ROLE OF NEUROGENIC INFLAMMATION IN THE PATHOGENESIS OF ACUTE PANCREATITIS AND ASSOCIATED LUNG INJURY

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SUMMARY

Substance P (SP) is an important neuropeptide implicated in neurogenic inflammation. The role of SP and neurokinin-1 receptor (NK1R) in experimentally-induced acute pancreatitis has been investigated using a NK1R antagonist. The results summarized below are the major findings of the present work.

1. The effects of neurokinin receptor antagonists, CP96,345 (NK1R antagonist), GR159897 (neurokinin-2 receptor antagonist) and SB-222200 (neurokinin-3 receptor antagonist), as well as calcitonin-gene related peptide (CGRP) receptor antagonist, CGRP (8-37), on caerulein-induced AP were investigated. CP96,345, the NK1R antagonist significantly reduced the plasma amylase level and tissue myeloperoxidase activities in the pancreas and the lungs. On the other hand, treatments with NK2R, NK3R and CGRP receptor antagonists did not produce any effect. The results suggested that the SP-NK1R system is important for the development of AP.

2. In the pancreas, CP96,345 treatment resulted in the suppression of the elevated SP concentration, preprotachykinin-A (PPT-A) gene mRNA expression, NK2R mRNA expression, and NK1R mRNA and protein expression. In the lungs, the antagonist was found to suppress the increase in SP concentration, PPT-A mRNA expression and preprotachykinin-C gene (PPT-C) mRNA expression. However, the antagonist treatment further promoted the accumulation of pulmonary NK1R mRNA and protein expression. These data have provided valuable information regarding the regulation of tachykinins and neurokinin receptors during AP.

3. Pancreatic acinar cells were isolated and incubated with caerulein, SP or CP96,345, alone or in combination. Incubation with 10^{-7} M caerulein for 1 hour caused the inhibition of amylase release and the elevation of PPT-A and NK1R mRNA expression. On the other hand, SP incubation resulted only in the increase of PPT-A mRNA expression. CP96,345 blocked PPT-A mRNA expression.

4. The effect of CP96,345 on the regulation of expression of the adhesion molecules ICAM-1, VCAM-1, E-selectin and P-selectin was investigated. The mRNA expression of these four adhesion molecules was upregulated in the pancreas during AP. Treatment with CP96,345 effectively reduced the mRNA expression of P-selectin and E-selectin but not ICAM-1 and VCAM-1. In the lung, ICAM-1, E- and P-selectin mRNA expression increased during AP. CP96,345 treatment suppressed this elevation. A similar expression pattern was seen by immunohistochemical (IHC) reactivity. This study has provided important information on the relationship between NK1R activation and the regulation of adhesion molecules.

5. Our findings on the difference in pancreatic and pulmonary expression of neurokinin receptors, tachykinins and adhesion molecules have suggested a differential regulation of inflammatory response in the pancreas and the lungs. It is remarkably interesting as this is the first evidence of the presence of a differential pattern of regulation in the inflammation in the pancreas and the lungs in AP.

6. The leukocyte-endothelial interaction in the pancreatic microcirculation in AP was visualized using intravital microscopy. Significant increases in rolling and

adherent leukocytes and a decrease in rolling velocity were observed in AP. Treatment with CP96,345 resulted in decreases in rolling and adherent leukocytes.

In conclusion, neurogenic inflammation (NI) plays a key role in the development of AP and associated lung injury. The blockade of SP-NK1R system, the major component of NI, has been proven to be effective in the treatment of AP and associated lung injury in a mouse model of AP.

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LIST OF ABBREVIATIONS

5-HT	5-hydroxytryptamine
ACE	Angiotensin-converting enzyme
AP	Acute pancreatitis
ARDS	Acute respiratory distress syndrome
CALC	Calcitonin receptor
CCK	Cholecystokinin
CCR1	Chemokine receptor 1
CGRP	Calcitonin gene–related peptide
CINC	Cytokine-induced neutrophil chemoattractant
CP	Chronic pancreatitis
CT scan	Computed tomographic scan
CTSB	Cathepsin B
EDNRB	Endothelin receptor type B
E-seletin	Endothelial adhesion molecule 1
ET-3	Endothelin-3
H&E	Hematoxylin and Eosin
HK-1	Hemokinin-1
ICAM-1	Intercellular adhesion molecule-1
ICAM-2	Intercellular adhesion molecule-2
IL-1β	Interleukin-1β
IL-10	Interleukin-10
IL-1ra	Interleukin-1 receptor antagonist
IL-6	Interleukin-6
IL-8	Interleukin-8
LFA-1	Lymphocyte function-associated antigen-1
L-selectin	Lymphocyte adhesion molecule 1
MAdCAM-1	Mucosal vascular addressin cell adhesion molecule 1
MCP-1	Monocyte chemoattractant protein-1
mM	Millimolar
MODS	Multiple organ dysfunction syndrome
NEP	Neutral endopeptidase
NF-κB	Nuclear factor kappa B
NI	Neurogenic inflammation
NK1R	Neurokinin 1 receptor
NK2R	Neurokinin 2 receptor
NK3R	Neurokinin 3 receptor
NKA	Neurokinin A
NKB	Neurokinin B
nM	Nanomolar
PAF	Platelet activating factor
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
PECAM-1	Platelet endothelial cell adhesion molecule-1
PPTA	Preprotachykinin-A
PPTB	Preprotachykinin B
PPTC	Preprotachykinin C
P-selectin	Platelet adhesion molecule 1
PSTI	Pancreatic secretory trypsin inhibitor

RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
Rh6G	Rhodamine 6G
RT-PCR	Reverse transcription polymerase chain reaction
SIRS	Systemic inflammatory response syndrome
sTNFR	Soluble tumor necrosis factor receptor
TACR1	Tachykinin receptor 1 gene
TBS	Tris buffered saline
TK	Tachykinins
TNF-α	Tumor necrosis factor-α
TRPV1	Transient Receptor Potential Vanilloid 1
VCAM-1	Vascular cell adhesion molecule-1
VR-1	Vanilloid receptor-1

CHAPTER 1

GENERAL INTRODUCTION

1.1 Overview

Pancreatitis is inflammation of the pancreas. This condition occurs when the pancreatic tissue is auto-digested by the digestive enzymes that are produced and abnormally activated within the pancreas. In severe cases, there may be bleeding into the gland, infection and cysts. Moreover, enzymes and toxins may enter the systemic circulation and cause subsequent injuries to vital organs such as the heart, lungs, liver and kidney, which might lead to death. This medical condition can be described as acute or chronic. It can occur as a sudden, painful attack; or a persistent condition progressing over a period of years in individuals with pancreatic damage from previous episodes of acute pancreatitis (AP). The acute form of pancreatitis occurs suddenly and it can become severe, with many life-threatening complications. In most cases, the patients recover completely. Nevertheless, if the pancreas is subjected to continual insult, such as persistent consumption of alcohol, severe acute and chronic forms of pancreatitis may develop.

The present literature review discusses the nature of acute and chronic forms of pancreatitis, the pathophysiology, the various inflammatory mediators implicated and the role of neurogenic inflammation in pancreatitis.

1.2 ACUTE PANCREATITIS (AP)

Numerous accounts of illnesses in history might have suggested the occurrence of AP, a unique example being the death of Alexander the Great in 323BC (Sbarounis, 1997; Breimer, 1998). A trend of rising incidence of this clinical condition has been recognized over the past several decades (McKay et al., 1999; Sandler et al., 2002). The incidence of AP varies considerably between regions and is estimated at 5-80 per 100,000 people (Mayerle et. al, 2005; Kingsnorth and O'Reilly, 2006). In Germany, the incident rate is about 2 cases per 10,000 people (Lankisch et al., 2002). In Finland, the frequency is about 7 cases per 10,000 people (Jaakkola and Nordback, 1993). It ranks 14th among the leading gastrointestinal causes of death in the United States, and it remains one of the top three causes of gastrointestinal inpatients hospital admissions in the United States (Russo et al., 2004; Shaheen et al., 2006). As a result, it imposes an enormous economic burden in the healthcare system, leading to more than $2 billion direct cost in United States annually (Sandler et al., 2002; Russo et al., 2004).

The majority of patients (75%) experience the mild form of AP, in which the pancreatic tissue (usually) recovers, as the condition is self-limited. Nevertheless, it causes death in 50 % of the remaining severe cases (Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a).

The cardinal symptom is upper abdominal pain that may last for a few days. Typically the pain is central, persistent, and 'boring' in nature, which sometimes radiates through to the back. It is usually aggravated by eating, especially foods high in fat, and worse when lying down, and partly relieved by sitting forward. Usually the pain builds up

over half to one hour, and remains stable for hours to days. This is often associated with nausea and vomiting. The upper abdomen of the patient is often tender and swollen. There are often other symptoms associated with AP, such as abdominal distension (fluid leaks into the space at the back of organs, pushing them to protrude forward), mild fever and increased heart rate. Respiratory problems can also occur in patients due to 'under-breathing'. The patient often feels and looks very sick. Bruising around the navel and along the flanks may be seen in severe attacks due to bleeding in the pancreas. The patient may become dehydrated and have hypotension, which can lead to shock, and even death due to organ failure (Kingsnorth and O'Reilly, 2006).

The diagnosis of AP is usually made using serological tests, for examples, serum lipase, serum amylase and serum liver enzymes profile (alanine aminotransferase/ aspartate aminotransferase) are elevated. The lipase assay is usually more sensitive and specific than the amylase assay. There may be abnormalities in the serum levels of ions like calcium, magnesium, sodium, potassium, and bicarbonate. Gallstones and swollen pancreas may be detected using abdominal ultrasound technique. However, in 50% of the case, the pancreas can not be visualized due to obesity and overlying bowel gas. A dynamic (bolus) computed tomographic (CT) scan of the abdomen can be performed when the diagnosis is uncertain, as well as in cases of moderate-to-severe pancreatitis (Neoptolemos et al., 1984).

1.2.1 Etiologies of AP

There are several causes known to trigger the development of AP. As shown in Table 1.1, etiologies of AP can be classified into six major categories (Buchler et al., 2004;

Steinberg WM, 1997). On the worldwide basis, gallstones / biliary tract disease (obstructive cause) is the primary etiologic cause, followed by alcoholism (toxins cause). Together, they account for about 80% of patients diagnosed with AP. The individual contribution of each appears to be related to the patient population being studied; that is, alcoholism is most common in inner-city populations, especially in Scandinavia and the United States; biliary tract disease is more common in the more affluent suburban areas. Gallstones are the most common cause accounting for 45% of cases in Western Europe, Asia and United States. Estimated population incidences for acute gallstone pancreatitis in the USA range from 45 to 65 per 10000 person years depending on the criteria used to diagnose gallstone pancreatitis (Schwartz J, 2004). The passing of gallstones into the bile duct and temporarily lodging at the sphincter of Oddi, causes pancreatic duct pressure built-up. The risk of a stone causing pancreatitis is inversely proportional to its size.

Pancreatitis is clearly linked to alcohol abuse. It occurs mainly in habitual drinkers who have been drinking for at least a decade. It typically manifests after a drinking binge or a heavy meal (Keddie and Corson, 1966). Although there have been reports describing a large amount of alcohol load precipitating a first attack, routine alcoholics remain the norm rather than the exception. However, there is no general explanation for the predisposition of certain alcoholics to this disease.

The remaining 10 to 20% of patients with AP have this disease either due to unknown causes (idiopathic), or in association with a variety of processes (miscellaneous causes like metabolic, drugs, vascular, hereditary/genetic, trauma etc). Vascular causes of the inflammation of the pancreas can be due to the formation of atherosclerotic emboli to

the pancreatic arterial tree, episodes of severe hypotension, or vasculitis (for example, in systemic lupus erythematosus). Infectious causes of pancreatitis are usually rare. They can be divided into those caused by viruses (for examples, mumps, cytomegalovirus, *etc*.) and bacteria (for examples, mycobacteria and leptospirosis). Fungi like *Cryptococcus* can also cause microabscesses in the pancreas. In underdeveloped countries where parasites like *Ascaris* are prevalent, it becomes a common source of pancreatitis. The hereditary type of AP is also known as familial pancreatitis. This is passed on by autosomal dominant transmission and it is rarely encountered. These patients often develop chronic pancreatitis over time and have an increased risk of developing pancreatic cancer.

1.2.2 Complications of AP

There are few complications in mild AP, while severe AP can be fatal. Complications of severe AP are either generalised (for examples, lung or kidney failure, sepsis, circulatory collapse, thrombosis, metabolic and haematological complications) or localised (for examples, pancreatic necrosis, pancreatic cyst formation, and development of fistulas) (Beckingham and Bornman, 2001; Kingsnorth and O'Reilly, 2006).

1.2.3 Treatment for AP

No causal treatments for pancreatitis are known. Patients with AP are primarily given symptomatic treatments like intravenous fluids and pain medications in the hospital. Patients in the intensive care units are closely monitored for heart, lungs and kidney

failure (Besselink et al., 2007). In cases where there is pancreatic necrosis, surgery may be necessary to remove the damaged tissue if a secondary infection develops. AP caused by gallstones may require surgical removal of the gallbladder by cholecystectomy. The pancreas usually recovers after the removal of the gallstones (Beckingham and Bornman, 2001; Kingsnorth and O'Reilly, 2006).

CATEGORY	EXAMPLES
Mechanical	**Gallstones***, duct obstruction / stenosis, pancreatic cancer, trauma, endoscopic retrograde cholangiopancreatography (ERCP)
Toxins / Metabolic	**Alcohol***, scorpion venom, organophosphorus insecticides, hyper-calcemia / lipidemia (types I, IV, V), drugs
Vascular	atheroembolism, ischemia, cardiovascular shock, vasculitis (Systemic Lupus Erythematosus, polyarteritis)
Infectious	Viruses, bacteria, fungi, parasites
Hereditary / Genetic	Autosomal dominant- abnormal gene / chromosome 7g, mutations in trypsin / trypsin inhibitor genes, cystic fibrosis transmembrane conductance regulator gene (CFTR)
Idiopathic	Undetermined cause, postoperative pancreatitis

Table 1.1 Etiologies of AP (* most commonly encountered).

1.3 PATHOPHYSIOLOGY OF AP

The physiological function of the exocrine pancreas includes the synthesis and secretion of digestive enzymes into the small intestine for carbohydrate, fat, and protein metabolism. Many of these enzymes are proteases that are synthesized as proenzymes (zymogens) that require a proteolytic activation through cleavage of their propeptide by the brush–border endoprotease enteropeptidase (enterokinase). Further activation of trypsinogen and other zymogens into their active forms is carried out by a positive feedback mechanism through the trypsin (active form). Thus, until it is activated for digestion in the small intestine, trypsinogen and other pancreatic proteases remain inactive under physiological conditions.

1.3.1 Early Events in AP

AP involves a complex cascade of events that starts in the pancreatic acinar cells. Despite advances in understanding of the disease, the exact pathogenesis of AP remains a subject of scientific debate. About a century ago, Chiari suggested that acute necrotizing pancreatitis was due to autodigestion of exocrine tissue by proteolytic and lipolytic enzymes (Chiari, 1896). From then onwards, the most common and widely accepted hypothesis has been based on the concept of an inappropriate activation of digestive zymogens in the pancreas. This is usually caused by an injury or disruption of the pancreatic acini, which results in the leakage of pancreatic enzymes (trypsin, chymotrypsin and elastase) into pancreatic tissue (Pandol, 2005; Bhatia et al., 2005; Halangk and Lerch, 2005; Halangk and Lerch, 2004; Steer, 1999). The activated proteases (trypsin and elastase) and lipase

autodigest pancreatic tissue and cell membranes, causing edema, vascular damage, hemorrhage and necrosis (Bhatia et al., 2005a).

Questions on why, where and how the activation of zymogen starts within the acinar cell still remain the topics of research interest and debate. So far, several pathways are postulated to be involved in the intracellular activation of pancreatic zymogens to active forms (Gorelick and Otani, 1999). These include: (1) trypsinogen autoactivation to trypsin, (2) cleavage of trypsinogen to trypsin by the lysosomal hydrolase cathepsin B (CTSB), (3) diminished activity of the intracellular pancreatic trypsin inhibitor, (4) leakage of zymogens and lysosomal enzymes into the cytoplasm and subsequent proteolytic activation, (5) shunting of zymogens into membrane-bound compartments that contain active proteases, (6) uptake and processing of secreted zymogens by endocytic pathways, and (7) enhanced susceptibility of zymogens to proteolysis because of oxidation or decondensation (Gorelick and Otani, 1999). Trypsinogen autoactivation (Figarella et al., 1988), CTSB activation of trypsinogen (Gorelick and Otani, 1999), inappropriate activation of trypsinogen (Naruse S., 2003) and disturbances in calcium (Ca^{2+}) signaling have received the most attention.

1.3.2 Trypsinogen Autoactivation

In the mechanism of trypsinogen autoactivation, trypsin-induced trypsinogen activation represents the event that initiates AP (Whitcomb et al., 1996). In the results of some recent studies, it is suggested that trypsinogen activation occurs intracellularly along the normal secretory pathway within small cytoplasmic vacuoles

that contain lysosomal markers and release trypsin into the cytoplasm in a time-dependent fashion (Bruno MJ, 2001). Further investigations are needed to better understand the role of these cytoplasmic vacuoles. Observations have suggested that progressive disassembly of microtubules and filaments in the acinar cells cause a blockage of luminal exocytosis, with subsequent accumulation of zymogen granules (Jungermann et al., 1995). However, in a recent study, the investigators concluded that autoactivation of trypsinogen is not an initiating factor for the intrapancreatic proteolytic cascade (Halangk et al., 2002). Using a cell-permeant, highly specific, and reversible trypsin inhibitor, researchers were able to completely inhibit trypsin activity in isolated rat pancreatic acini or lobules. The conversion of trypsinogen to trypsin in response to supramaximal caerulein remains completely unaffected by the presence of a specific trypsin inhibitor and thus by the presence or absence of free trypsin activity within the acinar cells. Thus, more studies are needed to establish the precise role of autoactivation of trypsinogen in AP.

1.3.3 CTSB Activation of Trypsinogen

There are many data that support the possible role of lysosomal cysteine proteinase CTSB in the molecular mechanisms of intracellular trypsinogen activation: 1) in vitro activation of trypsinogen by CTSB (Figarella et al., 1988); 2) Redistribution of CTSB into a zymogen granule-containing subcellular compartment was detected during the initial phase of AP (Hofbauer et al., 1998b); 3) Detection of lysosomal enzymes in secretory organelles by immunogold electron microscopy (for example, trypsinogen in the experimental AP) (Hofbauer at al., 1998b); 4) Reduced trypsin activity and pancreatic injury after in experimental secretagogue-induced pancreatitis in CTSB-

deficient mice (Halangk et al., 2000). These findings have provided evidence that CTSB may play an important role in trypsinogen activation within the pancreas and the initiation of AP. However, an incomplete protection against trypsinogen activation and pancreatic injury may suggest that other unknown mechanisms may be involved in the trypsinogen activation in early AP.

1.3.4 Inappropriate Activation of Trypsinogen

In the healthy pancreas, there are several safety mechanisms to cope with the risk of autoactivation of zymogens, which can lead to autodigestion (Naruse S, 2003). Usually, trypsinogen is activated by enterokinase only when it is secreted into the duodenum. At this point, trypsin is still able to autoactivate trypsinogen. Pancreatic secretory trypsin inhibitor (PSTI) is present in secretory granules of acinar cells, which binds to the active site of trypsin and inhibits trypsin activity. The molar ratio of PSTI to trypsin was estimated to be 1: 10 (Naruse, 2003). This inhibitory mechanism is no longer effective, when more than 10% of trypsinogen is activated. Thus, any disorders or agents that cause abnormalities in this natural protective mechanism can cause pancreatitis (Naruse, 2003).

1.3.5 Involvement of Ca^{2+} signaling

Ca^{2+} plays an important role in the early phase AP. Pancreatitis induced by caerulein hyperstimulation and by pancreatic duct obstruction has been shown to cause a rise in intracellular Ca^{2+} and a disruption of acinar cell Ca^{2+} signaling. This is associated with acinar cell vacuolization and the intracellular trypsinogen activation events that

occur in early AP (Bhatia et al., 2001; Raraty et al., 2000; Mooren et al., 2003). While there is clear evidence that intracellular Ca^{2+} takes part in intracellular trypsinogen activation, it is still unclear whether the change in Ca^{2+} signaling alone is sufficient for this effect. Incubation of pancreatic acini with thapsigargin (Ca^{2+}-ATPase inhibitor) resulted in trypsinogen activation in one (Raraty et al., 2000) but not another study (Saluja et al., 1999). Therefore, more investigation is required to clearly understand the role of Ca^{2+} in trypsinogen activation. Furthermore, acidic pH and presence of Ca^{2+} are important in trypsinogen autoactivation (Figarella et al., 1988). In addition, the affinity of PSTI is higher at neutral pH and is reduced at lower pH. Therefore, the generation of low-pH compartments within the acinar cell during experimental pancreatitis may be important to trypsinogen activation (Figarella et al., 1988).

1.4 INFLAMMATORY MEDIATORS

The pathogenesis of AP involves the interplay of local and systemic immune responses that are often difficult to characterize. There is an intricate balance between localized tissue damage with the systemic production of proinflammatory and anti-inflammatory mediators. The critical players in this interaction include the proinflammatory cytokines tumor necrosis factor-α (TNF-α), interleukin (IL)-1β, IL-6, platelet activating factor (PAF), chemokines, adhesion molecules and substance P (Rinderknecht H, 1988; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a). Anti-inflammatory mediators like IL-10 and complement component C5a, have also been shown to be intimately involved in the inflammatory response to AP. The expression of several cytokines (IL-1β, IL-6, and TNF-α) is mediated via receptor-

induced pathways, and are most commonly regulated by transcription factors such as nuclear factor kappa B (NF-κB) (Algul et al., 2002; Mercurio and Manning, 1999).

1.4.1 NF-κB

The exact cellular mechanism by which the cytokines are activated by NF-κB is not completely known, although the activity of NF-κB is mediated by the activation and translocation of NF-κB hetero- and homodimers into the nucleus (Algul et al., 2002; Thanos and Maniatis, 1995). Upon activation, degradation of the inhibitory element of NF-κB releases the latter, resulting in the translocation of NF-κB into the nucleus, where it activates gene transcription. Activation of NF-κB and decreased expression of the inhibitory element of NF-κB have been demonstrated in caerulein-induced pancreatitis in rats (Gukovsky et al., 1998; Han and Logsdon, 2000; Grady et al., 1997; Bhatia et al., 2002b). Considering the important role proinflammatory cytokines play in AP, NF-κB has been investigated by researchers as a potential therapeutic target. One report (Steinle et al., 1999) showed that inhibition of NF-κB enhanced tissue injury and inflammation while others found that this inhibition attenuates severity or even improves survival in different experimental models of AP (Ethridge et al., 2002; Satoh et al., 1999; Dunn et al., 1997). In a recent study, NF-κB was directly activated within the pancreas using adenoviral-mediated transfer of an active subunit, RelA/p65, delivered by intraductal injection (Chen et al., 2002). In this study, activation of NF-κB within the pancreas was sufficient for the initiation of an inflammatory response. Despite conflicting results with this transcription factor, NF-

κB remains an important eukaryotic transcription factor, whose precise function in the pathophysiology of AP has yet to be determined by future research.

1.4.2 TNF-α and IL-1β

Levels of both these proinflammatory mediators are elevated at the onset and during the progress of AP (Uhl et al., 2002; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a; Fink and Norman, 1997; Hirota et al., 2000; Norman et al., 1995). Naturally occurring soluble tumour necrosis factor (TNF) receptors (sTNFR) and IL-1ra, by neutralizing the activity of TNF-α and IL-1β, respectively, act as anti-inflammatory mediators (Uhl et al., 2002; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia et al., 2002a; Fink and Norman, 1997; Hirota et al., 2000; Norman et al., 1995). Pancreatic acinar cells have been shown to produce and release TNF-α (Gukovskaya et al., 1997). On induction of AP, knockout mice lacking receptors for IL-1β or/ and TNF-α have significantly improved survival when compared to wild-type mice (Denham et al., 1997). Moreover, the inhibition of IL-1β and TNF-α has been shown to attenuate the severity of pancreatitis in different experimental models of AP. Blockade of the IL-1 receptor before or soon after induction of pancreatitis is associated with decreased severity of pancreatitis and reduced intrinsic pancreatic damage. Also, neutralization of TNF-α with a polyclonal antibody significantly reduces the severity of AP in rats. Strategies that interfere with TNF-α or IL-1β translation, intracellular processing and release rather than antagonizing their effects also decrease the severity of an attack in experimental models (Hughes et al., 1996). On the other hand, combined augmentation of serum IL-1β and TNF-α in AP, exerts synergistic proinflammatory effects (Mayer et al., 2000).

1.4.3 IL-6

IL-6 is a proinflammatory cytokine that is produced by a wide range of cells including monocytes/macrophages, endothelial cells, fibroblasts and smooth muscle cells in response to stimulation by endotoxin, IL-1β and TNF-α (Uhl et al., 2002; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a). It is also produced by periacinar myofibroblasts in response to TNF-α and IL-1β (Jambrik et al., 2002). IL-6 levels are raised in a number of acute conditions such as burns, major surgery, and sepsis. Plasma levels of IL-6 correlate with hemodynamic abnormalities (cardiac output, left ventricular filling) characteristic in AP in the rabbit (Leser et al., 1991). Administration of IL-6 induces pyrexia. IL-6 levels are raised in patients with AP and correlate with disease severity (Pezzilli et al., 1998; Suzuki et al., 2000). Transgenic mice overexpressing human IL-6 are more susceptible to AP, and in these mice a monoclonal anti- IL-6 antibody has a protective effect (Shimada et al., 2002).

1.4.4 IL-8

IL-8 is a strong neutrophil attractant that belongs to the C-x-C chemokine family (Bhatia et al., 2000). It is produced by phagocytes and mesenchymal cells exposed to inflammatory stimuli, such as IL-1 or TNF, and activates neutrophils inducing chemotaxis, exocytosis and the respiratory burst (Baggiolini and Clark-Lewis, 1992). In AP, it has been reported that anti-IL-8 antibody reduces lung damage, but not the inflammatory condition in the pancreas (Bhatia et al., 2000). Its high concentrations have been observed in the lungs of patients with ARDS (Oppenheim et al, 1991;

Miller et al., 1992). It has been demonstrated that in bronchoalveolar lavage, levels of IL-8 are significantly higher in patients who have developed ARDS (Donnelly et al., 1993). Anti-interleukin 8 and interleukin-8 complexes has also been suggested to be a clinical marker for ARDS (Kurdowska et al., 2002).

1.4.5 IL-10

IL-10 is an anti-inflammatory cytokine (Uhl et al., 2002; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a). In cultured monocytes, IL-10 upregulates interleukin-1 receptor antagonist (IL-1ra) and sTNFR production, reduces IL-8 and monocyte chemoattractant protein-1 (MCP-1) levels (Seitz et al., 1995). Experimentally, IL-10 was found to reduce the extent of inflammation as well as the mortality associated with AP (Rongione et al., 1997a; Dembinski et al., 2001). Employing recombinant IL-10 in experimental models of AP, the animals were found to be protected to a significant extent (Rongione et al., 1997b; Van Laethem et al., 1995). Pre-treatment with synthetic IL-10 agonist in rabbits also lowers the extent of lung injury and mortality from this condition (Osman et al., 1998). Conversely, IL-10 gene deletion (Gloor et al., 1998), or administration of anti-IL-10 monoclonal antibody (Van Laethem et al., 1998) resulted in mice more susceptible to AP. In one study of clinical AP, serum IL-10 levels were found to be increased to a greater extent in the mild, as against severe, AP (Pizzelli et al., 1997). Yet, three other studies reported that IL-10 levels correlate with the severity of AP (Chen et al., 1999; Berney et al., 1999; Wereszczynska-Siemiatkowska et al., 2003). Two clinical trials involving IL-10 have been carried out in patients with postendoscopic retrograde cholangiopancreatography pancreatitis. In both trials, patients received either recombinant IL-10 or placebo

before endoscopic retrograde cholangiopancreatography. During the course of the condition, one study reported no significant difference in clinical outcome between the two groups (Dumot et al., 2001) while the other reported a significant protection by IL-10 (Deviere et al., 2001). Even in the study by Deviere et al. (Deviere et al., 2001), only 2 patients had severe AP (developed organ failure or had an inpatient stay greater than 10 days). Therefore it is at present uncertain if IL-10 will reduce the severity of AP in patients with severe disease from other causes.

1.4.6 PAF

Platelet-activating factor (PAF) is a potent low molecular weight pro-inflammatory phospholipid released by a number of cell types that include platelets, epithelial cells, mast cells, macrophages, endothelial cells (Uhl et al., 2002; Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a, Bhatia and Moochhala, 2004a). PAF has been linked to the pathogenesis of AP and associated lung injury (Hofbauer et al., 1998a). The study by Hofbauer and co-workers showed that an upregulation of PAF in blood, pancreatic and lung tissues during pancreatitis, and terminating PAF action by hydrolase significantly ameliorate acinar cell injury and necrosis (Hofbauer et al., 1998a). Similarly in other independent studies, it was observed that PAF plays an important role in the pathogenesis by causing or increasing the severity of AP (Zhou et al., 1993; Konturek et al., 1992; Emmanuelli G, 1989). Lexipafant, a PAF antagonist has been used to improve organ failure or mortality of pancreatitis patients in clinical trial as far as phase II study (Kingsnorth et al., 1995). However, its development was terminated in phase III trial, as the drug failed to demonstrate the favorable efficacy (McKay CJ, 2002).

1.4.7 C5a

C5a is a potent pro-inflammatory anaphylatoxin and chemoattractant that is derived from C5 through both the classical and alternative pathways of complement activation. It stimulates smooth muscle contraction, increases blood flow and enhances vascular permeability (Gerard and Gerard, 1994), thus generally viewed as a pro-inflammatory mediator (Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia M, 2002a; Hopken et al., 1996; Bozic et al., 1996). Paradoxically, C5a acts as an anti-inflammatory mediator during AP in mice (Bhatia et al., 2001b). In this study, removal of either C5a receptors or C5 resulted in a worsening of the severity of pancreatitis in mice, as measured from the parameters used to evaluate the severity of AP.

1.4.8 Chemokines

Chemokines are chemoattractant cytokines (8-10kDa) that are critical for the activation and directing leukocytes to areas of injury during inflammation (Baggiolini et al., 1997). Chemokines have been shown to play an important role in the pathophysiology of AP in several studies (Rau et al., 1997; Shokuhi et al., 2002). Levels of IL-8, GRO-α and ENA-78 are elevated during AP (Rau et al., 1997; Shokuhi et al., 2002). These chemokines are also good predictors of the severity of the disease (Rau et al., 1997; Shokuhi et al., 2002). Cytokine-induced neutrophil chemoattractant (CINC) is the rat homologue of the human chemokine GRO-α. The circulating levels of CINC are raised in severe experimental AP (Brady et al., 2002),

and treatment with neutralizing antibody against CINC attenuated pancreatitis-associated lung injury in rats (Bhatia et al., 2000b). Similarly, MIP-1α/RANTES receptor: CCR1 knockout mice were protected from pancreatitis-associated lung injury though there was little effect on pancreatic injury (Gerard et al., 1997). In addition, treatment with Met-RANTES, a CCR1 antagonist reduced the severity of pancreatitis-associated lung injury, with little effect on pancreatic damage (Bhatia et al., 2003c).

1.4.9 Adhesion molecules

Recruitment of leukocytes to sites of inflammation and tissue injury is characterized by a highly coordinated and well-regulated process that largely occurs in one region of the microvasculature, the post-capillary venules. Adhesion molecules expressed on the surface of endothelial cells in postcapillary venules and leukocytes serve to enable an orderly sequence of cell-cell interactions that sustain leukocyte adherence to vascular endothelium and the subsequent transendothelial migration into inflamed tissue. These adhesive interactions are regulated by sequential activation of three different families of adhesion molecules expressed on the surface of leukocytes and endothelial cells: the selectin family (E-, P-, and L-selectins), immunoglobulin G superfamily (ICAM-1&2, VCAM-1, PECAM-1 and MAdCAM-1), and the integrin family (LFA-1, Mac-1 and VLA-4) (Panes and Granger, 1998). It was demonstrated that levels and expression of P-selectin, E-seletin, ICAM-1, VCAM-1, LFA-1 and Mac-1 were upregulated and enhanced in AP (Lundberg et al., 2000; Sunamura et al., 1999; Uhlmann et al., 2001; Sun et al., 2006; Lau et al., 2007). The severity of AP can be effectively suppressed by immunoneutralization of adhesion molecules (Wang et

al., 1999). In rodent, inactivation of adhesion molecules by monoclonal antibodies significantly enhances capillary blood flow in the pancreas, reduces leukocyte rolling and stabilizes capillary permeability (Frossard et al., 1999). The interaction of leukocytes and endothelial cells via adhesion molecules seems to be an early and rate-limiting component of the microvascular dysfunction that accompanies the early events of AP. Although the therapeutic potential of inhibiting adhesion molecules is only beginning to be investigated, it is likely that new chemical agents that are designed to target this component of the inflammatory response will soon be tested in the clinics.

1.5 NEUROGENIC INFLAMMATION (NI)

In 1910, Bruce observed that the inflammatory response induced by the application of mustard oil in the conjuctival sac in experimental animals could be suppressed by sensory nerve ablation (Bruce, 1910; Bruce, 1913). These early studies of the sensory neuron–related inflammatory reaction led to the concept of neurogenic inflammation (NI), referring to both increased vascular permeability and vasodilatation caused by the release of neuropeptides by capsaicin-sensitive sensory neurons (Jancso et al., 1967). NI was recognized as a nervous system-induced physiological process of inflammation.

The neuropeptides released act on the vasculature at the peripheral level to cause the vasodilatation of arterioles, the extravasation of plasma protein from post-capillary venules, and the adhesion of leukocytes to endothelial cells of venules (Geppetti and Holzer, 1996). Additional tissue-specific responses produced by neurogenic

inflammatory mechanisms include smooth muscle relaxation/contraction in the urinary bladder, urethra and iris, inotropic and chronotropic effect on the heart, bronchoconstriction in the airways and other effects (Geppetti and Holzer, 1996).

Peptide-containing primary sensory neurons are characterized by their unique sensitivity to the pungent substance present in the plants of the genus Capsicum, known as capsaicin (Szallasi and Blumberg, 1999). The cloning of the channel operated by capsaicin, the 'vanilloid receptor-1' (VR-1) (Caterina et al., 1997) has clarified the molecular basis of the selective action of capsaicin on sensory neurons. This seven transmembrane domain protein is a non-selective cation channel, whose endogenous stimulants are heat (>43°C), protons (Bevan and Geppetti, 1994) and possibly high anandamide concentrations (Zygmunt et al., 1999). At low concentration, capsaicin has been used as a tool to study NI due to its ability to cause the release of sensory neuropeptides. However, capsaicin is neurotoxic at higher concentrations, and therefore wipes the source of neuropeptides that are required for NI responses (Szallasi and Blumberg, 1999). The specific excitatory/desensitizing effect of capsaicin on these neurons is the reason why they have been defined as 'capsaicin-sensitive' (Szolcsanyi and Mozsik, 1984).

A large variety of agents can stimulate both afferent and 'efferent' (neurogenic inflammation) functions of primary sensory neurons (Geppetti and Holzer, 1996; Geppetti P, 1993; Holzer P, 1988; Maggi CA, 1991). These stimuli include: autacoids (prostanoids, leukotrienes, histamine and serotonin) (Saria et al., 1983), changes in the extracellular milieu, such as increased temperature (Caterina et al., 1997), osmolarity (Piedimonte et al., 1993), lowering of the pH (Geppetti P et al., 1991), inflammatory

or tissue injury conditions like anaphylaxis (Bertrand and Geppetti, 1996). In particular, substance P (SP) produces inflammation in several important models of tissue injury in the airways (Advenier et al., 1997; Bertrand and Geppetti, 1996; Geppetti P, 1993). These inflammatory responses may be limited by the activation of inhibitory receptors on sensory nerves (Maggi CA, 1991). These receptors include histamine H3, adenosine A1, 5-HT1B/D, dopamine D2 receptors and many other receptors (Maggi CA, 1991). Agonists for these receptors may, thus, be considered as anti-inflammatory agents. On the other hand, tachykinin receptor antagonists are regarded as potential anti-inflammatory drugs. Besides SP, other neuropeptides that are implicated in NI include calcitonin gene-related peptide (CGRP), vasoactive intestinal polypeptide (VIP) and neuropeptide Y (NPY) (Karanth et al., 1991).

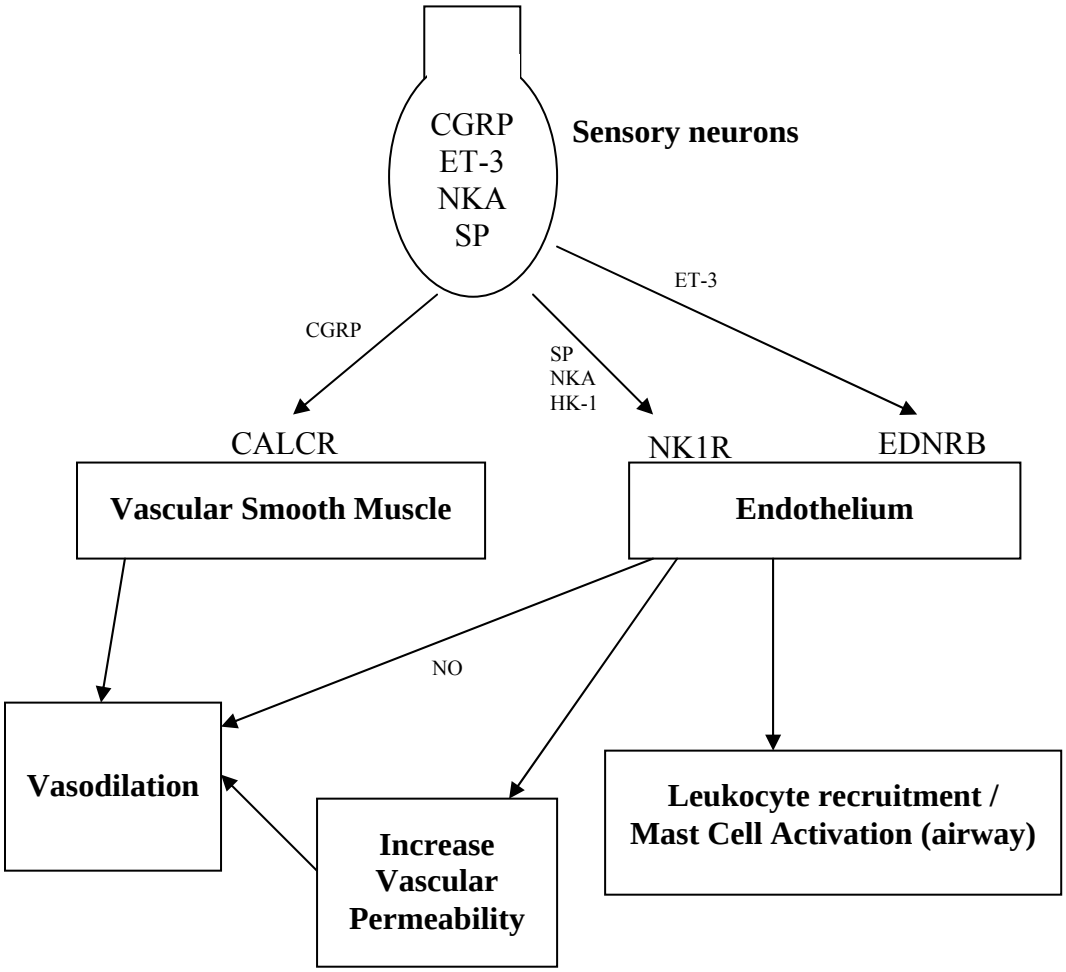


Figure 1.1 Molecular pathways of neurogenic inflammation. SP, substance P; NKA, neurokinin A; HK-1, hemokinin-1; ET-3, endothelin-3; CGRP, calcitonin gene–related peptide; NK1R, neurokinin-1 receptor; EDNRB, endothelin receptor type B; CALCR, calcitonin receptor; NO, nitric oxide.

1.6 TACHYKININS AND NEUROKININ RECEPTORS

1.6.1 Mammalian tachykinins (TK)

To date, there are six TKs identified in mammals. They are substance P (SP), neurokinin A, neurokinin B, neuropeptide K (NPK), neuropeptide γ (NPγ) and hemokinin-1. NPK and NPγ are N terminally extended forms of NKA. The primary structures of TKs are listed in Table 1.2. These closely related peptides are characterized by the presence of the common C-terminal, amino acid sequence FXGLM-NH2. Characteristically, all the TK peptides are aminopeptides with their C-terminal methionine amidated. While the amino acid sequence of SP, NKA and NKB is identical in all mammals where it has been studied, the sequence of HK-1 appears to vary among the mouse, rat and human (Zhang et al., 2000; Kurtz et al., 2002).

1.6.1.1 Substance P

Seventy five years ago, an unidentified chemical was described by von Euler and Gaddum in alcoholic extracts of equine brain and intestine that exhibited a strong stimulant effect on the jejunum and a hypotensive action in the rabbit that was different from any substance then reported to stimulate the gastrointestinal tract. The extract was referred to as "P" on the tracings and the protocols (van Euler and Gaddum, 1931). While numerous studies of its action were conducted using semi-purified preparations, much effort was made to isolate the active substance. Pure SP was isolated from bovine hypothalamus and its structure was established by Chang and Leeman 40 years later (Chang and Leeman, 1970). Over the first 50 years since its discovery, this peptide has been one of the most extensively studied active

peptides. For many years, SP was regarded as the only mammalian TK that was considered as a neuropeptide, until the isolation of neurokinin A and neurokinin B in 1983 (Kangawa et al., 1983; Kimura et al., 1983).

SP is a peptide that made up of 11 amino acids: HRPKPQQFFGLM-NH2 (MW: 1340). Its expression is almost exclusively confined to central and peripheral nervous systems, and it is involved in the transmission of pain, the rapid contractions of the gastrointestinal smooth muscle, and the modulation of inflammatory and immune reactions. It is derived from the product of pre-protachykinin-A (PPT-A) gene (Carter et al., 1994, Figure 1.2). SP is released from its precursor protein by the actions of convertases. Cleavage sites for the convertases on the PPT gene product are doublets of cationic residues. The COOH-terminal then undergoes amindation by the action of peptidyl-Gly-α-amidating monoxygenase using Gly as the amide donor after the cleavage of the Arg-Lys (Harmar et al., 1980; Harmar and Keen, 1982). SP is then transported into storage vesicles and axonally transported to terminal endings (Merighi et al., 1988; Plenderleith et al., 1990; Brimijoin et al., 1980).

Although a number of enzymes are involved in the in vitro metabolism of SP, neutral endopeptidase (NEP: metalloendopeptidase EC.3.4.24.11) and angiotensin-converting enzyme (ACE: EC.3.4.15.1) are probably the enzymes most commonly involved in the metabolism of SP within the periphery (Nadel JA, 1991). NEP has been demonstrated to be involved in the metabolism of SP in the brain (Hooper and Turner, 1987).

Tachykinins	*Peptide Sequence*
Substance P	RPKPQQFFGLM-NH2
Neurokinin A	HKTDSFVGLM-NH2
Neuropeptide K	DADSSI/VEKQVALLKALYGHGQISHKRHKTDSFVGLM-NH2
Neuropeptide V	DAGHGQISHKRHKTDSFVGLM-NH2
Neurokinin B	DMHDFFVGLM-NH2
Hemokinin-1 (Human)	TGKASQFFGLM-NH2
Hemokinin-1 (Rodent)	RSRTRQFYGLM-NH2

Table 1.2 Sequences of Mammalian Tachykinins

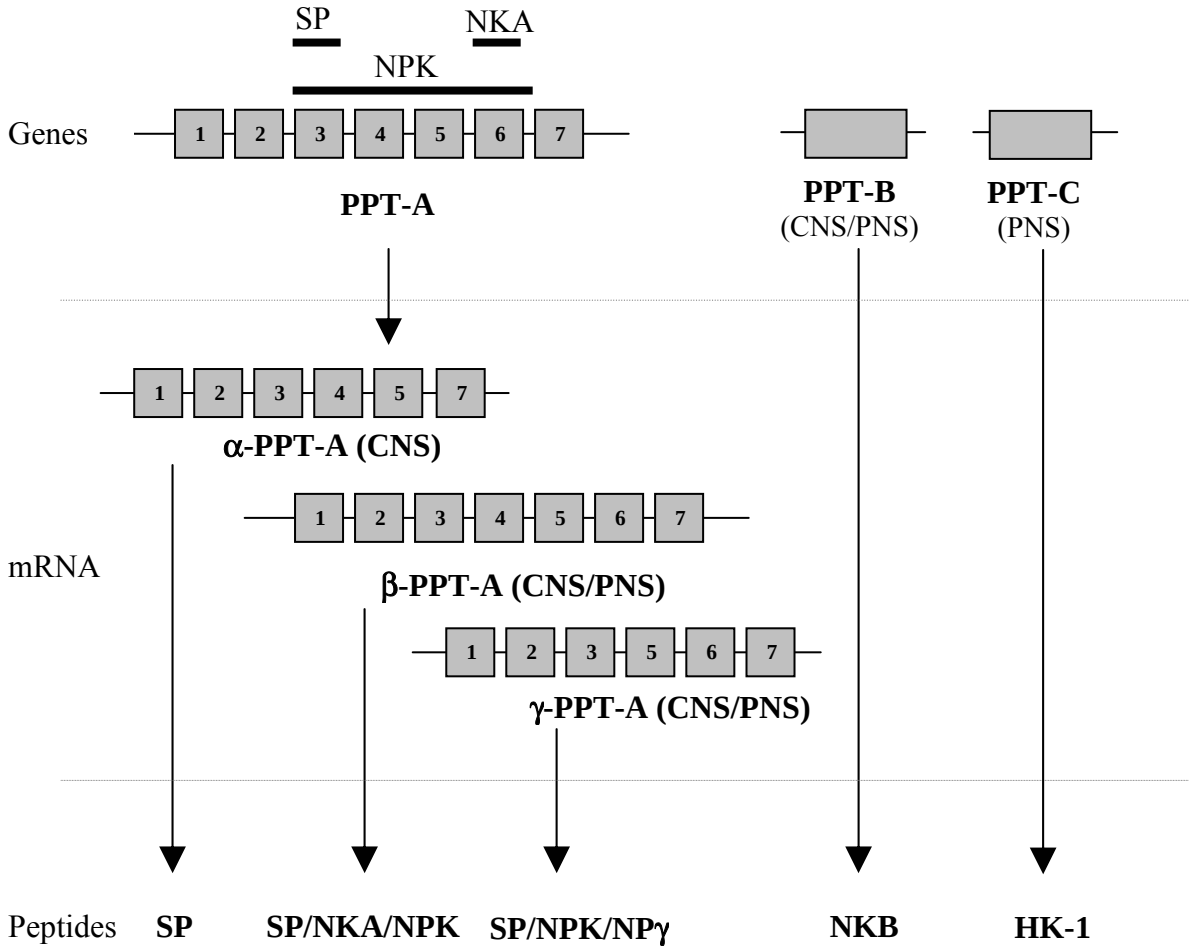


Figure 1.2 Schematic representation of the biosynthesis of SP and related peptides. PPT-A gene encodes for SP, NKA, NPK and NPγ, PPT-B gene encodes for NKB and PPT-C gene encodes for HK1. PPT-B, pre-protachykinin B; PPT-C, pre-protachykinin C; CNS, central nervous system; PNS, peripheral nervous system.

1.6.1.2 Neurokinin A (NKA)

The isolation and identification of NKA from porcine spinal cord was first reported by Kimura and co-workers in 1983 (Kimura et al., 1983). The peptide was also known as neurokinin α (Kimura et al., 1983), substance K (Nawa et al., 1984) or neuromedin L (Minamino et al., 1984). Both NKA and SP are derived from the same neurokinin precursors, the beta or gamma pre-protachykinin A (β-PPT-A and γ-PPT-A; Figure 1.2), and are expressed in and released from the peripheral sensory or autonomic neurons. The peptide sequence of this decapeptide is:

H- HKTDSFVGLM -NH_2.

Although NKA acts as an agonist on all three types of neurokinin receptors, it has the highest affinity towards the NK2R among the known TK (Regoli et al., 1990). It is a potent bronchocontrictor in guinea-pigs (Tatemoto et al., 1985) and human (Advenier et al., 1987). Besides, NKA has potent effect on micturition reflex and duodenum motility (Maggi et al., 1987). In isolated organs, NKA is a potent stimulant of the smooth muscle in the pulmonary artery, duodenum, gall bladder, bronchus, trachea, vas deferens and urinary bladder (Regoli et al., 1990). Other possible roles of NKA include immunomodulation (Zhang et al., 2006; Bost, 2004), inflammation (Marriott, 2004; Evangelista, 2001; Campos and Calixto, 2000) and neuromodulation (Chahl, 2006).

Clear differences exist between SP and NKA susceptibility to metabolism. A study has demonstrated that NKA is resistant to hydrolysis by both ACE and dipeptidyl(amino)peptidase IV (Hooper and Turner, 1987; Wang et al., 1991).

Although NKA is hydrolyzed by plasma (Wang et al., 1991) and endothelial aminopeptidase M (Wang et al., 1994), such hydrolysis gives rise to a smaller active metabolite NKA(4-10). The actual inactivation of circulating NKA may depend on the slow metabolism by low levels of plasma and endothelial NEP-24.11 and/or other unidentified enzymes (Wang et al., 1994).

1.6.1.3 Neurokinin B (NKB)

Besides NKA, Kimura and coworkers have also reported the presence of another TK peptide in the porcine spinal cord (Kimura et al., 1983). The peptide was named neurokinin β (Kimura et al., 1983) or neuromedin K (Kangawa et al., 1983). At the satellite symposium of the International Union of Pharmacology (IUPHAR, London) in 1984, the name neurokinin B (NKB) was recommended and this nomenclature is widely adopted today.

The peptide sequences of NKA and NKB only differ in the first three amino acids from the amino-end. However, NKB is derived from a different gene, pre-protachykinin B (PPT-B) (Bonner et al., 1987; Figure 1.2). This peptide is widely distributed in the CNS (Munekata E, 1991), particularly in the cerebral cortex (Munekata E, 1991). Besides it is also found in some of the sensory neurons in the peripheral tissues including the gastrointestinal system (Tateichi et al., 1990). The function of NKB in the brain remains unclear, although it has been suggested to be implicated in anxiety (Ribeiro et al., 1999) and sensory transmission (Zerari et al., 1997). In rats, NKB causes contraction of the hepatic portal vein (Mastrangelo et al., 1987), venoconstriction of the mesenteric beds (D'Orleans-Juste et al., 1991) and

increases the heart rate (Thompson et al., 1998). More recently, Page and co-workers (2000) identified the presence of NKB mRNA in the human placenta, suggesting the involvement of the peptide in women suffering from pre-eclampsia, with plasma NKB levels correlating well with blood pressure. The metabolism of NKB has not been studied but it was proposed that the peptide may also be a substrate for NEP and ACE (Khawaja and Rogers, 1996).

1.6.1.4 Hemokinin-1 (HK-1)

The fourth mammalian TK was discovered in 2000 in mouse and was named hemokinin-1 (HK-1, Zhang et al., 2000). This decapeptide has the peptide sequence of TGKASQFFGLM-NH_2 in human and RSRTRQFYGLM-NH2 in the rodents (Page NM, 2004). It is a product of the PPT-C gene (Figure 1.2), the third TK gene (Zhang et al., 2000). Strong expression of PPT-C was found mainly in the bone marrow, uterus and skeletal muscle (Page et al., 2003). Nevertheless, moderate tachykinin 4 gene (TAC4) expression was also detected in spleen, lungs, kidney and testis (Page NM, 2004). Although HK-1 and SP have almost equal affinity towards human NK1R, the decapeptide has stronger affinities towards human NK2R and NK3R than SP (Kurtz et al., 2002; Page et al., 2003; Duffy et al., 2003; Belluci et al., 2002).

The first reported pharmacological action of HK-1 was its ability to improve the survival of bone marrow cell in primary cultures and stimulate their proliferation. This effect was not observed with SP (Zhang et al., 2000), which led to speculation that a novel neurokinin receptor might exist (Page, 2004). However, HK-1 has been found to produce most of the effects of SP that are mediated by NK1R (Page NM,

2004). Due to the difference in the expression patterns of SP and HK-1 (nervous system *cf* peripheral tissues), HK-1 is often considered as the endogenous peripheral SP-like endocrine/paracrine agonist where SP is not expressed.

1.6.2 Neurokinin-1 receptor (NK1R)

The NK1R was cloned in 1989 (Yokota et al., 1989). The deduced amino acid sequence (407 amino acid residues) possesses seven putative membrane spanning domains and shows a sequence similarity to the members of G-protein-coupled receptors. Transfection of human NK1R in CHO cells has suggested that the receptor couples to $G_{q/11}$, $G\alpha_s$ and $G\alpha_0$. Cloned NK1R displayed a very high degree of sequence homology among species that include human, mouse, rats and guinea-pig (Gerard et al., 1993). Stimulation of the NK1R activates phospholipase C_β (PLC_β), which leads to intracellular inositol 1,4,5-trisphophate (IP_3) turnover and followed by subsequent elevation of intracellular Ca^{2+}. Therefore, the elevated Ca^{2+} would in turn regulate downstream gene transcriptions (Regoli et al., 1994).

NK1R is widely distributed in the central nervous system (CNS), with highest expression in the superior coliculus and caudate-putamen, and moderate to low levels in the hypothalamus, olfactory bulb, hypothalamus, inferior colliculus, hippocampus, substantia nigra, cerebral cortex, septum, striatum, mesencephalon and dorsal horn of the spinal cord (Shults et al., 1984; Dam and Quirion, 1986). In the peripheral nervous system (PNS), NK1R has been found in the intrinsic and extrinsic neurons of the gut (Sternini et al., 1995; Costa et al., 1986), in unmyelinated axons in glabrous skin (Carlton et al., 1996) and in rodent dorsal root ganglia (DRG) (Dray and Pinnock,

1982; Li and Zhao, 1998). In the gut, the cell bodies of the extrinsic enteric neurons are located in the nodose and DRG ganglia (Baron et al., 1983) and their terminals innervate primarily the arterial vascular system (Holzer and Holzer-Petsche, 1997b), and are thought to be involved in NI processes.

NK1R mediates most of the actions of SP and it has been an important target for drug discovery for the past twenty years. Antagonists of the receptor have been investigated for the treatment of various conditions including pain, depression, anxiety, migraine, chemotherapy-induced emesis and NI.

1.6.3 Neurokinin-2 receptor (NK2R)

The presence of NK2R (formerly known as the substance K receptor) was first reported by Buck and co-workers using radioligand binding assays (Buck et al., 1984). The cloning of NK2R was completed by Sasai and Nakanishi in 1989 (Sasai and Nakanishi, 1989). The rat NK2R consists of 390 amino acid residues (molecular weight, Mr = 43,851) and belongs to the family of seven transmembrane G-protein-coupled receptors. The receptor is also known as "neurokinin A-susceptive" due to its high affinity with NKA (Patacchini et al., 2004).

Although NK2R has limited presence in the CNS (Bensaid et al., 2001), it was found in the spinal cord and CNS (Hagan et al., 1993) and may be involved in, for example, pain processing (Xu et al., 1991; Nagy et al., 1993; Santucci et al., 1993) and anxiety (Stratton et al., 1993). The receptor is found mainly in the peripheral organs including the gastrointestinal tract and ferret airway smooth muscle (Tsuchida et al., 1990;

Meini et al., 1993). In the rat duodenum, the stimulation of NK2R causes smooth muscle relaxation (Giuliani et al., 1988). Due to its capability to regulate gastrointestinal motility and inflammatory response, NK2R has been a target for drug discovery for the treatment of diseases such as irritable bowel syndromes, chronic constipation, ulcerative colitis and Crohn's disease (Lecci et al., 2004; Lecci et al., 2006).

1.6.4 Neurokinin-3 receptor (NK3R)

The NK3R has been cloned from rat (Shigemoto et al., 1990, 452 amino acid residues) and human (Buell et al., 1992; Huang et al., 1992; Takahashi et al., 1992; 465 amino acid residues). The primary sequence of the two receptors shows 88% identity with 7 variant positions in putative TM segments (Gerard et al., 1993). Both the rat and human NK3R are considerably longer compared to NK1R and NK2R receptors. The information of the molecular determinants of interaction between ligands and the NK3R protein is almost confined to one study by Gether and co-workers (Gether et al., 1993a), providing information of the structural requirements of the rat NK3R protein which determine the binding affinity for NKB and senktide in a series of rat NK1/NK 3 chimeric receptor constructs.

The NK3R is defined as the mediator of those biological actions encoded by the C-terminal sequence of TK, for which NKB is a more potent agonist than NKA or SP. Studies on the expression of mRNA for NK3R have indicated a limited, albeit sizeable, expression in some peripheral tissues (Tsuchida et al., 1990). The expression and distribution of NK3R in the PNS is limited, as compared to that of NK1R and

NK2R. In sharp contrast, NK3R are abundantly expressed in the CNS and spinal cord: in various regions of the brain the expression of NK3R is clearly distinct from that of other neurokinin receptors, while in some areas NK3 and NK l receptors are co-expressed (Tsuchida et al., 1990; Stoessl and Hill, 1990; Otsuka and Yoshioka, 1993 for review). CNS areas enriched with NK3R are the cerebral cortex (laminae IV-V of frontoparietal cortex), the solitary nucleus, the interpeduncular nucleus, the habenula and the ventral tegmental area; in the spinal cord, the expression of NK3R seems limited to the dorsal horns, mRNA encoding for NK3R has also been detected in various regions of human brain while expression in peripheral tissues is very low or absent (Buell et al., 1992).

Functional responses to NK3R selective agonists have been detected in the longitudinal and circular muscle layers of the guinea-pig ileum and colon to produce indirect smooth muscle contraction/relaxation by releasing various mediators (Laufer et al., 1986; Laufer et al., 1988; Maggi et al., 1993e), direct smooth muscle contraction in the rat portal vein (Mastrangelo et al., 1986), rat oesophagus (Stables et al., 1991) and rat uterus (Barr et al., 1991) and neuronal depolarization in the guinea-pig superior cervical ganglia (Seabrook et al., 1992) and tracheobronchial ganglia (Myers and Undem, 1993).

1.7 Antagonism of Neurokinin receptors

The search for agents to inhibit the neurokinin-TK system started in the 1960's when Schroeder and co-workers reported weak peptide antagonists of SP (Schroeder et al., 1965). The first non-peptide antagonist, CP96,345 was synthesized by Pfizer Inc.

nearly thirty years later (Snider et al., 1991). CP96,345 is a selective antagonist of NK1R with low affinities for NK2R and NK3R (Snider et al., 1991). This was followed by the discoveries of other NK1R antagonists including CP99,994 (Pfizer Inc.), Vestipitant and Casopitant (GlaxoSmithKline Plc.), Aprepitant (EMEND; Merck Research Laboratories), Lanepitant (Lilly Research Laboratories), Dapitant (Sanofi-Aventis), FK888 (Fujisawa Pharmaceutical Co., Ltd.,) and so on. Although the receptor is implicated in a large number of pathological conditions, the developments of these antagonists have been limited to the treatment of pain, anxiety, depression, chemotherapy-induced emesis, psychosis and irritable bowel syndrome (IBS). So far, only Aprepitant, Vestipitant and Casopitant have been proven to be effective in the treatment of chemotherapy-induced emesis (FDA Center for Drug Evaluation and Research). The use of NK1R antagonists in depression and anxiety is also being investigated.

The human NK2R has been validated as an attractive target for the treatment of a number of conditions in the respiratory, gastrointestinal and genitourinary systems. The first selective NK2R antagonist, SR-48968 (Saredutant) has been in development by Sanofi-Synthélabo in 1992 (Emonds-Alt et al., 1992). The compound is currently in Phase III clinical trial for the treatment of depression and Phase IIb clinical trial for the treatment of irritable bowel syndrome (IBS) (Ashburn and Gupta, 2006). Menarini Pharmaceutical is also developing its NK2R antagonist, Nepadutant in Phase IIa for IBS. Pfizer Inc. is also developing its NK2R antagonist (UK-224671), but mainly for the treatment of urinary incontinence (MacKenzie et al., 2002).

SR-142801 represents the first non-peptide NK3R antagonist synthesized by Sanofi-Synthelabo in 1995 (Emonds-Alt et al., 1995). There are two NK3R antagonists that are currently in the late stage of the development, namely Talnetant (GlaxoSmithKline Plc) and Osanetant (Sanofi-Aventis), showing efficacy in the treatment of schizophrenia, anxiety and psychosis. However, these drugs are also being investigated for the treatment of IBS and over-reactive bladder (FDA Center for Drug Evaluation and Research).

1.8 Aims

Although there are a number reports that suggested a possible role of NI in AP, the pathway by which NI aggravates AP is still unknown. In light of a previous study that demonstrated the importance of tachykinins and neurokinin receptors in the development of AP and associated lung injury using PPT-A knockout mouse (Bhatia et al., 1998), I investigated the relationship between the activation of tachykinin-neurokinin receptors systems and the pathological events that take place during AP and associated lung injury. The following are the aims of the studies described in this thesis:

1. To examine the effects of CP96,345 (NK1R antagonist), GR159897 (NK2R antagonist), SB-222200 (NK3R antagonist) and CGRP (8-37) (CGRP receptor antagonist) on caerulein-induced AP. The goal is to understand the role of neuropeptide receptors in AP and associated lung injury and to examine the potential of using neurokinin receptors and CGRP receptor as potential therapeutic targets for the treatment of AP and the associated lung injury.

2. To study the effect of CP96,345 on the expression of TK and neurokinin receptors in AP using *in vitro* isolated acinar cells and an *in vivo* mouse model of AP. This will provide important information for the understanding of the role of SP and NK1R, as well as the contribution of other TK and neurokinin receptors in AP and associated lung injury.

3. To investigate the effect of CP96,345 on the expression of adhesion molecules in AP. This will enhance our understanding of the role of SP and NK1R in the regulation of the downstream inflammatory cascades mediated by adhesion molecules during AP and the associated lung injury.

CHAPTER 2

ROLE OF NEUROPEPTIDE RECEPTORS IN ACUTE PANCREATITIS AND ASSOCIATED LUNG INJURY

2.1 INTRODUCTION

It is widely recognized that NI is one of the most important processes in the development and aggravation of AP (Liddle and Nathan, 2004; Hegde and Bhatia, 2005; Nathan et al., 2002). The primary sensory neurons, which release neuropeptides and trigger the early events of AP, are characterized by their unique sensitivity to the pungent principle component contained in the plants of the genus Capsicum, known as the capsaicin (Szallasi and Blumberg, 1999). The molecular basis of the selective action of capsaicin on these sensory neurons has been solved by the cloning of the channel operated by capsaicin, the Transient Receptor Potential Vanilloid-1 (TRPV-1) (Caterina et al., 1997; Liddle and Nathan, 2004). TRPV1 is a nonselective-cation channel with a preference for Ca^{2+} that is expressed by a subset of primary spinal afferent neurons containing the neuropeptides such as TK and CGRP (Liddle and Nathan, 2004; Szolcsanyi J, 2004). It has been shown recently that TRPV-1 is important in mediating NI in caerulein-induced pancreatitis in rats, due to the release of neuropeptides upon its activation (Hutter et al., 2005).

Among the neuropeptides released from the primary sensor neurons, SP has attracted most attention. SP is a member of the TK family. Subsequent to its release, SP binds primarily, but not exclusively, to NK1R on the surface of effector cells and, in

addition to being a mediator of pain, acts as a pro-inflammatory mediator in many inflammatory states including asthma, immune-complex-mediated lung injury, experimental arthritis, and IBS (Bhatia et al., 2000a; Bhatia et al., 2001a; Bhatia, 2002a; Bhatia, 2003a; Bhatia and Moochhala, 2004a; Bhatia, 2004b). Knockout mice deficient in NK1R and in the PPT-A gene are protected against pancreatitis and associated lung injury (Bhatia et al., 1998a; Bhatia et al., 2003b; Grady et al., 2000). These findings suggest an important pro-inflammatory role for NI and NK1R in acute pancreatitis and associated lung injury. Besides SP, two other TK, NKA and NKB are also believed to be involved in inflammatory conditions of the gastrointestinal tract (Regoli et al., 1990; Marriott I, 2004; Evangelista S 2001; Campos and Calixto, 2000; Tateichi et al., 1990). However, their roles in AP are still unclear.

In addition to the TK, CGRP is another neuropeptide implicated in AP (Wick et al., 2006a; Warzecha et al., 2001; Warzecha et al., 1999). The peptide has been shown to produce a protective effect against AP in rats when given before the pancreatic injury (Warzecha et al., 1999; Dembinski et al., 2003). However, it aggravated the condition in rats when it was administered during AP (Warzecha et al., 2001, Warzecha et al., 2000). On the other hand, antagonism of rat CGRP receptors has been demonstrated to attenuate pain transmission in AP (Wick et al., 2006a; Wick et al., 2006b). Since the role of CGRP in AP was only investigated by two groups of scientists and the findings were controversial and inconclusive, further investigations of the action of this peptide are required.

Pancreatitis induced by supramaximal exocrine stimulation with the synthetic CCK analog caerulein, first described by Lampel and Kern in 1977, is characterized by

marked interstitial edema, leukocyte infiltration, hyperamylasemia, and severe disturbances of acinar cell morphology and the secretory process. Similar changes are seen in human pancreatitis, which in the great majority of cases presents as an edematous inflammatory process that regresses without development of fulminant necrotizing disease (Fernández-del, 1993). Pancreatic acinar cells have been shown to possess two classes or states of cholecystokinin (CCK) receptors – high affinity and low affinity receptors (Sankaran et al., 1982; Sankaran et al., 1980; Honda et al., 1987; Sato et al., 1989; Stark et al., 1989). The high affinity site is thought to mediate normal pancreatic secretion and growth, while the low affinity site is responsible for supramaximal inhibition of secretion and AP (Niederau et al., 1994). Because of its self-limited character, caerulein-induced pancreatitis offers the attractive possibility to observe the sequence of events that may lead to and cause premature protease activation before widespread autodigestion prevents any systematic investigation.

In this chapter, I have examined the potential of using neurokinin receptors and CGRP receptor as potential therapeutic targets for the treatment of AP and the associated lung injury in mice. To that end, I have investigated the effect of pharmacological intervention against the neurokinin receptors using CP96,345 (NK1R antagonist), GR159897 (NK2R antagonist), SB222200 (NK3R antagonist), and the CGRP receptor using CGRP (8-37) (CGRP antagonist), on pancreatic and lung injury in AP induced by caerulein hyperstimulation in mice.

2.2 MATERIALS AND METHODS

2.2.1 Antagonists and other chemicals

Caerulein, the decapeptide analog of cholecystokinin (CCK) was purchased from Bachem California (Torrance, CA), NK1R antagonist CP96,345 was a gift from Pfizer Diagnostics (USA), NK2R antagonist GR159897, NK3R antagonist SB-222200 and CGRP receptor antagonist CGRP(8-37) were purchased from Sigma-Aldrich Chemicals USA (St. Louis, Mo) (Table 2.1). Tetramethylbenzidine (TMB) was purchased from Kirkegaard & Perry Laboratories (KPL, Gaithersburg, MD), and pentobarbitone sodium (Nembutal®) was obtained from Ceva Chemicals (Ceva Chemicals Australia Pty. Ltd, NSW, Australia). Heparin Leo® was from Leo Pharma (Ballerup, Denmark). 10% pH-neutral, phosphate-buffered formalin solution was bought from JT Baker (NJ, USA), α-amylase reagent (Infinity^{TM} Liquid Amylase Reagent) was purchased from Thermo Trace Electron Corp (Waltham, MA, USA). Haemotoxylin, eosin solution, neo-clear and neo-mount were purchased from Merck KGaA (Darmstadt, Germany). 0.9% NaCl, hexadecylmethylammonium bromide, sulphuric acid, salmon testes DNA and Hoechst dye no. 33256 (bisBenzimidine) were purchased from Sigma-Aldrich Chemicals USA (St. Louis, Mo). Both caerulein and CP96,345 were freshly prepared in 0.9% NaCl, while GR159897, SB-222200 and CGRP8-37 were dissolved in dimethyl sulfoxide (DMSO) immediately before use.

2.2.2 Animal model and treatment protocol

All experiments were approved by the Animal Research Ethics Committee of National University of Singapore and performed in accordance with established International Guiding Principles for Animal Research. Experiments were performed on male Balb C mice weighing between 20-25g. Mice obtained from the National University of Singapore Laboratory Animal Centre were housed under climate-controlled conditions with a 12-hour alternate light/dark cycle. They were fed standard laboratory chow and allowed to drink water *ad libitum* throughout the experiment. Mice were randomly assigned to control or experimental groups. The experimental group was further subdivided into three groups with equal number of mice (n = 10 or more). The first group was given the respective antagonist half an hour before the first caerulein injection (prophylaxis treatment group), to simulate the clinical situation in which the prevention of AP (idiopathic) is important before its likely occurrence, for example, in patients who have undergone surgical procedures on or near pancreas. The second group was given the same antagonist one hour after the first caerulein injection immediately at the onset of AP (therapeutic treatment group) to simulate the clinical situation in which patients developed AP before admission to hospital. The last group was given caerulein-only to simulate untreated AP (caerulein group).

Caerulein was administered to mice in 10 consecutive intraperitoneal (i.p.) injections of saline (control) or saline containing a supramaximally stimulating concentration of caerulein (hourly injections of 50μg/kg). The time course of caerulein-induced AP has been investigated previously (Lau et al., 2005). The 10 hourly-injection group

represented the severe form of AP, in which secondary lung injury would be developed. CP96,345 at 2.5mg/kg has been found to block NK1R and consequent NI in several earlier studies (Sakamoto et al., 1993; Pothoulakis et al., 1994; Castagliuolo et al., 1997; Robledo and Witten, 1999). GR159897 at 0.12mg/kg, SB-222200 at 2mg/kg and CGRP (8-37) at 0.06mg/kg have been described in the literature and found to be effective *in vivo* (Beresford et al., 1995; Dakhama et al., 2005; Medhurst et al., 1997; Okajima et al., 2004). One hour after the last caerulein injection animals were sacrificed by an i.p. injection of a lethal dose of pentabarbitone sodium Nembutal® (90 mg kg^{-1}, three times the dose for surgical anesthesia). Blood samples were drawn from the right ventricles using heparinised syringe and centrifuged to obtain the plasma. These plasma samples were then kept for amylase activity determination. Besides, both the pancreas and lung were rapidly isolated and each of them was cut into two portions: 1) about 40mg of tissue, which was rapidly frozen in liquid nitrogen and kept at -70°C for myeloperoxidase (MPO) and DNA analyses; 2) the remaining tissues were fixed in 10% pH-neutral, phosphate-buffered formalin solution for histology study.

2.2.3 Plasma amylase activities determination

Plasma amylase activities were assayed using the substrate 4,6-ethylidene (G_7)-p-nitrophenyl (G_1)-α1-D-maltoheptoside (Thermo Trace Electron Corp., Waltham, MA, USA) following the manufacturer's instructions. In short, the substrate was added to the plasma sample in 40:1 volume ratio in a microplate and the absorbance at 405nm was measured at different time intervals using Tecan SpectraFluor Plus Multiple-Detection microplate reader (Austria Gesellschaft M.B.H). The amylase activity was

then calculated based on the milimolar absorption of p-nitrophenol at 405nm (Bhatia et al., 1998a; Bhatia et al., 2003b; Pierre et al., 1976).

2.2.4 Myeloperoxidase activity determination

Neutrophil sequestration in pancreas and lung in mice was quantified by measuring the respective tissue myeloperoxidase (MPO) activity. The determination was carried out as described previously (Bhatia et al., 1998a; Bhatia et al., 1998b; Bhatia et al., 2003b; Bhatia et al., 2000b; Bhatia et al., 2003c) with slight modifications. Briefly, each tissue sample initially stored at -70°C was thawed at 4ºC. The tissue was then homogenized in 20mM sodium phosphate buffer (pH 7.4) using a Polytron homogenizer. The homogenate was centrifuged at 13,000 *g* for 10 minutes at 4°C. The subsequent pellet was resuspended in 50mM sodium phosphate buffer (pH 6.0) comprising 0.5% hexadecylmethylammonium bromide (Sigma). The resultant suspension was subjected to four freeze-thaw cycles before it was sonicated for 40 seconds on ice. This is followed by centrifugation at 13,000 *g* for 10 min at 4°C to acquire the supernatant for MPO assay. The reaction mixture, consisting of the extracted enzyme, 1.6 mM TMB, 80 mM sodium phosphate buffer (pH 5.4) and 0.3 mM hydrogen peroxide, was incubated at 37°C for 2 minutes. Equal volume of 2N H_2SO_4 was added to quench the reaction. The absorbance at 405nm was measured using the Tecan spectrophotometry autoanalyzer. This absorbance was then corrected for the DNA content of the respective samples and results were expressed as fold increase over control group.

2.2.5 DNA content assay

The determination of the DNA quantity of the sample crude homogenates was performed fluorometrically by using Hoechst dye 33256 by the method described by Labarca and Paigen (Labarca and Paigen, 1980) using salmon testes DNA as standard. Briefly, both the samples and standards (0 to 0.4 µg/ml) were allowed to incubate with 1% Hoechst dye solution for 10 minutes in the dark before the absorbance was read on the Tecan spectrofluorometry autoanalyzer (excitation$_\lambda$ = 360nm, emission$_\lambda$ = 450nm). The standard curve was plotted using Microsoft Excel and the concentration of DNA in the samples was calculated by the linear equation obtained using linear regression.

2.2.6 Histological examination

The pancreas and lung tissues harvested were fixed overnight at room temperature in 10 % pH-neutral, phosphate-buffered formalin solution. The fixed tissues were subsequently processed using the Leica TP1020 automatic tissue processor (Leica Microsystems Nussloch GmbH, Germany), and embedded in paraffin blocks. Sections of 5 µm were cut using a Leica RM2125 manual rotary microtome (Leica Microsystems Nussloch GmbH, Germany) after which they were stained with haematoxylin and eosin (H&E). The slides were then coverslipped with Neo-mount before they were viewed under a Carl-Zeiss Axioskop 40 microscope system (Carl Zeiss, Göttingen, Germany), which was connected to an IBM personal computer. Images were examined using AxioVision Software for Life Sciences to qualitatively estimate tissue injury / necrosis characterized by: 1) oedema, vacuolization and

destruction of histoarchitecture of whole or parts of the acini and alveoli for pancreas and lung respectively; and 2) inflammatory cells infiltration at the necrotic areas in both pancreatic and lung tissues. This allows assessment of the degree of morphological changes during inflammation in both organs as previously described (Bhatia et al., 1998a; Bhatia et al., 1998b; Bhatia et al., 2003b; Bhatia et al., 2000b; Bhatia et al., 2003c).

2.2.7 Data analysis method

The results are expressed as mean ± standard error of mean (SEM). In all figures, vertical bars denote the SEM and the absence of such bars indicates that the SEM is too small to illustrate. The significance of changes was evaluated by analysis of variance (ANOVA) when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by using Tukey's method as a post hoc test for the difference between groups. A P-value of < 0.05 was considered to indicate a significant difference. All statistical analyses were performed using SPSS version 14.0 for Windows (Chicago, Illinois USA).

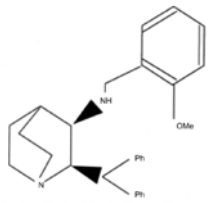
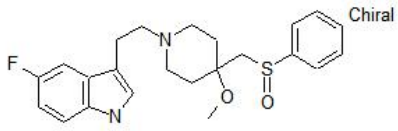
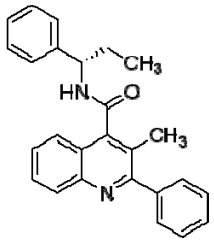
Receptor	Antagonist	MW	Chemical Structure
NK1R	CP96,345	412.67	
NK2R	GR159897	414.55	
NK3R	SB-222200	380.48	
CGRP	CGRP (8-37)	3125.59	VTHRLAGLLSRSGGVVKN-NFVPTNVGSKAF-NH2

Table 2.1 Chemical structures and molecular weights of antagonists of neurokinin receptors and CGRP receptor.

2.3 RESULTS

2.3.1 Effect of CP96,345 treatment on pancreatic injury in AP

Evidence of pancreatic injury in AP induced by intraperitoneal administration of caerulein at a dose of 50 μg/kg hourly for 10 h was confirmed by an 10-fold increase in plasma amylase (Fig. 2.1A), and 3-fold increase in pancreatic MPO activity as a measure of neutrophil infiltration (Fig. 2.1B) in mice treated with caerulein in comparison to control mice treated with i.p. normal saline. In animals administered CP96,345 either before starting caerulein administration or 1 hour after the first injection of caerulein and plasma amylase levels were significantly attenuated compared to animals treated with caerulein alone. MPO activity in the pancreas was also reduced in mice tested with CP96,345 either prophylactically or therapeutically. Histological examination of pancreas sections supported protection by CP96,345 treatment on AP (Fig. 2.2). Tissue damage in the pancreas of the caerulein treated group (Fig 2.2B) was demonstrated by the increase in neutrophil infiltration, edema and pancreatic acinar cell necrosis when compared to the untreated sections.

2.3.2 Effect of GR159897 treatment on pancreatic injury in AP

Neither prophylactic nor therapeutic treatment with NK2R antagonist, GR159897, produced any protective effect against caerulein-induced AP. Plasma amylase and pancreatic MPO activities remained elevated (Figure 2.4A&B). This was supported by histological examination of H&E stained pancreas sections (Figure 2.5).

2.3.3 Effect of SB-222200 treatment on pancreatic injury in AP

Treatment with SB-222200 had no effect on the plasma amylase level (Fig. 2.7A). When given as a prophylactic treatment, the quinolinecarboxamide compound did not change the degree of neutrophil infiltration as indicated by the pancreatic MPO activity (Fig 2.7B) and histological examination (Fig 2.8). Unexpectedly, significant higher (3-fold) MPO activity as observed when the antagonist was administered after the induction of AP. Pancreatic histological sections also appeared to show that the NK3R antagonist did not offer any protection against AP (Fig 2.8).

2.3.4 Effect of CGRP (8-37) treatment on pancreatic injury in AP

When given at 60μg/kg i.p., CGRP receptor antagonist did not affect the progression of AP. Elevation of plasma amylase level and pancreatic MPO activity was not affect by CGRP (8-37) given either prophylactically or therapeutically (Fig 2.10A&B). The pancreatitis morphology was not apparently altered by the inhibition of CGRP receptor (Fig 2.11).

2.3.5 Effect of CP96,345 treatment on AP-associated lung injury

Lungs from mice treated with caerulein had increased levels of MPO activity (2.3-fold) in comparison to control animals treated with saline suggesting neutrophil infiltration as a result of the pancreatitis (Fig. 2.1C). Administration of CP96,345 either 30 min before or 1 hour after the first caerulein injection significantly reduced

lung MPO levels (Fig. 2.1C). Alveolar thickening and infiltration of inflammatory cells in the lung following induction of AP was observed in the caerulein-only group. Treatment with CP96,345 were protect against AP-associated lung injury (Fig 2.3).

2.3.6 Effect of GR159897 treatment on AP-associated lung injury

The effect of GR159897 treatment on lung MPO activity is shown in Figure 2.4C. Caerulein treatment significantly increased the MPO activity and prophylactic treatment with the NK2R antagonist further increased the enzyme level (1.5-fold). However, histological examination did not reveal a similar trend (Figure 2.6). GR159897 did not affect the morphology that is characteristic of AP.

2.3.7 Effect of SB-222200 treatment on AP-associated lung injury

There was no significant reduction in the lung MPO activity in SB-222200 treated AP mice (Fig. 2.7C). H&E staining suggested that both the prophylactic and therapeutic treatment with the antagonist did not protect lung tissue from inflammatory damage (Fig. 2.9).

2.3.8 Effect of CGRP (8-37) treatment on AP-associated lung injury

Lung MPO activity (Fig 2.10C) increased significantly with caerulein treatment compared to the control group, and was not affected by the treatment with the peptide antagonist. Morphological examination demonstrated that the peptide had no effect on AP-associated lung injury (Fig. 2.12).

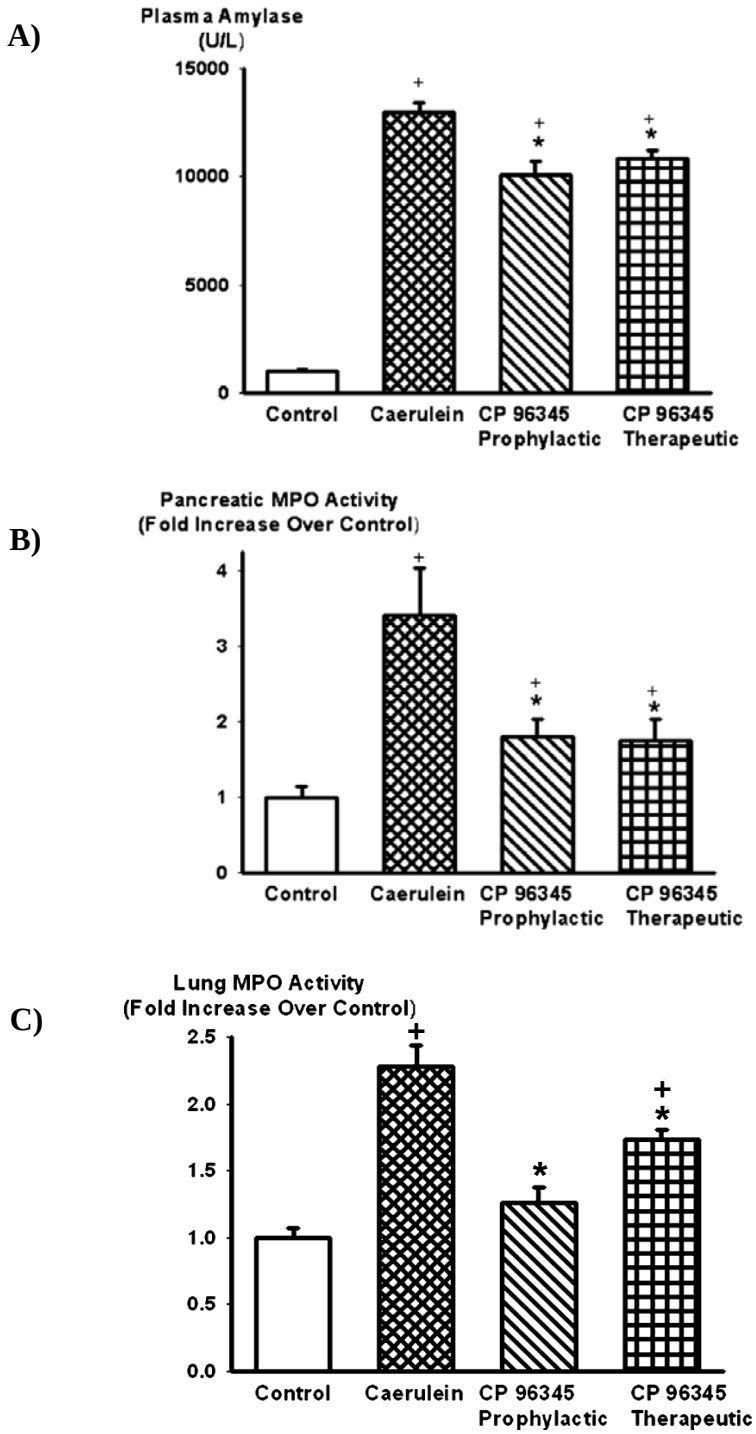


Figure 2.1 Effects of CP96,345 on A) Plasma amylase activity; B) Pancreatic MPO activity; C) Lung MPO activity in AP. Mice were given 10 hourly injections of caerulein (50μg/kg, i.p.). CP96,345 was administered at 2.5 mg/kg i.p. either 30 min before (prophylactic treatment) or 1 hour after (therapeutic treatment) the first caerulein injection. Bar charts represent mean value + SEM of at least 10 animals.

+ indicates P < 0.05 when caerulein-treated animals were compared with saline treated animals.

*** indicates P < 0.05 when CP96,345-treated animals were compared with caerulein-treated animals.**

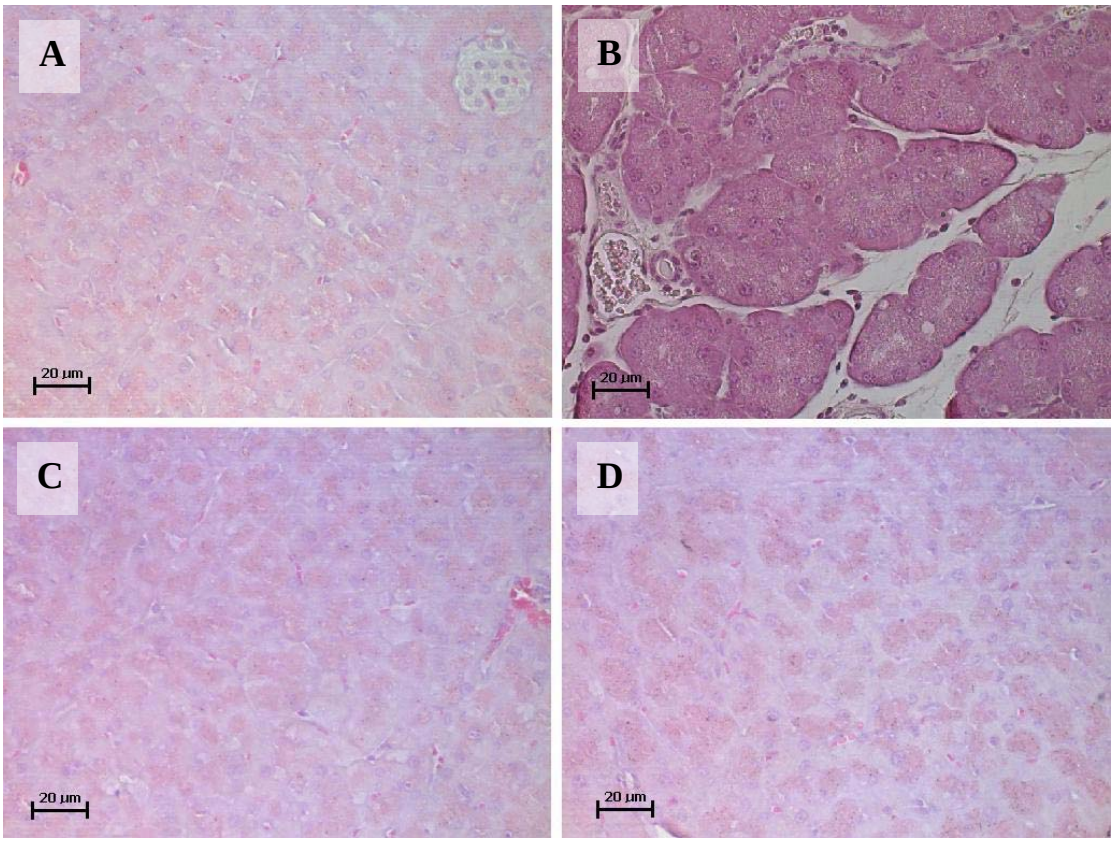


Figure 2.2 Morphological changes in mouse pancreas on induction of AP with/without treatment with CP96,345. Tissue damage in the pancreas of the caerulein treated group (B) was demonstrated by the increase in neutrophil infiltration, edema and pancreatic acinar cell necrosis. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered CP96,345 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered CP96,345 1 hour after the first caerulein injection. Scale bars = 20µm.

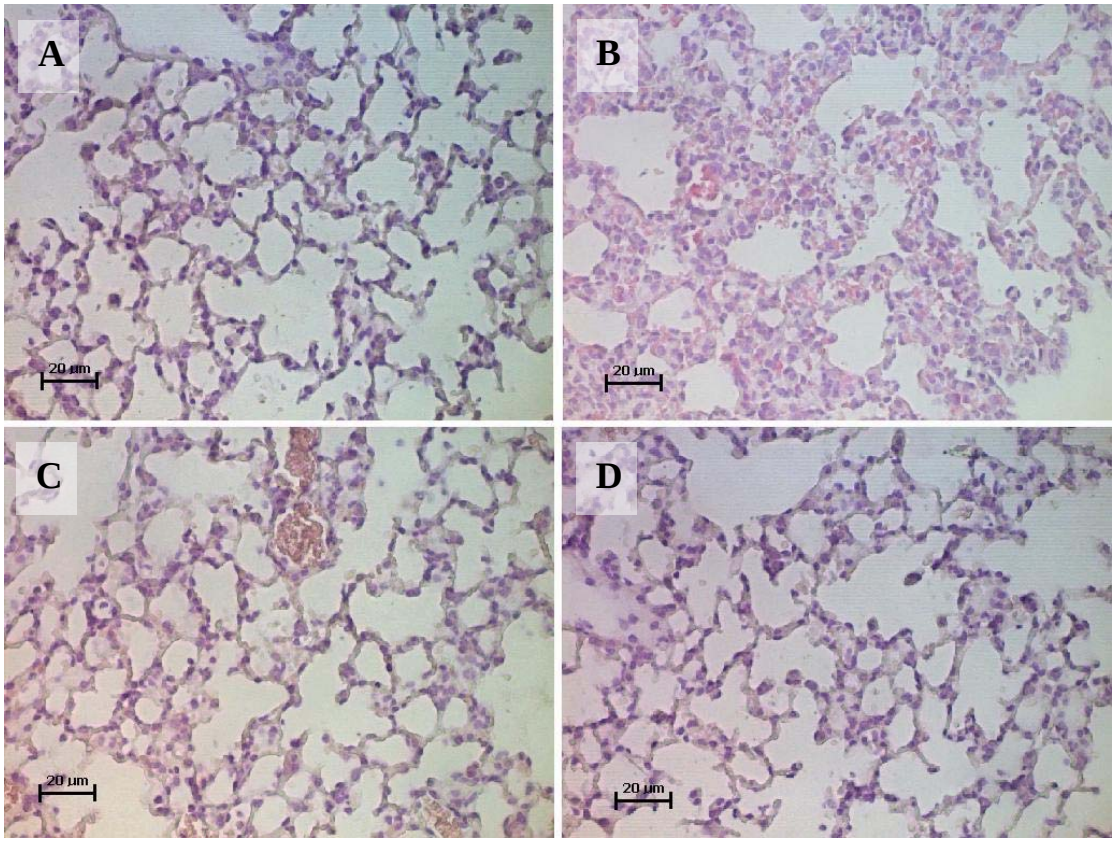


Figure 2.3 Morphological changes in mouse lung on induction of AP with/without treatment with CP96,345. Alveolar thickening and infiltration of inflammatory cells in the lung (B) following induction of AP. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered CP96,345 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered CP96,345 1 hour after the first caerulein injection. Scale bars = 20µm.

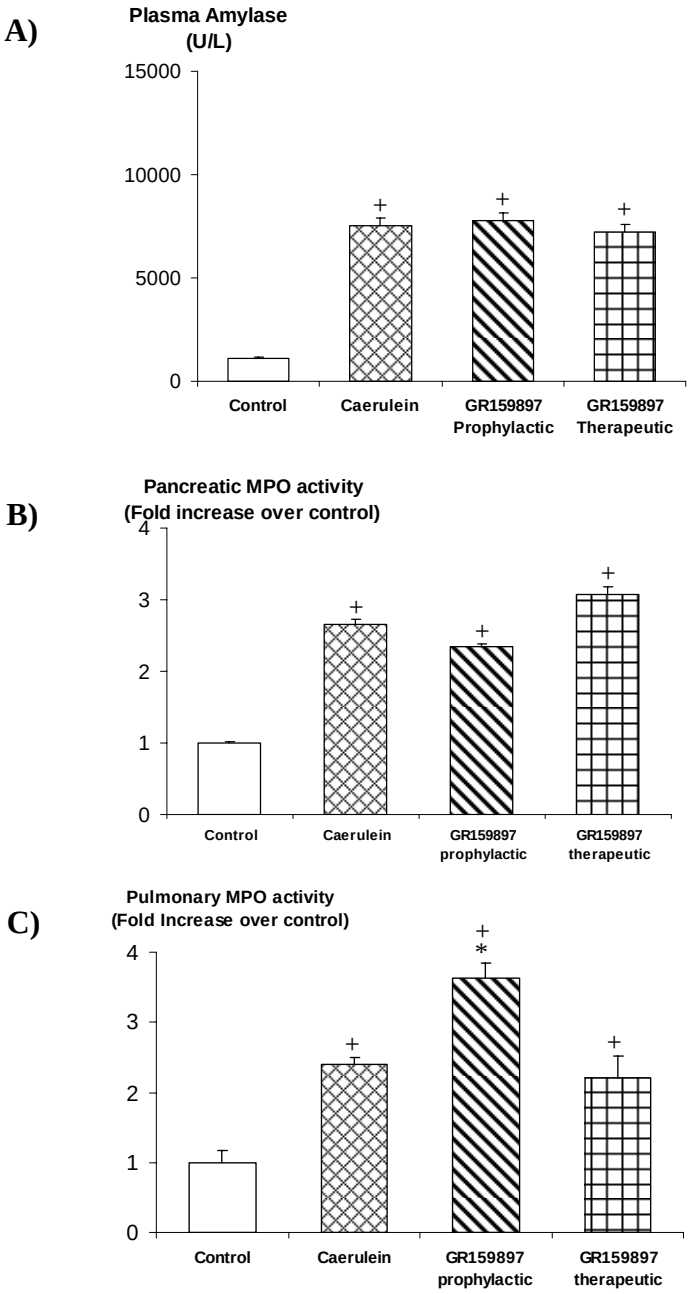


Figure 2.4 Effects of GR159897 on A) Plasma amylase activity; B) Pancreatic MPO activity; C) Lung MPO activity in AP. Mice were given 10 hourly injections of caerulein (50μg/kg, i.p.). GR159897 was administered at 0.12mg/kg i.p. either 30 minutes before (prophylactic treatment) or 1 hour after (therapeutic treatment) the first caerulein injection. Bar charts represent mean value ± SEM of at least 10 animals.

+ indicates P < 0.05 when caerulein-treated animals were compared with saline treated animals.

*** indicates P < 0.05 when GR159897-treated animals were compared with caerulein-treated animals.**

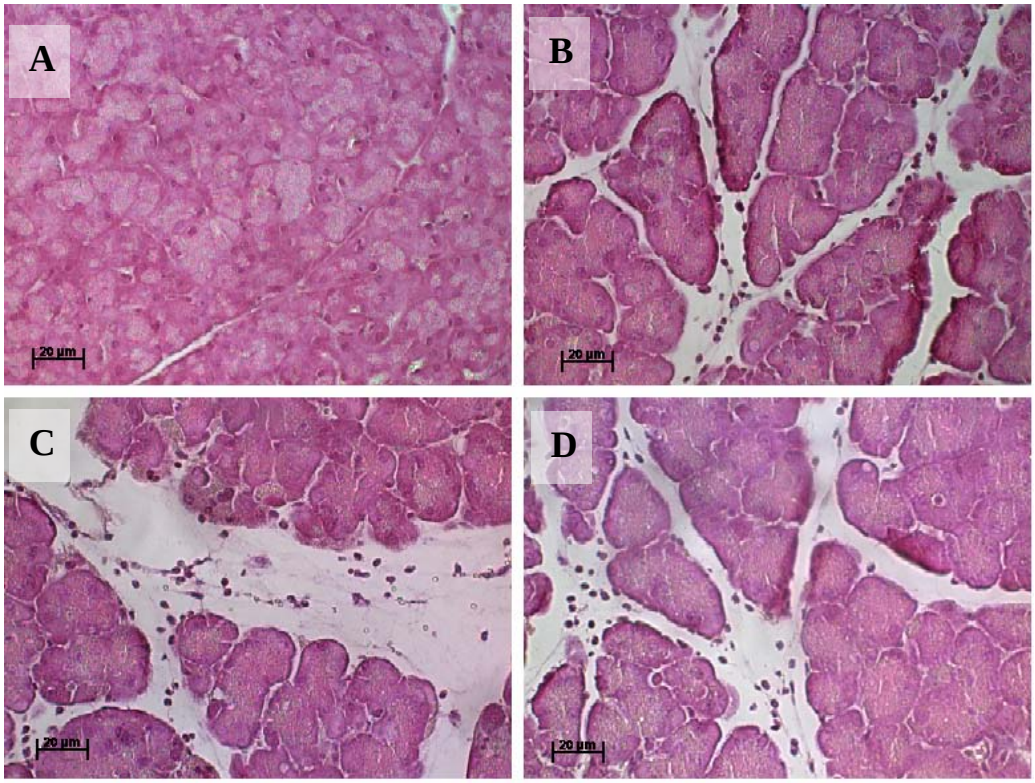


Figure 2.5 Morphological changes in mouse pancreas on induction of AP with/without treatment with GR159897. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered GR159897 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered GR159897 1 hour after the first caerulein injection. Scale bars = 20µm.

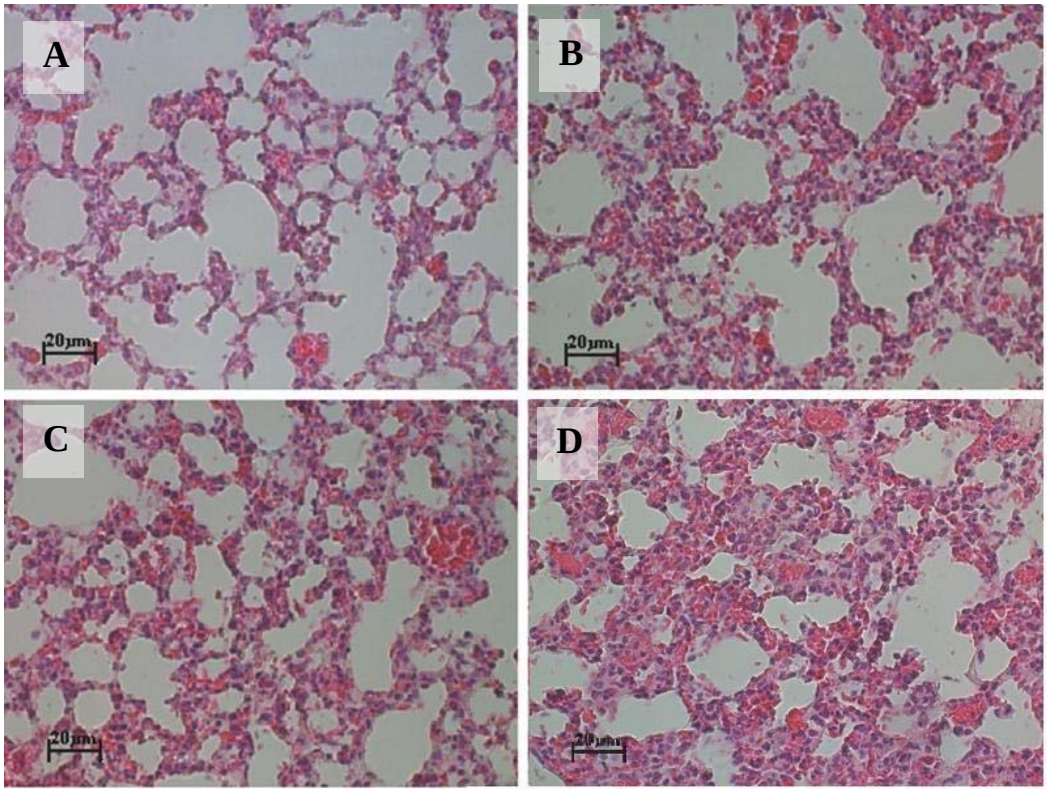


Figure 2.6 Morphological changes in mouse lungs on induction of AP with/without treatment with GR159897. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered GR159897 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered GR159897 1 hour after the first caerulein injection. Scale bars = 20μm.

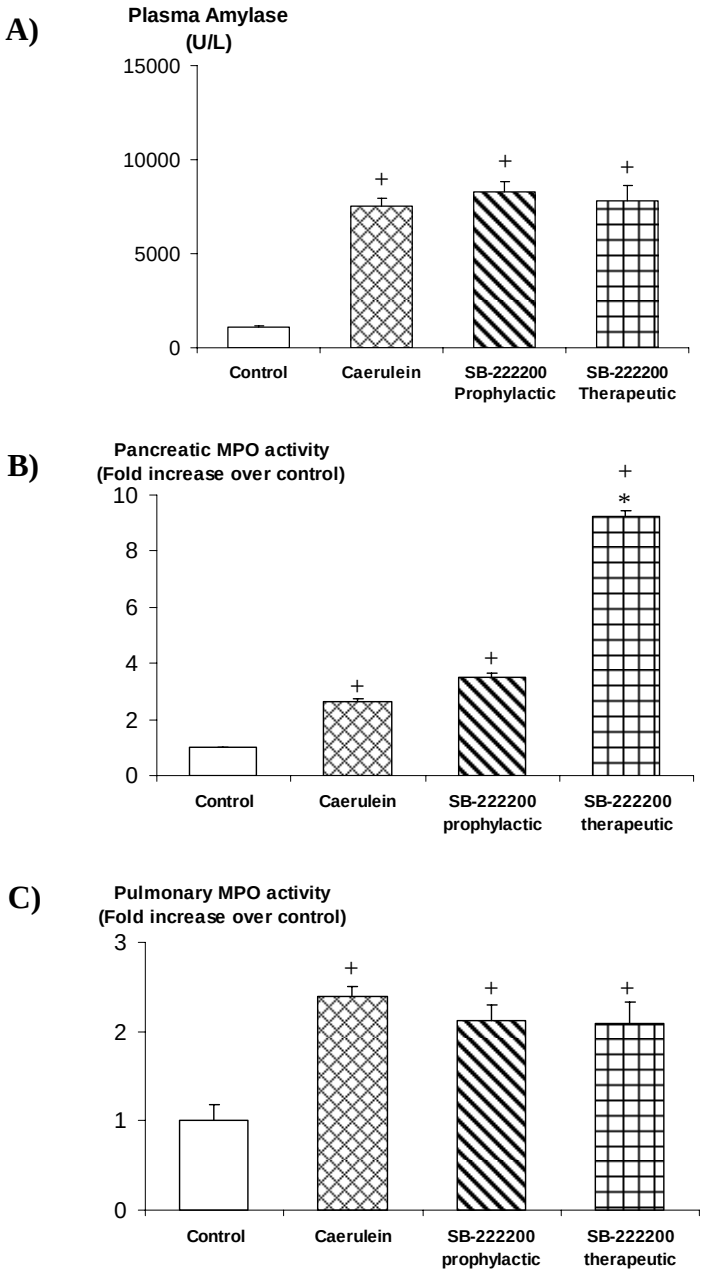


Figure 2.7 Effects of SB-222200 on A) Plasma amylase activity; B) Pancreatic MPO activity; C) Lung MPO activity in AP. Mice were given 10 hourly injections of caerulein (50µg/kg, i.p.). SB-222200 was administered at 2mg/kg i.p. either 30 minutes before (prophylactic treatment) or 1 hour after (therapeutic treatment) the first caerulein injection. Bar charts represent mean value ± SEM of at least 10 animals.

+ indicates P < 0.05 when caerulein-treated animals were compared with saline treated animals.

*** indicates P < 0.05 when SB-222200-treated animals were compared with caerulein-treated animals.**

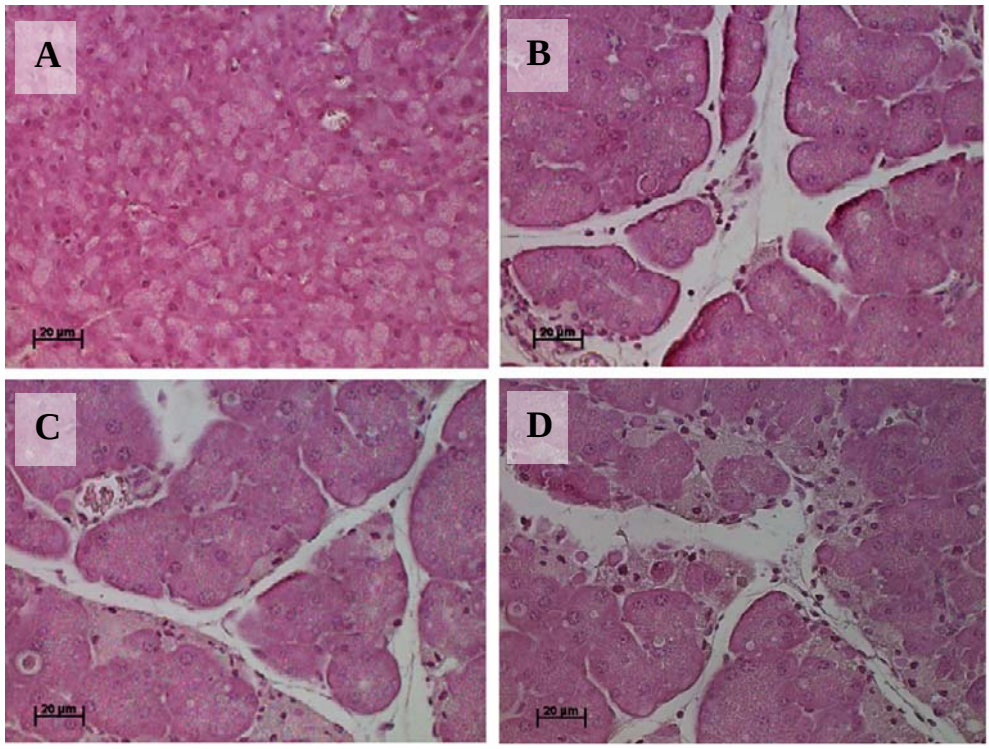


Figure 2.8 Morphological changes in mouse pancreas on induction of AP with/without treatment with SB-222200. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered SB-222200 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered SB-222200 1 hour after the first caerulein injection. Scale bars = 20μm.

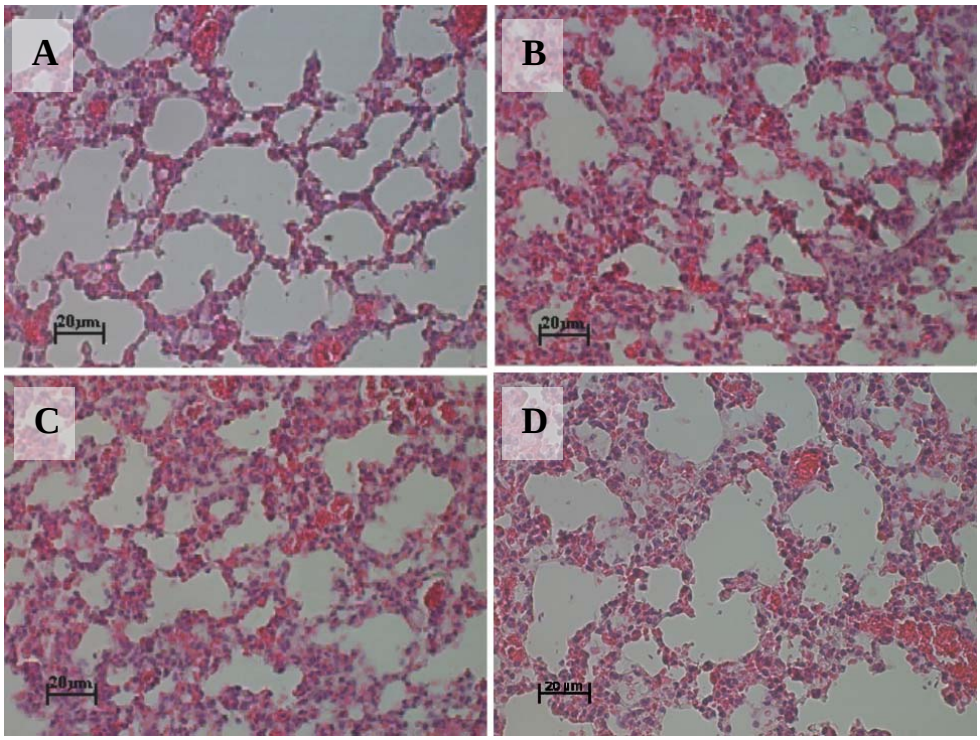


Figure 2.9 Morphological changes in mouse lungs on induction of AP with/without treatment with SB-222200. (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered SB-222200 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered SB-222200 1 hour after the first caerulein injection. Scale bars = 20μm.

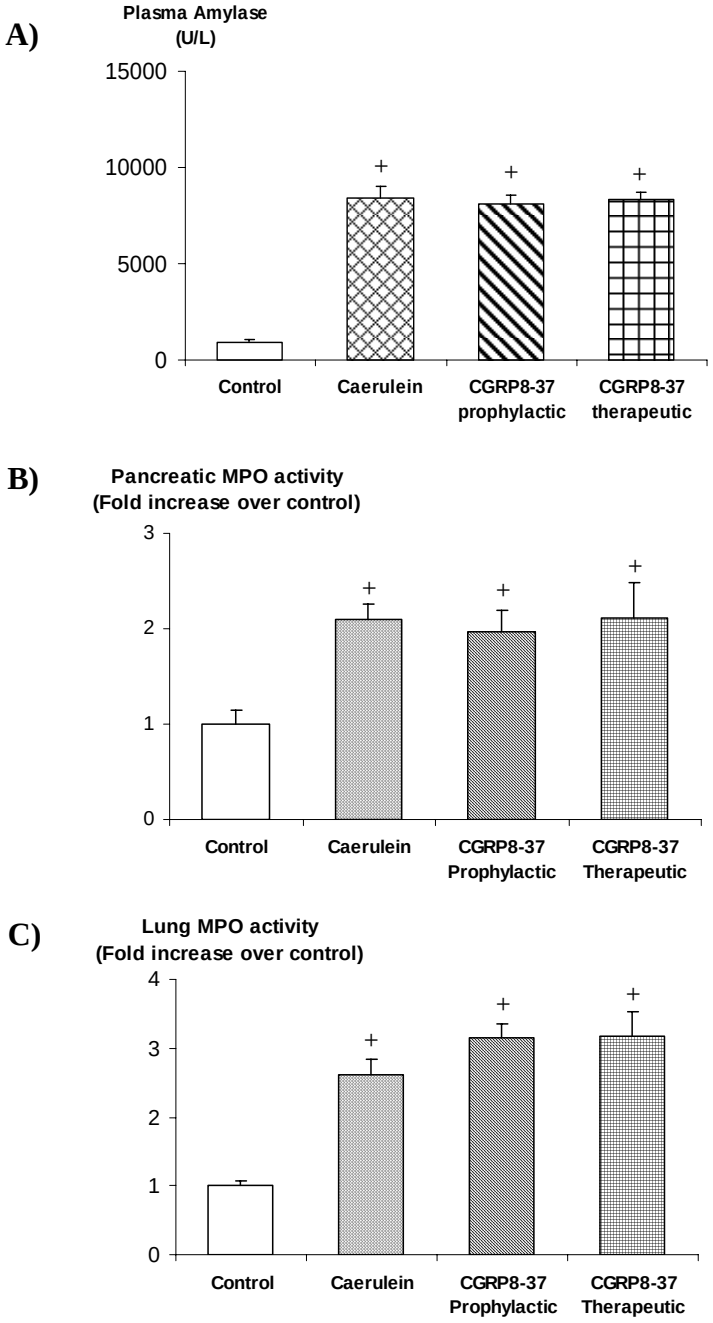


Figure 2.10 Effects of CGRP (8-37) on A) Plasma amylase activity; B) Pancreatic MPO activity; C) Lung MPO activity in AP. Mice were given 10 hourly injections of caerulein (50μg/kg, i.p.). CGRP (8-37) was administered at 0.06mg/kg i.p. either 30 minutes before (prophylactic treatment) or 1 hour after (therapeutic treatment) the first caerulein injection. Bar charts represent mean value ± SEM of at least 10 animals.

+ indicates P < 0.05 when caerulein-treated animals were compared with saline treated animals.

*** indicates P < 0.05 when CGRP (8-37)-treated animals were compared with caerulein-treated animals.**

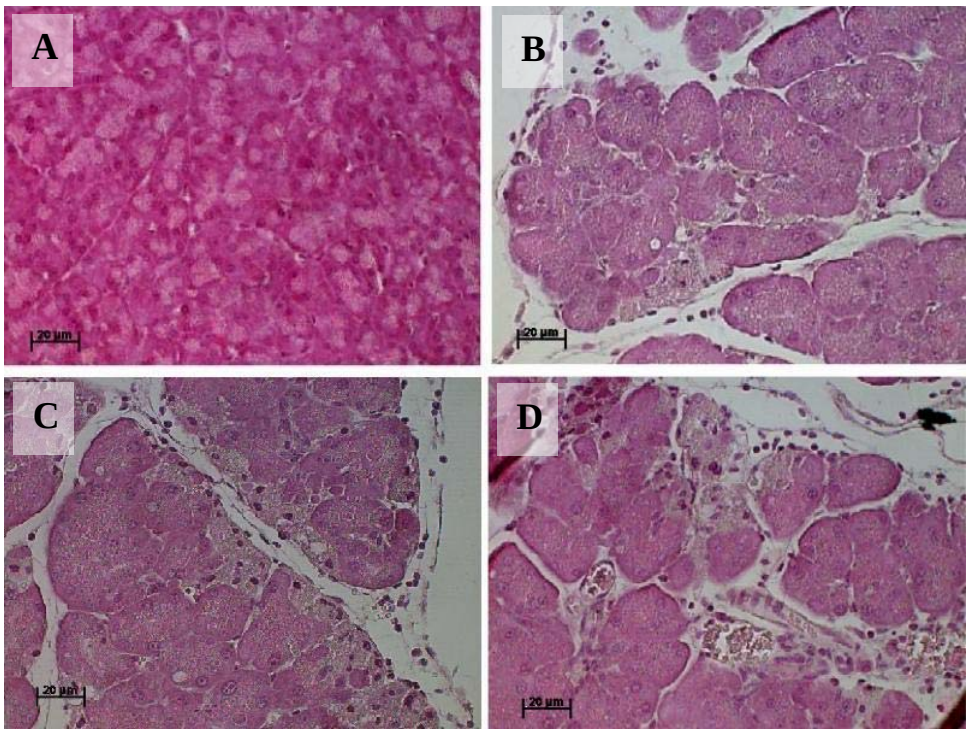


Figure 2.11 Morphological changes in mouse pancreas on induction of AP with/without treatment with CGRP (8-37). (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered CGRP (8-37) 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered CGRP (8-37) 1 hour after the first caerulein injection. Scale bars = 20μm.

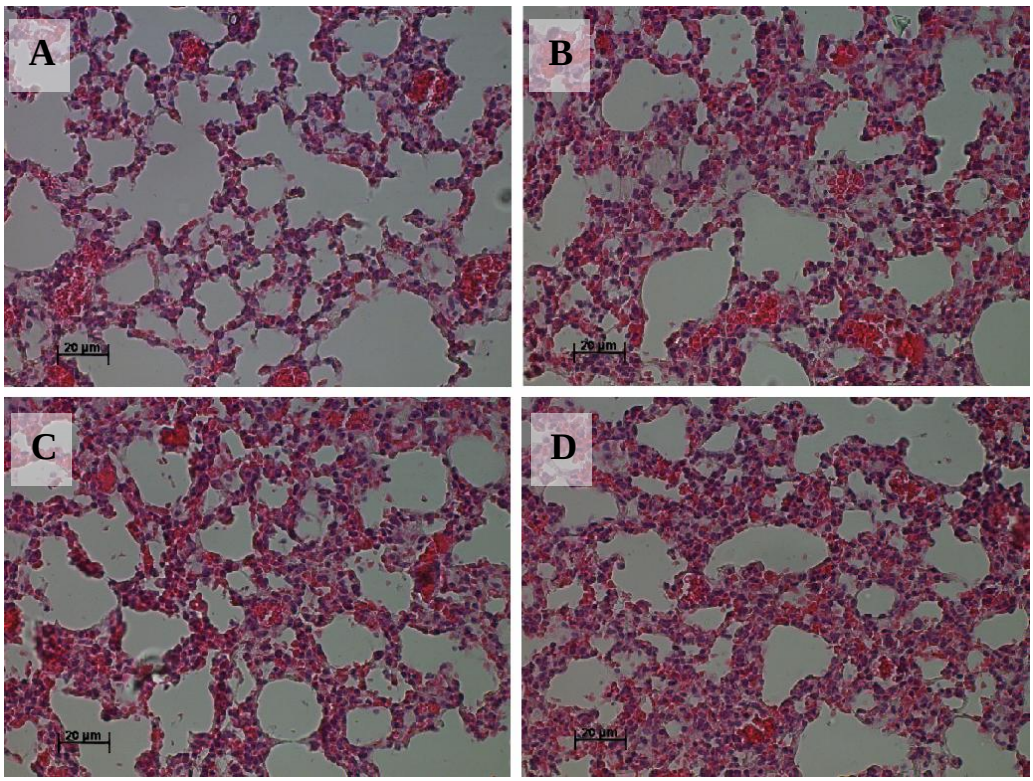


Figure 2.12 Morphological changes in mouse lungs on induction of AP with/without treatment with CGRP (8-37). (A) Control: no pancreatitis; (B) caerulein-induced AP; (C) caerulein-induced AP in mice administered CGRP (8-37) 30 minutes before the first caerulein injection; and (D) caerulein-induced AP in mice administered CGRP (8-37) 1 hour after the first caerulein injection. Scale bars = 20μm.

2.4 DISCUSSION

TK have been implicated in a number of inflammatory conditions (Liddle and Nathan, 2004; Bhatia, 2003a; Regoli et al., 1990; Marriott, 2004; Evangelista, 2001; Campos and Calixto, 2000; Tateichi et al., 1990). For this reason, their receptors, the neurokinin receptors have been important targets in drug discovery for the past thirty years. SP is a major mediator of NI in several tissues, including skin, cardiovascular tissue, cephalic structures, respiratory tract, genitourinary tract, and gastrointestinal tract (Bhatia, 2003a). NKA and NKB have also been shown to play a part in inflammatory conditions (Bhatia, 2003a; Regoli et al., 1990; Marriott, 2004; Evangelista, 2001; Campos and Calixto, 2000; Tateichi et al., 1990). On the basis of previous studies with NK1 receptor knockout mice and PPT-A knockout (SP- and NKA-deficient) mice we have proposed a pro-inflammatory contribution of SP and/or NKA in the pathogenesis of AP and associated lung injury. In those studies, mice genetically deficient in the NK1 receptor or the PPT-A gene were protected against AP and associated lung injury (Bhatia et al., 1998a; Bhatia et al., 2003b).

In this study, I have made use of the antagonists of neurokinin receptors to investigate the effect of receptor blockage on the development and progression of AP. CP96,345, GR159897 and SB-222200 are valuable compounds that are often used to study the NK1R, NK2R and NK3R systems, respectively (Snider et al., 1991; Beresford et al., 1995; Medhurst et al., 1997). The present findings have shown the effect of pharmacological treatment with CP96,345 (prophylactic and therapeutic) on the severity of AP and associated lung injury in mice. Prophylactic, as well as therapeutic, treatment with CP96,345 significantly protected the mice against AP. This was

evident by a substantial attenuation in hyperamylasemia and pancreatic MPO activity as well as by histological evidence of reduced pancreatic injury. On the other hand, treatment with GR159897 or SB-222200, either prophylactic or therapeutic, did not produce any protective effect against AP. Similar finding was observed in another study, where NK2R and NK3R blockade were unable to prevent pulmonary tissue injury during inflammation induced by hydrogen sulfide (Bhatia et al., 2006). One interesting finding is that when SB-222200 was given after the induction of AP, a further increase in pancreatic MPO activity was observed (Fig. 2.7B). However, an increase in leukocytes was not observed by histological examination. The significance of this finding requires further investigations.

Recently, two groups of scientists reported the involvement of CGRP and its receptor in the pathogenesis of AP (Wick et al., 2006a,b; Warzecha et al., 1999, 2000, 2001). Although the exact role of CGRP in AP has not been fully understood, it is clear that the peptide is a component of NI that might affect the severity of the condition. Therefore, in this study, we employed a peptide CGRP antagonist, CGRP (8-37), to investigate this component of NI. However, our results suggested that the involvement of CGRP in AP was minimal, as both the plasma amylase and the pancreatic MPO activity remained elevated even with the treatment of this antagonist (Fig 2.10A&B). These results were substantiated by histological examination of pancreas sections.

Severe AP was also associated with lung injury that is characterized by sequestration of neutrophils within the lung (that is, increased lung MPO activity) after 10 hourly injections of caerulein. Prophylactic, as well as therapeutic treatment with CP96,345,

but not GR159897, SB-222200 or CGRP (8-37), protected mice almost completely against AP-associated lung injury. This suggested that NK1R, but not NK2R, NK3R or CGRP receptor, is also implicated in systemic organ damage following AP.

The mechanisms by which SP acts to amplify the severity of pancreatitis are not clear. From studies probing the role of SP in inflammatory processes involving other tissues, one might suspect that this neuropeptide acts primarily on endothelial cells to increase vascular permeability and promote edema formation (Grady et al., 2000; Lei et al., 1992). Although this explanation could account for the finding that treatment with CP96,345 lessens the increased pulmonary vascular permeability noted in pancreatitis-associated lung injury, it would not account for some of the other effects of NK1 receptor deletion that we have noted including (i) diminished acinar-cell injury as evidenced by histological evidence from pancreas sections, (ii) decreased sequestration of neutrophils in the pancreas, and (iii) decreased sequestration of neutrophils in the lung. The observation that expression of NK1R in the pancreas is increased during caerulein-induced pancreatitis (Bhatia et al., 1998a), has suggested that the pro-inflammatory effects of SP may be directly exerted on the acinar cells. It is also tempting to speculate that this phenomenon could explain the role of SP and NK1 receptors in pancreatitis-associated lung injury, that is, that SP acting via NK1 receptors on acinar cells to enhance the severity of acinar-cell injury, and the subsequent increased release of pro-inflammatory mediators from the pancreas leads to increased lung injury. Results presented in this chapter strongly suggest that NK1R antagonists may potentially be of use for the treatment of AP and its systemic complications.

CHAPTER 3

EFFECT OF CP96,345 ON THE EXPRESSION OF TACHYKININS AND NEUROKININ RECEPTORS IN ACUTE PANCREATITIS

3.1 INTRODUCTION

The involvement of TK involvement in inflammatory conditions has received considerable attention (Maggi, 1997; Bhatia, 2003a; Campos and Calixto, 2000; Weinstock, 2004; Liddle and Nathan, 2004; O'Connor et al., 2004). SP, NKA and NKB are the primary TK, with each peptide showing high binding affinity for NK1R, NK2R and NK3R respectively (Patacchini et al, 2004). Among these, SP and NK1R have been implicated in the pathogenesis of AP (Bhatia et al., 2005a; Bhatia et al., 1998a; Bhatia et al., 2003b). SP is an eleven-amino acid peptide member of the TK family and is distributed throughout the central and peripheral nervous systems. This neuropeptide binds preferentially to NK1R on the vasculature and causes vasodilatation, plasma extravasation and leukocyte adhesion (Grady et al., 2000). Recent studies show that NI mediated by SP binding to NK1R plays a crucial role in the pathogenesis of AP and pancreatitis-associated lung injury in mice and rats (Grady et al., 2000; Maa et al., 2000). Knockout mice deficient in SP and NK1R were found to be resistant to the development of severe AP (Bhatia et al., 1998a; Bhatia et al., 2003b). However, the mechanism by which NK1R and SP amplifies the severity of pancreatitis is still unclear.

The method of isolating of pancreatic acinar cells using collagenases was first developed by Amsterdam and Jamieson in the early 1970s (Amsterdam and Jamieson, 1972; Amsterdam and Jamieson, 1974). Isolated acinar cells have been employed to study the effect of various biological and chemical agents on function and cell signaling at the level of individual cells. By incubating with high concentration of caerulein, isolated pancreatic acinar cells simulate some of the events found in AP *in vivo* (Leach et al., 1991). Pancreatic acinar cells have been shown to possess two classes or states of cholecystokinin (CCK) receptors – high affinity and low affinity receptors (Sankaran et al., 1982; Sankaran et al., 1980; Honda et al., 1987; Sato et al., 1989; Stark et al., 1989). The high affinity site is thought to mediate normal pancreatic secretion and growth, while the low affinity site is responsible for supramaximal inhibition of secretion and AP (Niederau et al., 1994).

In the present chapter, I investigated the mRNA expression of pre-protachykinin genes and neurokinin receptors, as well as the protein expression of NK1R during AP with the treatment of NK1R antagonist. This will provide important information for the understanding of the role of SP and NK1R, as well as the contribution of other TK and neurokinin receptors in AP. In addition, I also studied the effect of caerulein and SP on receptor and protein expression, as well as the secretory properties of isolated acinar cells. This was followed by an attempt to use a NK1R antagonist to modulate acinar cells responses.

3.2 MATERIALS AND METHODS

3.2.1 Materials

Trizol® reagent was bought from Invitrogen (Invitrogen, Carlsbad, CA USA) while chloroform, isopropyl alcohol, ethanol, methanol, trifluoroacetic acid were purchased from Merck KGaA (Darmstadt, Germany). Acetonitrile was obtained from Lab-Scan (Lab-Scan, Belfast, UK). Diethylpyrocarbonate (DEPC) and bovine serum albumin (BSA) were bought from Sigma-Aldrich Chemicals USA (St. Louis, Mo, USA). Ethidium bromide, iScript™ cDNA Synthesis Kit and iQ™ Supermix, certified molecular biology agarose and EZ load™ molecular ruler were bought from Bio-Rad (Bio-Rad, Hercules, CA, USA). Collagenase Type IV was purchased from Worthington Biochemical Corporation (Worthington Biochemical Corporation, Lakewood, NJ, USA). 0.01% soybean trypsin inhibitor and HEPES were obtained from Sigma (Sigma Chemical Co., St. Louis, MO, USA). MyCycler™ personal thermal cycler and Sub-Cell GT gel electrophoresis system were from Bio-Rad (Bio-Rad, Hercules, CA, USA). Primers for RT-PCR analysis were custom synthesized by Sigma-Proligo Singapore Pte. Ltd. 3,3 -diaminobenzidine (DAB) was purchased from Sigma-Aldrich Chemicals USA (St. Louis, Mo). SP enzyme immunoassay kit and C18 cartridge columns were purchased from Bachem California (Torrance, CA, USA). UVP GelDoc-It Imaging System and LabWorks Software were purchased from UVP (Upland, CA, USA). Applied Biosystems Prism® 7000 Sequence Detection System was purchased from Applied Biosystems (Foster City, CA, USA). Other reagents and chemicals were of analytical grade.

3.2.2 Induction of AP

AP was induced in mice as described in Section 2.2.2. One hour after the last caerulein injection animals were sacrificed by a lethal dose of i.p. pentabarbitone (90 mg/kg, three times the dose for surgical anaesthesia). Pancreas and lungs tissues were collected for total RNA extraction, SP analysis or immunohistochemistry.

3.2.3 Preparation of pancreatic acini

Pancreatic acini were obtained from Balb/C mouse (20-25g) pancreas (3 pancreas per study, 100-120mg per pancreas) by collagenase digestion method following a slightly modified Oliver's procedure (Oliver C., 1980). Briefly, mice were killed by instant cervical dislocation and the pancreas were isolated, dissected free of mesenteric fat and washed in sodium HEPES buffer (140 mM NaCl, 4.7 mM KCl, 1.13 mM $MgCl_2$, 10 mM glucose, 0.05% w/v soybean trypsin inhibitor and 10 mM HEPES; pH 7.2). The parenchyma of the pancreas was then infused with a syringe containing freshly prepared collagenase buffer (buffer A: sodium HEPES buffer containing 1 mM $CaCl_2$, 0.1% w/v BSA and 200 IU/ml collagenase type IV). Subsequently, the distended tissue were minced and incubated in a water bath at 37°C for 10 minutes, shaking at 180 cycles per minute to allow further tissue digestion. Detached acini were then dissociated by passing the suspension several times through a fine pipette tip. The dissociated pancreatic acinar cells was passed through a solution of 50 mg/ml BSA by centrifugation at 1,000 × g, 4°C for 1 minute. The pellet collected was washed three times with sodium HEPES buffer before the acinar cells were resuspended in the

same buffer for subsequent experiments. The freshly prepared acinar cells were kept on ice and used in an experiment within 2 hours after their extraction. Cell viability was determined by Trypan blue exclusion assay.

3.2.4 Pancreatic acini experiments

The prepared acinar cells were evenly distributed into microcentrifuge tubes containing buffer A. The cells were incubated with various doses of caerulein (from 10^{-13} M to 10^{-6} M) and/or SP (from 10^{-10} M to 10^{-5} M) for one hour at 37°C in a shaking water bath. At the end of the incubation, a small portion of the acinar cell suspension from each tube was taken out to check for cell viability by Trypan blue exclusion assay. The cell suspensions remaining in the tubes were spun at 3,000 × g at 4°C for 5 minutes. The resultant supernatants were used for immediate amylase assay, while the pellets were kept for DNA assay, SP ELISA assay, RT-PCR and immunofluorescence study. In order to study the role of NK1R in acini, acini were preincubated in the presence of CP96,345 (working concentration of 10^{-6} M) at 37°C in a shaker water bath for half an hour before the initiation of the treatment with caerulein and/or SP.

3.2.5 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total RNA was isolated from the pancreas and the lungs using Trizol® reagent according to the manufacturer's instructions with modifications (Bhatia et al., 2005b). Briefly, pancreatic or pulmonary tissues were isolated and rapidly ground and homogenized in 1.5 ml of Trizol® reagent using a Potter-Elvejhem homogenizer on

ice. The homogenate was centrifuged at 10,000 × g for 10 minutes at -10 °C to remove insoluble materials. 0.3ml of chloroform was added to the supernatant and shaken vigorously by hand for 15 seconds. The mixture was then centrifuged at 10,000 × g for 10 minutes at -10 °C to separate the aqueous and organic phases. The colourless upper aqueous phase was separated from the organic phase. 0.75ml of isopropanol was added to the aqueous phase and the solution was incubated at room temperature (approximately 20°C) for 10 minutes to allow precipitation of RNA. After RNA was pelleted by centrifugation (10 000 × g for 10 minutes at -10°C), the pellet was washed by resuspension in 70% v/v ethanol, re-pelleted and dissolved in RNase/DNase-free water.

Similarly, the extraction of total mRNA from the pelleted acinar cells was carried out using TriZol ® reagent. Briefly, pelleted acinar cells were treated with 1ml of TriZol® reagent and homogenized using a Potter-Elvejhem homogenizer at room temperature. 0.2ml of chloroform was added to supernatant and shaken vigorously by hand for 15 seconds. The mixture was then centrifuged at 10,000 × g for 10 minutes at 4°C to separate the aqueous and organic phases. 0.5ml of isopropanol was added to the aqueous phase and the solution was incubated at room temperature for 10 minutes to allow the precipitation of RNA. After RNA was pelleted and washed by resuspension in 70% v/v ethanol, repelleted and redissolved in RNase/DNase-free water. The amount of RNA was quantitated by absorbance at 260 nm (1 OD = 40 μg/ml) in triplicate measurements, and the integrity was verified by ethidium bromide staining of 18S and 28S rRNA bands on a denaturing agarose gel.

RT-PCR was performed using iScript™, according to the manufacturer's protocols. Briefly, total RNA (1μg) was added to the reaction mixture containing iScript reverse transcriptase, 5 × iScript reaction mix and nuclease-free water. The final volume was 20μl. Reverse transcription was carried out at 25°C for 5 minutes, 42°C for 30 minutes, followed by 85°C for 5 minutes. The cDNA synthesized from 1μg of total RNA was used as a template for a typical PCR amplification by iQ™ Supermix. The PCR primers (Table 3.1) for detection of NK2R, NK3R, PPT-A and PPT-C were synthesized by Sigma-Proligo Singapore Pte. Ltd. (Singapore). The primers were intron-spanning, such that genomic DNA contamination was excluded. cDNA synthesized from 1μg total RNA was included in a typical PCR. The reaction mixture was first subjected to 95°C for 3 minutes for the activation of iTaq DNA polymerase. This was followed by an optimal cycle of amplifications (Table 3.1), consisting of denaturation at 95°C for 30 seconds, optimal annealing temperature (Table 3.1) for 30 seconds and extension at 72°C for 30 seconds. r18S was chosen as the internal standard. PCR amplification was performed in MyCycler™ (Bio-Rad, Hercules, CA). The PCR products were analysed on 1% w/v agarose gels containing 0.05 mg/100 ml ethidium bromide running at 80V for 20 minutes. The gels were visualized using UVP GelDoc-It Imaging System and analyzed by LabWorks Software.

3.2.6 Real-time PCR of NK1R mRNA

Real-time-PCR was carried out as described previously with modifications (Shrikhande et al., 2001). The reactions were done on an Applied Biosystems Prism® 7000 Sequence Detection System. The synthesis of cDNA from the extract mRNA was carried out using Applied Biosystems (ABI) Taqman® Reverse Transcription

Reagents according to the manufacturer's instructions. Briefly, 1µg of the extracted RNA was incubated with the reverse transcription reaction mixture at 25°C for 10 minutes followed by 48°C for 30 minutes. The reaction was inactivated at 95°C for 10 minutes. The reverse transcription reaction mixture consisted of Taqman® RT buffer, 5.5mM $MgCl_2$, 500µM of each of the four dNTPs, 2.5µM random hexamers and 25U of MultiScribe™ Reverse Transcriptase in a final volume of 20µl. The product was then stored at 4°C for not longer than 1 week. Real time amplification of NK1R cDNA was carried out using Applied Biosystems Prism 7000 Sequence Detection System. The synthesized cDNA was used in the subsequent PCR amplification, which were carried out in a final volume of 25µl containing 12.5µl of Taqman® Universal PCR master mix, 900nM of each of the two primers (NK1R assay-on-demand), 250nM of Taqman® MGB probe and cDNA sythesised from 1µg of total RNA. The same thermal-cycling profile was used for both NK1R and r18S probes. The mixture was allowed to incubate for 2 minutes at 50°C via AmpErase® UNG for uracil N-glycosylase reaction to remove PCR carry-over decontamination (Longo et al., 1990). This was followed by 10 minutes activation of the AmpliTaq polymerase at 95°C, and 45 cycles of 95°C for 30 seconds and 60°C for 1 minute. All experiments were performed in triplicate. The threshold level was determined as 10 standard deviations above the mean of baseline fluorescence emission calculated from cycles 3 to 15. The threshold cycle (Ct) was defined as the cycle number at which the fluorescence emission level exceeds the threshold level (Heid et al., 1996). Endogenous reference was carried out using Taqman® Rodent r18S control reagent. The initial amount of NK1R cDNA normalized to the endogenous reference (r18S cDNA) was proportional to the value given by the expression: $2^{-\Delta Ct}$ (ΔCt represents the difference in Ct values

for the NK1R and the r18S). The arbitrary value was then compared for the value obtained from the control group and expressed as fold increase over control.

3.2.7 Measurement of SP levels

Pancreas and lung samples taken from *in vivo* studies were homogenized in 1.0 ml ice-cold assay buffer for 20 seconds. The homogenates were centrifuged (13, 000 × g, 20 minutes, 4°C) and the supernatants were collected. In studies using isolated acinar cells, pelleted acinar cells were homogenized in 2 ml ice-cold phosphate buffer (50mM sodium phosphate buffer (pH7.0), 150mM NaCl and 10nM sodium EDTA) for 20 seconds using a polytron homogenizer. The homogenates were centrifuged (13,000 × g, 20 minutes, 4 °C) and the supernatants were collected. The supernatants were then adsorbed on C18 cartridge columns as described (Bhatia et al., 1998a) and the adsorbed peptides were eluted with 1.5 ml 75% v/v acetonitrile. The samples were freeze-dried and reconstituted in assay buffer. SP content was then determined using an ELISA kit (Bachem) according to the manufacturer's instructions and expressed as ng/µg DNA. SP can be measured in the range of 0-10 ng/ml in this assay. This value was then corrected for the DNA content of the respective samples and results were expressed as fold increase over control group. DNA assay was performed fluorometrically, using Hoechst dye 33256 according to the method of Labarca and Paigen (Labarca and Paigen, 1980), using salmon testis DNA as standard (as described in Section 2.2.5)

3.2.8 Immunohistochemistry of pancreas and lung paraffin sections

The pancreas and lung tissues harvested were fixed overnight at room temperature in 10 % pH-neutral, phosphate-buffered formalin solution. The fixed tissues were subsequently processed using the Leica TP1020 automatic tissue processor (Leica Microsystems Nussloch GmbH, Germany), and embedded in paraffin blocks. Sections of 5 μm were cut using a Leica RM2125 manual rotary microtome (Leica Microsystems Nussloch GmbH, Germany) after which immunostaining was carried out. The paraffin sections were dewaxed in two changes of Histoclear, hydrated through a decreasing graded series of ethanol and finally washed with Tris buffered saline (TBS) immediately prior to use. The endogenous peroxidase activity was quenched with 0.3% H_2O_2. Then, sections were incubated with blocking buffer (3% BSA in TBS) for 1 hour, and washed for three times. This was followed by 2 hours incubation at room temperature with the rabbit polyclonal anti-mouse NK1R antibody (1:200, Chemicon, USA). Subsequently the slides were washed at the end of the first incubation. The sections were then incubated in horseradish peroxidase-conjugated secondary monoclonal anti-rabbit antibody (1:100, Sigma-Aldrich, USA) for 30 minutes at room temperature. After the second incubation, slides were washed again before treatment with the chromogen for visualization. Finally, slides were treated with 3,3'-diaminobenzidine (DAB) for 10 minutes, rinsed, counterstained with hematoxylin. The slides were then dehydrated in ehtanol, cleared in Histoclear, and coverslipped with Neo-mount before they were viewed under Carl-Zeiss Axioskop 40 microscope system (Carl Zeiss, Göttingen, Germany). Negative controls were included by replacing of the primary antibody with non-immune serum. Antibodies

were diluted in TBS at pH7.2. This buffer was also used in all washes (3 × 5 minutes), and incubations are all carried out at room temperature (25°C).

3.2.9 Immunofluorescence for NK1R in pancreatic acini

Pancreatic acini were centrifuged at 3,000 × g, 4°C for 5 minutes, after which the supernatant was kept for substance P-ELISA assay and amylase analysis. 500µl 3.7% paraformaldehyde was added to the cell pellet and resuspended. The mixture was then incubated at room temperature for 10 minutes to allow complete fixation of the acinar cells. This is followed by the addition of an excess amount of PBS to stop the fixation. The cells were then centrifuged and washed twice with PBS. At this stage, the fixed acinar cells were either stored in 4°C (for up to 1 week) or used immediate for immunofluorescence staining.

Fixed acinar cells were pipetted onto poly-lysine coated glass slides and centrifuged at 180 × g in a Medite Cytofuge (StatSpin Inc., USA) for 10 minutes at room temperature. The acinar cells attached on the slides were gently washed twice with PBS. Subsequently, the slides were blocked in blocking buffer (1% BSA in PBS) for 10 minutes at room temperature, followed by another round of washing (3 × 3 minutes) using PBS. Primary antibody (1:200; rabbit polyclonal anti-mouse NK1R antibody, Chemicon, USA) was then added to the slides and allowed to incubate for 2 hours in room temperature. The slides were washed three times in PBS and incubated with 1:200-diluted rhodamine-conjugated goat antirabbit IgG secondary antibody (Santa Cruz Biotechnology) for another 30 minutes. After another 3 washes with PBS,

the slides were coversliped and examined under a fluorescent microscope. The cell surface localization of NK1R was examined qualitatively.

3.2.10 Supernatant amylase activities determination

To determine the concentration of amylase released by acinar cells, the cell free supernatant was collected after the 1 hour incubation and analysed as described in Section 2.2.3.

3.2.11 Data analysis method

The results are expressed as mean ± standard error of mean (SEM). In all figures, vertical bars denote the SEM and the absence of such bars indicates that the SEM is too small to illustrate. The significance of changes was evaluated by analysis of variance (ANOVA) when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by using Tukey's method as a post hoc test for the difference between groups. A *P*-value of < 0.05 was considered to indicate a significant difference. All statistical analyses were performed using SPSS version 14.0 for Windows (Chicago, Illinois USA).

Gene	Primer Sequence	Optimal Conditions	Size
r18S	sense: 5’-GTAACCCGTTGAACCCCATT-3’ antisense: 5’-CCATCCAATCGGTAGTAGCG-3’	Lung: 22 cycles Pancreas: 22 cycles Annealing: 59°C	150 bp
NK2R	sense: 5’-TGCTGTCATCTGGCTGGTAG-3’ antisense: 5’-TCTTCCTCGGTTGGTGTCCC-3’	Lung: 42 cycles Pancreas: ND Annealing: 61°C	546 bp
NK3R	sense: 5’-TTCCTGGTAAACCTGGCTTTCTCC-3’ antisense: 5’-AGACCTGTTGGATGTATTTCCACCT-3’	Lung: ND Pancreas: ND Annealing:65°C	645 bp
PPT-A	sense: 5’-ACCTGCTCCACTCCTGCACCGCGGCCAAG-3’ antisense: 5’-GAACTGCTGAGGCTTGGGTCTTCGGGCGAT-3’	Lung: 43 cycles Pancreas: 42 cycles Anealing: 68°C	239 bp
PPT-C	sense: 5’-AACTGGCTTTTGGTGCAGAG-3’ antisense: 5’-AGTGCTACACGTTGCTGGTG-3’	Lung: 40 cycles Pancreas: ND Annealing: 64°C	322 bp

Table 3.1 PCR primer sequences, optimal amplification cycles, optimal annealing temperatures and product sizes.
ND: not detected at any annealing conditions.

3.3 RESULTS

3.3.1 Effect of CP96,345 on pancreatic and pulmonary SP levels and PPT-A mRNA expression in AP

Figure 3.1 and 3.2 show the SP concentration in the pancreas and lungs respectively. Treatment with CP96,345 alone did not affect the SP level in either pancreas or lungs. As expected, hyperstimulation of the pancreas by caerulein resulted in an increase of SP concentrations in both organs. Administration of CP96,345 significantly suppressed the accumulation of SP during caerulein hyperstimulation in the pancreas. Densitometry analysis of the PCR products on agarose gels shows that the pancreatic and pulmonary PPT-A mRNA expression increased three-fold in caerulein induced AP. Treatment with CP96,345 resulted in a significant decrease in gene expression (Figure 3.3).

3.3.2 Effect of CP96,345 on pancreatic and pulmonary NK1R mRNA and NK1R protein expression in AP

Expression of NK1R during AP was investigated using real-time PCR (Figure 3.4) and immunohistochemistry (Figures 3.5 & 3.6). Changes in NK1R mRNA expression correlated well with protein expression, in which NK1R was localized extensively on acinar and alveolar cells in the pancreas (Figure 3.5) and lungs (Figure 3.6), respectively, and endothelial cells in both tissues. Low level of NK1R was detected in normal pancreas and lung (Figures 3.5 & 3.6). During AP, increased localizations of NK1R on the cell surface of pancreatic acinar cells and alveolar cells were observed.

This was demonstrated by the increase in brown staining. A significant increase in NK1R mRNA and protein expression in both pancreas and lungs during AP was observed. However, treatment with CP96,345 led to differential changes in mRNA and protein expression in the two organs. In the pancreas, NK1R expression was suppressed when CP96,345 was administered prophylactically or therapeutically, with prophylactic CP96,345 treatment producing greater suppression. On the other hand, further increases in NK1R mRNA and protein expression were observed in the lungs when the mice with AP were treated with the antagonist. In general, the immunohistochemical examination of the relavant slides appeared to support the changes in NK1R mRNA data.

3.3.3 Pancreatic and pulmonary NK2R and NK3R mRNA expression

NK2R mRNA expression was not detected in normal pancreas (Figure 3.7). However, up-regulation of receptor mRNA expression was observed during AP. Treatment with the antagonist resulted in further accumulation of NK2R mRNA in the pancreas. In the lungs, down-regulation of NK2R mRNA expression was observed in mice with AP (Figure 3.8). Treatment with CP96,345 reversed the downregulation (Figure 3.8). NK3R mRNA was undetected in normal pancreas and lungs in both control mice and AP mice. However, strong NK3R mRNA expression was observed in the brain (Figure 3.10).

3.3.4 Effect of CP96,345 on pancreatic and pulmonary PPT-C mRNA in AP

PPT-C mRNA expression was observed in lungs (Figure 3.9) but not found in the normal or inflamed pancreas (Figure 3.9). During AP, increased PPT-C mRNA expression was detected in the lungs and CP96,345 treatment suppressed this increase, a profile similar to that of PPT-A.

3.3.5 Effect of caerulein, SP and CP96,345 on acinar cell amylase secretion

Figure 3.11 demonstrates the change in acinar cells amylase secretion in response to caerulein or SP. SP at any concentration between 10^{-13} M to 10^{-7} M did not affect the amylase concentration in the supernatant. However, there was a biphasic dose-response relationship between caerulein concentration and amylase secretion. At 10^{-10} M, caerulein caused the highest rate of amylase release from acinar cells. However, the secretion of amylase was inhibited at higher concentrations of caerulein. At the supramaximal concentration (10^{-7} M), caerulein inhibited the release of amylase from acinar cells. The treatment with NK1R antagonist did not affect the inhibition of amylase release caused by caerulein hyperstimulation (Fig. 3.12).

3.3.6 Effect of caerulein, SP and CP96,345 on acinar cell PPT-A mRNA expression

An inverted bell shape curve was observed with the PPT-A mRNA expression when acinar cells were incubated with increasing concentrations of caerulein (Fig. 3.13). Interestingly, maximum PTT-A mRNA expression was detected at 0.1μM – the supramaximal concentration of caerulein at which the release of amylase from acinar

cells was inhibited (Fig. 3.14). On the other hand, SP dose-dependently increased the expression of PPT-A mRNA in acinar cells and reached a plateau at concentrations higher that 1μM. Although both caerulein and SP caused an increase in PPT-A mRNA expression, their effects were not additive when both peptides were added at the same time (Fig. 3.14). Nevertheless, when acinar cells were pre-treated with CP96,345 30 minutes before the introduction of caerulein (10^{-7} M) and/or SP (10^{-6} M), no increase in PPT-A mRNA was detected. SP concentrations in the acinar cells and in the supernatant were measured in the caerulein and/or CP96,345 treated groups. However, no change in SP level was observed (Fig. 3.15).

3.3.7 Effect of caerulein, SP and CP96,345 on acinar cell NK1R mRNA expression

The change in NK1R mRNA expression with increasing caerulein or SP concentration is illustrated in Figure 3.16. Small, but significantly higher NK1R mRNA expression was again seen when the acinar cells were exposed to 0.1μM caerulein. SP treatment caused a small apparent increase in the mRNA expression but the results did not reach statistical significance. Although the small increase in NK1R mRNA was abolished in the presence of CP96,345, the changes (1.56 ± 0.27 fold *cf* 1.82 ± 0.25 fold), the difference was too small and therefore inconclusive (Fig. 3.17). Immunofluorescent staining did not show any significant effect of caerulein or SP on NK1R intensity on the acinar cell surface (Fig. 3.18). Low level of NK1R was fluorescently labeled on the cell surface of pancreatic acinar cells (Fig. 3.18). There was no significant difference in NK1R staining (fluorescence on cell surface – background fluorescent) observed among the treatment groups.

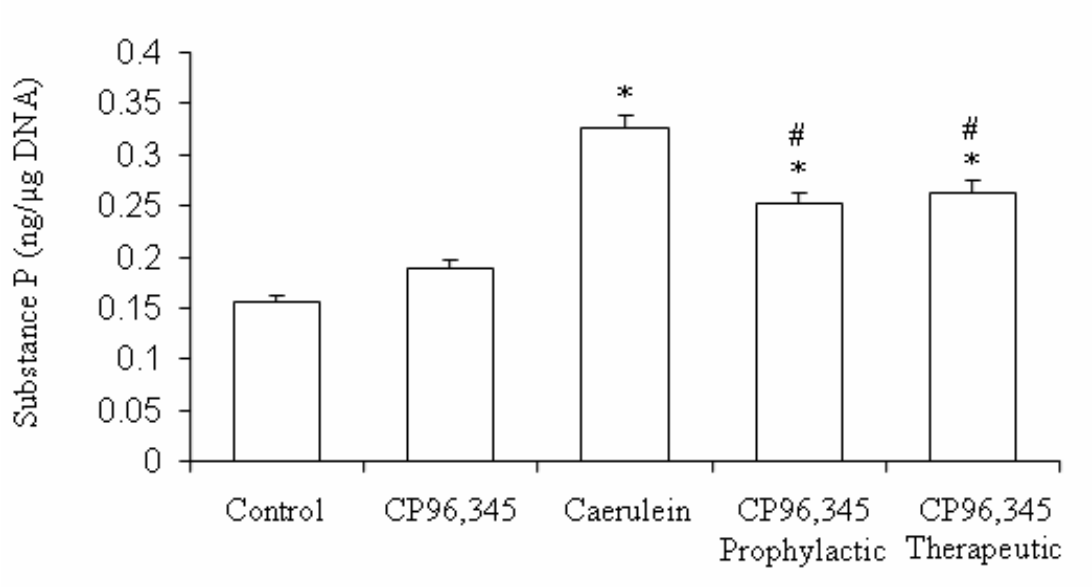


Figure 3.1 SP levels in pancreas of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

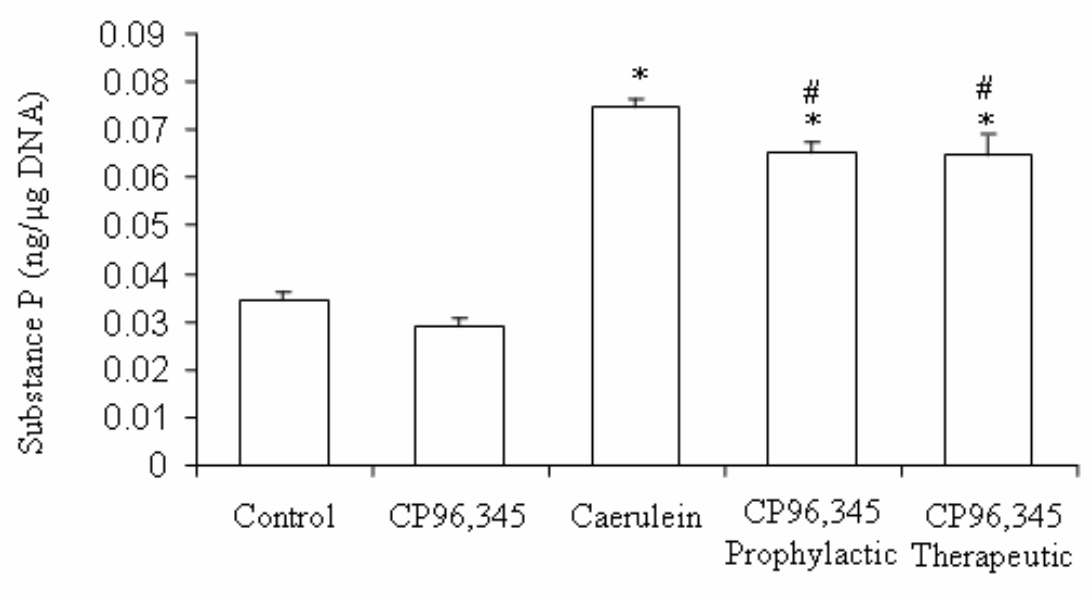


Figure 3.2 SP levels in the lungs of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

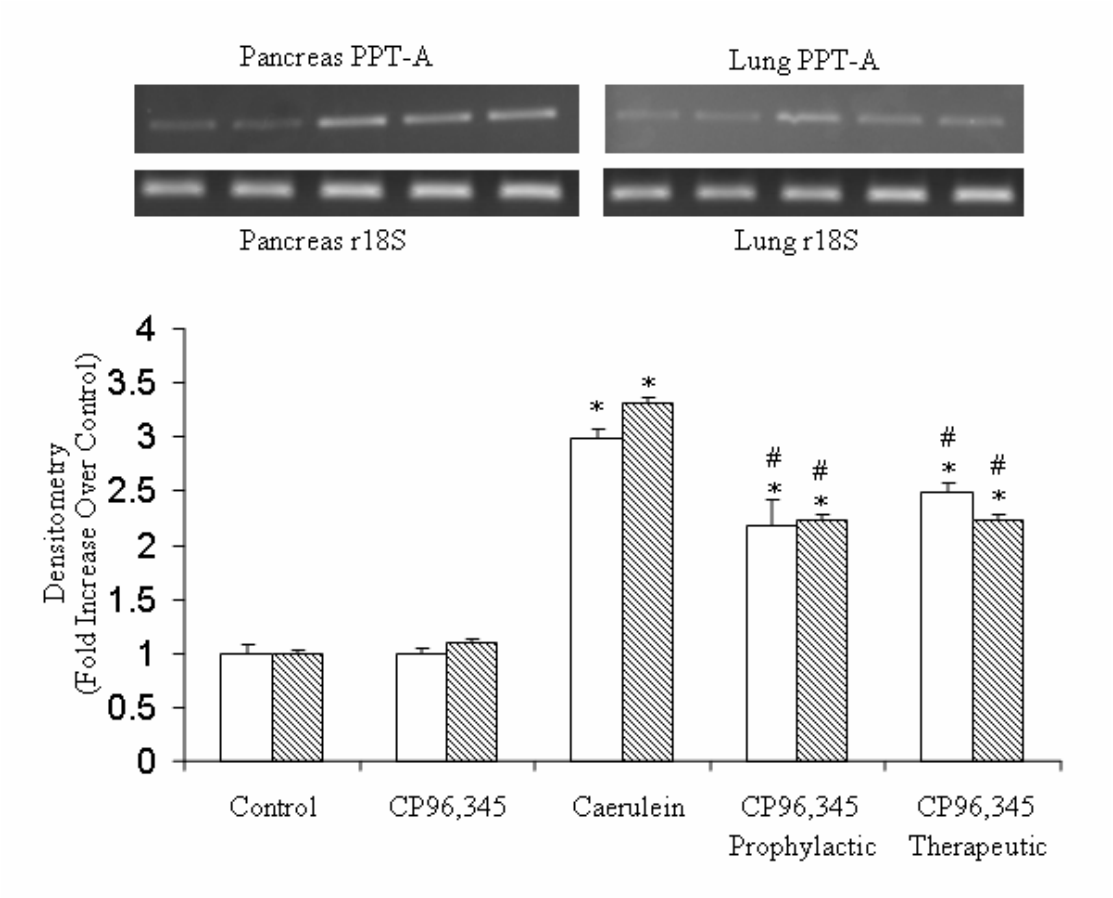


Figure 3.3 PPT-A mRNA expression in pancreas (hollow bars) and lungs (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

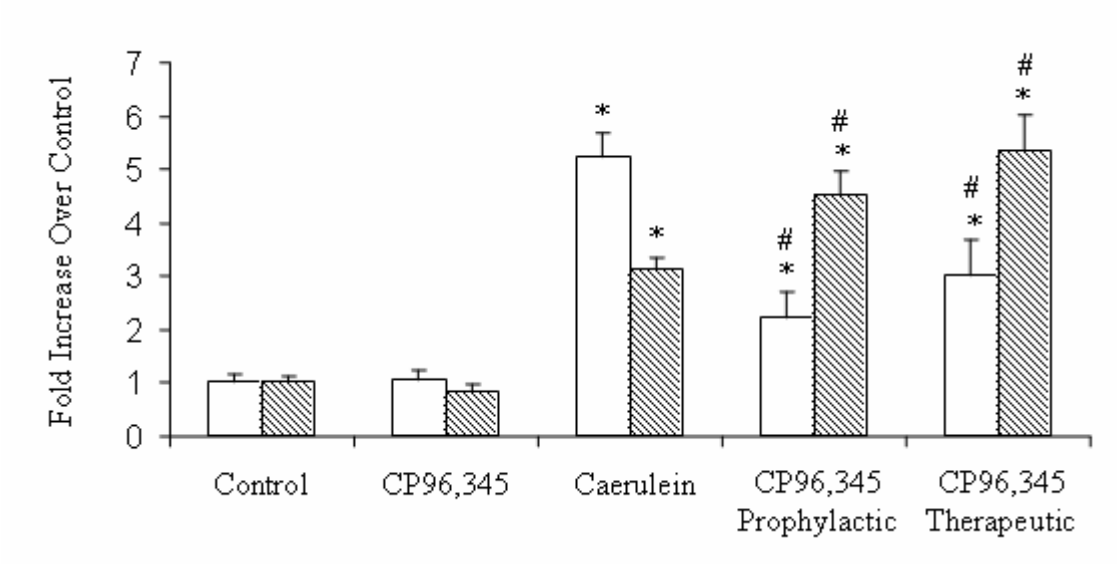


Figure 3.4 NK1 receptor mRNA expression in pancreas (hollow bars) and lungs (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

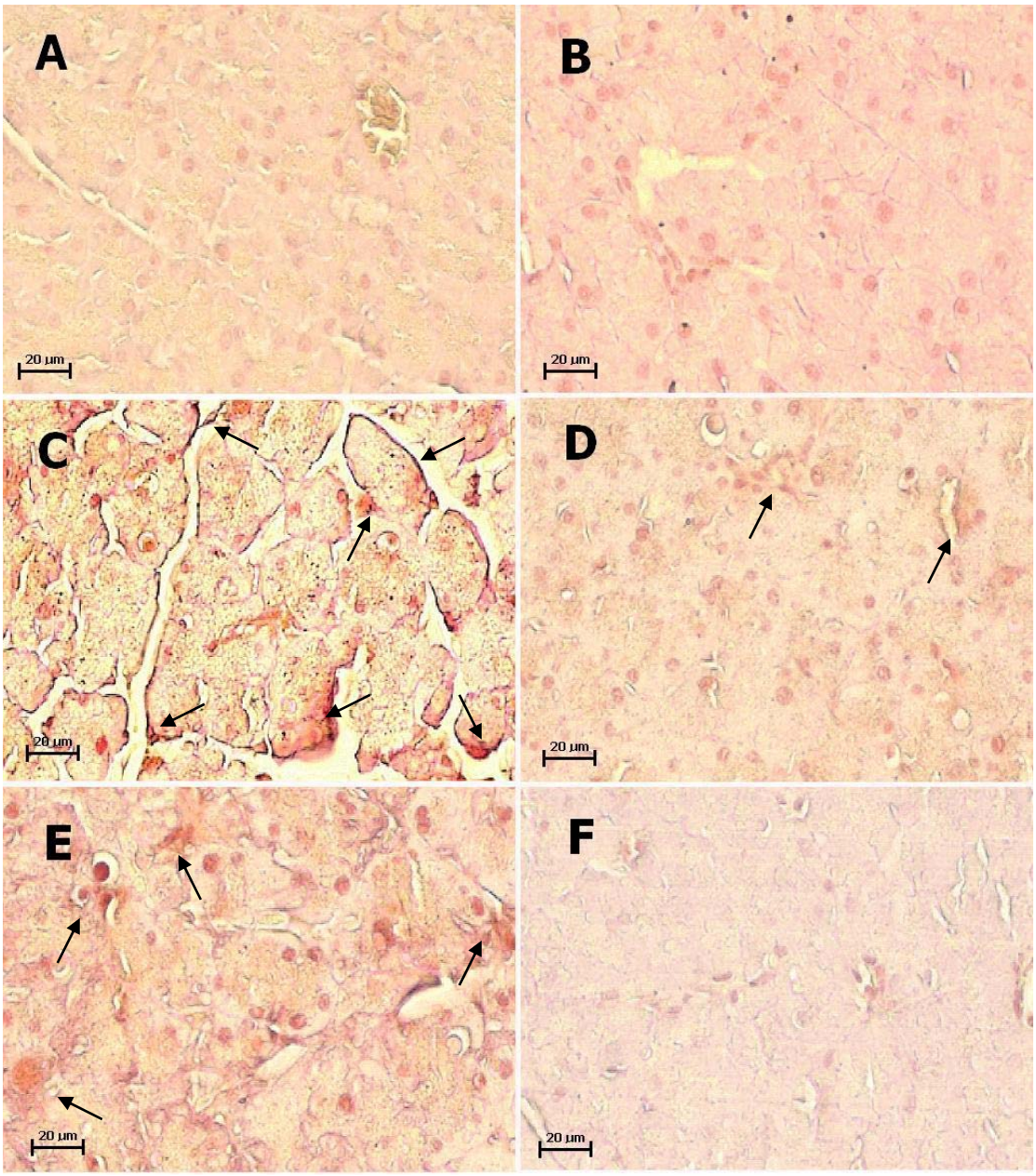


Figure 3.5 Immunohistochemistry staining of pancreas sections (5µm) using polyclonal anti-mouse NK1 receptor antibody. Low level of NK1R was detected in normal pancreas. During AP, increased localization of NK1R on the cell surface of pancreatic acinar cells was observed. This was demonstrated by the increase in brown staining in the caerulein-only group. (A) Saline only; (B) CP96345 (2.5mg/kg); (C) 10 consecutive hourly caerulein (50µg/kg/hr) injections; (D) CP96345 (2.5mg/kg) 30 minutes before caerulein injections; (E) CP96345 (2.5mg/kg) 60 minutes after first caerulein injection; (F) Negative control (absence of primary antibody). Scale bars = 20µm.

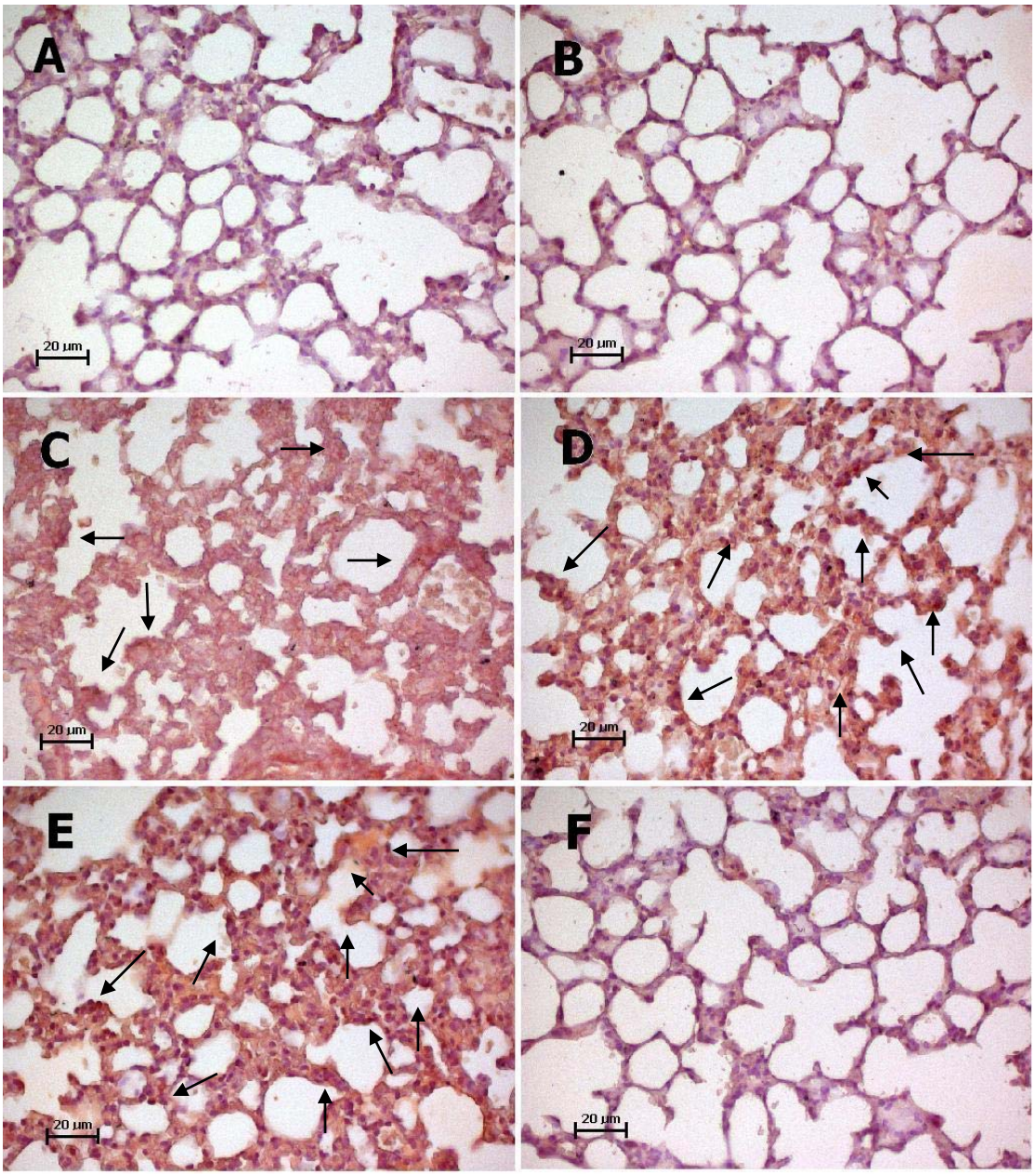


Figure 3.6 Immunohistochemistry staining of lung sections (5µm) using polyclonal anti-mouse NK1 receptor antibody. Low level of NK1R was detected in normal lung. During AP, increased localization of NK1R on the cell surface of alveolar cells was observed. This was demonstrated by the increase in brown staining in the caerulein-only group. (A) Saline only; (B) CP96345 (2.5mg/kg); (C) 10 consecutive hourly caerulein (50µg/kg/hr) injections; (D) CP96345 (2.5mg/kg) 30 minutes before caerulein injections; (E) CP96345 (2.5mg/kg) 60 minutes after first caerulein injection; (F) Negative control (absence of primary antibody). Scale bars = 20µm.

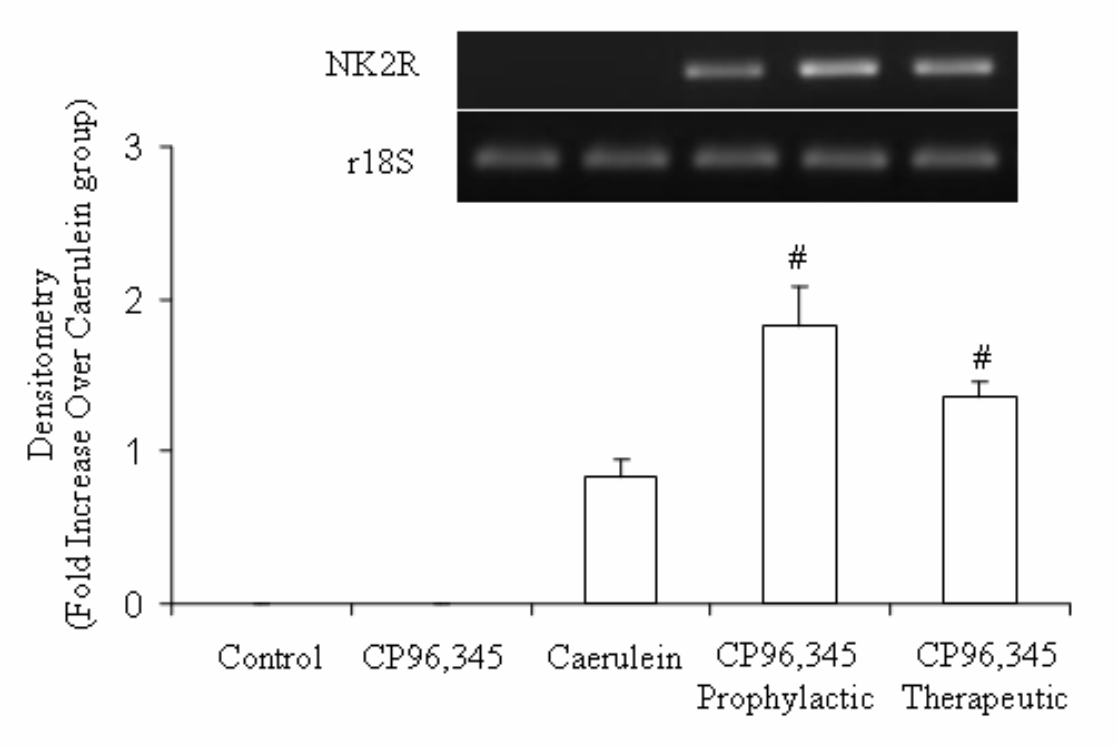


Figure 3.7 NK2 receptor mRNA expression in pancreas of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
 # indicates significantly different from the Caerulein group ($P < 0.05$).

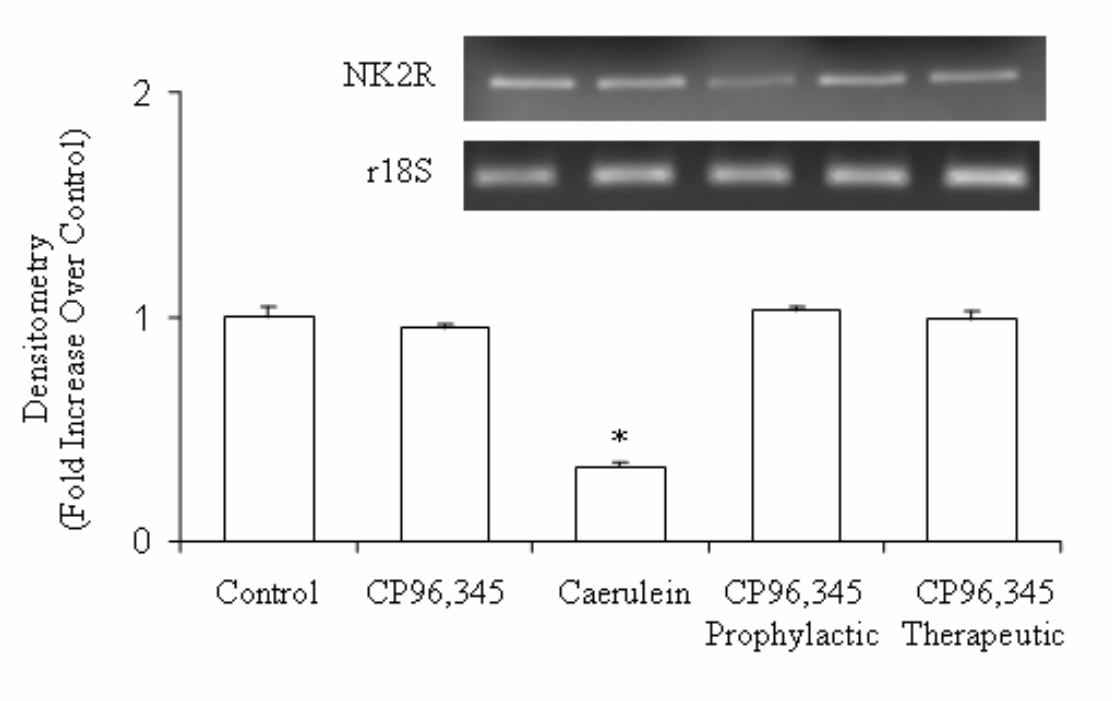


Figure 3.8 NK2 receptor mRNA expression in the lungs of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
 # indicates significantly different from the Caerulein group ($P < 0.05$).

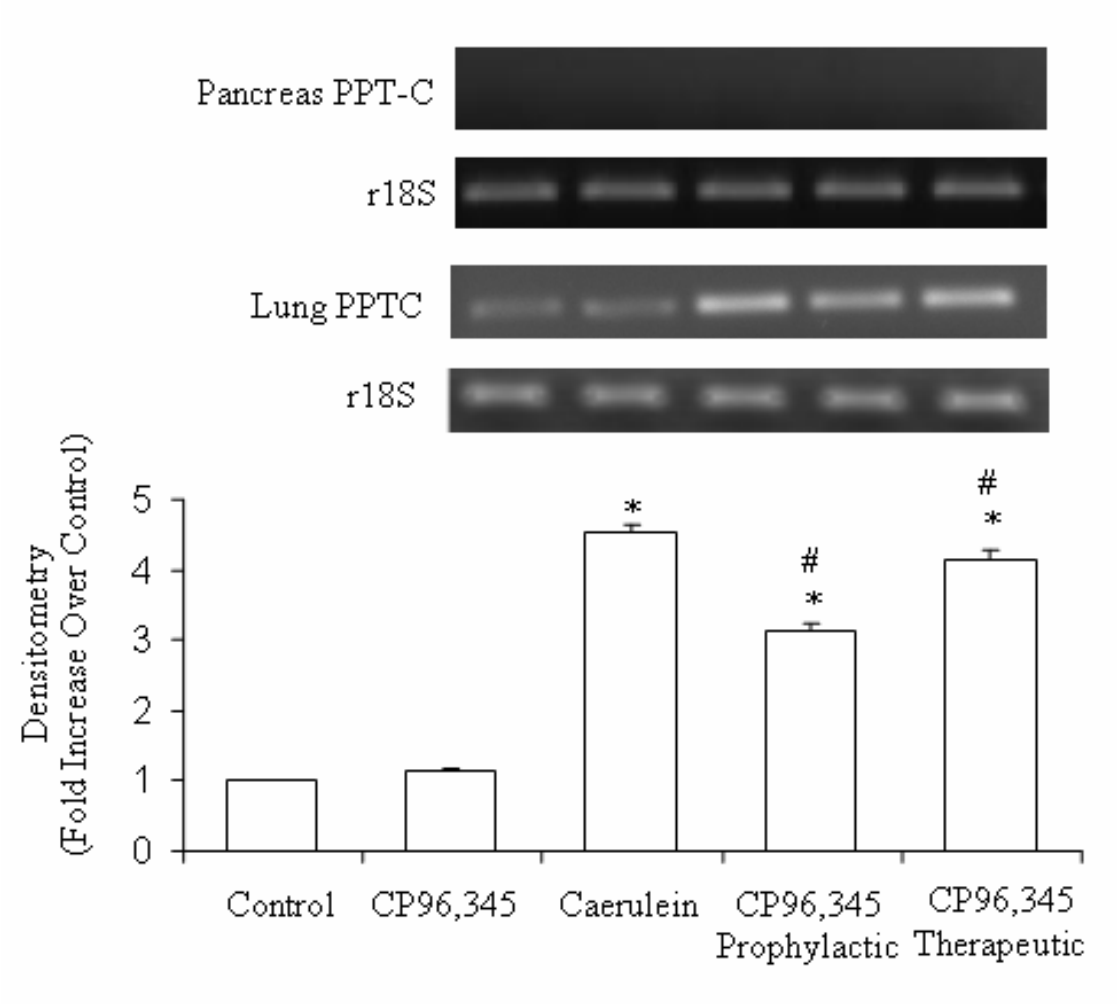


Figure 3.9 PPT-C mRNA expression in pancreas and lungs of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; CP96,345: CP96,345 (2.5mg/kg); Caerulein: 10 consecutive hourly caerulein (50µg/kg/hr) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. No expression of the gene was detected in pancreas. Bar charts represent mean value in lung ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

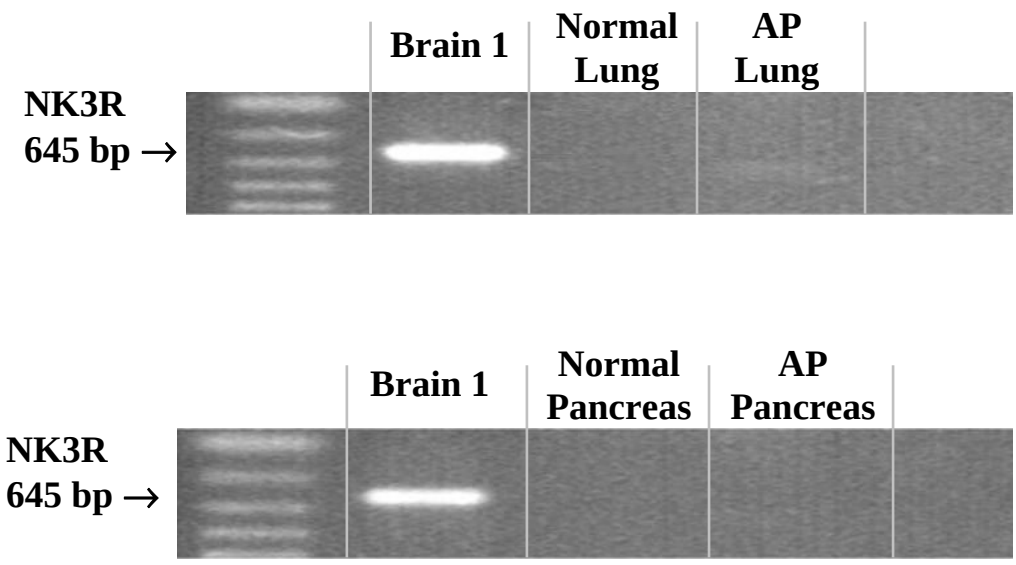


Figure 3.10 Absence of NK3R mRNA expression in normal and AP pancreas and lung of mouse.

Target Organs	CP96,345	mRNA expression											
		NK1R		NK2R		NK3R		PPT-A		PPT-C		SP (ELISA)	
		AP	*CP*	*AP*	*CP*	*AP*	*CP*	*AP*	*CP*	*AP*	*CP*	*AP*	*CP*
Pancreas	Prophylactic	↑	↓	↑	↑↑	ND	ND	↑	↓	ND	ND	↑	↓
	Therapeutic	↑	↓	↑	↑↑	ND	ND	↑	↓	ND	ND	↑	↓
Lung	Prophylactic	↑	↑↑	↓	↓	ND	ND	↑	↓	↑	↓	↑	↓
	Therapeutic	↑	↑↑	↓	↓	ND	ND	↑	↓	↑	↓	↑	↓

Table 3.2 Summary of the changes mRNA expression during AP. Upward arrows represent increase in mRNA expression. Downward arrows represent decrease in mRNA expression. ND: not detected.

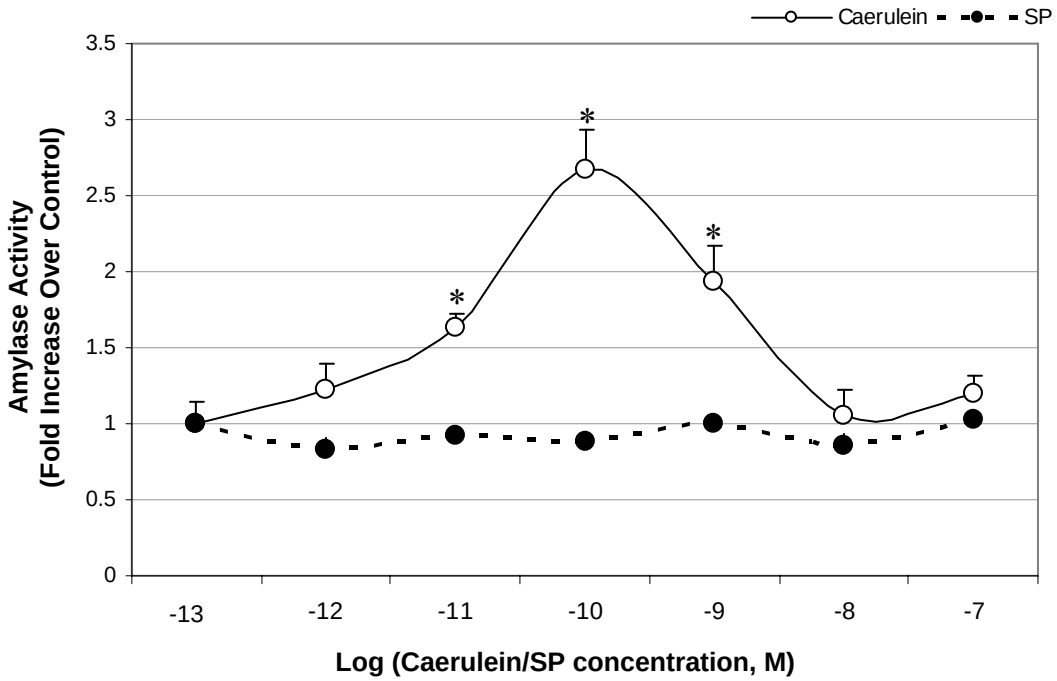


Figure 3.11 Dose-response relationship between the increasing caerulein/SP concentration (10^{-13} M to 10^{-7}M) and the release of amylase from pancreatic acinar cells. A bell-shaped curve was observed with the maximum amylase activity measured at 10^{-10} M caerulein. SP did not affect the amylase secretion. Data points represent mean value ± SEM of at least 8 animals.

*** indicates significantly different from control group (*P* <0.05).**

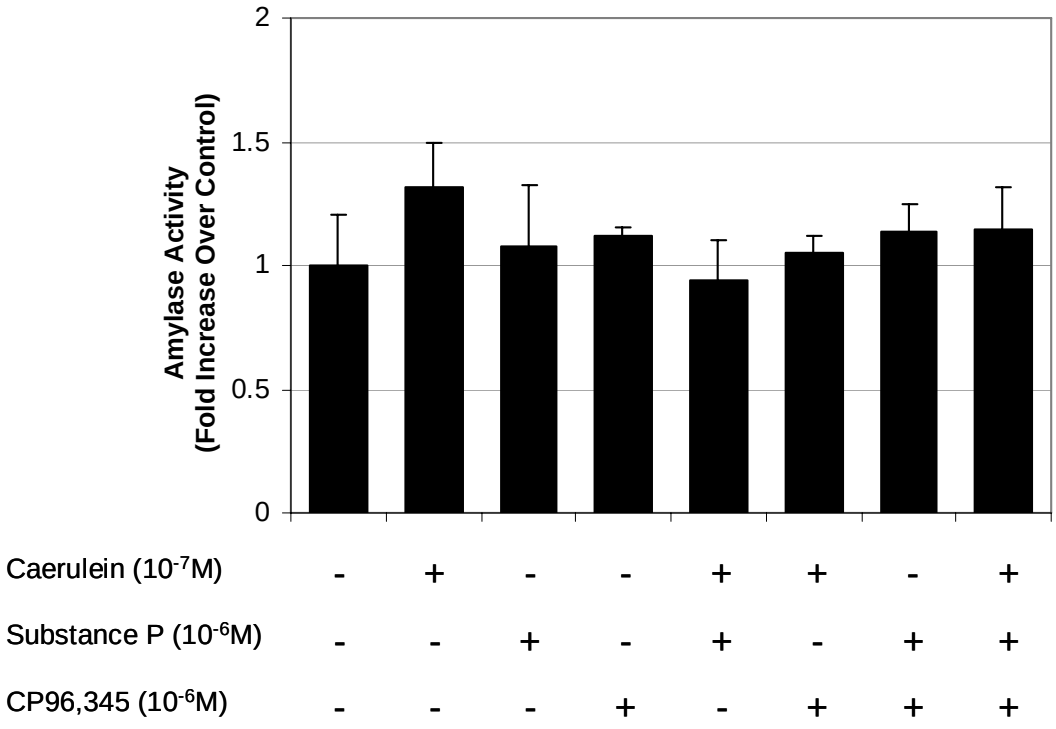


Figure 3.12 Effects of caerulein and/or SP incubation, in the presence or absence of CP96,345, on the release of amylase from the pancreatic acinar cells. Bar charts represent mean value ± SEM of at least 8 experiments.

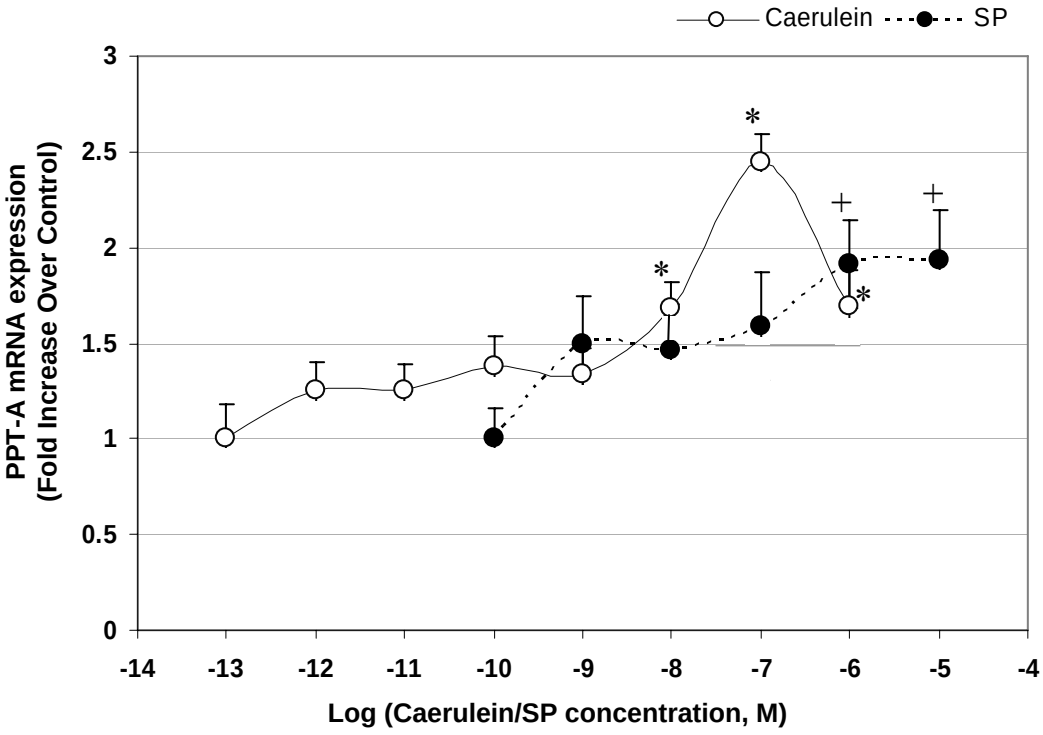


Figure 3.13 Dose-response relationship between the increasing caerulein (10^{-13} M to 10^{-6}M) / SP (10^{-10} M to 10^{-5}M) concentration and the change in PPT-A mRNA expression in pancreatic acinar cells. Data points represent mean value ± SEM of at least 8 animals.

*** indicates significantly different from control group (caerulein treatment) (*P* <0.05).**
+ indicates significantly different from control group (SP treatment) (*P* <0.05).

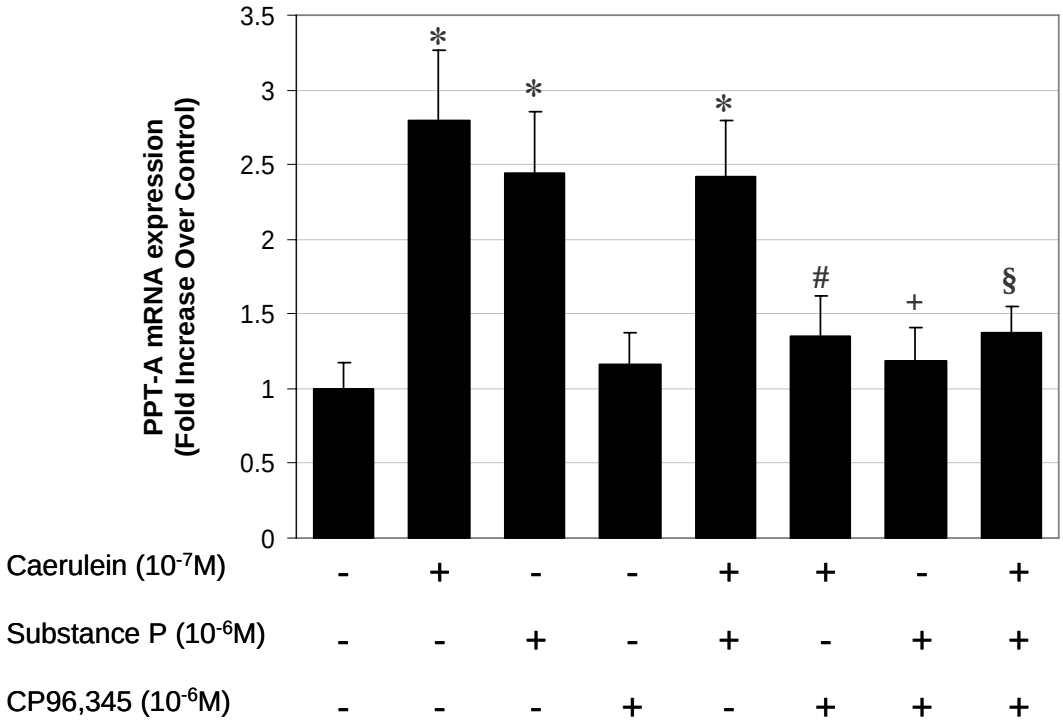


Figure 3.14 Effects of caerulein and/or SP incubation, in the presence or absence of CP96,345, on the expression of PPT-A mRNA. Significant increase in PPT-A mRNA was observed with caerulein and/or SP treatment, which could be blocked by NK1R antagonist. Bar charts represent mean value ± SEM of at least 8 experiments.

*** indicates significantly different from control group (*P* <0.05).**
 # indicates significantly different from caerulein-only group (*P* <0.05).
+ indicates significantly different from SP-only group (*P* <0.05).
§ indicates significantly different from caerulein/SP treated group (P <0.05).

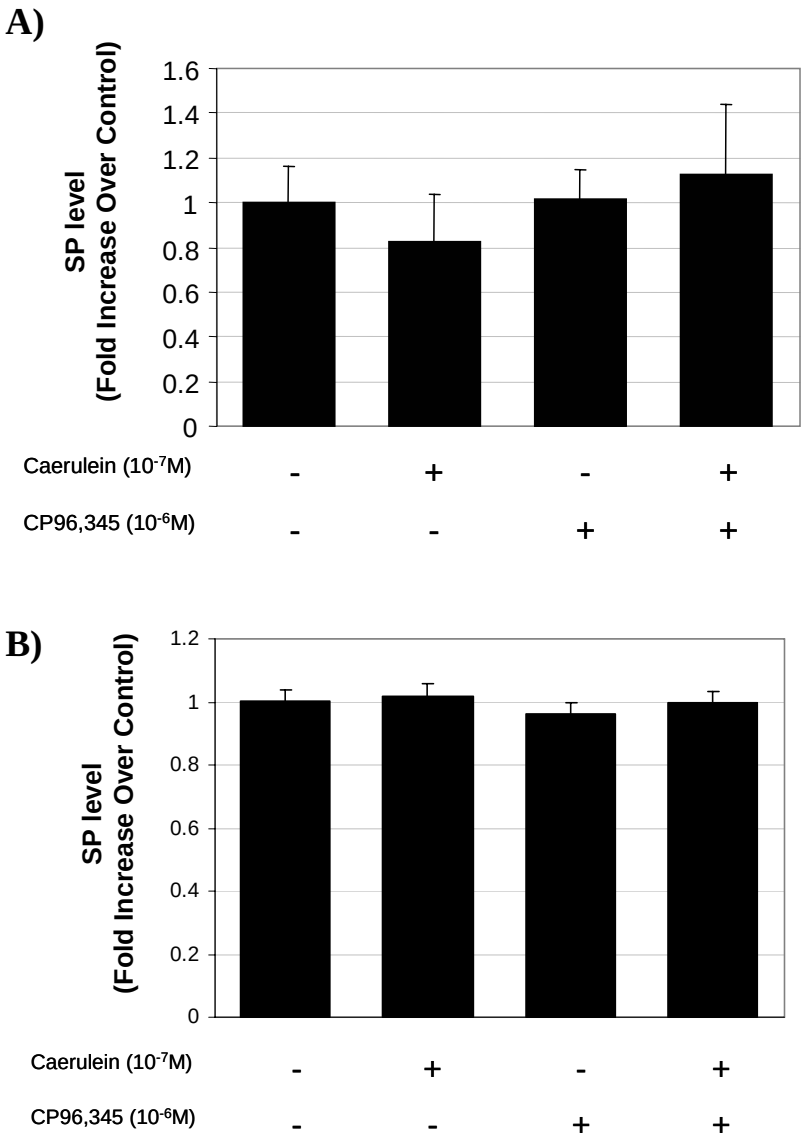


Figure 3.15 Effect of caerulein, in the presence or absence of CP96,345, on SP levels in the A) acinar cells; B) supernatant. Bar charts represent mean value ± SEM of at least 8 experiments.

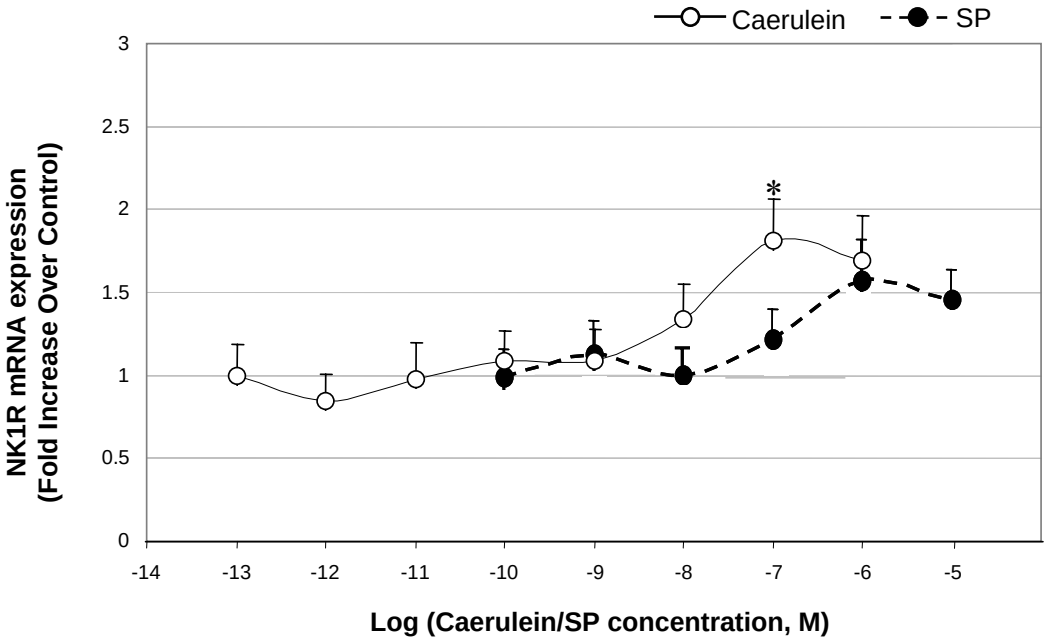


Figure 3.16 Dose-response relationship between the increasing caerulein (10^{-13} M to 10^{-6}M) / SP (10^{-10} M to 10^{-5}M) concentration and the change in NK1R mRNA expression in pancreatic acinar cells. Data points represent mean value ± SEM of at least 8 animals.

*** indicates significantly different from control group (caerulein treatment) (*P* <0.05).**

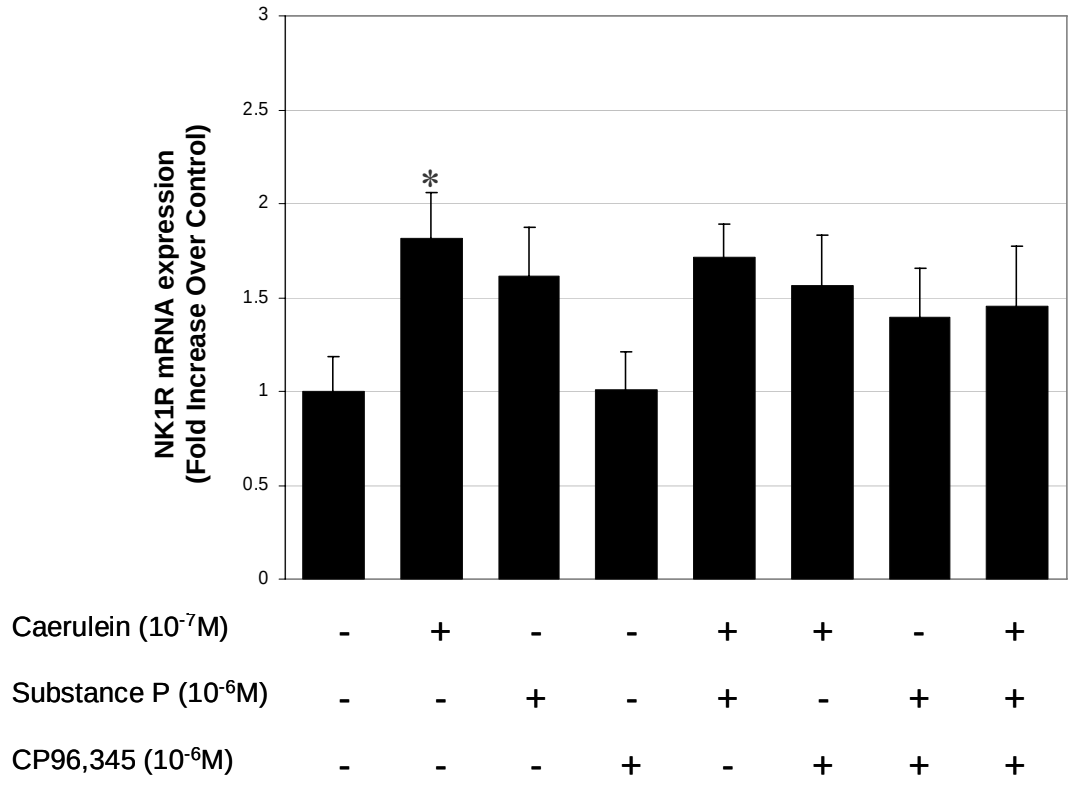


Figure 3.17 Effects of caerulein and/or SP incubation, in the presence or absence of CP96,345, on the expression of NK1R mRNA. Significant increase in NK1R mRNA was observed when the acinar cells were treated with caerulein. Bar charts represent mean value ± SEM of at least 8 experiments.

*** indicates significantly different from control group (*P* <0.05).**

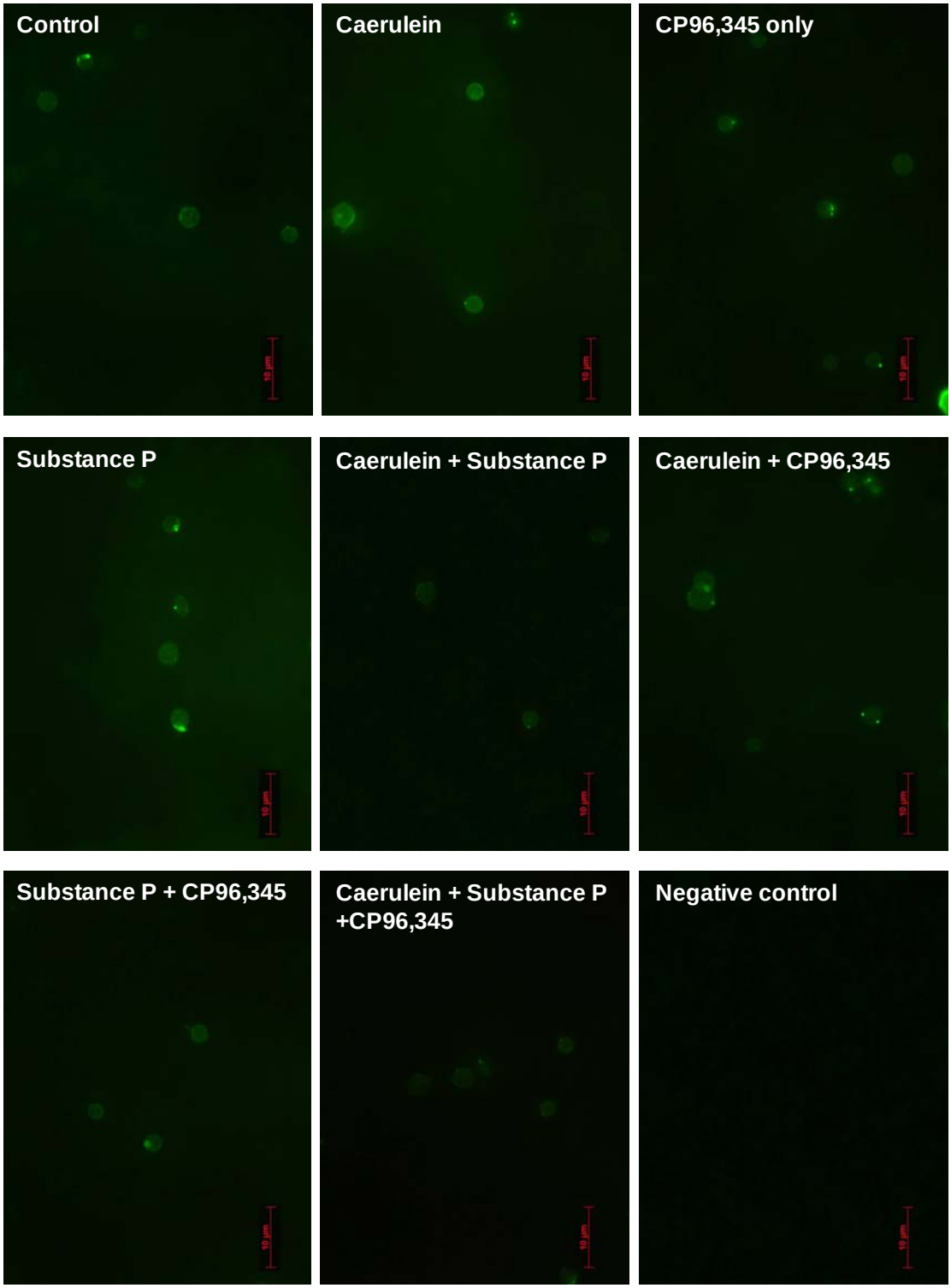


Figure 3.18 Effect of caerulein, SP and CP96,345, on NK1R expression. Low level of NK1R was fluorescently labeled on the cell surface of pancreatic acinar cells. There was no significant difference in NK1R staining (fluorescence on cell surface – background fluorescent) observed among the treatment groups.
Scale bars (red) = 10 μm

3.4 DISCUSSION

The up-regulation of SP levels in the pancreas and the lungs of AP mice induced by caerulein has been shown in Chapter 2. Caerulein, a CCK analog, at the physiological concentration activated the high affinity site that is believed to mediate normal pancreatic enzyme secretion (Fig 3.11). When given at supramaximal concentrations, caerulein stimulates the second low affinity receptor and resulting in the inhibition of enzyme secretion and AP (Fig 3.11) (Niederau et al., 1994). In the present study, I have further investigated the effect of the NK1R antagonist CP96,345 on SP levels in the pancreas and lungs. Patients with AP for the most part seek medical attention within 12–18 hours of the onset of pain, although some patients may present later. In patients with a severe attack, the effects of distant organ damage (such as lung injury) are often not fully established and only become apparent over the following 48 hours. Potentially, there is thus a therapeutic window between hospital presentation and the development of distant organ dysfunction. A similar therapeutic window exists in experimental models of AP (Bhatia et al., 2000a; Bhatia et al., 2005a; Bhatia et al., 2003b). Therefore, in this study CP96,345 was administered, either prophylactically or therapeutically, either before (prophylactic treatment) or after (therapeutic treatment) initiation of the pancreatic injury, but in both cases before the lung injury could set in.

We found that both prophylactic and therapeutic treatments of CP96,345 resulted in suppression of elevated SP levels in both organs (Fig 3.1 & 3.2). Similarly, there was an up-regulation of PPT-A gene expression in both pancreas and lungs (Fig 3.3). PPT-A is the gene that codes for SP (Holzer and Holzer-Petsche, 1997a) and its deletion

has been shown to offer protection against AP (Bhatia et al., 2003b). This finding suggests that SP levels and PPT-A gene expression in both the pancreas and lungs appear to be closely related to the severity of pancreatitis. The increases in SP and PPT-A gene expression were also detected some inflammatory conditions, such as allergic asthma (de Vries et al., 2006), arthritis (Garrett et al., 1995), trichinosis (Swain et al., 1992) and keratoconjunctivitis (Lambiase et al., 1997). In addition, the production of SP may be modulated by NK1R activation, as treatment with CP96,345 resulted in the inhibition of PPT-A gene expression and SP production. Although SP is mainly found in the neuronal cells, numerous reports have described its co-existence with NK1R in non-neuronal cells (Milner et al., 2004; Watanbe et al., 2002; Maghni et al., 2003; Lai et al., 2002; Bae et al., 2002; Qian et al., 2001; Germonpre et al., 1999) and have suggested that the possible role of endogenous SP production in the modulation of inflammation in an autocrine and/or paracrine pattern (Watanabe et al., 2002; Maghni et al., 2003; Qian et al., 2001, Germonpre et al., 1999). The regulation of SP production by the activation of NK1R was also observed in the *in vitro* studies where isolated acinar cells treated with caerulein or SP or in combination of the two peptides, acinar cells responded with increased PPT-A mRNA expression, which could be blocked by the co-administration of the NK1R antagonist, CP96,345 (Fig 3.14).

The expression profile of NK1R in AP using real time-PCR and immunohistochemistry was generated. Real-time PCR was employed to overcome the problem of low expression level of NK1R in the pancreas (Bhatia et al., 1998a). In the pancreas, caerulein-induced AP resulted in the elevation of NK1R mRNA expression, which could be impeded by treatment with CP96,345 (Fig 3.4). Up-regulation of

tissue NK1R expression has also been described in many inflammatory conditions, such as asthma (Adcock et al., 1993), irritable bowel syndrome (La et al., 2005, ter Beek et al., 2007) and allergic contact dermatitis (El-Nour et al., 2006). Immunohistochemistry of the pancreas with anti-NK1R antibody reinforced this finding, with the CP96,345-treated group showing apparently weaker immunostaining than the untreated AP group (Fig 3.5). Although the isolated acinar cell study demonstrated a similar trend, the change in NK1R expression over a 1 hour period was too small to reach any statistical significant (Fig 3.16 to 3.18). On the other hand, a different expression profile was observed in the lungs, with an elevation of NK1R mRNA in AP observed with administration of CP96,345 (Fig 3.4). This result was supported by immunohistochemistry (Fig 3.6).

In addition to NK1R, other members of the neurokinin receptor family were also examined in the mouse model of AP. NK3R mRNA expression was only detected in brain but not in the lungs and pancreas (Figure 3.10), suggesting that its main role is in the CNS. NK2R mRNA expression was detected in normal lungs (the alveolar cells) (Adcock et al., 1993) and inflamed pancreas (acinar cells) and lungs. In normal pancreas, NK2R mRNA expression was not detected (Fig 3.7). During AP, an increase in NK2R mRNA expression in the pancreas was observed but down-regulation of NK2R mRNA expression in the lungs was detected. In the pancreas, treatment with CP96,345 resulted in further NK2R mRNA expression. On the other hand, receptor mRNA expression in the lung was reversed to normal levels following treatment with CP96,345 (Fig 3.8). The significance of these findings is unknown and requires further investigation. However, these results show a differential regulation of

TK and neurokinin receptor expression in the pancreas and lungs in AP and point to a differential regulation of local and systemic (lung) inflammation in AP.

It is known that the PPT-A gene codes for SP and NKA, PPT-B codes for NKB (Patacchini et al., 2004) and PPT-C codes for the newly discovered HK-1 (Zhang et al., 2000). SP and haemokinin bind primarily to NK1R, NKA to NK2R and NKB to NK3R. Since NK3R was not detected in the pancreas and lungs, we did not include NKB/PPT-B expression was not evaluated in the present study. However, RT-PCR of PPT-C mRNA expression was carried out and the expression was detected in the lung but not the pancreas. The absence of PPT-C in normal mouse pancreas has been reported previously (Kurtz et al., 2002). Moreover, pulmonary but not pancreatic PPT-C mRNA expression was up-regulated during AP and CP96,345 treatment suppressed this elevation, further supporting the possibility of a differential regulation of inflammation in the pancreas and lungs during AP and associated lung injury.

The mechanism by which tachykinins participate in the pathophysiology of AP is unknown. However, it is clear that SP and the NK1R play an important role and the NK1R antagonist represents a promising therapeutic tool for the treatment of AP (Bhatia et al., 1998a; Bhatia et al., 2003b). The present study provides a deeper insight into the changes in and regulation of the TK system, during AP and in response to treatment with NK1R antagonist. The findings reinforce the key role of SP and NK1R in this inflammatory condition. However, the differential regulation of NK1R expression in pancreas and lung in response to the antagonist treatment might indicate a different nature of the inflammatory responses (neurogenic vs non-neurogenic) in the two organs (Fig 3.4). In addition to NK1R, the present results also

suggest the possible involvement of NK2R in the pathogenesis of AP, which is also expressed differentially in the pancreas and lungs during AP. On the other hand, the distribution of PPT-C expression suggests its importance in AP-associated lung injury, but not in the inflammatory insult to the pancreas.

A differential pattern of regulation of the inflammation is remarkably interesting, as this is the first evidence that, even though TK play a key role in the pathogenesis of pancreatitis and associated lung injury, in AP they may well be acting by different mechanisms in augmenting pancreatic and lung injury. In light of these results, we postulated the existence of two different organ-specific modulations of inflammatory response in the pancreas and the lungs during AP. In Chapter 4, we shall continue to investigate the consequences of these different organ-specific regulations on expression of several important cell surface adhesion molecules that are critical in directing inflammatory cells to the sites of injury in AP.

CHAPTER 4

EFFECT OF CP 96,345 ON THE EXPRESSION OF ADHESION MOLECULES IN AP

4.1 INTRODUCTION

AP is an acute inflammatory process of the pancreas, with variable involvement of other regional tissues or remote organ systems. It is a common inflammatory disorder with increasing incidence over the past few decades (Bhatia et al., 2000a). Most cases develop as a result of biliary disease or excess alcohol consumption. The severity of this disease ranges from mild, localized, self-limiting pancreatic inflammation to systemic inflammatory response syndrome (SIRS) with fatal consequences. Current knowledge on the pathophysiology of AP has been reviewed recently (Bhatia et al., 2005a). Despite advances in knowledge of the aetiologies and pathophysiology of AP, as well as advances in intensive care management for patients with severe AP, the mortality rate is still high and improvement in treatment is still unsatisfactory (Imrie and McKay, 1999).

SP and its receptor, NK1R have been implicated in the pathogenesis of AP by mediating NI. (Bhatia et al., 2005a; Bhatia et al., 1998a; Bhatia et al., 2003b). Studies have shown that NI mediated by the binding of SP to NK1R plays a crucial role in the pathogenesis of AP and pancreatitis-associated lung injury in mice and rats (Lau et al., 2005; Grady et al., 2000; Maa et al., 2000). Knock-out mice deficient in SP and NK1R were found to be resistant to the development of severe AP (Bhatia et al.,

1998a; Bhatia et al., 2003b). In addition, treatment with NK1R antagonists has beenshown to be effective in suppressing the progression of the inflammatory condition (Chapter 2 and 3). Nevertheless, the exact mechanism by which NK1R and SP amplifies the severity of pancreatitis remains to be elucidated. This work requires further investigation into the other components in the inflammatory cascade.

Leukocyte recruitment is a hallmark feature of inflammation and is characterized by a sequence of events that bring about extravasation of leukocytes, through leukocyte-endothelial interactions. Upon activation, leukocytes roll and adhere to the endothelium through the interactions between selectins (E- and P-selectins) or adhesion molecules (ICAM-1, VCAM-1) with their respective counter ligands. This is followed by the emigration of leukocytes to the site of injury under the influence of chemotactic agents. It was demonstrated that levels and expression of ICAM-1, VCAM-1, P- and E-selectin were upregulated and enhanced in AP (Lundberg et al., 2000a; Sunamura et al., 1999; Lundberg et al., 2000b; Uhlmann et al., 2001). Immunoneutralization of adhesion molecules has been proven to be effective in the treatment of experimental AP (Wang et al., 1999). Administration of monoclonal antibody against ICAM-1 to rats with acute severe pancreatitis significantly enhances capillary blood flow in the pancreas, reduces leukocyte rolling and stabilizes capillary permeability (Frossard et al., 1999). Moreover, blocking VCAM-1 decreases leukocyte adherence and recruitment into the lung, hence reducing lung injury in severe AP (Callicutt et al., 2003). However, the relationship between NK1R blockade and leukocyte activation during AP is largely unknown.

In this chapter, I have investigated the effect of the NK1R antagonist, CP96,345, on the mRNA and protein expression of various adhesion molecules implicated in AP. This will provide important information for the understanding of the role of SP and NK1R in the regulation of the downstream inflammatory cascades mediated by adhesion molecules during AP.

4.2 MATERIALS AND METHODS

4.2.1 Materials

Rat anti-mouse ICAM-1 (CD54) monoclonal antibody was purchased from Chemicon, CA, USA. Rat anti-mouse VACM-1 (CD106) polyclonal antibody was purchased from Serotec, UK. Rabbit anti-mouse E-selectin polyclonal antibody and goat anti-mouse P-selectin polyclonal antibody were obtained from Biovision, CA, USA.

4.2.2 Induction of AP

AP was induced in mice as described in Section 2.2.2. One hour after the last caerulein injection animals were sacrificed by a lethal dose of i.p. pentabarbitone (90 mg/kg, three times the dose for surgical anaesthesia). Pancreas and lungs tissues were collected for total RNA extraction, SP analysis or immunohistochemistry.

4.2.3 Semi-quantitative reverse transcription polymerase chain reaction (RT-PCR)

Reserve transcription polymerase chain reactions (RT-PCRs) were carried out as described in Section 3.2.5. The PCR primers (Table 4.1) for detection of ICAM-1, VCAM-1, E-selectin and P-selectin were synthesized by Proligo, Singapore. cDNA synthesized from 1 μg total RNA was included in a typical PCR. The reaction mixture was first subjected to 95 °C for 3 minutes for the activation of polymerase. This was followed by an optimal cycle of amplification (Table 4.1), consisting of 95 °C for 30

seconds, optimal annealing temperature (Table 4.1) for 30 seconds and 72 °C for 30 seconds. PCR amplification was performed in MyCycler™ (Biorad, Hercules, CA). PCR products were analysed on 1% w/v agarose gels containing 0.05 mg/100 ml ethidium bromide.

4.2.4 Immunohistochemistry with cryosections

One hour after the last caerulein injection, the mice were euthanized with sodium pentobarbital (90 mg/kg, three times the dose for surgical anesthesia) and perfused transcardially with heparinized 10 ml of 0.1M PBS at pH 7.4. Immediately after PBS perfusion, the pancreas was harvested, mounted and embedded in Optimal Cutting Temperature (OCT) compound (Ames Co., Division of Miles Laboratories, Elkhart, IN) in liquid nitrogen, and stored at -70°C for no more than 2 weeks. In addition, a mixture of OCT compound and PBS (1:2) was slowly infused into the trachea until the lungs were completely inflated under visual inspection, whereupon the lungs were removed and immediately mounted and embedded in OCT compound and placed in liquid nitrogen, and store at -70°C for no more than 2 weeks.

Pancreas or the lungs were sectioned on a Leica CM1800 cryostat (Leica Microsystems Nussloch GmbH, Germany) to obtain sections of 7µm thickness, which were then transferred to polylysine (0.1% v/v in distilled water) coated slides. The tissue slides were then fixed in ice-cold acetone for 10 minutes, washed, and air-dried. The sections were then kept at -70°C for no more than 1 month. During immunostaining, the cold sections were rinsed in TBS several times before the endogenous peroxidase activity was quenched with 0.3% H_2O_2. The slides were then

incubated with blocking buffer (3% BSA in TBS) for 1 hour to block non-specific binding sites, and washed with TBS. This was followed by incubation with the primary antibody diluted to the optimal working concentrations and in the optimal conditions (Table 4.2). After that, the sections were washed, and incubated in horseradish peroxidase-conjugated secondary antibodies for 30 minutes at room temperature. Finally, the slides were treated with DAB for 10 minutes, rinsed, counterstained with haematoxylin, dehydrated through graded alcohols, cleared in Histoclear, and coverslipped with Neomount. Negative controls were included by omitting the primary antibodies or substituting them with non-immune serum in order to check the specificity of the immunostaining (Figure 4.9). Antibodies were diluted in TBS at pH7.2. This buffer was also used in all washes (3 × 5 minutes), and all incubations are carried out at room temperature (25°C). The sections were then examined using Carl-Zeiss Axioskop 40 microscope system (Carl Zeiss, Göttingen, Germany) for qualitative assessment.

4.2.5 Data analytical method

Data are expressed as the mean ± SEM. In all figures, vertical error bars denote the SEM. The significance of differences between groups was evaluated by analysis of variance (ANOVA), with post hoc Tukey's test when comparing three or more groups. A P value of less than 0.05 was considered to indicate a statistically significant difference.

Gene	Primer Sequence	Optimal Conditions	Size
r18S	sense: 5’-GTAACCCGTTGAACCCCATT-3’ antisense: 5’-CCATCCAATCGGTAGTAGCG-3’	Lung: 22 cycles Pancreas: 22 cycles Annealing: 59°C	150 bp
ICAM-1	sense: 5’- CAACTGGAAGCTGTTTGAGCTG -3’ antisense: 5’- TAGCTGGAAGATCGAAAGTCCG -3’	Lung: 33 cycles Pancreas: 35 cycles Anealing: 60°C	437 bp
VCAM-1	sense: 5’- CCTCACTTGCAGCACTACGGGCT -3’ antisense: 5’- TTTTCCAATATCCTCAATGACGGG -3’	Lung: 34 cycles Pancreas: 36 cycles Annealing: 60°C	441 bp
E-selectin	sense: 5’- AGCTACCCATGGAACACGAC -3’ antisense: 5’- TGCAAGCTAAAGCCCTCATT -3’	Lung: 35 cycles Pancreas: 36 cycles Annealing: 60°C	622 bp
P-Selectin	sense: 5’- TACGAGCTGGACGGACCCG -3’ antisense: 5’- GGCTGGCACTCAAATTTACAGC -3’	Lung: 34 cycles Pancreas: 36 cycles Annealing: 60°C	181 bp

Table 4.1 PCR primer sequences, optimal amplification cycles, optimal annealing temperatures and product sizes for ICAM-1, VCAM-1, E- and P-selectins.

Antigen	Working dilution	Optimal Conditions	Duration
ICAM-1	1:200 (pancreas) 1:8000 (for lung)	Humidified chamber at room temperature	2 hours
VCAM-1	1:40	Humidified chamber at room temperature	2 hours
E-selectin	1:20	Humidified chamber at 4°C	24 hours
P-selectin	1:20	Humidified chamber at 4°C	24 hours

Table 4.2 Immunohistochemistry antibody working dilutions and optimal incubation conditions based on preliminary studies.

4.3 RESULT

4.3.1 Effect of CP96,345 treatment on pancreatic and pulmonary ICAM-1 mRNA expression and ICAM-1 protein expression in AP

Figure 4.1 shows the relative ICAM-1 mRNA level in the pancreas and the lungs. Hyperstimulation of the pancreas by caerulein resulted in an increase in ICAM-1 mRNA expression in both organs (2 to 3-fold). Administration of CP96,345 significantly attenuated the elevation of mRNA level in lung, but had no effect on the mRNA expression in pancreas. This is in contrast with the immunohistochemistry staining results (Figure 4.2), in which prophylactic CP96,345 treatment showed an apparent reduction in the immunoreactivity at the endothelial layer of the blood vessels in both the pancreas and the lungs.

4.3.2 Effect of CP96,345 treatment on pancreatic and pulmonary VCAM-1 mRNA expression and VCAM-1 protein expression in AP

In the pancreas, 10 consecutive hourly injections of caerulein results in a 2.5 fold increase in VCAM-1 mRNA expression (Figure 4.3). When CP96,345 was administered prophylactically, no significant change in the VCAM-1 expression was observed. Immunohistochemistry staining, however, demonstrated a apparent decline in VCAM-1 immunoreactivity in prophylactic treatment group (Figure 4.4). In the lungs, RT-PCR and immunohistochemistry (Figure 4.4) showed that VCAM-1 level was not affected by both caerulein and/or CP96,345 treatments.

4.3.3 Effect of CP96,345 treatment on pancreatic and pulmonary E-selectin mRNA expression and E-selectin protein expression in AP

E-selectin mRNA expression (Figure 4.5) correlated well with apparent protein expression (Figure 4.6). E-selectin mRNA and protein expression were not detected in normal pancreas. During AP, E-selectin was upregulated above 3.5-fold and localized extensively on the endothelial layer of the blood vessels in pancreas and the lungs, as well as the connective tissues between the acinar cells in the pancreas. Reduction of E-selectin mRNA and apparent protein expression in the pancreas and the lungs was observed in the CP96,345 prophylactic treatment group. Therapeutic treatment with the antagonist significantly reduced the E-selectin mRNA expression in the lungs but not in the pancreas.

4.3.4 Effect of CP96,345 treatment on pancreatic and pulmonary P-selectin mRNA expression and P-selectin protein expression in AP

A 2.2-fold increase in the P-selectin mRNA expression was observed in the pancreas during AP (Figure 4.7). Significant reduction in P-selectin expression was detected in mice treated with prophylactic CP96,345. Immunohistochemical analysis of P-selectin revealed a similar trend (Figure 4.8). An apparent increased P-selectin immunoreactivity was observed particularly in the endothelial layer and the surrounding connective tissues of the blood vessels in the pancreas. In the lungs, P-selectin mRNA expression increased 3.3-fold during AP and the prophylactic antagonist treatment significantly lowered the mRNA expression (Figure 4.7). Increased expression of P-selectin in the blood vessel endothelial layer and

surrounding connective tissue was observed during AP and the antagonist appeared to reduce the protein expression (Figure 4.8).

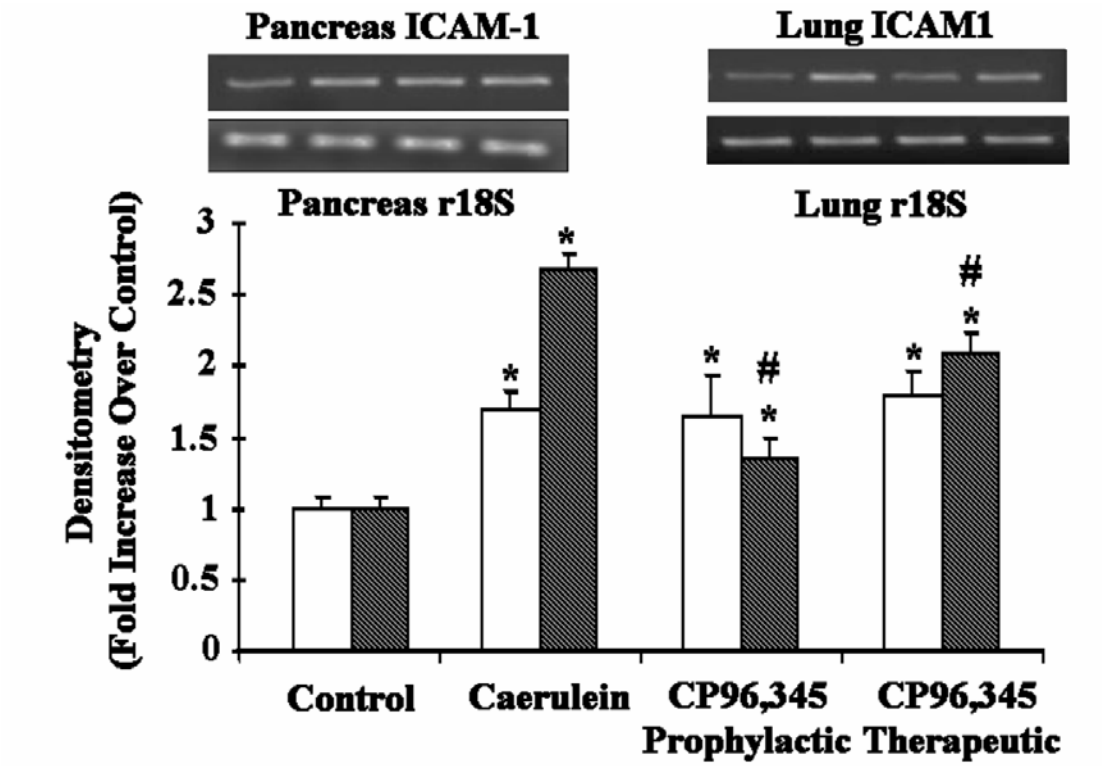


Figure 4.1 ICAM-1 mRNA expression in the pancreas (hollow bars) and lungs (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; Caerulein: 10 consecutive hourly caerulein (50μg/kg) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.

*** indicates significantly different from the Control group ($P < 0.05$).**

 # indicates significantly different from the Caerulein group ($P < 0.05$).

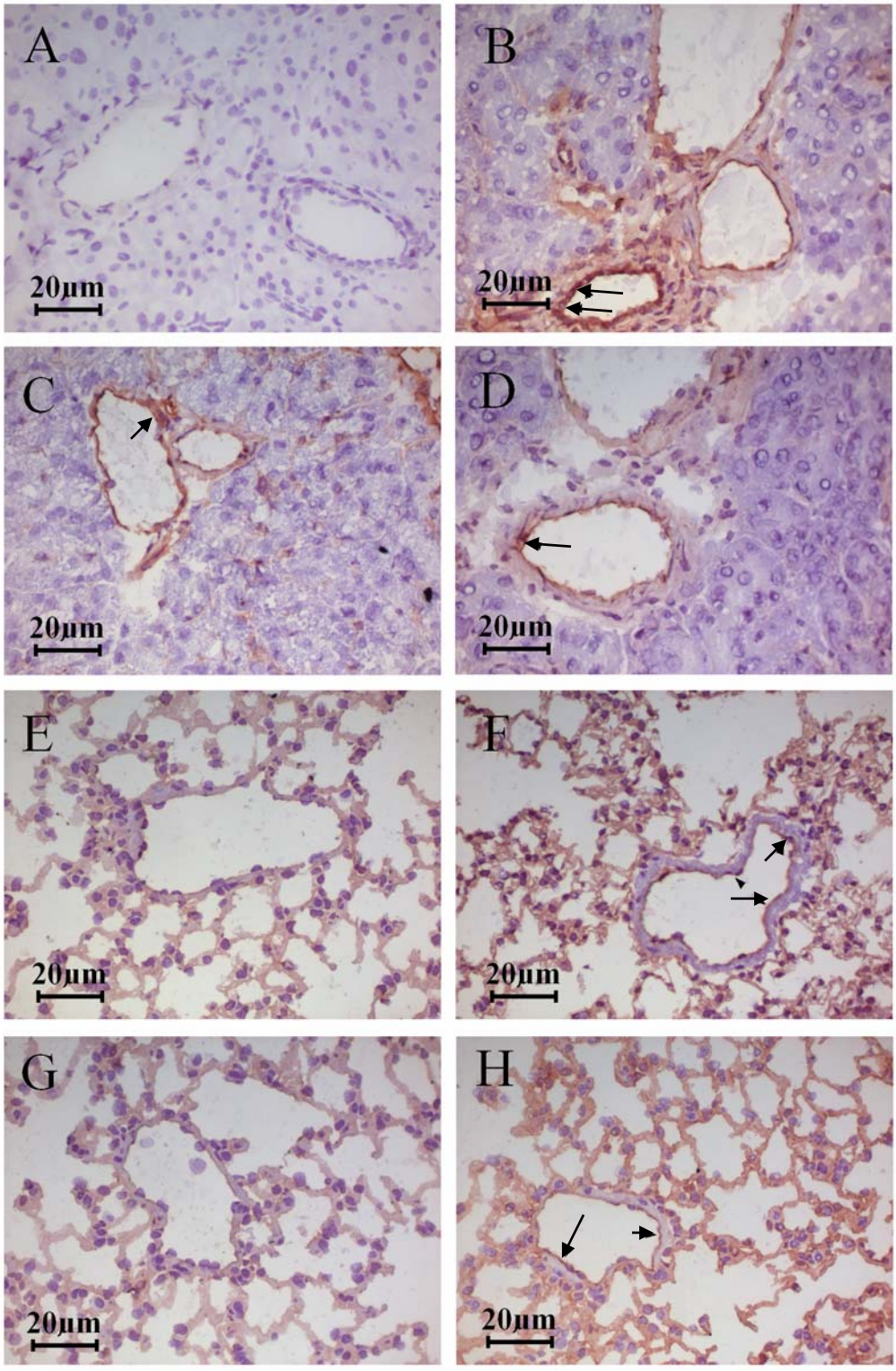


Figure 4.2 Immunohistochemistry staining of pancreas (A-D) and lung (E-H) sections (7µm) using monoclonal anti-ICAM-1 antibody. (A and E) Saline only; (B and F) 10 consecutive hourly caerulein (50µg/kg/hr) injections; (C and G) CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; (D and H) CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Scale bars = 20µm.

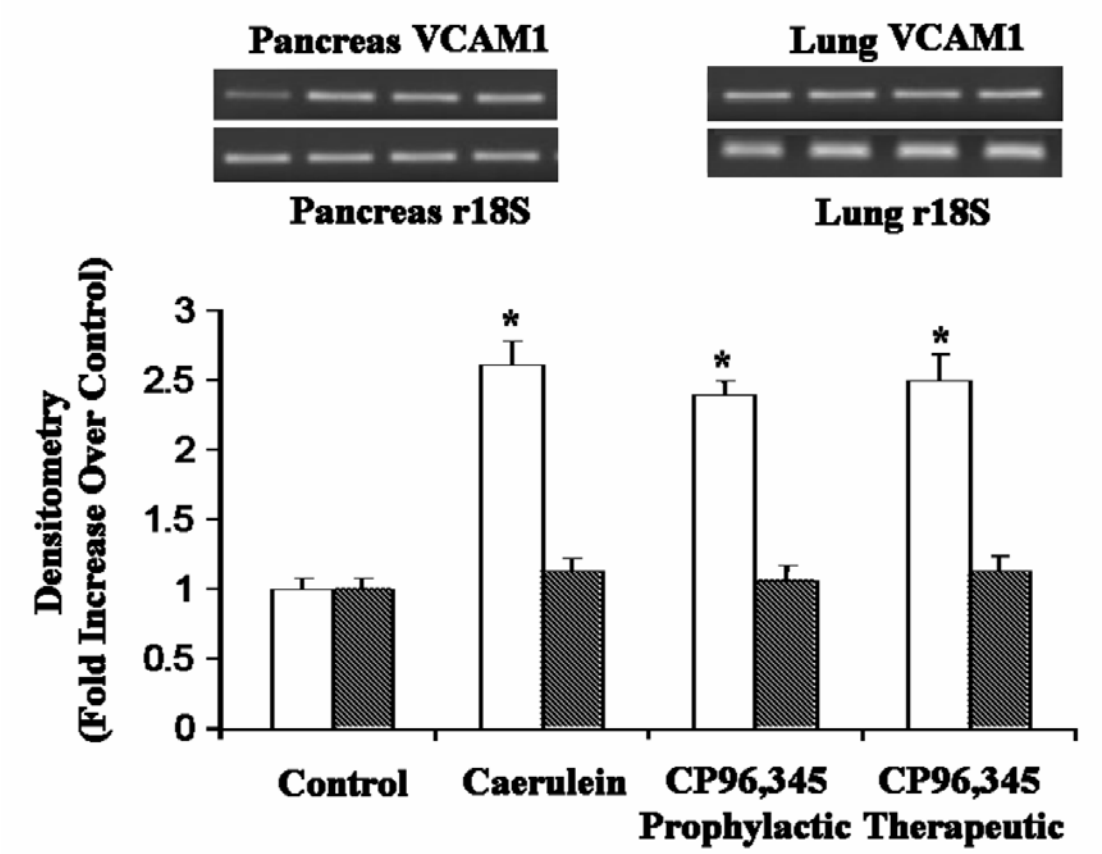


Figure 4.3 VCAM-1 mRNA expression in the pancreas (hollow bars) and lung (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; Caerulein: 10 consecutive hourly caerulein (50µg/kg) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.
*** indicates significantly different from the Control group ($P < 0.05$).**
 # indicates significantly different from the Caerulein group ($P < 0.05$).

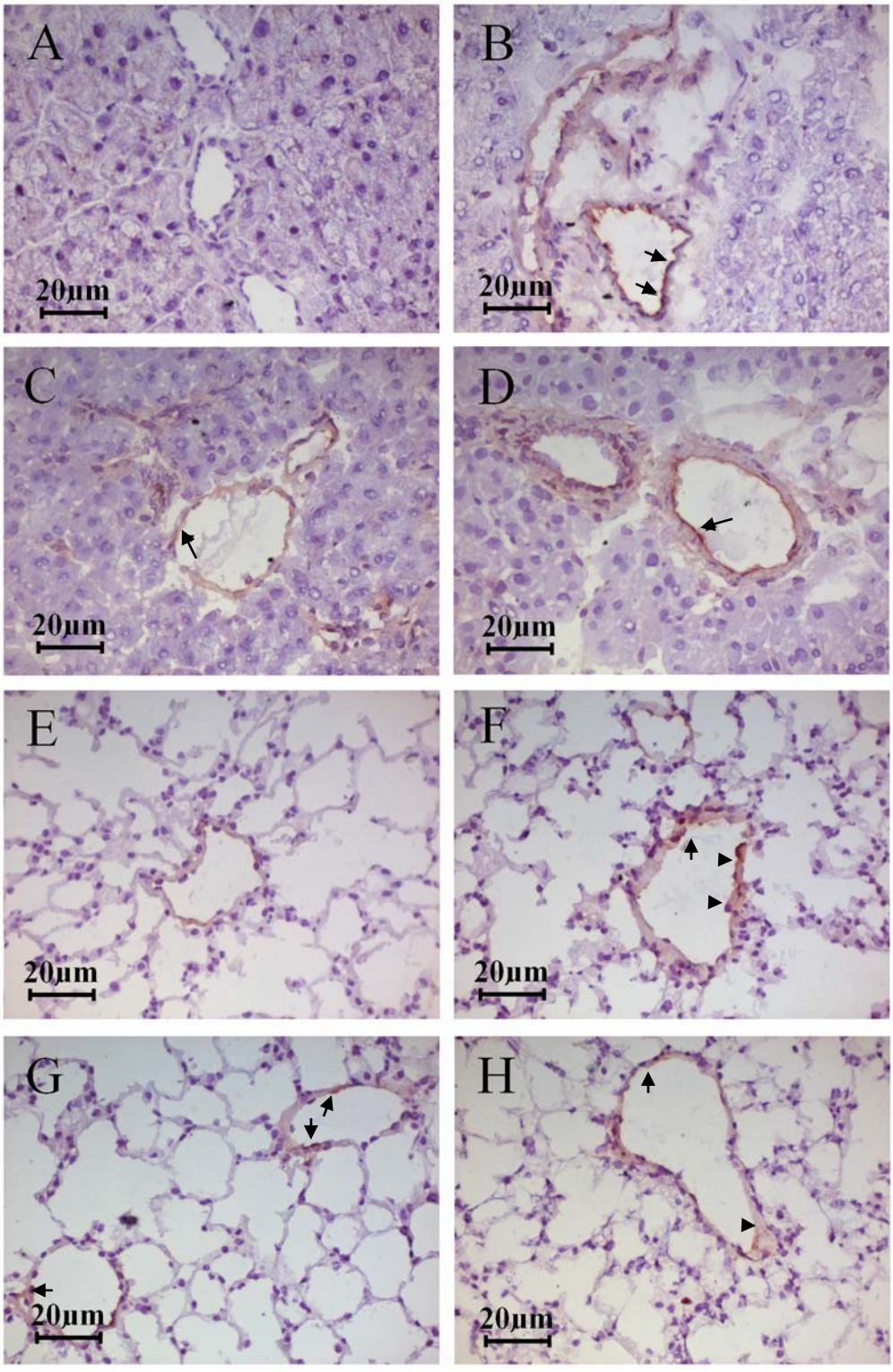


Figure 4.4 Immunohistochemistry staining of pancreas (A-D) and lung (E-H) sections (7µm) using polyclonal anti-VCAM-1 antibody. (A and E) Saline only; (B and F) 10 consecutive hourly caerulein (50µg/kg) injections; (C and G) CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; (D and H) CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Scale bars = 20µm.

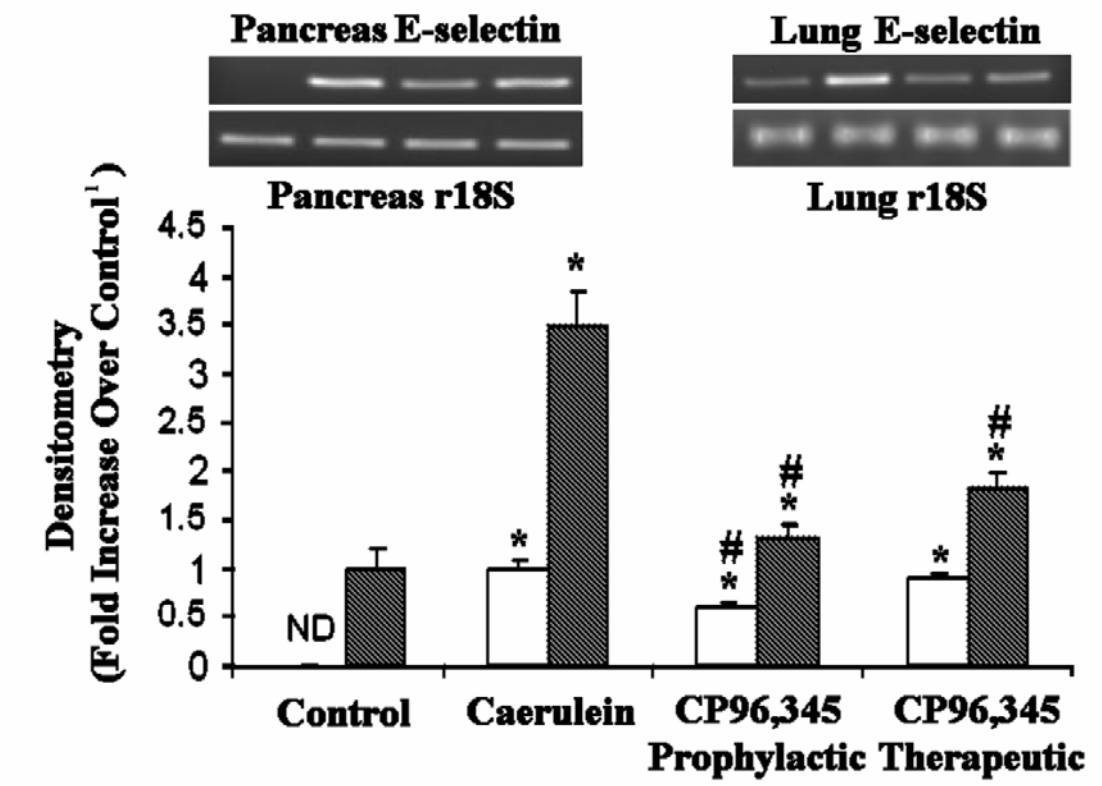


Figure 4.5 E-selectin mRNA expression in the pancreas (hollow bars) and lung (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; Caerulein: 10 consecutive hourly caerulein (50µg/kg) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals. ND: Not detected.

[1] E-selectin values of the pancreas are expressed as fold increase over caerulein group.

*** indicates significantly different from the Control group ($P < 0.05$).**

 # indicates significantly different from the Caerulein group ($P < 0.05$).

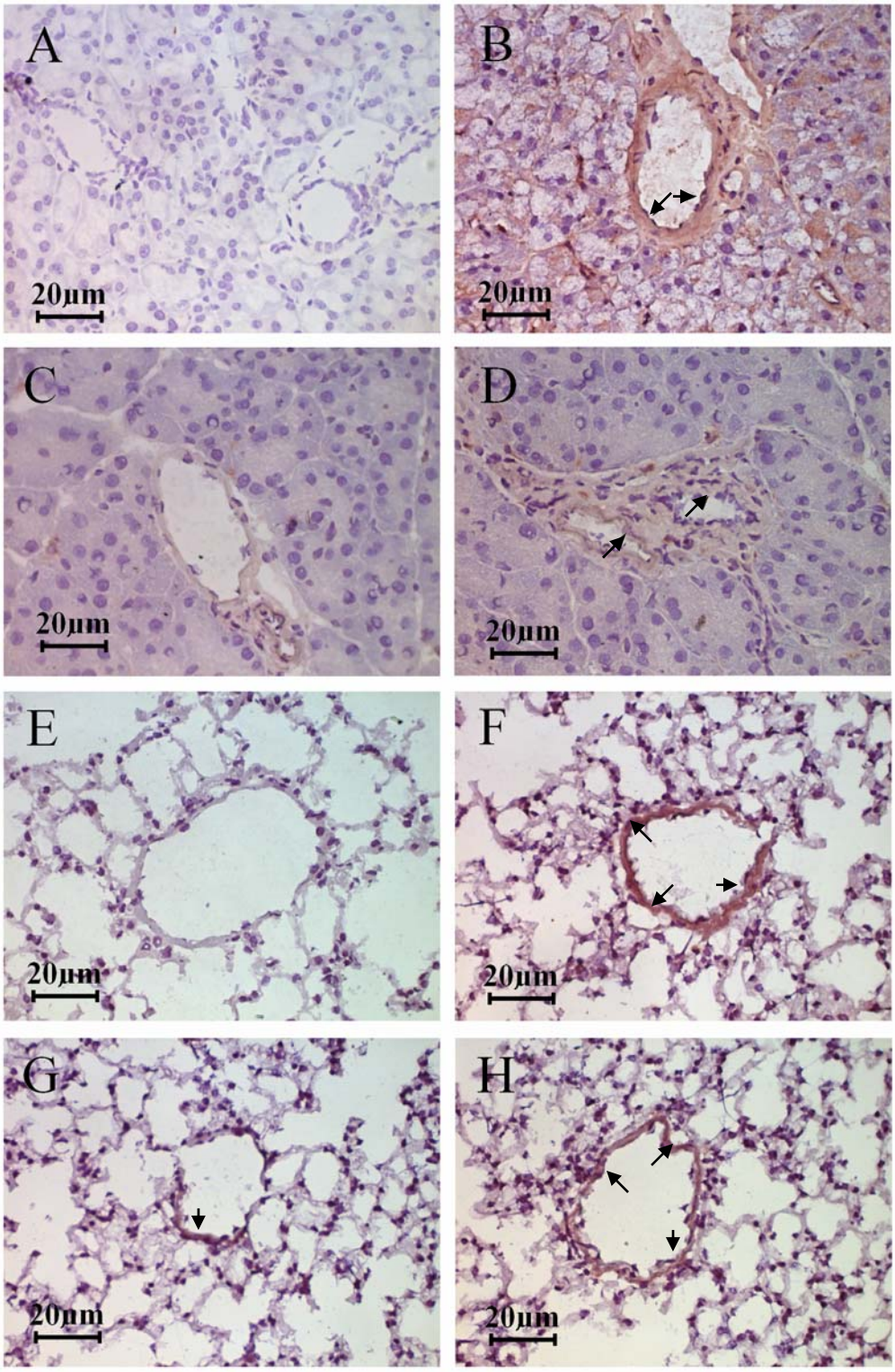


Figure 4.6 Immunohistochemistry staining of pancreas (A-D) and lung (E-H) sections (7µm) using polyclonal anti-E-selectin antibody. (A and E) Saline only; (B and F) 10 consecutive hourly caerulein (50µg/kg) injections; (C and G) CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; (D and H) CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Scale bars = 20µm.

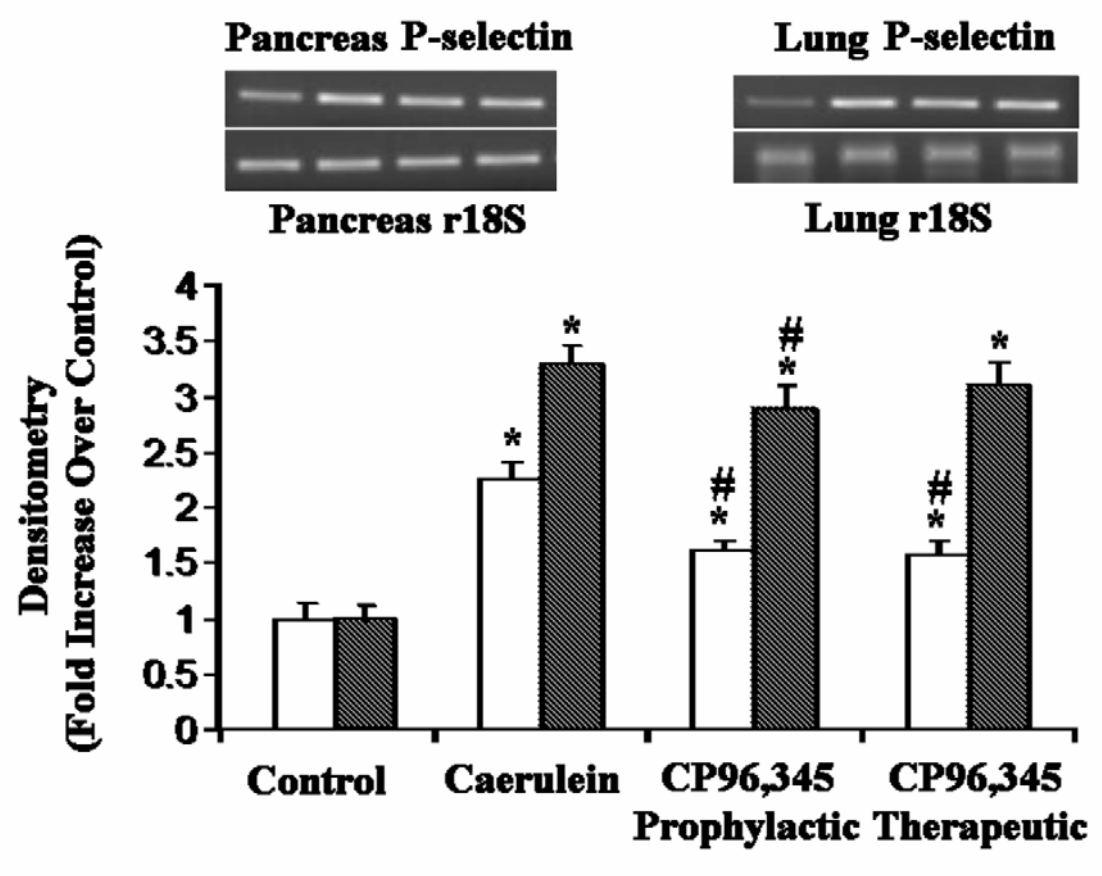


Figure 4.7 P-selectin mRNA expression in the pancreas (hollow bars) and lung (shaded bars) of mice with AP, treated with prophylactic/ therapeutic CP96,345. Control: Saline only; Caerulein: 10 consecutive hourly caerulein (50µg/kg) injections; CP96,345 Prophylactic: CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; CP96,345 Therapeutic: CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Bar charts represent mean value ± SEM of at least 8 animals.

*** indicates significantly different from the Control group ($P < 0.05$).**

 # indicates significantly different from the Caerulein group ($P < 0.05$).

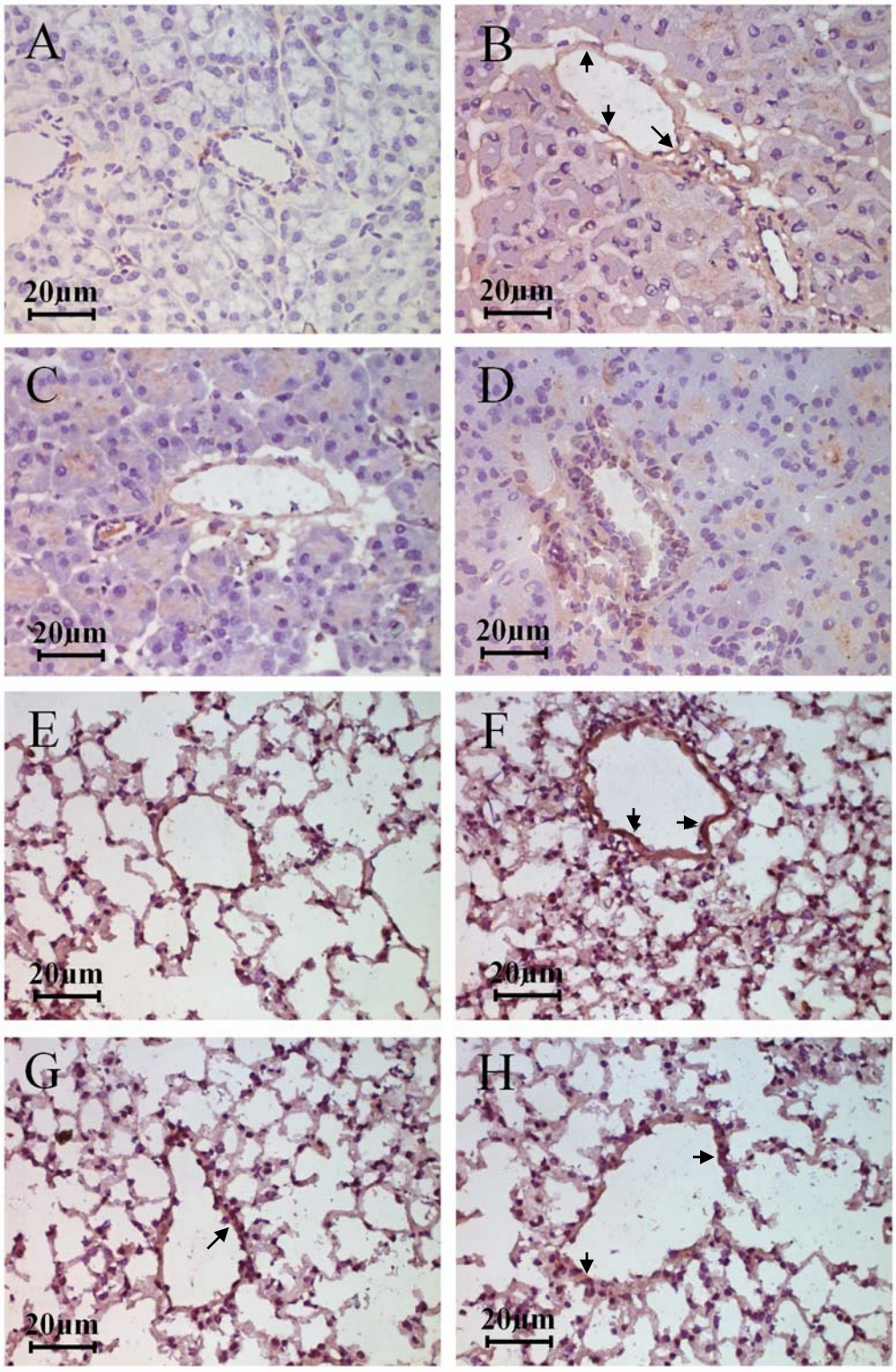


Figure 4.8 Immunohistochemistry staining of pancreas (A-D) and lung (E-H) sections (5µm) using polyclonal anti-P-selectin antibody. (A and E) Saline only; (B and F) 10 consecutive hourly caerulein (50µg/kg) injections; (C and G) CP96,345 (2.5mg/kg) 30 minutes before caerulein injections; (D and H) CP96,345 (2.5mg/kg) 60 minutes after first caerulein injection. Scale bars = 20µm.

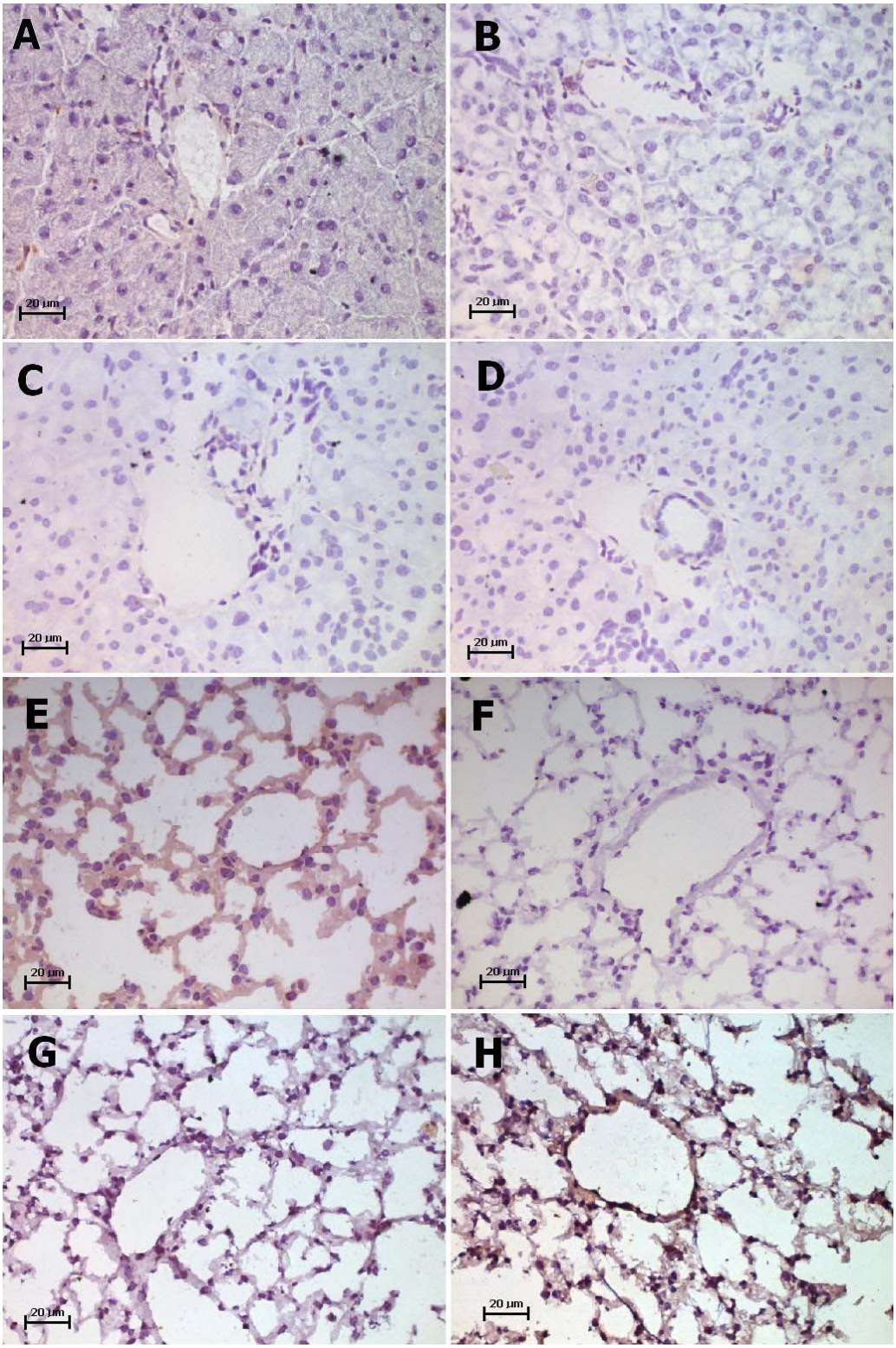


Figure 4.9 Negative controls slides of immunohistochemistry staining of pancreas (A-D) and lung (E-H) sections (5µm) in the absence of primary antibody. (A and E) absence of anti-ICAM-1 antibody; (B and F) absence of anti-VCAM-1 antibody; (C and G) absence of anti-E-selectin antibody; (D and H) absence of anti-P-selectin antibody. Scale bars = 20µm.

4.4 DISCUSSION

The recruitment of leukocytes to the site of tissue injury is an important component of inflammation. It is achieved by a complex interaction between adhesion molecules under the influence of various chemoattractants. This multi-step process involves leukocyte rolling, adhesion and emigration on the endothelium to reach the site of tissue injury. It is believed that leukocyte rolling is mediated by selectins (Rosen, 1993) and leukocyte adhesion is regulated by integrins (van der Flier and Sonnenberg, 2001). Since the treatment with CP96,345, an NK1R specific antagonist has been effective in reducing the severity of AP (Chapter 2 and Chapter 3), it is likely that the treatment with the NK1R antagonist may affect the regulation of integrin-associated ligands and selectins. Therefore, we investigated the effect of NK1R blockade on the expression of integrin-associated ligands (ICAM-1 and VCAM-1) and selectins (E-selectin and P-selectin) in the pancreas and lung during AP.

In the pancreas, ICAM-1, VCAM-1, E-selectin and P-selectin mRNA expression were upregulated during caerulein-induced AP. Previous studies have demonstrated similar findings (Genovese et al., 2006; Liu et al., 2005; Cuzzocrea et al., 2003). The mRNA expression of ICAM-1 and VCAM-1 were not affected by the antagonist treatment. However, their immunoreactivity appeared to be reduced with the antagonist treatment. ICAM-1 and VCAM-1 expression are known to be associated with the severity of organ injury in animal models of AP (Lundberg et al., 2000b; Frossard et al., 1999; Werner et al., 1999). ICAM-1, in particular, plays a crucial part in neutrophil adhesion to the endothelium (Lorant et al., 1991; Wetheimer et al., 1992). On the other hand, both prophylactic and therapeutic treatment with CP96,345

significantly reduced the mRNA and apparent protein expression of E- and P-selectins, with prophylactic administration of the antagonist producing a stronger effect. Selectins have been identified as important markers of AP (Miskovitz, 2001; Powell et al., 2001). E-selectin level has been shown to correlate well with the degree of organ dysfunction in AP patients, while P-selectin level is significantly higher in the non-survivors (Powell et al., 2001). Together, these results may imply that the blockade of NK1R has significant role on leukocyte rolling and adherence in pancreatic microcirculation. Therefore, it is clear that CP96,345 treatment is effective in preventing the progression of the disease by interfering with the expression of these pro-inflammatory molecules.

One of the major causes of death in AP patients is lung injury that clinically manifests as acute respiratory distress syndrome (ARDS). Therefore, the effect of CP96,345 treatment on the expression of the four adhesion molecules in the lungs were investigated. ICAM-1, E-selection and P-selectin mRNA and apparent protein expression were significantly elevated during AP. The treatment with the NK1R antagonist effectively reduced the elevated levels, with prophylactic administration of the compound offering a stronger effect. However, pulmonary VCAM-1 expression was not affected during AP and CP96,345 administration. It has been shown in a previous study that the treatment with NK1R receptor antagonist, SR140333, did not affect the VCAM-1 levels in the lungs in rat model of airway inflammation (Kang et al., 2002). Evidence of a differential regulation of the neurokinin system in pancreas and lung in caerulein-induced AP has been generated in Chapter 3. The results of the present study provide further evidence of the differential regulation of the

inflammatory responses in pancreas and lung in AP, especially in terms of the role of different adhesion molecules in the pancreas and lung.

In conclusion, these findings have shown that the NK1R antagonist, CP96,345, is effective in reducing pancreatitic and pulmonary expression of adhesion molecules implicated in AP. Therefore, NK1R blockade represents a promising therapeutic approach to the treatment of AP. Besides, these results have also demonstrated a differential regulation of the expression of adhesion molecules in the pancreas and the lung in AP.

CHAPTER 5

EFFECT OF CP96,345 ON LEUKOCYTE RECRUITMENT IN AP

5.1 INTRODUCTION

The recruitment of leukocytes from the circulation is an important process that occurs during inflammation. The trafficking of leukocytes cross the endothelium is divided into several distinct steps that are tightly regulated. This multi-step paradigm was originally proposed by Butcher (Butcher, 1991) and further elaborated on by Springer (Springer, 1994). The accumulation of leukocytes in an inflamed tissue is a result of the trans-endothelial migration cascade activated by the adhesive interactions between leukocytes and endothelial cells. During this process, the transient weak interaction between adhesion molecules on leukocytes and the endothelium will allow the leukocytes to slow down and roll along the endothelial surface. This is followed by the firm adherence of leukocytes onto the endothelial cells when the leukocytes come into close proximity with the endothelium. Upon the activation by chemotactic cytokines, leukocytes emigrate out of the vasculature and respond to the direction-specific stimuli that guide them to the inflammatory source (Foxman et al., 1997; van Buul and Hordijk, 2004).

In 1929, a German pharmacologist Philipp Ellinoer and a German anatomist, August Hirt, invented a modified fluorescence microscopy method to examine fluorescent-labeled structures in specimens from most living organs. The instrument constructed was later given the name - "intravital microscope", and it was considered as the first

epi-fluoresence (or incident-light excitation) microscope (Ellinoer and Hirt, 1929; Ellinoer and Hirt, 1932). Since then, intravital fluorescence video-microscopy has been established as a versatile technique for the study of leukocyte-endothelium interactions and blood flow at the level of the microcirculatory unit using fluorescent markers such as rhodamine 6G (Rh6G), which selectively attach to circulating leukocytes (Lehr et al. 1993a; Lehr et al. 1993b; Menger et al. 1992a,b).

In this chapter, I investigated the effect of 10 hourly injections of caerulein on the leukocyte-endothelium interaction in pancreatic post-capillary venules during AP, using Rhodamine 6G and an intravital microscope. It was found that the treatment with the NK1R antagonist effectively suppressed the recruitment of leukocytes into inflamed pancreatitic tissue.

5.2 MATERIALS AND METHODS

5.2.1 Materials

Rh6G was purchased from Sigma-Aldrich Chemicals USA (St. Louis, Mo). The mixture of ketamine and medetomindine was obtained from the Animal Holding Unit, National University of Singapore. Other reagents and chemicals were of analytical grade.

5.2.2 Induction of AP

AP was induced in mice as described in Section 2.2.2. One hour after the last caerulein injection animals were anaesthetized by a dose of i.p. ketamine and medetomindine mixuture (56mg/kg ketamine and 0.75mg/kg medetomindine). All experiments were approved by the Animal Research Ethics Committee of National University of Singapore and performed in accordance with established International Guiding Principles for Animal Research. Immediately after the examination of the pancreatic microcirculation, the animals (n=8 per treatment group) were euthanized by cervical dislocation.

5.2.3 Evaluation of pancreatic microcirculation

Microvascular changes of the pancreas during AP were assessed by intravital microscopy as described previously with modifications (Hartwig et al., 2000). For the *in vivo* staining of leukocytes, 0.01% w/v Rh6G was freshly prepared in 0.9% saline.

Briefly, after 10 hourly-caerulein injections, animals were given 0.1ml of the Rh6G solution through tail intravenous injection, 10 minutes before the start of the microcirculation examination. Anaesthetized animals were placed on a special anatomically constructed heating plate in order to maintain their body temperature at 37°C ± 1°C (Fig 5.1). A midline laparotomy was performed on the mice and the pancreas with the duodenal loop was gently exteriorized in a horizontal position. The organ was then kept hydrated by bathing it in an immersion chamber maintained at 37°C ± 1°C with Ringers solution (sodium chloride, 118mM; potassium chloride, 4.7mM; sodium bicarbonate, 25mM; calcium chloride, 2.5mM; magnesium sulphate, 1.2mM; glucose 10mM; EDTA 0.026mM) (Figure 5.1). The pancreas was gently laid flat and placed in between two glass slides that exert minimum pressure on the tissue while reducing the movements/vibrations due to breathing.

5.2.4 Intravital microscope system

The pancreatic microcirculation was examined under a fluorescence video-microscopy system (Eclipse PhysioStation E600FN, Nikon Corporation, Japan) with a xenon lamp, attached to a filter system for epi-illumination (Green light excitation$_\lambda$ =525nm, emission$_\lambda$ = 555nm). The pancreatic microcirculation was observed with nCFI Fluor ×20W water immersion objective lens (Nikon Corporation, Japan). Microscopic images were captured via CoolSNAP HQ monochrome cooled CCD video camera (Roper Scientific Inc., USA) and recorded onto a personal computer for subsequent offline analysis. This playback analysis of video recordings was performed in a blinded fashion using a computer-assisted microcirculation analysis program, Metamorph™ Imaging System (Molecular Devices Corporation, USA).

Single unbranched post-capillary venules with diameters ranging from 18 to 40 μm in three different areas on the same pancreas were investigated. Venular diameter was measured online using a video caliper in the computer program.

5.2.5 Offline video images evaluation

According to their interaction with the endothelial lining, the numbers of rolling and adherent neutrophils were determined offline during playbacks of the recorded images. Rolling neutrophils (rollers) were defined as those leukocytes that moved at a velocity less than two-thirds of that of the majority of the cells in the centerline of the venule. Leukocyte rolling velocity was determined from the time required for a leukocyte to traverse a given distance along the length of the venule. For leukocyte velocity measurements, the distance through which a labeled leukocyte traveled within two subsequent video frames was divided by the known video frame time interval. The mean leukocyte velocity expressed as μm/second in each venule was calculated by averaging the velocity of 10 to 15 leukocytes in the same venule. In addition, the mean white blood cell velocity in each area was calculated by averaging the mean velocity of the three areas in the same pancreas. A neutrophil was considered to be adherent (stickers) to venular endothelium if it did not move or detach from the endothelium within the 30-second observation period and were expressed as the number of cells per 100 μm of the vessel segment studied (Lehr et al., 1993a,b; Menger et al., 1992a,b)

5.2.6 Data analysis method

The data are expressed as mean ± SEM. In all figures, vertical bars denote the SEM and the absence of such bars indicates that the SEM is too small to illustrate. The significance of changes was evaluated by analysis of variance (ANOVA) when comparing three or more groups. If ANOVA indicated a significant difference, the data were analyzed by using Tukey's method as a post hoc test for the difference between groups. A P-value less than 0.05 was considered to indicate a significant difference. All statistical analyses were performed using SPSS version 14.0 for Windows (Chicago, Illinois USA).

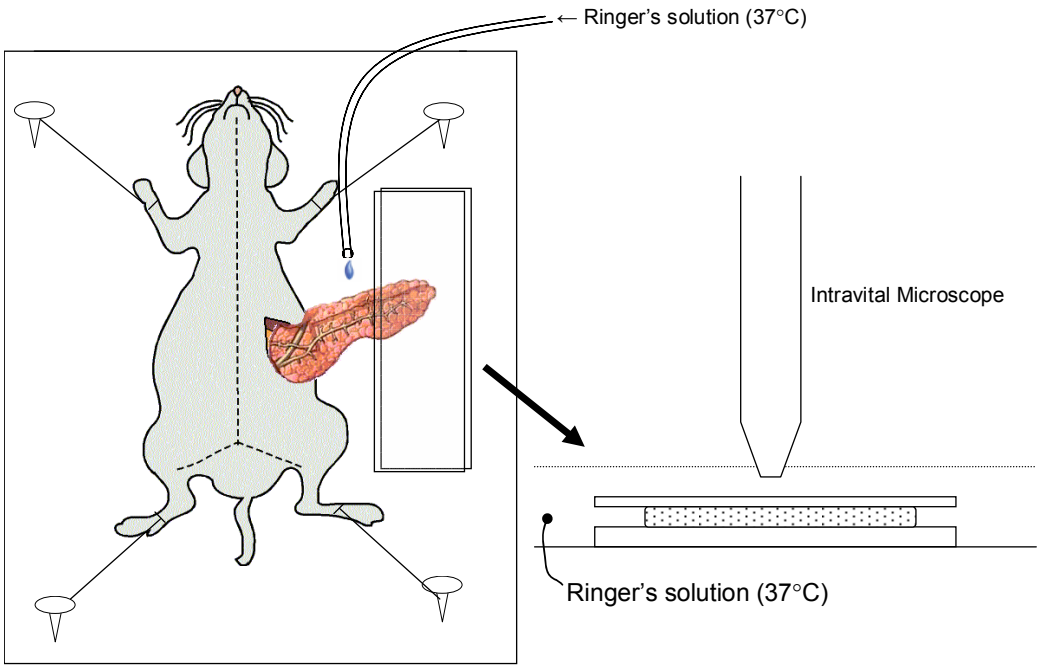


Figure 5.1 Examination of the pancreatic microcirculation in mice using intravital microscope.

5.3 RESULTS

5.3.1 Effect of CP96,345 treatment on leukocyte activation in the pancreas in AP

After 10 hourly caerulein injections, clear morphological characteristics of acute pancreatitis were observed. In the post-capillary venules of the pancreas, the number of rolling leukocytes increased by more than 25 times as compared to the saline treated animals (Table 5.1). In addition, these leukocytes were rolling at a significantly slower speed (2.5-fold) compared to the control group (Table 5.1). These changes increased the chance of leukocyte-endothelium interaction, and as a result, more leukocytes adhered to the endothelium of the blood vessel (Lehr et al., 1993a).

Animals treated with CP96,345 showed a reduced degree of inflammation in the pancreas. Treatments with CP96,345 effectively suppressed the pro-inflammatory behaviour of the leukocytes, with both prophylactic and therapeutic treatments producing similar effect on leukocyte-endothelium interaction. The number of rolling leukocytes in both groups was reduced to about 50% of that of the caerulein treated group. Additionally, the rolling velocity was normalized by blockade of NK1R (Table 5.1). Figure 5.2 shows the images of a section of a post-capillary venule taken at two different time points (3 seconds apart).

Parameters	Control	Caerulein	CP96,345 Prophylactic	CP96,345 Therapeutic
Rolling Leukocytes Flux (cells/minute)	9.13±2.15	232.50±11.50[a]	102.00±12.42[a,b]	132.75±10.48[a,b]
Rolling Velocity (μm/s)	21.53±1.09	10.56±1.21[a]	25.32±6.08[b]	23.88±3.92[b]
Adherent Leukocytes (cells/100μm)	0.33±0.21	7.00±1.46[a]	3.67±0.33[a,b]	4.67±0.91[a,b]

[a] indicates significantly different from the Control group ($P < 0.05$).

[b] indicates significantly different from the Caerulein group ($P < 0.05$).

Table 5.1: Leukocytes rolling and adherent properties in post-capillary venules during AP and the effect of CP96,345 (n=8).

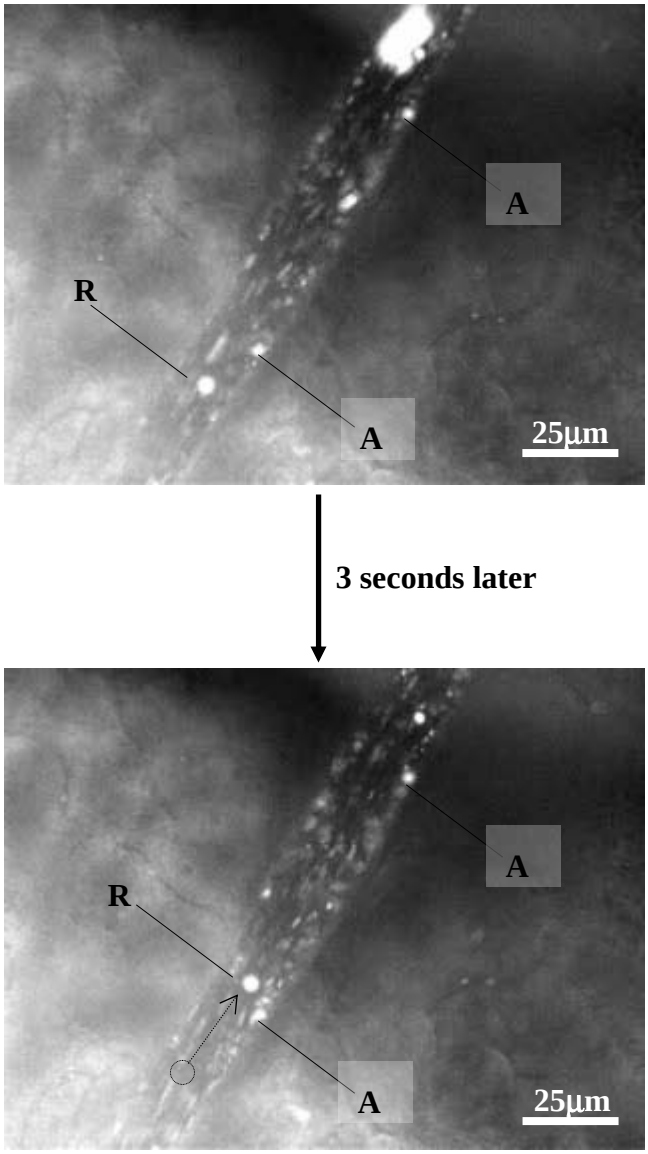


Figure 5.2 Post-capillary venule in the pancreas of an AP-mouse observed under intravital microscope at two different time points. Leukocytes were labeled using Rh6G. Rolling leukocytes (R) and adherent leukocytes (A) were classified and counted according to the pre-defined criteria. Two images were taken 3 seconds apart. The arrow in the lower image represents the movement path of the rolling leukocyte in the 3 minutes interval.

5.4 DISCUSSION

Intravital microscopy has been regarded as a valuable tool for the study of *in vivo* leukocyte behaviours during inflammation (Sumen et al., 2004; Broide and Sriramarao, 2001; Kubes and Ward, 2000; Ley K, 1996). The method has been used in the past to study the pancreatic microcirculatory impairment in experimentally induced AP (Zhou et al., 2002; Foitzik et al., 2002; Hartwig et al., 2000; Foitzik et al., 1999; Yamauchi et al., 1996). In the present study, this method was employed to visualize the effect of the NK1R antagonist on leukocyte-endothelium interaction in pancreatic microcirculation *in vivo*, by labeling the leukocytes using fluorescent Rh6G.

After 10 hourly caerulein injections, the mice developed severe AP. Real-time investigation revealed that the number of rolling leukocytes in the pancreatic post-capillary venules increased more than 25 times as compared to the saline treated animals (Table 5.1). These leukocytes rolled on the endothelial surface at a velocity less than two-thirds of that of the majority of the cells. Besides, they were rolling at a significantly slower speed and therefore had higher chance to interact and adhere to the endothelium. CP96,345 treatment significantly reduced the number of rolling leukocytes and restored the rolling speed to normal. This result correlates well with the reduction in neutrophils infiltration in antagonist treated animals described in Chapter 2 (section 2.3.1, MPO activity). Although the number of adherent leukocytes was also lowered by almost half in the antagonist treated groups, the reduction was approximately proportionate to the smaller number of rolling leukocytes. These results could suggest that NK1R signaling has a more pronounced effect on leukocyte

rolling than on leukocyte adhesion. Similar observation was noticed in Chapter 4 where the inhibition of SP receptor had a larger effect on the adhesion molecules responsible for rolling (E- and P-selectins; Lay K, 1989; Butcher EC, 1991; Springer TA, 1994; Lay and Tedder, 1995) than those related to adhesion (ICAM-1 and VCAM-1; Crook et al., 2002; Tamatani and Miyasaka, 1990; van der Flier and Sonnenberg, 2001).

The highly regulated process of leukocyte recruitment involves the participation of a wide range of adhesion proteins and signaling molecules. Comparing the present results with the adhesion molecule expression study in Chapter 4, there is an apparent discrepancy between the increase in the number of rolling leucocytes (observed in intravital microscopy) and the increase in the adhesion molecules expression (measured using immunohistochemistry and RT-PCR). A possible explaination for this observation is that the protective effect of CP96,345 is mediated by the change in the regulation of a number of adhesion molecules, and ICAM-1, VCAM-1, E-selectin and P-selectin, are only a few of the major contributors of the recruitment process. Indeed, SP has been shown to affect the levels of other adhesion molecules such as lymphocyte function-associated antigen-1 (LFA-1) (Reinke EK et al., 2006; Kang BN et al., 2004), integrin alpha-5 (Nakamura M et al., 1998) and complement receptor-associated OKM1 molecule (Dianzani C et al., 2003). The role and regulation of these molecules are being studied in other inflammatory conditions, and further investigation of the role of these molecules in AP is required.

In conclusion, this data once again demonstrates the effectiveness of a NK1R antagonist in the treatment of experimentally-induced AP in mice. The increase in

leukocyte-endothelium interaction (rolling and adhesion) could be significantly suppressed by the blockade of NK1R with CP96,345.

CHAPTER 6

GENERAL DISCUSSION

The involvement of the sensory nervous system in inflammation was first described more than a century ago (Bayliss WM, 1901). It was however, not until the 1960s when scientists re-visited the idea of NI and discovered the use of capsaicin to block NI (Chapman and Goodell, 1964; Jancso et al., 1967). In 1975, a group of Swedish scientists demonstrated the localisation of SP in some sensory nerves by immunohistochemical techniques (Hökfelt et al., 1975a; Hökfelt et al., 1975b). Subsequently, SP was shown to increase Evan-blue dye leakage from the circulation of rats (Chahl LA, 1977) and sensory nerves that innervate the pancreas were found to be SP-immunoreactive (Larsson LI, 1979). Clinical investigations have described the changes in gastrodoudenal mucosa and pancreas SP levels in patients with the chronic form of pancreatitis (Domschke et al., 1988; Buchler et al., 1992). However, the clear connection between SP and AP was first described only recently using NK1R knockout mice (Bhatia et al., 1998a).

While the importance of SP and NK1R has been shown using genetically modified mice, it is also important to find out whether targeting the NK1R in normal animals using a specific receptor antagonist will produce a beneficial effect. Beside NK1R, it is also useful to understand the possible roles of other TK peptides and their receptors in AP. Therefore, the effect of pharmacological intervention against the neurokinin receptors on pancreatic and lung injuries in AP in mice was investigated, using CP95,345, GR159897 and SB-222200, the specific antagonists of NK1R, NK2R and

NK3R respectively. AP induced in mice by hyperstimulation of caerulein responded positively to prophylactic and therapeutic treatments with CP96,345, as shown by lowered plasma amylase levels and tissue myeloperoxidase (MPO) activity. In addition, pulmonary injury caused by the systemic inflammatory response associated with AP was also abolished by the inhibition of NK1R. This has again demonstrated the important role of SP and NK1R in the development of AP. On the other hand, the blockade of NK2R and NK3R did not have any protective action. These results have eliminated the possible involvement of NKA-NK2R and NKB-NK3R systems in AP. A previous study, using the Evans blue and Monastral blue dye-leakage method, reported a protective effect of treatment with a NK1R antagonist, but not with NK2R and NK3R antagonists, in caerulein-induce AP in rats (Grady et al., 2000).

The changes in gene expression of inflammatory mediators have been observed in most inflammatory diseases, including AP (Iovanna JL, 1996; Keim et al., 1994). Therefore, it is crucial to recognize these changes in order to understand how the body reacts to the pathological condition. This has led me to investigate the effect of AP on the expression of neurokinin receptors and TK, since the activation of NK1R by SP has been shown to aggravate AP. It is known that the PPT-A gene codes for SP (Patacchini et al., 2004) and PPT-C codes for HK-1 (Zhang et al., 2000): both peptides act on NK1R. My results show that the PPT-A (pancreas and lungs) and PPT-C (lungs only) mRNA expression were upregulated during AP. In addition, using ELISA, an increase in SP concentrations in the pancreas and the lungs were also evident. HK-1 ELISA has not been described due to a lack of a specific antibody against HK-1 that does not cross react with SP. Since the release of SP (and probably HK-1) would worsen AP, this positive feedback mechanism is likely to be responsible

for the extended activation of NK1R. The treatment with CP96,345 suppressed the expression of PPT-A and PPT-C mRNA expression.

Since the early 1970s, scientists have been investigating the cellular physiology of acinar cells isolated from the pancreas using collagenase digestion (Amsterdam and Jamieson, 1972; Amsterdam and Jamieson, 1974). This has facilitated the study of the effects of various biological and chemical agents on the function and cellular signalling at the level of individual cells. Since then, many improved versions of the method have been reported and the protocol described by Oilver has been most widely employed (Oliver, 1980). By using this technique, isolated pancreatic acinar cells have been shown to simulate some of the events found in the *in vivo* model of AP in the presence of caerulein, (Leach et al., 1991). In this work, I have made use of the isolated acinar cells to study the effect of NK1R antagonism on the changes that occur during caerulein-induced AP. The biphasic dose-response relationship of caerulein on the release of amylase from acinar cells demonstrated in Chapter 3 has supported the reported existence of high affinity and low affinity CCK binding sites (Sankaran et al., 1982; Sankaran et al., 1980; Honda et al., 1987; Sato et al., 1989; Stark et al., 1989). At the supramaximal concentration (activation of the low affinity binding site), caerulein would cause the inhibition of enzyme secretion and AP (Niederau et al., 1994), which could be overcome by the pre-treatment of CP96,345. Besides, in order to compare the findings observed in *in vivo* animal model of AP (Chapter 3), the effect of caerulein, SP and CP96,345, when given alone or in combinations, on the expression of PPT-A and NK1R mRNA in acinar cells was also investigated. Elevation of PPT-A mRNA expression was observed as soon as 1 hour after caerulein and/or SP treatment which could be abolished by 30 minutes pre-

incubation of CP96,345. This is in agreement with the previous data. On the other hand, minimal change in NK1R mRNA expression was noted in the presence of caerulein. One explanation for the difference seen between the *in vivo* and the *in vitro* results is that the regulation of NK1R expression is a slow process, which requires more time before significant change to be detected.

I went on to investigate the changes in the expression of NK1R, NK2R and NK3R expression in AP and the effect of CP96,345 treatment on those changes. NK1R mRNA (real-time PCR) and possibly protein (immunohistochemistry staining) expression were elevated during AP in both pancreas and lungs. A similar pattern of NK1R immunoreactivity was observed previously (Bhatia et al, 1998a). This has represented an important mechanism that might facilitate the magnification of the initial signal of AP. Again, NK1R antagonist treatment resulted in significant reduction in NK1R mRNA and possibly protein expression. On the other hand, NK2R receptor mRNA expression was upregulated in the pancreas but downregulated in the lungs. Administration of CP96,345 has provided data that are not easily explainable: NK2R mRNA expression in pancreas was further upregulated, while the expression in lungs was reversed back to normal. The significance of these findings is not clear and requires further investigation, which is beyond the scope of this thesis. However, this points to a differential regulation of inflammatory response in the lungs and challenges the classical theory that equals pulmonary inflammation as an extension of pancreatitic inflammation. NK3R expression was not detected in both pancreas and lungs. Limited or negligible NK3R expression in peripheral tissues has been reported previously (Tsuchida et al., 1990; Buell et al., 1992).

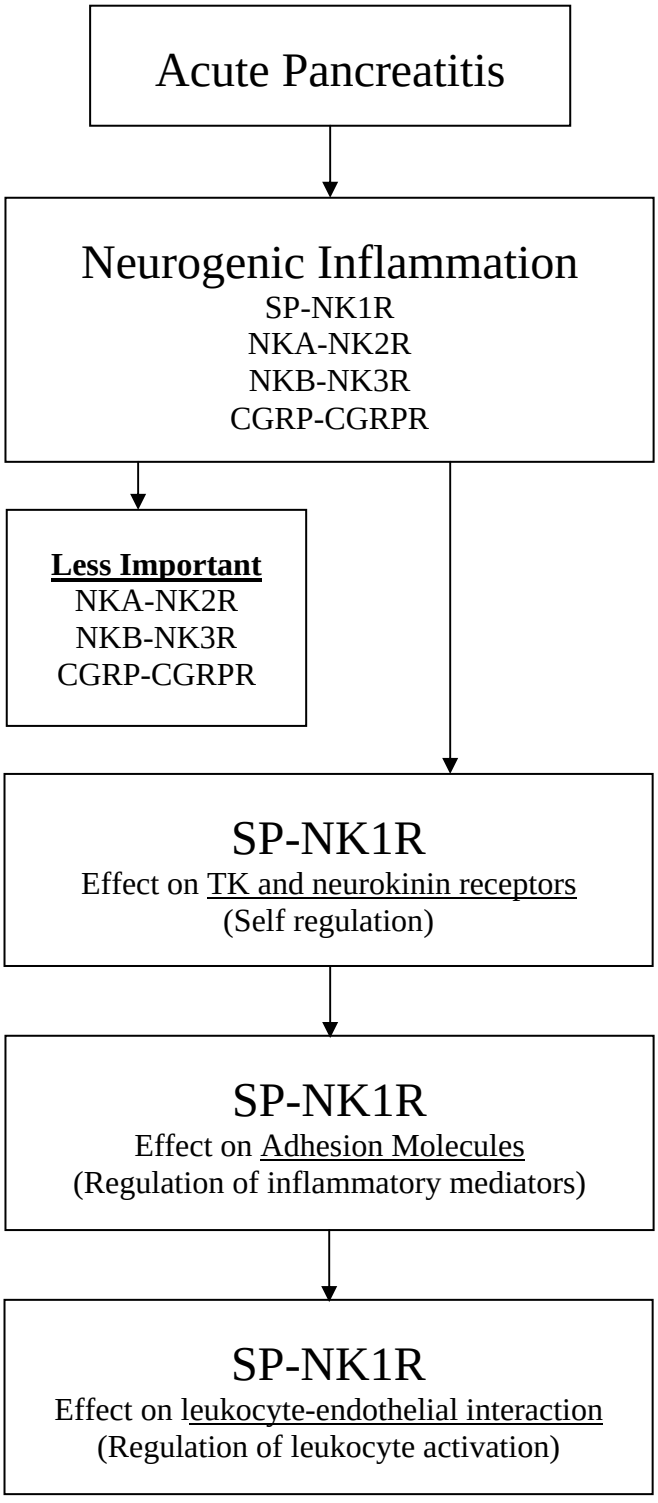


Figure 6.1 Summary of investigated events.

Leukocyte recruitment to the site of injury is a characteristic event during AP that consists of a series of events to bring about extravasation of leukocytes from the circulation. It requires the interaction between adhesion molecules that present on both the leukocytes and the endothelial cells under the influence of chemoattractants. Since the treatment with CP96,345 antagonist has demonstrated the protective effect against severe AP (Chapter 2 and 3), I postulated that NK1R antagonist may also influence the regulation of various adhesion molecules and affect the mode of leukocyte-endothelial interaction. Four well-known adhesion molecules from the immunoglobulin superfamily (ICAM-1 and VCAM-1) and the selectins family (E-selectin and P-selectin) were investigated. My findings have shown that, except for VCAM-1 in the lungs, all four adhesion molecules were upregulated in the pancreas and the lungs during AP. These results have served as a validation of our methods, since similar observations have been described previously (Lundberg et al., 2000a; Sunamura et al., 1999; Lundberg et al., 2000b; Uhlmann et al., 2001; Kang et al., 2002). Using these validated techniques, I have demonstrated that the treatment of CP96,345 has significantly suppressed the elevated selectins levels and had little effect on the expression of ICAM-1 and VCAM-1 in the pancreas. In the lungs, VCAM-1 level was not affected during AP and the treatment of NK1R antagonist inhibited the increase in ICAM-1, E- and P-selectins expression caused by AP-associated lungs injury.

Although the data from PCR and immunohistochemistry staining have provided invaluable information on the regulation of a few important adhesion molecules, it remained difficult to predict the outcome of the complex and not well-understood mechanism of leukocyte-endothelial interaction. However, the invention of the epi-

fluorescent intravital microscopy method has enabled scientists to examine samples in living animals under a microscope (Ellinger and Hirt, 1929; Ellinger and Hirt, 1932). In recent years, intravital microscopy has enabled study of leukocyte recruitment to the site of inflammation (Heit et al., 2006; Beck et al., 2007). Using Rh6G to label the circulating leukocytes, I have successfully visualized the leukocyte-endothelial interaction in the pancreatic post-capillary venules in AP with intravital microscopy. It was interesting to find that the number of rolling and adherent leukocytes increased significantly after 10 hourly injections of caerulein as compared to the normal animal. Besides, the average velocity of the rolling leukocytes was nearly half of that in saline treated animals, indicating a strong leukocyte-endothelial interaction. As expected, with the treatment of NK1R antagonist, significant reduction in rolling and adherent leukocytes was observed, and the rolling velocity was reversed to the normal level. However, even though the adherent leukocytes were reduced by 50% in the antagonist treated groups, it was roughly in proportion to the decrease in rolling leukocytes. This has indirectly suggested that NK1R inhibition has more significant influence on leukocyte rolling than on leukocyte adherence. These data were in line with my mRNA expression and immunohistochemistry findings where the blockade of NK1R had a larger effect on the adhesion molecules responsible for rolling (E- and P-selectins; Lay K, 1989; Butcher EC, 1991; Springer TA, 1994; Lay and Tedder, 1995) than those related to adhesion (ICAM-1 and VCAM-1; Crook et al., 2002; Tamatani and Miyasaka, 1990; van der Flier and Sonnenberg, 2001).

By comparing the difference in pancreatic and pulmonary expression of neurokinin receptors, TK and adhesion molecules, I have several lines of evidence showing a differential regulation of inflammatory response in the pancreas and the lungs. This

evidence includes (A) the upregulation of normally absent NK2R in the pancreas and the downregulation of NK2R in the lungs; (B) the upregulation of pulmonary PPT-C mRNA expression in AP compared to the absence of the mRNA in the pancreas; (C) the suppression of the elevated ICAM-1 in the lungs by CP96,345 which was not observed in the pancreas in AP; and (D) the upregulation of VCAM-1 in the pancreas and not in the lungs in AP. This is the first evidence of the presence of a differential pattern of regulation in the inflammation in the pancreas and the lungs in AP.

In summary, I have demonstrated that the prognosis of AP and associated lung injury can be improved by targeting the SP-NK1R system, the most well-known component of NI. We have successfully established the relationship between the NK1R activation and the expression of various genes and proteins that are related to the disease progression. The use of isolated pancreatic acinar cells has provided insight into the events that occur at the level of acinar cell in AP. In addition, we have also visualized the events of leukocyte recruitment using the intravital microscopy. Differential regulation of gene and protein expression in the pancreas and the lungs in AP has been observed, which could be due to the different mechanism of the inflammatory responses (neurogenic versus non-neurogenic) in the two organs. A better understanding of the role of NI in the pathogenesis of AP and associated lung injury will help in the development of new therapeutic options for the treatment of this life-threatening condition.

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