ and mitochondrial stimulus-metabolic coupling in isolated human pancreatic acinar cells* 135
3.4.2 *Effects of CCK-8 on secretion from isolated human PACs* 139

3.5 Discussion
3.5.1 *Isolated murine PACs responses to CCK-8 and CCK-58* 141
3.5.2 *Isolated human PACs responses to CCK-8 and CCK-58* 143

Chapter 4 Effects of Bak, Bcl-2 and Bax knock-out on murine caerulein-induced *in vivo* experimental pancreatitis

4.1 Introduction 147

4.2 Methods 149

4.3 Bak knock-out in murine experimental pancreatitis 156

4.4 Bcl-2 Knock-out in murine experimental pancreatitis 159

4.5 Bax knock-out in murine experimental pancreatitis 163

4.6 Caspase-3 staining and evidence of apoptosis in caerulein -induced pancreatitis in Bak KO and Bax KO 169

4.7 Discussion of caerulein-induced AP in Bak, Bax and Bcl-2 murine KO 173

Chapter 5 Actions of caffeine in murine caerulein-induced *in vivo* experimental pancreatitis

5.1 Introduction 177

5.2 Methods 178

5.3 Effect of caffeine on severity of caerulein-induced murine pancreatitis 182

5.4 Summary of effects of caffeine on severity in caerulein-induced murine pancreatitis 187

Chapter 6 Discussion

6.1 The need for research in acute pancreatitis 191

6.2 The relevance of the CCK-hyperstimulation model to human acute pancreatitis 194

6.3 Role of Bcl-2 family proteins in acute pancreatitis 197

6.4 Role of Caffeine as a potential therapeutic 202

6.5 Future directions 205

Statement of originality 206

Publications associated with this thesis 207

References 208

List of Tables

Table 1.1	Aetiology of acute pancreatitis	**16**
Table 1.2	Comparison of commonly used experimental models of acute pancreatitis	**28**
Table 1.3	Table 1.3 Transgenic studies of trypsin, trypsin inhibitor and trypsin receptor in experimental acute pancreatitis	**29**
Table 2.1	Caerulein injection schedule for different experimental time groups	**107**
Table 2.2	Criteria used to score histopathological severity	**112**
Table 3.1	Demographic details, surgical procedure and underlying pathology of patients donating human pancreatic tissue	**124**
Table 5.1	Protocol for timing and concentration of IP caffeine and caerulein	**180**
Table 5.2	Chart for calculating caffeine dose from prepared stock per animal weight to obtain a concentration of 10 mg/kg	**181**

List of figures

Figure 1.1	Schematic of apoptotic pathways	**46**
Figure 1.2	Representative diagram showing basic structure of mitochondrion	**51**
Figure 1.3	Mitochondria as central to cell death pathways	**53**
Figure 1.4	Mitochondria membrane permeability	**55**
Figure 1.5	EM and light microscopic images of PACs	**63**
Figure 1.6	Normal Ca^{2+} signalling	**66**
Figure 1.7	Calcium release at the endoplasmic reticulum	**69**
Figure 1.8	Response of zymogen granule to hyperstimulation	**78**
Figure 1.9	Challenge to the central role of trypsinogen activation	**79**
Figure 1.10	Common metabolites of caffeine	**87**
Figure 2.1	Photograph of Zeiss LSM510 confocal microscope	**95**
Figure 2.2	(A) Quinacrine fluorescence (B) Perfusion Chamber	**105**
Figure 3.1	Secretion in isolated murine PACs responding to ACh stimulation	**122**
Figure 3.2	Effects of physiological (10 pM) CCK-58 and CCK-8 on $[Ca^{2+}]_i$ in isolated murine PACs	**128**

Figure 3.3	Effects of supra-maximal (5 nM) CCK-58 and CCK-8 on $[Ca^{2+}]_i$ in isolated murine PACs	**129**
Figure 3.4	Representative trace showing stimulus-Metabolic coupling induced by 10 pM CCK-58	**130**
Figure 3.5	Representative traces of apical quinacrine loss from isolated murine PACs by secretion in response to physiological (A) CCK-8 and (B) CCK-58	**133**
Figure 3.6	Meaned quinacrine fluorescence responses to physiological CCK-8 and CCK-58	**134**
Figure 3.7	Effects of physiological (2-20 pM) CCK-8 (A) and CCK-58 (B) on cytosolic calcium concentration, $[Ca^{2+}]_i$, in isolated human pancreatic acinar cells	**136**
Figure 3.8	Effects of hyperstimulation with CCK-8 on $[Ca^{2+}]_i$ in isolated human PACs	**138**
Figure 3.9	Apical quinacrine secretion in response to physiological CCK-8 (10 pM) in isolated human PACs	**140**
Figure 4.1	(A) oedema (B) amylase in C57BL/6 caerulein AP	**150**
Figure 4.2	Histology score in C57BL/6 caerulein AP	**152**
Figure 4.3	Slides showing typical histology in C57BL/6 caerulein AP	**153**
Figure 4.4	Representative images of caspase-3 IHC stain	**155**
Figure 4.5	Pancreatic oedema and amylase in Bak null AP	**157**

Figure 4.6	Total histology score in Bak null AP	**158**
Figure 4.7	Pancreatic oedema and amylase in Bcl-2 AP	**161**
Figure 4.8	Total histology score in Bcl-2 AP	**162**
Figure 4.9	Pancreatic oedema and amylase in Bax null AP	**164**
Figure 4.10	Total histology score in Bax null AP	**165**
Figure 4.11	Breakdown of histology score into (A) oedema, (B) inflammation and (C) necrosis	**166**
Figure 4.12	Caspase-3 IHC staining in Bak null and Bax null AP	**170**
Figure 4.13	Representative slides of IHC staining in C57BL/6, Bak null and Bax null AP	**172**
Figure 5.1	Pancreatic oedema and amylase in caffeine AP	**184**
Figure 5.2	Histology scores in caffeine AP	**185**

List of abbreviations

ACh	Acetylcholine
ANT	Adenine nucleotide translocase
AP	Acute pancreatitis
APAF-1	Apoptotic peptidase activating factor – 1
APO-1	Apoptosis antigen 1 (See FasR)
ARDS	Adult respiratory distress syndrome
ATP	Adenosine-5'-triphosphate
BAD	Bcl-2 associated death promoter
Bak	Bcl-2 homologous antagonist/killer
Bax	Bcl-2 associated X protein
Bcl-2	B-cell lymphoma 2
BH3	Bcl-2 homology domain 3
BID	BH3 interacting-domain death agonist
cADPR	Cyclic adenosine diphosphate ribose
Ca^{2+}	Calcium ions
$[Ca^{2+}]_c$	Cytosolic calcium ion concentration
CCK	Cholecystokinin
CDE	Choline-deficient, 0.5% DL-ethionine-supplemented
CFTR	Cystic fibrosis transmembrane conductance regulator
CHB	1-Cyano-2-hydroxy-3-butene (crambene)
CPBD	Common pancreatic biliary duct
CT	Computer Tomography
CyP-D	Cyclophilin D
E. coli	*Escherichia coli*
EGTA	Ethylene glycol tetra-acetic acid
ER	Endoplasmic reticulum
FADD	FAS-associated protein with death domain
FAEE	Fatty acid ethyl ester
FasR	FAS receptor (also called APO-1 and CD95)
H&E	Haematoxylin and eosin
GJC	Gap junction communication
Gpbar-1	G protein-coupled bile acid receptor-1
IAP	Inhibitor of apoptosis proteins
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
IMM	Inner mitochondrial membrane
I.P.	Intra-peritoneal
IP_3	Inositol triphosphate
IP_3R	Inositol triphosphate receptor
i.v.	Intravenous

KO	Knock out
LPS	Lipopolysaccharide
MAC	Mitochondrial apoptosis-induced channel (see MPTP)
MMP	Mitochondrial membrane permeabilisation
MODS	Multi-organ dysfunction syndrome
MOMP	Mitochondrial outer membrane pore
MPTP	Mitochondrial permeability transition pore
NAADP	Nicotinic acid adenine dinucleotide phosphate
NADH	Nicotinamide adenine dinulceotide
Na-GDC	Sodium glycodeoxycholic acid
Na-TC	Sodium taurocholate
Na-TDC	Taurodeoxycholate
NF-kB	Nuclear factor-kappa B
OMM	Outer mitochondrial membrane
PACs	Pancreatic acinar cells
PAF	Platelet activating factor
PAR-2	Proteinase-activated receptor-2
PBS	Phosphate buffered solution
PD	Pancreatic duct
PMCA	Plasma membrane calcium ATPase
ROS	Reactive oxygen species
RyR	Ryanodine receptor
SAP	Severe acute pancreatitis
SERCA	Sarco/endoplasmic reticulum calcium ATPase
SIRS	Systemic inflammatory response
SOC	Store operated calcium channels
TLC-S	Taurolithocholic acid 3-sulphate
TNF	Tumour necrosis factor
TNFR	Tumour necrosis factor receptor
VDAC	Voltage dependent anion channel
VIP	Vasoactive intestinal peptide
ZG	Zymogen granule
$\Delta\Psi m$	Mitochondrial membrane potential

CHAPTER 1 – INTRODUCTION

1 Introduction

1.1 The burden of human acute pancreatitis

Acute pancreatitis in humans is a common and debilitating condition in which inflammation of the pancreas occurs, that can lead to considerable morbidity and mortality. Whilst the majority of patients present with mild, self-limiting inflammation, in a sizable minority there is severe disease, which is associated with multi-organ injury (Pandol et al., 2007). Currently therapy is limited in its ability to stop or reverse the development of a severe episode of disease. Patients with acute pancreatitis usually present with a short history of severe abdominal pain (typically central), nausea and/or vomiting and have a measurable serum amylase at least three times the normal upper limit (though values need to be interpreted in the light of onset from symptoms) (UK Working Party on Acute Pancreatitis, 2005). Patients are admitted for observation and supportive interventions with the intention of reducing injury by ameliorating the systemic inflammatory response system (SIRS). Patients with severe disease may require transfer of care to an intensive care setting. In patients with severe disease the initial clinical diagnosis is usually confirmed with a computer tomography (CT) scan, at least in part to determine the extent of pancreatic necrosis. Much work has been done to identify patients with severe disease early.

Mortality is higher in patients with necrotising pancreatitis compared to those with milder, oedematous pancreatitis (17 percent *versus* 3 percent), and is higher still (approximately 30 percent) in those with infected necrosis (Banks and Freeman, 2006)(Pandol et al., 2007). The prevalence of infected necrosis has fallen over recent years from 35 percent to nearer 15-20 percent. This improvement in survival is likely multi-factorial, and is most likely to reflect better intensive organ support and the use of CT-guided fine-needle aspiration for accurate microbiology sampling in infective necrosis (with application appropriate antibiotics). More patients present with oedematous pancreatitis than necrotising pancreatitis (85 percent *versus* 15 percent).

The incidence of acute pancreatitis in the UK has historically been show to be rising over the last few decades. Three studies have shown this trend: in Scotland (between 1984-1995) (McKay et al., 1999) and in the south of England (Corfield et al., 1985)(Toh et al., 2000). From these studies the incidence of acute pancreatitis in the UK has been shown to range from 150 - 420 per million population.

In the USA acute pancreatitis accounts for over 220,000 hospital admissions per year, but here the role of alcohol appears to be more substantial, and shows a sex preference, being more common among men (Whitcomb, 2006). Gallstone-related disease is highest among white women over sixty years. About 20 percent of adult cases have severe disease and the overall mortality is approximately 5 percent (Banks & Freeman 2006).

UK guidelines state that mortality should be less than 10 percent overall and less than 30 percent in severe (complicated) pancreatitis (UK Working Party on Acute Pancreatitis, 2005). However some specialist units would argue that this overall rate should be lower. Changing therapies may contribute to a drop in mortality; 19 percent is achievable in patients undergo minimally invasive surgical techniques to debride necrotic pancreatitis, rather than traditional 'open' techniques (Raraty et al., 2010).

1.1.1 Aetiology of human disease

The two commonest aetiological factors associated with acute pancreatitis in the western world are gallstone disease and alcohol abuse, though there is a myriad of other rarer, and aetiologically disparate, causes (see Table 1.1).

The fact that so many unrelated aetiologies can lead to the same disease strongly suggests a final common pathway(s). The dominance of the two major aetiological factors, gallstones and alcohol, differs between populations. For example the majority of cases in the USA are attributed to alcohol, whilst the majority of cases in the UK are attributed to gallstone and biliary disease. In 1979 a study in Bristol reported 4 percent of patients having an alcohol aetiology (Trapnell et al., 1979), whilst a contemporary study in Atlanta, USA report 92 percent secondary to excess alcohol (Satiani et al., 1975) (as reported by Schmid et al., 1999) . In Finland between 1970-1989 an increase in incidence from 466 to 734 per million population was found to correlate strongly with an increase nationally in alcohol consumption (Jaakkola & Nordback, 1993).

In 1901 Opie reported his theory that gallstones caused acute pancreatitis by one of two proposed mechanisms (Opie 1901). In the first (Opie 1 or common channel theory) it was proposed that gallstones impacted the ampulla and an increase in pressure forced bile to reflux into the pancreatic duct and that this initiated acute pancreatitis. In the second (Opie 2 or obstructed pancreatic duct) there is obstruction at the ampulla but no bile refluxing into the pancreatic duct.

Duct Obstruction	Gallstone obstruction Focal area of necrosis leading to fibrosis Congenital abnormalities -pancreatic divisum -choledochal cyst Neoplasia -intra-papillary mucinous neoplasia -adenocarcinoma of head of pancreas -cholangiocarcinoma ERCP Sphincter of Oddi dysfunction Trauma -blunt injury -surgical injury to pancreas
Toxic/metabolic	Alcohol Drug-induced -thiazides, azathioprine, steroids, valproate, frusemide Hypercalcaemia Hyperlipoproteinaemia (types I and V) Hyperparathyroidism Scorpion venom
Infection	Viral: coxsackie B, CMV, HIV, viral hepatitis, mumps Bacterial: mycoplasma, leptospira, salmonella Fungal: aspergillus, toxoplasma, ascaris
Genetic	Familial defects of cationic trypsinogen (N291, R117H) Mutation of serine protease inhibitor Kazal type 1 cystic fibrosis (CTFR gene defect)
Others	Ischaemia -SMA thrombosis/embolism, coronary bypass Inflammatory -primary autoimmune (IgG4-related disease spectrum) -secondary autoimmune (polyarteritis nodosa, systemic lupus erythematosus, vasculitis, inflammatory bowel disease Idiopathic

Table 1.1: Aetiology of acute pancreatitis (adapted from Dominguez-Munoz 2005; Carter et al., 2009)

In support of the common channel theory experimental work in animals has shown that bile acids perfused into the pancreatic duct will cause pancreatitis (Pandol et al., 2007) and recent studies have demonstrated uptake of bile acids by pancreatic acinar cells with subsequent Ca^{2+} release from the endoplasmic reticulum store coupled with inhibition of calcium uptake pumps (Voronina et al., 2002). The supramaximal level of intracellular calcium leads to mitochondrial dysfunction, and the death of the cell by necrosis. It is important to note that these animal models of disease have yet to be confirmed in human disease.

Gallstone obstruction at the ampulla can cause obstruction of the pancreatic duct without bile reflux into the pancreas. Animal models of pancreatic duct obstruction without bile reflux (i.e. by ligation of the pancreatic duct) differ between animal species as to whether or not pancreatitis is induced, with the opossum model being notable for its resultant necrotising pancreatitis (Chan and Leung, 2007). Obstruction of the pancreatic duct by malignancy is a suspected cause of acute pancreatitis in humans.

Although excessive alcohol intake is a major cause of acute pancreatitis, pancreatitis only occurs in a small minority of patients who regularly drink

excessive alcohol. One suggestion is that excessive alcohol intake increases the sensitivity of the pancreas to other aetiological factors and several mechanisms are proposed including: ethanol affecting the inflammatory signalling system; decrease in the expression of caspases involved in apoptosis; decreased microperfusion of the exocrine pancreas and increased cathepsin-B activity (Pandol et al., 2007). The combination of these effects is to result in increased necrosis when the pancreas is stressed and/or damaged. Recently there has been evidence that genetic variations in the PRSS1-PRSS2 gene (which codes for cationic human trypsinogen) and in the claudin-2 (CLDN-2) gene may be linked with higher risk for both alcohol-related and sporadic pancreatitis (Whitcomb et al., 2012). Homozygous (or hemizygous in males) CLDN2 genotype carries the higher risk, and may explain while alcohol-related pancreatitis is higher in men than women (male hemizygote frequency is 0.26 versus 0.07 in women).

There is experimental evidence that ethanol has more direct actions on the pancreas itself, linked to the formation of fatty acid ethyl esters (FAEEs) during metabolism of ethanol in the pancreas. In animal models FAEEs are capable of producing pancreatic oedema and intracellular trypsin activation, and have also been shown to cause sustained increases in cytosolic Ca^{2+} via inositol trisphosphate receptors leading to mitochondrial injury and loss of

ATP production, predisposing the pancreatic acinar cell to necrosis (Criddle et al., 2006).

1.1.2 Does aetiology influence severity?

Some older studies have previously suggested a greater prevalence of increased severity with biliary pancreatitis as opposed to alcoholic pancreatitis. However more recent work did not detect any statistical difference in severity in acute pancreatitis due to differing aetiologies. One study of 190 patients with acute pancreatitis looked at several clinically measurable parameters including prognostic scoring, mortality, sepsis, jaundice, ileus and pulmonary, renal and cardiorespiratory insufficiency (Uhl et al., 1996) and grouped results into three categories: alcoholic aetiology, biliary aetiology and other aetiology. Other than jaundice and a transient rise in the liver enzyme aspartate aminotransferase seen in biliary aetiology, the course of disease was very similar in all three aetiological groups. The authors concluded that after initiation of pancreatitis the course was not influenced by the aetiological factor (provided the aetiologically was not still present, causing a persistent stimulus).

Another group looking at 602 patients again found no significant differences between aetiological groups with the exception of a higher rate of pancreatic pseudocysts in alcoholic aetiology (Lankisch et al., 1996). However this finding of increased rates of pancreatic pseudocysts was not seen in other, contemporary studies, where rates were the same for differing aetiologies (Nguyen et al., 1991).

Interpretation of studies investigating whether aetiology influences severity of acute pancreatitis is made difficult by differences between the studies themselves; differences in definitions of the various aetiologies; differences in collection of data (retrospective, prospective, etc.); and differences between specific population groups. However it would seem to be reasonable to state that there is no clear difference in the severity of acute pancreatitis between groups with differing aetiological causes.

1.1.3 Severity scoring systems

Attempting to identify those patents with severe disease early has led to the development of several prognostic scoring systems. The aim is to accurately, rapidly, cheaply and safely identify those patients for whom

therapies for organ support may reduce morbidity and mortality. For scoring systems to be applied widely there first has to be an agreement on the definition of acute pancreatitis and its sub-types. In this way studies and trials can be compared across international centres.

Prognostic scoring systems have been widely employed since Ranson published his famous system for scoring patients admitted with acute pancreatitis caused by alcohol (Ranson et al., 1974). This scoring system uses easily measured biochemical markers (except for the measurement of Blood Urea Nitrogen, which is not routinely available in the UK, though can be calculated from serum Urea levels). Imrie further refined this to allow application to all cases of acute pancreatitis in the development of the Glasgow scoring system (Imrie et al., 1978) and by Ranson again in 1979. The Glasgow score is still widely employed today, as it is easy to calculate. Other scoring systems such as the Acute Physiological and Chronic Health Score (APACHE)(Knaus et al., 1985)(Domínguez-Muñoz et al., 1993) and APACHE-O which combines with BMI (obesity engendering a higher risk of severe disease)(Martinez et al., 2006) are not specific to pancreatitis but give a detailed measurement of physiological injury. They are wieldy to perform and are usually confined to intensive care and research centre settings.

Other scoring systems proposed have been based on radiological criteria (Balthazar et al., 1985), simple physiological measures (BISAP score) (Layer, 2008)(Papachristou et al., 2010), and measurements of organ failure (Marshall score). Persistent organ failure in the first 48 hours has been linked to mortality greater than 50% (Buter et al., 2002)(Johnson and Abu-Hilal, 2004).

In 1992 an international symposium of experts met in Atlanta, USA and produced a clinically based classification system (Bradley EL III, 1993). This introduced international uniformity to the classification system, with the intention of allowing greater comparison between studies. As well as a definition of acute pancreatitis, further definitions were also given for mild and severe disease, and the local complications of 'acute fluid collection', 'acute pseudocyst', 'pancreatic necrosis' and 'pancreatic abscess'. Following criticism this classification has since been revised to update the terminology and provide simple functional clinical and morphologic classifications (Thoeni, 2012)(Vege et al., 2009). The modifications: i) address the clinical course and severity of disease, ii) divide acute pancreatitis into interstitial edematous pancreatitis and necrotizing pancreatitis, iii) distinguish an early phase and a late phase (before/after the 1st week) and iv) emphasize systemic inflammatory response syndrome and

multisystem organ failure. Acute collections in the first 4 weeks are called 'acute necrotic collections' or 'acute peripancreatic fluid collections', and further sub-divided on presence or absence of necrosis. Once an enhancing capsule develops, persistent acute peripancreatic fluid collections are referred to as 'pseudocysts'; and acute necrotic collections, as 'walled-off necrosis' (which can be sterile or infected). Although it is the only widely accepted classification system, it is not without critics and terms abandoned by the Atlanta system (such as 'Phlegmon') are still found frequently in the literature (Bollen et al., 2008). Not all national guidelines for managing acute pancreatitis recommend the Atlanta system.

In the last year a new 'Determinant-Based classification of Acute Pancreatitis Severity' has been published (International Multidisciplinary Consultation, 2012). This involved a consultative survey of pancreatologists from 49 countries who recommended 4 gradings: i) mild AP– absence of both necrosis and organ failure; ii) Moderate AP – presence of sterile necrosis and/or transient organ failure; iii) Severe AP – presence of either infected necrosis or persistent organ failure; iv) Critical AP – presence of infected necrosis and persistent organ failure. It should be noted that these gradings may have some use in quantifying results, especially between centres, but may be difficult to distinguish in clinical practice.

1.1.4 Improvements in management of acute pancreatitis

There has been a decrease in morbidity and mortality in acute pancreatitis over recent decades. However this is due to improvements in organ support and timing of interventions and not to the application of a specific drug therapy, which is still lacking (Petersen and Sutton, 2006).

Clinical management of severe acute pancreatitis has improved over recent decades due to advances in: early radiology with CT scanning, including correct microbiology from fine needle aspiration (Bakker et al., 2009); improved supportive care (Gardner et al., 2009)(Warndorf et al., 2011); nutritional support; haemodialysis (Yang et al., 2010). In biliary disease, in particular, there is the need to balance early intervention with endoscopic retrograde cholangiopancreatography (ERCP) and cholecystectomy (for removal of further gallstones) with the clinical suitability of the patients to undergo these procedures. A meta-analysis of 450 patients concluded that early ERCP in patients with mild and predicted severe biliary pancreatitis (but without cholangitis), did not lead to a significant reduction in the risk of overall complications and mortality (Petrov et al., 2008). Definitive treatment by cholecystectomy should be either on the same admission or within two weeks of discharge on patients fit for surgery (UK working party

for the management of acute pancreatitis, 2005). Finally surgery may be necessary to remove infected necrosis. While still high risk, recently this has been shown to have better outcomes if performed in a minimally invasive intervention (Raraty et al., 2010).

There have been trials of therapeutics aimed at ameliorating severe acute pancreatitis. Despite initial encouraging results large studies using anti-proteases (e.g. gabexate)(Buchler et al., 1993), anti-secretory agents (e.g. octreotide)(Uhl et al., 1999) and anti-inflammatory (e.g. lexipafant - a platelet activating factor receptor antagonist)(Johnson et al., 2001) have all proved disappointing. Antioxidant therapy was investigated in a well-conducted study which clearly showed not only no benefit from antioxidants but actually showed a trend toward adverse outcomes (Siriwardena et al., 2007). Several studies have shown no benefit from prophylactic use of antibiotics, given in an attempt to decrease infection of necrosis (Dellinger et al., 2007)(Wittau et al., 2011). The failure of so many different approaches attests to the complexity of the disease and the need for further understanding of the underlying mechanisms involved.

1.2 Experimental models of acute pancreatitis

The intracellular mechanisms involved in the development of severe acute pancreatitis are still unclear. In order to effect better therapy, to ameliorate disease progress, further work with living pancreatic tissue is vital. Human samples are difficult to obtain. Sampling of human pancreas during an episode of active pancreatitis risks worsening the disease by introducing infection, and is clearly unethical. Human samples are usually obtained during elective surgery on the pancreas (for pathologies other than pancreatitis), and this work is invaluable (Murphy et al., 2008). However even in specialist units the availability of human tissue remains limited and to progress understanding at a faster pace animal models are still needed. Animal models not only allow an understanding physiology but also allow testing of new potential therapies before human trials. It is a criticism of animal models that inter-species differences may lead to a difference in the mechanisms involved in pancreatitis, so that parallel experiments in animal and human tissue are also important.

1.2.1 Animal models of acute pancreatitis

Animal models of acute pancreatitis have been used for over a century. Historically most common mammalian species have been investigated and

although recently porcine pancreas tissue has been considered a close match to human pancreas tissue (Zhao et al., 2001) it is the rodent model that is most prevalent. While rodents are easier to handle and cheaper to breed, it is in their relative ease of use in production of genetically altered animals (transgenic and knock-outs), that are bred to test how specific genes and proteins are involved in the overall pathobiological mechanism, that makes them an ideal animal model.

The problems with experimental models currently employed is that none are direct replications of human disease and the different models produce a spectrum of severity from mild to severe. Some models show effects on other organs making any assessment of MODS harder to interpret. In the invasive models injury from surgery may have a role in the severity of disease. See Table 1.2 for summary of current common experimental models.

Table 1.2: Comparison of commonly used experimental models of acute pancreatitis

Experimental model	Clinical Relevance	Animals	Comments	References
Secretagogue (CCK, Caerulein)	Very rarely (Scorpion bites)	Rats Mice	Non-invasive (injections) Adjustable severity from mild oedematous to necrosis In standard model does not cause death (7 x hourly 50µg/kg)	Lampel and Kern 1977, Niederau et al., 1985, Saluja et al., 2007
L-arginine	Limited	Rats Mice Rabbits	Non-invasive (diet) Adjustable severity from mild to severe disease Some systemic inflammation	Dawra et al., 2007
CDE diet	No	Mice	Non-invasive (diet in female mice) Haemorrhagic pancreatitis with massive fat necrosis Effects other organs (liver, brain)	Lombardi et al. 1975, Chan and Leung 2007,
Vascular induced	No	Rats Dogs	Rapid withdrawal of 30-30% blood volume or invasive specialist surgical procedure to ligate inflow/outflow. Injury not specific to pancreas. Relevant to post surgical AP and splenic/portal vein thrombosis?	Barzilai et al., 1987 Waldner H 1992
Duct obstruction (PD) (CPBD)	Gallstone obstruction	Rats/Mice Opossum	Invasive procedure needing specialist surgery Produces moderate to severe pancreatitis (adjustable) Does produce systemic effects	Ohshio et al., 1991 Lerch et al 1993 Runzi et al., 1995 Samuel et al., 1994 +2010
Duct perfusion	Biliary	Rats Mice	Invasive procedure needing specialist surgery Applicable to human disease Adjustable moderate to severe pancreatitis depending on bile acids and their concentrations Can be combined with other stresses/stimulations (e.g. secretagogue)	Lichtenstein et al., 2000 Schmidt et al., 1992 Perides et al., 2010a&b

CDE: Choline deficient and 0.5% DL-ethionine supplemented diet; PD: Pancreatic duct; CPBD: Common pancreatic biliary duct. (Adapted from Huang 2011).

Table 1.3 Transgenic studies of trypsin, trypsin inhibitor and trypsin receptor in experimental acute pancreatitis

Transgene or KO	Model	Comments	References
Cathepsin B (KO)	Caerulein (mice)	Largely decreased trypsin activity and acinar cell necrosis.	(Halangk et al., 2000)
PSTI (overexpression)	Caerulein (mice)	Decreased trypsin activity and histological appearance of the pancreas.	(Nathan et al., 2005)
MAM and/or MUG (KO)	CDE-diet (mice)	The clinical symptoms were most severe in MAM-/- mice (mortality 70%), compared to 56% in double KO mice and 25% in Wt mice.	(Umans et al., 1999)
PAR-2 (KO)	Caerulein (mice)	Increased severity of pancreatitis but no changes in liver, lung and kidney.	(Singh et al., 2007)
Trypsinogen isoform 7 (KO)	Caerulein (mice)	A 50% reduction in acinar necrosis but had similar degrees of local and systemic inflammation during AP progression, and comparable levels of intra-acinar NF-κB activation in T7-/- mice.	(Dawra et al., 2011)

NOTE. KO, knock out; MAM: mouse alpha2-macroglobulin and MUG: murinoglobulin, they are inhibitor of proteinases, including pancreatic trypsin and chymotrypsin; PSTI, pancreatic secretory trypsin inhibitor; CDE-diet, choline-deficient and 0.5% DL-ethionine supplemented diet; PAR-2, protease activated receptor-2; Na-TC, sodium taurocholate; NF-κB, nuclear factor-kappaB; PACE-tryp^{on}, paired basic amino acid-cleaving enzyme–trypsinogen. (Adapted from Huang 2011)

1.2.2 Secretagogue-induced models

Secretagogues are chemicals that when administered at physiological levels cause normal pancreatic exocrine secretion and when give in supra-maximal levels induce acute pancreatitis. Secretagogues used for inducing AP include cholecystokinin (CCK), its analogue caeruelin, carbachol (Chaudhuri et al., 2005), anticholinesterase (Dressel et al., 1982), and scorpion venom (Pantoja et al., 1983)(Novaes et al., 1989).

Caerulein is a decapeptide with a very similar chemical structure to porcine cholecystokinin (CCK) and its action on gastrointestinal secretion also resembles CCK. It acts via CCK receptors to produce hyperstimulation of pancreatic acinar cells (Saluja A 1999). It was first isolated from the skin of the Australian frog *Hyla caerulea* in 1967 (Anastasi et al., 1967) and was used by Lampel and Kern in 1977 to induce acute pancreatitis in rats by giving a high dose intravenous infusion (Lampel and Kern, 1977). It was subsequently found to be effective at producing pancreatitis in many animals including mice, rats, opossums, and dogs (Niederau et al., 1985, Renner et al., 1986, Kahle et al., 1991).

Caerulein has become the most extensively used in experimental models of pancreatitis because of several advantageous factors. It is easy to administer, especially if via the intra-peritoneal route (50 μg/kg/hr), or by the intravenous infusion (5 μg/kg/hr) and is less invasive than some surgical modes of induction, such as ductal infusion, and is reproducible giving consistent results (Niederau et al., 1985). The histological changes it produces are similar to those seen in human AP, and in addition it also causes acute lung injury. It has proved useful for assessment of potential therapeutic agents and for the examination of events early in the disease.

Within the first 30 minutes after administration abnormal vacuoles are formed, a feature of secretagogue pancreatitis. It was postulated that this might be due to fusion of condensing vacuoles and zymogen granules (Lampel and Kern, 1977). It was seen in some cells that these abnormal vacuoles fused with the baso-lateral membrane instead of the apical membrane, suggesting discharge into the interstitium of the pancreas. These initial changes were subsequently described in greater detail (Wantanabe et al., 1984; Saluja et al., 1985; Niederau et al., 1990). Intracellular activation of enzymes was shown to occur in caerulein pancreatitis *in vivo*, and together with the co-localisation of zymogen granules and lysosomes (Saluja et al., 1987; Steer et al., 1988; Willemer et al., 1990) the inhibition

of digestive enzyme secretion (Saluja et al 1989) and the hypothesis of excessive Ca^{2+} signalling (Ward JB et al 1995) several mechanisms of how caerulein induces pancreatitis have been postulated.

Caerulein and CCK produce stimulation by action on CCK receptors. In the pancreas of rodents CCK1 receptors, but not CCK2, are expressed on pancreatic acinar cells (PACs) (Wank et al., 1994). In human PACs the situation is reversed, with CCK2 receptors being more prevalent (Nishimori et al., 1999). Until recently it was controversial if these CKK receptors functioned the same way in humans, as in animal models. If proven this would have seriously harmed the validity of the hypersecretion animal model. This controversy was settled when work from work from our group categorically demonstrated that application of CCK-8 or CCK-58 at physiological levels caused typical intracellular changes in isolated human PACs (namely elevated cytosolic Ca^{2+}, mitochondrial nicotinamide adenine dinucleotide phosphaste (NADPH) generation and pancreatic enzyme secretion) (Murphy et al., 2008). Conducting these experiments in the presence of either atropine or tetrodotoxin to ensure other signalling pathways were not involved further strengthened the evidence.

Hyperstimulation of PACs with supra-physiological doses of CCK/caerulein leads to $[Ca^{2+}]_c$ overload, co-localisation of lysosomes and trypsinogen, and abnormal secretion of activated trypsin into the pancreatic interstitium. Local pancreatic injury is followed by distant organ injury (esp. lung) (Saluja et al., 2007)

The disadvantage of caerulein as an experimental model of pancreatitis is whether it can be used as a comparative model for human disease. It has been suggested as a demonstrating a mechanism found in rare clinical cases such as exposure to venom of certain scorpions or accidental ingestion of anti-cholinesterase insecticides (Buchler et al 1999). It is still uncertain what role hyperstimulation plays in human acute pancreatitis, but human pancreatic acinar cells have been found to have CCK receptors that respond *in vitro* to secretagogues the same way that murine pancreatic acinar cells do (Murphy J., et al 2008).

1.2.3 Other non-invasive experimental models

In 1975 it was reported that female mice (less than 20g) when fed with a choline-deficient diet with 0.5% DL-ethionine supplement (CDE diet)

developed acute pancreatitis with massive fat necrosis. The mortality rate was 100% after 4 days (Lombardi et al., 1975), but severity can be altered with different feeding regimes. The exact mechanism is unclear, but male mice given oestrogen exhibited similar showed similar changes (Rao et al., 1982). Larger animals have been shown to develop very mild pancreatitis only. Although it is the most non-invasive experimental model it is limited by its application only in female mice, by its toxic effects on other organs (mainly brain and liver) and by the length of onset (days). Larger animals have only shown very mild pancreatitis (Chan and Leung, 2007).

Amino-acid induced AP models include L-arginine, L-lysine (Dawra et al., 2007)(Biczo et al., 2011) and L-ornithine (Rakonczay et al., 2008). The L-arginine experimental model is the most widely employed and is relatively non-invasive, administered usually with one or two intra-peritoneal injections (Dawra et al., 2007). The degree of severity depends on the dose, with 2.5 mg/kg leading to mild, oedematous pancreatitis and 5 mg/kg being enough to induce massive, necrotising pancreatitis in rats (Mizunuma et al., 1984)(Kishino et al., 1984), rabbits and mice (Zhao et al., 2004)(Darwa et al., 2007). L-arginine also has little effect on other organs, so that its specificity, dose-dependent severity and ease of use make it a commonly used experimental model. How L-arginine produces its effects is unclear,

but experimental evidence suggests protein synthesis inhibition as the most likely mechanism: specifically suppression of ornithine decarboxylase, which ultimately leads to a reduction in nucleic acid and protein synthesis. As the pancreas is highly metabolic it is vulnerable to this toxicity (Chan and Leung, 2007). Its disadvantage is its lack of relevance to clinical practice.

Alcohol abuse is an important aetiology in human APs, however the mechanism is unclear. Animal experiments administering ethanol alone (either i.v. or orogastric) have failed to show evidence of AP (Letko et al., 1991), unless simultaneously given with a physiological does of another agent such as CCK and secretin (Siech et al., 1991). When ethanol is given with caerulein (Foitzik et al., 1994) or bile acid (Andrzejewska et al., 1998) the AP seen in animal models more severe, suggesting that ethanol is a potentiator of AP as opposed to a primary cause. This would seem to correlate with human AP related to alcohol where only a small number of patients abusing alcohol actually develop AP (compared with, say, liver disease).

1.2.4 Invasive experimental models

Several invasive animal experimental models of AP exist and all require surgical intervention, needing specialist expertise and resources. The models described are grouped as: vascular/ischaemic models; closed duodenal loop; duct ligation models; duct perfusion models.

Vascular impedance can cause pancreatitis and there are several ways to achieve this, though the relevance to human disease is doubtful. Hypovolaemic shock by sudden loss of 30-35% blood volume can cause pancreatitis in dogs (Brasilia et al., 1987) and has a clinical counterpart in human disease with AP seen after hypotension in surgery (esp. cardiac bypass). Injection of microspheres in rodents causes AP (Pfeffer et al., 1962)(Reddha et al., 1990). Others studies have ligated the arteries supplying the pancreas (superior pancreatico-duodenal artery/ superior mesenteric artery) to demonstrate experimental AP (Spormann et al., 1989)(Waldner 1992). Alternatively venous outflow can be impeded by occlusion of the splenic/gastro-duodenal veins, which can lead to SAP (Sjovall et al., 1988). Overall vascular models are not widely employed. They often have traumatic effects on other organs not directly from AP but from systemic injury instigated by surgery or rapid blood loss (Chang and

Leung et al., 2007). There is also limited evidence human AP is caused by vascular impedance.

The closed duodenal loop model was originally described in 1910 (Seidel 1910) but was investigated in more detail half a century later in 1957 (Pfeffer et al., 1957). This model requires extensive surgery: the duodenum is divided distally and proximally to the insertion of the pancreatic duct PD, creating a blind loop, with the bowel being rejoined in a gastro-jejunostomy (Chang and Leung, 2007). The model investigates if duodenal content reflux into the PD causes AP. The model has been modified slightly over the years (Sugimoto et al., 2004). The model has lent weight to bile involvement in SAP (Chetty et al., 1980). However there is no evidence this is how human AP occurs, and, taken with the complexity of the surgery required, means this is not a widely used experimental model.

1.2.5 Duct obstruction-induced model

The duct obstruction experimental model mimics human gallstone pancreatitis and, although it requires surgery and is invasive, it is relatively

straight forward technically, requiring only ligation of the pancreatic duct (PD) with vertical cannulation or balloon tip catheter in the rat (Chan and Leung, 2007) The opossum is an interesting model as it has also has a common biliopancreatic duct (CBPD)(Lerch et al., 1992). The opossum model produces a SAP with over 50% necrosis, compared to approximately 10% in the rat. (Kaiser et al., 1995). It is postulated that bile reflux damages pancreatic acinar cells, but it is also suspected that a build up of pressure in the PD from pancreatic secretions also has a role as it is observed that the longer the obstruction is applied the more severe the AP. Removal of the obstruction after 24 hours in rodents invariably results in complete resolution (Azima et al., 1996), but a more recent model in mice showed 100% mortality after four days of PD ligation (Samuel et al., 2010), again suggesting a need for build-up of pancreatic secretion and pressure. Duct obstruction with concurrent additional stimulus (e.g. secretagogue) invariably worsens AP compared to duct obstruction alone (Yoshinaga et al., 2000)(Chang and Leung, 2007). This model is strengthened by its clinical relevance.

1.2.6 Duct perfusion-induced model

Cannulation and perfusion of the pancreatic duct with chemicals linked to AP (usually bile salts but also ethanol metabolites) will elicit AP in animal models, and has been extensively used as a proxy for human disease. The commonly used bile salts are: sodium taurocholate (Na-TC), taurolithocholic acid 3-sulphate (TLC-S) (Perides et al., 2010a&b), sodium glycodeoxycholic acid (Na-GDC) (Schimdt et al., 1992), and taurodeoxycholate (Na-TDC) (Jin et al., 2008 and Jin et al., 2011). This model requires surgical intervention, and as large animals show AP changes, but not MODS, it is the rodent model that predominates, as it shows both changes. Being a smaller animal model the technique of PD cannulation and perfusion can be technically demanding. The infusion pressure must be carefully controlled (usually with a pump) as a raised PD duct pressure with saline can cause mild AP by itself (Lichtenstein et al., 2000).

Na-TC in rodents is the most widely used PD bile salt-infusion model and induces oedema, haemorrhage and necrosis depending on the dose infused (usually 3-6%) (Chan and Leung, 2007). It has also been shown to cause MODS, with injury especially in the lungs (Chen et al., 2008) as is seen in

human disease. It can be combined with other models, including LPS (simulating septic shock)(Segersvard et al., 2004); e.coli (representing an infective component in human disease) (Zhou et al., 2008) and with trypsin (Yamano et al., 1998) generally resulting in more severe disease. More recently a non-lethal murine model has been described (Laukkarinen et al., 2007 and Perides et al., 2010b) generally affecting the head of the pancreas only. Thus a spectrum of severity now exists in Na-TC rodent models.

The so-called *Boston model* (Schimdt et al., 1992) combined caerulein with PD infusion of Na-GDC leading to SAP with MODS. The pattern of disease, onset of symptoms over 24 hours and a mortality of about 20%, mirrors human disease.

TLC-S in used extensively in *in vitro* work with isolated pancreatic acinar cells (PACs), where it has been shown to be taken up by G protein-coupled bile acid receptor-1 (Gpbar1). Hyperstimulation of isolated PACs with TLC-S leads to increased $[Ca^{2+}]_i$ which in turn injures the mitochondria and, depending on level of damage, to either apoptosis or necrotic pathways. This been confirmed in animal models using genetic knock-out (KO) mice Gpbar1 -/-. These animals were protected against TLC-S induced AP, with

no change seen in either *in vivo* (PD infusion models or *in vitro* models (isolated PACs) compared with controls (Perides et al., 2010a).

Despite being technically demanding the PD-infusion model of AP has many parallels to human gallstone AP. It is a current popular experimental model, especially in rodents.

1.3 Apoptosis and necrosis in acute pancreatitis

Events that regulate the severity of acute pancreatitis are unknown and may be multi-factorial; for instance the induction of an inflammatory reaction with recruitment of inflammatory cells, such as neutrophils, may be responsible for local damage. How the pancreatic acinar cells (PACs) themselves react to damage early in the course of the disease is likely to be important to the severity of the outcome. Several studies over the last 15 years have shown that 'programmed' cell death by apoptosis appears to be protective whilst 'uncontrolled' death by necrosis is associated with more severe disease. Some experimental animal models have sought to induce apoptosis in PACs prior to using an established model of experimental pancreatitis (as listed in the previous section) and have shown a milder outcome (Bhatia et al., 1998). However the mechanisms involved in PACs apoptosis are currently uncertain, though identification of mechanisms that might *'switch'* necrosis to apoptosis could improve outcome (Pandol et al., 2007). Until recently apoptosis was seen as an organised and complex cell death process, with necrosis as an uncontrolled process, but this view of necrosis is slowly changing (Golstein and Kroemer, 2007).

1.3.1 Morphology in apoptosis and necrosis

Apoptosis was first described in 1972 for a morphologically distinct form of cell death (Kerr et al., 1972). The typical morphological features of apoptosis are cytoplasmic and nuclear shrinkage, chromatin margination and fragmentation, and breakdown of the cell to several residual *'apoptotic bodies'* (Majno and Joris, 1995)(Bhatia 2004). These bodies are recognised by macrophages, through externally located phosphatidylserine moieties (Criddle et al., 2007a), and removed from the tissues with minimal inflammation (Pandol et al., 2007).

Necrosis is triggered by pathological, extrinsic factors (e.g. toxic or traumatic insults) and in the context of AP this includes secretagogue hyperstimulation (CCK, Caerulein), bile salts (Vornina et al., 2002), ethanol metabolites (esp. FAEE) (Criddle et al., 2006), Ca^{2+} overload (Ward et al., 1995)(Kruger et al. 2000)(Petersen and Sutton, 2005)(Criddle et al., 2007a+b), excessive reactive oxygen species (ROS) and depletion of Adenosine Trisphosphate (ATP) through loss of mitochondrial membrane potential ($\Delta\Psi m$) (Criddle et al., 2006). Necrosis is characterised by mitochondrial swelling and dysfunction, followed by rapid swelling of the cell and nuclear pykinosis due to loss of cell membrane integrity. The

resultant release of lysosomal and granular contents into local tissues causes inflammation (Majno and Joris, 1995)(Barros et al., 2001)(Bhatia 2004)(Gukovsky et al., 2011).

1.3.2 Caspase cascade

Apoptosis is a highly co-ordinated process and is regulated by a family of aspartate-specific cysteine proteases, called caspases. Caspases are initially stored in an inactive form (pro-caspase) and require activation. The peptide cleavage required to convert pro-caspases to active caspases is specific and occurs at aspartate residues, meaning that other caspases are responsible and leading to the theory of a caspase cascade. As a further control cells also synthesise a range of inhibitor proteins (e.g. *'inhibitor of apoptosis proteins'* or IAP) (Slee et al., 1999)(Mace et al., 2010). Caspases are divided into two active groups: *'effectors/executioners'* (inc. caspases 3, 6 and 7) which actively disassemble the cell structures; and *'initiators/activators'* (inc. caspases 2, 8 and 9) which trigger cleavage and activation of the *'effectors/executioners'* (Slee et al., 1999)(Criddle et al., 2007a)(Pandol et al, 2007).

Initiator/activator caspases are activated either by an extrinsic pathway or an intrinsic pathway. In the extrinsic pathway *'death receptors'* (e.g. TNF-Receptor family such as CD95/FasR/APO-1) situated in the cell membrane are activated and, via 'adaptor molecules' (e.g. FADD), recruit membrane-bound caspases (esp. caspase-2 and caspase-8). These caspases are then activated and initiate the caspase cascade. In the intrinsic pathway the mitochondria is central to the process, with mitochondrial membrane permeabilization (MMP) a universal trigger and 'point of no return' in cell death pathways (Gukovsky et al., 2011). The stimulating group in the intrinsic pathway is diverse, including radiation, heat stress and cytotoxic drugs that ultimately results in the protein cytochrome c release from the mitochondria. Once in the cytosol cytochrome c combines and interacts with *'apoptotic peptidase activating factor 1'* (APAF-1) and procaspase-9 forming an *'apoptosome'*, with activation of caspase-9. Caspases-9 activates effector caspases, especially caspase-3 and caspase-7. Caspase-3 appears to have greater importance as studies of loss of caspase-3 show that other 'downstream' caspases (i.e. caspase-2 and caspase-6) are not activated (Slee et al., 1999). Any process leading to cytochrome c release can activate the intrinsic pathway, so that the intrinsic and extrinsic pathways can be connected. Figure 1.1 shows a simplified representation of the intrinsic and extrinsic pathways.

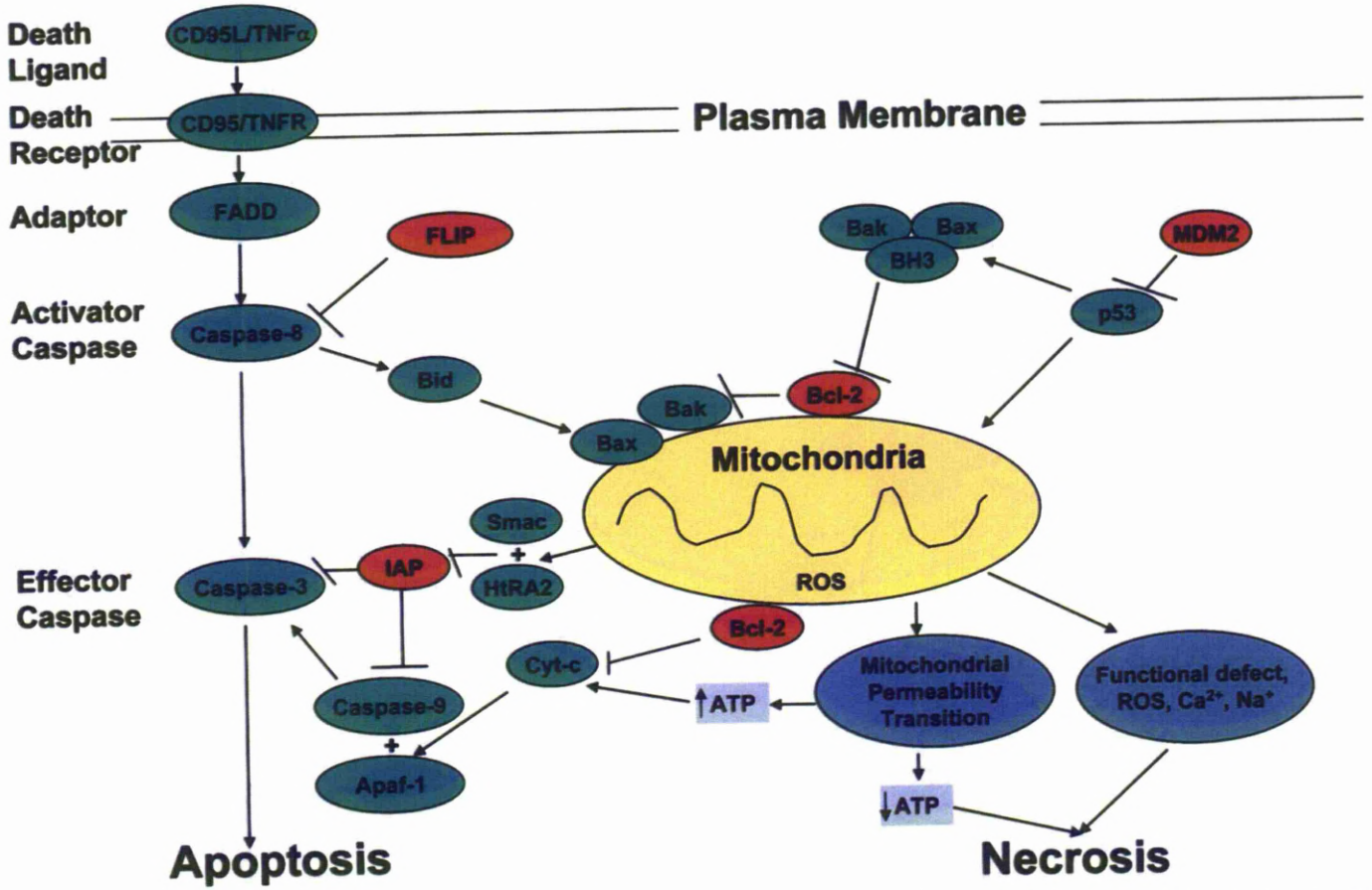


Figure 1.1: Overview of cell-death signaling pathways, apoptosis vs. necrosis. Components in red inhibit apoptosis, whereas those in green promote apoptosis. Factors (in blue) such as mitochondrial permeability transition and reactive oxygen species (ROS) can cause either apoptosis or necrosis, depending on other variables such as ATP supply. FLIP, FLICE-like inhibitory protein; IAP, inhibitor of apoptosis proteins; Apaf-1, apoptotic protease activating factor; Cyt-c, cytochrome c; TNFR, TNF receptor; BH3, Bcl-2 homolgy domain 3. (from Bhatia, 2004)

There may be crossover between these pathways, as Caspase-8, although activated via the extrinsic pathway, can also engage the intrinsic (mitochondrial) pathways by activation of BID. BID in turn affects MMP and leads to release of cytochrome c (Slee et al., 1999)(Gukovsky et al., 2011).

1.3.3 Bcl-2 family proteins

Other key molecules regulating cell death are proteins of the Bcl-2 (B-cell lymphoma 2) family (Kroemer et al., 2007). The name derives from Bcl-2 first being described in follicular lymphoma. The Bcl-2 family are subdivided into 3 groups based on their Bcl-2 homology (BH) domains and on whether they are pro-survival (anti-apoptotic) or pro-apoptotic (Kroemer et al., 2007)(Chipuk et al., 2008)(Gukovsky 2011). The pro-survival members (Bcl-2 and Bcl-xL) contain 4 domains (BH1-4), the pro-apoptotic members (Bax and Bak) contain 3 domains (BH1-3) and, finally, the BH3 members, also considered pro-apoptotic, contain one domain (e.g. BAD, BID or Puma).

Bak (Bcl-2 homologous antagonist/killer) and Bax (Bcl-2 associated x protein) are described as 'pro-apoptotic' and are involved in the formation of the mitochondrial outer membrane pore (MOMP), through which cytochrome c is released. Bak is predominantly localised to mitochondria, whereas Bax is found in the cytosol. Activated Bax promotes apoptosis in two ways. First by inserting into the outer mitochondrial membrane (OMM) with Bak to form the MOMP. Second by inducing opening of the separate mitochondrial permeability transition pore (MPTP), which also leads to release of cytochrome c (Galluzzi et al., 2009)(Gukovsky et al., 2011).

Pro-survival (or anti-apoptotic) Bcl-2 and Bcl-xL act by inhibiting Bak and Bax, stopping the formation of the MOMP, or by inhibition of cytochrome c (Bhatia, 2004)(Gukovsky et al., 2011). However this may not be entirely straight forward as more recently it also appears that Bcl-2 and Bcl-xL have a role in regulating necrosis, through effects on mitochondrial membrane potential ($\Delta\Psi m$); namely the difference in their effects on $\Delta\Psi m$ *versus* their effects on cytochrome c inhibition (Sung KF et al., 2009). In experimental *in vitro* models of CCK-induced changes it was demonstrated that inhibiting Bcl-2 and Bcl-xL increases loss of $\Delta\Psi m$ and facilitates necrosis, whilst also

inhibiting CCK-induced caspase-3 activation and apoptosis. The net result, surprisingly, being an increase in necrosis and not apoptosis as expected.

The other pro-apoptotic BH3 proteins support the role of Bak/Bax either by interacting directly and promoting MOMP formation (e.g. BID), or by interfering with the inhibitory actions of Bcl-2/Bcl-xL (e.g. BAD).

1.3.4 Role of the mitochondria in pancreatic acinar cell death

Mitochondria have long been known to be the main organelle supplying energy to the cell in the form of ATP. In the last 15 years it has become clear that they play a central role in cell death as well (Kroemer et al., 2007), determining whether apoptotic pathways or necrotic pathways are followed. They are central to the activation of the intrinsic pathway of apoptosis, whereas any inability to supply ATP may lead cells to a necrotic pathway. The structure of mitochondria, with different compartments, is important to their function. These compartments are the: outer membrane, inter-membrane space, inner membrane and the cristae and matrix (Hermann and Neupert, 2000) (see figure 1.2). The outer membrane

contains channel-forming proteins called porins, which allow molecules less than 5000 Daltons to diffuse across the membrane; for larger molecules there are active transport mechanisms. Cytochrome c is contained in the inter-membrane space and disruption of the outer membrane will lead to its release and activation of the intrinsic pathway. The inner membrane encloses the matrix, which is the site of ATP production. The inner membrane maintains a mitochondrial membrane potential ($\Delta\Psi m$) of 150-180 mV across the inner mitochondrial membrane by actively pumping protons into the inter-membrane space (Mukherjee et al., 2008).

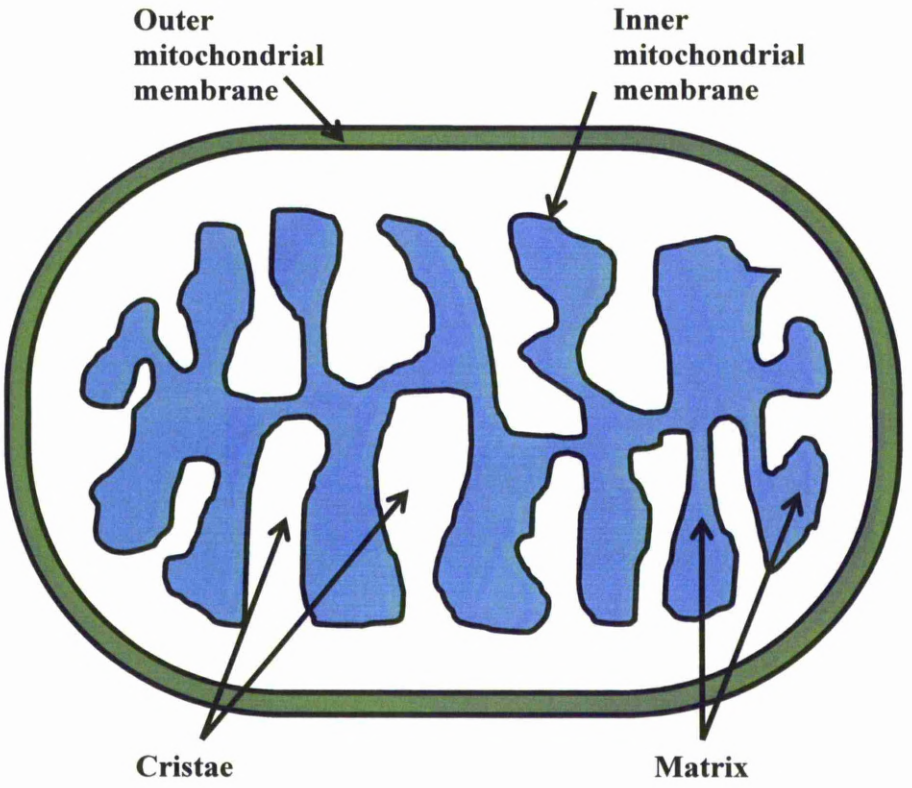


Figure 1.2: Representative diagram showing basic structure of mitochondrion. In particular demonstrating the outer mitochondrial membrane (OMM), the inter-membrane space and the inner mitochondrial membrane (IMM). The matrix is further demonstrated as contained within the IMM.

1.3.5 Role of Calcium signalling and mitochondrial function

The $\Delta\Psi m$ is important for many of the functions of mitochondria, and one of these is concentration of Ca^{2+} within the matrix (where Ca^{2+} has a role in activating Kreb's cycle enzymes). Loss of $\Delta\Psi m$ (second to an abnormal rise in $[Ca^{2+}]_i$ triggered by toxins (further described in Ca^{2+} signalling section)) (Petersen and Sutton, 2005)(Criddle et al., 2007a)(Mukherjee et al., 2008), leads to loss of ATP production and ultimately death by necrosis (see figure 1.3). Experimental evidence supporting the role of toxic Ca^{2+} signalling in loss of mitochondrion function is found by either excluding Ca^{2+}, following toxic signals, with a Ca^{2+} chelator (Voronina et al., 2002)(Criddle et al., 2004), or by providing supplementary *in vitro* intracellular ATP after a toxic rise in $[Ca^{2+}]_i$ (Criddle et al., 2006), both of which allow survival of the pancreatic acinar cell (PACs).

Mitochondria have an important role in maintaining PACs Ca^{2+} homeostasis, which is critical for PACs function (Park et al., 2001)(Voronina et al., 2002). Mitochondria are important in buffering the Ca^{2+} signals released in the apical section of the cells from the rest of the cell and provide ATP for energy dependent termination of Ca^{2+} signals by uptake by Sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) and plasma

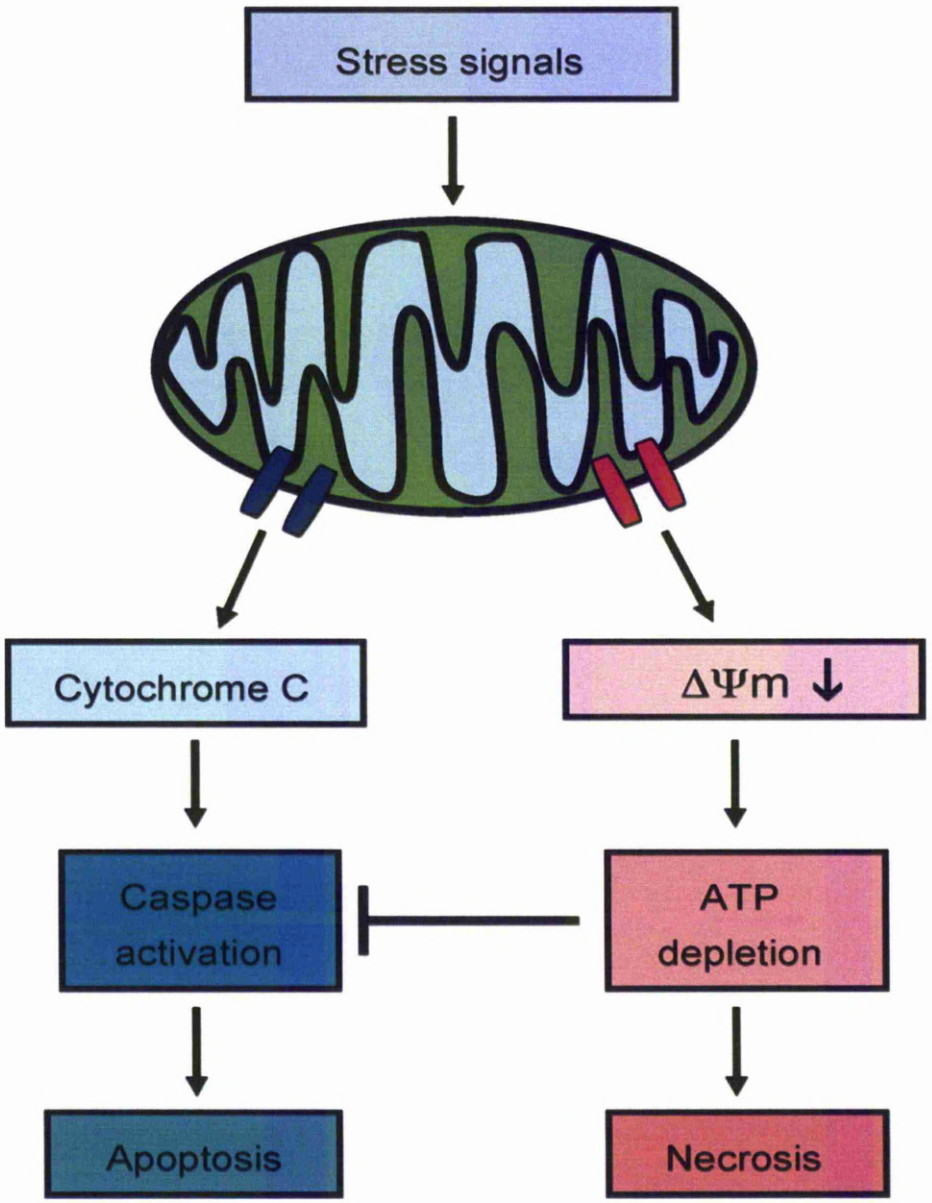


Figure 1.3: Mitochondria as central to cell death pathways. Toxic Ca^{2+} levels and other stresses, lead to cytochrome c release from the inner mitochondrial membrane, activating caspases (and inducing further Ca^{2+} release via IP_3Rs). These events may be controlled by Bax/Bak channels (shown in blue). Caspase activation has a protective role in pancreatitis, but requires adequate supply of ATP. Marked loss of mitochondrial membrane potential, notably with induction of the MPTP (shown in red), inhibits ATP production, which in turn inhibits apoptosis and leads to necrosis. (From Mukherjee et al., 2008)

membrane Ca^{2+} ATPase (PMCA) activity (see section on Ca^{2+} signalling) (Petersen 2005 & Petersen et al., 2006).

1.3.6 Mitochondrial membrane permeabilisation

Mitochondria regulate apoptosis/necrosis in two ways. Firstly by mitochondrial membrane permeabilisation (MMP), which is a key 'point of no return' in apoptosis/necrosis (Galluzzi et al., 2009)(Gukovsky et al., 2011) and is brought about by activation of two separate membrane systems: the mitochondrial permeability transition pore (MPTP) and the mitochondrial outer membrane pore (MOMP), both of which lead to release of cytochrome c. Secondly by loss of production of ATP which ultimately leads to death by necrosis.

The MPTP is thought to determine cell fate from injury and is linked to the degree of loss of $\Delta\Psi m$, with a potentially small, recoverable loss of $\Delta\Psi m$ leading to apoptosis and an unrecoverable loss of $\Delta\Psi m$ leading to loss of ATP production and death by necrosis (Mukherjee et al., 2008). MPTP opening across the inner mitochondrial membrane (IMM) leads to loss of $\Delta\Psi m$ and ATP production. It is formed from at least 3 components: Voltage

dependent anion channel (VDAC) in the outer membrane, adenine nucleotide translocase (ANT) in the inner membrane and Cyclophilin-D (CyP-D) within the matrix (see figure 1.4a below) (Kroemer et al., 2007)(Mukherjee et al., 2008). CyP-D is thought to be the predominant regulator and high concentrations of Ca^{2+} induce conformational changes that lead to these proteins forming the MPTP (Kroemer et al., 2007) with subsequent release of cytochrome c.

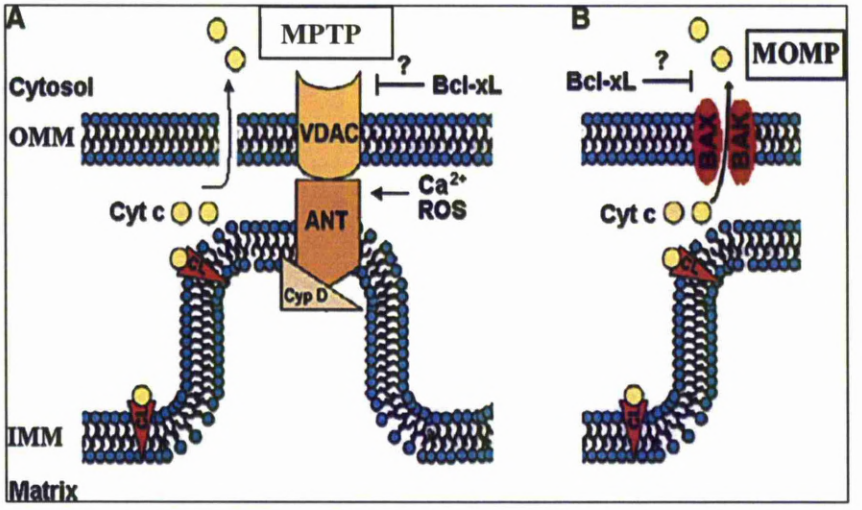


Figure 1.4. Mitochondrial membrane permeability. (A) The MPTP is a large nonselective channel the components of which include Cyp D, VDAC, and ANT. MPTP opening is stimulated by Ca^{2+} and ROS and leads to release of cytochrome c. (B) The MOMP channel is formed by the pro-apoptotic Bcl-2 proteins Bax and Bak. There is evidence that the prosurvival Bcl-xL protein may inhibit both MPTP and MOMP.(Abbreviations: Cyp D, cyclophilin D; VDAC, voltage-dependent anion channel; ANT, adenine nucleotide translocase; ROS, reactive oxygen species; Cyt c, cytochrome c; CL, cardiolipin. (figure from Gukovsky et al., 2011)).

Overload of signals (esp. from toxic levels of $[Ca^{2+}]_i$) leads to a sustained opening of the MPTP, with uncontrolled entry of solutes and water with subsequent leakage and swelling of the mitochondria. This leads to a loss of ATP and ultimately cell necrosis (review figure 1.4). Studies inhibiting MPTP function (with bongkrekic acid) have shown a reduction in apoptosis (Gerasimenko et al., 2002), which supports its role in the intrinsic pathway. However there must more than one way to induce the intrinsic pathway as cyclophilin D (CyP-D) knock-out transgenic mice show no protection from a range of apoptosis inducing stimuli (Nakagawa et al., 2005)(Mukherjee et al., 2008). Indeed further collaborative work from our group in Liverpool has shown that CyP-D ablation greatly reduced necrosis in 3 dissimilar models of pancreatitis proving further evidence that the MPTP is also involved in necrosis, and perhaps more so than it is involved in apoptosis (Gukovsky et al., 2011). These results suggest that apoptosis may actually be mediated more by another MMP pore: the mitochondrial outer membrane pore (MOMP).

The MOMP are large channels formed by the pro-apoptotic Bak and Bax proteins, though the full mechanism of action is currently unknown. When activated they release cytochrome c and activation of the intrinsic pathway (see figure 1.4b). There may be overlap between the MPTP and the MOMP.

1.3.7 Evidence that apoptosis is protective in acute pancreatitis

Analysis of experimental models of AP show that severe acute pancreatitis (e.g. that seen in opossum PD ligation, or by caerulein hyperstimulation in the mouse) is associated more with necrosis, and that milder experimental models are seen to have much higher relative levels of apoptosis (Kaiser et al., 1995)(Bhatia, 2004). Looking specifically at the hyperstimulation model, it has been shown by one group that caerulein can induce apoptosis by stimulating platelet-activating factor (PAF), which in turn regulates apoptosis (Sandoval et al., 1996). Subsequently CCK has been shown to stimulate death-signalling pathways in rat PACs by activating caspases, releasing cytochrome c and by mitochondrial depolarisation (Gukovskaya et al., 2002). Caspases also help inhibit necrosis and intra-PACs trypsin activation. One group used a plant nitrile extract called crambene (1-Cyano-2-hydroxy-3-butene (CHB)), which induces apoptosis (Bhatia et al. 1998). When caerulein hyperstimulation was administered to mice pre-exposed to crambene, they were protected against AP. However, as the exact mechanism of crambene action is unclear, caution should be shown before attributing all of crambene's protective effects as simply pro-apoptotic.

Crambene could be affecting pancreatitis severity through actions other than on apoptosis. Crambene may affect gap junction communication (GJC). Mice deficient in a gene coding for GJC (Connexin 32) are not protected from caerulein hyperstimulation AP despite the administration of crambene (Frossard et al., 2003).

1.4 Pancreatic acinar cells and calcium signalling

1.4.1 Pancreatic exocrine function

Everyday the human pancreas secrets about 1.5 litres of fluid, rich in digestive enzymes (called zymogens), bicarbonate and water. The bicarbonate and water are predominantly produced by the cells of the pancreatic duct (PD). The relatively high pH helps to neutralise the acidic chyme that exits from the stomach into the duodenum, providing an optimal pH for digestive enzymes to function. The pancreatic acinar cells (PACs) are grouped together in clusters called acini (*singular: acinus),* and produce and secrete zymogens into the lumen of the acini. Zymogens are capable of breaking down the vast majority of proteins (e.g. trypsin, chymotrypsin), fats and phospholipids (e.g. lipase, phospholipase) and carbohydrates (e.g.

amylase, carboxypeptidase) that are ingested. In order to protect the PACs from self-digestion, these zymogens are stored in an inactive state (pro-zymogens) within PACs, inside localised secretory granules (also called zymogen granules) (Raraty et al., 2000). These secretory granules are clustered in the apex of the PACs, adjacent to the acini lumen ready for secretion. Trypsin is thought to be the first enzyme activated and subsequently leads to activation of the others zymogens as part of an enzyme activation cascade. Under physiological conditions this activation occurs in the duodenum where trypsinogen is converted to active trypsin by the enzyme enteropeptidase (formerly called enterokinase). Enteropeptidase is produced by cells lining the duodenum. Premature activation of trypsinogen in the apex of PACs is now widely acknowledged as the key event in the autodigestive process. This premature activation has been shown to be triggered by PD hypertension, bile salts, ethanol, ischaemia, hypercalcaemia, hyperlipidaemia, drugs and toxins (Sutton et al., 2003). Premature activation within and around acinar cells leads to cytoskeleton disruption, co-localisation of zymogens and lysosomes (Gorelick and Matovcik, 1995), vacuole formation and pro-inflammatory cytokine expression, ultimately resulting in acute pancreatitis (Ward et al., 1996)(Sutton et al., 2003). The exact process of the mechanisms leading to activation are still unclear, but work from our group and others have shown

that toxic Ca^{2+} signalling plays a critical role (Ward et al., 1995)(Raraty et al., 2000)(Sutton et al., 2003)(Criddle et al., 2007b). PACs have some protection against low-level inadvertent premature activation of trypsinogen to trypsin, through inhibitory mechanisms such as pancreatic secretory trypsin inhibitor (PSTI) (Nathan et al., 2005). The importance of trypsinogen activation has been further clarified by studies on families with hereditary pancreatitis, which is caused by mutations of the cationic trypsinogen (PRSS1) gene. The resultant abnormal trypsinogen is thought to cause pancreatitis by being more likely to undergo auto-activation, and by being more resistant to normal inhibition from PSTI (Whitcomb et al., 1996) (Pfutzer et al., 2000)(Howes et al., 2005)(Crocock et al., 2010). Although currently demonstrated to be the most important prematurely activated enzyme, trypsinogen may not be alone, as there is evidence to support premature activation of other enzymes such as cathepsin B and granular elastase (Halangk et al., 2000)(Sutton et al., 2003).

Secretion from PACs is controlled by both neural and hormonal pathways (Gorelick and Jamieson, 1994). Neural control is via cholinergic and peptidergic pathways, with messengers including acetylcholine (ACh) vasoactive intestinal peptide (VIP) and gastrin-releasing peptide (GRP) (Holst 1993). Hormonal control is via cholecystokinin (CCK) and secretin.

CCK is released by duodenual I cells (Chey et al., 1993) and causes secretion of zymogens from PACs. Secretin acts to stimulate water and bicarbonate secretion from pancreatic ductal cells.

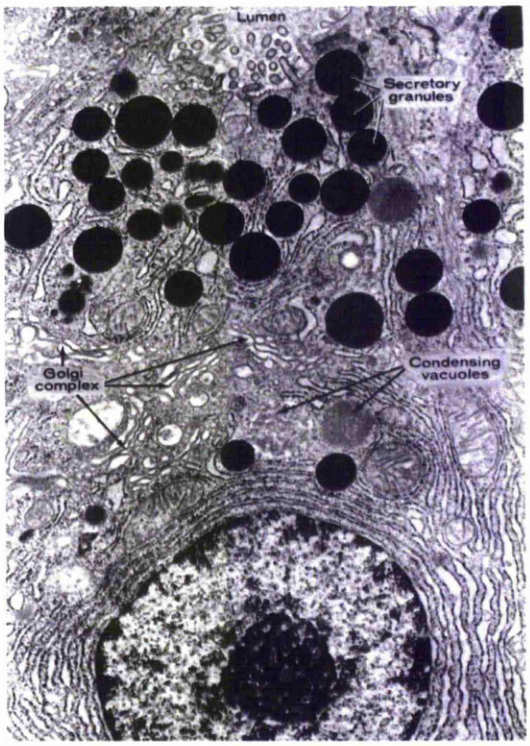
Under physiological conditions vagal stimulation is recognised in an early 'cephalic phase' of PACs secretion. This is followed by a 'gastric phase' in which stretch receptors in the stomach activate a vagally-controlled reflex. In the last 'intestinal phase' both neural and hormonal pathways lead to pancreatic exocrine secretion. Secretion is inhibited by somatostatin and its analogues.

1.4.2 The pancreatic acinar cell

Pancreatic acinar cells (PACs) have several structural arrangements that are particular to their role as a secretory cell. They are bound together in acinar clusters by tight junctions. PACs show marked structural polarity with secretory granules (containing zymogens) clustered at the apical pole, on the acinar lumen side. The endoplasmic reticulum (ER) is extensive, reflecting

the role of PACs in the manufacture of zymogens. The ER is predominantly found in the basolateral region of the cell, but has projections into the apical region, where secretory granules bud off from the ER (Park et al., 2000). The nucleus is found in the baso-lateral region. The PACs are also noted for particular groupings of mitochondria. In particular surrounding the nucleus and, more notably, forming a buffer zone between the secretory granules and the rest of the cell (peri-granular mitochondrial belt) (Tinel et al., 1999), where they have been show to play an important role in reducing toxic Ca^{2+} signals (Criddle et al., 2007b). The golgi apparatus is found between the ER and the peri-granular mitochondrial belt. Isolated PACs used in experimental work retain this particular structural arrangement (see figure 1.5).

A



B

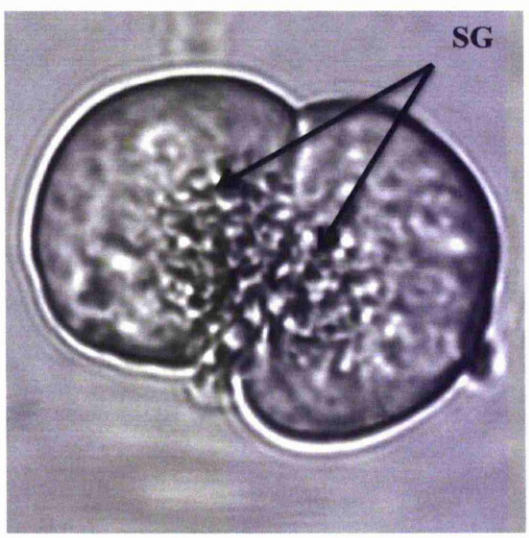


Figure 1.5: EM and light microscopic images of PACs. A. Shows an electronmicroscopic image of a pancreatic acinar cell. Note the clustering of secretory granules at the apical pole and the extensive basol-lateral endoplasmic reticulum. Also indicated are golgi apparatus and condensing vacuoles. (From Bloom and Fawcett, 1975). B Shows a typical view of isolated murine pancreatic acinar cells when viewed with a light microscope. In this image a doublet is shown. Note the secretory granules again clustered at the apical poles. (Taken by myself, using a two-photon light microscope).

1.4.3 Normal Ca^{2+} signalling in pancreatic acinar cells

Ca^{2+} is a universal second messenger found in many diverse cell types. In PACs Ca^{2+} signals are triggered by neurotransmitters (e.g. ACh) and hormones (esp. CCK). As detailed in the previous section, these Ca^{2+} signals are linked to secretion of the zymogens from secretory granules in the apical part of the PACs. This process, where a stimulant applied to a cell leads to a chain of events resulting in secretion, is called 'stimulus-secretion coupling' and was first described five decades ago (Douglas et al., 1968). Under normal conditions there exists a Ca^{2+} concentration gradient within the PACs, with a cytosolic concentration ($[Ca]_i$) in the range 10^{-7} M, compared to a range of 10^{-4} within the ER (intra-cellular stores) and 10^{-3} M in the extra-cellular fluid (Sutton et al., 2003)(Raraty et al., 2005). This concentration gradient is maintained between the cytosol and the extra-cellular fluid by the Mg^{2+} dependent plasma membrane Ca^{2+} ATPase pumps (PMCA), and between the cytosol and the ER by sarco/endoplasmic reticulum Ca^{2+} ATPase pumps (SERCA). This low concentration of $[Ca^{2+}]_i$ allows small, transient, oscillatory rises in Ca^{2+} to be used as a signalling messenger (Sutton et al., 2003)(Raraty et al., 2005).

Following ACh/CCK stimulation transient oscillations of $[Ca^{2+}]_i$ are actually first detected in the apical pole in the same area as the secretory granules (Ashby et al., 2002)(Criddle et al., 2007b). These spikes of $[Ca^{2+}]_i$ then spread towards the baso-lateral region of the ER, but are limited by uptake by the peri-granular mitochondria which act as a buffer taking up Ca^{2+} (Tinel et al., 1999)(Park et al., 2001). The SERCA and PMCA pumps then restore $[Ca^{2+}]_i$ by transporting Ca^{2+} into the ER and out of the cell respectively (Petersen, 2005). Even without signalling there is a low-level continuous leak of Ca^{2+} from the ER, which the SERCA pumps counter. See figure 1.6.

Each transient rise in $[Ca^{2+}]_i$ is associated with an increase in mitochondrial NADH production and ATP production (Voronina et al., 2002), thus supporting the role of cytosolic Ca^{2+} signals as part of physiological energy-dependent PACs secretion (Criddle et al., 2007b). The positioning of mitochondria as a peri-granular buffer, and also in close proximity to the ER, indicates these are areas of high metabolic demand (Park et al., 2001), with Ca^{2+} uptake by the mitochondria necessary for Krebs cycle activity and ATP production.

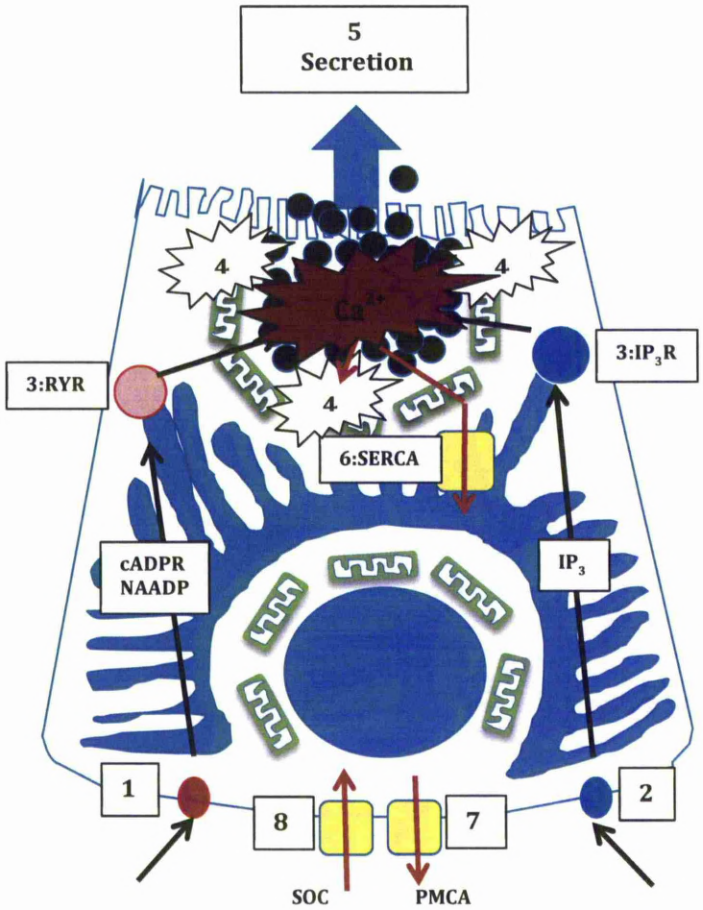


Figure 1.6: Normal Ca^{2+} signalling in pancreatic acinar cells. Diagram shows a representative PAC, with zymogen granules (black circles) in apex, surrounded by peri-granular mitochondrial belt (green) and an extensive endoplasmic reticulum (blue) 1) CCK binds to specific CCK_1 receptors, which leads to release of second messengers cADPR and NAADP. 2) ACh binds to muscarinic receptors, which leads to release of IP_3. 3) cADPR and NAADP bind to RyR in the ER membrane and IP_3 binds to IP_3Rs in the ER membrane *and* in ZG membranes. 4) The combined action so of 3) leads to rapid rise in apical Ca^{2+} levels. This wave spreads to the perigranular mitochondria, where some Ca^{2+} is taken up to activate ATP production. 5) ATP drives secretion of ZG from the apex of the PACs. 6) Ca^{2+} is actively taken up by SERCA, pumps in the ER membrane. Ca levels are returned to baseline by 7) actively pumping of Ca^{2+} out of the cell by plasma membrane Ca^{2+} activated pump (PMCA) and 8) diffusion of Ca^{2+} through Store-operated channels (SOC).

As described above, following ACh/CCK stimulation, transient Ca^{2+} oscillations are first seen in the apical pole of the PACs. However neuro-hormonal receptors are located in the baso-lateral membranes of the PACs, so secondary messengers are required to transmit the neuro-hormonal signals from the baso-lateral membrane to the apex. These secondary messengers have been identified as inositol trisphosphate (IP_3), cyclic adenosine dinucleotide phosphate ribose (cADPR) and nicotinic acid adenine dinucleotide phosphate (NAADP) (Cancela et al., 2002)(Yamasaki et al., 2005). These secondary messengers cause Ca^{2+} release from ER stores by interaction with receptors in the apical portion of the ER, adjacent to the secretory granules. These receptors function as Ca^{2+} channels, and when activated release Ca^{2+} into the apical portion of the PACs. The receptors are the IP_3 receptor (IP_3R) and the ryanodine receptor (RyR) (Petersen, 2005). ACh stimulation acts predominantly via IP_3, with actions on the IP_3R (Petersen, 2005)(Raraty et al., 2005), whereas CCK stimulation acts via NAADP and cADPR which are thought to act on RyR (Yamasaki et al., 2005)(Raraty et al., 2005)(Criddle et al., 2007b) though it is possible NAADP may have its own receptor as well. Whichever receptor is activated first is then capable of activating the other receptors in a process termed 'calcium-induced calcium release' (see figure 1.7). The consequence of these two separate second messenger pathways is that they converge to

induce a local, rapid magnification of Ca^{2+} release. This release ends when Ca^{2+} levels peak and then show a negative feedback inhibitory action on these ER Ca^{2+} release channels (Raraty et al, 2005). In combination with the previously described perigranular mitochondria buffering effect, ER uptake and PMCA extrusion from the cell, local $[Ca^{2+}]_i$ levels return to normal, resting levels (refer back to figure 1.6). When this buffering ability is overwhelmed, toxic Ca^{2+} signalling results.

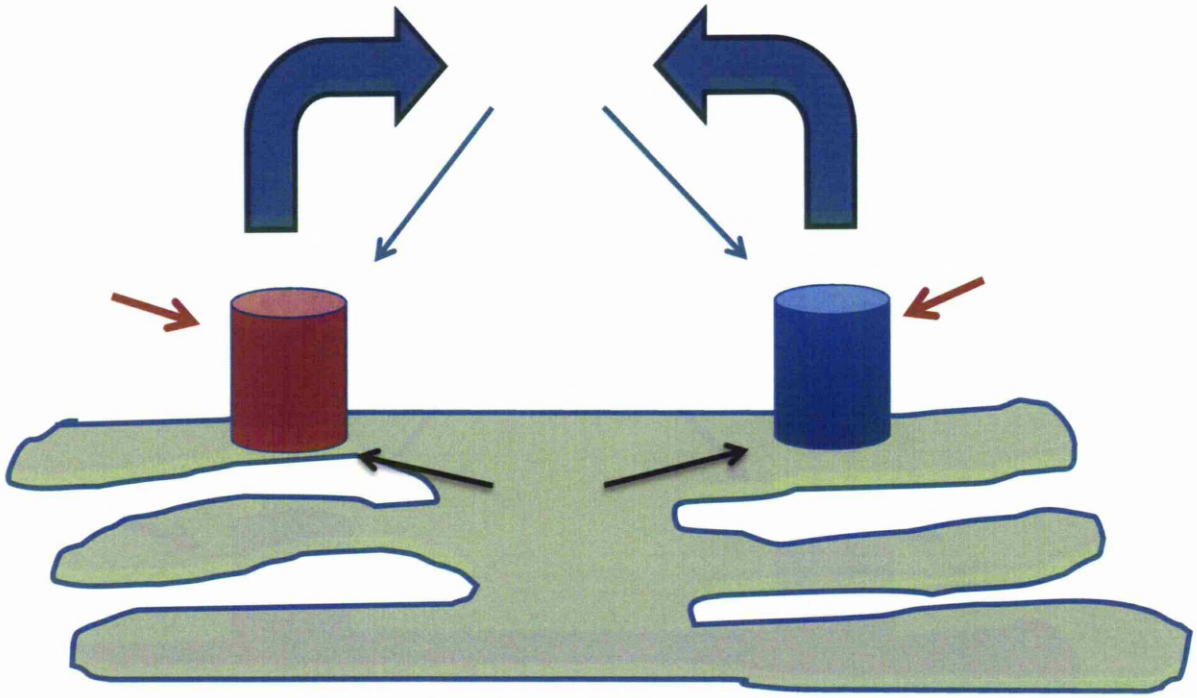


Figure 1.7: Calcium release at the endoplasmic reticulum. The two Ca^{2+} release channels, IP_eR and RyR are activated by second messengers IP_3 and NAADP/cADPR respectively. Ca^{2+} from either channel can act to open the other via calcium-induced calcium release (CICR). This mechanism is also capable of inhibition at higher levels. (Adapted from Petersen and Sutton, 2006)

1.4.4 Toxic Ca^{2+} signalling in pancreatic acinar cells

When PACs are stimulated with very high doses of neuro-hormonal transmitters (e.g. Ach or CCK) instead of the localised, transient rises in $[Ca^{2+}]_i$ seen in normal signalling, a dramatic, sustained, global rise in $[Ca^{2+}]_i$ occurs (Cancela et al., 2002)(Ward et al., 1999)(Raraty et al., 2005)(Petersen, 2005)(Criddle et al, 2007b). After the initial global rise in $[Ca^{2+}]_i$ there is a fall to an elevated baseline, but not to normal resting cytosolic Ca^{2+} levels. This disruption in cell signalling is thought to lead to inappropriate enzyme activation, vacuole formation, cytoskeletal damage, mitochondrial depolarisation, loss of plasma membrane integrity, cytokine expression, culminating in the events of acute pancreatitis (Raraty et al., 2005)(Sutton et al., 2003). Experiments showing the critical role of sustained $[Ca^{2+}]_i$ include those loading PACs with BAPTA (a Ca^{2+} chelator). BAPTA loading prevents global Ca^{2+} rises and PACs show no signs of premature enzyme activation (Raraty et al., 2000). Other compelling evidence for the role of Ca^{2+} comes from hyperstimulation experiments in PACs in the absence of Ca^{2+} in the extra-cellular fluid. No evidence of Ca^{2+} spikes or PACs damage is shown, suggesting that sustained Ca^{2+} entry into the cell must play an important role in toxic Ca^{2+} signalling (Raraty et al., 2000)(Voronina et al., 2002)(Criddle et al., 2007b).

Further evidence is seen in experiments involving the enzyme phosphatidylinositol 3-kinase (PI_3K), which has an inhibitory action on SERCA. Pharmacological inhibition or knockout of the PI_3K gene leads to protective effects in experimental pancreatitis (Singh et al, 2001)(Lupia et al., 2004)(Criddle et al., 2007b).

This toxic response to hyperstimulation is also seen with other, more clinically relevant, stimulants in experiments on isolated PACs. These include bile salts and non-oxidative ethanol metabolites (in the form of fatty acid ethyl esters - FAEEs) (Voronina et al, 2002a)(Criddle et al., 2006) and in pancreatic duct obstruction experiments (Mooren et al., 2003). It has been demonstrated that bile acids and fatty ethyl esters induce a sustained increase in $[Ca^{2+}]_i$ in part through action on IP_3R calcium release. This effect can be blocked by caffeine (Voronina et al., 2002a)(Criddle et al., 2006). As well as IP_3R action FAEEs accumulate in mitochondria, causing damage and reduction in ATP necessary to drive SERCA and PCMA pumps (Criddle et al., 2006). Likewise inhibition of RyR both *in vitro* and *in vivo* has been shown to reduce premature intracellular enzyme activation (Criddle et al., 2007b).

A fuller understanding of the mechanisms involved in Ca^{2+} cell signalling is therefore of great importance, as a number of potential therapeutic targets are possible, involving either inhibition of Ca^{2+} entry into PACs or inhibition of toxic release from PACs organelles.

1.4.5 Premature trypsinogen activation and enzyme co-localisation

As previously discussed premature trypsinogen activation within the PACs has been demonstrated to be a central event in most models of toxic premature enzyme activation. However, more recently this central role has been challenged, at least in part.

Supporting evidence for the role of intracellular, abnormal activation of trypsinogen leading to pancreatitis comes from studies observing events when trypsinogen, or the mechanisms that regulate activation, are abnormal. This includes: i) mutations of cationic trypsinogen (PRSS1 - the dominant human form) that renders trypsinogen more likely to activate and be resistant to normal degradation resulting in the human condition of hereditary pancreatitis (Whitcomb et al., 1996)(Howes et al., 2005); ii) a

variant of anionic trypsinogen (PRSS2 – the second most frequent human form) which leads to rapid autocatalysis is also protective against pancreatitis (Criddle et al., 2007b); iii) Mutations of a trypsin inhibitor serine protease inhibitor Kazal type 1 (SPINK 1) increase the risk of pancreatitis in transgenic mice (Witt et al., 2000), and in trypsin activation in human cultured cells (Kiraly et al., 2007) through loss of inhibition of activated trypsinogen. Overexpression of SPINK 1 ameliorates pancreatitis in transgenic mice (Nathan et al., 2005).

It is important to accept that while trypsinogen activation appears to be central in most models, recent evidence shows it may not be the only route to premature zymogen activation. As recently described, mutations in PRSS1-PRSS2 and in CLDN2 already predispose to pancreatitis, which is realised when exposure to a suitable aetiological factor occurs (e.g. alcohol) (Whitcomb et al., 2012). This forms part of a so-called 'double-hit hypothesis'. In experiments with trypsinogen KO mice (specifically isoform 7 -/- mice: the equivalent gene of human cationic trypsinogen) there was indeed a significant reduction in PAC necrosis and severity of pancreatitis, but changes associated with pancreatitis *still* occurred (Dawra et al., 2011). Interestingly local and systemic inflammatory response was largely unaffected and it was suggested this was because Nuclear factor kappa B

(NF-kB) levels were maintained. NF-kB plays an important role in the cytokine release associated with inflammation (see later section on cytokines). It is also not clear if trypsinogen-7 is the only isoform in mice, suggesting the possibility of other isoforms.

There is still debate about how premature trypsinogen activation occurs. Following hyperstimulation with caerulein in experimental models it has been shown that vacuoles are formed in the apical (or granular) section of PACs. These vacuoles result from fusion of lysosomes and zymogen granules, and the contents are acidic. Lysosome enzymes, particularly Cathepsin B, are co-localised with pro-zymogens and then might be involved in initiation of premature activation of trypsinogen (Kukor et al., 2002) resulting in an activation cascade. However the role of cathepsin B is controversial. Evidence supporting its role arises from experimental work, where administration of cathepsin B inhibitors, or use of cathepsin B knock-out mice, leads to a reduction in trypsinogen activation, and a reduction in the severity of pancreatitis (Saluja et al., 1999). However it is important to note than pancreatitis still occurred in these models, it was just less severe. Also there was no decrease in the systemic inflammatory response, meaning other organs were still affected (Halangk et al., 2000). Further uncertainty for the role of cathepsin B is from older work showing that zymogens and

cathepsin B co-localise under physiological conditions without premature activation within PACs (Tooze et al., 1991). Cathepsin B does appear to have a role in apoptosis and necrosis, secondary to cytochrome c release from the mitochondria (at least experimentally in liver hepatocytes) (Guicciardi et al., 2000). It may have a similar effect in PACs, which could ameliorate the severity of pancreatitis. Inhibition of cathepsin B significantly decreases apoptosis, but not necrosis, in experimental models of pancreatitis (Pandol et al., 2007). Taken together the evidence for the importance of the role of cathepsin B is still uncertain.

It had been suggested that, under toxic conditions, damage to the normal secretory function of exocytosis allows for a build up of activated zymogens within PACs (Perides et al., 2005), especially once co-localisation occurs. This disorder of secretion may contribute to pancreatitis. Inhibition and retention of activated zymogens is seen in studies affecting the trypsin-activated proteinase-activated receptor-2 (PAR-2) (Pandol et al., 2007). These are G-coupled transmembrane receptors, which aid in secretion of activated trypsin, and are therefore considered protective in acute pancreatitis (Olejar et al., 2001). Studies of PAR-2 knock-out mice demonstrate more severe pancreatitis (Sharma et al., 2005). Furthermore giving a PAR-2 activating agent in CCK-induced experimental pancreatitis

is also protective (Singh et al., 2007). These studies strongly suggest a detrimental effect from inability of PACs to excrete activated zymogens.

Ca^{2+} has a central role in premature trypsinogen activation. This activation does not occur in PACs that have had BAPTA (Ca^{2+} chelator) applied. Zymogen granules have high levels of Ca^{2+}, which are thought to be important in stabilising pro-zymogens in an inactive state (Nguyen et al., 1998)(Yoo et al., 2000). They also have IP_3Rs and RyRs on their membranes (Gerismenko et al., 1996)(Thrower et al., 2006) and it is through activation of these Ca^{2+} channels by second messengers IP_3 and cADPR that zymogen granules lose Ca^{2+} and the pro-enzymes activate prematurely (Yang et al., 2007). Ca^{2+} release activates K^+ entry and a rise in zymogen granule (ZG) pH and prepares zymogens for secretion (Thrower et al., 2006)(Petersen and Sutton, 2006). If toxic signalling occurs then this process may become excessive and disordered, leading to premature zymogen activation (see figure 1.8).

There are still many aspects to premature zymogen activation that need further investigation, such as the role of acidic compartments in premature trypsinogen activation, when trypsinogen is more readily activated in

alkaline conditions (as found in the duodenum). Also intriguing is the possibility of endocytosis, where active trypsin is brought back into the PAC from the lumen of the acini and then co-localised with lysosomes leading to vacuole formation (Sherwood et al., 2007)(Vornina et al., 2007).

The exact mechanisms of premature trypsinogen activation are still unclear. Enzymes co-localisation in acidic vacuoles and toxic Ca^{2+} signalling have been demonstrated to play a role. However the idea of premature trypsinogen activation as the only pathway to PACs injury and pancreatitis is less certain in recent years. It maybe only one possible pathway (albeit a very commonly utilised and important pathway) of a more complex response of PACs to injury (see figure 1.9).

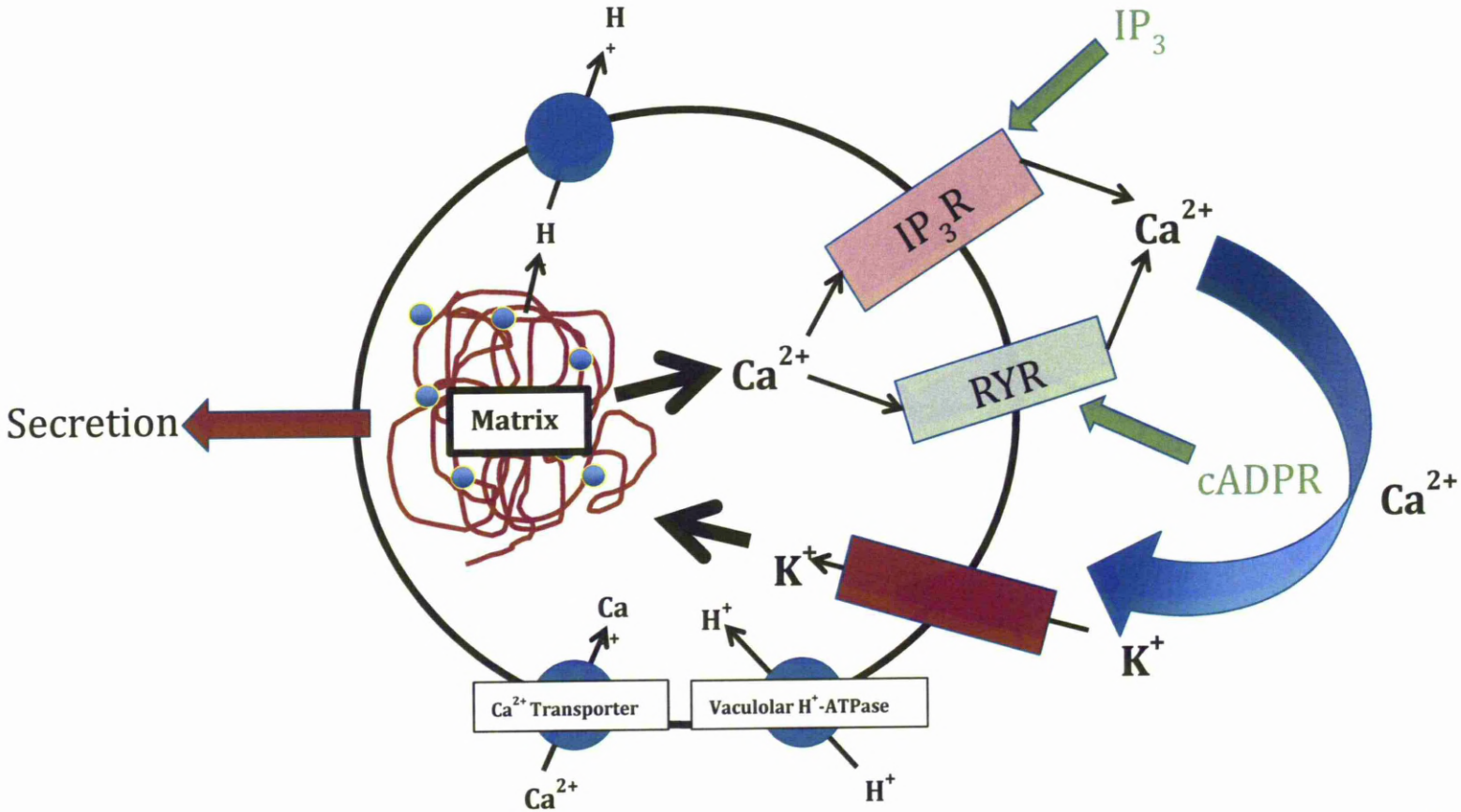


Figure 1.8: Diagram showing response within a zymogen granule to hyperstimulation with secretagogues. Ca^{2+} release via IP_3 or RyR receptors causes a rise in the extra-granular $[Ca^{2+}]_i$ which leads to activation of Ca^{2+}-dependent K^+ channels. K^+ diffuses into the ZG and displaces Ca^{2+} and H^+ bound with pro-zymogens in the matrix, leading to a drop in ZG pH, matrix disaggregation and secretion of (?activated) zymogens. (Adapted from Petersen and Sutton, 2006).

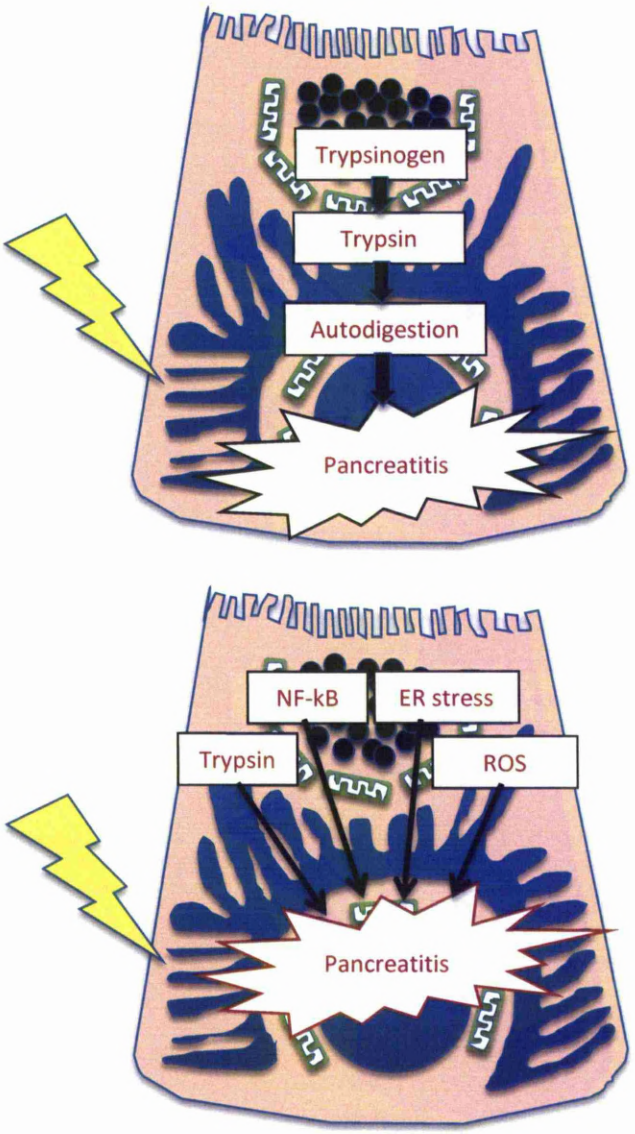


Figure 1.9: Challenge to the central role of trypsinogen activation. A) Diagram represents the view that trypsinogen alone leads to PAC damage and pancreatitis. B) Diagram represents some of the more recent uncertainties that do not require trypsinogen activation to cause PAC damage, and perhaps pancreatitis. These include the role of NFkB in release of cytokines, the role of reactive oxygen species (ROS) and ER stress.

1.4.6 CCK stimulation of pancreatic acinar cells

The peptide hormone cholecystokinin (CCK) exists in several forms and is an important regulator of the gastrointestinal system. To date the form most used in experimental models of pancreatitis is CCK-8, but evidence now suggests that CCK-58 may be the more physiologically relevant variant (Criddle et al., 2009). CCK-58 is the principal circulating type in humans (Eberlein et al., 1987), dogs and rats (Reeve et al., 2003). As discussed previously, physiological levels of CCK-8 stimulate zymogen secretion form PACs by inducing Ca^{2+} signalling. Hyperstimulation leads to toxic levels of $[Ca^{2+}]_i$ and the events leading to premature enzymes activation.

Interestingly until recently it was proposed that in humans CCK did not act directly on PACs, and acted via stimulation of parasympathetic vagal pathways, with release of ACh on PACs leading to enzymes secretion (Ji et al., 2001)(Owyang and Logsdon, 2004). This is important as it called into question the relevance of experimental work with CCK and its analogues (esp. Caerulein) in animal models if human disease was not related to CCK stimulation. This position was based on experimental work showing lack of Ca^{2+} signalling within isolated human PACs exposed to CCK (Ji et al, 2001).

However subsequent work showed that human PACs do exhibit CCK receptors on their surface membranes (Galindo et al., 2005). This was followed up by work from our group at Liverpool that showed isolated human cells do respond to both CCK-8 and CCK-58, at physiological and supra-maximal levels. We suggested that the quality of the human cells at the time of experimentation was vital (Murphy et al., 2008). These results confirmed the similarity between murine and human models in respect to the role of CCK as a regulator of PACs zymogen secretion, and the validation of experimental animal models in studying pancreatitis.

1.4.7 The role of cytokines in acute pancreatitis

Events that occur after acinar cell injury may determine the severity of acute pancreatitis. A severe inflammatory response, initially with accumulation of neutrophils within the pancreas itself, but subsequently involving other organs, is seen in severe acute pancreatitis. These systemic effects are brought about by release of a 'cytokine storm'. PACs cytokines include tumour necrosis factor alpha (TNF-a), interleukins 1 and 6 (IL-1, IL-6) and

platelet activating factor (PAF) (Pandol et al., 2007). Cytokines are immune modulating cell-signalling protein molecules, that are released from cells when they are damaged, and recruit inflammatory cells such as neutrophils and macrophages. The release of cytokines appears to be independent from trypsinogen activation, but maybe dependent on toxic Ca^{2+} signalling. Their release is regulated by signalling systems including: nuclear factor kappa B (NFkB), activator protein-1 (AP-1) and phosphatidylinositol-3 kinase (PI-3 kinase). They recruit leucocytes to the site of injury on order to deal with damaged cells, and initiate the reparative process. However during a cytokine storm this process is overstimulated and the inflammatory cells recruited release further cytokines leading to a positive feedback loop and more damage within the pancreas.

Attempts to inhibit the signalling systems initiating cytokine release have previously studied NF-kB (Rakonczay et al., 2008b). Inhibition of NF-kB has demonstrated mixed results: in most cases NF-kB activation was harmful (Pandol et al., 2007), but in one study it was protective (Steinle et al., 1999). NF-kB also has others roles in the inflammatory response, including upregulating adhesion molecule ICAM-1 (Zaninovic et al., 2000). Some pancreatic zymogens themselves may activate NF-kB, though the evidence for this is weak at present (Pandol et al., 2007).

Eventually the process may be counteracted by anti-inflammatory cytokines (e.g. IL-10, IL-1 receptor antagonist) and become self-limiting. But in severe disease the balance of pro- and anti-inflammatory cytokines favours pro-inflammatory cytokines and allows progression to a systemic illness, affecting organs distant to the pancreas, and leading to the systemic inflammatory response syndrome (SIRS) (Simovic et al., 1999). This most frequently affected organ outside of the pancreas is the lung, and the resulting adult respiratory distress syndrome (ARDS) is a well-recognised cause of death in severe pancreatitis in humans.

The mechanisms involved in cytokine release are complex and there are multiple interactions between various pathways compounding a fuller understanding. It would seem to be that an ability to stop pro-inflammatory cytokine release and decrease the severity of the disease would be beneficial in acute pancreatitis.

1.4.8 Reactive oxygen species and cell stress

Reactive oxygen species (ROS) are highly reactive molecules containing oxygen ions and peroxides that form as a by-product of aerobic respiration

and have roles in cell signalling. However when cells, including PACs, are damaged ROS levels can rise rapidly leading to the condition *oxidative stress* (or cell stress), where the PACs are unable to nullify ROS levels. This is turn leads to excess free radicals and peroxides directly damaging the proteins, lipids and DNA that make up the functioning cell machinery. Experiments show this happens early in the course of experimental pancreatitis (Weber et al., 1998). Experimentally anti-oxidants have been shown to decrease the severity of pancreatitis in animal models, however this has not been seen in corresponding human trials (Siriwardena et al., 2007). Indeed these human trials have even shown an *adverse* outcome with use of anti-oxidants. Currently the mechanisms of ROS-mediated damage are still unclear.

Finally there maybe a relation between ER Ca^{2+} levels and cell stress, resulting in mal-production of proteins in the ER. This is turn has been suggested to lead to excessive Ca^{2+} release from the ER into the cytosol and the familiar effects of sustained, toxic $[Ca^{2+}]_i$ levels, as well as increasing NF-kB levels (Pahl, 1999)(Panchen, 2001).

1.5 Caffeine

Caffeine is a xanthine alkaloid that acts a stimulant in mammals and is commonly consumed by humans in infusions of coffee, tea, some soft drinks and is present is some foods, such as chocolate. It is absorbed rapidly from the gastro-intestinal tract and passes freely across all biological membranes (Lachance et al., 1983). Peak plasma caffeine concentration is reached between 15 and 120 minutes after oral ingestion in humans (Arnuad and Welsch, 1982) and a single cup of coffee is though to provide a dose of 0.4-2.5 mg/kg (which equates to 1-10 μM). For doses lower than 10mg/kg the half-life in rodents and murine models has been found to be approximately 1 hour (Bonati et al., 1984) and approximately 3 hours in adult humans (Arnaud, 1987). A cup of coffee is thought to contain approximately 80-135 mg caffeine, with tea containing 22-74 mg (Chin et al., 2008).

It has been shown that caffeine has effects on $[Ca^{2+}]_i$ signalling, and thus on potentially on acute pancreatitis (Petersen and Sutton, 2006)(Gerasimenko et al., 2009). As previously detailed a range of stimuli, associated with triggering the events of acute pancreatitis, lead to elevated, sustained $[Ca^{2+}]_i$ levels. This pathological Ca^{2+} signalling requires activation of IP_3R channels (sited on endoplasmic reticulum), which release Ca^{2+} stores from the ER and potentiate the toxic elevation of $[Ca^{2+}]_i$ (Gerasimenko 2009).

Caffeine acts an IP_3R inhibitor (Petersen and Sutton, 2006) (Criddle et al., 2007b) and may also inhibit the production of the second messenger IP_3 (Toescu et al., 1992). Caffeine may have others routes of action on PACs including: i) inhibition of cyclic adenosine monophosphate phosphodiesterase, interfering with cAMP recycling (Wu et al., 2009); ii) inhibition of adenosine A1 receptor, which has been shown to be protective in experimental pancreatitis (Fredholm et al., 1999)(Satoh et al., 2000) and may be involved in cystic fibrosis transmembrane conductance regulator type pancreatitis (DiMagno et al., 2010)(Steiner et al., 2011); Caffeine has been shown by our group to inhibit Ca^{2+} entry through store-operated channels (SOC) in the PACs membrane (Cane et al., unpublished data). This body of work suggests caffeine has theoretical potential as a therapeutic in human pancreatitis, if administration *in vivo* produces the same inhibition seen *in vitro*.

Caffeine is metabolised in the liver by the cytochrome p450 oxidase enzyme system. The main metabolites are paraxanthine (1,7-dimethylxantine), theobromine (3,7-dimethylxanthine) and theophylline (dimethylxanthine) (see figure 1.10). Paraxanthine is the most commonly produced metabolite. Is is possible that the protective effects in pancreatitis may be related to

metabolite activity. The results chapter details experiments with caffeine in caerulein murine model of *in vivo* acute pancreatitis.

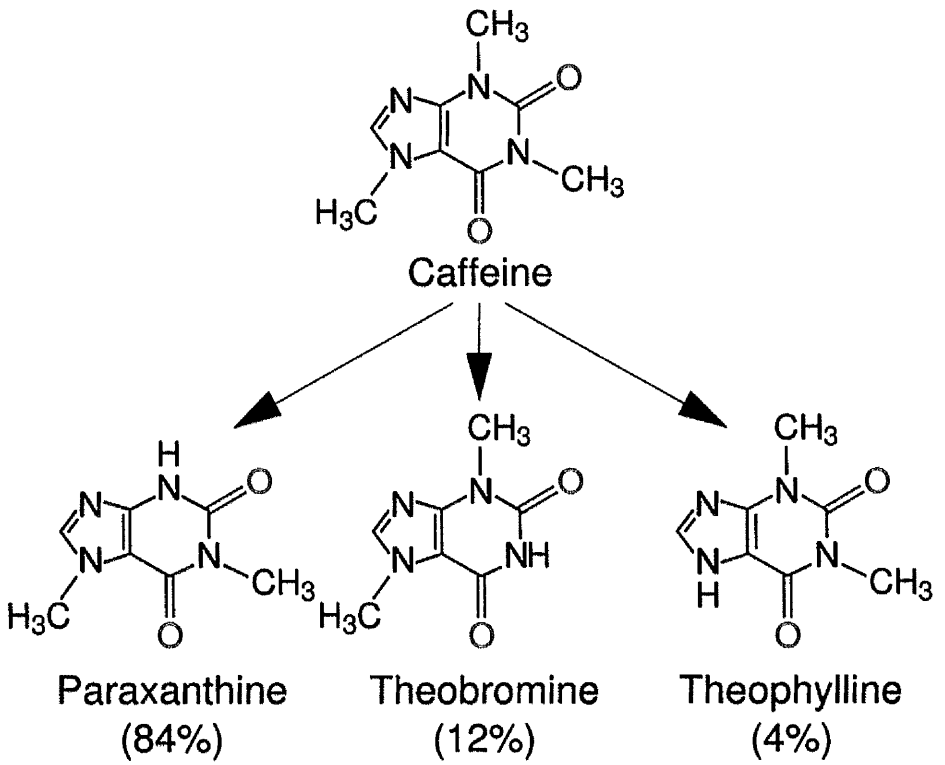


Figure 1.10: Common metabolites of caffeine. Paraxanthine is most commonly formed, at 84%.

1.6 Summary

Acute pancreatitis is a complex disease that so far has resisted attempts at amelioration with therapeutics. Although understanding is increasing there are still many areas that remain unclear. Much of our current understanding has been drawn from the many varied animal models, mostly from murine and rodent models. Although there is much commonality between mammalian models, it is also clear that many inter-species differences do exist, meaning pathways and mechanisms can vary between species. This is the likely explain why many promising treatments, that ameliorate disease in experimental animal models, do not to translate to the treatment of human disease. Nevertheless continued use of animal models is vital to rapidly push forward understanding and highlight promising targets of therapy in human disease.

However work must been done in parallel with human tissue, as the ability to replicate findings from animal tissue would help identify plausible therapeutic targets. Work with human tissue will clarify which mechanisms are shared between mammalian species, and thus which mechanisms need to be focused on, and which are not found in human tissue and are not relevant. Whilst obtaining human tissue is technically and ethically

problematic, it is vital to further understanding of acute pancreatitis. This is ideally undertaken in the setting off a multidisciplinary collaboration involving basic scientists and clinicians with an appropriate spread of technical expertise.

The role of apoptosis and necrosis, and the interconnected importance of mitochondrial metabolism, has been recognised as important in determining the severity of acute pancreatitis. However previous opinion that apoptosis is protective, and necrosis detrimental, in acute pancreatitis maybe simplistic. Apoptosis *per se* may not be protective in all circumstances and, while many of the components of cell death pathways have been elucidated, a full understanding of all the possible interactions of these components is far from clear. Some of these mechanisms have been elucidated *in vitro*, but there are still many aspects of these pathways that are unclear. In addition it is unknown if these pathways work the same way *in* vivo as *in vitro*, and so experimental work is required *in vivo* to further explore their importance in living animals.

Caffeine has promising inhibitory effects on mechanisms known to cause pancreatitis *in vitro* (namely toxic Ca^{2+} signalling). To date its role in experimental *in vivo* pancreatitis has not been tested. As caffeine is

currently used safely as a therapy in human disease (for the unrelated neonatal apnoea syndrome) it has potential to be used as a therapy to ameliorate acute pancreatitis.

1.7 Aims and objectives

1) To test whether cholecystokinin (CCK) stimulates signalling responses directly on isolated murine and human pancreatic acinar cells and whether this stimulation ultimately leads to secretion. In addition to test whether CCK-58 stimulates similar responses to the more established CCK-8.

The null hypothesis is that CCK does not act directly on pancreatic acinar cells and another pathway is involved.

2) To test the effects of Bcl-2 protein family gene knock-out on caerulein-induced murine experimental pancreatitis.

The hypothesis to be tested is that Bak and Bax are considered pro-apoptotic, and apoptosis is considered protective in acute pancreatitis. So Bak or Bax knock-out should result in decreased apoptosis and worsening

of severity of induced pancreatitis. The null hypothesis is that Bak or Bax knockout has no effect or protects against acute pancreatitis.

The hypothesis to be tested is that Bcl-2 is considered anti-apoptotic so that knock-out should result in an increase in apoptosis and improvement in severity of induced pancreatitis. The null hypothesis is that Bcl-2 knock-out has no effect or shows increased severity in acute pancreatitis.

3) To test the effects of administering caffeine in caerulein-induced murine experimental pancreatitis. Caffeine is known to inhibit Ca^{2+} signalling and stops changes in isolated pancreatic acinar cells associated with pancreatitis. The hypothesis to be tested is that caffeine is protective against caerulein-induced murine experimental pancreatitis.

The null hypothesis is that caffeine has no effect or increases the severity of caerulein-induced murine experimental pancreatitis.

CHAPTER 2:

Materials and Methods

CHAPTER 2: MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

The water bath used for incubation where indicated was a Grant OLS-200, Grant instruments, Cambridge. A Fisher PF-203 was used for determining mass of reagents in the making of solutions and in the weighing of sections of pancreas. A Heraeus Biofuge Stratos (Kendro Laboratory Products, Sollentum, Germany) was used for centrifugation where necessary during all experiments. A Fluorescence Spectrometer LS50B (PerkinElmer, Massachusetts, USA) in conjunction with a Circulator C-85A, Techne instruments (Bibby Scientific, Stone, Staffs) was employed for the analysis of trypsin activity. Bradford assays were performed utilising a spectrophotometer. Light microscope was used for analysis of murine pancreatic tissue.

2.1.2 Confocal microscopy

Confocal microscopy allows measurement of narrow focal sections of an object, rather than collecting all available light as in standard fluorescence microscopy. Standard fluorescence microscopy is not sensitive enough to detect the small, localised changes occurring within a cell, such as localised Ca^{2+} signals. Measuring fluorescence is performed by excitation of the target molecule (fluorophore) with laser light of a specified wavelength, exciting the fluorophore to a higher state. This is followed by collection of the emitted photons from the fluorophore when it returns to a lower energy state. The emitted light is always of a lower energy level (Stoke's shift) (and therefore longer wavelength of light). This longer wavelength allows emitted light to be separated from the excitation light, by use of an emission pinhole. Only light from the narrow focal plane passes through to the detector. In the context of isolated cell work this means very specific localised regions of the cell can be studied, such as zymogen granules.

The two confocal microscopes used in our group were a Zeiss LSM 510 system and a Leica TCS SP2 (AOBS). Both systems are laser scanning confocal microscopes. Fluorescent signals were recorded using a 63x water immersion objective lens with a numerical aperture of 1.2 (see figure 2.1).

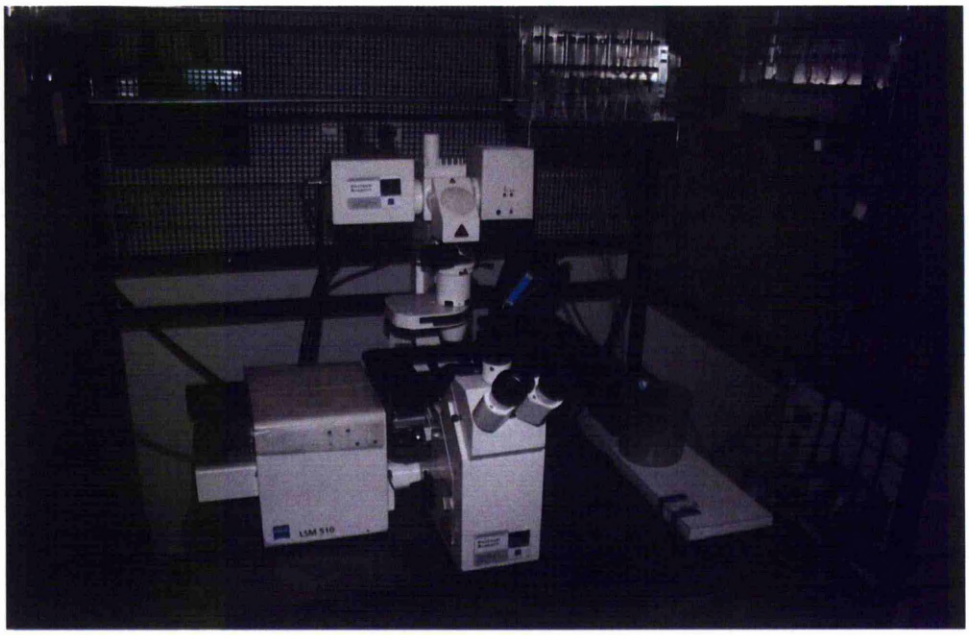


Figure 2.1 : Photograph demonstrates the Zeiss LSM 510 confocal microscope, housed in a Faraday cage and placed on an air table to reduce vibrations. The perfusion system is seen attached to the cage in the upper left hand of the image.

2.1.3 Consumables

Coverslips from Merck Ltd, UK

Cuvettes for fluorescence spectroscopy from Sigma-Aldrich

Eppendorf Vials from Eppendorf, Hamburg, Germany

Forceps from Sigma-Aldrich Company Ltd, Poole, UK

Magnetic stirrers (small) from Sigma-Aldrich Co.

Needles (25G) from Becton Dickson UK Ltd, Oxford, UK

Pippettes from Anachem

Pipette tips from Anachem

Scissors from Sigma-Aldrich Co.

Syringes from Beckton Dickinson UK Ltd, Oxford, UK

Teflon/glass homogeniser

Formalin pots

2.1.4 Chemicals and Reagents

Albumin (bovine serum) from Sigma-Aldrich Co.

Anti-mouse Caspase 3 anitbody (rabbit) from R&D systems, Germany

Bradford protein assay reagent from Biorad, Hemel Hampstead UK

Boc-Gln-Ala-Arg-MCA Peptide substrate from Peptide International Inc, Kentucky, USA

Caerulein from Sigma-Aldrich Co.

Calcium Chloride ($CaCl_2$) from Sigma-Aldrich Co.

Caffeine from Sigma-Aldrich Co.

Citric Acid Buffer from Sigma-Aldrich Co.

3,3-Diaminobenzidine (DAB) from Sigma-Aldrich Co.

Dimethyl Sulfoxide (DMSO)

Ethanol from Sigma-Aldrich Co.

Formalin 10% solution from Sigma-Aldrich Co.

Goat serum from Dako, Glostrup, Denmark

Haematoxylin solution from Sigma-Aldrich Co.

Hydrochloric acid (HCl) from Sigma-Aldrich Co.

Methanol from Sigma-Aldrich Co.

MOPS (3-(N-morpholino)propanesulfonic acid) from Sigma-Aldrich Co.

Mouse serum from Dako, Glostrup, Denmark

PBS (Phosphate Buffered Solution) constituents from Sigma-Aldrich Co.

Sodium chloride (NaCl) from Sigma-Aldrich Co.

Sodium hydroxide from (NaOH) from Sigma-Aldrich Co.

Tris(hydroxymethyl)aminomethane from Sigma-Aldrich Co.

Tris Buffered Saline (TBS) components from Sigma-Aldrich Co.

Tween-20 from Sigma-Aldrich Co.

Trypsin (Bovine) from Sigma-Aldrich Co.

Vector Stain (Elite ABC kit) from Vector Laboratories Ltd, Peterborough, UK

Xylene from Sigma-Aldrich Co.

2.1.5 Animals

All experimental mice were bred and housed in clean conditions in the Biomedical Services Unit at constant temperature (23 ± 2^0C) with 12-hour light/dark cycle and free access to standard laboratory pellet feed and water. All experiments were conducted in accordance with a valid project licence from the Home office under the terms of the Animals (Scientific Procedures) Act 1986.

C57BL/6 male mice were purchased through the Biomedical Services Unit at the University of Liverpool, from a licenced supplier (Charles River UK Ltd, Margate).

Knockout mice (Bcl-2, Bak and Bax) were used from the inbred stock at the Biomedical Services Unit at the University of Liverpool, in collaboration with the Division of Gastroenterology.

Bak wild-type and homozygously null mice were obtained from the Division of Gastroenterology, from a stock bred in the Biomedical Services Unit, University of Liverpool. These were originally derived from

embryonic stem cells microinjected into C57BL/6 blastocysts (Pritchard et al, 1999) and were considered close enough to C57BL/6 stock for the latter to be used as controls.

Bcl-2 wild-type and homozygously null mice were obtained from the Division of Gastroenterology, from a stock bred in the Biomedical Services Unit, University of Liverpool. These were originally derived from 129Sv E14 and D3 embryonic stem cells microinjected into C57BL/6 blastocysts (Pritchard et al, 1999).

The Bax wild-type and homozygously null mice were obtained from the Division of Gastroenterology, from a stock bred in the Biomedical Services Unit, University of Liverpool. These were originally derived from 129Sv RW-4 embryonic stem cells implanted into C57BL/6 blastocysts and maintained on this background (Pritchard et al, 1999).

2.2 Methods for isolated murine and human PACs experiments

2.2.1 Isolation of murine pancreatic acinar cells

Freshly isolated murine PACs were obtained from the pancreases of adult male CD1 mice (age 21-30 days and weight 30-35g). The mice were sacrificed by a schedule 1 action, with a stunning blow to the head and cervical dislocation, in accordance with the Animal (Scientific Procedures) Act 1986. The flank was washed with 100% ethanol to wet the fur and prevent contamination. The pancreas was removed through a laparotomy, dissected from surrounding structures and placed into extracellular solution. The extracellular solution contained (in mM): 140 NaCl, 4.7 KCl, 1.13 $MgCl_2$, 1 $CaCl_2$, 10 D-glucose and 10 HEPES (adjusted to a pH of 7.35 using NaOH). All chemicals were of the highest grade, obtained from Sigma, Gillingham, UK. Collagenase (Worthington, Lorne laboratories, Reading) was then injected into the pancreas tissue at a dose of 200U/ml, and the pancreas was incubated in a water bath at 37°C for 15 minutes. At the end of the incubation period the pancreas was transferred into a small falcon tube, containing 5 ml fresh extracellular solution, and agitated with a pipette to break the pancreas down into smaller constituent parts. The sample was purified for PACs by: centrifugation of the suspension at 1000 revs for 1 minute. The supernatant was discarded, the pellet resuspended in extracellular fluid and filtered, before being further centrifuged and the

process repeating 2-3 cycles. The final pellet containing PACs as single cell, doublet and smaller clusters was then re-suspended in 2ml of extra-cellular solution. All experiments were performed at room temperature and within 3 hours of isolation.

2.2.2 Isolation of human pancreatic acinar cells

Human pancreatic tissue was obtained from patients undergoing pancreatic resections at the Royal Liverpool University Hospital, Liverpool, UK, between Jan 2004 and Feb 2008. The study had approval from the Liverpool Adult Local Research Ethics Committee (Ref:03/12/242/A). All patients were provided with verbal and written information regarding the study on the night of admission (usually the day prior to surgery) and then informed consent was taken on the day of surgery. The sample specimen was taken from the resected pancreas specimen, so that no unnecessary extra pancreatic tissue was taken from the patient. Ideally this was taken away from the area of pathology, to ensure tissue was as normal as possible (other means to optimise collection of healthy tissue are described below, page 101). It was found that sub-optimal pancreas tissue came from patients with disease obstructing the pancreatic duct and/or jaundice (bile-duct

obstruction), suggesting these pathologies elicit damage throughout the pancreas.

Freshly isolated human PACs were prepared in a similar manner as to murine isolated PACs as detailed above. However several modifications were required to ensure good quality cells. Human pancreas samples were obtained fresh from within the operating theatre at the time of surgery. Once the pancreas resection was performed a small section was cut, without delay, by the surgeon from the resected specimen. This was performed with a scalpel blade, and not with a diathermy instrument, to reduce damage from heat injury. The specimen was immediately washed of blood (to reduce the chance of residual inflammatory cells), placed in extracellular fluid and transported on ice. In order to maintain the specimen in an optimal condition the extracellular solution contained enzyme inhibitors (protease inhibitors (ROCHE, USA) and the trypsin inhibitor Benzamidine (Worthington Biochemical)) and also pyruvate to optimise mitochondrial function. The time to transfer to the lab was under 10 minutes.

Once in the lab the human pancreas was injected at several points with 200U/ml collagenase, with 1mM benzamidine, and then cut into small

sections and incubated at 37^0C form 15 minutes. The collagenase was then drained and washed off and the sample resuspended in extracellular solution and purified by serial centrifugation, discarding the supernatant, resuspending the pellet, filtering and then repeating the process usually 3 times (as per the method used in the preparation of murine isolated cells). All experiments were performed within 3 hours of preparation of the cells, and cells kept at 4^0C until used.

2.2.3 Loading of fluorescent dyes and measurement of secretion

Once the isolated PACs were prepared, as described in the previous two sections, they were loaded with fluorescent dyes (dyes dissolved in dimethyl sulphoxide (DMSO)). This was achieved by adding an appropriate dose of dye to the 2ml of suspended isolated PACs, covering with light excluding foil and incubating at room temperature with gentle agitation. At the end of the loading period the dye was washed out with more extracellular fluid and the cells centrifuged and resuspended.

Quinacrine dye was loaded into the PACs at a concentration of 10μM (2μl of 10μm dye to a 2ml suspension of isolated PACs), and incubated at 37^0C

for 10 minutes, and washed out as outlined. Quinacrine was preferentially concentrated in zymogen granules (see figure 2.2) and was expelled from the zymogen granules during secretion. Its fluorescence was measured using a confocal microscope (figure 2.2) and the loss of fluorescence when the PACs were stimulated indicated secretion (Myoung et al., 2004). Fluorescence measurements were expressed either as absolute values (where appropriate) or as normalized changes from basal fluorescence (called the F/F_0 ratio), where F_0 represents the fluorescence recorded at the specific time points. Other dye used was Fluo-4, for detecting Ca^{2+} signalling and loading is further detailed in Chapter 3.

During experiments the PACs suspension were placed on a thin glass coverslip, which was then fixed to a Perspex perfusion chamber. A short time period is allowed to that the PACs could settle and fix to the coverslip. Extracellular solution was then fed into the chamber via a gravity-fed perfusion system, consisting of plastic syringes (with two-way taps to control flow) connected to the perfusion chamber by thin silicon tubing. Low power suction tubing is applied to the other end of the chamber to provide a continuous one-way direction of flow. Stimulating agents (i.e. CCK-8, CCK-58) and/or receptor inhibitors were added during the experiments and their actions on the PACs secretion observed.

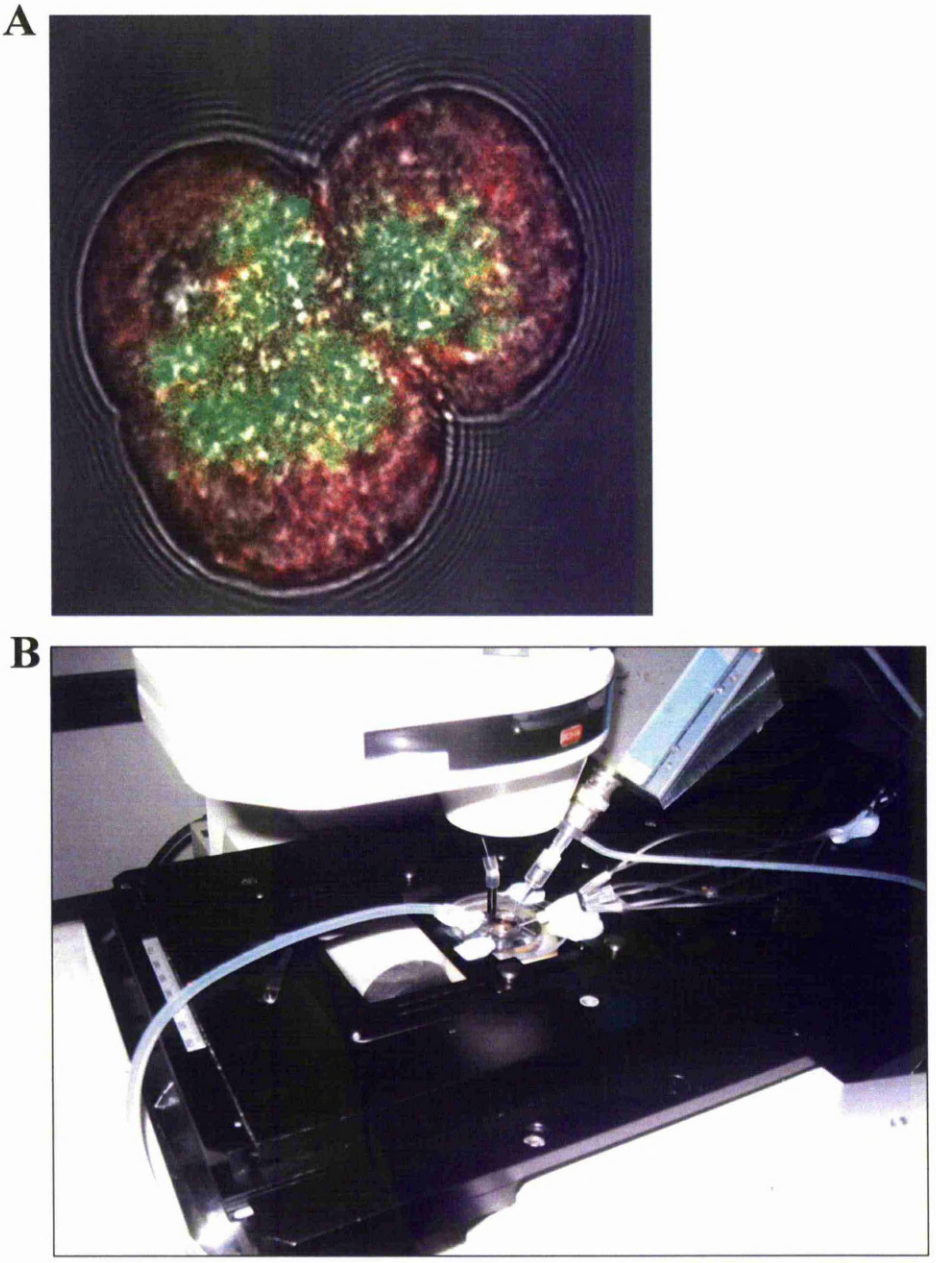


Figure 2.2: A) Quinacrine fluorescence. Typical view of murine pancreatic acinar cells loaded with quinacrine and fluorescence captured using a confocal microscope. Quinacrine is concentrated in the zymogen granules, which are concentrated in the apices of the PACs. B) Perfusion chamber set up on microscope stage with multiple perfusion tubing running in from the right and low-power suction tubing leading away to left.

2.3 Methods for experimental *in vivo* murine pancreatitis models

2.3.1 Induction of experimental pancreatitis *in vivo*

Mice were fasted over night before induction of pancreatitis (between 12-16 hours), but received free access to water. Experimental pancreatitis was induced through the well described method of hourly intra-peritoneal caerulein (Sigma) administration. The required concentration of caerulein is 50μg/kg/hr (Niederau et al., 1985). Caerulein was dissolved in 0.9% saline to a concentration of 10μg/ml. Thus a 30g mouse would require 1.5μg per injection, or 0.15 ml. Injections were administered with a 1ml syringe to facilitate the accurate administration of such small volumes. Opioid analgesia was given with the 1st injection (Buprenorphine 0.1mg/kg).

To administer intra-peritoneal injections mice were held by the neck scruff, between forefinger and thumb, and the left hind leg held straight with the ring and little finger. An alcohol swab was applied to the abdomen before each injection, and the injection site placed in either the left or right lower lateral parts of the abdomen, to avoid injuries to the liver or stomach. Injections were given using a small 25G needle.

With the exception of the control group, all mice received hourly intra-peritoneal injections. Those mice in the four hour experimental group received only four injections. Those in the eight, twelve and twenty-four hour experimental groups all received seven hourly injections, with the timing for the experiment starting with the first injection at zero hour (see Table 2.1 below).

	0hr	1hr	2hr	3hr	4hr	5hr	6hr	7hr	8hr	9hr	10hr	11hr	12hr	24hr
4hr	+	+	+	+	end									
8hr	+	+	+	+	+	+	+	-	end					
12hr	+	+	+	+	+	+	+	-	-	-	-	-	end	
24hr	+	+	+	+	+	+	+	-	-	-	-	-	-	end

Table 2.1: Caerulein injection schedule for different experimental time groups. (+) denotes one injection of caerulein 50µg/kg given. (-) denotes no injection given. (end) denotes end of experiment with harvest of pancreas.

2.3.2 Removal of pancreas

Mice were euthanised by CO_2 inhalation, in accordance with the Animal (Scientific Procedures) Act (1986). The pancreas was removed from each mouse by an incision in the left flank. It was identified by its location between the duodenum and the spleen. It was carefully cut from surrounding tissues and immediately weighed. This weight was used to obtain pancreas weight as a percentage of whole body weight: a proxy for oedema. The pancreas was divided into three approximately equal portions: head, body and tail. The weight of each portion was recorded. The head section was used for histology, the body of the pancreas used for trypsin activity analysis and the tail for calculation of wet weight.

2.3.3 Measurement of oedema

After weighting the tail section of the pancreas was placed onto a dry glass slide and placed in an oven at 80^0C for 72hrs (until desiccated). The dry weight of the desiccated pancreas section was measured and the water loss calculated as the difference between the original weight and the dry weight. The pancreatic tissue wet to dry weight ratio was expressed as:

(wet weight-dry weight)/wet weight x100%

2.3.4 Analysis of serum amylase

Mice were euthanised by CO_2 inhalation, in accordance with the Animal (Scientific Procedures) Act (1986). This method was chosen because of the requirement of a sufficient quantity of blood for serum amylase analysis. In pilot studies stunning and cervical neck dislocation had resulted in internal bleeding with subsequent drop in circulating blood volume, making sufficient blood collection difficult. Use of CO_2 allowed the vascular system to remain intact. Cardiocentesis could be performed using a 1ml syringe with a 23G needle under the sternum. This resulted in a 0.5-1.0 ml sample, depending on the size of the individual mouse. The samples were placed in an eppendorf vial on ice and subsequently centrifuged at 1500g for 20 minutes. Serum was then pipetted off into a new, cooled eppendorf vial. Serum samples were stored in the freezer (-20^0C) until analysed. Analysis of serum amylase was carried out by the Department of Clinical Chemistry in The Royal Liverpool University Hospital, using a kinetic method with Roche automated clinical chemistry analyzers (GMI, Leeds, UK). The results were expressed as IU/L.

2.3.5 Preparation of histological slides

The body section of the pancreas was placed into vials containing 10% formalin (Sigma-Aldrich Co.) for fixation. The fixed tissue was embedded in paraffin, then sectioned at 5μm. Two slides were prepared per block. One was stained with haematoxylin and eosin (H&E) for histological grading and the other left unprepared for immunohistochemistry staining with anti-caspase 3 antibody. Slide preparation and H&E staining was carried out by the Department of Pathology in the University of Liverpool.

2.3.6 Histological scoring of severity of pancreatitis

A histological score was developed to quantify morphological changes induced by caerulein in murine pancreas. Most groups with a consistently recognised high standard of work in experimental pancreatitis use similar morphological changes in pancreatic tissue (Niederau et al., 1985; Spormann et al., 1989; Dembinski et al., 2003; Nathan et al., 2005). The key standard components of these scores are inclusion of: (1) rating the degree of histological changes, notably oedema, neutrophil infiltration and acinar necrosis; (2) blinded assessment of severity; and (3) some form of quality

assurance (e.g. two blinded assessors, with measures to ensure conformity in rating; checking similar changes in other parameters e.g. serum amylase). Some groups have assessed the degree of vacuolisation, but this change is more dependent on the timing of sampling so this was not used. The scoring system adopted used the three principal separate morphological changes (adapted from previous work e.g. Nathan et al 2005): oedema, inflammatory infiltration and necrosis. Each parameter was scored from 0-3, as set out in the Table 2.2 below.

Parameter	Morphological Change	Score
Oedema	Nil	0
	Interlobular	1
	Interlobular and moderate intralobular	2
	Severe interlobular and intralobular (acinar disruption	3
Inflammatory infiltration	Absent	0
	Perivascular infiltration only	1
	Perivascular and scanty diffuse infiltration	2
	Diffuse infiltration	3
Necrosis	Absent	0
	<5%	1
	5-25%	2
	>5%	3
Total		(0-12)

Table 2.2: Criteria used to score histopathological severity of caerulein-induced experimental murine AP (using H&E stained slides).

This provided a combined histological severity score ranging from 0-12. Two blinded assessors, who had previously received training in grading histological severity in pancreas tissue, scored slides. Using a light microscope, with two separate viewing ports, assessors simultaneously viewed slides, at a magnification of x100. Assessors were blinded to the nature of each slide, did not communicate during scoring and viewed approximately 30 slides per session, to ensure a mix of experiments. 10 separate fields were scored per slide and then meaned for an overall slide score. The meaned combined score of both assessors was the final score for the slide. This is 'interval data' and an assumption is made it is parametric (accepting that the groups have small sample sizes).

2.3.7 Immunohistochemistry for caspase-3 staining

To identify apoptotic cells, and discriminate them from necrotic cells, detection by immunohistochemical labelling of active caspes-3 was employed. Slides containing 5 μm sections of pancreas tissue, embedded in paraffin, were prepared by the Department of Pathology in the University of Liverpool. Staining for caspase-3 was carried out by the author for the first batches of slides and whilst the protocol was being developed. Subsequently slides were stained by the Department of Pathology, University of Liverpool

using the same primary antibody, supplied by the author. The protocol for staining of murine pancreatic tissue for caspase-3 is as follows.

The slides were dewaxed in Xylene for 10 minutes then immersed in 100% ethanol for 3 minutes. Next they were immersed in 3% H_2O_2 in methanol for 45 seconds. A rehydration series in alcohol followed (90%, 70%, 40% for 2 minutes each). The slides were then washed in water followed by Phosphate Buffer Solution (8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, 1 litre H_2O [pH 7.4]) (PBS). Epitope retrieval was performed by immersing in citric acid buffer (2.94 g/L [pH 6.0] covering and heating at maximum power in 800w microwave for 20 minutes (stopping half way to ensure buffer solution covering slide and adding more buffer if necessary). Slides were cooled in running water and, after two further 5 minute washes in PBS, the tissue was blocked with 10% goat serum in TBS-Tween (1 litre dH_2O, 6.05 g Tris, 8.5 g NaCl, 2ml tween-20 [7.4]) for 45 minutes at room temperature. The excess liquid was washed off and the slides were incubated with the primary antibody (active caspase-3 antibody in 1:1000 solution with 10% goat serum/TBS-Tween20) overnight at 4°C.

After overnight incubation the slides were first washed in TBS-Tween20 then incubated with the secondary antibody (biotin labelled anti-rabbit secondary antibody in 1:200 solution with 5% mouse serum/TBS-tween) for 40mins at room temperature. After two further TBS-Tween20 washes the slides were incubated with Vectastain ABC elite for 30 minutes at room temperature. After washing with TBS-Tween20 and then PBS the slides were incubated with DAB 0.07% in PBS for 3 minutes at room temperature. After a further PBS wash the slides are immersed in 80% methanol for 10 minutes then transferred to haemotoxylin and counter-stained with agitation for 4 minutes. After rinsing with water slides were dehydrated by immersion first in 90% ethanol and then 100% ethanol. Lastly slides were transferred to xylene for 5 minutes before being mounted with slide covers and dried.

Ten random fields were imaged at magnification 200x from each slide. The images were processed using ImageJ software that splits positive caspases-3 stained nuclei/cytoplasm into one channel and all other stained nuclei into another channel. The proportion of apoptotic cells was defined by the mean ratio of positively stained cells out of the total nuclei.

2.3.8 Use of caffeine in experimental models

Caffeine powder (Sigma-Aldrich) was dissolved in extracellular solution and made into 2 mg/ml stock. During murine experimental models the appropriate dose was made by further dilution in extracellular solution depending on the weight of the individual animal. Concentrations used were 1 mg/kg and 10 mg/kg. Higher doses (50 mg/kg and 100 mg/kg) were trialled, but side effects were too severe and experiments ceased. Caffeine was administered IP hourly, with caerulein, and the animal sacrificed at 8 hours. Further details regarding caffeine administration are described in chapter 5.

2.3.9 Statistical analysis

Results in figures are expressed as mean ± standard error of mean (SEM). Normally distributed parameters (e.g. pancreatic oedema, amylase and trypsin measurements) were analysed using student's *t*-test. Statistical significance was set at <0.05. Microsoft Excel, Origin 2.6 and SPSS 2.0 were used for statistical processing and graphics.

RESULTS

Chapter 3

Direct activation of Ca^{2+} signalling and zymogen secretion from isolated murine and human pancreatic acinar cells in response to stimulation by cholecystokinin-8 and cholecystokinin-58

3.1 Introduction

Cholecystokinin-8 (CCK-8) and its analogues (caerulein) are still one of the most widely employed experimental models. Extensive use of CCK-8/caerulein in murine and rodent models have shown that physiological levels (range 1-20 pM) of neuro-hormonal stimulants (e.g. Ach, CCK-8) elicit Ca^{2+} signalling that in turn controls zymogen secretion (Petersen and Tepikin, 2008).

However there has been some controversy as to the relevance of CCK stimulation in human disease. There is doubt regarding a direct clinical equivalent of CCK-induced human pancreatitis and there has been controversy as to whether CCK acts directly on PACs or acts distally via stimulation of vagal pathways, which in turn stimulate PACs (Ji et al., 2001)(Owyang et al., 2004). Both scenarios provide some difficulty in continued employment of CCK/caerulein experimental models. To date there has been little work with isolated human PACs to determine if human PACs have the same mechanisms as murine PACs, mainly due to the obvious difficulty in safely sampling live human pancreas tissue (Miller, 1996). In addition there is some debate regarding which subtype of CCK is most relevant. CCK-8 is most widely employed in murine models, though there has been some evidence that CCK-58 may be more active

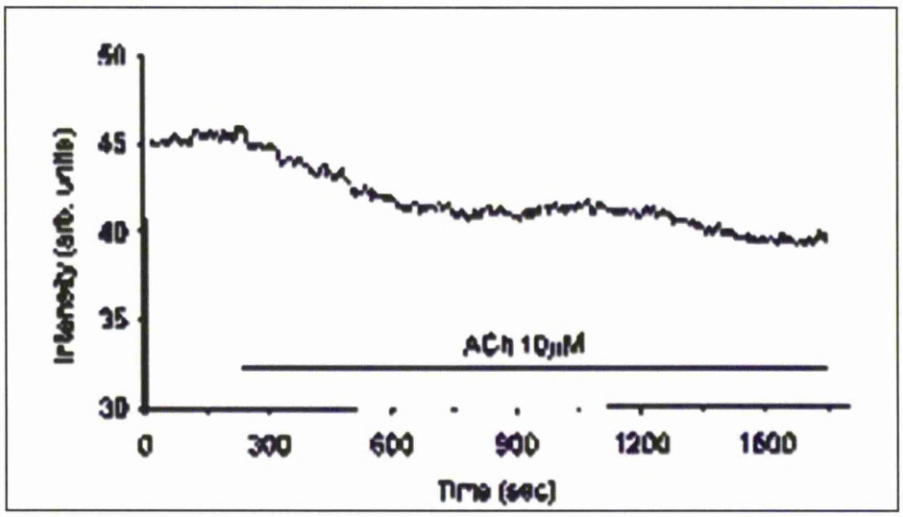
physiologically and has previously been shown to circulate in humans (Eberlein et al., 1987) and is the only form found in rats (Reeve et al., 2003). In murine models at physiological levels (1-20 pM) CCK-8 induces focal apical Ca^{2+} signalling coupled to mitochondrial energy production and secretion. However at supra-maximal levels of stimulation (10nM) it leads to pancreatitis and is, thus, widely employed as a pancreatitic model. It is necessary to compare CCK-8 and CCK-58 effects on isolated murine and human PACs to further ensure relevance of the sub-types. This chapter details experiments that investigated CCK-58 effects on murine isolated PACs and compared to previously established CCK-8 effects. Ca^{2+} signalling, mitochondrial metabolism and quinacrine excretion were all recorded at both physiological and supra-maximal doses of CCK-8 and CCK-58

In order to investigate human PACs response to CCK-8 and CCK-58 it was necessary to work in collaboration. The experimental work was undertaken by our group at the University of Liverpool and we collaborated with the University of California and University of Texas Health science centre, USA, which supplied CCK-58. Within our group, work was undertaken on different mechanisms of PACs stimulation in parallel. Part of our group worked on demonstrating that Ca^{2+} signalling was indeed induced by CCK

stimulation. It is necessary to provide evidence that this signalling is in turn linked to release of zymogens from PACs, making it necessary to demonstrate secretion in response to CCK stimulation. This chapter details the experiments undertaken to show this secretion, using quinacrine loading of zymogen granules in isolated human PACs and recording changes in the levels of fluorescence when PACs were stimulated by CCK. Similar techniques have been reported previously in isolated murine PACs, which when stimulated with ACh showed a drop in apical quinacrine secretion of approximately 20 per cent (Belan et al., 1996)(Myoung et al., 2004) (See figure 3.1). Secretion is an active process and (to strengthen the case for CCK stimulation leading to secretion) recording autofluorescence of NADH in perigranular mitochondrial belts also assessed mitochondrial activation. All experiments took place in the presence of atropine and tetrodotoxin (which blocks voltage-gated Na^+ channels) to eliminate possible effects from neuro-transmitter pathways other than CCK (e.g. vagal pathways).

These experiments showed that CCK-8 and CCK-58 produce very similar Ca^{2+} signalling, mitochondrial metabolism and ZG secretion in murine PACs and that very similar responses were seen when repeated in human isolatedPACs.

A



B

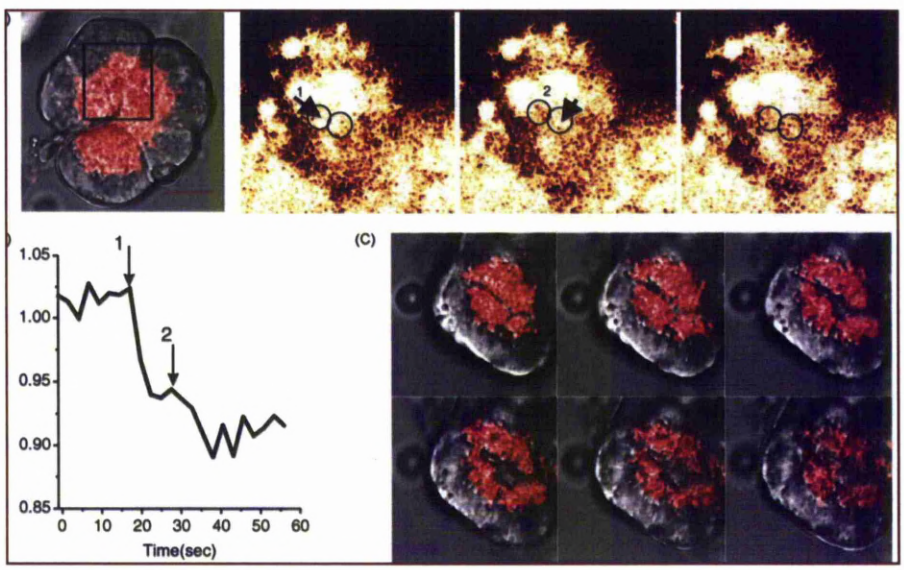


Figure 3.1: Secretion in isolated murine PACs responding to Ach stimulation A) Taken from Belan et al. (1996) this graph shows typical loss of fluorescence in zymogen granules (ZG) loaded with quinacrine and stimulated with Ach. The drop was approximately 10 per cent of baseline. B) Figure from Myoung et al. (2004), which shows quinacrine loading of zymogen granules. Individual ZG show a drop in fluorescence when stimulated of approximately 10 per cent from baseline. This is an indication of secretion from the PACs.

3.2 Materials and methods

3.2.1 Isolation of murine and human PACs

Murine PACs were isolated as previously described in Methods Chapter. Human PACs were obtained from 14 patients (median age 59yrs; interquartile range 57-68yrs) undergoing surgery between January 2004 and February 2008 (See table 3.1). It was discovered that it was necessary to be selective with the patients, and only take samples from patients whose underlying disease induced no pancreatic or biliary duct obstruction. Preliminary work with human PACs, from patients undergoing surgery for pancreatic cancer, showed poor morphology and poor responses to secretagogues. Such patients frequently had disease-related obstruction of the pancreatic and/or biliary ductal system. However when patients with unobstructed pancreatic disease had tissue sampled, human PACs with excellent morphology were obtained. As a result many patients donating tissue reported here underwent surgery involving the left side of the pancreas, and had no obstruction of the main pancreatic duct. Interestingly the poor morphological PACs obtained from obstructed disease looked very similar to images of isolated human PACs in publications, which recorded no response to CCK stimulation (Ji et al., 2001). This strongly suggests that previous work by other groups had been undertaken with suboptimal cells.

Table 3.1: Demographic details, surgical procedure and underlying pathology of patients donating human pancreatic tissue (from Murphy et al., 2008)

Age	Sex	Surgical procedure[a]	Final diagnosis[b]	Main duct[c]	Pancreas[d]
57	F	Pancreatoduodenectomy	IPMN[e]	Normal	No fibrosis
58	F	Pancreatoduodenectomy	Duodenal adenoma	Normal	No fibrosis
59	M	Pancreatoduodenectomy	Simple squamous cyst	Normal	No fibrosis
66	M	Pancreatoduodenectomy	Ampullary adenoma	Normal	Mild fibrosis
68	M	Pancreatoduodenectomy	Ampullary carcinoma	Normal	Mild fibrosis
71	F	Enucleation[f]	Insulinoma	Normal	No fibrosis
25	F	Left pancreatectomy	Gastrinoma	Normal	Mild fibrosis
27	M	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
55	M	Left pancreatectomy[g]	Endocrine carcinoma	Normal	No fibrosis
58	F	Left pancreatectomy[g]	Adenosquamous carcinoma	Normal	Mild fibrosis
59	F	Left pancreatectomy	Mucinous cystadenoma	Normal	No fibrosis
65	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
72	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis
82	F	Left pancreatectomy	Serous cystadenoma	Normal	No fibrosis

[a]Pancreatoduodenectomy was pylorus-preserving Kausch-Whipple resection
[b]Histology of resected specimen
[c]Data from Computerised Tomography and/or histology
[d]Histology of non-neoplastic pancreatic parenchyma at transection line in resected specimen
[e]Intraductal papillary mucinous neoplasm
[f]Pancreatic sample taken from normal parenchyma near to, but not contiguous with, completely excised insulinoma
[g]With splenectomy

3.2.2 Solution and chemicals

Extracellular solution contained (mM): NaCl 140, KCl 4.7, $MgCl_2$ 1.13, $CaCl_2$ 1, D-glucose 10, HEPES 10, adjusted to pH 7.35. As described in methods chapter protease inhibitor tablet (1 tablet, Roche, Germany) and benzamidine (1mM, Sigma-Aldrich) were added to human PACs extracellular solution to reduce spontaneous zymogen activation. Pyruvate, a substrate for mitochondrial metabolism was added to keep cells in optimal condition (100μm). CCK-8 and CCK-58 were added to extracellular solution to make physiological preparations (2pM and 10pM) and supra-maximal preparations (10nM). Human synthetic CCK-58 was supplied by UCLA peptide synthesis facility (Dr J.R. Reeve), and was synthesized using an Applied Biosystems Peptide Synthesizer (Foster City, CA), unblocked and purified to >90 per cent (Reeve et al., 2004).

Atropine (1 μM) was added to extracellular solutions, as it prevents activation muscarinic receptors and responses to Ach, but not to CCK (Petersen, 1992). Tetrodotoxin (100 nM) blocks voltage-gated $Na^{=}$ channels and other potential neuro-transmitters.

Fluo 4-AM (3μM) was loaded into murine cells by incubating for 30 minutes at 37^0C and used to measure changes $[Ca^{2+}]_i$. Fluorescence was

excited by an argon 488 nm laser and emission collected between 505-550 nm.

Mitochondrial metabolism was assessed simultaneously by monitoring NADH autofluorescence in the perigranular mitochondrial belt (excitation 363 nm; emission 390-450 nm).

Quinacrine (10μM) was loaded into murine and human PACs by incubating for 10 minutes at 37^0C, then washing out. Confocal imaging of cells loaded with fluorescent quinacrine was performed using a Zeiss LSM510 system (Carl Zeiss, Germany). Excitation was at 488nm; emission >505 nm.

Images were composed of 256x256 pixels, and optical sections were 5-6μm. Image analysis was undertaken with LSM510 software.

3.3 Isolated murine PACs responses to CCK-8 and CCK-58

3.3.1 Effects of CCK-8 and CCK-58 on $[Ca^{2+}]_i$ and mitochondrial stimulus-metabolic coupling in isolated murine pancreatic acinar cells

Stimulation of isolated murine PACs with physiological levels of CCK-58 (10 pM) produced typical oscillatory, focal spikes of $[Ca^{2+}]_i$ starting in the apical, granular region and spreading basally (12 out of 15 cells). These were very similar to those typically seen in response to CCK-8 stimulation (see figure 3.2).

Hyperstimulation with supra-maximal doses of CCK-58 (5-10 nM) led to a single large spike in $[Ca^{2+}]_i$, that subsequently declined to an elevated baseline (10 out of 10 cells). Removal of Ca^{2+} from the extracellular solution, and addition of Ca^{2+} chelator ethylene glycol tetra-acetic acid (EGTA), led to a fall in $[Ca^{2+}]_i$ back to baseline, and demonstrated that this toxic rise in $[Ca^{2+}]_i$ was dependent on Ca^{2+} entry to the cell. Again these responses were practically identical to those seen in CCK-8 hyperstimulation (see figure 3.3).

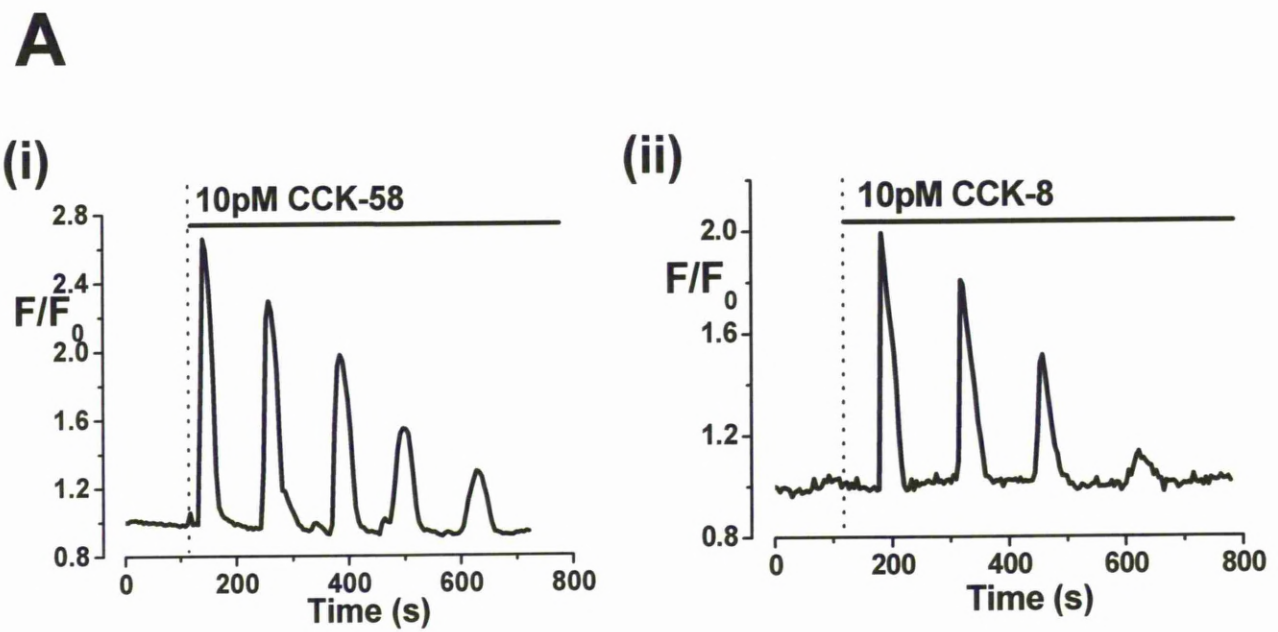


Figure 3.2: Effects of physiological (10 pM) CCK-58 (i) and CCK-8 (ii) on cytosolic Calcium concentration, $[Ca^{2+}]_{i,}$ in isolated murine pancreatic acinar cells. Representative traces show the typical oscillatory spikes in $[Ca^{2+}]_i$ in response to stimulation. F/F_0 represents ratio of change in fluorescence from baseline (F_0) at given time point (F).

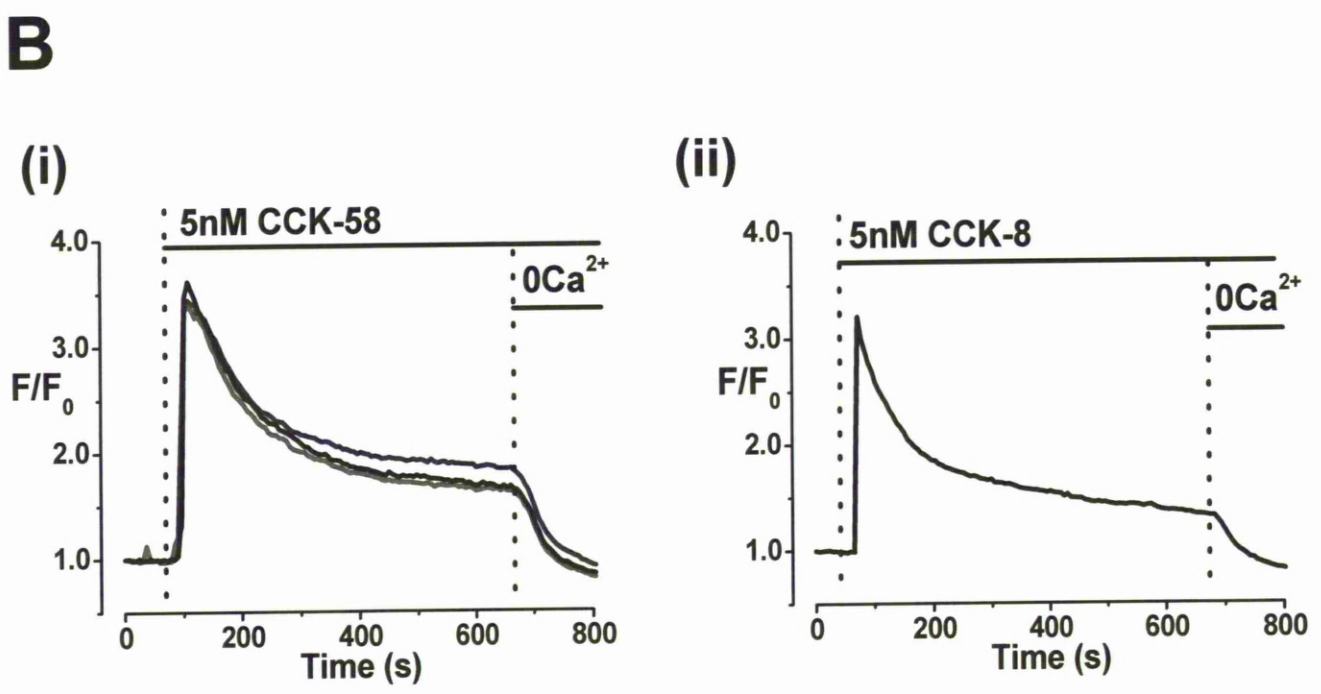


Figure 3.3: Effects of supra-maximal (5nM) CCK-58 (i) and CCK-8 (ii) on cytosolic Calcium concentration, $[Ca^{2+}]_{i,}$ in isolated murine pancreatic acinar cells. Both CCK-58 (i) and CCK-8 (ii) show a single spiked elevation in $[Ca^{2+}]_i$ followed by a sustained, elevated baseline. When Ca was removed from the extracellular solution (and EGTA added) this plateau reverted to basal levels. F/F_0 represents ratio of change in fluorescence from baseline (F_0) at given time point (F). i) shows response from several different murine PACs. Reproduced with permission Dr D Cridddle (from Criddle et al., 2009).

Additionally each spike in $[Ca^{2+}]_i$ at physiological stimulation was associated with a rise of mitochondrial NAD(P)H autofluorescence in the perigranular region that denoted stimulus-metabolism coupling, generating ATP necessary for zymogen secretion (see figure 3.4).

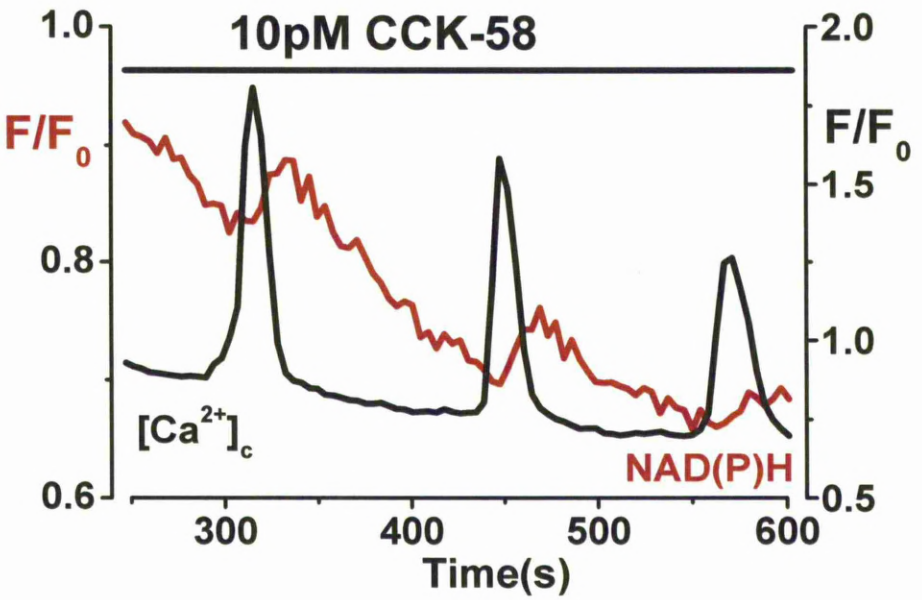


Figure 3.4: Representative trace showing stimulus-metabolic coupling induced by 10 pM CCK-58. After each elevation of $[Ca^{2+}]_i$ a slower rise in NAD(P)H (red trace) was recorded. Similar responses were seen with CCK-8 in 6 cells. Reproduced with permission Dr D Cridddle (from Criddle et al., 2009).

3.3.2 Effects of CCK-8 and CCK-58 on secretion in isolated murine PACs

Quinacrine loading of zymogen granules, and subsequent drop in fluorescence levels in response to CCk-8 and CCK-58 stimulation, was used to measure isolated murine PACs secretion. Quinacrine is an acidophilic fluorescent dye that is preferentially taken up by secretory granules and has been used to detect and quantify exocytosis in previous isolated cell work (Campos-Toimil et al., 2000)(Park et al., 2004). Typical quinacrine uptake staining was shown in figure 2.x (Materials and Methods Chapter).

The apical area of the PACs, containing quinacrine loaded ZGs, were selected (using the confocal imaging software) for continuous recording of fluorescence throughout the duration of the experiment. Separately the basolateral area was also selected, to give a baseline level for comparison. The basolateral area did not show significant quinacrine uptake. Figure 3.5 shows typical representative traces from isolated murine PACs stimulated with physiological levels of CCK-8 (Figure 3.5A) and CCK-58 (figure 3.5B). The fluorescence of the selected ZG containing apical area drops, representing loss of quinacrine (along with the rest of the contents of the ZGs) during secretion. It was observed that no changes occurred in the

basolateral area during this process, indicating quinacrine was being lost from the PACs, into the lumen. This process was repeated in multiple isolated murine PACs, over several different experiments and on different days. The mean results were (see figure 3.6A & B). The loss of quinacrine fluorescence from apical regions in response to physiological CCK-8 or CCK-58 was very similar with no significant difference between CCK-8 2pM (n=13), CCK-8 10 pM (n=11) or CCK-58 (n=16). In each case the drop was about a third from the initial level.

There was no significant quinacrine secretion when cells were hyperstimulated with supra-maximal levels of CCK-8 or CCK-58 (10-20 nM). Indeed continuous perfusion with supra-maximal CCK-8 or CCK-58 over a period of about 20minutes, led to PACs damage, with evidence of membrane breakdown.

These experiments demonstrate that CCK-8 and CCK-58 have very similar effects on isolated murine PACs secretion *in vitro*.

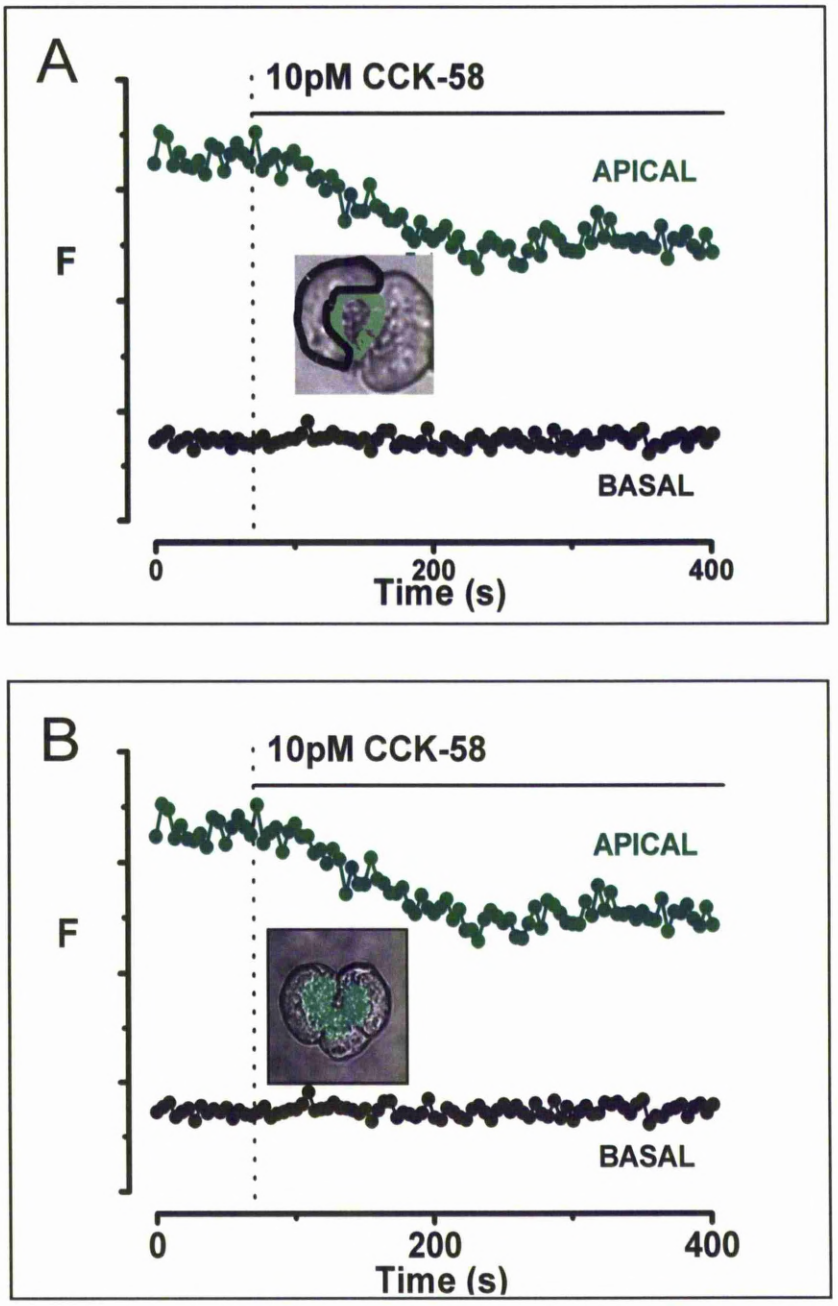


Figure 3.5: Representative traces of apical quinacrine loss from isolated murine PACs by secretion in response to physiological (A) CCK-8 and (B) CCK-58. The inset image in both diagrams shows acinar cells loaded with quinacrine and selection of regions for analysis during CCK stimulation (green for apical; black for basolateral). On stimulation both demonstrated a decrease in fluorescence of about a third. In both figures arbitrary units of fluorescence were applied. B reproduced with permission from Dr D Criddle.

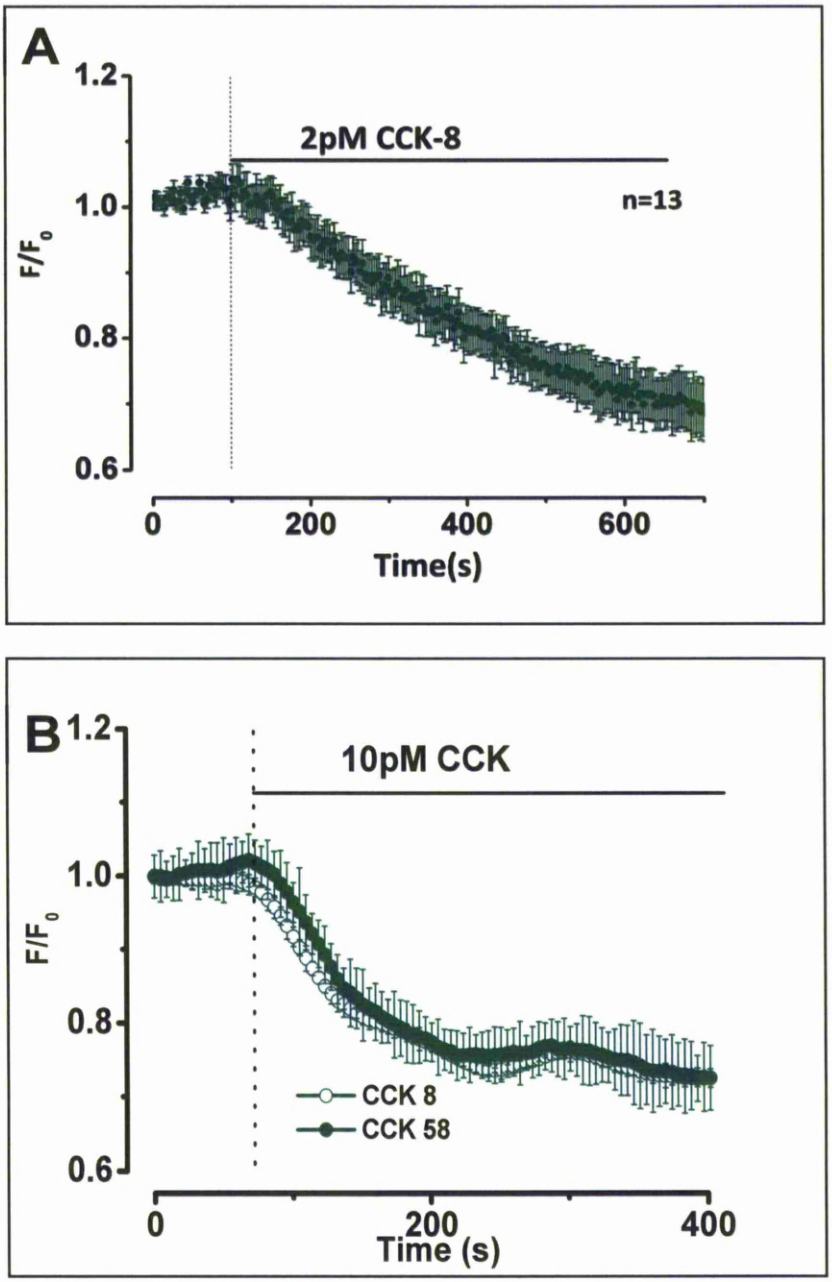


Figure 3.6: Meaned quinacrine fluorescence response to physiological CCK-8 and CCK-58. (A) shows meaned decrease in quinacrine fluorescence in apical regions of isolated murine PACs in response to CCK-8 2 pM (number of cells (n) = 13). (B) Shows overlayed apical quinacrine responses in isolated murine PACs to CCK-58 (n = 16) and CCK-8 (n=11) both at 10 pM. The responses are virtually identical. In all groups drop in fluorscence was apporximately a third. Fluorescence normalised from baseline (F/F_0). Both figures show standard error bars. B reproduced with persmission from Dr D Criddle.

3.4 Isolated human PACs responses to CCK-8 and CCK-58

3.4.1 Effects of CCK-8 and CCK-58 on $[Ca^{2+}]_i$ and mitochondrial stimulus-metabolic coupling in isolated human pancreatic acinar cells

Working within a collaboration our group showed that stimulation of isolated humans PACs with physiological (2-20 pM) CCK-8 (63 cells in total) or CCK-58 (19 cells) led to clear spikes of $[Ca^{2+}]_i$, that included oscillatory responses. To ensure only CCK responses were recorded, atropine (1 μM) and atropine with tetrodotoxin (100 nM) were included in extracellular solutions (see figure 3.7). The spikes of $[Ca^{2+}]_i$ in response to CCK-8 or CCK-58 were not inhibited, demonstrating that their effects were not due to ACh or other neurotransmitter.

The $[Ca^{2+}]_i$ spikes demonstrated apical to basolateral progression and a simultaneous rise in NADH autofluorescence (indicating stimulus-metabolic response). Again this is in keeping with the well-established murine model. There were no significant differences between CCK-8 and CCK-58 in type or scale of $[Ca^{2+}]_i$ oscillatory spikes.

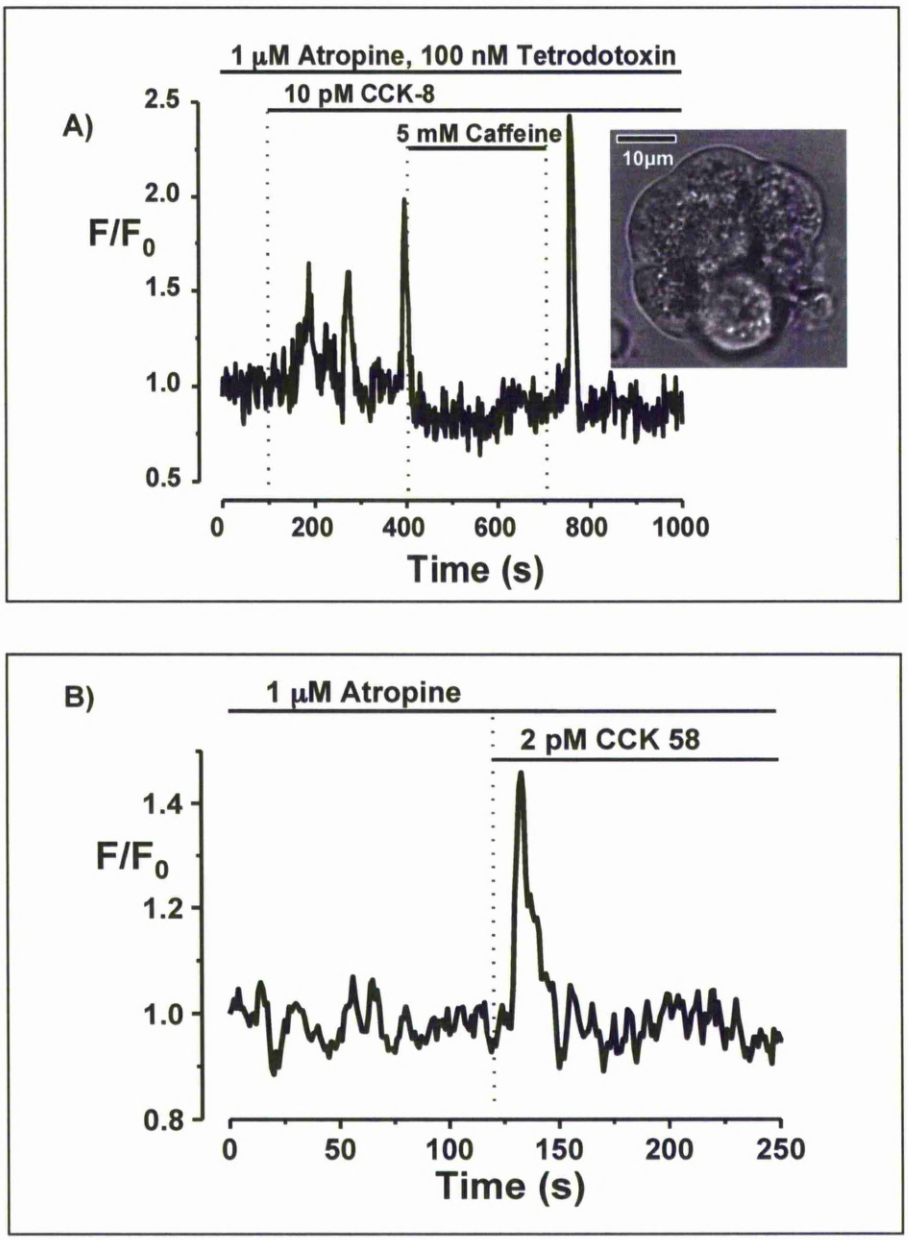


Figure 3.7: Effects of physiological (2-20 pM) CCK-8 (A) and CCK-58 (B) on cytosolic calcium concentration, $[Ca^{2+}]_{i,}$ in isolated human pancreatic acinar cells. A cluster of human PACs in shown in inset image in (A), which also shows the typical oscillatory spikes in $[Ca^{2+}]_i$ in response to stimulation. (A) additionally demonstrates caffeine application reversibly inhibits Ca^{2+} signalling. F/F_0 represents ratio of change in fluorescence from baseline (F_0) at given time point (F).

These oscillatory $[Ca^{2+}]_i$ spikes could be reversibly inhibited with caffeine. When physiological levels (2-20 pM) of both CCK-8 (15 cells) and CCK-58 (11 cells) were added to extracellular solutions, reversible inhibition of stimulation was elicited by caffeine (5 mM). Caffeine is a membrane-permeable inhibitor of IP_3R and is known to inhibit both ACh and CCK stimulated Ca^{2+} release, by acting as a reversible antagonist to Ca^{2+} releasing-IP_3R channels on the endoplasmic reticulum (Criddle et al., 2006)(Petersen et al., 2008). Ceasing caffeine stimulation led to prompt return of $[Ca^{2+}]_i$ spikes (see figure 3.7A).

Hyperstimulation with supra-maximal levels of CCK-8 (5-10 nM) led to single spikes in $[Ca^{2+}]_i$ with an elevated baseline, again as also seen in isolated murine PACs (see figure 3.8). These elevations returned to baseline when the stimulus was stopped.

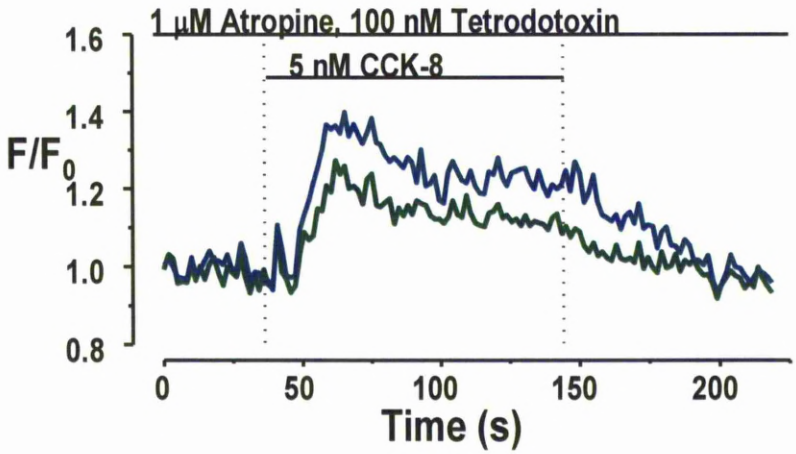


Figure 3.8: Effects of hyperstimulation with CCK-8 on $[Ca^{2+}]_i$ in isolated human PACs. Supra-maximal levels of CCK-8 (5 nM) were applied to human PACs, in the presence of atropine and tetrodotoxin. Hyperstimulation produced an elevation, which remained at an elevated plateau until hyperstimulation ceased, when levels returned to baseline. F/F_0 represents ratio of change in fluorescence from baseline (F_0) at given time point (F).

3.4.2 Effects of CCK-8 on secretion from isolated human PACs

As in the murine isolated PACs experiments detailed above, Quinacrine loading of zymogen granules, and subsequent drop in fluorescence levels in response to CCk-8 and CCK-58 stimulation, was used to measure isolated human PACs secretion. Due to the controversy regarding direct CCK stimulation of human PACs, steps were taken to inhibit possible responses to other residual neurotransmitters and/or nerve endings. These measures included adding tetrodotoxin (100 nM) and atropine (1 μM) to all extracellular solutions, so that responses to CCK stimulation could be shown to be from direct activation of CCK receptors.

Figure 3.9A shows a representative trace of quinacrine and NADH fluorescence in response to physiological CCK-8 (10 pM), from one human PAC. Quinacrine shows a similar drop in fluorescence in human as in murine PACs. In all cases the loss is approximately 20 per cent from initial levels. There is simultaneous evidence of stimulus-metabolic coupling, shown by a rise in NADH autofluorescence, showing this is active secretion. Figure 3.9B shows the meaned results from 7 separate human PACs, again demonstrating an approximate loss of fluorescence of 20 per cent.

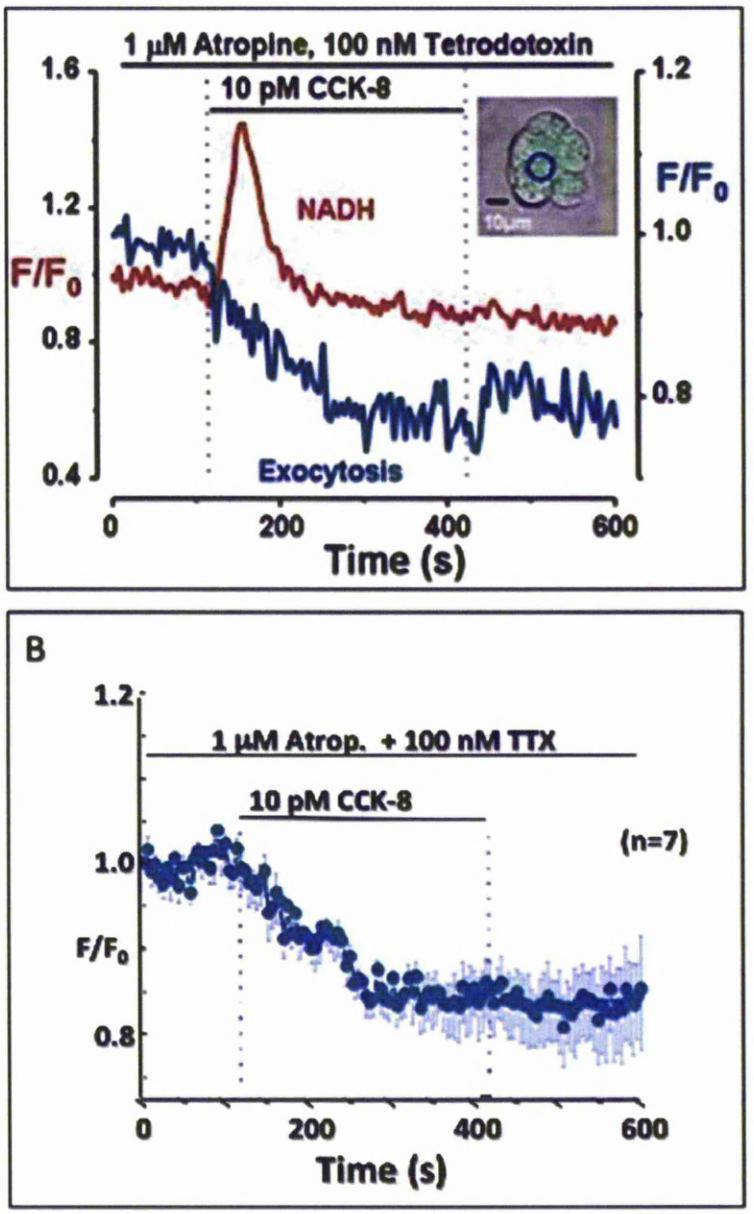


Figure 3.9: Apical quinacrine secretion in response to physiological CCK-8 (10 pM) in isolated human PACs. A) Representative apical quinacrine trace, and simultaneous NADH recordings, in human PAC when stimulated with 10 pM CCK-8, in the presence of atropine and tetrodotoxin. Inset image shows cluster of human PACs from which trace was taken. Note F/F_0 for quinacrine is on the right y-axis, and that for NADH on the left y-axis B) Meaned apical quinacrine loss from 7 separate isolated human PACs (Mean values ± S.E. bars).

3.5 Discussion

3.5.1 Isolated murine PACs responses to CCK-8 and CCK-58

The experiments undertaken with isolated murine PACs demonstrated markedly similar responses from CCK-8 and CCK-58. At physiological concentrations (1-20 pM) CCK-58 induced oscilatory $[Ca^{2+}]_i$ spikes, which were generated at the apical pole and spread basolaterally. Such responses are typical of rodent and murine iolsated PACs with CCK-8 (Maruyama et al., 1993)(Petersen and Tepikin, 2008). $[Ca^{2+}]_i$ spikes were associated with metabolic activity of peri-granular mitochondria, with uptake of Ca^{2+} leading to stimulation of Ca^{2+}-dependent dehydrogenase enzymes that generate NADH and ATP. This was detected by NAD(P)H autofluorescence. The consequence of this stimulus-metabolic couplong was zymogen granules secretion. This was detected by the drop in measured fluorescence of quinacrine-loaded ZGs. Again there was no difference between CCK-8 and CCK-58 stimulated cells. Our group also observed exocytotic activity by measuring amylase secretion during stimulation, which was also increased (Murphy et al., 2008).

Hyperstimulation of isolated murine PACs with CCK-8/caerulein is a well-described model of experimental *in vitro* acute pancreatitis. Suprisingly

recent work had shown CCK-58 did *not* induce pancreatitis *in vivo* experimental models (Yamamoto et al., 2007). However in our *in vitro* experiments CCK-58 and CCK-8 produced near-identical responses when isolated murine PACs were hyperstimulated (5-20 nM). Both peptides elicited typical elevations of $[Ca^{2+}]_i$ with subsequent evidence of PACs damage. Our group measured several parameters of PACs damage not included in the results section (but presented in the groups publication: Murphy et al., 2008). These parameters include: trypsinogen activation detected using dye BZiPAR; increased proportion of necrotic cells after hyperstimulation with both CCK-8 and CCK-58; and morphological changes associated with pancreatitis, such as membrane blebbing and breakdown. The differences between *in vivo* and *in vitro* responses to CCK-58 may represent alternate pathways of action for CCK-58 in the whole animal, that aren't available in isolated cell work.

Otherwise these experiments showed remarkedly similar effects for CCK-8 and CCK-58 on isolated murine cells.

3.5.2 Isolated human PACs responses to CCK-8 and CCK-58

The experiments described with human isolated PACs demonstrated, for the first time, that physiological concentration of CCK-8 and CCK-58 elicit $[Ca^{2+}]_i$ spikes, mitochondrial activation and secretion. The concentrations of CCK-8 and CCK-58 were equivalent to to that *in vivo* (Walsh, 1987), and elicited $[Ca^{2+}]_i$ spikes that were oscillatory and spread from apical to basolateral regions. These $[Ca^{2+}]_i$ signals were linked to activation of mitochondrial metabolism, providing the ATP needed for secretion of zymogen granules from the apices. These findings are very similar to those previously reported in murine and rodent isolated PACs (Petersen et al, 2008). There is debate as to which form of CCK is the most physiologically active, as CCK-58 has been found to be the most dominant circulating form in several species (Sun et al., 1992)(Reeves et al., 2003). All CCK subtypes share the same C-terminal hepapeptide amide sequence, that is necessary for binding to CCK receptor (Dufresne et al., 2006). These experiments found that effects of physiological concentrations of CCK-8 and CCK-58 had very similar effects on isolated human PACs.

The effects off CCK-8 and CCK-58 were conclusively not produced by Ach mediaited action, as suggested by other groups (Ji et al., 2001)(Owyang et

al., 2004), as the responses ocurred in the presence of atropine and tetrodotoxin. However as this work was in isolated human PACs *in vitro*, it is not possible to say if CCK also activates vagal pathways separately *in vivo*.

The selection of human tissue from donors with unobstructed pancreatic disease, which results in less pancreatic fibrosis and damage, and sussequent rapid transport to the lab (in optimised extracellular solution) was demonstrated to be critical in obtaining viable human PACs for experimental *in vivo* work. Preliminary work with tissue from obstructed disease showed PACs were morphologically poor and responses were poor. Our group has postulated that the failure of other groups (Ji et al., 2001) to obtain CCK responses was due to not optimising the cells and using poor quality human PACs.

Caffeine works by reversibly inhibiting IP_3R channels, which are found on the endoplasmic reticulum and on the membranes of zymogen granules. By showing that caffeine reversibly inhibits response of human PACs to CCK stimulus it is seen that human PACs have mechanisms in common with murine PACs. This action suggests a possible thearpeutic role for caffeine, which needs further exploration.

Finally, by demonstrating such similarity between murine and human PACs mechanisms, the argument for the continued use of CCK/caerulein experimental animal models is strengthened, and translating findings in these models to human disease becomes more valid.

CHAPTER 4

Effects of BAK, Bcl-2 and BAX knock-out on murine caerulein-induced *in vivo* experimental pancreatitis

4.1 Introduction

Members of the Bcl-2 family of proteins regulate cell death pathways, and the balance between apoptosis and necrosis is thought to be critical in determining the severity of pancreatitis (Bhatia, 2004)(Pandol et al., 2007)(Golstein and Kroemer, 2007).

Previous work has suggested that Bak and Bax are pro-apoptotic (Bhatia, 2004)(Kroemer et al., 2007)(Chipuk et al., 2008) and have a protective role in pancreatitis. Both are involved in cytochrome c release from mitochondria, by formation of the mitochondrial outer membrane pore (MOMP) and by action on opening of the mitochondrial permeability transition pore (MPTP. It postulated that by knock-out of these genes there should be less apoptotic activity and cell death pathways would shift to necrosis with an increase in severity of pancreatitis.

By contrast Bcl-2 has been suggested as pro-survival (or anti-apoptotic) in its action, stopping the formation of the MOMP and inhibition of cytochrome c (Bhatia, 2004)(Gukovsky et al., 2011). However it may also have a role in regulating necrosis directly, and not indirectly just by negative actions on apoptosis (Sung KF et al., 2009). In *in vitro* models of cholecystokinin-induced pancreatitis it was shown that inhibiting Bcl-2 (and

Bcl-xL) increases loss of $\Delta\Psi m$ (mitochondrial membrane potential) and facilitates necrosis, whilst also inhibiting CCK-induced caspase-3 activation and apoptosis. The net result was *increase* in necrosis and severity. This makes Bcl-2's role more ambiguous.

There is wide acceptance that knowledge surrounding the interactions of these proteins is far from complete. The current sub-divisions into 'anti-apoptotic' and 'pro-apoptotic' may be too simplistic. Despite their importance in elucidating cellular mechanism in pancreatitis, *in vitro* work may not translate to living organism. Further investigation of the actions of these important proteins *in vivo*, using experimental models of acute pancreatitis, has not been reported before and is important to help further explore the mechanisms involved in severity. This chapter details experiments investigating the role of Bcl-2, Bak and Bax genes in determining the severity of caerulein-induced murine experimental in *in vivo* acute pancreatitis.

4.2 Methods

Acute pancreatitis was induced in mice by means of the standard caerulein-induced model, in which 7 hourly intra-peritoneal (IP) injections of caerulein 50 μg/kg were administered. End points for the experiments were 8, 12 and 24 hours after the first injection. C57BL/6 male mice (aged 8-12 weeks) were used as the controls for the Bak knock-out (KO) mice and for the Bcl-2 and BAX groups their own wild types (WT) were used as controls. Each experimental group contained 6 animals (unless otherwise stated). Buprenorphine analgesia was administered IP with the first dose of caerulein.

The development of acute pancreatitis was evaluated by measurement of pancreatic oedema (pancreatic wet-to-dry weight ratio), serum amylase, grading of H&E stained histology slides and scoring of slides stained by immunohistochemistry (IHC) for caspase-3 (a measure of apoptotic activity) (as detailed in Chapter 2 – Methods).

Initial work was undertaken on the C57BL/6 animals, to ensure the model produced expected results in the control group. Pancreatic oedema and serum amylase measurements were typical (see figure 4.1).

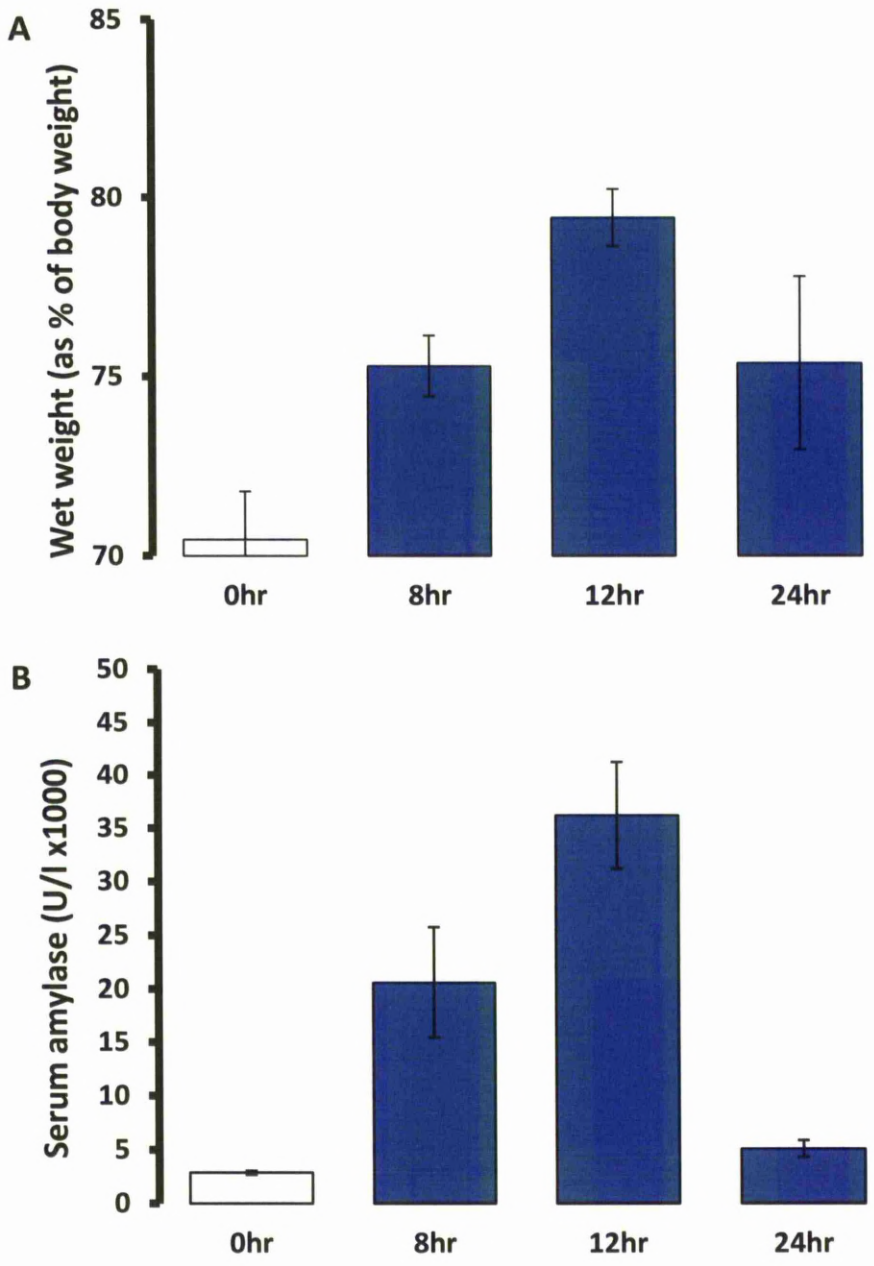


Figure 4.1: Caerulein-induced acute pancreatitis in C57BL/6 mice with measurements at 8, 12 and 24 hrs. (A) Shows mean scores for pancreatic oedema. Progression over time points typical (except 24 hr lower than expected). (B) Shows progression of serum amylase as expected. All groups contained 6-8 animals. Figures show mean values with SEM bars.

Histopathological scoring was undertaken by two blinded assessors, who simultaneously (but without communication) scored 10 fields per slide. The mean score over the ten fields for each assessor was combined into a total mean score per slide. Mean scores for assessors were very similar and showed no significant difference (see figure 4.2a). The combined scores demonstrated typical progression over the time periods the reach at maximum at 24 hours (see figure 4.2b).

7 hourly IP injections of Caerulein induced typical histopathological changes, which progress with time up to 24 hours. At eight hours, early oedema, perivascular inflammatory cell recruitment and occasional, focal necrosis were observed (figure 4.3c). At 12 hours oedema was maximal and there was moderate inflammatory infiltration of pancreas tissue, with increasing necrosis. By 24 hours there was extensive inflammatory cell infiltration and marked areas of necrosis (see figure 4.3e&f).

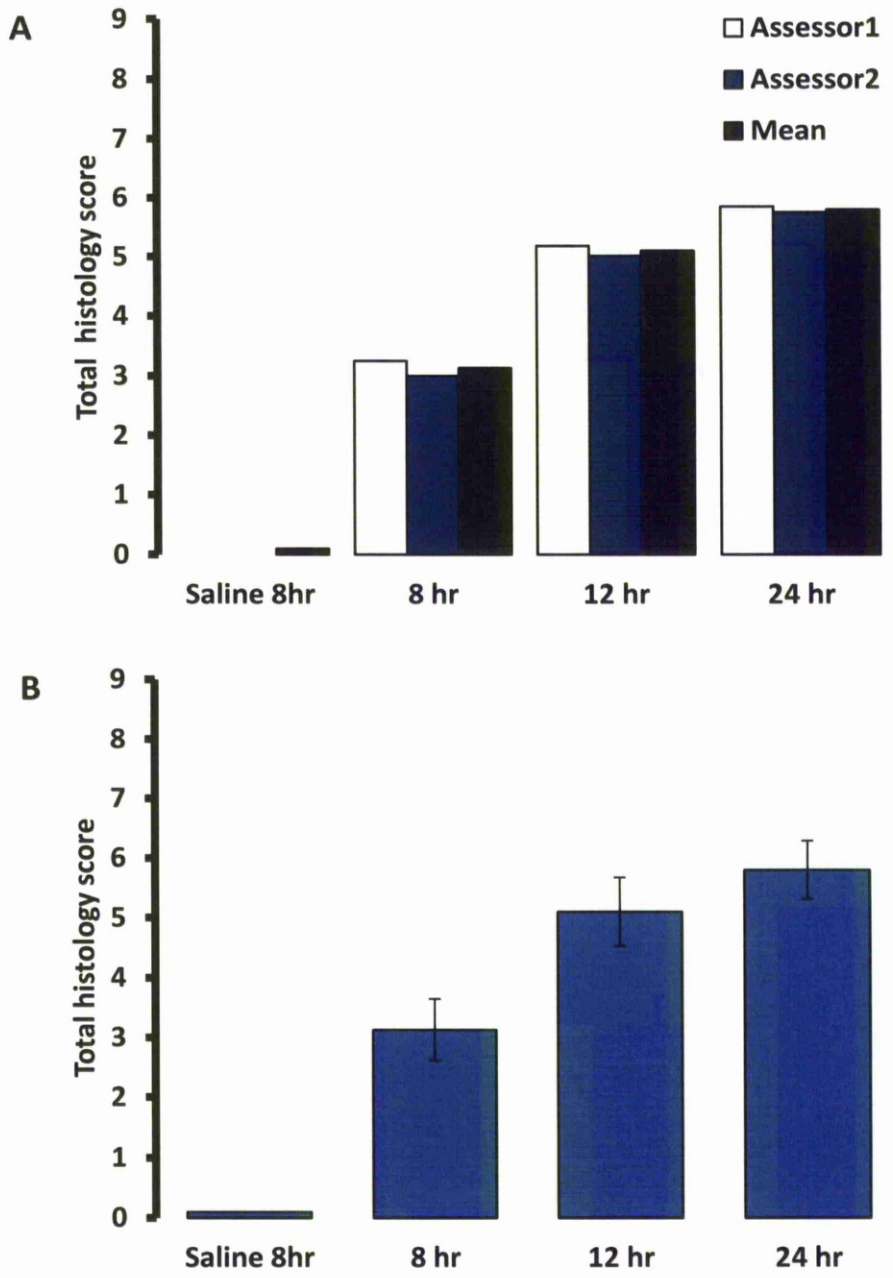


Figure 4.2: Caerulein-induced acute pancreatitis in C57BL/6 mice with measurements at 8, 12 and 24 hrs. (A) Shows comparison between mean scores for each assessor (no significant difference). (B) Shows total mean scores over the time points. All groups contained 6-8 animals. Figures show mean values with SEM bars.

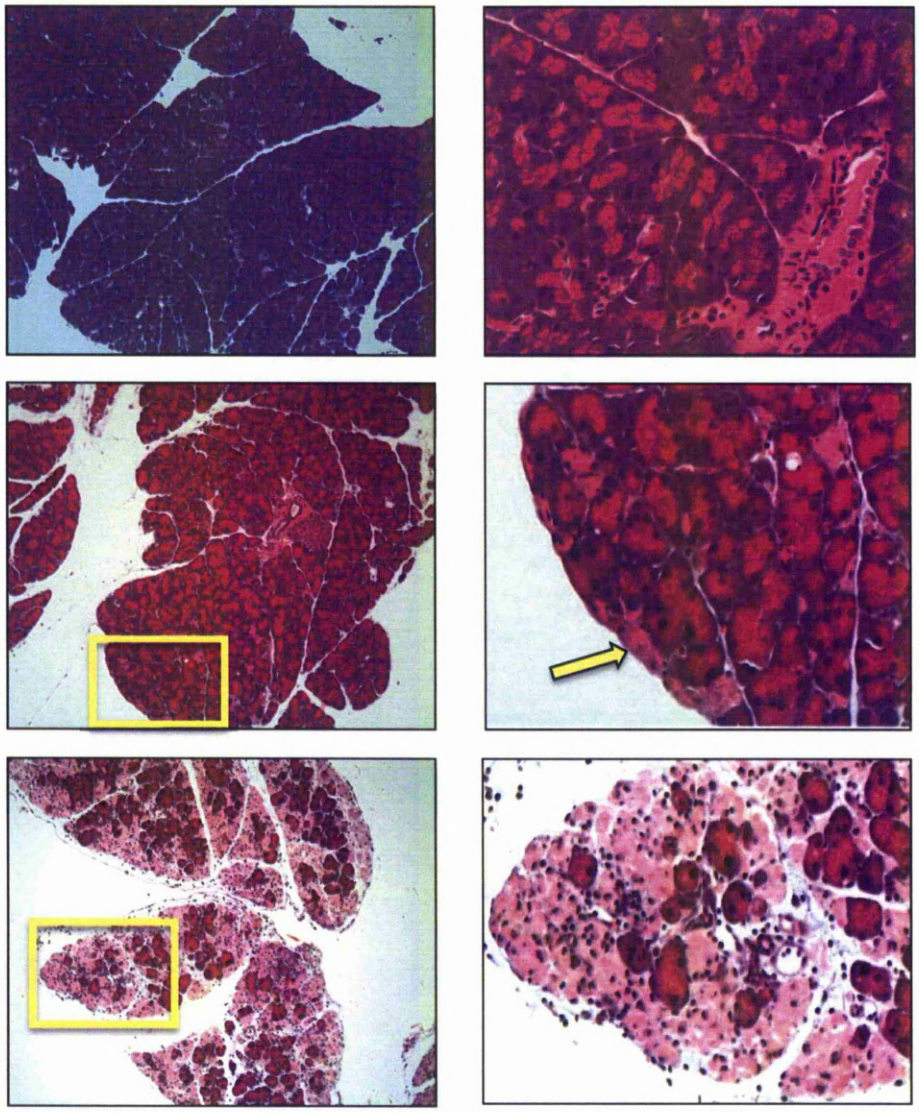


Figure 4.3: Typical appearance of C57BL/6 murine pancreas after 7 hourly IP injections of caerulein (or saline in controls). (A)&(B) Show normal appearances of pancreatic tissue. (C) Shows typical appearances at 8hrs with some oedema and focal necrosis. (D) Shows enlarged boxed area from (C) - with focal necrosis arrowed. (E) Shows severe changes after 24 hr, with extensive inflammatory cell infiltrate and necrosis (F) Shows enlarged boxed area from (E). Magnification x100 (except B & F at Magnification x200).

Caspase-3 is an upstream protease in both the extrinsic and intrinsic apoptotic pathways. As such activation of caspase-3 is a key point in apoptosis, and measurement of caspase-3 is widely used to quantify apoptotic activity in other organs (Przemeck et al., 2007). Caspase-3 was stained by IHC in pancreas tissue and the slides subsequently scored using ImageJ software, and results expressed as a ratio of caspase-3 staining cells to normal cells. Typical capase-3 IHC staining of murine pancreas tissue is shown in figure 4.4. In C57BL/6 mice there were no appreciable levels of apoptotic activity until 12-24 hours.

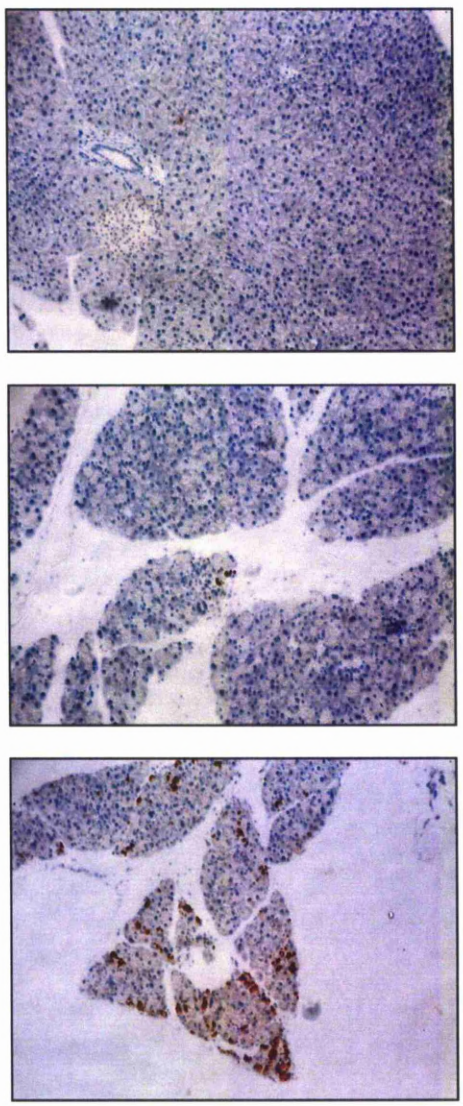


Figure 4.4: Representative images of caspase-3 IHC staining in murine acute pancreatitis. Time points are: A. 0hr; B. 8hr; C 24hr. Caspase-3 stains brown ,and is most obvious in C. (Magnification x 200).

4.3 Bak knock-out in murine experimental pancreatitis

Acute pancreatitis was induced in Bak knock-out (KO) mice by means of the standard caerulein-induced model. End points for the experiments were 8, 12 and 24 hours after the first injection. Each time group contained 6-8 animals and one group had sham injections of saline only. Results for Bak KO were compared against C57bL/6 (the wild-type for Bak KO).

Bak KO mice showed significantly lower pancreatic wet-to-dry weight ratio at 12 hours, but not at other time points. Both C57BL/6 and Bak KO mice showed lower ratios at 24 hours, which was contra to expectations, as oedema should be maximal at 24 hours (See figure 4.5a).

Serum amylase was significantly lower in the Bak KO group at 12 hours compared to the C57BL/6 group. Other time points showed no significant difference, although the 8 hours group did show a higher mean value in the Bak KO group (see figure 4.5b).

The histopathological score was lower in the Bak KO group at all three experimental time points (see figure 4.6). These results provide evidence that Bak KO is protective in caerulein-induced murine experimental pancreatitis.

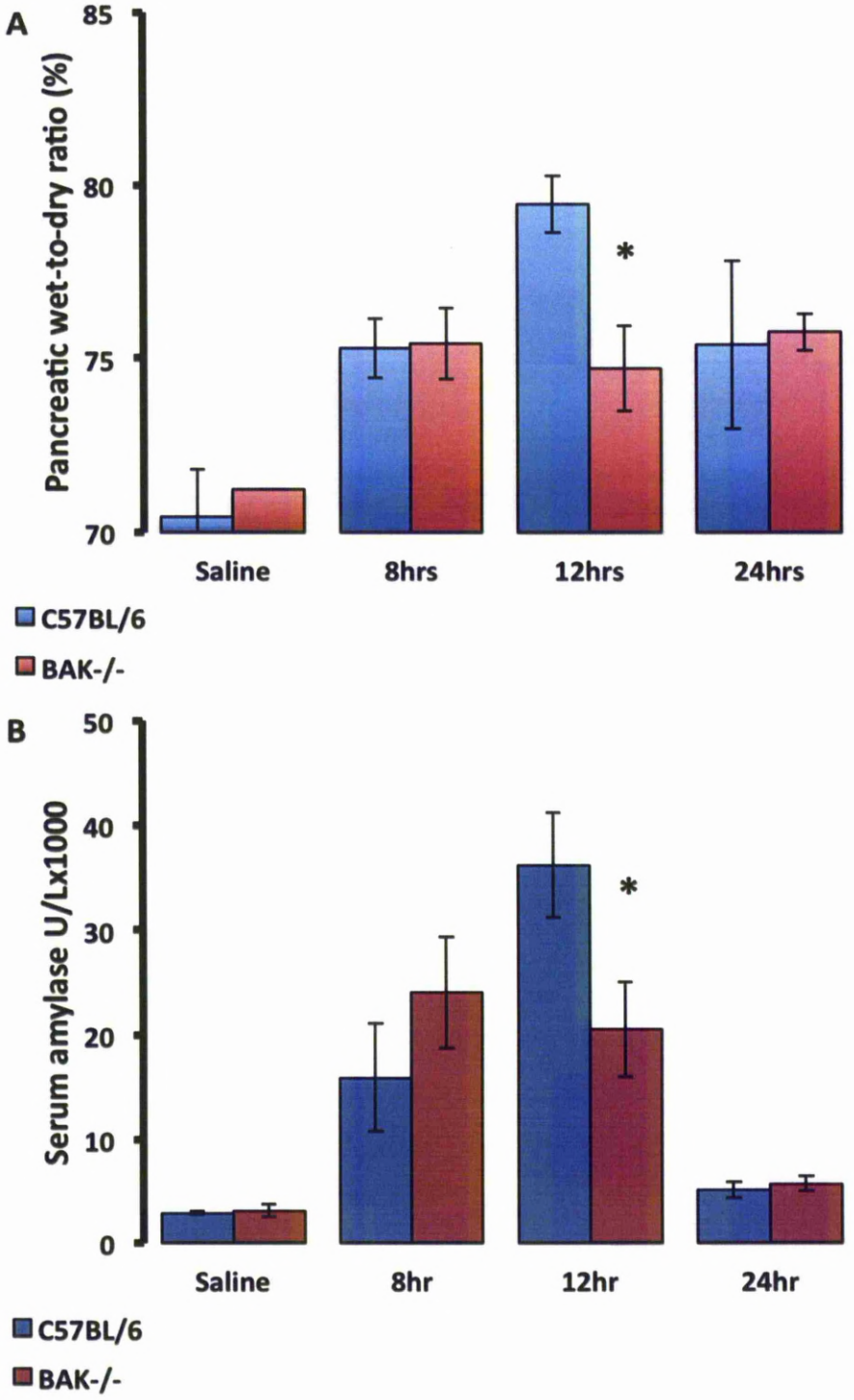


Figure 4.5: Comparing pancreatic oedema (A) and serum amylase (B) in caerulein-induced pancreatitis in Bak knock-out (red) and C57BL/6 (blue) mice. * significant difference at <0.05 level. Mean values ± SEM.

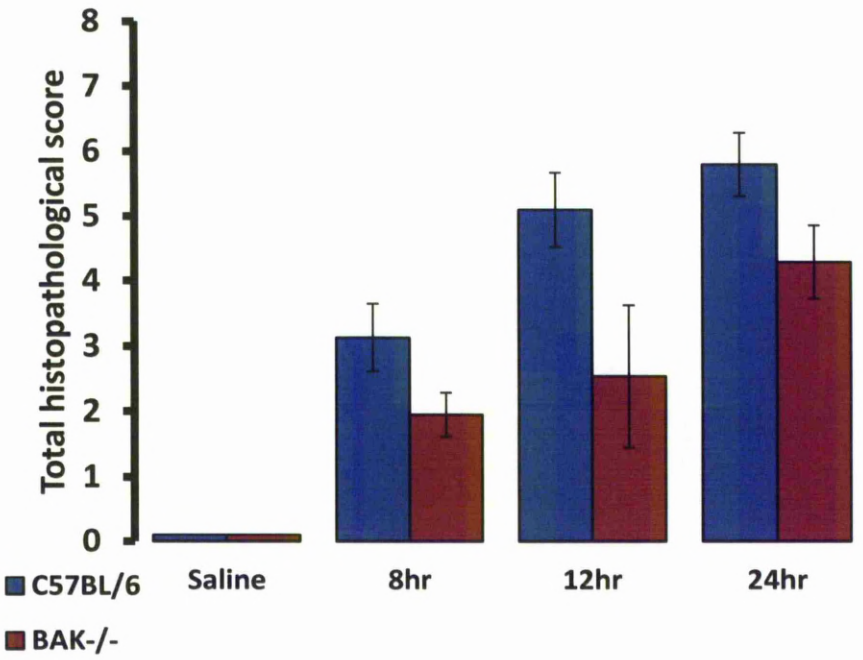


Figure 4.6: Difference in pancreatitis histopathological score between C57BL/6 (blue) and Bak -/- mice (red) at 8, 12 and 24 hr (with a saline group at 8hr). Standard mean values ± SEM.

4.4 Bcl-2 knock-out in murine experimental pancreatitis

Acute pancreatitis was induced in Bcl-2 knock-out (KO) mice by means of the standard caerulein-induced model. End points for the experiments were 8, 12 and 24 hours after the first injection. Each time group contained 6 animals and one group had sham injections of saline only. Results for Bcl-2 KO were compared against wild-type (WT - control group).

Bcl-2 KO are much smaller and weaker than WT. Approximately half die before 6 weeks (Nakayama et al., 1994). Abnormalities include: small ears; greying of fur at 4 weeks; low lymphocyte count; polycystic kidney disease. As a consequence it was harder to breed viable adults. Due to cost, and time, constraints the 24 hours time group only contained two animals, so conclusion from this group have to be treated with caution.

There was no significant difference in pancreatic wet-to-dry weight ratio between the Bcl-2 KO and the WT controls. Even so there was trend towards a higher pancreatic wet-to-dry weight ratio in the Bcl-2 KO group at 12 hours and 24 hours (see figure 4.7a).

Serum amylase was significantly higher in the Bcl-2 KO group at 12 hours compared to the WT group. This was also the case at 24 hours, though this group only contained 2 animals (see figure 4.7b).

Over 24 hours the histopathological score showed no clear difference between the groups. At early stages (8 hours) the Bcl-2 KO group histopathological score was lower than the WT group, and when the breakdown of the score was analysed this was due to a lower inflammatory and necrosis score in the Bcl-2 group. It should be noted, however, that the score in the WT group is still relatively low (2.03 out of 9.0), and in the early stages small differences can lead to large differences between groups. If this finding is correct then it suggests Bcl-2 KO are slower to develop an inflammatory response than wild types, yet by 12-24 hours this has made no difference to the severity of pancreatitis.

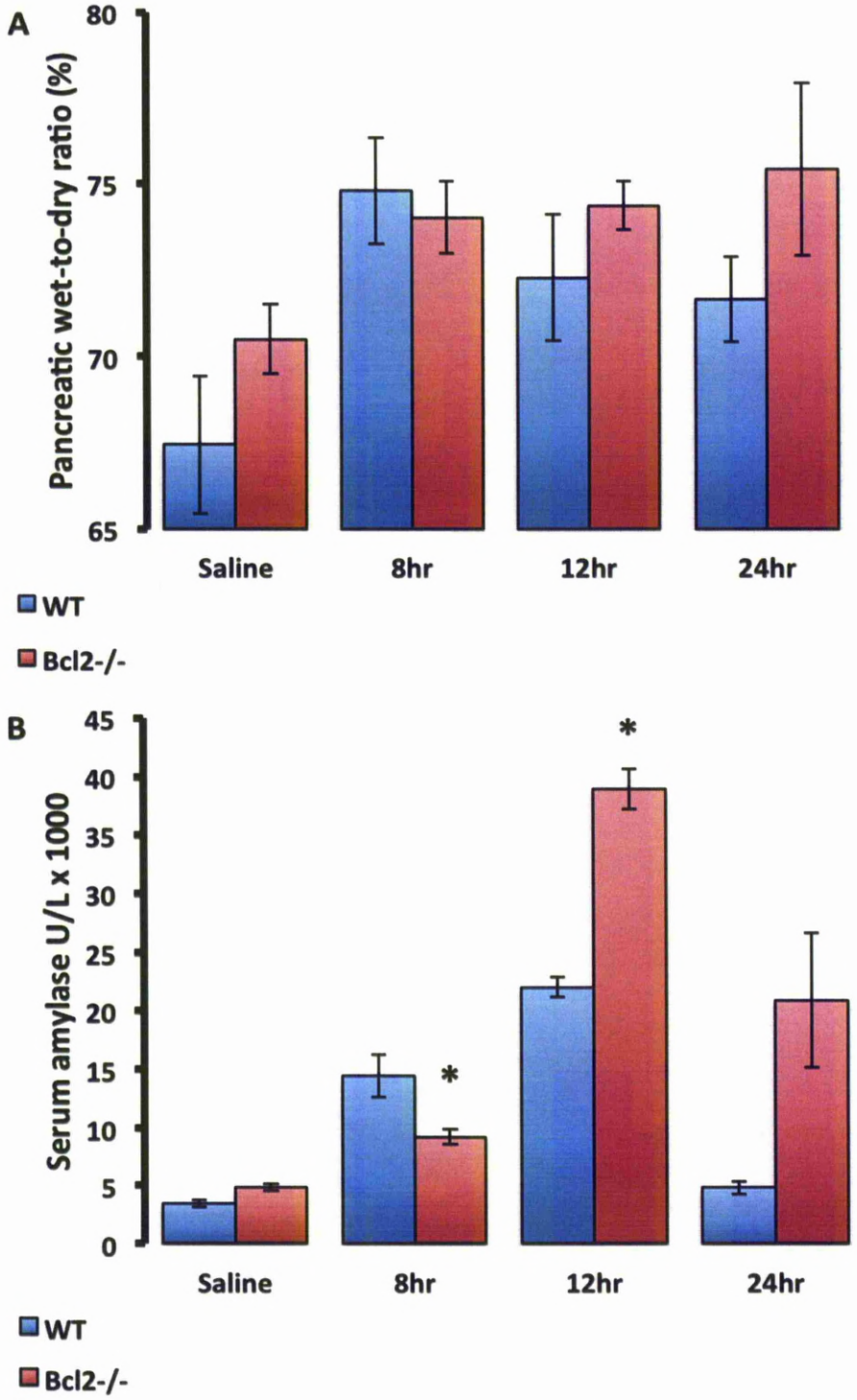


Figure 4.7: Comparing pancreatic oedema (A) and serum amylase (B) in caerulein-induced pancreatitis in Bcl-2 knock-out (red) and wild type (WT) (blue) mice. * significant difference at <0.05 level. 24 hours Bcl-2 group contained only 2 animals. Mean values ± SEM.

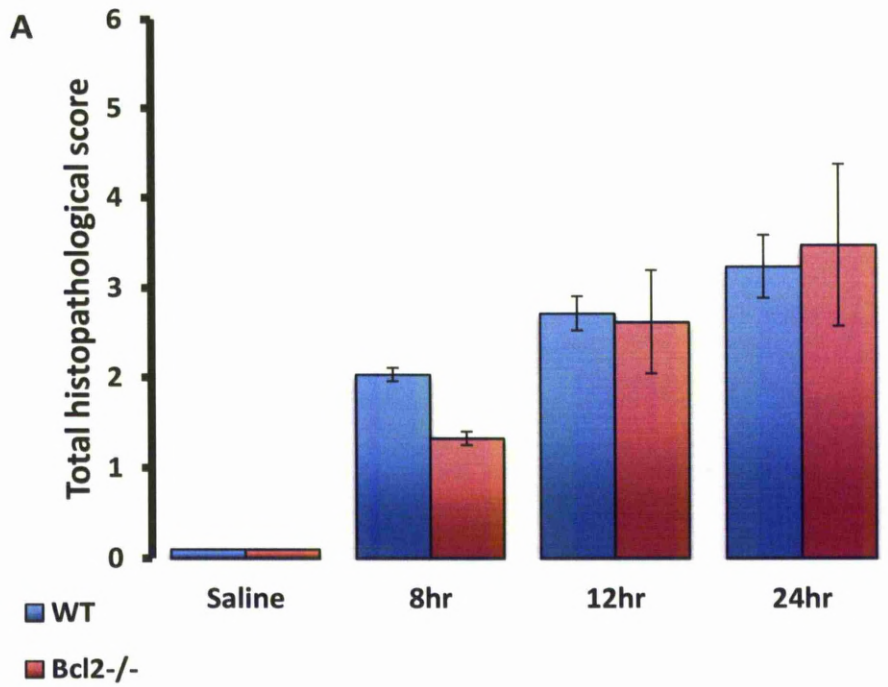


Figure 4.8: Difference in pancreatitis histopathological score between WT (blue) and Bcl-2 -/- mice (red) at 8, 12 and 24 hr (with a saline group also at 8hr). Only 2 animals in the 24 hr group.

4.5 Bax knock-out in murine experimental pancreatitis

Acute pancreatitis was induced in Bax knock-out (KO) mice by means of the standard caerulein-induced model. End points for the experiments were 8, 12 and 24 hours after the first injection. Each time group contained 6-8 animals and one group (8 hours) had sham injections of saline only. Results for Bax KO (Bak -/-) were compared against wild-type (Bax +/+).

There was no significant difference in pancreatic wet-to-dry weight ratio between the Bax KO and the WT controls (see figure 4.9a).

Serum amylase in the Bax KO group, compared to WT controls, was significantly higher at 12 hours, but was otherwise very similar at other time points (see figure 4.9b).

Bax KO mice showed evidence of a more severe early stage of pancreatitis compared to WT controls (see figure 4.10). At both 8 and 2 hours the overall severity score was higher in the Bax KO groups. By 24 hours the control group's score had reached similar levels. The breakdown of the scores in shown in figure 4.11(A,B,C). Bax KO have overall higher oedema, inflammatory infiltrate and (interestingly) even necrosis at a much earlier stage than WT controls.

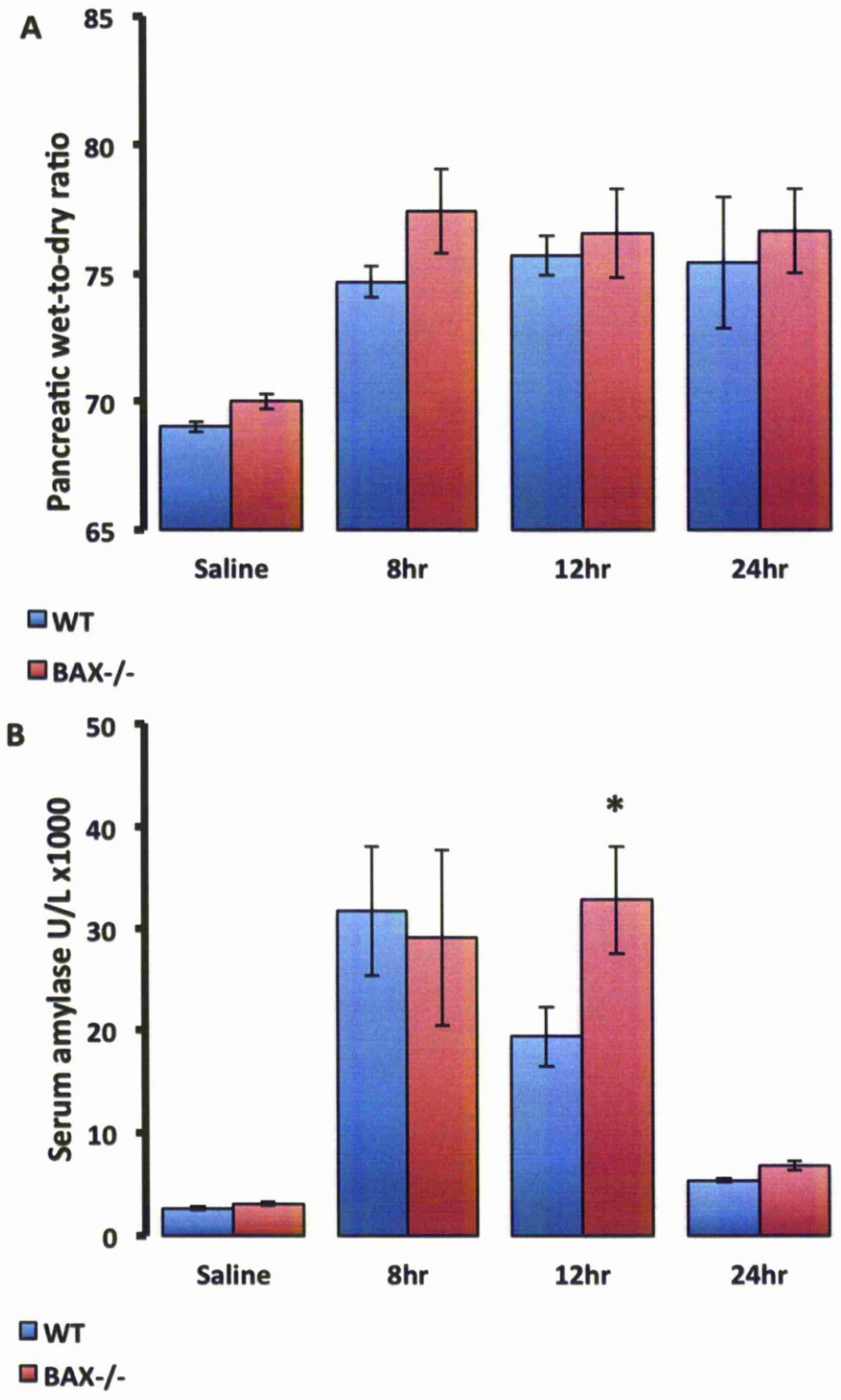


Figure 4.9: Comparing pancreatic oedema (A) and serum amylase (B) in caerulein-induced pancreatitis in Bax knock-out (red) and wild type (WT) (blue) mice. * significant difference at <0.05 level. Mean values ± SEM.

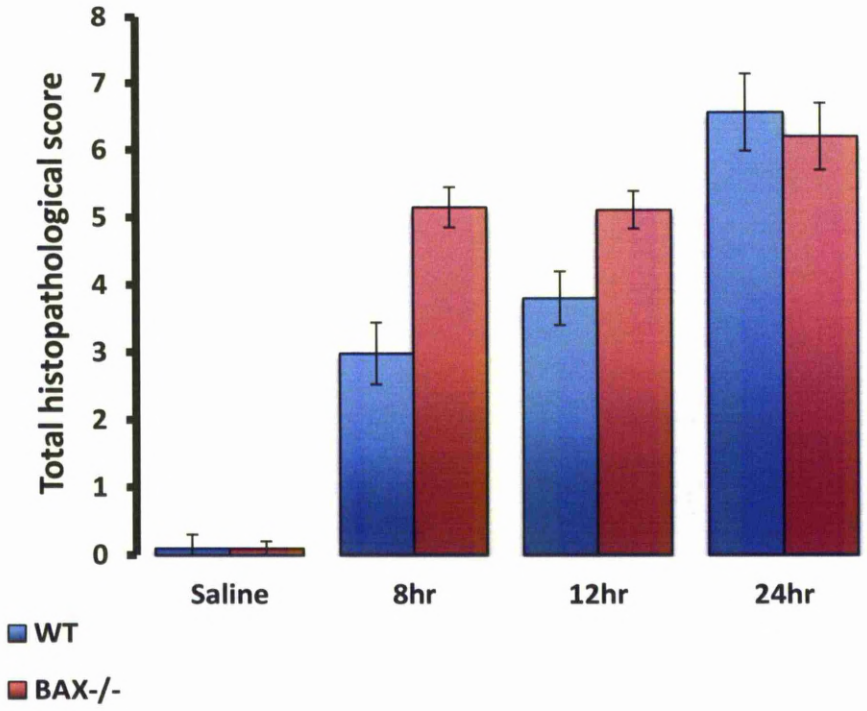


Figure 4.10: Difference in pancreatitis histopathological score between WT (blue) and Bax -/- mice (red) at 8, 12 and 24 hr (with a saline group also at 8hr) (4-6 animals per group). Mean values ± SEM.

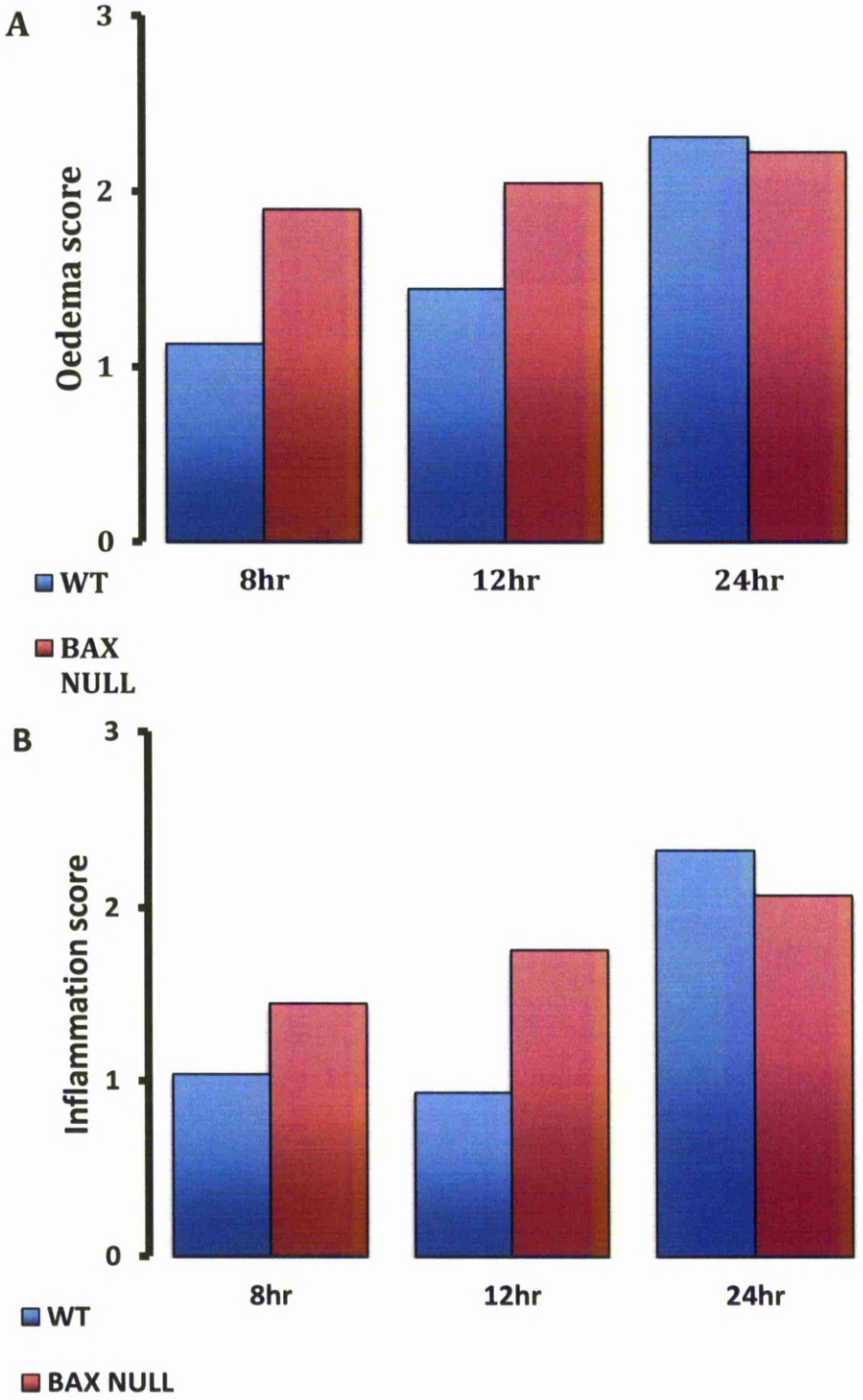


Figure 4.11: Breakdown of histopathological score in caerulein-induced AP in Bax knock-out (red) mice compared to WT controls (blue) at 8, 12 and 24 hours. Bax KO demonstrate higher oedema (A) and inflammation (B) scores in the first 12 hours, but by 24 hours levels are similar to controls. 4-6 animals per group. Mean values per group ± SEM.

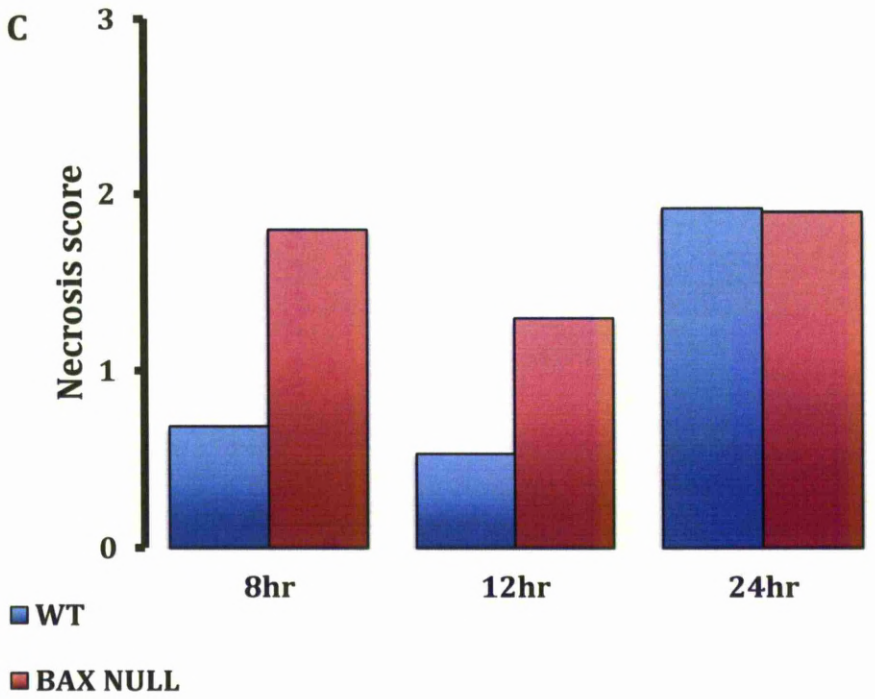


Figure 4.11C: Breakdown of histopathological score in caerulein-induced AP in Bax knock-out (red) mice compared to WT controls (blue) at 8, 12 and 24 hours. Bax KO demonstrate higher levels of necrosis from a much earlier time points than controls, but by 24 hours levels are similar in both groups. 4-6 animals per group. Mean values per group ± SEM.

The WT groups showed a steadier increase of severity over the time points (similar to the C57BL/6 groups from the previous section) and by 24 hours had reached the same level of severity as the Bax KO group.

This difference is interesting and suggests Bax KO increases the potential for pancreatic acinar cells injury in hypersensitivity-induced pancreatitis, which is the hypothesis to be tested. However the hypothesis also stated that this would be due to decreased apoptosis and increased necrosis, yet in the next section it will be described how apoptosis appeared to *increase* in caerulein-induced pancreatitis in Bax KO mice.

4.6 Caspase-3 staining and evidence of apoptosis in caerulein induced pancreatitis in Bak KO and Bax KO mice

All three knock-out mice (Bak KO, Bcl-2 KO and Bax KO) had pancreatitic tissue removed for caspase-3 immunohistochemistry (IHC) following induction of caerulein-induced pancreatitis as already described. Tissue was taken at 8, 12 and 24 hours time points and fixed on slides for assessing after IHC staining. Each slides had 5 representative fields at of view digitally imaged (x 200). The images were then processed using ImageJ software to obtain a ratio for caspase-3 stained-to-normal cells. The five fields were meaned to give a ratio per slide. Each animal group contained 4-6 animals (except the Bcl-2 KO 24 hr group, which only contained 2 animals) and the total mean value per group was calculated (see figure 5.2). The Bcl-2 KO groups' results were very similar to the C57BL/6 and Bak KO. Because of this, and because the 24 hours group was too small to be included, the Bcl-2 KO group has not been shown in the figure.

There was very little caspase-3 activity, in any group, until 12 hours. C57BL/6, Bak KO, and Bcl-2 KO all showed very similar levels of caspase-3 activity, which increased slightly over the 24 hours. Bcl-2 KO appeared to have no effect on apoptosis, as measured by caspase-3 activity, and had

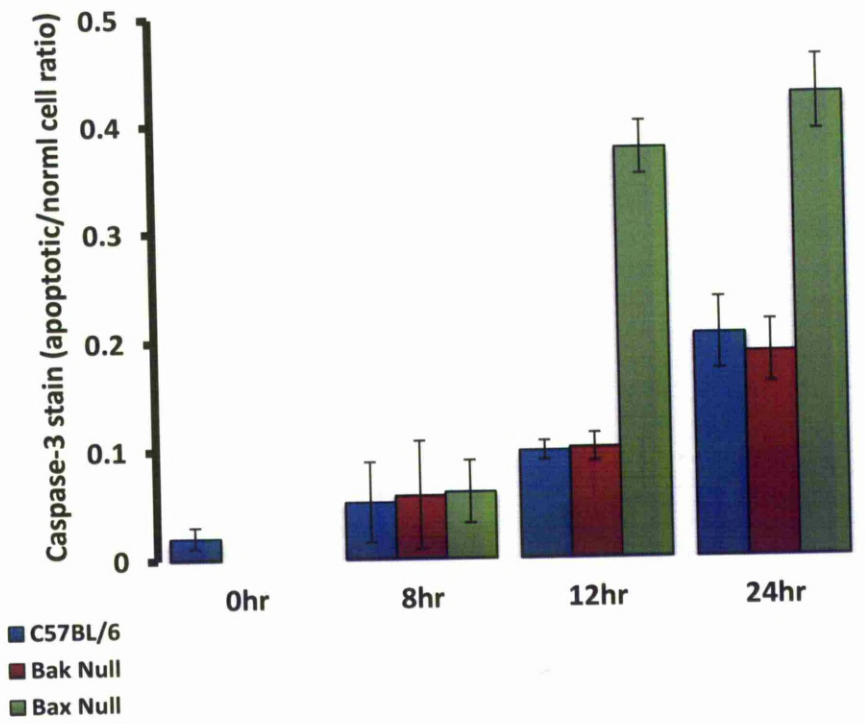


Figure 4.12: Caspase 3 IHC staining. C57BL/6 (blue), Bak Null (red) and Bax Null (green) mice are compared at 8, 12 and 24 hours after induction of caerulein-induced acute pancreatitis in. The y-axis is caspase-3 IHC stained cells (calculated as the ratio of stained cells to normal cells per field of view at x200 magnification). 5 fields of view per animal are combined for an overall mean, and there were 4-6 animals per group. Values presented are mean group values ± SEM.

already demonstrated no difference in histopathological severity from WT controls. Although Bak KO had less severe histopathological severity from controls at all time points, there was no evidence this was related to apoptotic activity, as caspase-3 activity was the same as controls.

Bax KO showed significantly elevated caspse-3 activity compared to the other groups. This was a surprising result. The histopathological severity was much greater in Bax KO than in controls, and it was expected that apoptosis (and thus caspase-3 activity) would be *decreased.* Bax is considered pro-apoptotic and to find an *increase* in apoptosis, with more severe pancreatitis, was unexpected and suggests the role of Bax in cell death pathways is more complicated than previously thought.

Figure 4.13 shows representative slides of C57BL/6, Bak KO and Bax KO at 8, 12 and 24 hours.

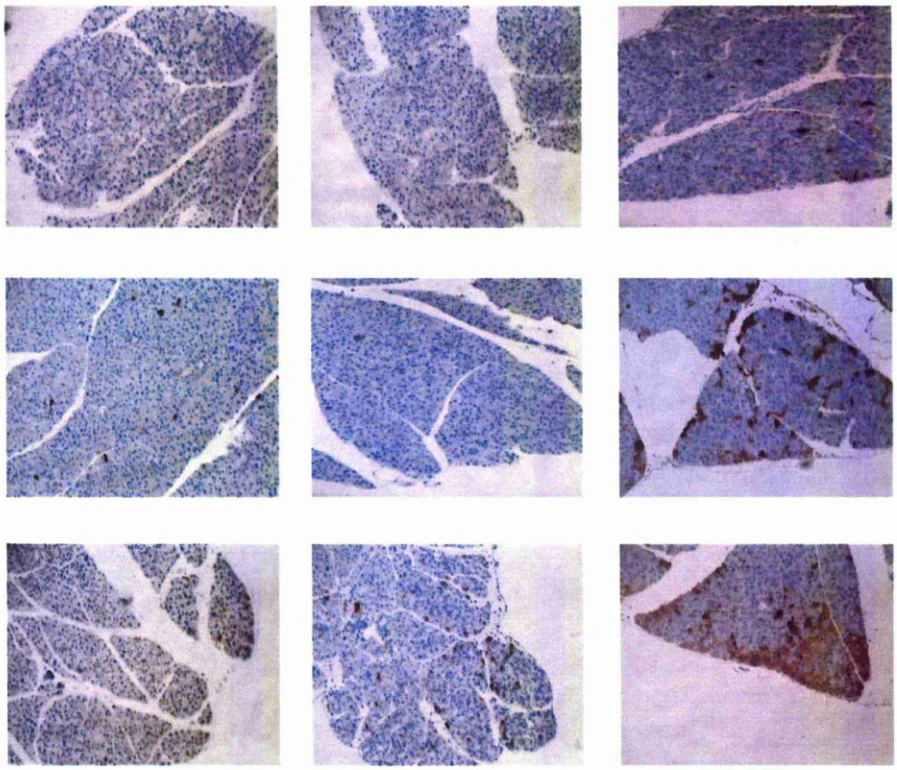


Figure 4.13: Representative slides stained by IHC for caspase-3 from C57Bl/6 (left column), Bak Null (middle column) and Bax Null (right column) mice. All slides show pancreas specimens after induction of caerulein experimental acute pancreatitis. Experiment time points were 8 hr (top row), 12 hrs (middle row) and 24 hrs (bottom row). Caspase-3 stains brown and increased activity can be seen in Bax Null at 12 an 24 hrs. Light microscope magnification x200.

4.7 Discussion of caerulein-induced acute pancreatitis in Bak, Bax and Bcl-2 murine knock-outs

The Bcl-2 family proteins have been shown to have important roles regulating cell death pathways, and are thought to be through interactions with mitochondria (Bhatia, 2004). Bak and Bax are thought to be important in facilitating cytochrome c release from mitochondria, through formation of mitochondrial OMM pores (Gukovsky et al., 2011), and thus are important to the intrinsic apoptotic pathway. Until recently their activity was thought to be purely 'pro-apoptotic' yet there their full mechanisms of action are still unclear. If their actions are purely pro-apoptotic, then pancreatitis in Bak and Bax KO mice should lead to reduced apoptosis and more severe pancreatitis. The previous results showed that the histopathology of Bax KO was indeed more severe than WT controls in the first 12 hours of pancreatitis, though severity was similar at 24 hours. However Bak KO showed *less* severe histopathology at all times points, against C57BL/6 controls. These results suggest a few points in the context of caerulein murine AP: i) that loss of Bak is protective; ii) that loss of Bak does not appear to influence apoptosis; iii) that loss of Bax is detrimental; iv) that loss of Bax leads to increased apoptosis *and* necrosis in the early stages. These results suggest that the current understanding of the roles of

Bak and Bax may be simplistic and there are more complex interactions occurring. In particular Bax interactions are suggested to be more important than Bak in preventing severe disease. Also apoptosis *per se* may not always be protective in AP.

Bcl-2 is considered as 'anti-apoptotic' (Bhatia, 2004)(Pandol et al., 2007)(Gukovsky et al., 2011) and loss, through knock-out, was expected to be protective in caerulein murine AP. However Bcl-2 KO did not demonstrate either a change in severity of AP or a change in apoptotic activity from wild types. This may fit with recent evidence that the role of Bcl-2 is not just simply 'anti-apoptotic' and its effect may depend on the balance of a number of different interactions (e.g. cytochrome c inhibition; effecting mitochondrial ATP production). It should be noted that, due to difficulty in breeding these frail animals, some Bcl-2 KO groups had less than optimal numbers from which to draw results.

These experiments measured parameters of pancreas damage during caerulein-induced murine AP. In these experiments pancreatic wet-to-dry weight ratio was not found to be an accurate way of measuring pancreatic oedema. Serum amylase levels were useful to confirm pancreatitis, but did not always accurately reflect severity. Histopathological score was the most

useful parameter and caspase-3 IHC staining gave useful insight into apoptotic activity.

These experiments could be improved by using other parameters of pancreatic injury and also of concurrent systemic injury. Attempts were made to measure pancreatic trypsin activity and lung histopathology, but for technical reasons were unsuccessful.

Chapter 5

Actions of caffeine in murine caerulein-induced *in vivo* experimental pancreatitis

5.1 Introduction

Caffeine has been shown to reversibly inhibit Ca^{2+} signalling in isolated murine PACs, by inhibition of IP_3R channels (Petersen and Sutton, 2006)(Criddle et al., 2007b), sited on endoplasmic reticulum and zymogen granule membranes (see Introduction chapter). It may also have other effects on cAMP recycling (Wu et al., 2009), inhibition of adenosine A1 receptor (Fredholm et al., 1999)(Satoh et al., 2000) and inhibition of store-operated Ca^{2+} channels (SOC). As detailed in results chapter 3, our group has shown that caffeine had similar inhibitory affects to physiological (1-20 pM) CCK-8 and CCK-8 stimulation in isolated humans PACs. Caffeine is metabolised in the liver, by action of cytochrome P450, into several metabolites: paraxanthine, theobromine and theophylline. It is possible they too have potential therapeutic action in pancreatic acinar cells.

Caffeine therefore has potential as a therapeutic agent in acute pancreatitis. Caffeine is used as a therapeutic agent in some circumstances in humans. It is used to treat neonatal apnoea in premature infant, though the mechanisms of action are unclear (Erenberg et al., 2000). Caffeine stimulates increased breathing frequency (possibly through increasing the sensitivity of

chemoreceptors to CO_2), enabling premature infants to be weaned off ventilators quicker (Steer et al., 2004). The maintenance dose is 5-10 mg/kg.

Caffeine has been shown to have a half-life in murine models of approximately 1 hour (Bonati et al., 1984) and is lethal to mice at doses of 100 mg/kg (Fredholm et al., 1999). This causes a concern as our group used doses of caffeine at a concentration of 2-5 mM (equivalent to 50-100 mg/kg) in our isolated cells work. In preliminary work it was confirmed that 100 mg/kg was lethal to mice and 50 mg/kg induced significant symptoms of caffeine toxicity (tremors, poor motor function and signs of distress). Concentrations of 1 mg/kg and 10 mg/kg were used, which produced no adverse side effects in mice, and were an equivalent to concentrations used in human neonatal disease.

5.2 Methods

A caerulein-induced murine experimental model of acute pancreatitis was used. Male C57BL/6 male mice (8-12 weeks old) were given 7 hourly IP injections of caerulein were given at a dose of 50 μg/kg and 8 hourly caffeine IP injections to achieve either 1 mg/kg or 10 mg/kg concentrations

in vivo. was also administered via hourly IP injections, and as caffeine has a half-life of approximately 1 hour, it was necessary to calculate a loading dose and then subsequent hourly doses to maintain an average serum concentration. For an average concentration of 10 mg/kg: 13.5 mg/kg was given at 0 hours, followed by 7 hourly injections of 6.75 mg/kg to maintain an average concentration of 10 mg/kg (more precisely 10.125 mg/kg, these concentrations were chosen for ease of use)(see Table 5.1). Caffeine was made and stored (under sterile conditions) at a stock concentration of 2 mg/ml. A reference chart was created to determine the volume of stock that would be needed per individual animal's weight, to maintain a correct concentration of 10 mg/kg caffeine (see Table 5.2). The volumes required were appropriate for the size of the animals. Buprenorphine analgesia was administered IP with the first injection. Parameters measured included pancreatic wet-to-dry ratio, serum amylase, trypsin activity, histopathological scoring and caspase 3 IHC scoring.

Experiment time point (hr)	IP caffeine injection conc (mg/kg)	Average circulating caffeine conc (mg/kg)	IP Caerulein conc (50 µg/kg)
0	13.5	10	50
1	6.75	10	50
2	6.75	10	50
3	6.75	10	50
4	6.75	10	50
5	6.75	10	50
6	6.75	10	50
7	6.75	10	-
8	End of experiment – pancreas harvested		

Table 5.1 Protocol for timing and concentration of IP caffeine and caerulein. Shows doses required of IP caffeine to maintain an average concentration of 10 mg/kg. Caffeine was continued for an extra hour after caerulein had stopped, to maintain a circulating volume. In total 8 hourly IP injections of caffeine and 7 hourly IP of caerulein were given.

Caffeine Dose (mg/kg)	Mouse Weight (kg)	Amount required (mg)	Caffeine Stock conc. (mg/ml)	Volume required (ml)
Initial injection				
13.5	0.02	0.27	2	0.14
	0.021	0.28	2	0.14
	0.022	0.30	2	0.15
	0.023	0.31	2	0.16
	0.024	0.32	2	0.16
	0.025	0.34	2	0.17
	0.026	0.35	2	0.18
	0.027	0.36	2	0.18
	0.028	0.38	2	0.19
	0.029	0.39	2	0.20
	0.03	0.41	2	0.20
Subsequent injections				
6.75	0.02	0.14	2	0.07
	0.021	0.14	2	0.07
	0.022	0.15	2	0.07
	0.023	0.16	2	0.08
	0.024	0.16	2	0.08
	0.025	0.17	2	0.08
	0.026	0.18	2	0.09
	0.027	0.18	2	0.09
	0.028	0.19	2	0.09
	0.029	0.20	2	0.10
	0.03	0.20	2	0.10

Table 5.2: Reference chart for calculating volume (ml) of caffeine stock (2 mg/ml) required for individual mice by weight (0.020 – 0.030 kg) to maintain an average serum caffeine concentration of 10 mg/kg. The initial injection is 13.5 mg/kg with subsequent doses of 6.75 mg/

5.3 Effect of caffeine on severity of caerulein-induced murine pancreatitis

As described in Chapter 4, C57BL/6 mice were divided into groups (six animals) and received IP caerulein (50 μg/kg) injections hourly for 7 hours. They were sacrificed at 8 hours from the first injection. Caerulein induced acute pancreatitis (AP) by hyperstimulation, as evidenced by pancreatic oedema, rise in serum amylase, and histopathological changes including: vacuolisation, inflammatory cell infiltrate and necrosis. One group received sham injection with saline only, and showed no evidence of pancreatitis in the parameters measured.

In the interventional groups (again containing six C57BL/6 mice) caffeine was administered hourly, with caerulein. One group received 1mg/kg and the other 10 mg/kg. Both groups were sacrificed at 8 hours and compared with the caerulein only groups. In addition two animals received 50 mg/kg caffeine, but toxic side effects were severe and this experimental group was not repeated. Although only representing these two animals, the results after 50 mg/kg were interesting and some results are included in the figures.

Compared to saline-only, caerulein administration caused a marked increase in pancreatic oedema (as measured by pancreatic wet-to-dry ratio) and serum amylase. Caffeine did not show a significant effect on these two parameters (see figure 5.1). Although the mean value of both pancreatic oedema and serum amylase was slightly lower in the 10 mg/kg compared to caerulein alone, these results were not significant. If the caffeine groups were taken alone, then in both categories caffeine 10 mg/kg showed less severe pancreatitis than caffeine 1 mg/kg.

Histopathology scoring revealed a difference when 10 mg/kg of caffeine was administered with caerulein compared to the caerulein-alone group (See figure 5.2). The mean total histopathological score for the 10 mg/kg group was 1.98 ± 0.26, while the caerulein-only group was 3.13 ± 0.52. In the breakdown of the score, 10 mg/kg showed markedly less histopathological evidence of oedema and inflammation than the caerulein-only group. There was no difference noted between the caffeine 1 mg/kg and caerulein-alone group, though of note was the finding of less histopathological evidence of oedema. Interestingly the caffeine 50 mg/kg group (though only two animals) showed a very low histopathological score, much lower than the 10 mg/kg group.

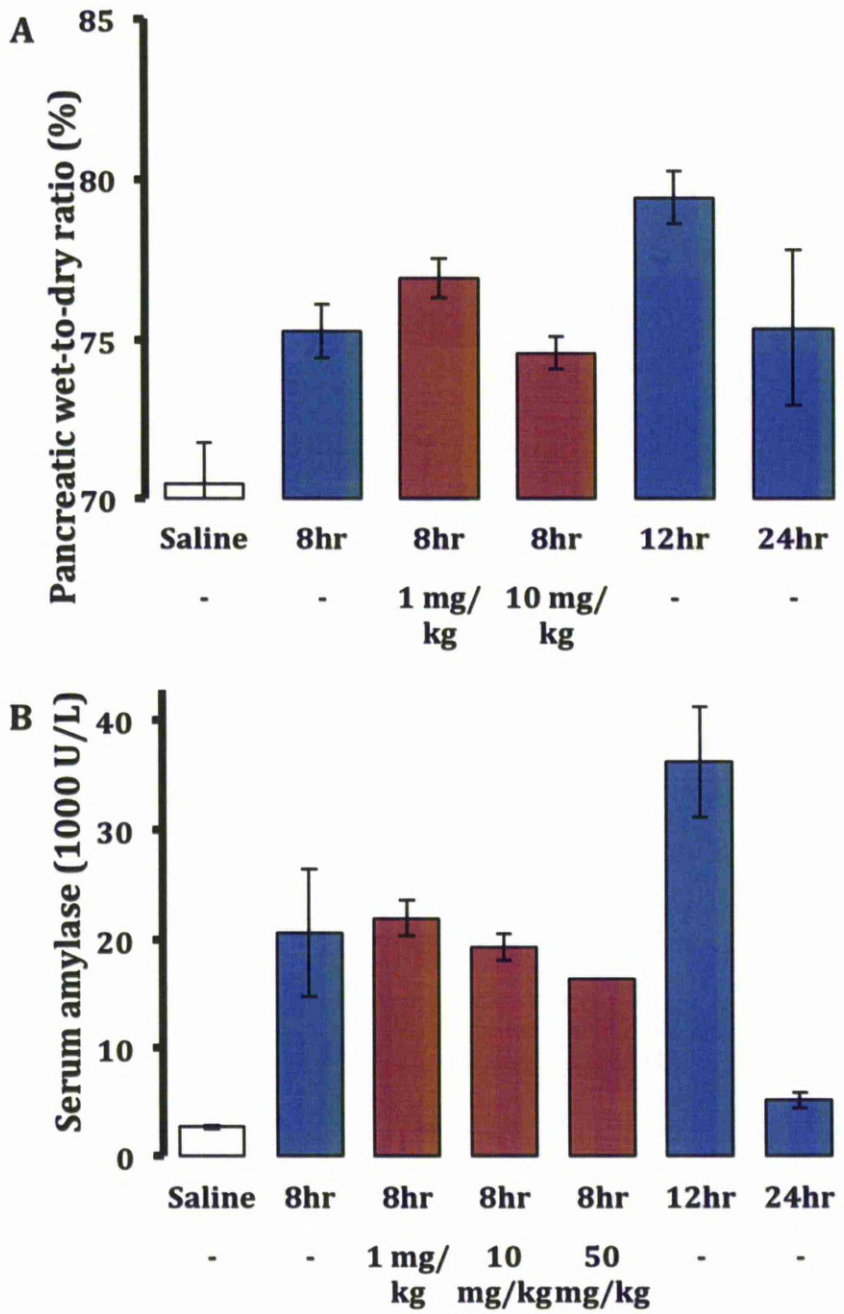


Figure 5.1: Effects of caffeine on pancreatic oedema (A) and serum amylase (B) in caerulein induced pancreatitis. All animals (6 per group) received 7 hourly injections of caerulein (blue), except saline controls (white). Treatment groups (red) received caffeine 1 or 10 mg/kg (red) (and two animals given 50 mg/kg). Animals were sacrificed at 8 hours (12 and 24 caerulein results included for comparison). Bars are mean values ± SEM.

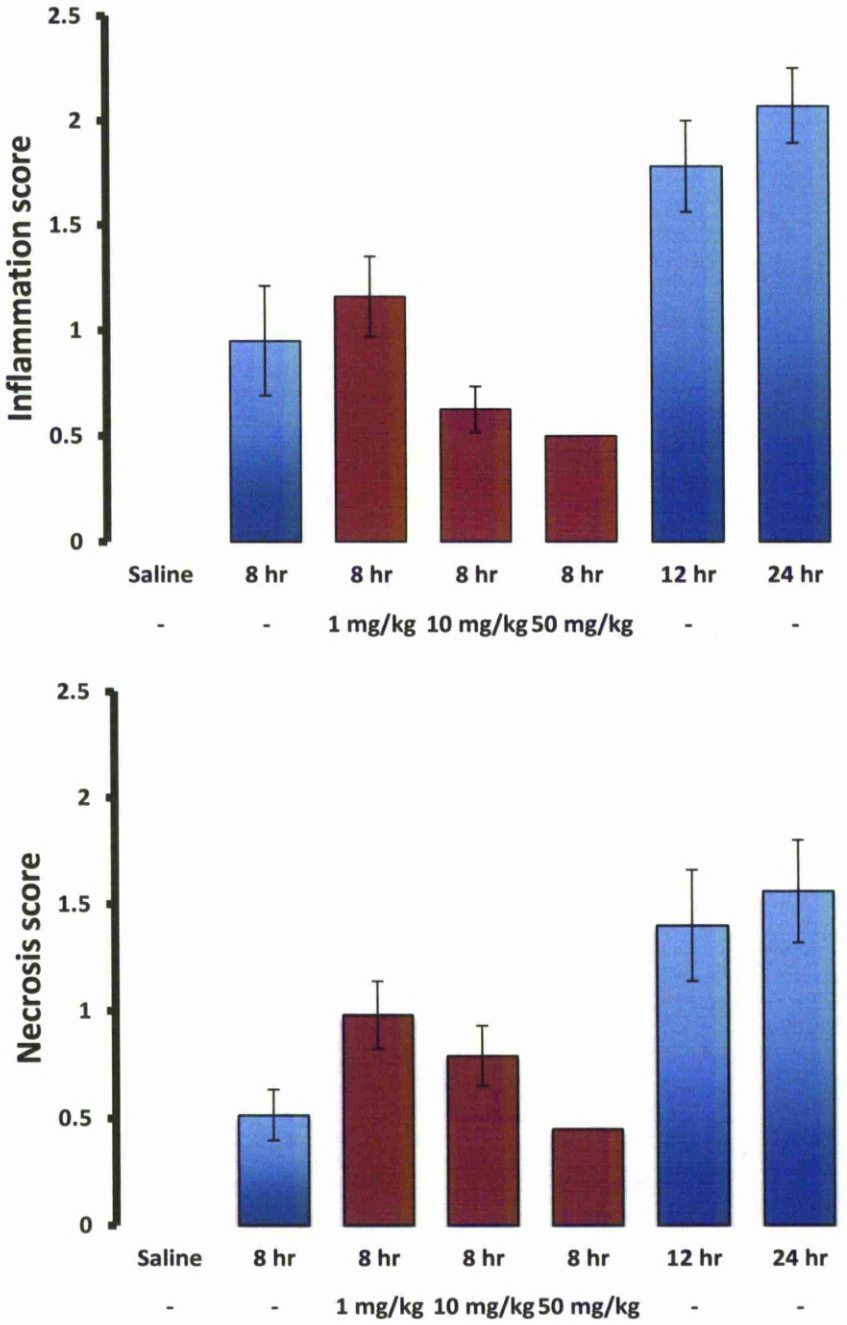


Figure 5.2: Effects of caffeine on histopathological features of caerulein induced pancreatitis. All animals (6 per group) received 7 hourly injections of caerulein (blue), except saline controls (white). Treatment groups (red) received caffeine 1 or 10 mg/kg (red) (and two animals given 50 mg/kg). Animals were sacrificed at 8 hours (12 and 24 caerulein results included for comparison). Graphs show overall histopathological score (top) and oedema component score (bottom). Bars are mean values ± SEM.

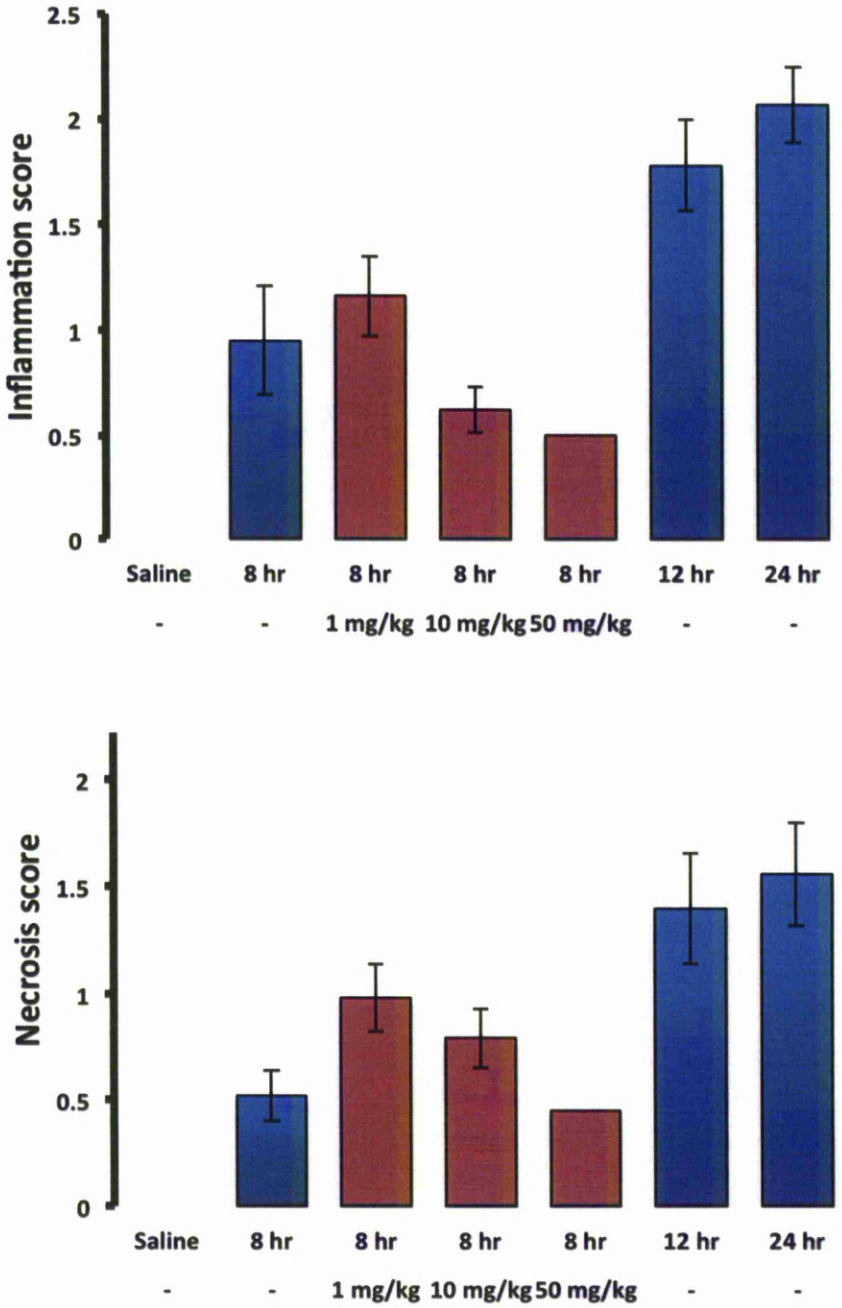


Figure 5.2 continued: Effects of administering caffeine in caerulein-induced pancreatitis. Inflammation (top) and necrosis (bottom) component of histopathological scores shown. Bars are mean values ± SEM.

5.4 Summary of effects of caffeine on severity in caerulein-induced murine pancreatitis

These results describe, for the first time, experiments investigating the potential protective effect of caffeine in *in* vivo caerulein-induced pancreatitis. Caffeine at a concentration of 1 mg/kg did not demonstrate any effects in reducing severity of acute pancreatitis in a caerulein-induced murine experimental model. However increasing the concentration of caffeine to 10 mg/kg showed a significantly lower histopathological score. Both these concentrations produced no evidence of toxic side-effects in live mice. There was a slighter lower pancreatic wet-to-dry weight ratio and serum amylase in the 10 mg/kg group, but these results were not statistically significant. Interestingly the pancreatic wet-to-dry weight ratio (as proxy for pancreatic oedema) showed no difference with 10 mg/kg caffeine, yet the oedema component of the histopathological score showed a marked decrease in mean value. It is difficult to reconcile these two results, but of all the parameters the histopathological score is usually taken as the 'gold standard' for severity assessment and carries more weight. It is more likely a reflection of the difficulty inherent in utilising the pancreatic wet-to-dry ratio parameter. It involves handling and measuring the weight of very small

pieces of pancreas tissue. Very small errors in weighing and reweighing samples can produce widely vary results. As such the pancreatic wet-to-dry ratio should be treated with some caution, and the histopathological score given greater weight.

One potential criticism that could be levelled at the methodology is the administration of caffeine simultaneously with caerulein, as an hourly i.p. injection (albeit in separate syringes and separate i.p. sites). There is a small possibility caffeine interacts with caerulein directly within the murine peritoneal cavity, prior to absorption. In order to show this is not the case the experiments would need to be repeated with caffeine given a few hours after caerulein (i.e. at the mid-point of the experiment) to allow hypersecretion pancreatitis to begin and then demonstrate if caffeine can still ameliorate the disease.

The results for 50 mg/kg were included because of the dramatic effect this concentration showed in reduction of severity of caerulein-induced acute pancreatitis, as evidenced by the reduction of the histopathological score and (to a lesser extent) the amylase score. Even though caution must be employed in drawing conclusion from only two animals, these results show that increasing doses of caffeine seem to have a protective effect in

caerulein-induced pancreatitis. However 50 mg/kg produced significant toxic side effects.

As previously described caffeine reduces Ca^{2+} signalling by reversibly inhibiting IP_3R Ca^{2+} channels (Petersen and Sutton, 2006) and inhibition of Ca^{2+} signalling is already a well established mechanism of reducing pancreatitis. It is of course entirely possible that other pathways are involved. Caffeine is known to reduce cyclic adenosine monophosphate (cAMP) by inhibiting one of the necessary activating enzymes, cAMP-phosphodiesterase (cAMP-PDE)(Wu et al., 2009). cAMP is involved in the initiation of the inflammatory response. It has previously been shown that inhibiting cAMP-PDE by using specific inhibitors such as pentoxifylline (Chooklin et al., 2008)(Matheus et al., 2009)(De Campos et al., 2008) and rolipram (Sarmiento et al., 2010) reduces the severity of pancreatitis and caffeine could be working in a similar manner.

These initial results suggest a concentration of caffeine between 20-30 mg/kg may produce protective effects without significant side effects. In addition the main metabolites of caffeine should be tested to determine if the protective mechanism is regulated by one of these specifically.

CHAPTER 6

DISCUSSION

6.1 DISCUSSION

6.1 The need for research in acute pancreatitis

The human disease of acute pancreatitis (AP) currently has limited therapy which can ameliorate or halt the progression of severe disease (Pandol et al., 2007). Severity of human AP is determined not only by localised pancreatic acinar cell (PAC) injury, but also by systemic effects, regulated by the systemic inflammatory response and potentially leading to multiple organ injury/failure. This systemic process takes time to develop, certainly over many hours, if not days, suggesting a potential therapeutic window for amelioration of the disease process. Indeed pancreatic necrosis, consistently associated with severe disease, is usually not present for the first 24-48 hours after disease onset. There is still a great deal that is unknown during the initiation of PAC injury and so research has tended to focus on the response of PACs to injury and try to elucidate the cellular mechanisms involved in the hope of identifying therapeutic targets.

Multiple mammalian experimental *in vivo* and *in vitro* models have been develop to discover possible therapeutic targets. In particular is the extensive use of the murine experimental model, which in recent decades

has allowed great advances in understanding of pancreatic acinar cell responses. Many different murine models have been developed to test specific hypotheses of disease initiation. These range from responses to toxic stimulation, to testing responses of genetic knockouts and transgenic models to examine the actions of specific components of cellular pathways. Many of these models seek to emulate an aspect of human disease; for example perfusion of pancreatic ducts with bile salts emulates conditions during gallstone pancreatitis (a common cause of human disease). Other models are less amenable to direct human correlation. One such example of a controversial model is the hyperstimulation model, typically utilising cholecystokinin or its analogue caerulein. Although this model is widely employed in research into AP, (and indeed has been employed in this body of work), there has been debate regarding its correlation to human disease. Chapter 3 of this thesis describes work undertaken to further explore the relevance of the murine hyperstimulation model to human disease, and is discussed in more detailed subsequently.

In fact this point of clinical relevance can be expanded to many other more 'clinically relevant' models: that findings in all experimental models need to be confirmed by repeating experiments (where possible) with human tissue. Inter-species differences in PACs responses exist in animal models, and

there is no reason to suppose that differences exist in human disease too. This likely explains the failure of previous human clinical trials of therapeutics, failing to demonstrate responses that had been shown in animal models. However there is still an overarching greater similarity between the various mammalian models, and identification of suitable targets for human therapeutics are much more rapidly generated by continued animal research. This work should be increasing undertaken in parallel with work on human tissue (subject to all appropriate ethical and legal controls).

Recent advances in basic science research have provided insight into the mechanisms and pathways involved. Our own group, in Liverpool, has shown the importance of abnormal $Ca^{2=}$ signalling, in response to various toxins and stressors (inc. FAEE, ROS), in the development of AP (Criddle et al., 2006)(Petersen et al., 2011). Other important work has explored the role of other mechanisms, such as mitochondrial injury (Mukherjee et al., 2008)(Gukovsky et al., 2011), and premature trypsinogen activation (Dawra et al., 2011).

What is clear is that AP is a very complex process, with many potential interacting mechanisms and pathways. The potential crossover between all

the various participants is huge and adds to this complexity. Work must continue to further clarify these processes, and likely uncover previously unknown actions, if a therapy is to be found.

6.2 The relevance of the CCK-hyperstimulation model to human acute pancreatitis

The hyperstimulation model, utilising neuro-hormones such as ACh and CCK (or is analogue caerulein), has been controversial as an experimental model, due to the uncertainty surrounding clinical relevance. It is widely employed in both *in vivo* and *in* vitro work, partly due to reliability and convenience. Hyperstimulation human acute pancreatitis does exist, although it is rare. It occurs in exposure to a species-specific (Tityus trinitatis) scorpion venom and has been described in exposure to organophosphates. The role of CCK in commoner aetiology (i.e. gallstone disease and alcohol) in human AP is unclear. In addition published work had suggested CCK had no direct effect on isolated human PACs (Ji et al., 2001)(Owyang et al., 2004), which seriously undermined the continued use of this model.

In Chapter 3 the direct effects of CCK-8 (and an important sub-type CCK-58) was explored in human tissue. Working within a collaboration our group definitively demonstrated that CCK-8 and CCK-58 do elicit direct responses from human isolated PACs (Murphy et al., 2008). This was confirmed by undertaking experiments in the presence of inhibitors of other neuro-hormonal pathways, so that responses must have been through activation of CCK receptors only. We found that human tissue had to be selected for optimal viability, which invariably meant coming from donors with pancreatic diseases not obstructing the pancreatic duct. We also optimised the transport solution and found that rapid transport to the laboratory for immediate preparation of cells was vital. CCK stimulation led to responses in isolated human tissue that closely mimicked that seen in murine PACs, from transient $[Ca^{2+}]_i$ spikes with physiological concentrations to elevated, persistent toxic signalling with hyperstimulation. It is important to show that these responses lead to real secretion mechanisms. This was accomplished experimentally by loading zymogen granules with quinacrine and demonstrating stimulus-metabolic-secretion coupling.

Work was also undertaken to explore differences in effects between CCK-8 and CCK-58, as previous work had suggested that CCK-58 might be more physiologically relevant (Reeve et al., 2003 and 2004). Experiments in both

human and murine PACs showed remarkably similar responses to both CCK-8 and CCK-58.

Taken together this work has justified the continued use of the murine hyperstimulation model, with CCK/caerulein, in further investigate of acute pancreatitis. It has also demonstrated the importance of undertaking animal experimental animal work in parallel with human tissue to confirm common mechanisms.

Future work should continue to utilise human tissue (guided by all appropriate ethical and legal obligations) whenever possible. Tissue slices may provide more useful interpretations than isolated cells, as the latter are, by their nature of preparation, less physiological and more prone to unintended injury.

6.3 Role of Bcl-2 family proteins in acute pancreatitis

Whilst there is strong evidence that necrosis is always detrimental in acute pancreatitis (Pandol et al., 2007) the role of apoptosis may not be entirely straightforward. For most of the last two decades it has been held that apoptosis is protective in pancreatitis (Bhatia et al., 2004), which has been supported by experimental work. Such work included observations that severe AP models histologically show more evidence of necrosis (e.g. duct ligation in the opossum, caerulein hyperstimulation in mice), whilst milder AP models are found on histological examination to have evidence of more apoptosis (e.g. PD ligation and caerulein hyperstimulation in rats). More direct experimental work involved exposing murine models to agents known to induce apoptosis (such as 1-Cyano-2-hydroxy-3-butene (CHB)) and then inducing AP via the caerulein model (Bhatia et al., 1998). The effects of AP were ameliorated, but then it is possible that CHB could be acting via many other pathways and not just via the apoptotic pathway.

Apoptosis is regulated by two pathways: the extrinsic pathway (involving death receptor activation) and the intrinsic pathway (regulated by cytochrome c release and is closely linked to mitochondrial injury). These two pathways are not mutually exclusive, and there is cross-talk between

them. So numerous, complex interactions with participating agents (often having both positive and negative feedback behaviour) exist and the full extent of all these interactions is very unclear. The Bcl-2 family of proteins are well-known regulators of the intrinsic pathway, but recent evidence suggests possible action on the extrinsic pathway (He et al., 2009). Several members of the Bcl-2 family have been described in terms of their apoptotic activity as pro-apoptotic (Bak and Bax) or pro-survival/anti-apoptotic (Bcl-2 and Bcl-xL) (Bhatia, 2004)(Kroemer et al., 2007). However that view is being recognised as possibly simplistic (Sung KF et al., 2009)(Gukovsky et al., 2011). Apoptosis may not be protective in every situation. We undertook experiments to further investigate the role of Bak, Bax and Bcl-2 in acute pancreatitis. It is generally believed from *in vitro* work that Bak and Bax have overlapping roles (Brooks et al., 2007) in apoptosis and mitochondrial injury. Their role in mitochondrial injury, and formation of the mitochondrial permeability transition pore (MPTP), is important for optimal cytochrome c release and intrinsic pathway activation. However some cytochrome c release can occur in the absence of both genes (Brooks et al., 2007). Bak and Bax also regulate the IP_3R on the endoplasmic reticulum surface and have important effects on Ca^{2+} release needed during apoptosis (Oakes et al., 2005). Isolated cells deficient in Bak and Bax have markedly reduced stores of ER Ca^{2+} and have been shown to be resistant to

Ca^{2+}-dependent death stimuli (Oakes et al., 2005). To date no work has been reported in *in vivo* Bcl-2 family knockouts.

The work described in Chapter 4 details experiments with caerulein-induced acute pancreatitis in Bak, Bax and Bcl-2 KO mice. The results were contra to apoptosis always being protective. In Bak KO and Bax KO it was hypothesised that AP would be more severe and this would be related to less apoptosis. However in Bak KO less severe pancreatitis was found at all time points, and with no measurable effect on apoptotic activity as compared to controls. Bax KO produced an even more complex picture with more severe disease in the first 12 hours. There was evidence of necrosis from a very early time point (8 hours) but was then followed with *increased* apoptotic activity between 12 and 24 hours. These results are unexpected and difficult to reconcile with current understanding of cell death pathways in AP. Possible explanations are:

i) Bax is important in the mitochondrial response to injury and in activation of the intrinsic pathway of apoptosis via cytochrome c release. In Bax KO these roles are missing and early phase (within first 8 hours) mitochondrial injury is much more likely to lead to necrosis than to apoptosis. This would also explain why necrosis is seen on histology at 8 hours (which is not seen

so early in any other experimental group), and why the overall severity of AP is higher.

ii) The increased apoptotic activity (as observed through caspase-3 IHC staining) may be due to activation of the extrinsic pathway of apoptosis. This could be triggered by the necrosis already occurring in some of the damaged PACs, and would suggest that under normal conditions Bax may even have an inhibitory function on the extrinsic pathway, that is lost in Bax KO. The development of necrosis leading onto extrinsic pathway activation would explain the time delay of apoptosis appearing by 12 hours.

ii) This is an experimental error in interpretation of the caspase-3 IHC staining data. The histopathological score was reasonably consistent, and fitted with the original hypothesis that Bax KO should develop more severe AP. What was surprising was the degree of apoptotic activity at 8 and 12 hours, as measured by caspase-3 staining. The concern would be that areas of necrosis are staining for caspase-3 and confounding results. Histological appearances do not immediately suggest this, and in many slides it was observed that non-staining, clearly necrotic regions were adjacent to heavily staining, and assumed apoptotic, regions. It is possible that these heavily stained areas could be PACs that initiated apoptosis, but were unable to

complete and switched to necrosis. The time points of 12 and 24 hours could coincide for when this switch is occurring. This would not immediately explain why necrosis is seen early on in histopathology, yet caspase-3 staining takes 12 hours to rise significantly.

The results from *in vivo* AP experiments in Bcl-2 family KO mice are the first undertaken and are intriguing. If genuine they suggest that there are any aspects of the roles of Bax and Bak that are not fully understood, and that the prevailing view that apoptosis is protective in all settings may not be true. Bak KO showed decrease in severity of AP, but without any significant change in apoptotic activity. This could be explained by relation to the finding in *in vitro* work that Bak regulates IP3R function and Bak KO leads to reduced Ca^{2+} signalling, preventing toxic signalling and resistance to Ca^{2+}-related death pathways (Oakes et al., 2005). Bcl-2 KO showed no significant change in either severity or apoptotic activity from controls. Bax KO showed more dramatic, and more challenging, results by demonstrating an increase in early severity, with necrosis, followed 12 hours later with a dramatic increase in apoptotic activity.

Further work is needed to clarify the roles of Bcl-2 family proteins. In particular the Bax gene appears to play a critical role in severity, and

warrants further investigation. *In vitro* work with Bax KO models would help clarify the mix of necrosis and apoptosis demonstrated in these experiments. Bak KO led to less severe disease do that agents that target Bak function would be of potential interest.

6.4 Role of caffeine as a potential therapeutic

Caffeine was investigated for its potential therapeutic value. This potential was derived from caffeine's action in preventing toxic Ca^{2+} signalling in isolated murine PACS, and (since the work described in Chapter 3) in isolated human PACs. Caffeine has been shown to act on the IP_3R receptor Ca^{2+} channels found on the ER surface, and to a lesser extent on ZGs. Two subtypes, IP_3R2 and IP_3R3, are found in PACs (Wojcikiewicz et al., 1995), with IP_3R2 being most critical to IP_3-induced Ca^{2+} release (Park et al., 2008). Our group has shown that caffeine inhibits IP_3R and IP_3 production (Wakui et al., 1990) and specific inhibition, or knockout, of IP_3R markedly reduces toxic Ca^{2+} and premature trypsinogen activation *in vitro* (Gerasimenko et al., 2009).

The work described in Chapter 5 is the first, to our knowledge, to test the effects on caffeine on murine experimental models of caerulein-induced acute pancreatitis *in vivo*. It was found that a doses-dependent protective effect of caffeine did exist in this experimental model. At a concentration of 1 mg/kg, caffeine showed no effects on the course of caerulein-induced pancreatitis. However increasing the dose to 10 mg/kg showed a protective effect with significantly less evidence of histopathological disease at 8 hours. There were no observable detrimental side effects in the animals at either of these concentrations. A higher concentration of 50 mg/kg appeared to be even more effective, but produced severe side-effects of caffeine toxicity in the animals tested and experiments with this dose were abandoned. Also only two animals received 50 mg/kg, so some caution needs to be employed when considering results from such a small group. Even so the preliminary findings are encouraging.

Caffeine produces its effects on Ca^{2+} signalling *in vitro* through inhibitory action on IP_3R. This was the rationale for these *in vivo* experiments. However it is possible it acts through other pathways. Caffeine is known to act on phosphodiesterase (PDE) and inhibition of this pathway may be a factor in decreasing severity of disease, through an unknown mechanism (Camello et al., 1996).

The protective effects of caffeine need to be further explored with *in vivo* murine models. Suggestions for future work include:

i) Repeating experiments with a caffeine concentration of 20-30 mg/kg. This may provide further protective effect whilst keeping toxic side effects within acceptable levels.

ii) Repeating experiments at longer times points (either 12 and 24 hours) as this will demonstrate whether caffeine protection lasts over a time frame that caerulein-induced pancreatitis is known to progress.

iii) Measure serum caffeine levels to ensure the estimated concentration of about 0.5 mM is correct. This serum level may be too high for human use.

iv) Measure other parameters associated with pancreatitis both locally (e.g. trypsin activity) and systemically (e.g. lung histology, IL-6 levels)

v) Repeating experiments with caffeine metabolites (theophylline, paraxanthine, theobromine) to assess if any of these are actually producing the protective effect.

vi) Giving caffeine at the mid-point of the experiment, to counter any suggestion that it interferes with caerulein absorption when they are administered together.

Caffeine represents an intriguing possible therapeutic agent for the treatment of acute pancreatitis. It is already used as therapeutic in neonatal apnoea, but much experimental work remains to clarify its mechanism and optimal effective dosage before human trials could be contemplated. It might be that a caffeine analogue may have similar protective effects, at much smaller concentrations, reducing the risks of toxic side effects

6.5 Future directions

Currently AP is without specific therapy, yet rapid advances have been made especially in the last two decades with regards to understanding of underlying mechanisms. This understanding has been brought in large part by basic science research with animal models. There is an increasing need for parallel work in human tissue, to validate which mechanisms translate across species, and thus are suitable targets for therapy. Once suitable agents have demonstrated efficacy in animal models they can be considered for the various phases of human clinical trials, and find an effective treatment for this common debilitating disease.

Statement of originality

The work undertaken and described in this thesis was carried out while I was employed as a clinical research fellow within the Division of Surgery and Oncology (later School of Cancer Studies) and the Department of Physiology (under the Institute of translational medicine), University of Liverpool. Most of the experimental work was carried out between Feb 2005 and Apr 2008. This thesis has been written entirely by myself.

The majority of experimental work was carried out by myself, except for some sections of Chapter 3, which were produced by joint effort within our collaboration. Some figures were produced by other members of our group and are reproduced with permission. These include figure 3.7 (Mr J Murphy and Dr M Chanov) and figure 3.8 (Dr J Gerasimenko). Some work in chapter 3 was undertaken jointly by myself and Dr D Criddle (figures 3.3, 3.4, 3.5, 3.6 and 3.9). Serum amylase measurements were performed by the Department of Biochemistry and H&E slide preparation by the Department of Pathology at the Royal Liverpool University Hospital. Bak, Bax and Bcl-2 animals were originally supplied by Prof. M. Pritchard and Prof. A. Watson, Division of Gastroenterology. Some IHC staining was performed by the Department of Immunohistochemistry, University of Liverpool.

Publications associated with this thesis

Criddle D, Booth D, Mukherjee R, McLaughlin E, Green G, Sutton R, Petersen O, Reeve J. CCK-58 and CCK-8 exhibit similar actions on calcium signaling, zymogen secretion, and cell fate in murine pancreatic acinar cells. Am J Physio Gastro Liver Physiol 2009; 297: G1085-G1092

Murphy JA, Criddle DN, Sherwood M, Chvanov M, Mukherjee R, McLaughlin EW, Booth D, Gerasimenko J, Raraty MG, Ghaneh P, Neoptolemos JP, Gerasimenko OV, Tepikin AV, Green GM, Reeve JR, Petersen OH, Sutton R. Direct activation of cytosolic Ca2+ signaling and exocytosis by cholecystokinin in human pancreatic acinar cells. Gastroenterology 2008; 135(20): 632-641

Criddle DN, McLaughlin E, Murphy JA, Petersen OH, Sutton R. The pancreas misled: signals to pancreatitis. Pancreatology 2007; 7: 436-46

Raraty MG, Murphy JA, McLoughlin E, Smith D, Criddle D, Sutton R. Mechanisms of acinar cell injury in acute pancreatitis. Scand J Surg. 2005; 94(2): 89-96.

References

Anastasi A, Erspamer V, and Endean R. Isolation and structure of caerulein, an active decapeptide from the skin of Hyla caerulea. Experientia 1967; 23: 699-700

Andrzejewska A, Dlugosz JW, Jurkowska G. The effect of antecedent acute ethanol ingestion on the pancreas ultrastructure in taurocholate pancreatitis in rats. Exp Mol Pathol 1998; 65: 64-77.

Ashby MC, Craske M, Park MK, Gerasimenko OV, Burgoune RD, Peterson OH, Tepikin AV. Localised calcium uncaging reveals polarised distribution of calcium sensitive calcium release sites: mechanism of unidirectional calcium waves. J Cell Biol 2002; 158: 283 – 92

Azima B, Kao RL, Youngberg G, et al. A new animal model of reversible acute pancreatitis. J Surg Res 1996; 63: 419-424

Bakker OJ, van Santvoort HC, Besselink MG, van Der Harst E, Hofker HS, Gooszen HG. Prevention, detection, and management of infected necrosis in severe acute pancreatitis. Current Gastroenterol Rep 2009; 11: 104-110

Banks PA & Freeman ML. Practice guidelines in acute pancreatitis, American Journal of Gastroenterology 2006; 101:2379-400

Balthazar EJ, Ranson JHC, Naidich DP, Megibow AJ, Caccavale R, Cooper MM. Acute pancreatitis: prognostic value of CT. Radiology 1985; 156:767-772

Barros LF, Hermosilla T, Castro J. Necrotic volume increase and the early physiology of necrosis. Camp Biochem Physiol A 2001; 130: 401-409

Barzilai A, Ryback BJ, Medina JA, Toth L, Dreiling DA. The morphological changes of the pancreas in hypovolemic shock and the effect of pretreatment with steroids. International Journal of Pancreatology 1987; 2: 23-32

Belan PV, Gerasimenko OV, Tepikin AV, Peterson OH. Localisation of calcium extrusion sites in pancreatic acinar cells. J Biol Chem 1996; 271: 7615-9

Bhatia M, Wallig M, Hofbauer B, Lee HS, Frossard JL, Steer ML, Saluja AK. Induction of apoptosis in pancreatic acinar cells reduces the severity of acute pancreatitis. Biochemical and biophysical Research Communications 1998; 246: 476-483

Biczo G, Hegyi P, Dosa S, Shalbuyeva N, Berczi S, Sinervirta R, Hracsko Z, Siska A, Kukor Z, Jarmay K, Venglovecz V, Varga IS, Ivanyi B, Alhonen L, Wittmann T, Gukovskaya A, Takacs T, Rakonczay Z. The Crucial Role of Early Mitochondrial Injury in L-Lysine-Induced Acute Pancreatitis. Antioxid Redox Signal 2011;15(10):2669-2681

Bloom, W and Fawcett, DW, A Textbook of Histology 10th ed., WB Saunders, Philadelphia, 1975, p. 115

Bollen TL, van Santvoort HC, Besselink MG, van Leeuwen MS, Horvath KD, Freeny PC, Gooszen HG. The Atlanta Classification of acute pancreatitis revisited. British Journal of Surgery 2008; 95: 6-21

Bonati M, Latini R, Tognoni G, Young JF, Garattini S. Interspecies comparison of in vivo caffeine pharmacokinetics in man, monkey, rabbit, rat, and mouse. Drug Metab Rev 1984; 15: 1355-83.

Bradley EL III. A clinically based classification system for acute pancreatitis. Summary of the International Symposium on Acute Pancreatitis, Atlanta, GA, September 11 through 13, 1992. Arch Surg 1993; 128: 586-590

Bradley III. Acute pancreatitis: Novel Concepts in Biology and Therapy. Blackwell Science 1999. Ch. 21: A natural history-based clinical classification system for acute pancreatitis.

Brooks C, Wei Q, Feng L, Dong G, Tao Y, Mei L, Xie ZJ, Dong Z. Bak regulates mitochondrial morphology and pathology during apoptosis by inertacting with mitofusins. PNAS 2007; 104(28): 11649-11654

Buchler M, Malfertheiner P, Uhl W, Schölmerich J, Stöckmann F, Adler G, Gaus W, Rolle K, Beger HG. Gabexate mesilate in human acute pancreatitis. German Pancreatitis Study Group. Gastroenterology 1993; 45: 97-104

Buter A, Imrie C, Carter CR, Evans s, McKay CJ. Dynamic nature of early organ dysfunction determines outcome in acute pancreatitis. British Journal of Surgery 2002; 89: 298-302

Camello PJ, Petersen OH, Toescu EC. Simultaneous presence of cAMP and cGMP exert a co-ordinated inhibitory effect on the agonist-evoked Ca2+ signal in pancreatic acinar cells. Pflugers Arch 1996; 432: 775-81

Campos-Toimil M, Edwardson JM, Thomas P. Real-time studies of zymogen granule exocytosis in intact rat pancreatic acinar cells. J Physiol 2000; 528: 317-326

Cancela JM, Van Coppenolle F, Galione A, Tepikin AV, Peterson OH. Transformation of local calcium spikes to global calcium transients: the combinatorial roles of multiple calcium releasing messengers. EMBO J 2002; 21: 909 -19

Carter CR, McKay CJ, Dickson EJ. Ch 13. Acute pancreatitis. Companion to specialist surgical practice: Hepatobiliary and pancreatic surgery. Fourth edition. Saunders, Elsevier. Edited by OJ Garden. 2009

Chan YC, Leung PS. Acute pancreatitis: animal models and recent advances in basic research. Pancreas 2007; 34: 1-14

Chaudhrui A, Kolodecik TR, Gorelick FS. Effects of increased intracellular cAMP on carbachol-stimulated zymogen activation, secretion, and injury in the pancreatic acinar cell. American Journal of Physiology Gastroinest Liver Physiol 2005; 228: G235-G243

Chen X, Li SL, Wu T, Liu JD. Proteasome inhibitor ameliorates severe acute pancreatitis and associated lung injury of rats. World J Gastroenterology 2008, 14, 3249-53

Chetty U, Gilmour HM, Taylor TV. Experimental acute pancreatitis in the rat-a new model. Gut 1980; 2: 115-7

Chey, WY. (1993). Hormonal control of pancreatic exocrine secretion. In: The Pancreas: Biology, Pathobiology and Disease, Eds Go, VLW, Dimango EP, Gardner, J., Lebenthal, E., Reber, HA, Scheele, GA. pp 403-424. Raven Press New York

Chin, Jenna M.; Merves, Michele L.; Goldberger, Bruce A.; Sampson-Cone, Angela; Cone, Edward J. "Caffeine Content of Brewed Teas". J Anal Toxicol 2008; 32(8): 702-704

Chooklin S, Pereyaslov A, Bihalskyy I. Pathogenic role of myeloperoxidase in acute pancreatitis. Hepatobiliary Pancreat Dis Int 2009; 8: 627-31

Corfield AP, Cooper MJ, Williamson RCN et al. Prediction of severity in acute pancreatitis: a prospective comparison of three prognostic indices. Lancet 1985;II: 403-406

Criddle DN, Raraty MG, Neoptolemos JP, Tepikin AV, Petersen OH, Sutton R. Ethanol toxicity in pancreatic acinar cells: mediation by nonoxidative fatty acid metabolites. Procedures of The National Academy of Science USA 2004: 101: 10738-10743

Criddle DN, Murphy J, Fistetto G, Barrow S, Tepikin AV, Neoptolemos JP, Sutton R, Petersen OH. Fatty acid ethyl esters cause pancreatic calcium toxicity via inositol trisphosphate receptors and loss of ATP synthesis. Gastroenterology 2006; 130:781-793

Criddle DN, Gerasimenko JV, Baumgartner HK, Jaffar M, Voronina S, Sutton R, Petersen OH, Gerasimenko OV. Calcium signalling and pancreatic cell death: apoptosis or necrosis? Cell Death Differ 2007a; 14: 1285-94

Criddle DN, McLaughlin E, Murphy JA, Petersen OH, Sutton R. The pancreas misled: signals to pancreatitis. Pancreatology 2007b; 7: 436-46

Criddle D, Booth D, Mukherjee R, McLaughlin E, Green G, Sutton R, Petersen O, Reeve J. CCK-58 and CCK-8 exhibit similar actions on calcium signaling, zymogen secretion, and cell fate in murine pancreatic acinar cells. Am J Physio Gastro Liver Physiol 2009; 297: G1085-G1092

Grocock CJ, Rebours V, Delhaye MN, et al. The variable phenotype of the p.A16V mutation of cationic trypsinogen (PRSS1) in pancreatitis families. Gut 2010; 59(3): 357-363

Dawra R, Sharif R, Phillips P, Dudeja V, Dhaulakhandi D, Saluja AK. Development of a new mouse model of acute pancreatitis induced by administration of L-arginine. American journal of Physiology: Gastroenterology and Liver Physiology 2007; 292: G1009-1018

Dawra R, Sah RP, Dudeja V, Rishi L, Talukdar R, Garg P, Saluja AK. Intra-Acinar Trypsinogen Activation Mediates Early Stages of Pancreatic Injury but not Inflammation in Mice with Acute Pancreatitis. Gastroenterology 2011; 141: 2210-2217

De Campos T, Deree J, Martins JO, Loomis WH, Shenvi E, Putnam JG, Coimbra R. Pentoxifylline attenuates pulmonary inflammation and neutrophil activation in experimental acute pancreatitis. Pancreas 2008; 37: 42-9

Dellinger EP, Tellado JM, Soto NE, Ashley SW, Barie PS, Dugernier T, Imrie CW, Johnson CD, Knaebel HP, Laterre PF, Maravi-Poma E, Kissler JJ, Sanchez-Garcia M, Utzolino S. Early antibiotic treatment for severe acute necrotizing pancreatitis: a randomized, double-blind, placebo-controlled study. Annals of Surgery 2007; 245: 674-683

Dimagno MJ, Lee SH, Owyang C, Zhou SY. Inhibition of acinar apoptosis occurs during acute pancreatitis in the human homologue DeltaF508 cystic fibrosis mouse. Am J Physiol Gastrointest Liver Physiol 2010; 299: G400-12

Domínguez-Muñoz JE, Carballo F, García MJ, de Diego JM, Campos R, Yangüela J, de la Morena J. Evaluation of the clinical usefulness of APACHE II and SAPS systems in the initial prognostic classification of acute pancreatitis: a multicenter study. Pancreas 1993; 8(6):682-686

Dominguez-Munoz. Ch. 20: Diagnosis of acute pancreatitis: any news or still amylase? Acute Pancreatitis: Novel Concepts in Biology and Therapy. Eds Büchler MW, Uhl W, Friess H, Malfertheiner P. Berlin; Blackwell Wissenschafts-Verlag, 1999, pp 171-179

Douglas WW. Stimulus-secretion coupling: the concept and clues from chromaffin and other cells. Br Jour of Pharm 1968; 34: 451-474

Dressel TD, Goodale RL Jr, Zweber B, Borner JW. The effect of atropine and duct decompression on the evolution of Diazinon-induced acute canine pancreatitis. Ann Surg, 1982; 195: 424-34

Dufresne M, Seva C, Fourmy D. Cholecystokinin and gastrin receptors. Physiol Rev 2006; 86: 805-847

Eberlein GA, Eysselein VE, Hesse WH, Goebell H, Schaefer M, Reeve JR Jr. Detection of CCK-58 in human blood by inhibition of degradation. Am J Physiol Gastrointest Liver Physiol 1987; 253: G477-G482

Elman R, Arneson N, Graham. Value of blood amylase estimations in the diagnosis of pancreatic disease. Arch Surg 1929; 19: 943-965

Erenberg A, Leff RD, Haack DG. Caffeine citrate for the treatment of apnea of prematurity: a double-blind placebo-controlled trial. Pharmacotherpay 2000; 20: 644-652

Fitz RH. Acute pancreatitis: A consideration of pancreatic haemorrhage, haemorrhagic suppurative, and gangrenous pancreatitis and of disseminated fat necrosis. Boston Ned Surg Joun (1889);120: 181-187, 205-207, 229-235

Foitzik T, Lewandrowski KB, Fernandez-Del Castillo C, Rattner DW, Klar E, Warshaw AL. Exocrine hyperstimulation but not pancreatic duct obstruction increases the susceptibility to alcohol-related pancreatic injury. Arch Surg 1994; 129: 1081-5

Formela LJ, Galloway S, Kingsnorth AN. Inflammatory mediators in acute pancreatitis. Br J Surg 1995; 82:6-13

Fredholm BB, Battig K, Holmen J, Nehlig A, Zvartau EE. Actions of caffeine in the brain with special reference to factors that contribute to its widespread use. Pharmacol Rev 1999; 51: 83-133

Frossard JL. Trypsin activation peptide (TAP) in acute pancreatitis: From pathophysiology to clinical usefulness. JOP. J.Pancreas (online) 2001; 2(2): 69-77

Frossard JL, Rubbia-Brandt L, Wallig MA, Benathan M, Ott T, Morel P, Hadengue A, Suter S, Willecke K, Chanson M. Severe acute pancreatitis and reduced acinar cell apoptosis in the exocrine pancreas of mice deficient for the Cx32 gene. Gastroenterology 2003; 124: 481-493

Galindo J, Jones N, Powell G, Hollingsworth S, Shankley N. Advanced qRT-PCR technology allows detection of the CCK 1 receptor (CCK1R) expression in human pancreas. Pancreas 2005; 31(4): 325-331

Galluzzi L, Blomgren K, Kroemer G. Mitochondrial membrane permeabilisation in neuronal injury. Nat Rev Neurosci 2009; 10: 481-494

Gardner TB, Vege SS, Chari ST, Petersen BT, Topazian MD, Clain JE, Pearson RK, Levy MJ, Saar MG. Faster rate of initial fluid resuscitation in severe acute pancreatitis diminishes in-hospital mortality. Pancreatology 2009; 9: 770-776

Gerasimenko OV, Gerasimenko J, Belan PV, Petersen OH. Inositol trisphosphate and cyclic ADP-ribose-mediated release of Ca^{2+} from single isolated pancreatic zymogen granules. Cell 1996; 84: 473-480

Gerasimenko JV, Gerasimenko OV, Palejwala A, Tepikin AV, Petersen OH, Watson AJ. Menadione-induced apoptosis: roles of cytosolic Ca(2+) elevations and the mitochondrial permeability transition pore. J. Cell 2002; 115: 485-497

Gerasimenko, JV, Lur G, Sherwood MW, Ebisui E, Tepikin AV, Mikoshiba K, Gerasimenko OV & Petersen, O. H. 2009. Pancreatic protease activation by alcohol metabolite depends on Ca2+ release via acid store IP3 receptors. Proc Natl Acad Sci U S A, 2009; 106, 10758-63

Gijs J. D. van Acker, Ashok K. Saluja, Lakshmi Bhagat, Vijay P. Singh, Albert M. Song, and Michael L. Steer. Cathepsin B inhibition prevents trypsinogen activation and reduces pancreatitis severity Am J Physiol Gastrointest Liver Physiol; 2002; 283(3): G794-G800

Golstein P, Kroemer G. Cell death by necrosis: towards a molecular definition. Trends Biochem Sci 2007; 32: 37-43

Gorelick F.S. and Jamieson J.D. (1994). The pancreatic acinar cell: structure – function relationship. In: The Physiology of the Gastrointestinal Tract, ed. Johnson, L. pp 1353-1376. Raven Press, New York.

Gorelick FS, Matovcik LM. Lysosomal enzymes and pancreatitis. Gastroenterology 1995; 109: 620-625

Guicciardi ME, Deussing J, Miyoshi H, Bronk SF, Svingen PA, Peters C, Kaufmann SH, Gores GJ. Cathepsin B contributes to TNF-alpha-mediated hepatocyte apoptosis by promoting mitochondrial release of cytochrome c. J Clin Invest. 2000;106(9):1127-37

Gukovskaya AS, Gukovsky I, Jung Y, Mouria M, Pandol SJ. Cholecystokinin induces caspase activation and mitochondrial dysfunction

in pancreatic acinar cells. Roles in cell injury processes of pancreatitis. J Biol Chem 2002; 277: 22595-22604

Gukovsky I, Pandol SJ, Gukovskaya AS. Organellar dysfunction in the pathogenesis of pancreatitis. Antioxidants and Redox Signaling 2011; 15(10): 2699-2710

Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C, Deussing J. Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. J Clin Invest 2000; 106: 773-781

He S, Wang L, Miao L, Wang T, Du F, Zhao L, Wang X. Receptor interacting protein kinase-3 determines cellular necrotic response to TNF-alpha. Cell 2009; 137(6): 1100-1111

Hedstrom J, Korvuo A, Kenkimaki P, Tikanoja S,Haapiainen R, Kivilaakso E, et al. Urinary trypsinogen-2 test strip for acute pancreatitis. Lancet1996; 347:729-30.

Hermann JM, Neupert W. Protein transport into mitochondria. Curr Opin Microbiol 2000; 3(2): 210-214

Holst, JJ. (1993). Neural regulation of pancreatic exocrine function. In The Pancreas: Biology, Pathobiology and Disease, eds. Go, VLW., Dimango EP, Gardner, J., Lebenthal, E., Reber HA., & Scheele, GA., pp. 381-402. Raven Press, New York

Howes N, Greenhalf W, Stocken DD, Neoptolemos JP. Cationic trypsinogen mutations and pancreatitis. Clin Lab Med. 2005 Mar;25(1):39-59

Huang W. Development of therapy directed at cell signalling abnormalities in experimental acute pancreatitis. 2011. PhD Thesis, University of Liverpool

International multidisciplinary consultation: Dellinger EP, Forsmark CE, Layer P, Lévy P, Maraví-Poma E, Petrov MS, Shimosegawa T, Siriwardena AK, Uomo G, Whitcomb DC, Windsor JA. Determinant-Based Classification of Acute Pancreatitis Severity: An International Multidisciplinary Consultation. Annals of Surgery 2012; Aug 8: 1-6

Imrie CW, Benjamin IS, McKay AJ, MacKenzie I, O'Neill J, Blumgart LH. A single centre double blind trial of Tranylol therapy in primary acute pancreatitis. Br J Surg 1978; 65: 337-341

Johnson CD, Kingsnorth A, Imrie CW, McMahon MJ, Neoptolemos JP, McKay C, Toh SK, Skaife P, Leder PC, Wilson P, Larvin M, Curtis LD. Double bind, randomised, placebo controlled study of a platelet activating factor antagonist, lexipafant, in the treatment and prevention of organ failure in predicted severe acute pancreatitis. Gut 2001; 48: 62-69

Johnson CD, Abu-Hilal M. Persistent organ failure during the first week as a marker of fatal outcome in acute pancreatitis. Gut 2004; 53: 1340-1344

Ji B, Bi Y, Simeone D. Mortensen RM, Logsdon CD. Human pancreatic acinar cells lack functional responses to CCK and gastrin. Gastroenterology 2001; 121: 1380-1390

Jin HT, Lamsa T, Hyvonen MT, Sand J, Raty S, Grigorenko N, Khomutov AR, Herzig KH, Alhonen L, Nordback I. A polyamine analog bismethylspermine ameliorates severe pancreatitis induced by intraductal infusion of taurodeoxycholate. Surgery 2008; 144: 49-56

Jin HT, Lamsa T, Nordback PH, Hyvonen MT, Raty S, Nordback I, Herzig KH, Alhonen L, Sand J. Polyamine Catabolism in Relation to Trypsin Activation and Apoptosis in Experimental Acute Pancreatitis. Pancreatology 2011; 11: 83-91

Kahle M, Lippert J, Willemer S, et al. Effects of positive end-expiratory pressure (PEEP) ventilation on the exocrine pancreas in minipigs. Res Exp Med (Berl). 1991; 191: 309-325

Kaiser AM, Saluja AK, Sengupta A, Saluja, Stee ML. Relationship between severity, necrosis, and apoptosis in five models of experimental acute pancreatitis. Am J Physiol 1995; 269: C1295-304

Kemppainen E, Sainio V, Haapiainen R, Kivisaari L, Kivilaakso E, Puolakkainen P. Early localization of necrosis by contrast-enhanced computed tomography can predict outcome in severe acute pancreatitis. Br J Surg 1996; 83:924-929

Kemppainen EA, Hedstrom JI, Puolakkainen PA, Sainio VS, Haapiainen RK, Perhoniemi V, et al. Rapid measurement of urinary trypsinogen-2 as a

screening test for acute pancreatitis. N Engl J Med 1997; 336:1788-93

Kerr JF, Wyllie AH, Currie AR. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. British journal of Cancer 1972; 26: 239-257

Király O, Wartmann T, Sahin-Tóth M. Missense mutations in pancreatic secretory trypsin inhibitor (SPINK1) cause intracellular retention and degradation. Gut 2007; 56: 1433-1438

Kishino Y, Kawamura S. Pancreatic damage induced by injecting a large dose of arginine. Virchows Arch B Cell Pathol Incl Mol Pathol 1984; 47: 147-55

Knaus WA, Draper EA, Wagner DP, Zimmerman JE. APCHE II: a severity of disease classification system 1985; 13(10): 818-829

Kroemer G, Galluzzi L, Brenner C. Mitochondrial membrane permeabilization in cell death. Physiol Review 2007; 87: 99-163

Kukor Z, Mayerle J, Kruger B, Toth M, Steed PM, Halangk W, Lerch MM, Sahin-Toth M. Presence of cathepsin B in the human pancreatic secretory pathway and its role in trypsinogen activation during hereditary pancreatitis. J Biol Chem 2002; 277: 21389-21396

Kwon R, Banks P. Clinical Pancreatology for Practising Gastroenterologists and Surgeons. Blackwell Publishing 2005. Ch. 4: How should acute pancreatitis be diagnosed in clinical practice?

Lampel M, Kern HF. Acute interstitial pancreatitis in the rat induced by excessive doses of a pancreatic secretagogue. Virchows Arch a Pathol Anat Histol 1977; 373: 97-117

Lankisch PG, Burchard S, Reckert M, Petersen M, Lehnick D, Schirren CA, Stockmann F, Kohler H. Etiology and age have only a limited influence on the course of acute pancreatitis. Pancreas 1996; 13: 344-349

Larvin M. Assessment of clinical severity and prognosis. The pancreas. Blackwell Science 1998. P. 489

Layer P. BISAP – A simple index on the day of admission which predicts outcome in acute pancreatitis. Gut 2008; 57: 1645-1646

Lerch MM, Saluja AK, Runzi M, Dawra R, Saluja M, Steer, ML. Pancreatic duct obstruction triggers acute necrotizing pancreatitis in the opossum. Gastroenterology 1993; 104: 853-61

Letko G, Nosofsky T, Lessel W, Siech M. Transition of rat pancreatic juice edema into acute pancreatitis by single ethanol administration. Pathol Res Pract 1991; 187: 247-250

Lichtenstein A, Milani R Jr, Fernezlian SM, et al. Acute lung injury in two experimental models of acute pancreatitis: infusion of saline or sodium taurocholate into the pancreatic duct. Critical care medicine 2000; 28: 1497-1502

Lombardi B., Estes LW. Ongnecker DS. Acute hemorrhagic pancreatitis (massive necrosis) with fat necrosis induced in mice by DL-ethionine fed with a choline-deficient diet. American Journal of Pathology 1975; 79: 465-480

Lupia E, Goffi A, De Giuli P, Azzolino O, Bosco O, Patrucco E, Vivaldo MC, Ricca M, Wymann MP, Hirsch E, Montrucchio G, Emanuelli G. Ablation of phosphoinositide 3-kinase-y reduces the severity of acute pancreatitis. Am J Pathol 2004; 165: 2003-2011

Mace PD, Shirley S, Day CL. Assembling the building blocks: structure and function of inhibitor of apoptosis proteins. Cell Death Differ 2010; 17:46-53

Majno G, Joris I. Apoptosis, oncosis, and necrosis: an overview of cell death. American Journal of Pathology 1995: 146: 3-15

Markhija R, Kingsnorth AN. Cytokine storm in acute pancreatitis. J Hepato-biliary-pancreat surg 2002; 9:401-410

Martinez J, Johnson CD, Sanchez-Paya J, de Madaria E, Robles-Diaz G, Perez-Mateo M. Obesity is a definitive risk factor of severity and mortality in acute pancreatitis: an updated meta-analysis. Pancreatology 2006; 6(3): 206-9

Maruyama Y, Inooka G, Li YX, Miyashita Y, Kasai H. Agonist-induced localized Ca2+ spikes directly triggering exocytotic secretion in exocrine pancreas. EMBO J 1993; 12: 3017-3022

Matheus AS, Coelho AM, Sampietre S, Jukemura J, Patzina RA, Cunha JE, Machado MC. Do the effects of pentoxifylline on the inflammatory process and pancreatic infection justify its use in acute pancreatitis? Pancreatology 2009; 9: 687-93

Miller LJ. Does the human pancreas have a type A or B personality? Gastroenterology 1996;111:1767-1770

Mizunuma T, Kawamura S, Kishino Y. Effects of injecting excess arginine on rat pancreas. J Nutr 1984; 114: 467-471

Mooren FC, Hlousechek V, Finkes T, Turi S, Weber IA, Singh J, Domscke W, Schnekenburger J, Kruger B. Lerch MM. Early changes in pancreatic acinae cell calcium signalling after pancreatic duct obstruction. J Biol Chem 2003; 278: 9361-9369

Mukherjee R, Criddle DN, Gukovskaya A, Pandol S, Petersen OH, Sutton R. Mitochondrial injury in pancreatitis. Cell Calcium 2008; 44: 14-23

Murphy JA, Criddle DN, Sherwood M, Chvanov M, Mukherjee R, McLaughlin EW, Booth D, Gerasimenko J, Raraty MG, Ghaneh P, Neoptolemos JP, Gerasimenko OV, Tepikin AV, Green GM, Reeve JR, Petersen OH, Sutton R. Direct activation of cytosolic Ca2+ signaling and exocytosis by cholecystokinin in human pancreatic acinar cells. Gastroenterology 2008; 135(20): 632-641

Myoung P, Misun L, Petersen O. Morphological and functional changes of dissociated single pancreatic acinar cells: testing the suitability of the single cell as a model for exocytosis and calcium signaling. Cell 2004; 35: 367

Nakagawa T, Shimizu S, Watanabe T, Yamaguchi O, Otsu K, Yamagata H, Inohara H, Kubo T, Tsujimoto Y. Cyclophilin D-dependent mitochondrial permeability transition regulates some necrotic but not apoptotic cell death. Nature 2005; 434: 652-658

Nakayama K, Nakayama KI, Negishi I, Kuida K, Sawa H, Loh D. Targeted disruption of Bcl-2aß in mice: Occurrence of gray hair, polycystic kidney disease, and lymphocytopenia. PNAS 1994; 91: 3700-3704

Nathan JD, Romac J, Peng RY, Peyton M, Macdonald RJ, Liddle RA. Transgenic expression of pancreatic secretory trypsin inhibitor-I ameliorates

secretagogue-induced pancreatitis in mice. Gastroenterology 2005; 128: 717-27

Neoptolemos J, Kemppainen E, Mayer J et al. Early prediction of severity in acute pancreatitis by urinary trypsinogen activation peptide: a multicentre study. Lancet 2000; 355:1955-1960

Nguyen BL, Thompson JS, Edney JA, Bragg LE, Rikkers LF. Influence of the aetiology of pancreatitis on the natural history of pancreatic pseudocysts. Am J Surg (1991); 162:527-530

Nguyen T, Chin WC, Verdugo P. Role of Ca^{2+}/K^{+} ion exchange in intracellular storage and release of Ca^{2+}. Nature 1998; 395: 908-912

Niederau C, Ferrell LD, Grendell JH. Caerulein-induced acute necrotizing pancreatitis in mice: protective effects of proglumide, benzotript, and secretin. Gastroenterology 1985; 88:1192-204

Niederau C, Liddle RA, Ferrel LD, Grendell JH. Beneficial effects of CCK-receptor blockade and inhibition of proteolytic enzyme activity in experimental acute haemorrhagic pancreatitis in mice. J Clin Invest 1986;78:1056-63

Novaes G, Cabral AP, De Falco CN, De Queiroz AC. Acute pancreatitis induced by scorpion toxin, tityustoxin. Histopathological study in rats. Arq Gastroenterology 1989; 26: 9-12

Oakes SA, Scorrano L, Opferman JT, Bassik MC, Nishino M, Pozzani T, Korsmeyer S. Proapoptotic Bax and Bak regulate the type 1 inositol trisphosphate receptor and calcium leak from the endoplasmic reticulum. PNAS 2005; 102(1): 105-110

Ohshio G, Saluja A, Steer, ML. Effects of short-term pancreatic duct obstruction in rats. Gastroenterology 1991; 100: 196-202.

Olejar T, Matej R, Zadinova M, Pouckova P. Expression of proteinase-activated receptor 2 during taurocholate-induced acute pancreatic lesion development in Wistar rats. Int J Gastrointest Cancer 2001; 30: 113-121

Opie E. The etiology of acute haemorrhagic pancreatitis. Johns Hopkins Hospital Bulletin 1901; 12:182-188

Owyang C, Logsdon CD. New insights into neurohormonal regulation of pancreatic secretion. Gastroenterology 2004; 127: 957-969

Pahl HL. Signal transduction from the endoplasmic reticulum to the cell nucleus. Physiol Rev 1999; 79: 683-701

Pandol SJ, Saluja AK, Imrie CW, Banks PA. Acute pancreatitis: Bench to bedside. Gastroenterology 2007; 132:1127-1151

Pantoja JL, Renner IG, Abramson SB, Edmondson HA. Production of acute hemorrhagic pancreatitis in the dog using venom of the scorpion, Buthus quinquestriatus. Dig Dis Sci 1983; 28: 429-439

Papachristou GI, Muddana V, Yadav D, O'Connell M, Sanders MK, Slivka A, Whitcomb DC. Comparison of BISAP, Ranson's, APACHE-II, and CTSI scores in predicting organ failure, complications, and mortality in acute pancreatitis. American Journal of Gastroenterology 2010 Feb; 105(2): 435-41

Park MK, Petersen OH, Tepikin AV. The endoplasmic reticulum as one continuous calcium pool: visualisation of rapid calcium movements and equilibration. EMBO J 2000; 19: 5729-5739

Park MK, Ashby MC, Erdemli G, Petersen OH, Tepikin AV. Perinuclear, perigranular and sub-plasmalemmal mitochondria have distinct functions in the regulation of cellular calcium transport. EMBO Journal 2001; 20: 1863-1874

Park HS, Betzenhauser, MJ, Won JH, Chen J, Yule DI. The type 2 inositol (1,4,5)-trisphosphate (InsP3) receptor determines the sensitivity of InsP3-induced Ca2+ release to ATP in pancreatic acinar cells. J Biol Chem 2008; 283: 26081-8

Paschen W. Dependence of vital cell function on endoplasmic reticulum levels: implications for the mechanisms underlying neuronal cell injury in different pathological states. Cell Calcium 2001; 29: 1-11

Perides G,A.Sharma ,A.Gopal ,X.Tao ,K.Dwyer ,B.Ligon, M.Steer. Secretin Differentially Sensitizes Rat Pancreatic Acini To The Effects Of Supramaximal Stimulation With Caerulin. Am Journ Physiol in press (May 26, 2005)

Perides G, Laukkarinen JM, Vassileva G, Steer ML. Biliary acute pancreatitis in mice is mediated by the G-protein-coupled cell surface bile acid receptor Gpbar1. Gastroenterology 2010a; 138: 715-725

Perides G, Van Acker GJ, Laukkarinen JM, Steer ML. Experimental acute pancreatitis induced by retrograde infusion of bile acids into the mouse pancreatic duct. Nat Protoc 2010b; 5: 335-341

Petersen OH. Stimulus-secretion coupling: cytoplasmic calcium signals and the control of ion channels in exocrine acinar cells. J Physiol 1992; 448: 1-51

Petersen OH. Ca^{2+} signalling and Ca^{2+}-activated ion channels in exocrine acinar cells. Cell Calcium 2005; 38: 171-200

Petersen OH, Sutton R. Ca2+ signalling and pancreatitis: effects of alcohol, bile and coffee. Trends Pharmacol Sci 2006; 27: 113-120

Petersen OH, Sutton R, Criddle D. Failure of calcium microdomain generation and pathological consequences. Cell Calcium 2006; 40: 593-600

Petersen OH, Tepikin AV. Polarized calcium signalling in exocrine gland cells. Annu Rev Physiol 2008; 70:273-299

Petersen OH, Gerasimenko OV, Gerasimenko J.V. Pathobiology of acute pancreatitis: focus on intracellular calcium and calmodulin. F1000 Med Rep 2011; 3: 15

Petrov MS, van Santvoort HC, Besselink MG, van der Heijden GJ, van Erpecum KJ, Gooszen HG. Early endoscopic retrograde cholangiopancreatography versus conservative management in acute biliary pancreatitis without cholangitis: a meta-analysis of randomized trials. Ann Surg 2008; 247(2): 250-257

Pezzili R, Billi P, Miniero R, et al. Serum IL-6, IL-8, and beta 2-microglobulin in early assessment of severity of acute pancreatitis. Comparison with CRP. Dig Dis Sci 1995; 40:2341-2348

Pezzilli R, Morselli-Labate AM, Barbieri AR, Platè L. Clinical usefulness of the serum carboxypeptidase B activation peptide in acute pancreatitis. JOP. JPancreas (Online) 2000; 1:58-68.

Pfeffer RB, Lazzarini-Robertson A, Jr., Safadi D, Mixter G, Jr., Secoy CF, Hinton JW. Gradations of pancreatitis, edematous, through hemorrhagic, experimentally produced by controlled injection of microspheres into blood vessels in dogs. Surgery 1962; 51: 764-769

Pfutzer RH, Barmada MM, Brunskill AP, Finch R, Hart PS, Neoptolemos JP, Furey WF, Whitcomb DC. SPINK/PSTI polymorphisms act as disease modifiers in familial and idiopathic chronic pancreatitis. Gastroenterology 2000; 119: 615-623

Przemeck SMC, Duckworth CA, Pritchard DM. Radiation-induced gastric epithelial apoptosis occurs in the proliferative zone and is regulated by p53, bak, bax and bcl-2. Am J Gastrointest Liver Physiol 2007; 292: G620-G627

Rakonczay Z, Jr., Hegyi P, Dosa S, Ivanyi B, Jarmay K, Biczo G, Hracsko Z, Varga IS, Karg E, Kaszaki J, Varro A, Lonovics J, Boros I, Gukovsky I, Gukovskaya AS, Pandol SJ, Takacs T. A new severe acute necrotizing pancreatitis model induced by L-ornithine in rats. Crit Care Med 2008; 36: 2117-2127

Rakonczay Z Jr, Hegyi P, Takacs T, Mccarroll J, Saluja AK. The role of NF-kappaB activation in the pathogenesis of acute pancreatitis. Gut 2008b; 57: 259-67

Ranson HJC, Rifkind KM, Roses DF, Fink SD, Eng K, Spencer FC. Prognostic signs and the role of operative management in acute pancreatitis. Surg Gynecol Obstet 1974; 139:69-81

Ranson HJC. The timing of biliary surgery in acute pancreatitis. Ann Surg 1979; 189:654-662

Rao KN, Eagon PK, Okamura K, Van Thiel DH, Gavaler JS, Kelly RH, Lombardi B. Acute hemorrhagic pancreatic necrosis in mice. Induction in male mice treated with estradiol. American Journal of Pathology 1982: 109; 8-14

Reeve JR Jr, Green GM, Chew P, Eysselein VE, Keire DA. CCK-58 is the only detectable endocrine form of cholecystokinin in rat. Am J Physiol Gastrointest Liver Physiol 2003; 285: G255-G265

Reeve JR Jr, Wu SV, Keire DA, Faull K, Chew P, Solomon TE, Green GM, Coskun T. Differential bile-pancreatic secretory effects of CCK-58 and CCK-8. Am J Physiol 2004; 286: G395-402

Renner IG, Wisner JR Jr. Ceruletide-induced acute pancreatitis in the dog and its amelioration by exogenous secretin. International Journal of Pancreatology. 1986; 1: 39Y49

Raraty M, Ward J, Erdemli G, Vaillant C, Neoptolemos JP, Sutton R, Peterson OH. Calcium-dependent enzyme activation and vacuole formation in the apical granular region of pancreatic acinar cells. PNAS; 2000: 97(24): 13126-13131

Raraty MG, Murphy JA, Mcloughlin E, Smith D, Criddle D, Sutton R. Mechanisms of acinar cell injury in acute pancreatitis. Scand J Surg. 2005; 94(2): 89-96

Raraty MG, Halloran CM, Dodd S, Ghaneh P, Connor S, Evans J, Sutton R, Neoptolemos JP. Minimal access retroperitoneal pancreatic necrosectomy: improvement in morbidity and mortality with a less invasive approach. Ann Surg 2010 May; 251(5): 787-793

Redha F, Uhlschmid G, Ammann RW, Freiburghaus AU. 1990. Injection of microspheres into pancreatic arteries causes acute hemorrhagic pancreatitis in the rat: a new animal model. Pancreas 1990; 5: 188-193

Reeve JR Jr, Wu SV, Keire DA, Faull K, Chew P, Solomon TE, Green GM, Coskun T. Differential bile-pancreatic secretory effects of CCK-58 and CCK-8. Am J Physiol 2004; 286: G395-402

Runzi M, Saulja AK, Kaiser A. Biochemical and morphological changes that characterise recovery from necrotising biliary pancreatitis in the opossum. Gut 1995; 37: 427-433

Saluja A K, E A Donovan, K Yamanaka, Y Yamaguchi, B Hofbauer, M L Steer. Cerulein-induced in vitro activation of trypsinogen in rat pancreatic acini is mediated by cathepsin B. Gastroenterology 1997; 113(1): 304-10

Saluja AK, Bhagat L, Lee HS, Bhatia M, Frossard JL, Steer ML. Secretagogue-induced digestive enzyme activation and cell injury in rat pancreatic acini. Am J Physiol 1999; 276: G835-42

Saluja AK, Lerch MM, Phillips PA, Dudeja V. Why does pancreatic overstimulation cause pancreatitis? Annu Rev Physiology 2007; 69: 249-69

Saluja AK, Dudeja V. Heat shock proteins in pancreatic diseases. Journal of gastroenterology and heptaology 2008; 23(1): S42-S45

Samuel I, Tourmi Y, Yokko H, Wilcockson DP, Trout JJ, Joehl RJ. Ligation-induced acute pancreatitis in rats and opossums: a comparative morphological study of the early phase. Journal of Surg Res 1994; 57: 299-311

Samuel I, Yuan Z, Meyerholz DK, Twait E, Williard DE, Kempuraj D. A novel method of severe gallstone pancreatitis: murine pancreatic duct ligation results in systemic inflammation and substantial mortality. Pancreatology 2010; 10: 536-544

Sandoval D, Gukovskaya A, Reavey P, Gukovsky S, Sisk A, Braquet P, Pandol SJ, Poucell-Hatton S. The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. Gastroenterology 1996; 111: 1081-1091

Sarner M. Pancreatitis definitions and classifications. In: Go VLW, Dimagno EP, Gardner JD, Lebenthal E, Reber HA, Scheele GA, eds. The Pancreas: Biology, Pathobiology, and disease. 2nd Edition. New York: Raven Press, 1993: 575-80

Sarmiento N, Sanchez-Bernal C, Perez N, Sardina JL, Mangas A, Calvo JJ, Sanchez-Yague J. Rolipram and SP600125 Suppress the Early Increase in PTP1B Expression During Cerulein-Induced Pancreatitis in Rats. Pancreas 2010; 39(5): 639-645

Satoh A, Shimosegawa T, Satoh K, Ito H, Kohno Y, Masamune A, Fujita M, Toyota T. Activation of adenosine A1-receptor pathway induces edema formation in the pancreas of rats. Gastroenterology 200; 119:z`` 829-36

Schmid SW, Uhl W, Büchler MW. Chapter 22: Does the etiology influence the severity of acute pancreatitis? in: Acute Pancreatitis: Novel Concepts in Biology and Therapy. Eds Büchler MW, Uhl W, Friess H, Malfertheiner P. Berlin; Blackwell Wissenschafts-Verlag, 1999, pp 190-198

Schmidt J, Rattner DW, Lewandrowski K, Compton CC, Mandavilli U, Knoefel WT, Warshaw AL. A better model of acute pancreatitis for evaluating therapy 1992; Annals of Surgery; 215: 44-56

Segersvard R, Sylvan M, Herrington M, Larsson J, Permert J. Obesity increases the severity of acute experimental pancreatitis in the rat. Scandinavian Journal of Gastroenterology 2001; 36, 658-63

Sharma A, Tao X, Gopal A, Ligon B, Andrade-Gordon P, Steer ML, Perides G. Protection against acute pancreatitis by activation of protease-activated receptor-2. Am J Physiol Gastroinest Liver Physiol 2005; 228: G388-G395

Sherwood MW, Prior IA, Voronina SG, Barrow SL, Woodsmith JD, Gerasimenko OV, Petersen OH, Tepikin AV. Activation of trypsinogen in large endocytic vacuoles of pancreatic acinar cells. Proc Natl Acad Sci U S A. 2007 Mar 27; 104(13): 5674-9

Siech M, Heinrich P, Letko G. Development of acute pancreatitis in rats after single ethanol administration and induction of a pancreatic juice edema. Int J Pancreatol 1991; 8: 169-175.

Simovic MO, Bonham MJ, Abu-Zidan FM, Windsor JA. Anti-inflammatory cytokine response and clinical outcome in acute pancreatitis. Crit Care Med 1999; 27: 2662-5

Singh VP, Saluja AK, Bhagat L, Van Acker GJ, Song AM, Soltoff SP, Cantley LC, Steer ML. Phosphatidylinositol 3-kinase-dependent activation of trypsinogen modulates the severity of acute pancreatitis. J Clin Invest 2001; 108: 1387-1395

Singh VP, Bhagat L, Navina S, Sharif R, Dawra R, Saluja AK. PAR-2 protects against pancreatitis by stimulating exocrine secretion. Gut 2007; 56(7): 958-964

Siriwardena AK, Mason J, Balachandra S, Bagul A, Galloway S, Formela L, Hardman J and Jamdar S. Randomised, double blind, placebo controlled trial of intravenous antioxidant (n-acetylcysteine, selenium, vitamin C) therapy in severe acute pancreatitis. Gut 2007; 56: 1439-1444

Sjovall S, Holmin T, Evander A, Stenram U. 1988. Splenic and gastro-duodenal vein occlusion--influence on the pancreatic gland and on the outcome of experimental pancreatitis. Int J Pancreatol 1988. 3; 143-149

Slee EA, Adrain C, Martin SJ. Serial killers: ordering caspases activation events in apoptosis. Cell Death and Differentiation 1999; 6: 1067-1074

Spormann H, Sokolowski A, Letko G. Effect of temporary ischemia upon development and histological patterns of acute pancreatitis in the rat. Pathol Res Pract 1989; 184: 507-513

Steiner B, Rosendahl J, Witt H, Teich N. Keim V, Schulz HU. Pfutzer R, Lohr M, Gress TM, Nickel R, Landt O, Koudova M, Macek M Jr, Farre A, Casals T, Desax MC, Gallati S, Gomez-Lira M, Audrezet MP, Ferec C, Des Georges M, Claustres M, Truninger K. Common CFTR haplotypes and susceptibility to chronic pancreatitis and congenital bilateral absence of the vas deferens. Hum Mutat 2011; 32: 912-20

Steinle AU, Weidenbach H, Wagner M, Alder G, Schmid RM. NF-kB/Rel activation in cerulein pancreatitis. Gastroenterology 1999; 116: 420-430

Steer P, Flenady V, Sharman A, et al. High dose caffeine citrate for extubation of preterm infants; a randomised controlled trial. Arch Dis Child 2004; 89: F499-503

Sugimoto M, Takada T, Yasuda T. A new experimental pancreatitis by incomplete closed duodenal loop: the influence of pancreatic microcirculation on the development and progression of induced severe pancreatitis in rats. Pancreas 2004; 28: e112-119

Sutton R, Criddle D, Raraty MG, Tepikin A, Neoptolemos JP, Petersen OH. Signal transduction, calcium and acute pancreatitis. Pancreatology. 2003;3(6):497-505

Sun G, Chang TM, Xue WJ, Wey JF, Lee KY, Chey WY. Release of cholecystokinin and secretin by sodium oleate in dogs: molecular form and bioactivity. Am J Physiol 1992; 262: G35-43

Sung KF, Odinokova IV, Mareninova OA, Rokonczay Z Jr, Hegyi P, Pandol SJ, Gukovsky I, Gukovskaya AS. Prosurvival Bcl-2 proteins stabilize pancreatic mitochondria and protect against necrosis in experimental pancreatitis. Exp Cell Res 2009; 315: 1975-1989

Tenner S, Fernandez del Castillo C, Warshaw AL et al. Urinary trypsinogen activation peptide (TAP) predicts severity in patients with acute pancreatitis. Int J Pancreatology 1997; 21:105-110

Thoeni RF. The revised Atlanta Classification of Acute Pancreatitis: its importance for the radiologist and tis effect on treatment. Radiology 2012; 262:751-764

Thrower EC, Diaz de Villalvilla AP, Kolodecik TR, Gorelick FS. Zymogen activation in a reconstituted pancreatic acinar cell system. Am J Physiol Gastrointest Liver Physiol. 2006; 290: G894-G902

Tinel H, Cancela J, Gerasimenko JV, Gerasimenko OV, Tepikin AV, Peterson OH. Active mitochondria surrounding the pancreatic acinar granule region prevents spreading of inositol – evoked local cytosolic calcium signals. EMBO J 1999; 18: 4999-5008

Toescu EC, O'neill SC, Petersen OH, Eisner DA. 1992. Caffeine inhibits the agonist-evoked cytosolic Ca2+ signal in mouse pancreatic acinar cells by blocking inositol trisphosphate production. J Biol Chem, 1992; 267: 23467-70

Tooze, J., Hollinshead, M., Hensel, G., Kern, H.F., and Hoflack, B. Regulated secretion of mature cathepsin B from rat exocrine pancreatic cells. Eur. J. Cell Biol. 1991; 56: 187-200

Uhl W, Isenmann R, Curti G, Vogel R, Beger HG, Buchler MW. Influence of etiology on the course and outcome of acute pancreatitis. Pancreas 1996; 13:335-343

Uhl W, Büchler MW, Malfertheiner P, Beger HG, Adler G, Gaus W and the German Pancreatitis Study Group. A randomised, double blind, multicentre trial of octreotide in moderate to severe acute pancreatitis. Gut 1999; 45: 97-104

UK working party on acute pancreatitis. UK guidelines for the management of acute pancreatitis. Gut 2005; 54: 1-9

Van Santvoort HC, Besselink MG, De Vries AC, Boermeester MA, Fischer K, Bollen TL, Cirkel GA, Schaapherder AF, Nieuwenhuijs VB, Van Goor H, Dejong CH, Van Eijck CH, Witteman BJ, Weusten BL, Van Larrhoven CJ, Wahab PT, Tan AC, Schwartz PD, Gooszen HG, Van Erpecum KJ.

Early endoscopic retrograde cholangiopancreatography in predicted severe acute biliary pancreatitis: a prospective multicenter study. Annals of Surgery 2009; 250: 68-75

Van Santvoort HC, Bakker OJ, Bollen TL, Besselink MG, Ahmed Ali U, Schrijver AM, Boermeester MA, Van Goor H, Dejong CH, Van Eijck CH, Van Ramshorst B, Schaapherder AF, Van Der Harst E, Hofker S, Nieuwenhuijs VB, Brink MA, Kruyt PM, Manusama ER, Van Der Schelling GP, Karsten T, Hesselink EJ, Laarhoven CJ, Rosman C, Bosscha K, De Wit R, Houdijk AP, Cuesta MA, Wahab PJ, Gooszen HG. Dutch Pancreatitis Study Group. A conservative and minimally invasive approach to necrotizing pancreatitis improves outcome. Gastroenterology 2011; 141(4): 1254-1263

Vege SS, Gardner TB, Chari ST, Munukuti P, Pearson RK, Clain JE, Petersen BT, Baron TH, Farnell MB, Sarr MG. Low mortality and high morbidity in severe acute pancreatitis without organ failure: a case for revising the Atlanta classification to include "moderately severe acute pancreatitis". American Journal of Gastroenterology 2009; 104(3):710-715

Voronina S, Longbottom R, Sutton R, Petersen OH, Tepikin A. Bile acids induce calcium signals in mouse pancreatic acinar cells: implications for bile-induced pancreatic pathology. J Physiol 2002a; 540:49-55

Voronina S, Sukhomlin T, Johnson PR, Erdemli G, Peterson OH, Tepikin AV. Correlation of NADH and calcium signals in mouse pancreatic acinar cells. J Physiol 2002b; 539: 41-52

Voronina SG, Sherwood MW, Gerasimenko OV, Petersen OH, Tepikin AV. Visualizing formation and dynamics of vacuoles in living cells using contrasting dextran-bound indicator: endocytic and nonendocytic vacuoles. Am J Physiol Gastrointest Liver Physiol. 2007 Dec; 293(6): G1333-8

Wakui M, Osipchuk YV, Petersen OH. Receptor-activated cytoplasmic Ca2+ spiking mediated by inositol trisphosphate is due to Ca2(+)-induced Ca2+ release. Cell 1990; 63: 1025-32

Waldner H. Vascular mechansims to induce acute pancreatitis. Eur Surg Res. 1992; 24(1): 62-67

Walsh JH. Gastrointestinal hormones. In: Johnson LR (ed) Physiology of the gastrointestinal tract, 2nd edition, Raven Press, New York, 1987; pp 181-253

Wantanabe O, Baccino FM, Steer ML, Meldolesi J. Supramaximal caerulein stimulation and ultrastructure of rat pancreatic acinar cell: early morphological changes during development of experimental pancreatitis. Am J Physiol 1984; 246: G457-67

Ward JB, Petersen OH, Jenkins SA, Sutton, R. 1995. Is an elevated concentration of acinar cytosolic free ionised calcium the trigger for acute pancreatitis? Lancet 1995; 346: 1016-1019

Ward JB, Sutton R, Jenkins SA, Petersen OH. Progressive disruption of acinar cell calcium signaling is an early feature of caerulein-induced pancreatitis in mice. Gastroenterology 1996; 111: 481-491

Warndorf MG, Kurtzman JT, Bartel MJ, Cox M, Mackenzie T, Robinson S, Burchard P, Gordon SR, Gardner TB. Early fluid resuscitation reduces morbidity among patients with acute pancreatitis. Clin Gastroenterol Hepatol 2011; 9: 705-709

Willemer S, Bialek R, Adler G. Localization of lysosomal and digestive enzymes in cytoplasmic vacuoles in caerulein-pancreatitis. Histochemistry 1990; 94: 161-70

Whitcomb DC, Gorry MC, Preston RA, Furey W, Sossenheimer MJ, Ulrich CD, Martin SP, Gate LK Jr, Amann ST, Toskes PP, Liddle R, McGrath K, Uomo G, Post JC, Ehrlich GD. Hereditary pancreatitis is caused by a mutation in the cationic trypsinogen gene. Nat Genet 1996; 14: 141-145

Whitcomb DC, LaRusch J, Krasinskas AM, et al. Common genetic variants in the *CLDN2* and *PRSS1-PRSS2* loci alter risk for alcohol-related and sporadic pancreatitis. Nat Genetics 2012; 44: 1349-1356

Witt H, Luck W, Hennies HC, Classen M, Kage A, Lass U, LAndt O, Becker M. Mutations in the gene encoding the serine protease inhibitor Kazal type 1 are associated with chronic pancreatitis. Nat Genet 2000; 25: 213-216

Wittau M, Mayer B, Scheele J, Henne-Bruns D, Dellinger EP, Isenmann R. Systematic review and meta-analysis of antibiotic prophylaxis in severe acute pancreatitis. Scandanavian Journal of gastroenterology

Wojcikiewicz RJ. Type I, II, and III inositol 1,4,5-trisphosphate receptors are unequally susceptible to down-regulation and are expressed in markedly different proportions in different cell types. J Biol Chem 1995: 270: 11678-83

Wu Bu, Johannes RS, Sun X, Tabak Y, Conwell DL, Banks PA. The early prediction of mortality in acute pancreatitis: a large population-based study. Gut 2008 Dec; 57(12): 1698-703

Yamamoto M, Reeve JR jr., Green SM. Supramaximal CCK-58 does not induce pancreatitis in the rat: role of pancreatic water secretion. Am J Physiol Gastrointest Liver Physiol 2007; 292: G964-G974

Yamano M, Umeda M, Miyata K, Yamada T. Protective effect of the combined treatment of pancreatic and neutrophil elastase inhibitors on acute pancreatitis elicited by lipopolysaccharide in rats given intraductal injection of taurocholate plus trypsin. Naunyn Schmiedebergs Arch Pharmacol 1998; 357: 558-64

Yamasaki M, Thomas JM, Churchill GC, Garnham C, Lewis AM, Cancela JM, Patel S, Galione A. Role of NAADP and CADPR in the induction and maintenance of agonist-evoked Ca^{2+} spiking in mouse pancreatic acinar cells. Curr Biol 2005; 15: 874-878

Yang C, Guanghua F, Wei Z, Zhong J, Penghui J, Xin F, Xiping Z. Combination of hemofiltration and peritoneal dialysis in the treatment of severe acute pancreatitis. Pancreas 2010; 39:16-19

Yang K, Ding YX, Chin WC. K+-induced ion-exchanes trigger trypsin activation in pancreas acinar zymogen granules. Arch Biochem Biophys 2007; 459: 256-263

Yoo SH, So SH, Kweon HS, Lee JS, Kang MK, Jeon CJ. Coupling of the inositol 1,4,5-triphosphate receptor and chromogranins A and B in secretory granules. J Biol Chem 2000; 275:12553-9

Yoshinaga K, Wasizuka M, Segawa Y. Cholecystokinin acts as an essential factor in the exacerbation of pancreatic bile duct ligation-induced rat

pancreatitis model under non-fasting condition. Jpn J Pharmacol 2000; 84: 44-50

Zaninovic V, Gukovskaya AS, Gukovsky I, Mouria M, Pandol SJ. Cerulein upregulates ICAM-1 in pancreatic acinar cells, which mediates neutrophil adhesion to these cells. Am J Physiol Gastrointest Liver Physiol 2000; 279: G666-G676

Zhao X, Han J, Tang C. Primary culture of porcine pancreatic acinar cells. Journal of the Pancreas (JOP) online 2001; 2(2): 78-82

Zhao QL, Huang CY, Huang Y, et al. Study on acute pancreatitis-associated lung injury by L-arginine in mice. Sichuan Da Xue Xue Bao Yi Xue Ban. 2004; 35: 839-842