REGULATION OF SUBSTANCE P AND NEUROKININ-1 RECEPTOR EXPRESSION IN A MOUSE MODEL OF ACUTE PANCREATITIS

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DECLARATION

I hereby declare that the thesis is my original work and it has been written by me in its entirety. I have duly acknowledged all the sources of information which have been used in the thesis.
This thesis has also not been submitted for any degree in any university previously.

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Koh Yung Hua
25 June 2012

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SUMMARY

The neuropeptide substance P (SP) has been identified as a key pro-inflammatory mediator in experimental acute pancreatitis (AP). SP is a product of the *preprotachykinin-A* (*PPTA*) gene, and it binds mainly to neurokinin-1 receptor (NK1R). SP and NK1R were previously detected in isolated pancreatic acinar cells, and up-regulation of pancreatic SP/NK1R was observed upon induction of AP in mice. Despite this knowledge, mechanisms that regulate the expression of SP and NK1R in AP remain elusive. In this thesis, possible mechanisms that caused SP/NK1R up-regulation after induction of AP were examined using both *in vitro* and *in vivo* murine models of AP.

The effect of caerulein, a cholecystokinin analogue, on SP/NK1R expression in isolated pancreatic acinar cells was first investigated. In these cells, both gene and protein expression of SP/NK1R responded to supraphysiological concentrations of caerulein (10^{-7}M). The effect of caerulein on SP up-regulation could be blocked by pre-treatment of a CCK_A receptor antagonist, devazepide. Caerulein also induced the phosphorylation of several downstream signaling kinases, which include PKCα, PKCδ, ERK1/2 and JNK. Caerulein also induced DNA-binding activity of transcription factors AP-1 and NF-κB. With the use of specific signaling molecule inhibitors, we identified that caerulein up-regulated the expression of SP/NK1R via a PKCα/PKCδ – JNK/ERK1/2 – NF-κB/AP-1 dependent pathway.

Apart from caerulein, it was found that activation of NK1R by SP (10^{-6}M) or GR73,632, a selective NK1R agonist, significantly increased gene and protein expression of SP/NK1R in murine pancreatic acinar cells. These effects were abolished by pre-treatment of a selective NK1R antagonist, CP96,345. Pre-treatment

with specific inhibitors of PKCα, PKCδ, ERK1/2, JNK and NF-κB significantly inhibited SP-induced up-regulation of SP/NK1R. Therefore, activation of NK1R may up-regulate the expression of SP/NK1R through mechanisms similar to those induced by caerulein. The findings also suggest a possible auto-regulatory mechanism on SP/NK1R expression, which might contribute to elevated SP bioavailability.

A third mechanism that explained increased SP levels was described using a mouse model of caerulein-induced AP. Caerulein suppressed neutral endopeptidase (NEP) activity and protein expression, which caused diminished degradation of SP. The role of NEP in AP was examined in two opposite ways. Further inhibition of NEP activity by pre-treatment with phosphoramidon or thiorphan raised SP levels, and exacerbated AP-induced inflammation in mice. Meanwhile, the severity of AP, determined by histological examination, tissue water content, myeloperoxidase activity and plasma amylase activity, was markedly decreased in mice that received exogenous NEP treatment. Our results suggest that NEP has a protective effect in AP, mainly by suppressing the pro-inflammatory activity of SP.

In summary, the present study described three different mechanisms that might regulate the expression of SP and NK1R in caerulein-induced AP. Caerulein can directly up-regulate the expression of SP and NK1R through CCK_A receptor–PKCα/PKCδ - ERK/JNK- NF-κB/AP-1 dependant pathway. Activation of NK1R also elevated SP/NK1R expression in murine pancreatic acinar cells, forming a positive feedback loop that enables further expression of SP/NK1R. Furthermore, a decrease in SP degradation, as shown by decreased NEP activity, may also contribute to elevated SP-NK1R interaction by increasing SP bioavailability.

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ABBREVIATIONS

AP	Acute pancreatitis
AP-1	Activator protein-1
BSA	Bovine serum albumin
Cae	Caerulein
CCK	Cholecystokinin
cDNA	Complementary deoxyribose nucleic acid
DMSO	Dimethyl sulfoxide
ELISA	Enzyme-linked immunosorbent assay
ERK	Extracellular signal regulated kinase
GPCR	G-protein-coupled receptor
HEPES	*N*-2-hydroxyethylpiperazine-*N*'-2- ethanesulfonic acid
HPRT	Hypoxantine-guanine phosphoribosyl transferase
ICAM	Intracellular adhesion molecule
IL-	Interleukin
IκB	I kappa B
i.p.	Intraperitoneal
i.v.	Intravenous
JNK	c-Jun N-terminal kinase
MAPK	Mitogen activated protein kinase
MCP	Monocyte chemoattractant protein
MEK	Mitogen-activated protein kinase Kinase
MIP	Macrophage inflammatory protein
MPO	Myeloperoxidase
mRNA	Messenger ribose nucleic acid
NEP	Neutral endopeptidase

NF-κB	Nuclear factor kappa B
NK1R	Neurokinin-1 receptor
NKA	Neurokinin A
NKB	Neurokinin B
PBS	Phosphate buffered saline
PBST	0.05% Tween-20 in PBS
PKC	Protein kinase C
PLC	Phospholipase C
PPTA	*Preprotachykinin-A* gene
PCR	Polymerase chain reaction
RIPA	Radio-immunoprecipitation assay
SEM	Standard error of the mean
SIRS	Systemic inflammatory response syndrome
SP	Substance P
TNF	Tumor necrosis factor
TRPV1	Transient receptor potential vanilloid 1
VCAM	Vascular cell adhesion molecule

PUBLICATIONS

Original reports

1. **Koh YH**, Tamizhselvi R, Bhatia M. Extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase, through nuclear factor-kappaB and activator protein-1, contribute to caerulein-induced expression of substance P and neurokinin-1 receptors in pancreatic acinar cells. *J Pharmacol Exp Ther.* 2010; 332(3):940-8.
2. **Koh YH**, Tamizhselvi R, Moochhala SM, Bian JS, Bhatia M. Role of Protein Kinase C in Caerulein Induced Expression of Substance P and Neurokinin-1-Receptors in Murine Pancreatic Acinar Cells. *J Cell Mol Med.* 2011; 15(10):2139-49.
3. **Koh YH**, Moochhala SM, Bhatia M. Activation of Neurokinin-1 receptors Up-regulates Substance P and Neurokinin-1 receptor in Murine Pancreatic Acinar Cells. *J Cell Mol Med.* 2011; doi: 10.1111/j.1582-4934.2011.01475.x.
4. **Koh YH**. Moochhala SM, Bhatia M. The Role of Neutral Endopeptidase in Caerulein-Induced Acute Pancreatitis. *J Immunol.* 2011;187(10):5429-39.
5. Tamizhselvi R, Sun J, **Koh YH**, Bhatia M. Effect of hydrogen sulfide on the phosphatidylinositol 3-kinase-protein kinase B pathway and on caerulein-induced cytokine production in isolated mouse pancreatic acinar cells. *J Pharmacol Exp Ther.* 2009; 329(3):1166-77.
6. Tamizhselvi R, **Koh YH**, Sun J, Zhang H, Bhatia M. Hydrogen sulfide induces ICAM-1 expression and neutrophil adhesion to caerulein-treated pancreatic acinar cells through NF-kappaB and Src-family kinases pathway. *Exp Cell Res.* 2010; 316(9):1625-36.
7. Hegde A, **Koh YH**, Moochhala SM, Bhatia M. Neurokinin-1 receptor antagonist treatment in polymicrobial sepsis: molecular insights. *Int J Inflam.* 2010; 2010:601098.
8. Tamizhselvi R, Shrivastava P, **Koh YH**, Zhang H, Bhatia M. Preprotachykinin-A gene deletion regulates hydrogen sulfide-induced toll-like receptor 4 signaling pathway in cerulein-treated pancreatic acinar cells. *Pancreas.* 2011; 40(3):444-52.

Webpage reviews

1. **Koh YH**, Bhatia M. (2011). Substance P (SP). The Pancreapedia: Exocrine Pancreas Knowledge Base, DOI: 10.3998/panc.2011.23

International conference presentations

1. **Koh YH**, Moochhala SM, Bian JS, Bhatia M. Protective effects of neutral endopeptidase in mouse model of caerulein-induced acute pancreatitis. The 3rd EMBO meeting, 2011, Vienna, Austria.

CHAPTER 1 INTRODUCTION

1.1 GENERAL OVERVIEW

The pancreas is an oblong-shaped organ that has both endocrine and exocrine functions. The function of endocrine pancreas is well known, as it is responsible for secreting vital hormones such as insulin, glucagon and somatostatin, which regulate blood sugar and appetite. Irregularities of the endocrine pancreas' function are associated with diabetes and blood sugar disorders. Endocrine pancreas consists of Islets of Langerhans, and is interspersed throughout the pancreas, contributing to only 2-3% of the total pancreatic mass (Brannon, 1990). On the other hand, the exocrine pancreas contains clusters of enzyme-producing cells called the pancreatic acinar cells. Pancreatic acinar cells, along with pancreatic duct cells and other minor exocrine-related cell types, form up to more than 90% of the total pancreatic mass. Pancreatic acinar cells produce large amount of proteases, lipases and amylase that are secreted into the small intestine to aid digestion. These powerful digestive enzymes are produced by the pancreas as an inactive molecule called zymogens, and activated in the small intestine by enteropeptidases. These digestive enzymes can damage tissue when activated. Therefore, in the pancreas, a number of inhibitors are responsible to repress their activation before reaching the small intestine.

1.2 ACUTE PANCREATITIS

1.2.1 Etiology and epidemiology of acute pancreatitis

Inflammation is an immunological response characterized by redness, swelling, heat, and pain localized to a tissue. A rapid and prominent increase in pancreatic inflammation is a hall-mark of acute pancreatitis (AP). A majority of AP cases can be attributed to gallstones and alcohol abuse, making up for more than 80% of total cases (Sakorafas and Tsiotou, 2000; Gullo et al., 2002). Other less encountered causes of AP include drug use, endoscopic retrograde cholangio-pancreatography, hyperlipidemia, trauma, viral infection, and autoimmune diseases. Cigaratte smoke has also recently been idenfied as a risk factor of AP, and its effects may be synergistic with alcohol consumption (Alexandre et al., 2011). Despite better knowledge on the pathogenesis of AP, up to 10% of cases remain idiopathic. It is notable that although a number of situations can cause AP in humans, only a small fraction of patients with these predisposing factors develop the disease. There is no clear association between gender and the occurrence of AP.

AP is a fairly common clinical disorder. In the United States, approximately 210,000 patients seek treatment for AP annually, placing a huge burden of more than USD2.2 billion in hospitalization costs (Fagenholz et al., 2007). In this study, blacks and the elderly were reported to have a higher incidence rate of AP, while whites and Hispanics have a relatively lower risk (Fagenholz et al., 2007). In another Swedish study, the incidence was reported to be about 38 per 100,000, which is similar to the average of Americans (Appelros and Borgstrom, 1999). In recent years, the incidence rate continued to rise at a rapid rate, widely believed to be caused by increasingly fatty diet and increased alcohol consumption (Yadav and Lowenfels, 2006). The

incidence rate in Asian population is reportedly lower than in the western world, but a similar uptrend in incidence rate was also observed.

1.2.2 Mild vs. severe acute pancreatitis

Patients with AP are roughly divided into two categories, mild AP and severe AP. Mild AP usually consists of interstitial edematous pancreatitis, where damage is limited within the pancreas and requires minimal medical attention. Mild AP usually recovers within a week without further complications. On the other hand, a necrotizing pancreatitis usually results in a severe form of AP. The initial assault deteriorates into pancreatic necrosis and the exaggerated inflammation sometimes cause systemic complications, resulting in multi-organ injury and a much higher mortality rate than observed with mild disease. Regardless of the severity, there is no correlation between the different etiologies and the severity of AP.

As the outcome differs greatly between mild AP and severe AP, effective identification of AP and differentiating the severity during point of admission is important in determining the treatment required. As there is no single biological marker that accurately diagnose AP, initial diagnosis is based on the presence of at least 2 or 3 features, which include abdominal pain, increased serum amylase/lipase/trypsin levels and imaging tests (Smotkin and Tenner, 2002). Amylase is normally produced in the pancreas and salivary glands, and is responsible for digesting carbohydrates. During acute pancreatic injury, plasma amylase levels may rise up to 10 times above the normal level, and recover to normal levels within a week. However, the use of serum amylase alone does not offer sufficient sensitivity and

specificity. A previous study reported that plasma amylase levels occasionally remain at basal levels even in severe AP (Orebaugh, 1994). Further, the levels of serum amylase or lipase do not correspond to disease severity (Lankisch et al., 1999). Detection of serum lipase levels are often used in conjunction with serum amylase levels for initial assessment of AP. This fat digesting enzyme is also produced in the pancreas and an injury to the pancreas acutely raises serum lipase levels and peaks within 24 hours. Serum lipase levels reportedly offers better selectivity and sensitivity than amylase levels (Orebaugh, 1994; Smith et al., 2005). A serum trypsin level test is thought to be the most sensitive blood test for pancreatitis, although it is still not widely available and routinely used.

Enzyme assays alone cannot accurately assess the severity or cause of AP. After initial diagnosis of AP using serum enzyme activity tests, a series of physiological parameters should be taken and assessed for severity. These include a complete blood count, measurement of blood glucose, C-reactive protein and calcium levels, determination of liver function (including bilirubin and liver enzymes). Furthermore, imaging methods such as magnetic resonance cholangiopancreatography and computed tomography scans are used to observe abnormalities in the abdomen. These collected parameters are then used to evaluate for severity using Ranson's score (Ranson et al., 1974), Acute Physiology and Chronic Health Evaluation II (APACHE II) score (Larvin and McMahon, 1989), or modified Glasgow Coma score (Williams and Simms, 1999). Among these scoring systems, the APACHE II scoring system reportedly has a better prediction value for severe AP (Yeung et al., 2006; Gravante et al., 2009).

In about 80% of cases, patients suffer mild pancreatic edema and local pancreatic inflammation. Other cases, which consist of 20-30% of all patients in AP,

experience a severe attack with a high mortality rate. In recent years, medical advances in critical care and management of AP patients have resulted in an improvement of outcome of AP. Despite this, the mortality rate remained persistently high for severe AP patients.

1.2.3 Pathophysiology of acute pancreatitis

Despite well-recognized etiologies of AP, the molecular mechanisms involved in the pathogenesis of AP remains incompletely understood. It is now commonly believed that AP originates from an injury in enzyme secreting pancreatic acinar cells. Inactive pancreatic zymogens are produced in pancreatic acinar cells and then secreted and eventually activated by enteropeptidases in the duodenum and small intestine. Abnormal activation of these digestive enzymes within the pancreas could cause a chain reaction that cause massive activation of zymogens within the pancreas, resulting in an injury to the organ and triggers a complex cascade of events (Bhatia et al., 2005; Hirota et al., 2006).

Activation of trypsinogen by the lysosomal hydrolase cathepsin B is now held to be an initiating event in acute pancreatitis. Lysosomal dysfunction also occurs in acute pancreatitis and appears to reduce the intracellular degradation of activated proteases (Halangk et al., 2000). Trypsin is a powerful protease that hydrolyses the C-terminal side of lysine or arginine residues in a peptide chain, except when either is followed by a proline residue. Trypsin is responsible for cleaving several other zymogens, which include pro-enzymes for phospholipase A_2, chymotrypsin and elastase in pancreatic acinar cells. Phospholipase A_2 and elastase are particularly harmful in terms of direct cell damage, as they are capable of breaking down the

cellular membranes and blood vessels respectively (Niederau et al., 1995). In addition, release of pancreatic lipase by injured cells causes lipolysis of adipocyte triglycerides, which ultimately exacerbates pancreatic damage (Navina et al., 2011). These powerful enzymes, when activated together, leads to auto-digestion of the pancreatic tissue and release of noxious products into the surrounding tissue and system (Grady et al., 1998; Gorelick and Otani, 1999). A massive activation of digestive enzymes also overwhelm the inhibitory mechanisms that keep enzyme activity in check, causing a reaction that tilts towards more destruction (Gorelick and Otani, 1999).

After the initial assault, the events within the pancreatic acinar cell follows an unpredictable path that either result in mild, local interstitial inflammation or severe necrosis. Pancreatic injury release chemokines that attract leukocytes, which in turn aggravates inflammation and further damage surrounding healthy tissue. Therefore, it is important to understand the factors and underlying mechanisms that determine the manifestation of AP.

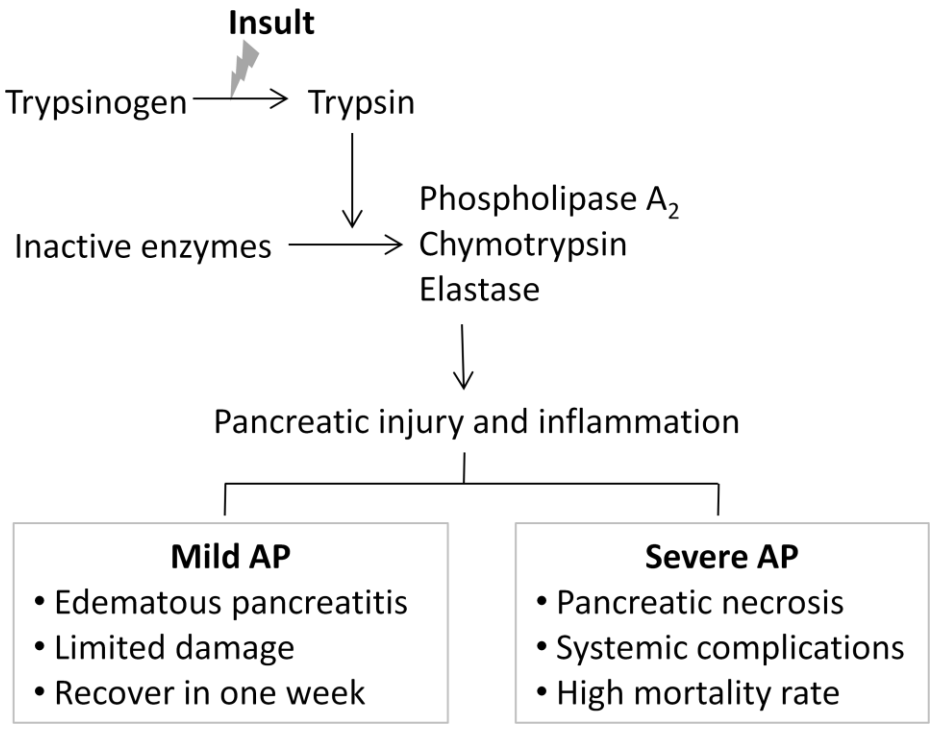

Figure 1.1 Schematic illustration of the pathogenesis of AP. An abnormal event causes activation of trypsin and subsequent activation of pancreatic digestive enzymes in pancreatic acinar cells. The resulting injury causes acute pancreatic inflammation. In severe cases, the exaggerated inflammation causes systemic complications accompanied with a high mortality rate.

1.2.4 Severe acute pancreatitis and pancreatitis associated distant organ injury

In severe AP, harmful substances such as activated pancreatic enzymes and reactive oxygen species can spill over to other organs through the cardiovascular system, causing systemic inflammation. Complications frequently manifest as necrosis and organ failure in the pulmonary system, cardiovascular system and renal system, but pancreatitis-associated lung injury is most commonly observed (Beger et al., 1997; Browne and Pitchumoni, 2006). Among these noxious substances released by pancreatic injury, elastase appeared to be one of the most detrimental substance responsible for lung damage (Day et al., 2005). The lungs contain an abundant amount of elastin, but the breakdown of elastins by elastase could severely affect pulmonary function. This leads to acute lung injury and eventually causes acute respiratory distress syndrome. In fact, decreased pulmonary function and early onset of pleural effusion is associated with a poor outcome of AP (Browne and Pitchumoni, 2006). Acute renal failure may ensue secondary to cardiovascular collapse and hypotension, resulting in acute tubular necrosis.

Most complications of AP resolve within the first two weeks of onset. If severe AP is not resolved within this period, secondary pancreatic infection by microbes may ensue. Bacterial infection of necrotic tissues is now known to have a very high rate of mortality, accounting for nearly 80 percent of deaths (Beger et al., 1997). Bacteriologic analysis of necrotic tissue revealed a higher proportion of gram-negative germs such as *Escherichia coli*, and also gram-positive bacteria and fungi

(Tsui et al., 2009). In such cases, bacterial infection causes excessive cytokine secretion into the bloodstream by infiltrating leukocytes, causing uncontrollable inflammation and sepsis.

1.2.5 Experimental models of acute pancreatitis

Despite medical advancements in the management of AP, there remains much to be understood about the underlying mechanisms of AP. Currently, studies of severe acute human pancreatitis can be performed only with restrictions and are tissue samples are relatively inaccessible. Therefore, experimental models of AP that resemble the human situation are important tools to help understand the disease and to devise better treatment options. To date, a variety of animal models have been developed to produce features that are similar to human AP cases.

Animal models of AP can generally be divided into two categories: Invasive models, which require surgery on experimental animals, and non-invasive models. These models produce AP that range from mild edematous pancreatitis to severe necrotizing pancreatitis, and they have proven to be valuable models to help understand the pathogenesis of AP.

1.2.5.1 Caerulein-induced acute pancreatitis

Caerulein is a short peptide with an amino acid sequence of Pglu-Gln-Asp-Tyr[SO_3H]-Thr-Gly-Trp-Met-Asp-Phe-NH_2. Originally isolated from the skin of an Australian frog (Anastasi et al., 1967), caerulein is a structural homolog of the endogenous hormone cholecystokinin (CCK). Caerulein/CCK is a ligand of cholecystokinin receptors CCK_A and CCK_B; where CCK_A receptors are predominantly located in the gastrointestinal tract and CCK_B receptors are primarily located in the central nervous system (Noble et al., 1999). The CCK_A receptors were

further found to contain a high-affinity binding state and a low-affinity binding state for its ligand. Physiological concentrations of CCK/caerulein stimulate enzyme secretion from the pancreatic acinar cells via stimulation of high-affinity CCK_A receptors (Dufresne et al., 2006). On the other hand, when a supraphysiological dose of CCK/caerulein is given to the animals, the activation of low-affinity CCK_A receptors lead to a distinctly different downstream signaling mechanism which blocked enzyme secretion from the pancreatic acinar cells (Saluja et al., 1989; Dufresne et al., 2006). The inability to secrete activated zymogens out of the pancreatic acinar cells will raise intracellular protease activities to a critical level that ultimately causes auto-activation of digestive enzymes within these cells (Saluja et al., 1985; Saito et al., 1987).

This feature of caerulein-induced AP has similarities with current knowledge on the pathogenesis of AP, where an abnormal activation of trypsinogen in pancreatic acinar cells caused subsequent inflammatory responses. Furthermore, treatment of animals with caerulein can induce many features that resemble clinical AP, which include hyperamylasemia, pancreatic edema, cellular necrosis, zymogen activation, and severe inflammation of the pancreas (Grady et al., 1998; Gorelick and Otani, 1999). Caerulein-induced AP is also highly reproducible, and it has been successfully shown to induce pancreatitis in different animals such as mice (Bhatia et al., 1998), rats (Wisner and Renner, 1988), rabbits (Klar et al., 1994), and dogs (Morita et al., 1998). Its convenience, reproducibility, and its non-invasive nature of induction of pancreatitis have encouraged its popularity in studying the pathogenesis of AP.

Animals that received caerulein treatment rapidly develop AP. Significant physiological changes occur rapidly within three hours after caerulein administration into animals, and the diseased state is most significant after twelve hours of infusion

(Lampel and Kern, 1977). Caerulein-induced AP also induces pulmonary damage in its later stages, which is useful to study pancreatitis-induced distant organ damage. The pancreas of caerulein-induced AP also exhibit similar histological presentations to those found in the early phase of AP in humans (Dabrowski et al., 1999). Interestingly, infusion of caerulein into animals for a prolonged period of time (>12 hours) did not cause further inflammation. Instead, it was found that pancreatic edema is largely reabsorbed after a 24 hour treatment, and the pancreas remained in an indurated state (Lampel and Kern, 1977; Adler et al., 1979). Therefore, one major drawback of caerulein-induced AP is that this model does not cause severe AP. Several important features of severe AP, such as hemorrhage and saponification of the lipids or death were not observed in caerulein-induced AP models. Finally, over stimulation of CCK receptors is not a recognized cause of human AP. Despite these drawbacks, caerulein-induced AP remains to be one of the most popular methods and the use of this method shaped the current knowledge of AP.

1.2.5.2 Diet-induced acute pancreatitis

Mice fed with a diet deficient in choline and supplemented with ethionine (CDE-diet) develop severe AP (Lombardi et al., 1975). The toxic effects of ethionine caused intraparenchymal activation of zymogens, and the toxic effects were greatly potentiated by a diet deficient in choline. Besides massive inflammation and cellular necrosis in the pancreas, diseased mice also develop injuries characterized by hemorrhage and fat necrosis throughout the peritoneal cavity (Lombardi et al., 1975). Therefore, this experimental model exhibits similar characteristics with human AP cases with acute hemorrhagic pancreatitis and fat necrosis. Interestingly, the outcome of CDE-diet model depends greatly on gender (Lombardi and Rao, 1975). Female mice died within four to five days after being fed with a CDE-diet, but male mice

develop AP that has inconsistent severity and a much lowered mortality rate. Therefore in female mice, the CDE-diet induced AP model is a cheap, highly reproducible and non-invasive alternative to the milder caerulein-induced model, which is suitable for studying of hemorrhagic AP and mortality studies.

1.2.5.3 Amino acid (L-arginine and L-ornithine) induced acute pancreatitis

Arginine is essential for protein synthesis and L-arginine is one of the most common natural amino acids. It was first observed in 1984 that injection of excess arginine into rats caused pancreatic acinar cell injury, without affecting the Islet of Langerhans (Mizunuma et al., 1984). The exact mechanisms by which arginine initiates AP is still unclear, but the involvement of reactive oxygen species (Czako et al., 1998) and nitric oxide (Takacs et al., 2002) are potential mechanisms that lead to the progression of AP. This model of AP is characterized by acute pancreatic inflammation, pancreatic edema, infiltration of leukocytes and capillary dilatation. Mortality rate is relatively low, averaging 2.5% in rat models (Hegyi et al., 2004). Interestingly, it is possible to modify the severity of AP by changing the dose of arginine administered to the animals (Hegyi et al., 2004). Animals that receive a larger dose of arginine is used for studies on the mechanisms of severe AP, and a smaller dose of arginine causes mild AP that could be used to characterize the regenerative processes of AP. Long term administration of arginine was also performed to study the mechanisms of chronic pancreatitis (Weaver et al., 1994).

Administration of excess L-ornithine to animals was also recently proposed to induce severe AP (Rakonczay et al., 2008a). L-ornithine is a metabolic product of L-arginine and is physiologically produced as an intermediate molecule in the urea cycle. Similar to L-arginine induced AP, administration of exogenous L-ornithine to rats caused massive interstitial edema, apoptosis/necrosis of acinar cells and

infiltration of neutrophil granulocytes. It was reported that L-ornithine induced a more severe form of pancreatitis, when compared with L-arginine induced models (Rakonczay et al., 2008a). However, the underlying mechanisms that leads to this difference remains to be investigated.

1.2.5.4 Pancreatic duct ligation induced acute pancreatitis

Since gallstone obstruction of the pancreatic ducts is a major risk factor of AP, the pancreatic duct ligation model is perhaps one of the more clinically relevant model available for studying the pathogenesis of AP. Pancreatic duct ligation was successfully applied in opossums (Lerch et al., 1993), mice (Samuel et al.) and rats (Walker, 1987), with the opossums consistently produce a particularly severe form of AP. Pancreatic juices containing zymogens are normally secreted to the digestive tract through a common biliopancreatic channel. An obstruction of pancreatic outflow through pancreatic duct ligation causes accumulation of pancreatic zymogens within the pancreas. The end result is an auto-activation of zymogens that initiate pancreatic injury and inflammation. Despite the clinical relevance, the pancreatic duct ligation model is technically difficult to perform, and also yields inconsistent results in the mice and rat models.

1.2.5.5 Alcohol-induced acute pancreatitis

Although excessive alcohol consumption is a well known risk factor of AP, chronic alcohol feeding alone failed to induce AP in animal models (Schneider et al., 2002). Prior sensitization or additional procedures is required to produce a significant alcohol-induced injury to the pancreas. When rats were co-treated with alcohol and CCK, these animals exhibited pathological changes that resemble AP (Pandol et al., 2003). Alcohol-induced AP can also be triggered by direct infusion of ethanol into the pancreatic duct (Schneider et al., 2002). However, the invasive nature of this model

would result in pancreatic injury and contribute to AP. Because of these observations, ethanol was thought to sensitize the pancreas to injury and inflammatory responses, rather than being a triggering factor itself (Pandol et al., 2003). Ethanol-induced AP models exhibit pathological features that are common to all AP models, which include zymogen activation and decreased pancreatic blood flow (Schneider et al., 2002).

Since alcohol consumption is considered a major risk factor for AP, these models provided a valuable tool to investigate the pathogenesis of AP in a more clinical relevant way.

1.2.5.6 Pancreatic duct infusion model

Various compounds have been infused into the pancreatic duct to induce AP. Infusion of a bile salt, sodium taurocholate, is most commonly performed in such experiments (Aho et al., 1980; Wittel et al., 2008). This model requires invasive surgery of the animal, and then the bile salts are injected into the pancreatic duct via a retrograde manner. After retrograde infusion, a severe, rapidly evolving and lethal variety of acute hemorrhagic pancreatitis is formed. However, the pancreatic duct infusion model is technically challenging as extra care is required to maintain a standard degree of injection pressure and surgery quality.

1.2.5.7 Concluding remarks

There has been great progress on uncovering the underlying mechanisms that dictate the initiation, progression and recovery of AP by using these various animal models. However, none of the commonly used models exactly mimic the inciting event of human cases of pancreatitis. Nonetheless, the pathologic features of these models of AP are very similar.

In this study, we chose caerulein-induced AP as a model for a variety of reasons. Firstly, caerulein-induced AP produces pancreatic acinar cell changes and pulmonary injuries that resemble human AP cases. Furthermore, caerulein-induced AP is very reproducible and was considered non-invasive. Finally, caerulein-treated pancreatic acinar cells were widely recognized as a cellular model of AP. With other considerations such as costs and time, we believe that the use of caerulein-induced AP model could best achieve the aims of the proposed study.

1.2.6 Pancreatic acinar cells as an in vitro model of acute pancreatitis

Pancreatic acinar cells form the bulk of pancreatic mass, and they are responsible for synthesis, storage and secretion of digestive enzymes. Evidence from animal models of AP suggested that pancreatic injury originated from dysfunction of these enzyme producing cells. Premature activation of zymogens leads to massive inflammation that ensued. Thus, isolated pancreatic acinar cells are widely considered to be a valid model to investigate pathological changes in pancreatitis.

Caerulein treated pancreatic acinar cells is one of the best characterized cellular models of AP. Both isolated pancreatic acinar cells and the pancreas respond similarly when treated with CCK or its analogue caerulein. A low dose of CCK stimulates enzyme secretion from the pancreatic acinar cells. On the other hand, stimulation of these isolated cells with a high concentration of CCK/caerulein causes pathological changes that resemble the responses observed in animal models of AP (Thrower et al., 2008). For example, a supramaximal concentration of caerulein (10^{-7}M) causes intra-acinar cell activation of trypsinogen and increased trypsin activity (Hofbauer et al., 1998). Besides being the initiation site of injury in pancreatitis, pancreatic acinar cells also express cytokines and chemokines which might play a

role in inflammation. Treatment of isolated pancreatic acinar cells with caerulein caused up-regulation of noxious products which include pro-inflammatory interleukins (IL-1, IL-6) and reactive oxygen species, which (Yu et al., 2002; Samuel et al., 2006). Therefore, the pancreatic acinar cells represent a well suited *in vitro* system to investigate the pathogenesis of AP and the underlying signaling mechanisms involved.

Two sources of pancreatic acinar cells were being used to investigate the pathophysiology of AP. The pancreatic acinar cell tumoral cell line, AR42J cells, proliferate rapidly and produce digestive enzymes in a way similar to normal pancreatic acinar cells. However, the regulation of exocrine function is different due to mutations in this tumoral cell line (Christophe, 1994). On the other hand, pancreatic acinar cells could be obtained from collagenase dispersion of the pancreas. Due to significant difficulties in culturing isolated pancreatic acinar cells for a prolonged period of time, freshly isolated cells are essential in order to study their functions.

1.3 SUBSTANCE P

Substance P (SP) is an undecapeptide with an amino acid sequence of Arg-Pro-Lys-Pro-Gln-Gln-Phe-Phe-Gly-Leu-Met-NH2. It was first isolated in 1931 by Ulf von Euler and John H. Gaddum, who described the "preparation P" as a substance that cause intestinal contraction and lowered blood pressure (US and Gaddum, 1931). However, it was 1971 when the amino acid sequence of SP was determined and the ligand successfully synthesized (Chang et al., 1971; Tregear et al., 1971). This breakthrough permitted a larger and more accurate research on the physiological functions of SP. Forty years has passed since the successful identification of SP, and it is now known to be an important molecule which is involved in a myriad of biological functions.

1.3.1 Tachykinin family of peptides

Since the successful identification of SP, subsequent research has identified several other peptides that share similarities with SP. This family of short peptides were named "tachykinins", which consist of SP, neurokinin A (NKA) (Kangawa et al., 1983; Nawa et al., 1984), neurokinin B (NKB) (Kanazawa et al., 1984; Kimura et al., 1984), and two elongated forms of NKA, neuropeptide K (Tatemoto et al., 1985) and neuropeptide γ (Kage et al., 1988). Within this family of peptides, the biological functions of SP were most extensively studied. Tachykinins are characterized by a common C-terminal sequence, Phe-X-Gly-Leu-Met-NH2, where X is either an aromatic or an aliphatic amino acid (Severini et al., 2002). Tachykinins are produced by three genes in mammals, namely preprotachykinin A (*PPTA*), preprotachykinin B (*PPTB*), and preprotachykinin C (*PPTC*). The preprotachykinin gene encodes long precursor proteins, which are then cleaved by proteases to yield smaller peptides.

Tachykinins are important neurotransmitters; therefore members of the tachykinin family were also known as neuropeptides. Tachykinins elicit a wide spectrum of physiological effects, with reports showed their implications on cardiovascular system, intestinal motility and secretions, respiratory system, urogenital tract, immune system, central nervous system, and nociception. In more recent reports, tachykinins were also reported to have significant effects on inflammation (Severini et al., 2002).

1.3.2 Sources and distribution of SP

SP is encoded by the *PPTA* gene. It is synthesized as a larger protein in the ribosomes, and then enzymatically converted to the much shorter but biologically active undecapeptide and stored (Hokfelt and Kuteeva, 2006). SP is highly expressed in the peripheral nervous system (Pickel et al., 1983) and central nervous system (McCarthy and Lawson, 1989), but also shown to be induced or expressed in other cell types such as monocytes (Ho et al., 1997), macrophages (Ho et al., 1997), lymphocytes (Lai et al., 1998), pancreatic acinar cells (Tamizhselvi et al., 2007), Leydig cells (Chiwakata et al., 1991) and various tumors (Esteban et al., 2009; Gonzalez-Moles et al., 2009). A number of studies indicate control plasma SP levels in humans fall in the range between 30pg/ml to 500pg/ml (Reynolds et al., 1988; Lee et al., 1997; Bondy et al., 2003). Neuronal sources, mainly non-myelinated C-fiber sensory nociceptive neurons, represent a primary source of SP release in the periphery. The stored SP is released from the nerve endings upon activation of transient receptor potential vanilloid 1 (TRPV1) located on the surface of these C-fibres. Although SP was widely known as a neurotransmitter, the role of SP in inflammation has gained attention in recent years.

1.3.3 Neurokinin-1 receptor (NK1R)

The biological actions of tachykinins are mediated by three distinct G-protein coupled receptors (GPCRs), NK1R, NK2R and NK3R. SP binds with high affinity to NK1R. SP can also bind and activate NK2R and NK3R, albeit with much less affinity and its physiological effects were less understood. NK1R is expressed in all major organs in the body, including pancreas and lungs (O'Connor et al., 2004). Depending on the cell type used, activated NK1R can couple to downstream Gq/11, Gαs and Gαo proteins (Nishimura et al., 1998; Roush and Kwatra, 1998), which in turn activates the phospholipase C (PLC) pathway or adenylate cyclase pathway. NK1R might also function as an auto-receptor, as SP was found to modulate its own release (Malcangio and Bowery, 1999; Holzer and Holzer-Petsche, 2001). This auto-regulation feature may be important in the pathophysiology of inflammation, nerve injury or noxious stimuli.

1.3.4 Pro-inflammatory effects of SP

The role of SP on inflammatory diseases has now become clearer. As the primary sensory nerve endings are a major source of SP release, an inflammatory response evoked by SP is popularly termed "neurogenic inflammation". SP-NK1R interaction was proposed as a major pro-inflammatory process and many studies have now shown that disruption of the SP-NK1R interaction helps reduce inflammation.

SP has been shown to play an early and important role in the inflammatory cascade and promote excessive activation of inflammatory cells. SP can activate NF-κB, a transcription factor that is known to control expression of pro-inflammatory cytokines and chemokines (Lieb et al., 1997; Sun et al., 2008). In T-lymphocytes,

neutrophils and macrophages, SP up-regulated the expression of several cytokines that are well known pro-inflammatory mediators, including tumor necrosis factor-alpha (TNF-α) interleukin 1-beta (IL-1), interleukin 2 (IL-2) and interleukin 6 (IL-6) (Delgado et al., 2003). SP also up-regulated chemokine receptor expression in primary mouse neutrophils (Sun et al., 2008), which might increase neutrophil responsiveness to the noxious compounds released from an inflamed site. On the other hand, SP also causes plasma extravasation, which contributes to localized edema in inflamed tissues (Figini et al., 1997).

Interestingly, elevated levels of both SP and NK1R were observed in inflammatory disease, which might increase SP-NK1R interaction and aggravate inflammation. An elevated expression of SP receptor binding sites has been observed in the inflamed colon of patients suffering from inflammatory bowel disease (Goode et al., 2000). Similarly, increased NK1R expression was observed in lymphoid aggregates, small blood vessels, and enteric neurons in Crohn's disease and a bacteria-induced model of colitis (Mantyh et al., 1995; Mantyh et al., 1996). On the other hand, elevated systemic SP levels have been reported in postoperative septic patients (Beer et al., 2002). In addition, infiltrating leukocytes and induced SP production in the inflamed site could also contribute to elevated SP levels in the local tissue during inflammation, thus representing a non-neuronal source of SP.

1.3.5 SP in acute pancreatitis

SP-NK1R interaction was shown to be a key mediator in the pathogenesis of experimental AP. The role of SP in AP and associated lung injury has been extensively studied. It was found that in wild type mice, SP and NK1R expression in the pancreas are both increased during caerulein-induced AP (Bhatia et al., 1998; Lau

and Bhatia, 2006). Genetic deletion of either *NK1R* or *PPTA* protected mice against experimental pancreatitis. This was demonstrated by a significant reduction of the magnitude of hyperamylasemia, neutrophil sequestration in the pancreas, and pancreatic acinar cell necrosis in *NK1R*$^{-/-}$ mice and *PPTA*$^{-/-}$ mice, when compared with their wild type controls. Moreover, pancreatitis associated lung injury was almost completely abolished when *NK1R* or *PPTA* were knocked out, as shown by reduced intrapulmonary sequestration of neutrophils and pulmonary microvascular permeability (Bhatia et al., 1998; Bhatia et al., 2003). Similar protective effects were also observed in CDE-diet induced hemorrhagic pancreatitis in NK1R$^{-/-}$ mice (Maa et al., 2000a). These results showed that *PPTA* gene products, as well as NK1R, are critical pro-inflammatory mediators in AP and the associated lung injury. SP-NK1R interaction is also a determinant of inflammatory edema in acute interstitial pancreatitis (Maa et al., 2000b). Furthermore, mice treated with CP96,345, a specific NK1R antagonist, either prophylactically or therapeutically, were significantly protected against caerulein-induced AP (Lau et al., 2005). These results point to a key role of SP-NK1R interaction in AP and associated lung injury.

Primary sensory neurons that innervate the tissues contain an abundance of neurotransmitters, including SP. TRPV1 channels located on these neurons, when activated, causes neuronal release of stored SP. It was demonstrated *in vivo* that capsazepine, a TRPV1 antagonist, significantly reduced inflammation and pancreatic injury in caerulein-induced AP (Nathan et al., 2001). On the other hand, activation of TRPV1 by capsaicin caused release of SP, and exaggerated caerulein-induced SP (Hutter et al., 2005). Pre-treatment of capsazepine or CP96,345 before administration of capsaicin showed reduced severity of SP, highlighting the importance of TRPV1 and NK1R (Hutter et al., 2005). High doses of Resiniferatoxin caused disruption of

the celiac ganglion and inhibited SP release, and showed protective effects against caerulein-induced pancreatitis in rats (Noble et al., 2006).

Besides pro-inflammatory effects of SP in AP, it also mediates nociception in animal models of AP. Induction of necrotizing pancreatitis by L-arginine caused a large increase in c-fos expressing spinal neurons, suggesting activation of nociceptive pathways. Intrathecal administration of SR140333, a specific NK1R antagonist, was shown to suppress pancreatitis pain (Wick et al., 2006). In another study, intraperitoneal injection of CP99,994, another specific NK1R antagonist, attenuated nociceptive behaviours in dibutyltin dichloride induced AP (Vera-Portocarrero and Westlund, 2004).

Table 1.1 Evidence of SP-NK1R interaction in the pathogenesis of AP.

	SP-NK1R interaction	Severity of AP	Reference
PPTA gene knockout	Decrease	Decrease	Bhatia et al., 2003
NK1R gene knockout	Decrease	Decrease	Bhatia et al., 1998 Maa et al., 2000
NK1R antagonist	Decrease	Decrease	Lau et al., 2005
TRPV1 antagonist	Decrease	Decrease	Nathan et al., 2001
TRPV1 agonist	Increase	Increase	Hutter et al., 2005
Disruption of celiac ganglion	Decrease	Decrease	Noble et al., 2006

1.3.6 Metabolism of SP

Compared to the well known pro-inflammatory effects of SP, the mechanisms that terminate its effects were lesser known. SP-NK1R interaction could be reduced by blocking with NK1R antagonists, but also by reducing SP bioavailability. Degradation of SP is one of the mechanisms that reduce SP levels in the system.

Currently, a few enzymes were implicated in the metabolism of SP, which include neutral endopeptidase (NEP), angiotensin-converting enzyme (ACE), dipeptidyl aminopeptidase IV, prolyl endopeptidase, cathepsin-D and cathepsin E (Harrison and Geppetti, 2001). However, only NEP and ACE were more commonly reported in the metabolism of SP *in vivo* (Harrison and Geppetti, 2001).

NEP, also known as enkephalinase, neprilysin, common acute lymphoblastic leukemia antigen (CALLA) or CD10, is a membrane-bound enzyme known to degrade a variety of short peptides in the extracellular fluid, including SP. NEP cleaves SP at Gln6-Phe7, Phe7-Phe8, and Gly9-Leu10, thereby preventing SP from binding to its receptor (Skidgel et al., 1984). NEP is also known to degrade amyloid beta, a protein that is best known as a molecule implicated in Alzheimer's disease.

NEP is capable of modulating inflammatory responses by degradation of SP. This is supported by studies showing that NEP knockout or inhibition potentiates inflammation, but was prevented by co-treatment with NK1R antagonists (Sturiale et al., 1999). On the other hand, administration of exogenous recombinant NEP to animals is protective against inflammatory disorders, such as intestinal inflammation and burns (Neely et al., 1996; Sturiale et al., 1999; Kirkwood et al., 2001). Current evidence supports that NEP plays an anti-inflammatory role.

ACE is a circulating enzyme that is most widely known for its role in the renin-angiotensin system. ACE inhibitors lower plasma angiotensin II levels and is widely applied as an anti-hypertensive drug. In comparison, the physiological role of ACE on SP is poorly understood. Purified ACE was found to inactivate SP by cleaving it at Phe8-Gly9 and Gly9-Leu10 position (Skidgel et al., 1984). Inhibition of ACE by captopril or enalapril potentiated SP-induced bronchoconstriction in guinea

pigs (Subissi et al., 1990). ACE was also found to play a part in SP-induced inflammation. In a model of ACE-knockout mice, an increased SP level was observed and subject animals were more susceptible to cutaneous inflammatory responses to allergens (Scholzen et al., 2003).

1.3.7 SP and NK1R in isolated pancreatic acinar cells

Expression of SP and NK1R has been detected in isolated pancreatic acinar cells. Their expression can be rapidly induced by caerulein, but its physiological significance is unclear (Tamizhselvi et al., 2007). On the other hand, exogenous SP is able to stimulate the expression of chemokines in isolated primary pancreatic acinar cells. Exposure of mouse pancreatic acini to SP significantly increased synthesis of monocyte chemotactic protein-1 (MCP-1), macrophage inflammatory protein-1 alpha (MIP-1α), as well as macrophage inflammatory protein-2 (MIP-2). The stimulatory effect of SP was mediated through the NF-κB pathway (Ramnath and Bhatia, 2006). SP, acting specifically through NK1R, induced the activation of protein kinase C-δ (PKCδ), ERK and JNK with a time-dependent manner in isolated pancreatic acinar cells (Ramnath et al., 2007; Ramnath et al., 2008). Inhibition of PKCδ, ERK or JNK with their respective specific inhibitors attenuated SP-induced chemokine production (Ramnath et al., 2007; Ramnath et al., 2008). More recently, a critical role of SRC family kinases (SFKs) has been demonstrated. SFKs mediated the activation of mitogen activated protein kinases (ERK, JNK), transcription factors (NF-κB, AP-1, STAT3), and production of chemokines in pancreatic acinar cells (Ramnath et al., 2009).

Besides playing a role in pancreatic acinar cell gene expression, SP might also be involved in pancreatic secretory mechanisms. In isolated guinea pig pancreatic

acinar cells, SP caused a twofold increase in amylase secretion (Patto et al., 1992). Experiments done on AR42J cells also showed evidence of increased exocrine secretion when these cells were treated with SP (Gorr and Tseng, 1995). On the other hand, SP inhibited bicarbonate secretion from guinea pig pancreatic ducts, probably by inhibition of a Cl^-/HCO_3 exchanger on the apical membrane of pancreatic duct cells (Hegyi et al., 2003).

1.3.8 Therapeutic options targeting SP-NK1R pathway

Although the pathological role of SP has been studied for over forty years, therapeutic options that target the SP-NK1R pathway are still very limited at this stage. Currently, the focus is on the development of clinically effective NK1R antagonist. Emend (also called Aprepitant or MK869), as a selective antagonist of NK1R, was the first drug of this class to be approved for clinical use in the United States (Patel and Lindley, 2003). Emend was described as an antiemetic, and being used as a prophylactic treatment for chemotherapy-induced emesis since its approval in 2003. Several NK1R antagonists underwent clinical trials for use in central nervous system disorders and irritable bowel syndrome, but further trials are needed to ensure their safety (Quartara and Altamura, 2006; Zakko et al., 2011). NK1R antagonists might also be a potential target for cancer therapy (Munoz et al., 2010).

Clinical results derived from the use of NK1R antagonists were promising. However, other mechanisms that interfere with SP-NK1R interaction, such as inhibition of SP release from nerve endings, have not gained sufficient attention to date. Therefore, there is still a lot of potential to develop therapeutic options that target the SP-NK1R pathway.

1.4 OBJECTIVES

At present, the mechanisms that propagate inflammation in AP are still incompletely understood. Evidence derived from animal models of AP suggested that SP-NK1R interaction may play an important role in the pathogenesis of AP. Both SP and NK1R were found to be expressed in murine pancreatic acinar cells (Tamizhselvi et al., 2007). In addition, their expression was up-regulated in the local inflamed tissue. An elevated SP and NK1R expression could potentially increase SP-NK1R interaction and contribute to exaggerated inflammation.

In light of this information, the focus of the present study is to investigate the expression of SP and NK1R in caerulein-induced AP models, and the underlying mechanisms involved. More specifically, by using a murine *in vitro* and *in vivo* AP model, this study sought to examine:

- Expression levels of SP and NK1R in caerulein-stimulated pancreatic acinar cells.
- The signaling mechanisms that mediate the expression of SP and NK1R in caerulein-stimulated pancreatic acinar cells.
- The expression and activity of NEP using both cellular and animal models of caerulein-induced AP.
- To determine how NEP activity affects SP expression and the outcome of AP in caerulein-induced AP.

CHAPTER 2: CAERULEIN UP-REGULATES SUBSTANCE P AND NEUROKININ-1 RECEPTORS IN MURINE PANCREATIC ACINAR CELLS

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Yung-Hua Koh, Ramasamy Tamizhselvi, and Madhav Bhatia. Extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase, through nuclear factor-kappaB and activator protein-1, contribute to caerulein-induced expression of substance P and neurokinin-1 receptors in pancreatic acinar cells. *J Pharmacol Exp Ther*. 2010; 332(3):940-8.

Yung-Hua Koh, Ramasamy Tamizhselvi, Shabbir Moochhala, Jin-Song Bian and Madhav Bhatia. Role of protein kinase C in caerulein induced expression of substance P and neurokinin-1-receptors in murine pancreatic acinar cells. *J Cell Mol Med*. 2011; 15(10):2139-49.

2.1 INTRODUCTION

It is getting clear that SP binding to NK1R is involved in pro-inflammatory effects of experimental pancreatitis. Despite the clear pathogenic role of SP in AP, mechanisms that regulate their expression levels were not clear during the course of AP. In a previous study, gene expression of *PPTA* and *NK1R* was detected in isolated pancreatic acinar cells (Tamizhselvi et al., 2007). Protein levels of SP were also detected in acinar cell lysates, using an ELISA based method. However, the effects of caerulein on pancreatic acinar cells were not thoroughly investigated. As pancreatic acinar cells represent the bulk of pancreatic mass, SP produced by these cells might contribute significantly to local availability of SP. Moreover, activation of NK1R was found to up-regulate chemokines, which include MIP-1α, MIP-2 and MCP-1, in pancreatic acinar cells(Ramnath and Bhatia, 2006). Therefore, we aim to investigate the effects of caerulein on mouse pancreatic acinar cells, to see its effect of the expression of SP and its receptor, NK1R.

2.2 MATERIALS AND METHODS

2.2.1 Animals and chemicals.

All experimental procedures were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Swiss mice (Male, 25-30 grams) were acclimatized in a controlled environment with an ambient temperature of 23^{o}C and a 12:12-hour light-dark cycle. Caerulein, a CCK analogue, was purchased from Bachem California (Torrance, CA) and dissolved in normal saline. The CCK_A receptor antagonist, devazepide, was purchased from Tocris Bioscience (Ellisville, MO) and dissolved in dimethyl sulfoxide (DMSO). Glucose,

N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), and soybean trypsin inhibitor were obtained from Sigma-Aldrich (St.Louis, MO). Type IV collagenase was purchased from Worthington (Freehold, NJ). All chemicals were purchased with the highest purity available.

2.2.2 Preparation of Pancreatic Acini.

Primary pancreatic acini were obtained from mouse pancreas by a collagenase dispersion method. Swiss mice were first euthanized by a lethal dose of sodium pentobarbitone (150mg/kg, i.p.). Fresh pancreas were infused with ***buffer A*** (mM: 140 NaCl, 4.7 KCl, 1.13$MgCl_2$, 1$CaCl_2$, 10 glucose, and 10 HEPES, and 0.5mg/ml soybean trypsin inhibitor, pH 7.3) containing 200IU/ml Type IV collagenase. The pancreas was then minced and placed in 12mL of ***buffer A*** containing 200IU/ml Type IV collagenase, and incubated in a shaking water bath for 10 minutes at 37ºC. To obtain dispersed acini, the digested tissue was passed through small pipette tips. The cells were then passed through a solution of 50mg/ml bovine serum albumin (Dissolved in ***buffer A***) and then washed twice with ***buffer A*** before further experiments. The viability of pancreatic acinar cells was determined by trypan blue exclusion assay. Cell preparations with at least 95% viability were used for further experiments.

2.2.3 Treatment of Pancreatic Acinar Cells.

Isolated pancreatic acinar cells were treated with caerulein. In concentration-dependent studies, cells were treated with caerulein at concentrations ranging from 10^{-12}M to 10^{-7}M for 60 minutes in a 37ºC water bath. To study the time-dependent effect of caerulein, pancreatic acinar cells were incubated with caerulein (10^{-7}M) for 0-120 minutes. For other experiments, cells were pre-treated with a selective CCK_A antagonist, devazapide, for 30 minutes before addition of caerulein (10^{-7}M).

2.2.4 Substance P extraction and detection.

SP was concentrated and detected according to manufacturer's instructions (Bachem, CA). Briefly, treated pancreatic acinar cells were homogenized in ice-cold SP assay buffer (Bachem). A small amount of supernatant was kept for subsequent DNA assay, and the remaining supernatant was adsorbed on C_{18} cartridge columns (Bachem). The adsorbed peptides were eluted with 1.5ml of 75% acetonitrile (Bhatia et al., 1998). They were freeze-dried overnight and reconstituted with 55µl of SP assay buffer. SP content was then determined with an ELISA kit (Bachem) according to manufacturer's instructions. The results were quantified by spectophotometry at 450nm. The results were then normalized with DNA content of the acinar cell samples. SP expression was corrected and presented as nanograms per microgram of DNA.

2.2.5 DNA assay

Determination of DNA concentration in the samples was performed fluorometrically as described by Labarca and Paigen (Labarca and Paigen, 1980). Briefly, cellular or tissue homogenates were incubated with Hoechst dye 33258 (Sigma-Aldrich, Singapore). Calf thymus DNA of known concentration was used as a standard. The fluorescence intensity was determined under a wavelength of 340nm. The DNA concentration of the sample was then determined with reference to a linear standard curve.

2.2.6 RNA isolation and reverse transcription

Total RNA from the pancreatic acinar cells was extracted with TRIzol reagent (Invitrogen, Singapore) according to the manufacturer's instructions. All the steps were done on ice-cold conditions. Briefly, cells were homogenized in 1ml of TRIzol reagent and mixed with chloroform. After centrifugation, the transparent aqueous

layer was collected and mixed with isopropanol in a 1:1 ratio in order to precipitate RNA. After centrifugation, the resulting pellet was washed twice with 70% ethanol and dried. The dried pellet was then dissolved in RNase-free water for determination of RNA concentration and subsequent PCR analysis. The integrity of RNA was verified by ethidium bromide staining for the presence of distinct 28S and 18S bands on a 1.2% agarose gel (Figure 2.1). One microgram (1μg) of total RNA was reverse transcribed using iScript cDNA synthesis kit (Bio-Rad, USA) into a total volume of 20μl, according to manufacturer's instructions.

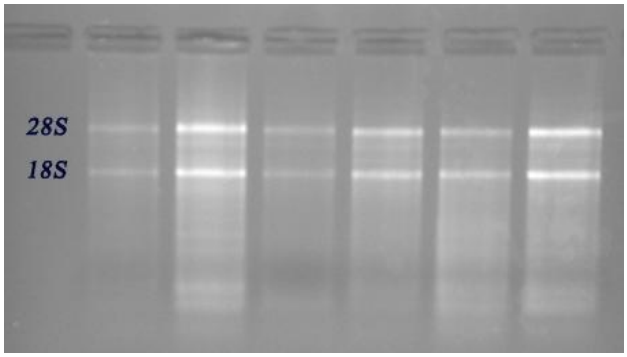

Figure 2.1 RNA integrity was determined by the presence of distinct 28S and 18S rRNA bands.

2.2.7 Semi-quantitative RT-PCR analysis.

2μl of cDNA was used as a template for PCR amplification by using PCR master mix from iDNA (Singapore). No reverse-transcriptase controls and no template control were performed to ensure that no genomic DNA and PCR reagent contamination is present. 3mM Mg^{2+} was added to the PCR reactions. PCR reaction mix was first denatured at 95°C for 3 minutes, followed by a optimal cycle number which is in the linear range of amplification. Each cycle consisted of of 95°C for 30 seconds, annealing step (Table 2.1) for 30 seconds, and a 72°C elongation step for 45 seconds. The final extension was at 72°C for 10 minutes. PCR products were analyzed on 1.2% w/v agarose gels containing 0.5μg/ml ethidium bromide. The

ribosomal RNA, *r18S*, was used as a housekeeping gene to normalize the mRNA expression of *PPTA* and *NK1R*.

The primer sequences are as follows:

Table 2.1 PCR primer sequences, amplification cycles, annealing temperatures, and product sizes for semi-quantitative RT-PCR

Gene (GenBank Accession No.)	Primer sequence	Amplification conditions	Size (bp)
r18S	Sense: 5'- GTAACCCGTTGAACCCCATT-3'	23 cycles	150
(NR_003278)	Antisense: 5'-CCATCCAATCGGTAGTAGCG-3'	Annealing: 59℃	
NK1R	Sense: 5'- CTTGCCTTTTGGAACCGTGTG-3'	36 cycles	501
(NM_009313)	Antisense: 5'- CACTGTCCTCATTCTCTTGTGGG-3'	Annealing: 57.5℃	
PPTA	Sense: 5'- CGCGATGCAGAACTACGAAA-3'	36 cycles	282
(NM_009311)	Antisense: 5'- GCTTGGACAGCTCCTTCATC-3'	Annealing: 57.5℃	

2.2.8 Quantitative real-time PCR analysis.

2μl of cDNA was used as a template for PCR amplification by using SYBR-green PCR master mix from Roche Diagnostics (Singapore). No template controls and intron spanning primers were used. All reactions were done in duplicates. PCR reaction mix was first subjected to 95°C for 5 minutes, followed by a 45 cycles of amplification. Each cycle consisted of 95°C for 30 seconds, annealing temperature of 55^{o}C for 15 seconds, and elongation temperature of 72°C for 15 seconds. *β-actin* was used as a housekeeping gene to normalize the mRNA expression for real-time PCR experiments. The primer sequence of NK1R was re-designed to shorten its product size and better suit real-time PCR conditions. Expression of *PPTA*, *NK1R*, *NEP* and

β-actin was determined using the "crossing point (Cp)" of the sample, where Cp is the point (cycle number) at which the fluorescence of a sample rises above the background fluorescence.

The primer sequences are as follows:

Table 2.2 PCR primer sequences, amplification cycles, annealing temperatures, and product sizes for quantitative real-time PCR

Gene (GenBank Accession No.)	Primer sequence	Size (bp)
β-actin	Sense: 5'- TGTTACCAACTGGGACGACA-3'	165
(NM_007393)	Antisense: 5'- GGGGTGTTGAAGGTCTCAAA-3'	
NK1R	Sense: 5'- GCTGCCCTTCCACATCTTCT-3'	223
(NM_009313)	Antisense: 5'- TTCCAGCCCCTCATAATCAC-3'	
PPTA	Sense: 5'- CGCGATGCAGAACTACGAAA-3'	282
(NM_009311)	Antisense: 5'- GCTTGGACAGCTCCTTCATC-3'	
NEP	Sense: 5'- CAGCCTCAGCCGAAACTACA-3'	156
(NM_008604.3)	Antisense: 5'- GCAAAAGCCGCTTCCACATA-3'	

2.2.9 Whole cell lysate preparation and Western blot analysis.

After treatment of pancreatic acinar cells, they were homogenized on ice in radioimmunoprecipitation assay (RIPA) lysis buffer supplemented with protease inhibitor cocktail (Roche, Switzerland) and phosphatase inhibitor cocktail (Sigma-Aldrich). Protein concentrations were determined by the Bradford protein assay (Bio-rad, CA). Protein samples (80 µg) were separated by 10% SDS-polyacrylamide gels and electrophoretically transferred to polyvinylidene difluoride (PVDF) membranes. Non-specific binding was blocked by 1-hour incubation of the membranes in 5%

nonfat dry milk in PBST (0.05% Tween 20 in PBS). The blots were then incubated overnight with primary antibody NK1R (Abcam, UK) at 1:1000 dilutions in the buffer containing 2.5 % nonfat dry milk in PBST. After which they were washed four times with PBST, and finally incubated for 2 h with goat anti-rabbit HRP-conjugated secondary antibody (Santa Cruz Biotechnology, CA) at 1:2000 dilutions in the buffer containing 2.5 % nonfat dry milk in PBST. Membranes were washed and then incubated in SuperSignal™ West Pico chemiluminescent substrate (Pierce, IL) before exposure to X-ray films (CL-Xposure™, Pierce). Hypoxanthine-guanine phosphoribosyltransferase (HPRT) (Santa Cruz Biotechnology) was applied as an internal control to normalize protein loading. The intensity of bands was quantified using LabWorks™ Image Analysis software (UVP, CA).

2.2.10 Statistical analysis.

The data were expressed as the mean ± standard error of mean (SEM). The significance of changes among groups was evaluated by using ANOVA with a Tukey's post hoc test. A p value ≤ 0.05 was considered as statistically significant.

2.3 RESULTS

2.3.1 Caerulein induces *PPTA* mRNA expression and SP protein expression

Freshly isolated pancreatic acinar cells were assessed for *PPTA* and SP expression after treatment with caerulein. Cells were treated with caerulein for different concentrations (10^{-12}M, 10^{-11}M, 10^{-10}M, 10^{-9}M, 10^{-8}M, and 10^{-7} M; 60 minutes) and time (0, 30, 60, 120 minutes; 10^{-7}M caerulein) to determine the time- and concentration- dependant response. After treatment, RNA was extracted for detection of *PPTA* mRNA expression by semi-quantitative RT-PCR (Figure 2.2A) or quantitative real time-PCR (Figure 2.2B). SP was detected using a commercially available ELISA kit. Our results showed that caerulein increased expression of both gene and protein expression of SP. Caerulein-induced SP up-regulation was evident at a dose from 10^{-8}M and there was a 3 fold increase at a concentration of 10^{-7}M (Figure 2.2C). Densitometric analysis of PCR products on agarose gel showed that *PPTA* mRNA expression showed an increasing trend at a dose of 10^{-8}M, and was significantly different to the expression of the control when caerulein concentration was 10^{-7}M (Figure 2.2A). Although statistically significant, the increase in *PPTA* mRNA (1.5 fold) transcription appeared small relative to SP. After determining the effective concentration for *PPTA* stimulation, we performed a time-dependant study to investigate how rapidly *PPTA* responded to caerulein. Cells were treated with caerulein (10^{-7}M) for 0, 30, 60, and 120 minutes, and our results showed that mRNA expression of *PPTA* was up-regulated between 30-60 minutes and is significantly different from the control at the 60 minute time point. The up-regulated response was sustained until the 120 minute mark (Figure 2.2B).

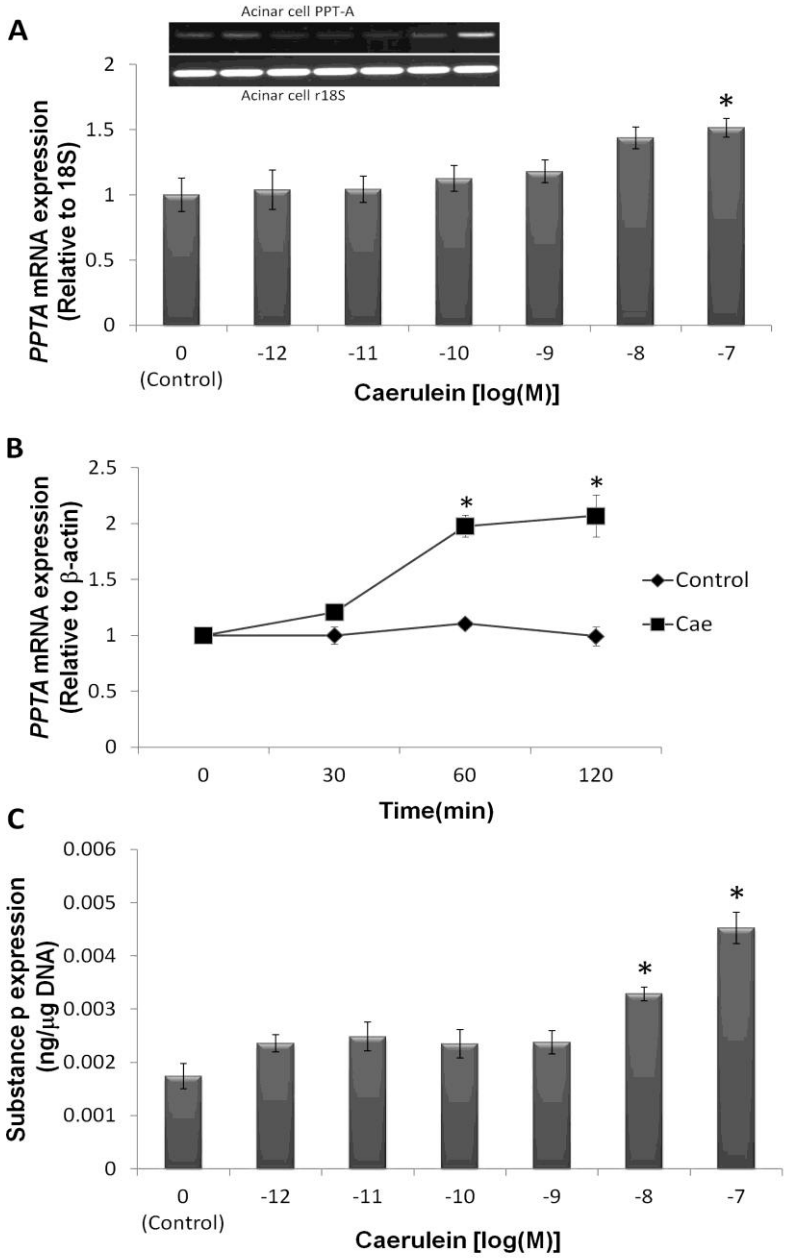

Figure 2.2 Caerulein induces PPTA mRNA expression, and also SP peptide expression in pancreatic acinar cells. Freshly prepared cells were treated with caerulein (10^{-12}M to 10^{-7}M; A) for 60 minutes. *PPTA* expression was determined with RT-PCR (Fig 2.2A) or real-time PCR (Fig 2.2B). SP expression was determined by a commercially available ELISA kit. *PPTA* expression was normalized with *r18S*/*β-actin* expression and SP levels were normalized with DNA content in the samples. A) Concentration response study on *PPTA* mRNA expression. B) Time response study on *PPTA* mRNA expression. C) Concentration response study on SP expression. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control.

2.3.2 Caerulein induces *NK1R* mRNA and protein expression

The effect of caerulein on expression of NK1R, a high affinity receptor of SP, was also assessed using our pancreatic acinar cell model. Freshly isolated pancreatic acinar cells were stimulated with caerulein (10^{-12}M, 10^{-11}M, 10^{-10}M, 10^{-9}M, 10^{-8}M, and 10^{-7} M) for 60 minutes. After treatment, RNA was extracted for detection of *NK1R* mRNA expression by RT-PCR. NK1R protein expression was detected using Western blot. Our results showed that caerulein increased both the expression of mRNA and protein of NK1R. Caerulein-induced *NK1R* up-regulation was evident only at a high concentration of 10^{-7}M, while lower concentrations showed minimal effects (Figure 2.3A). The time-dependent response of NK1R mRNA expression was similar to time-dependent response of *PPTA* mRNA expression, which showed significant increases in 1 hour (Figure 2.3B). In contrast, protein expression of NK1R required a longer stimulation time of 2 hours to show significant increases (Figure 2.3C). Overall, our preliminary results showed that caerulein concentrations below 10^{-8}M did not induce up-regulation of SP and NK1R. Therefore, a concentration of 10^{-7}M was chosen in further experiments for maximum stimulation.

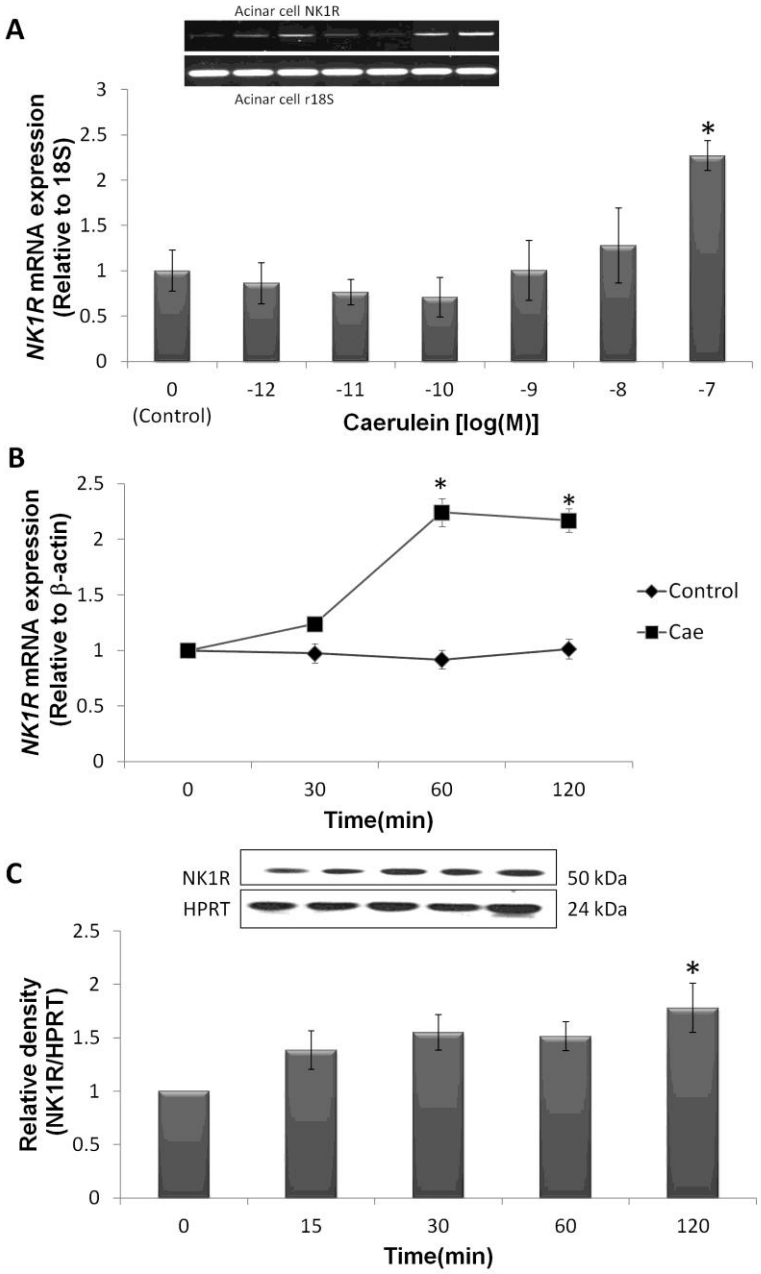

Figure 2.3 Caerulein induces NK1R gene and protein expression in the pancreatic acinar cells. Freshly prepared cells were treated with caerulein (10^{-12}M to 10^{-7} M; A) for 60 minutes or caerulein (10^{-7}M; B) for 0-120 minutes. *NK1R* gene expression was determined with RT-PCR (Figure 2.3A) or real-time PCR (Fig 2.3B). NK1R protein expression was determined by Western blot. *NK1R* mRNA expression was normalized with *r18S/β-actin* expression and NK1R protein levels was normalized with HPRT expression in the samples. A) Concentration response study on *NK1R* mRNA expression. B) Time response study on *NK1R* mRNA expression. C) Time response study on NK1R protein expression. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control.

2.3.3 SP expression is mediated via CCK_A receptors

Previous studies have mostly attributed the effect of caerulein to the stimulation of CCK_A receptors in pancreatic acinar cells. In this study, we tested whether caerulein binding on CCK_A receptors mediates SP up-regulation in pancreatic acinar cells. Pancreatic acinar cells were pre-treated with different concentrations of devazepide, a specific CCK_A antagonist, for 30 minutes before stimulated with caerulein (10^{-7}M) to induce maximum response. Devazepide treatment is able to abolish the up-regulation of SP. Devazepide concentrations less than 10 nM showed a trend to decrease SP levels in the pancreatic acinar cells, but they were not statistically significant. However, pretreatment of cells with more than 10 nM devazepide showed significant reversal of SP up-regulation, whereas a concentration of 1 μM completely abolished the increase in SP production caused by caerulein stimulation (Figure 2.4).

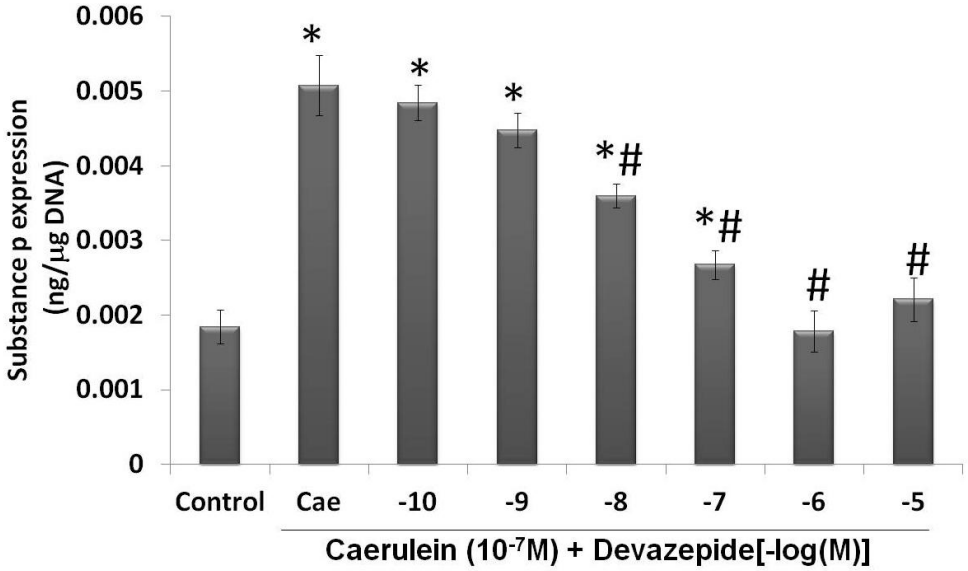

Figure 2.4 Caerulein induced SP up-regulation is mediated by CCK_A signaling. Pancreatic acinar cells were pre-treated with different concentrations of devazepide for 30 minutes before caerulein (10^{-7}M) stimulation for 60 minutes. SP expression was determined by a commercially available ELISA kit. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

2.4 DISCUSSION

In experimental AP models, SP has been shown to be up-regulated and plays a proinflammatory role (Lau and Bhatia, 2006). SP and NK1R are expressed in the pancreatic acinar cells, and this expression could contribute to the overall outcome of caerulein-induced AP.

Our results showed that treatment of pancreatic acinar cells with caerulein up-regulated the gene expression of *PPTA* and *NK1R*, and also the protein levels of SP. The changes were found to be concentration-dependent. At a physiological concentration of caerulein (10^{-10}M), the expression of *PPTA*, *NK1R*, and SP remained at the basal level. However, at a supramaximal concentration of caerulein (10^{-7}M), mRNA expression of *PPTA* and *NK1R* was significantly up-regulated. On the other hand, SP expression was significantly increased at a lower concentration of 10^{-8}M. The up-regulation of SP was also substantially stronger than the up-regulation of its gene, *PPTA* (3- to 1.5-fold compared with control). It is possible that RNA stabilization, increased translational activity or reduced degradation of the SP product by neutral endopeptidases might contribute to the results observed, although these hypotheses remain to be elucidated (Estival et al., 1991; O'Connor et al., 2004). The 3-fold increase in SP levels is in line with previous experiments done *in vivo*, in which SP is increased with a similar magnitude in the pancreas after induction of AP (Lau and Bhatia, 2006). A time dependent study using supramaximal concentrations of caerulein also clearly showed a slow but steady increase of NK1R protein expression in mouse pancreatic acinar cells, and is statistically significant after two hours of caerulein exposure. These findings showed that the up-regulation of SP/NK1R was rapid upon induction of AP, further showing its importance in mediating the early phase of the disease.

Administration of a selective CCK_A antagonist, devazepide, inhibited the expression of SP. A concentration of 10 nM is able to cause a significant down-regulation of SP, whereas a concentration of 1μM completely reversed SP expression to basal levels. Because the effective inhibitory concentration of devazepide is well below the IC_{50} for CCK_B, along with the very low expression of CCK_B receptors in mouse pancreas, we suggest that caerulein acts through CCK_A receptors to mediate the up-regulation of SP (Lay et al., 2000; Dufresne et al., 2006).

CHAPTER 3: CAERULEIN UP-REGULATES SUBSTANCE P AND NEUROKININ-1 RECEPTOR VIA A PKC-MAPK-NF-κB/AP-1 PATHWAY

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Yung-Hua Koh, Ramasamy Tamizhselvi, and Madhav Bhatia. Extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase, through nuclear factor-kappaB and activator protein-1, contribute to caerulein-induced expression of substance P and neurokinin-1 receptors in pancreatic acinar cells. *J Pharmacol Exp Ther.* 2010; 332(3):940-8.

Yung-Hua Koh, Ramasamy Tamizhselvi, Shabbir Moochhala, Jin-Song Bian and Madhav Bhatia. Role of protein kinase C in caerulein induced expression of substance P and neurokinin-1-receptors in murine pancreatic acinar cells. *J Cell Mol Med.* 2011; 15(10):2139-49.

3.1 INTRODUCTION

Caerulein-induced activation of downstream secondary messengers was extensively studied in both cellular and animal models of AP. Caerulein was found to be able to activate three main members of the mitogen activated protein kinases (MAPK), which include extracellular signal-regulated kinase (ERK), c-Jun NH_2-terminal kinase (JNK) and p38. The activation of ERK and JNK is rapid. Both molecules were found to be induced by caerulein within 15 minutes and maintained their activated state for 2 hours in the rat pancreas (Namkung et al., 2008). When the animals received pre-treatment with an ERK or JNK inhibitor, they exhibit reduced pancreatic damage (Namkung et al., 2008). In another study, treatment of rats with CEP-1347, a JNK inhibitor, protected mice against caerulein-induced pancreatitis (Wagner et al., 2000).

Protein kinase C (PKC) was also activated in caerulein-induced pancreatic acinar cells. Treatment of cells with caerulein causes translocation of PKC to the cell membrane (Bruzzone et al., 1988). However, the exact role of PKC in caerulein-induced pancreatic acinar cells was not clear. Two members of the PKC family, PKCδ and PKCε, were found to be activated and involved in the modulation of zymogen activation in pancreatic acinar cells (Thrower et al., 2008). On the other hand, caerulein was also able to cause activation of eIF4E-binding protein and S6 ribosomal protein by a protein kinase B dependent pathway (Sans et al., 2003).

In this study, we have focused on the role of PKC, MAPK and transcription factors NF-κB and AP-1 in the regulation of SP/NK1R expression in pancreatic acinar cells.

3.1.1 Mitogen activated protein kinases

The MAPKs are a family of serine/threonine kinases that are widely implicated in physiological and pathological responses. MAPKs can be divided into six distinct groups, in which the ERK, JNK, and p38-MAPK are best characterized (Krishna and Narang, 2008). MAPK activation cascades are organized in which phosphorylation of an upstream kinase leads to sequential activation of the MAPK downstream molecules (MAPKKK → MAPKK → MAPK) (Chang and Karin, 2001). In other words, phosphorylation and dephosphorylation of MAPKs at activation sites enable it to regulate cellular functions. The MAPKs respond to growth and stress signals, and is implicated in the control of cell division, cell death, and pathological responses. In experimental pancreatitis models, three members of the MAPK, which include ERK, JNK and p38, were found to be activated. Treatment with MAPK inhibitors down-regulates MAPK signaling and showed protective effects, suggesting the important role of MAPKs in the pathogenesis of AP (Samuel et al., 2006; Namkung et al., 2008).

Activated MAPKs will then phosphorylate an array of downstream kinases, and eventually lead to the activation of gene regulating transcription factors. In this present study, we have focused on the role of ERK and JNK pathways, and also the transcription factors NF-κB and AP-1, in caerulein induced SP/NK1R expression in pancreatic acinar cells.

3.1.2 Transcription factors NF-κB and AP-1

Nuclear factor-kappa B (NF-κB) and activator protein-1 (AP-1) are transcription factors frequently associated with inflammatory response in AP (Gukovsky et al., 2003; Ramnath and Bhatia, 2006; Rakonczay et al., 2008b). In the cytoplasm, NF-κB is inhibited by IκB. During a stimulus, phosphorylation of IκB by IκB kinase (IKK) leads to IκB degradation through the 26S proteosome. The nuclear

translocation signal of NF-κB was then exposed and translocated into the nucleus. NF-κB is composed of homodimers or heterodimers of two subunits from a family of Rel proteins, which include p50, p52, p65, RelB, and c-Rel. In particular, the p65 subunit was suggested to be responsible for a strong transcription activity of NF-κB (Schmitz and Baeuerle, 1991).

AP-1 is a heterodimer which consisted of c-Jun, c-Fos, or ATF. The subunits of AP-1 share a leucine zipper motif that facilitates dimerizaton, and also a DNA binding domain is responsible for transcriptional activity. AP-1 was well known to be activated by JNK, but other members of the MAPKs, such as ERK, were also found to be able to activate AP-1 (Xu et al., 2006; Guedea et al., 2011). The activation of these transcription factors is rapid, allowing a fast response of transcriptional activity when a stress signal is available. Both NF-κB and AP-1 are widely implicated in the regulation of genes that code for inflammatory mediators. In neuronal models, NF-κB regulates the expression of *PPTA* and it is known that there are multiple AP-1 response elements in the proximal region of its promoter (Paterson et al., 1995; Fiskerstrand et al., 1997). The flanking region of the NK1R gene also has a NF-κB transcriptional regulation site (O'Connor et al., 2004).

3.1.3 Protein kinase C

In recent years, the role of protein kinase C (PKC) has been extensively studied in the pathogenesis of AP. There are more than 10 isoforms of PKC divided into three different classes, namely, conventional, novel and atypical PKC. The conventional PKC isoforms α, β, and γ are regulated by Ca^{2+} and diacylglycerol (DAG). On the other hand, novel PKC isoforms δ, ε, ν, and θ respond to DAG, but do not involve Ca^{2+}. The atypical PKC isoforms ζ, λ/ι and μ are different as their

activation does not need the presence of Ca^{2+} and DAG (Mellor and Parker, 1998). In the pancreatic acinar cells, the conventional PKCα and novel PKCδ, ε and atypical ζ isoforms had been identified (Bastani et al., 1995). In general, the activation of PKC is characterized by the phosphorylation of the molecule, followed by translocation of distinct intracellular compartments in which it performs its specific function (Shirai and Saito, 2002). The individual roles of PKCs in the regulation of pancreatic acinar function have been reported in various studies, where PKCδ is the most studied. PKCδ is activated and translocated to the plasma membrane and participates in CCK/caerulein-stimulated secretion (Li et al., 2004) and protease activation (Thrower et al., 2009) in pancreatic acinar cells. PKCδ was also found to modulate inflammatory molecule expression in the SP-induced pancreatic acinar cells (Ramnath et al., 2008). The role of conventional PKCα is less understood, but with CCK treatment stimulates increases in Ca^{2+} and DAG in the pancreatic acinar cells, a potential role of PKCα cannot be ruled out (Williams, 2001).

3.1.4 Concluding remarks

In chapter 2, we have elaborated the concentration and time dependent effect of caerulein on SP/NK1R expression in isolated murine pancreatic acinar cells. In this chapter, we investigated intracellular mechanisms that might lead to SP/NK1R up-regulation, using specific pharmacological inhibitors. To our knowledge, this is the first report of the detailed mechanisms that regulate the expression of SP and NK1R in pancreatic acinar cells. Elucidation of key signaling molecules involved in the regulation of SP/NK1R pathway contribute to understanding how inflammatory mediators were regulated in an event of AP and provides the basis for therapeutic intervention.

3.2 MATERIALS AND METHODS

3.2.1 Animals and chemicals.

Refer to section 2.2.1. Swiss mice (Male, 25-30 grams) were used for isolation of pancreatic acinar cells. PD98059 (Mitogen-activated protein kinase kinase (MEK) inhibitor), SP600125 (JNK inhibitor), Bay11-7082 (NF-κB inhibitor), Gö6976 (PKCα/β inhibitor) and rottlerin (PKCδ inhibitor), were purchased from Calbiochem (Darmstadt, Germany). They were dissolved in DMSO. All chemicals were purchased with the highest purity available.

3.2.2 Preparation and treatment of pancreatic acinar cells.

Refer to section 2.2.2 and 2.2.3. For concentration dependent studies, pancreatic acinar cells were treated with different concentrations of caerulein for 60 minutes; for time dependent studies, cells were incubated with caerulein (10^{-7}M) for 0-120 minutes. In order to study the signaling mechanisms involved, isolated pancreatic acinar cells were pre-treated with PD98059, SP600125 or Bay11-7082 for 30 minutes before treatment with caerulein (10^{-7}M, 60 minutes). Similarly, cells were pre-treated with rottlerin or Gö6976 for 45 minutes before stimulation with caerulein.

3.2.3 Substance P extraction and detection.

Refer to section 2.2.4.

3.2.4 Whole cell lysate preparation and Western blot analysis.

Refer to section 2.2.9. Cells were lysed with RIPA lysis buffer and protein concentrations were determined by Bradford protein assay. 50-80 µg of total cell lysate from each sample were subjected to Western blot analysis. The blots were incubated overnight with primary antibody of phospho-ERK (Thr^{202}/Tyr^{204}), total ERK, phospho-JNK (Thr^{183}/Tyr^{185}), total JNK, phospho-PKCα (Thr^{638}), phospho-

PKCδ (Thr^{505}), phospho-IκB (Ser^{32}), total IκB, or HPRT at 1:1000 dilutions. After washing away the primary antibodies, the membranes were incubated for 2 hours with goat anti-rabbit HRP-conjugated secondary antibody at 1:2000 dilutions. Protein expression was then detected by adding SuperSignal™ West Pico chemiluminescent substrate before exposure to X-ray films.

3.2.5 Nuclear cell extract preparation and NF-κB/AP-1 DNA-binding activity.

Nuclear cell extracts were prepared by using Nuclear Extract Kit (Active Motif, CA). In brief, cells were washed in ice-cold PBS in the presence of phosphatase inhibitors. After centrifugation, the cell pellets were re-suspended in a hypotonic buffer, treated with detergent and centrifuged at 14,000 g for 30 sec. After removing of the cytoplasmic fraction, the pellet (nuclei) was lysed with lysis buffer containing protease inhibitors and nuclear proteins solubilise in the lysis buffer. Nuclear protein concentrations were determined by Bradford protein assay (Bio-Rad).

The binding of nuclear NF-κB and AP-1 to DNA was measured with ELISA based NF-κB p65 assay kit and AP-1 c-Jun assay kit respectively (Active Motif). The plates were pre-coated with an unlabeled oligonucleotide containing the consensus-binding site (NF-κB p65: 5'-GGGACTTTCC-3'; AP-1 c-Jun: 5' –TGAGTCA-3'). Nuclear proteins (20 μg) were used for the assay and the results were quantified by spectophotometry at 450nm.

3.2.6 Semi-quantitive RT-PCR analysis and Quantitative real time PCR analysis.

Refer to section 2.2.6, 2.2.7 and 2.2.8.

3.2.7 Statistical analysis.

Refer to section 2.2.10.

3.3 RESULTS

3.3.1 Caerulein stimulates ERK and JNK phosphorylation in a concentration dependent manner

To investigate the possible involvement of MAPKs, a concentration-response study was done on the phosphorylation of ERK and JNK. Cells were stimulated with caerulein (10^{-12}M, 10^{-11}M, 10^{-10}M, 10^{-9}M, 10^{-8}M, and 10^{-7} M) for 60 minutes and whole cell lysates were subjected to Western blot analysis. Both ERK1/2 and JNK were significantly activated only at a concentration of 10^{-7}M, as shown by the increased phosphorylation. Lower concentrations of caerulein do not appear to affect the phosphorylation of ERK and JNK (Figure 3.1A, 3.1B). The concentration of caerulein to stimulate phosphorylation of ERK and JNK was in line with the concentration required for *NK1R*, *PPTA* and SP up-regulation (Figure 2.2A, 2.2C, 2.3A).

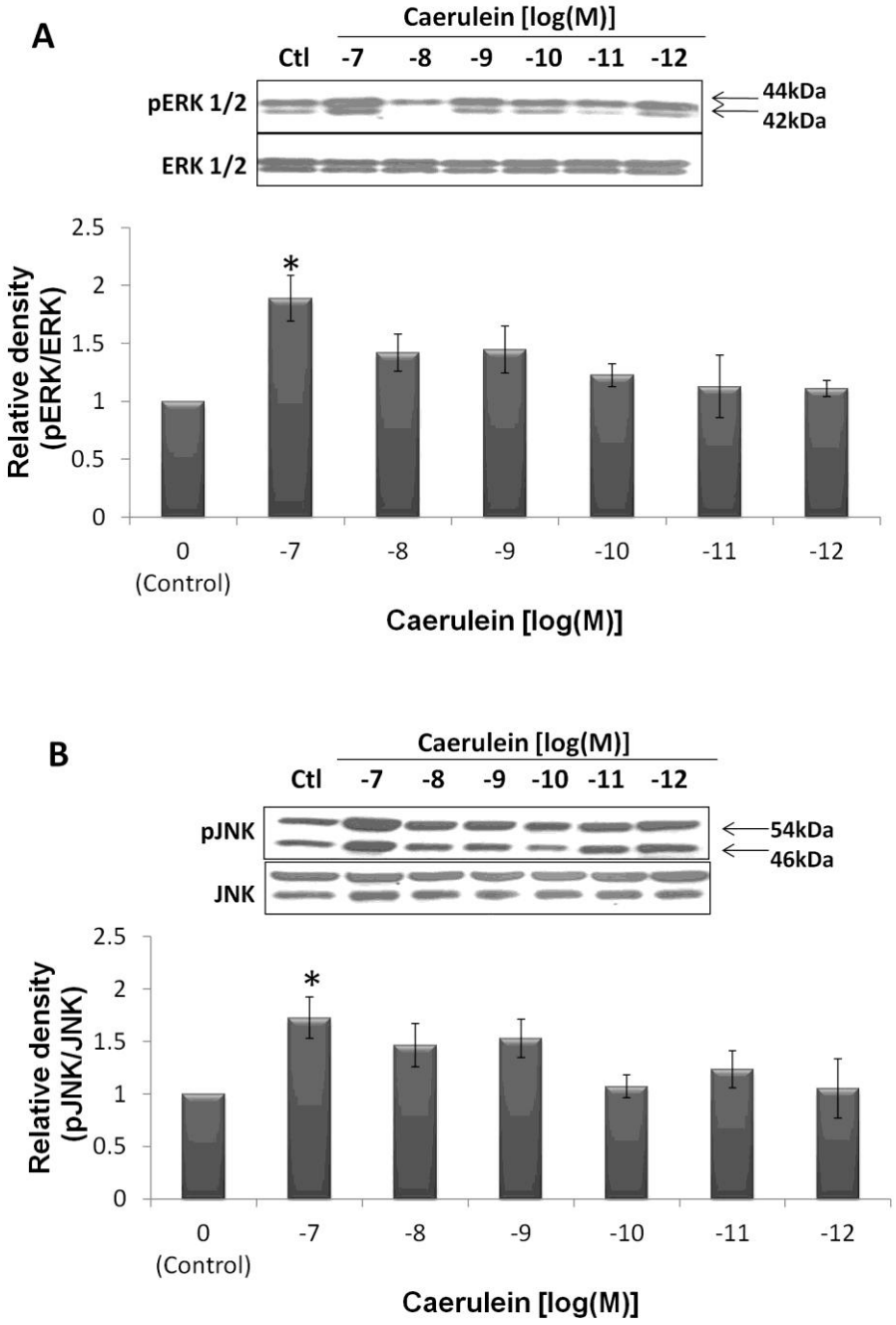


Figure 3.1 Caerulein treatment activates ERK and JNK in pancreatic acinar cells. Cells were treated with caerulein (10^{-12}M to 10^{-7}M) for 60 minutes. ERK and JNK activation was determined by Western blotting. Whole cell lysates were prepared for Western Blot analysis of phospho-JNK(Thr^{183}/Tyr^{185}), JNK, phospho-ERK (Thr^{202}/Tyr^{204}), and ERK. A) Concentration response study of ERK activation. B) Concentration response study of JNK activation. Results are expressed as the means± SEM from four to six independent experiments.*$P<0.05$vs. control

3.3.2 PD98059 and SP600125 inhibits ERK and JNK respectively in the pancreatic acinar cells

PD98059 and SP600125 are known inhibitors of ERK and JNK respectively. The effective concentrations for ERK and JNK inhibition in caerulein-stimulated cells were investigated. Cells were pre-treated with PD98059 or SP600125 for 30 minutes to allow permeation into the cells. PD98059 and SP600125 attenuated caerulein-induced phosphorylation of ERK and JNK, respectively, in a concentration dependent manner (Figure 3.2A, 3.2B). The inhibition of ERK phosphorylation was significant when cells were pre-treated with 30μM and 50 μM of PD98059 (Figure 3.2A). On the other hand, concentrations of 30 μM and 50 μM were found to inhibit JNK phosphorylation significantly (Figure 3.2B).

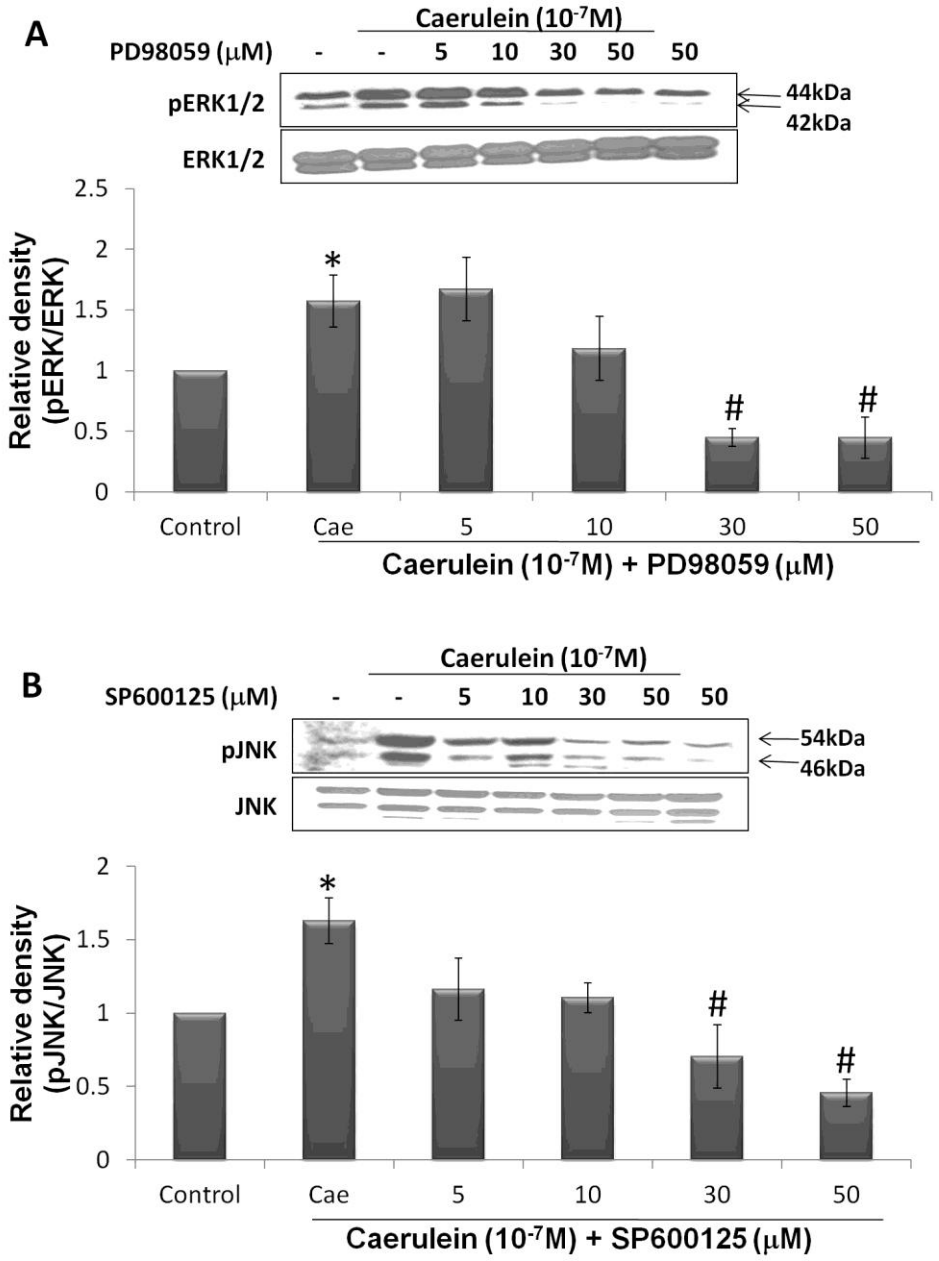


Figure 3.2 PD98059 and SP600125 inhibited ERK and JNK activation respectively in pancreatic acinar cells. Cells were pre-treated with PD98059 or SP600125 for 30 minutes before treatment with caerulein (10^{-7}M) for 60 minutes. ERK and JNK phosphorylation was determined by Western blotting. Whole cell lysates were prepared for Western Blot analysis of phospho-ERK (Thr^{202}/Tyr^{204}), ERK, phospho-JNK (Thr^{183}/Tyr^{185}) and JNK. A) ERK phosphorylation after treated with PD98059. B) JNK phosphorylation after treated with SP600125. Results are expressed as means ± SEM. from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein

3.3.3 Caerulein-induced *PPTA*/SP up-regulation is dependent on JNK activation, but not ERK activation

We have previously shown that a caerulein concentration of 10^{-7}M significantly up-regulated *PPTA* mRNA transcription and SP expression in pancreatic acinar cells. We tested whether inhibition of ERK or JNK could attenuate *PPTA*/SP up-regulation. It was found that treatment of cells with 30μM SP600125 significantly attenuated the up-regulation of *PPTA*/SP (Figure 3.3A, 3.3B). This concentration is similar to the concentration required to inhibit JNK (Figure 3.2B). However, treatment with PD98059 does not appear to cause any significant reduction of *PPTA*/SP expression (Figure 3.3A, 3.3B).

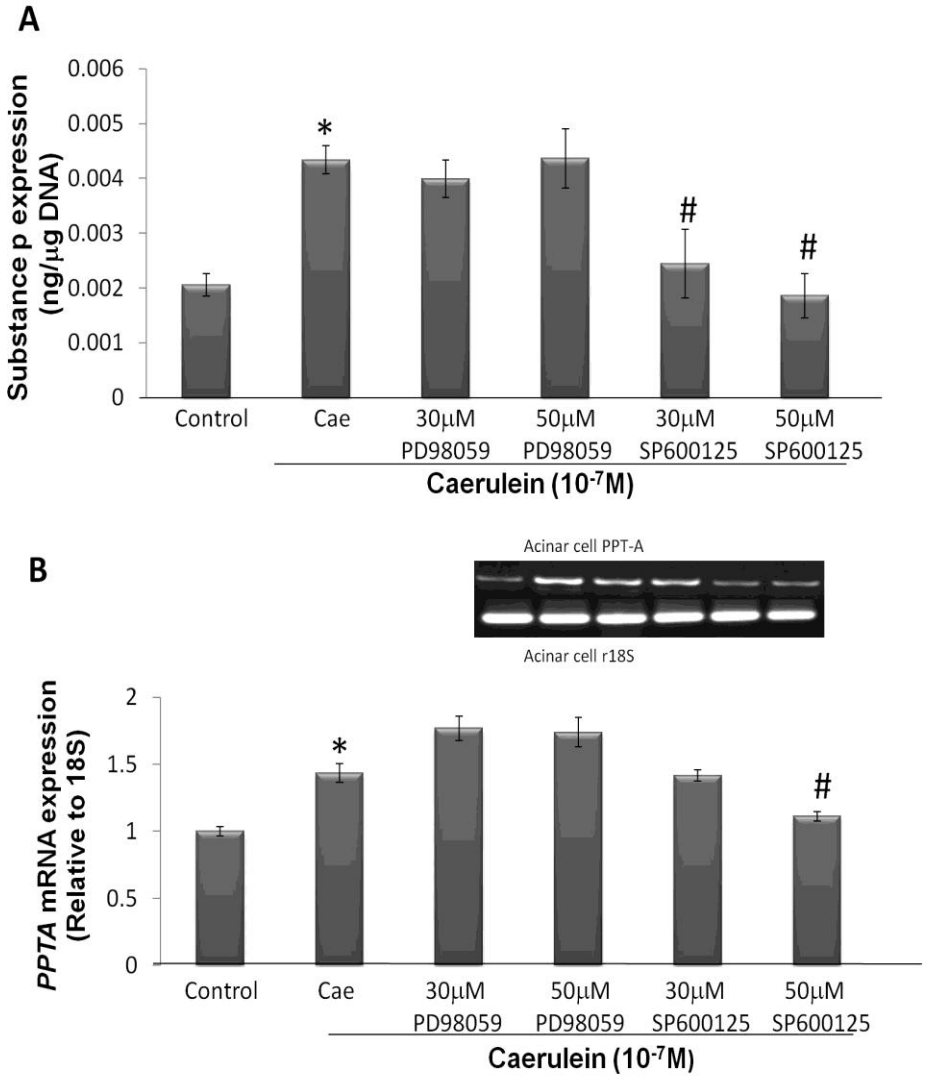


Figure 3.3 The role of ERK and JNK pathways in mediating the increased expression of PPTA and SP. Cells were pre-treated with PD98059 or SP600125 for 30 minutes before treatment with caerulein (10^{-7}M) for 60 minutes. *PPTA* mRNA expression was determined with RT-PCR and normalized with *r18S* expression. SP levels were determined by a commercially available ELISA kit and normalized with DNA content in the samples. A) SP expression. B) *PPTA* mRNA expression. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.4 Caerulein treatment induces NK1R gene expression via ERK and JNK dependent pathways

We also tested the possible role of ERK and JNK in *NK1R* mRNA expression. As opposed to the expression of *PPTA*/SP, pre-treatment of pancreatic acinar cells with both 50µl of PD98059 or SP600125 significantly inhibited *NK1R* expression (Figure 3.4).

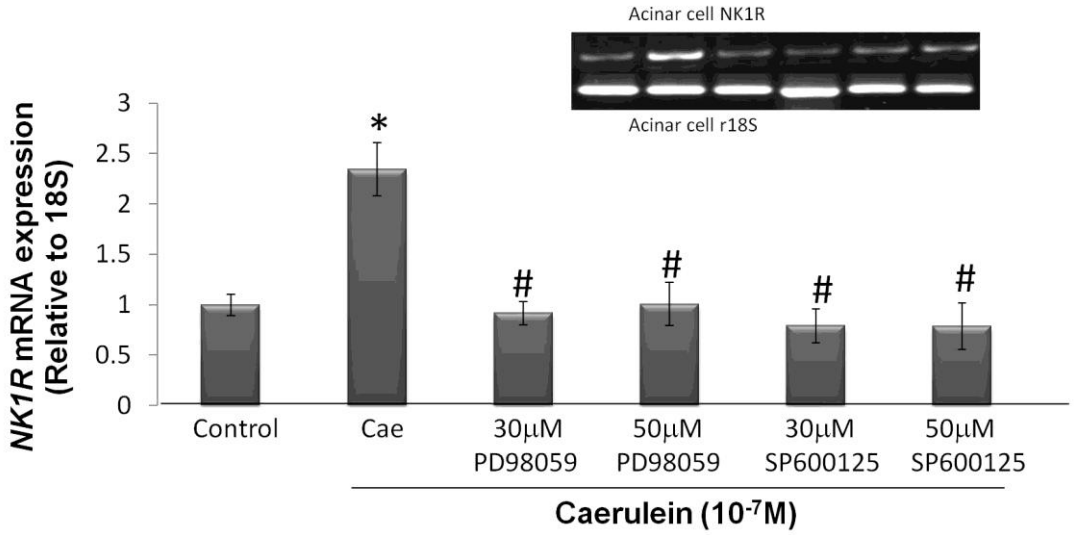


Figure 3.4 The role of ERK and JNK pathways in mediating the increased expression of NK1R. Cells were pre-treated with PD98059 or SP600125 for 30 minutes before treatment with caerulein (10^{-7}M) for 60 minutes. *NK1R* mRNA expression was determined with RT-PCR and was normalized with *r18S* expression. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.5 Caerulein stimulates NF-κB and AP-1

Given the importance of NF-κB and AP-1 transcription factors in inflammatory molecule expression, we examined the activation profile of NF-κB and AP-1 in caerulein-treated pancreatic acinar cells. Phosphorylation and subsequent degradation of IκB is required for NF-κB activation. Therefore, IκB degradation was also examined. Caerulein treatment induced substantial activation of NF-κB and AP-1, as reflected by the increased DNA binding activity (Figure 3.5A, 3.5B). IκB degradation was also significant in pancreatic acinar cells treated with supraphysiological concentrations of caerulein (Figure 3.5C). Caerulein concentrations below 10^{-7}M do not appear to activate NF-κB and AP-1 DNA binding activity.

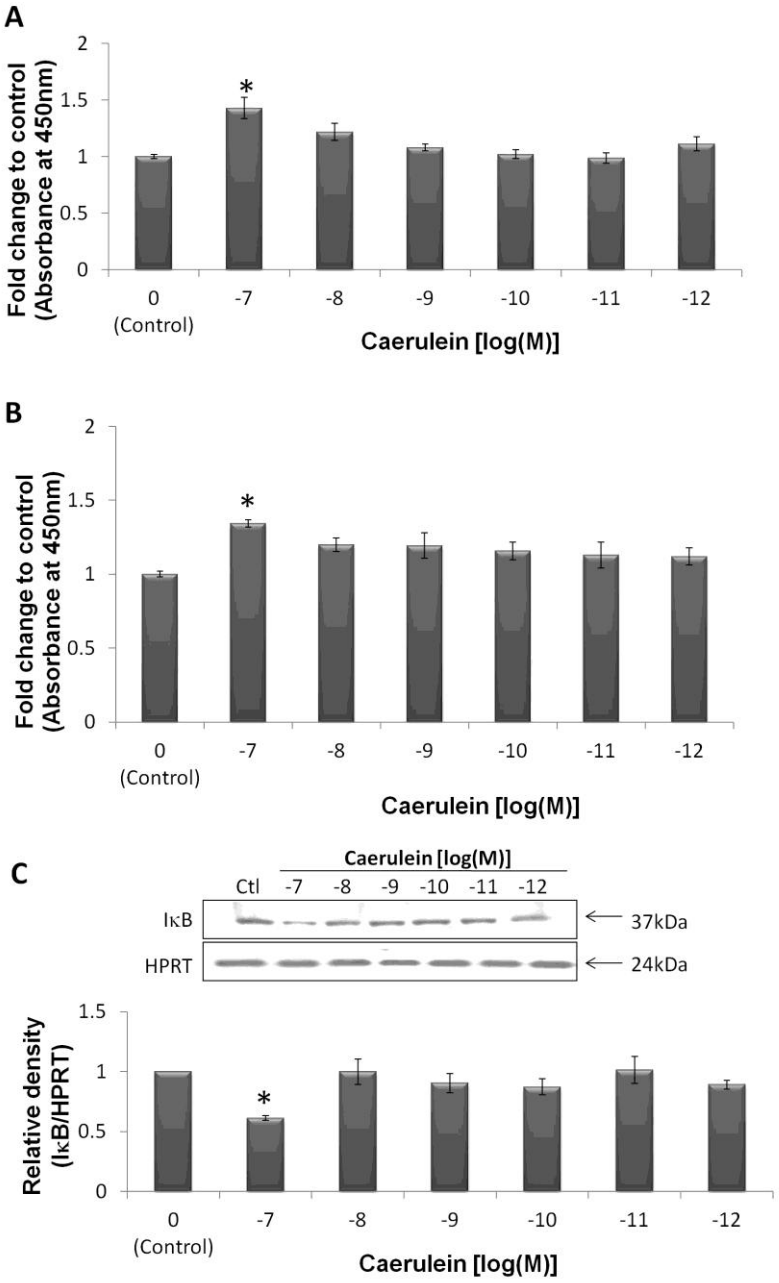


Figure 3.5 Caerulein induces AP-1 and NF-κB activity in the pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with caerulein (10^{-12}M to 10^{-7}M) for 60 minutes. Whole-cell lysates were prepared for Western blot analysis of IκB and HPRT. Nuclear NF-κB and AP-1 DNA binding activity was determined by using a commercially available ELISA kit. A) Concentration response study of AP-1 DNA binding activity. B) Concentration response study of NF-κB DNA binding activity. C) Concentration response study of IκB degradation. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control.

3.3.6 Effect of PD98059 and SP600125 on DNA binding activity of NF-κB and AP-1

The ERK and JNK pathways were found to be upstream activators of NF-κB and AP-1 in SP-induced chemokine production in the pancreatic acinar cells. To investigate the possibility that the ERK/JNK pathway contributes to NF-κB/AP-1 activation in caerulein-treated pancreatic acinar cells, we investigated the effects of PD98059 and SP600125 on NF-κB/AP-1. The DNA binding activity of NF-κB/AP-1 was significantly suppressed after treatment with PD98059 and SP600125 (Figure 3.6A, 3.6B). PD98059 and SP600125 also reduced the amount of phosphorylated IκB in whole cell lysates, suggesting a reduced activation of NF-κB (Figure 3.6B).

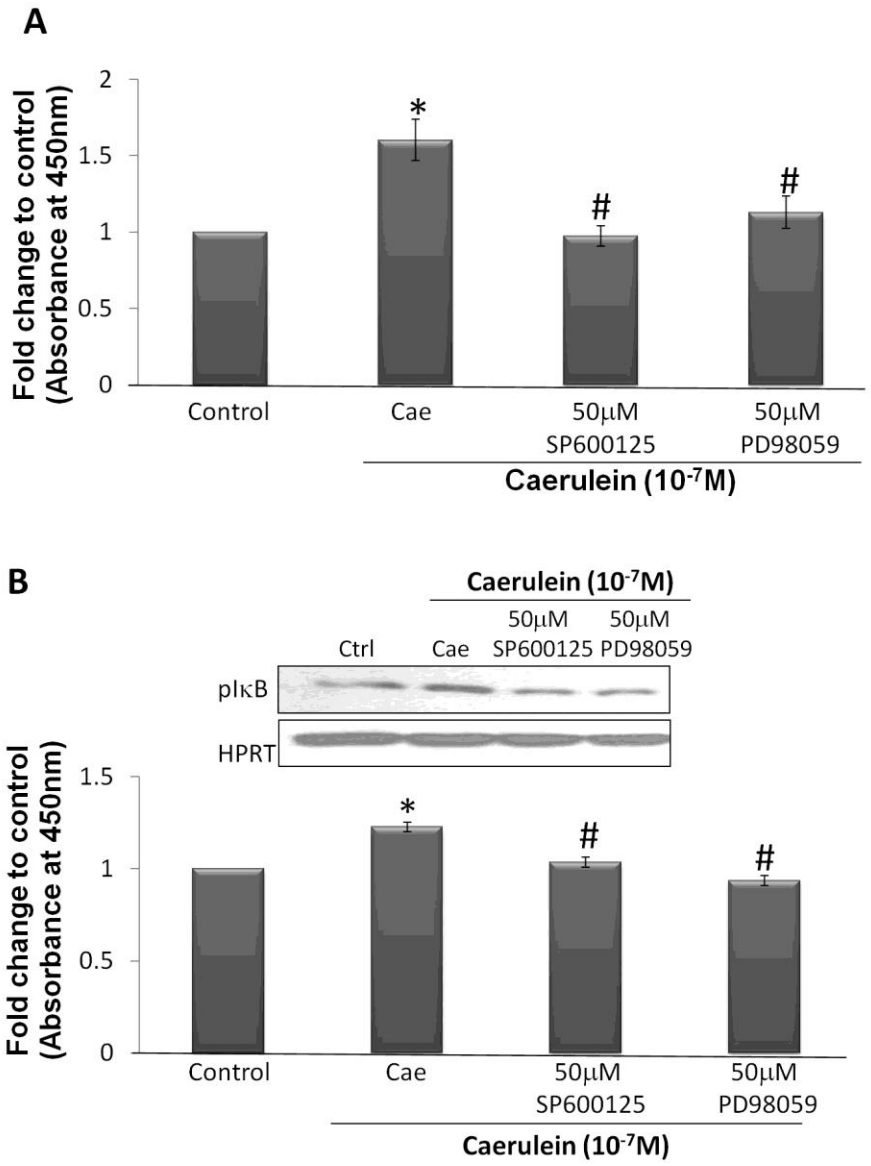


Figure 3.6 ERK and JNK activation is involved in the DNA binding activity of NF-κB and AP-1. Pancreatic acinar cells were pre-treated with PD98059 or SP600125 for 30 minutes before stimulation with caerulein (10^{-7}M, 60 minutes). Subsequently, nuclear extracts was obtained and AP-1 and NF-κB DNA binding ability was determined by using a commercially available ELISA kit. Whole cell lysates were prepared for Western blot analysis for phospho-IκB and HPRT. A) AP-1 DNA binding activity. B) NF-κB DNA binding activity and representative blot for phospho-IκB levels in whole cell lysates. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.7 Effect of Bay 11-7082 on the expression of SP, *PPTA* and NK1R.

We investigated the effect of the NF-κB inhibitor Bay 11-7082 on the expression of SP, *PPTA* and NK1R. It was found that treatment with Bay 11-7082 significantly attenuated the up-regulation of SP, *PPTA* and NK1R, although the effective concentrations are different (Figure 3.7 A-C). Bay 11-7082 at a 10μM concentration significantly reduced the expression of *NK1R*, whereas a concentration of 30μM to 50μM is required to inhibit the expression of SP and *PPTA*.

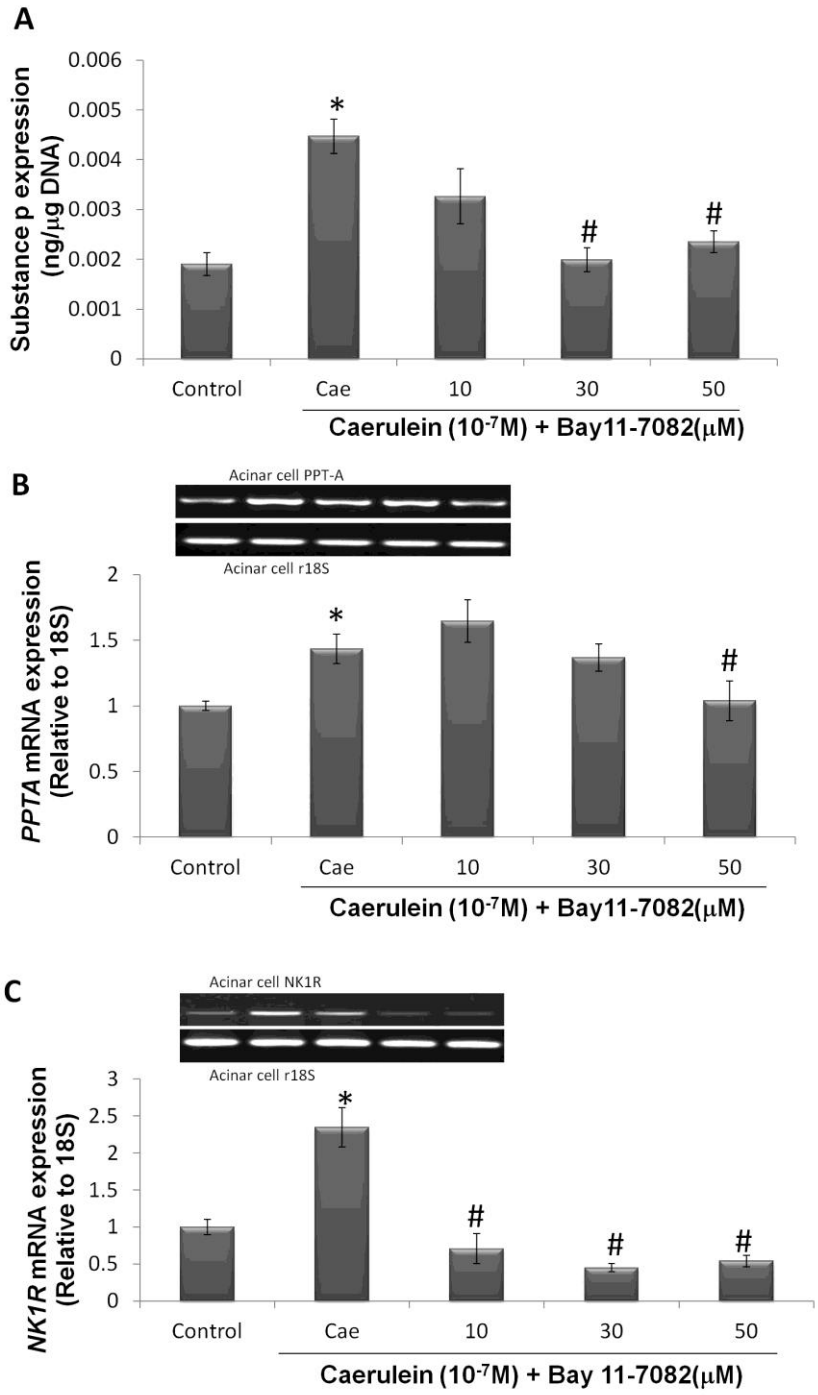


Figure 3.7 Bay 11-7082, a NF-κB inhibitor, inhibited the expression of SP, PPTA and NK1R. Freshly prepared pancreatic acinar cells were pre-treated with Bay 11-7082 for 30 minutes before stimulation with caerulein (10^{-7}M, 60 minutes). *NK1R* and *PPTA* mRNA expression was determined with RT-PCR and SP levels were determined by a commercially available ELISA kit. NK1R and *PPTA* expression was normalized with *r18S* expression and SP levels were normalized with DNA content in the samples. A) SP expression. B) *PPTA* mRNA expression C) *NK1R* mRNA expression. Results are expressed as the means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.8 Caerulein induces phosphorylation of PKCα and PKCδ in mouse pancreatic acinar cells

Caerulein and its analogue CCK are known to activate PKC family in the pancreatic acinar cells. We examined the time-dependant phosphorylation of PKCα and PKCδ in our model of caerulein-induced pancreatic acinar cells. To do this, freshly isolated pancreatic acinar cells were treated with caerulein (10^{-7}M) for 2, 5, 15, 30 and 60 minutes. Western blot analysis of the whole cell lysates showed a modest but significant increase in phosphorylation of both PKCα and PKCδ. Both PKCα and PKCδ are rapidly activated, with significant increases after 2 minutes of caerulein incubation. The activation of PKCα was transient, as the phosphorylated molecules decreased after one hour of incubation (Figure 3.8B). On the other hand, PKCδ remained phosphorylated throughout the studied time course (Figure 3.8C). HPRT was used as an internal control for this experiment.

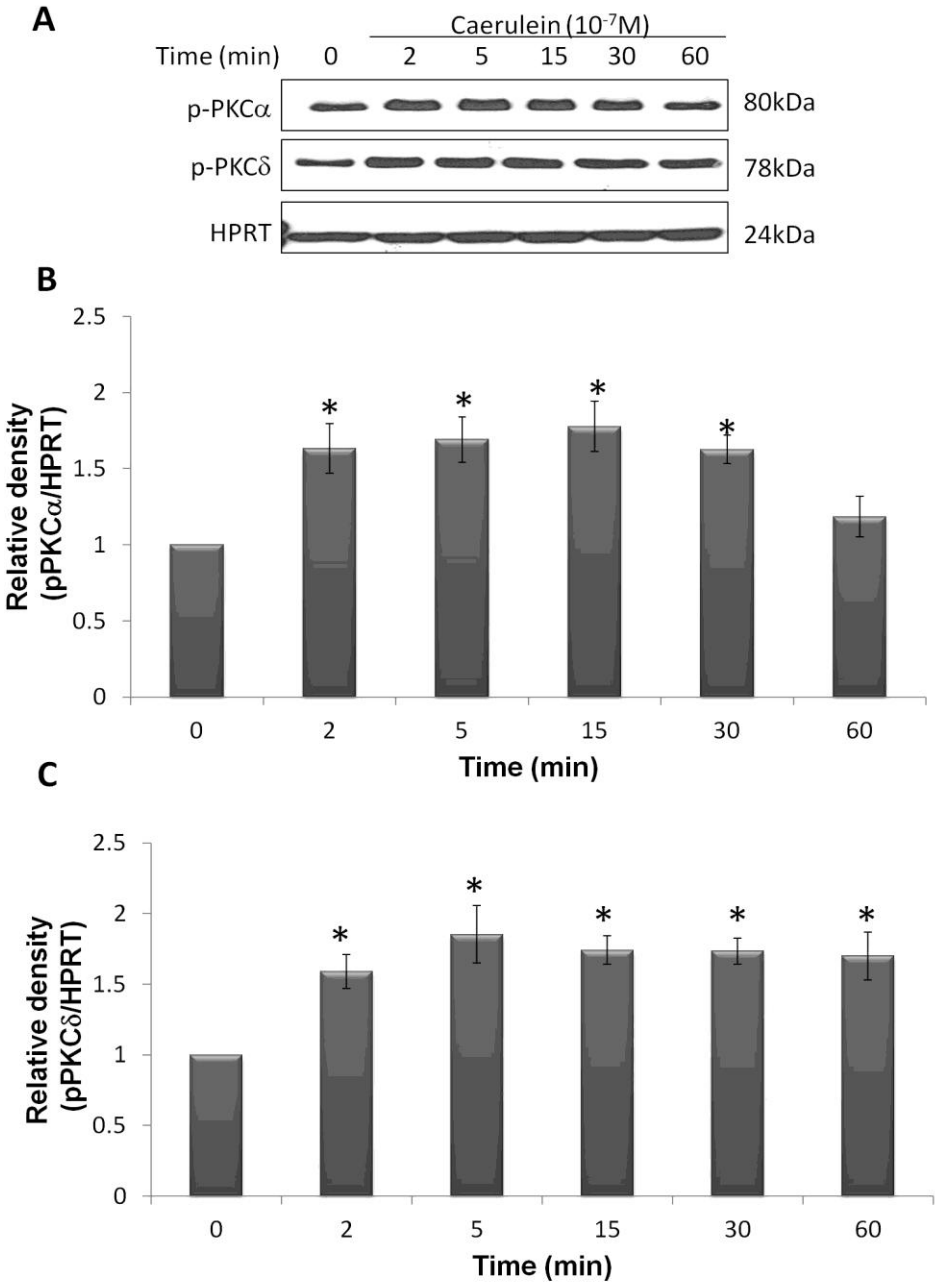
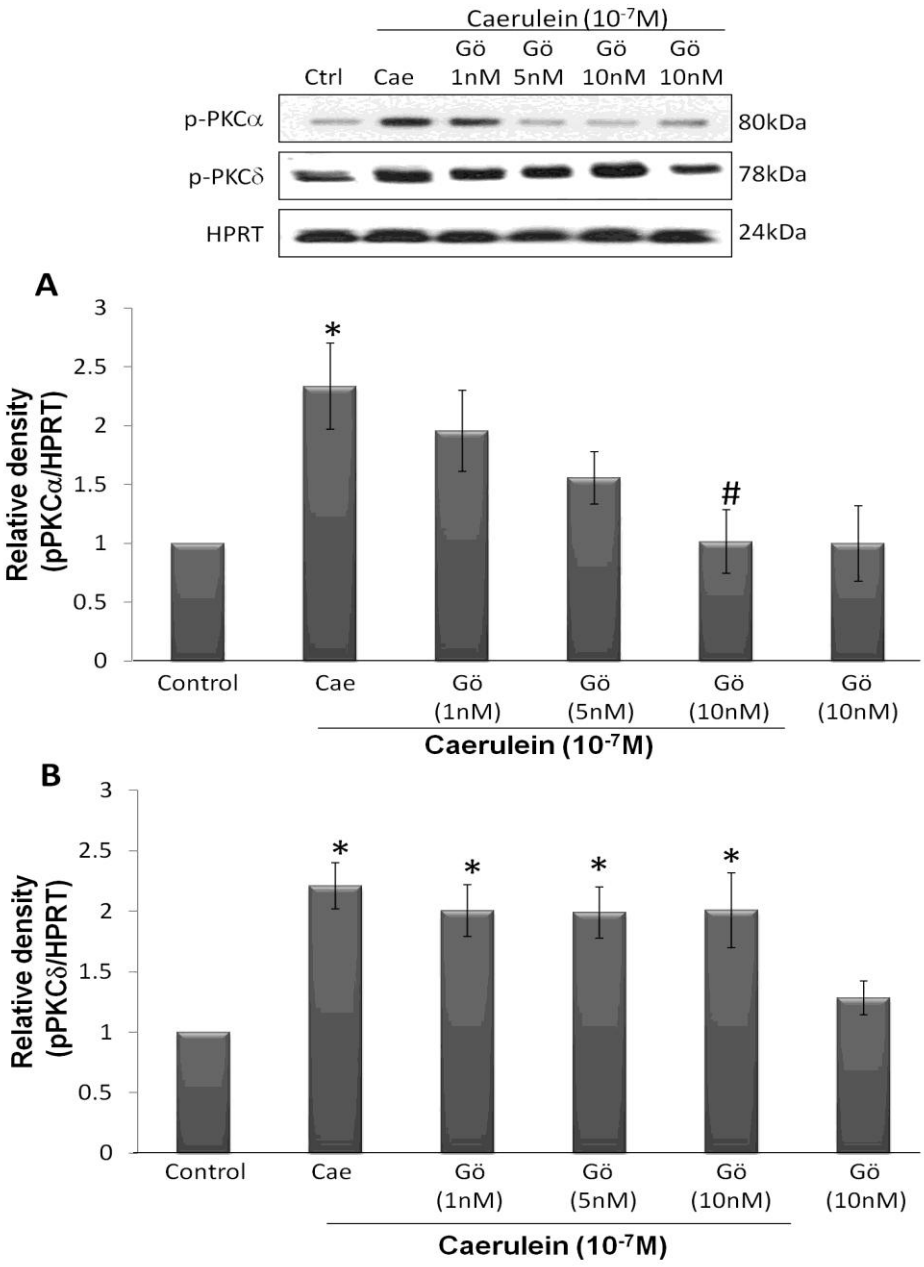


Figure 3.8 Caerulein induces phosphorylation of PKCα and PKCδ in mouse pancreatic acinar cells. Freshly prepared cells were treated with caerulein (10^{-7}M) for 2, 5, 15, 30, and 60 minutes. Whole cell lysates were prepared and 50μg of protein were fractionated on 10% SDS page gels for Western Blot analysis of phospho-PKCα (Thr^{638}), phospho-PKCδ (Thr^{505}) and HPRT. A) Representative blots of phospho-PKCα and phospho-PKCδ from 6 independent experiments. B) Time response study on PKCα phosphorylation. C) Time response study on PKCδ phosphorylation. Results are expressed as means ± SEM from 6 independent experiments. *P<0.05 vs. control.

3.3.9 Effect of Gö6976 and Rottlerin on PKCα and PKCδ phosphorylation

After determining the time course response of PKCα and PKCδ, we proceed to investigate the effect of Gö6976 and rottlerin on PKCα and PKCδ activation. From the previous data, we chose a stimulation timing of 15 minutes, as the response at this time is greatest and more stable for both types of studied PKC. Pancreatic acinar cells were pre-treated with Gö6976 (1, 5 and 10nM) or rottlerin (1, 5 and 10) for 45 minutes followed by caerulein (10^{-7}M) stimulation. Western blot analysis indicate that Gö6976 (IC_{50}=2.3nM) showed a concentration dependent inhibition of PKCα and PKCα phosphorylation is completely reversed at a concentration of 10nM (Figure 3.9A). However, Gö6976 does not inhibit PKCδ at the concentrations used (Figure 3.9B). On the other hand, rottlerin (IC_{50}=3μM) significantly inhibited PKCδ phosphorylation even at a low concentration of 1μM (Figure 3.9C). Rottlerin pre-treatment also showed a very small decrease of PKCα activation when compared to the caerulein treated group but they are far from statistical significance (Figure 3.9D). Treatment of cells with the used inhibitors alone does not significantly affect the phosphorylation of PKCα and PKCδ.


Caerulein (10-7M)
Ctrl
Cae
Gö 1nM
Gö 5nM
Gö 10nM
Gö 10nM
p-PKCα
p-PKCδ
HPRT
80kDa
78kDa
24kDa
A
Relative density (pPKCα/HPRT)
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)
B
Relative density (pPKCδ/HPRT)
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)

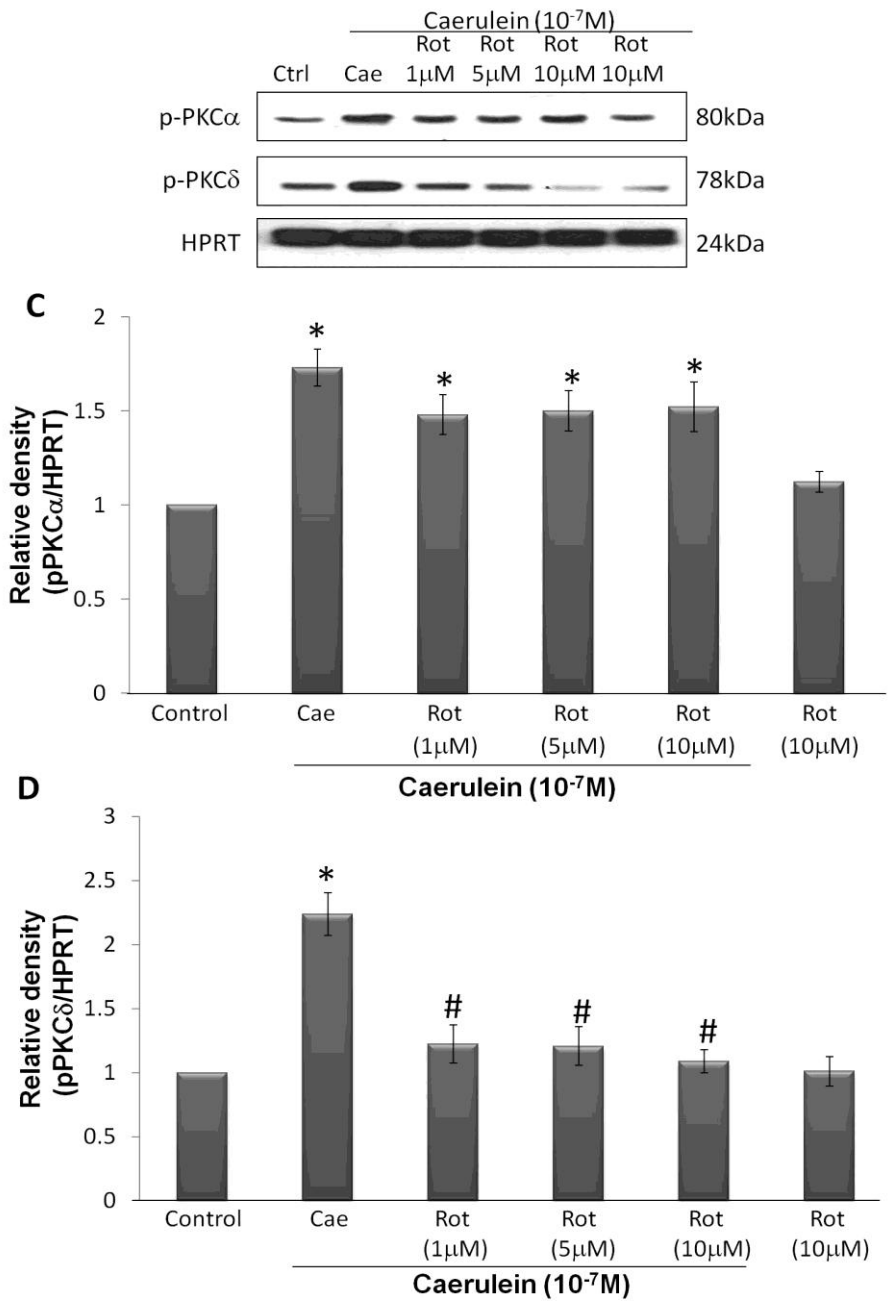
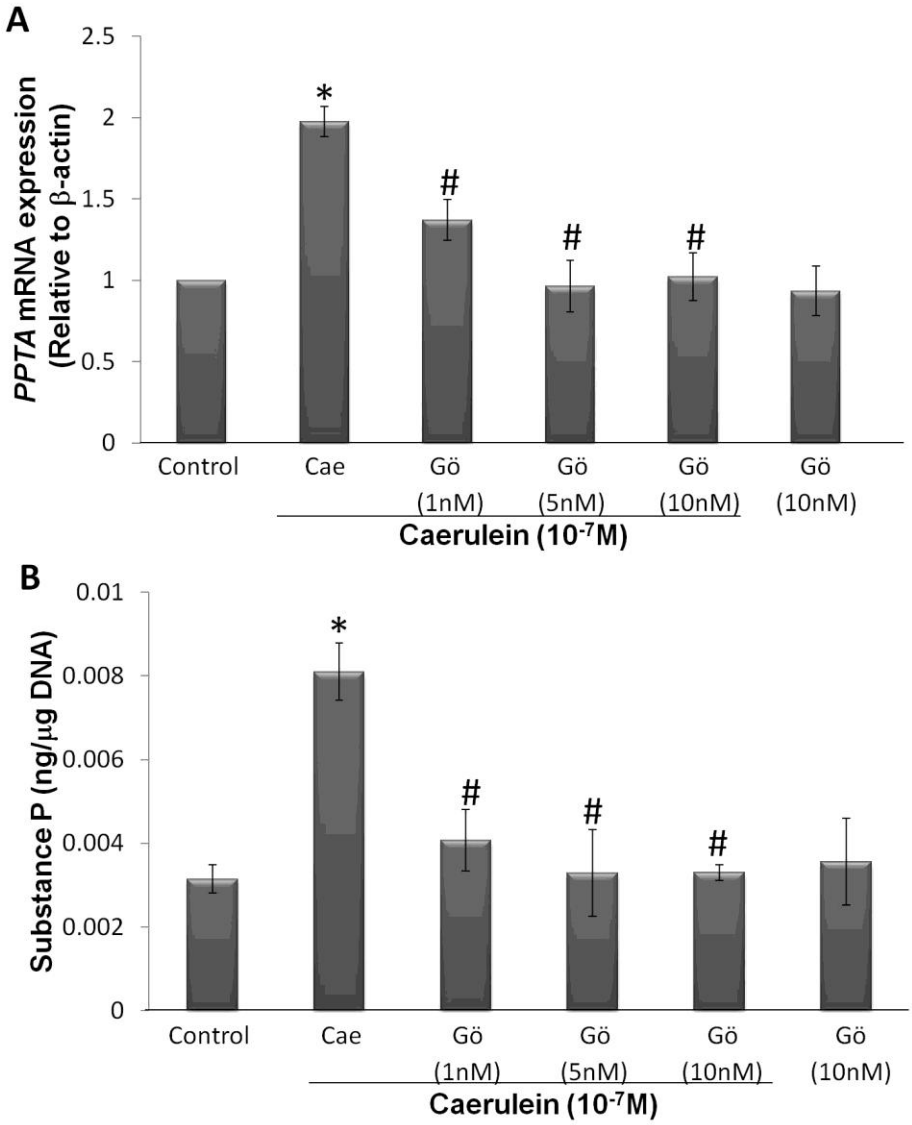


Figure 3.9 The effect of rottlerin and Gö6976 on PKCα and PKCδ phosphorylation. Pancreatic acinar cells were pre-treated with Gö6976 (1-10nM) or rottlerin (1-10μM) for 45 minutes before caerulein (10^{-7}M) stimulation for 15 minutes. Whole cell lysates were prepared and 50μg of protein were fractionated on 10% SDS page gels for Western blot analysis of phospho-PKCα (Thr^{638}), phospho-PKCδ (Thr^{505}) and HPRT. A) Effect of Gö6976 on PKCα phosphorylation. B) Effect of Gö6976 on PKCδ phosphorylation. C) Effect of rottlerin on PKCδ phosphorylation. D) Effect of rottlerin on PKCδ phosphorylation. Results are expressed as means ± SEM from 6 independent experiments. *P<0.05, vs. control, #P<0.05 vs. caerulein.

3.3.10 PKCα and PKCδ are involved in caerulein-induced SP up-regulation in mouse pancreatic acinar cells

We have previously shown that caerulein hyperstimulation significantly up-regulated SP peptide expression and its gene, *PPTA*, in mouse pancreatic acinar cells. To examine the involvement of PKCα and PKCδ in this up-regulation, we pre-treated cells with Gö6976 and rottlerin. It was previously shown that Gö6976 and rottlerin are specific to PKCα and PKCδ respectively (Figure 3.9A-D). By using quantitative real time PCR, caerulein (10^{-7}M) significantly up-regulated *PPTA* mRNA expression when compared to the control. When the cells were pre-treated with Gö6976 or rottlerin, the gene expression of *PPTA* was significantly reduced (Figure 3.10A, 3.10C). In line with the observations for *PPTA*, the peptide expression of SP was significantly reversed with pre-treatment of Gö6976 or rottlerin, indicating that *PPTA* mRNA expression is one of the factors that mediate SP levels in pancreatic acinar cells (Figure 3.10B, 3.10D). Generally, there is a slight concentration dependant inhibitory effect observed for the experiments done, as there was a more complete inhibition when higher concentrations of inhibitors were used.


A
PPTA mRNA expression (Relative to β-actin)
2.5
2
1.5
1
0.5
0
*
#
#
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)
B
Substance P (ng/μg DNA)
0.01
0.008
0.006
0.004
0.002
0
*
#
#
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)

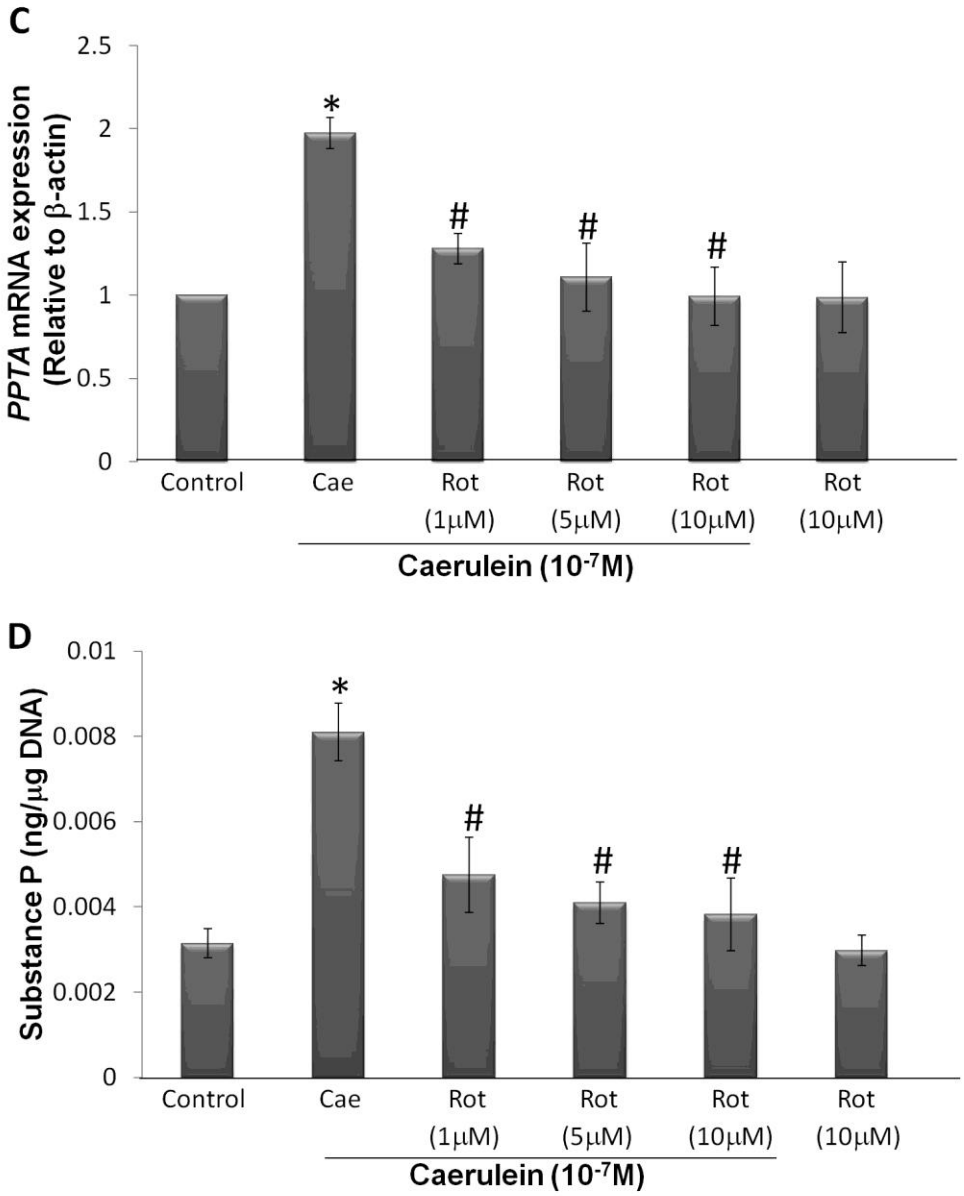
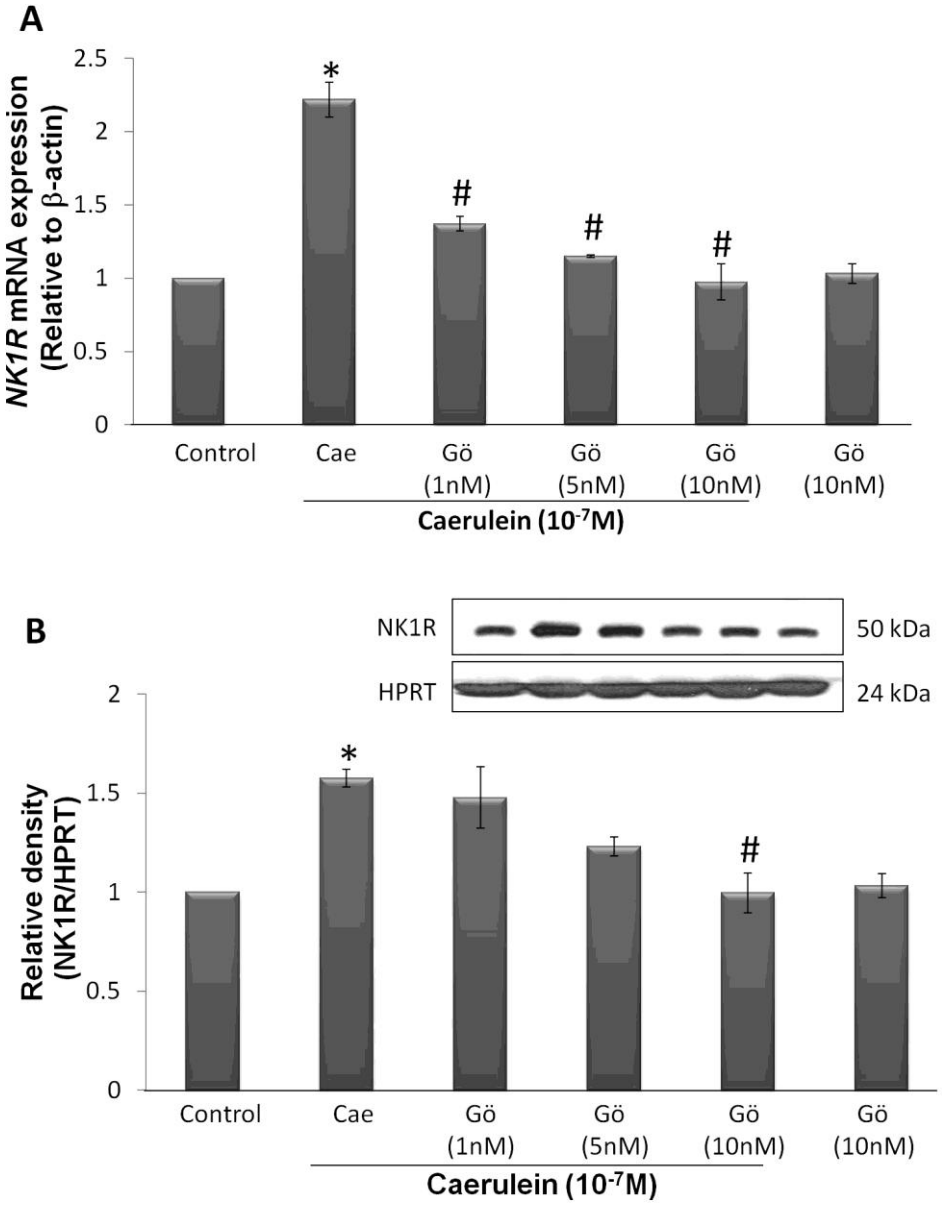


Figure 3.10 Caerulein stimulates PKCα and PKCδ mediated SP gene and protein expression. Freshly prepared pancreatic acinar cells were pre-treated with Gö6976 (1-10nM) or rottlerin (1-10μM) for 45 minutes before caerulein (10^{-7}M) stimulation for 60 minutes. *PPTA* mRNA expression was determined with real time PCR and SP levels were determined by a commercially available ELISA kit. *PPTA* mRNA expression was normalized with *β-actin* expression and SP levels was normalized with DNA content in the samples. A) *PPTA* mRNA expression of cells treated with Gö6976. B) SP expression of cells treated with Gö6976. C) *PPTA* mRNA expression of cells treated with rottlerin. D) SP expression of cells treated with rottlerin. Results are expressed as means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.11 PKCα and PKCδ are involved in caerulein-induced NK1R up-regulation in mouse pancreatic acinar cells

With also investigated the effect of PKC inhibition on the expression of NK1R. Using quantitative real time PCR, caerulein (10^{-7}M) significantly up-regulated *NK1R* mRNA expression when compared to the control. When the cells were pre-treated with Gö6976 or rottlerin, the gene expression of *NK1R* was significantly reduced (Figure 3.11A, 3.11C). Pre-treatment with either Gö6976 or rottlerin also inhibited the increased expression of NK1R protein (Figure 3.11B, 3.11D).


A
NK1R mRNA expression (Relative to β-actin)
2.5
2
1.5
1
0.5
0
*
#
#
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)
B
NK1R
50 kDa
HPRT
24 kDa
Relative density (NK1R/HPRT)
2
1.5
1
0.5
0
*
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)

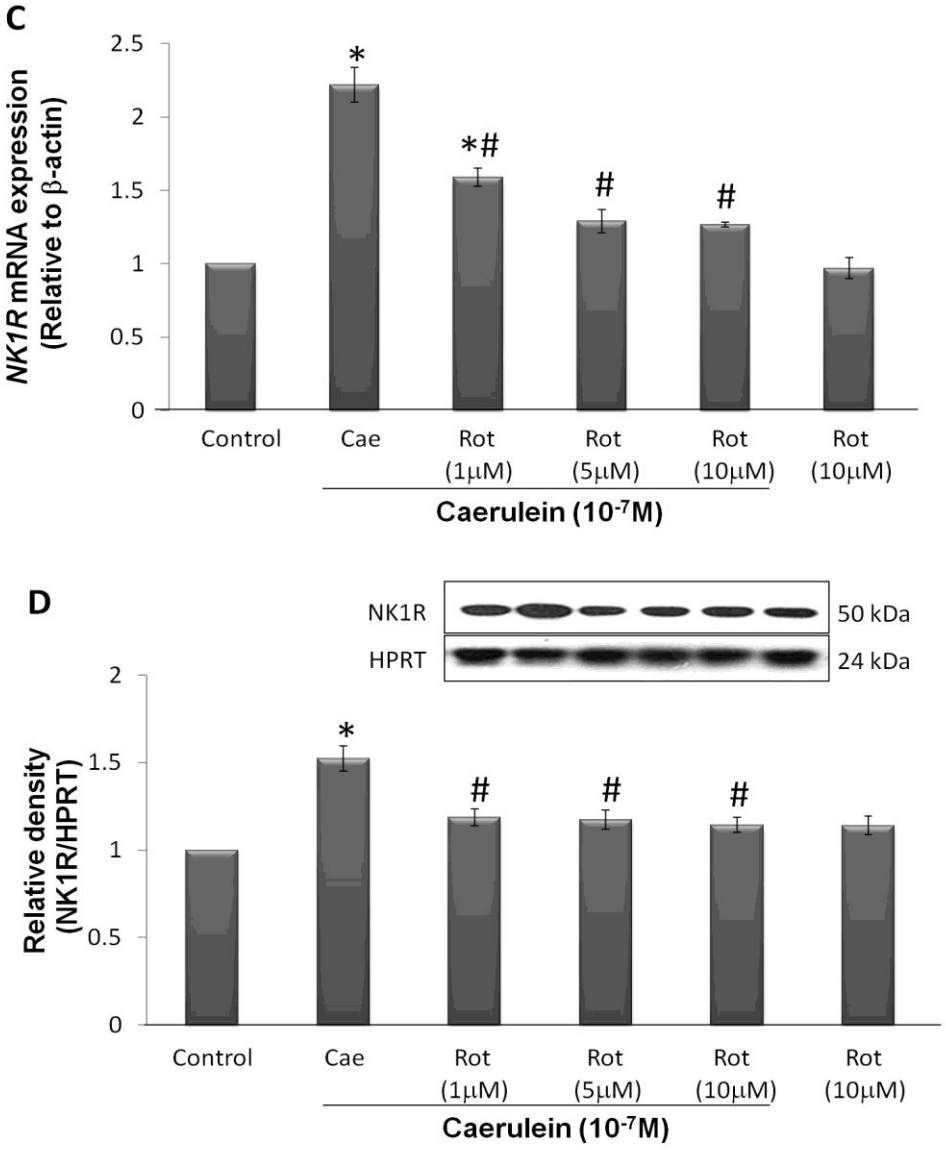
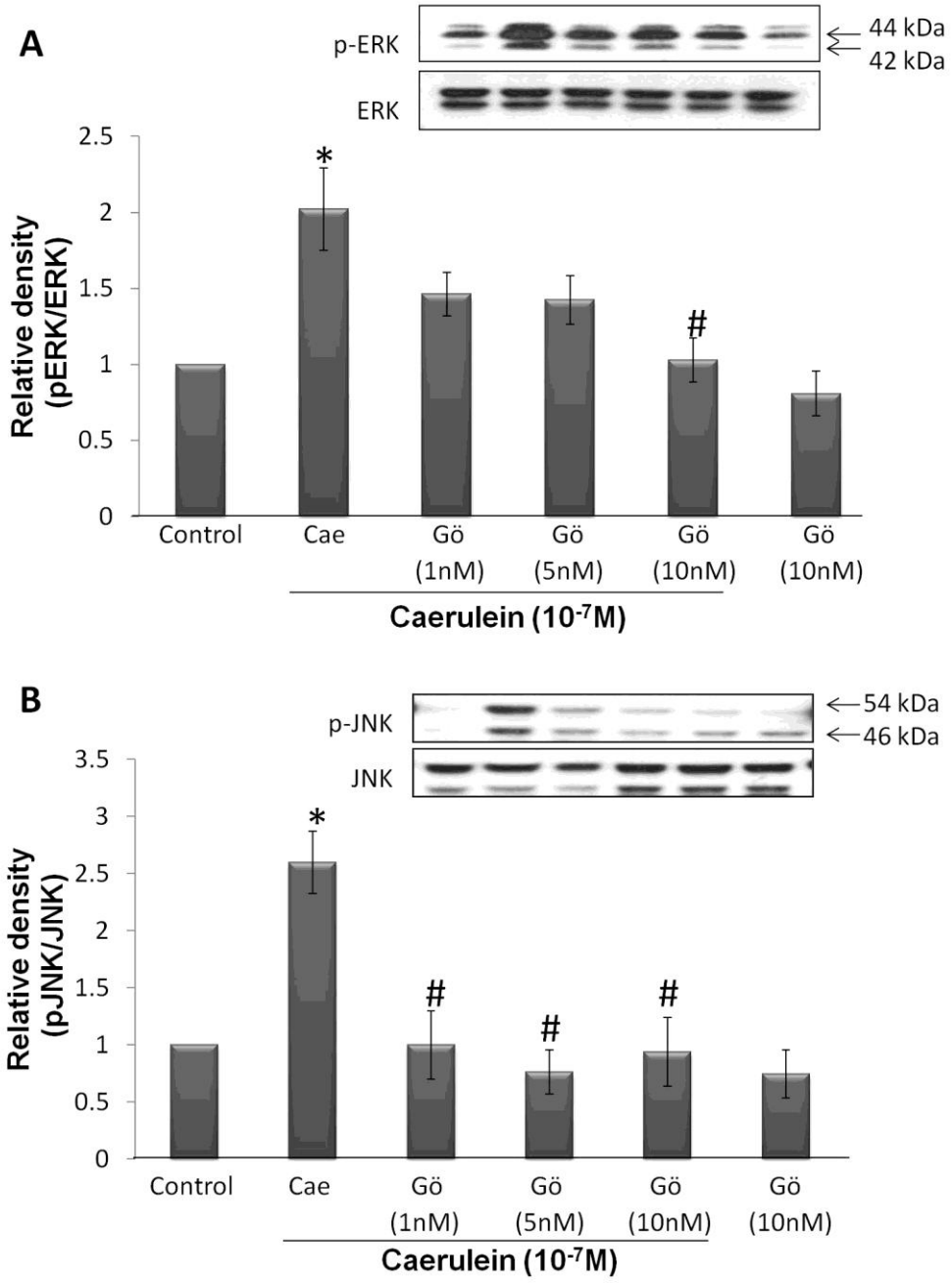


Figure 3.11 Caerulein stimulates PKCα and PKCδ mediated NK1R gene and protein expression. NK1R mRNA expression was determined with real time PCR and protein expression was determined by Western blotting. *NK1R* mRNA expression was normalized with *β-actin* expression and protein expression was normalized with HPRT expression in the samples. Freshly prepared pancreatic acinar cells were pre-treated with Gö6976 (1-10nM) or rottlerin (1-10μM) for 45 minutes before caerulein (10^{-7}M) stimulation for 60 minutes (mRNA expression) or 120 minutes (protein expression). (A) *NK1R* mRNA expression of cells treated with Gö6976. (B) NK1R protein expression of cells treated with Gö6976. (C) *NK1R* mRNA expression of cells treated with rottlerin. (D) NK1R protein expression of cells treated with rottlerin. Results are expressed as means ± SEM from 4-6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.3.12 PKCα and PKCδ are involved in caerulein induced ERK and JNK activation in mouse pancreatic acinar cells

The role of PKCs in caerulein induced SP/NK1R activation was further studied by looking at possible downstream signalling molecules. Our previous results have shown that the up-regulation of SP and NK1R involves the activation of the MAP kinases ERK and JNK. To determine if PKCα and PKCδ are involved in the activation of ERK and JNK, pancreatic acinar cells were treated with Gö6976 (1-10nM) or rottlerin (1-10μM) followed by caerulein stimulation. Western blot analysis of whole cell lysates revealed that ERK and JNK were significantly activated after stimulated with caerulein. Our results also showed that both Gö6976 and rottlerin inhibited the phosphorylation of ERK and JNK (Figure 3.12A-D). Furthermore, rottlerin treatment strongly inhibits JNK phosphorylation such that the rottlerin treated groups fell below control levels (Figure 3.12D). However, this decrease in JNK phosphorylation was not statistically significant in our study.


A
p-ERK
ERK
44 kDa
42 kDa
Relative density (pERK/ERK)
2.5
2
1.5
1
0.5
0
*
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)
B
p-JNK
JNK
54 kDa
46 kDa
Relative density (pJNK/JNK)
3.5
3
2.5
2
1.5
1
0.5
0
*
#
#
#
Control
Cae
Gö (1nM)
Gö (5nM)
Gö (10nM)
Gö (10nM)
Caerulein (10-7M)

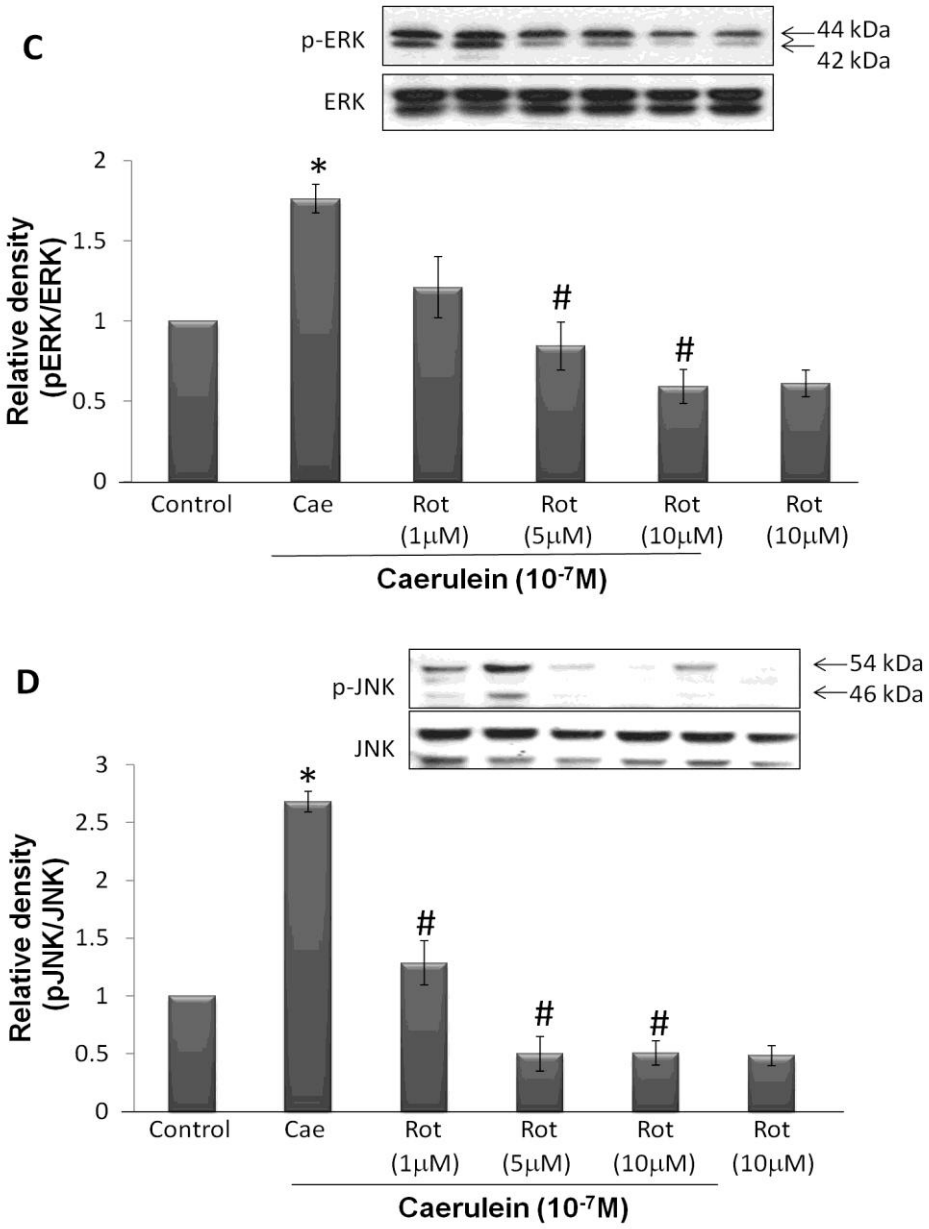


Figure 3.12 The activation of ERK and JNK in mouse pancreatic acinar cells is dependent on both PKCα and PKCδ. Freshly prepared pancreatic acinar cells were pre-treated with Gö6976 (1-10nM) or rottlerin (1-10μM) for 45 minutes before caerulein (10^{-7}M) stimulation for 60 minutes. Whole cell lysates were prepared and 50μg of protein were fractionated on 10% SDS page gels for Western blot analysis of phospho-JNK (Thr^{183}/Tyr^{185}), JNK, phospho-ERK(Thr^{202}/Tyr^{204}), and ERK. A) ERK activation of cells treated with Gö6976. B) JNK activation of cells treated with Gö6976. C) ERK activation of cells treated with rottlerin. D) JNK activation of cells treated with rottlerin. Results are expressed as means ± SEM from 4-6 independent experiments. *P<0.05vs. control, #P<0.05 vs. caerulein

3.3.13 Inhibition of PKCα and PKCδ attenuates caerulein induced NF-κB and AP-1 activation in mouse pancreatic acinar cells

We examined the effect of Gö6976 and rottlerin on transcription factor NF-κB and AP-1 in isolated pancreatic acinar cells. Nuclear fractions of treated cells were extracted. DNA binding activity was performed using an ELISA based method using nuclear fractions. Treatment of caerulein for 60 minutes significantly increased NF-κB and AP-1 DNA binding activity and this increase is attenuated by pre-treatment of cells with Gö6976 or rottlerin (Figure 3.13B, 3.13C). Due to a small difference observed in NF-κB DNA binding activity, we used an indirect method, the phosphorylation of IκB to further confirm NF-κB activation. Whole cells lysates were used to perform Western blot, and the results shown a significant increase in IκB phosphorylation, which was attenuated with Gö6976 or rottlerin pre-treatment (Figure 3.13A). The trend of IκB phosphorylation correlated with NF-κB binding activity, confirming the role of PKCα and PKCδ in the activation of transcription factor NF-κB.

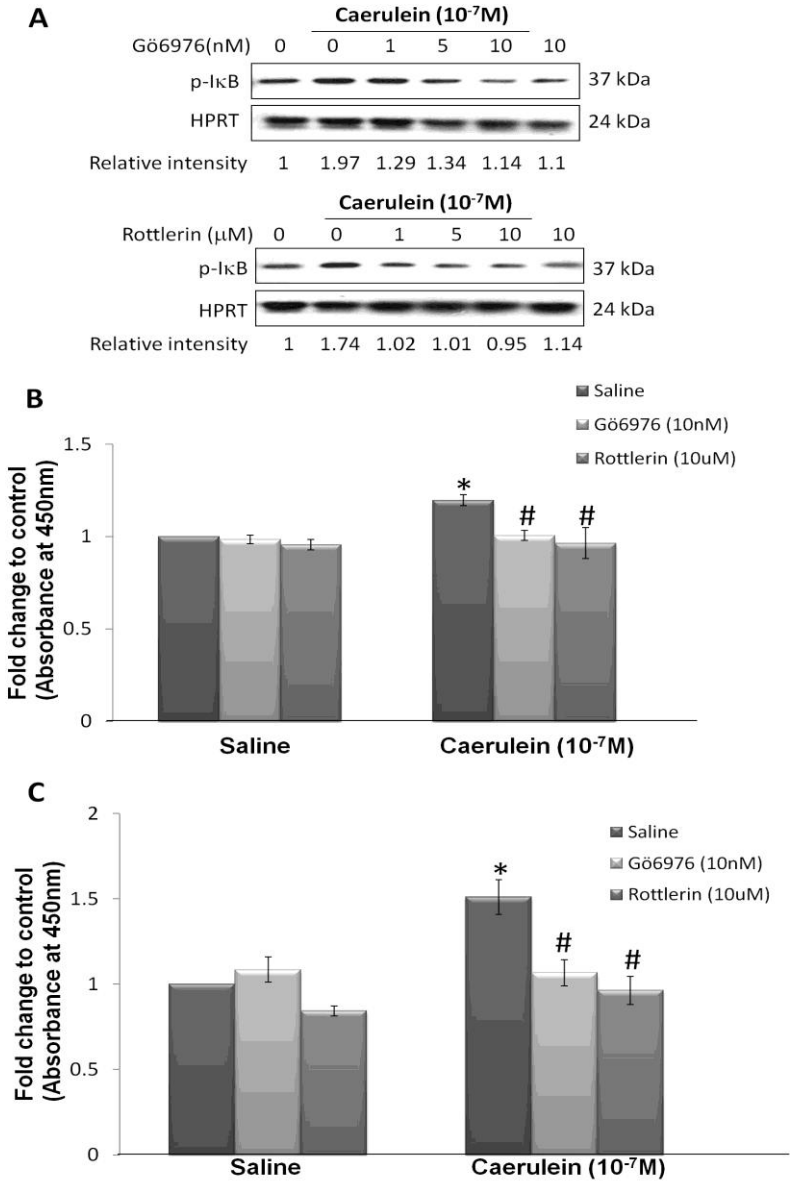


Figure 3.13 PKCα and PKCδ activation is involved in the DNA binding activity of NF-κB and AP-1. Pancreatic acinar cells were pre-treated with Gö6976 (1-10nM) or rottlerin (1-10μM) for 45 minutes before caerulein (10^{-7}M) stimulation for 60 minutes. Subsequently, nuclear extracts was obtained and AP-1 and NF-κB DNA binding ability was determined by using a commercially available ELISA kit. Whole cell lysates were prepared for Western blot analysis of phospho-IκB (Ser^{32}) and HPRT. A) Representative phospho-IκB Western blot results and relative intensity are averages of 6 independent experiments. B) NF-κB DNA binding activity. C) AP-1 DNA binding activity. Results are expressed as the means ± SEM from 6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

3.4 DISCUSSION

Intracellular signaling molecules such as the MAPKs, cyclic AMP (cAMP), protein kinase C (PKC) and NF-κB are known to be activated upon induction of AP (Tando et al., 1999; Lu et al., 2003; Bi and Williams, 2004; Thrower et al., 2008). Previous studies had showed that activation of ERK and JNK induced cytokine production and inhibition of ERK and JNK showed protective effects (Samuel et al., 2006; Namkung et al., 2008; Samuel et al., 2008). In agreement with previous studies, we demonstrated that supraphysiological concentrations of caerulein treatment induced ERK and JNK phosphorylation. The observed results suggested that induction of ERK and JNK occurred when the cell was experiencing stress and injury; whereas at physiological stimulations the phosphorylation of ERK and JNK remained at basal levels.

Studies utilizing small molecule MAPK inhibitors directly demonstrated the involvement of these molecules in SP/NK1R up-regulation. The MEK inhibitor PD98059 and JNK inhibitor SP600125 significantly inhibited the phosphorylation of ERK and JNK respectively in stimulated pancreatic acinar cells. We found that pre-treatment of PD98059 on caerulein-stimulated pancreatic acinar cells attenuated the transcription of *NK1R* gene. On the other hand, SP600125 treatment attenuated the transcription of both *PPTA* and *NK1R* gene, as well as SP peptide. These results suggest that caerulein-induced up-regulation of SP and NK1R goes through different pathways. Activation of ERK is involved in NK1R expression and activation of JNK is involved in both NK1R and SP expression. This observation is not surprising as *PPTA* and *NK1R* are encoded by two entirely different genes, and thus their activation mechanism would be different.

The regulation of MAPKs depends on the phosphorylation and dephosphorylation at its activation site (Kondoh and Nishida, 2007; Boutros et al., 2008). We found that higher concentrations of PD98059 and SP600125 tended to downregulate ERK and JNK activation when compared to the control. However, these observations were not statistically significant. On the other hand, basal phosphatase activity continued to dephosphorylate ERK and JNK. Therefore, a shift in thebalance towards dephosphorylation caused a decrease of MAPK activity, and this might affect other cellular activities should the effect is significant.

NF-κB and AP-1 are transcription factors frequently linked to pathological inflammation and the up-regulation of inflammatory mediators (Ghosh and Hayden, 2008; Zenz et al., 2008). Supraphysiological concentrations of caerulein treatment caused significant degradation of IκB in the pancreatic acinar cells, but did not occur at lower concentrations of caerulein. We also observed that DNA binding activity of NF-κB p65 subunit complies with caerulein-induced IκB degradation in the pancreatic acinar cells. Our observations suggest that the conventional pathway of NF-κB activation, which involves IκB degradation, is involved in caerulein-stimulated pancreatic acinar cells. Another important transcription factor, AP-1, was found to have a dose-dependent activation pattern similar to that of NF-κB activation.

We also looked at the possible relationship between the intracellular kinases (ERK and JNK) and the transcription factors (NF-κB and AP-1). JNK is widely known to be an activator of AP-1 (Karin and Gallagher, 2005), but other possible interactions between ERK, JNK, NF-κB and AP-1 are poorly understood. Kook *et al*. reported that inhibitors of ERK and JNK inhibited AP-1 DNA binding and c-Fos/c-Jun activation in human periodontal ligament fibroblasts (Kook et al., 2009). Jiang *et al*. also showed an ERK dependent activation of NF-κB in IL-1β induced rat vascular

smooth muscle cells (Jiang et al., 2004). In agreement with Ramnath *et al*, our results pointed as well to an ERK/JNK-dependent activation of NF-κB and AP-1 in pancreatic acinar cells (Ramnath et al., 2007). We demonstrated that administration of PD98059 and SP600125 significantly inhibited both NF-κB p65 subunit and AP-1 DNA binding activity in caerulein stimulated pancreatic acinar cells. These observations suggest a possible role of MAPKs in regulating the nuclear translocation that lead to the activity of NF-κB p65 subunit and AP-1 during stimulation by caerulein. Our results also add to the growing evidence about MAPKs in regulating the activity of NF-κB and AP-1 (Kundu et al., 2006; Bladh et al., 2009).

NF-κB is generally known to control the expression of numerous inflammatory mediators. We used an irreversible inhibitor of IκB phosphorylation, Bay 11-7082 to investigate the role of NF-κB in caerulein-stimulated SP/NK1R up-regulation. Our results showed directly that NF-κB is involved in the transcription of *NK1R* and *PPTA*, as inhibition of NF-κB by Bay 11-7082 abolished the up-regulation of these products. The increased transcription of *PPTA* is probably contributed to the increased expression of SP. These observations were in agreement with the results done on other models, which also highlighted the involvement of NF-κB activation in the increased expression of *PPTA* and *NK1R* (Guo et al., 2004; Reed et al., 2005; Corcoran and Rameshwar, 2007).

Our results had shown that inhibition of ERK phosphorylation by PD98059 caused inhibition of DNA binding activity of NF-κB. Interestingly, pre-treatment with NF-κB inhibitor Bay 11-7082, but not ERK inhibitor PD98059, inhibited the up-regulation of *PPTA* and SP in caerulein stimulated pancreatic acinar cells. These results suggest that the up-regulation of *PPTA* and SP is independent of ERK

activation, but might be dependent on NF-κB activation. This may seem inconsistent, but we should take into consideration that it is still unclear how the activation of ERK lead to the activation of NF-κB. It is possible that when ERK is inactivated, an alternative mechanism is available to maintain the high expression of *PPTA* and SP in caerulein stimulated pancreatic acinar cells. Another possibility that Bay 11-7082 may have non-specific effects in our caerulein model. Thus, we have found that AP-1 DNA binding ability was inhibited when high concentrations of Bay 11-7082 were used (Data not shown). Therefore, there is a possibility that AP-1, but not NF-κB, is involved in the up-regulation of *PPTA* and SP. This explanation requires further confirmation as there is little evidence to challenge the specificity of Bay 11-7082. On the contrary, the ERK dependent NF-κB activation is probably involved in mediating the gene expression of *NK1R*, as inhibition of ERK by PD98059, as well as inhibition of NF-κB activation by Bay 11-7082, reversed the up-regulation of *NK1R* mRNA expression.

In the second part of this chapter, we investigate the role of PKCs on caerulein-mediated SP/NK1R expression in murine pancreatic acinar cells.

We first identified that both PKCα and PKCδ are activated in a time dependent manner during supramaximal caerulein exposure. This is shown by a rapid phosphorylation at key activation sites of the molecule. The phosphorylation of PKCα is transient, as we observed a decrease of phosphorylation around 60 minutes of caerulein treatment, when compared with earlier time points. Caerulein has previously been shown to activate PKCδ for various cellular responses in pancreatic acinar cells. However, the activation of PKCα is still not clear in high concentrations of CCK/caerulein used. Satoh *et al.* did not observe increased PKCα translocation to

the membrane after 30 minutes of supramaximal CCK stimulation (Satoh et al., 2004). On the other hand, Cosen-Binker *et al*. found significant increase in PKCα activity after treating pancreatic acinar cells with CCK (Cosen-Binker et al., 2007). Previous studies have shown an excessive rise in Ca^{2+} levels in caerulein-induced pancreatic acinar cells (Okada et al., 1998). Therefore, the Ca^{2+} dependent activation of PKCα is very likely to occur. The different incubation time, animal strain used, or detection methods might explain discrepancies between studies, and further investigation will be needed to address the issue.

In order to differentiate the role of PKCα and PKCδ in caerulein stimulated cells, we employed the use of isozyme specific pharmacological inhibitors. Gö6976 (IC_{50}=2.3nM) is a Ca^{2+} dependent PKC isozyme inhibitor, and therefore it was used to block the actions of PKCα in our study (Martiny-Baron et al., 1993). Rottlerin (IC_{50}=3μM) has some selectivity towards PKCδ and is widely used to investigate the cellular mechanisms mediated by PKCδ (Gschwendt et al., 1994; Li et al., 2004; Ramnath et al., 2008). In an attempt to avoid cross-inhibition of PKCα and PKCδ, we tested the inhibitors with reference to the reported IC_{50} values. The efficacy and specificity of the two inhibitors was tested and confirmed in our results. Gö6976 showed some inhibition of PKCα at a concentration of 1nM and further inhibited PKCα phosphorylation when the concentration was increased to 10nM. On the other hand, rottlerin showed a complete inhibition of PKCδ phosphorylation when a concentration of 1-10μM was used. At these concentrations used, Gö6976 and rottlerin did not significantly block the other form of studied PKC. This could then be applied to differentiate the role of conventional PKCα and novel PKCδ in caerulein-induced SP/NK1R up-regulation in our model. Despite the specificity shown in PKC

inhibition, rottlerin has also recently been shown to affect several PKC-independent mechanisms, mostly related to mitochondrial uncoupling pathway (Soltoff, 2001; Tapia et al., 2006). Therefore, it is important to control the concentration of rottlerin used and caution exercised when interpreting the results.

We then employed the use of selective PKCα inhibitor Gö6976, and selective PKCδ inhibitor rottlerin, to observe the role of PKCs on SP/NK1R expression. Our data indicated that pre-treatment with either Gö6976 or rottlerin significantly attenuated caerulein-induced SP up-regulation. Furthermore, real time PCR analysis revealed that mRNA expression of *PPTA* was significantly reduced with Gö6976 or rottlerin pre-treatment. Studies done on NK1R expression also showed similar results that pre-treatment of pancreatic acinar cells with either Gö6976 or rottlerin significantly attenuated *NK1R* mRNA and protein expression. The concentration of Gö6976 and rottlerin required to reverse SP and NK1R up-regulation was also in line with the concentration required to inhibit phosphorylation of PKCα and PKCδ. These observations suggested that the PKCs play a role in mediating the gene expression of *PPTA* and *NK1R*, which in turn cause an increase in protein expression of SP and NK1R in the mouse pancreatic acinar cells. Overall, our data showed that activation of both conventional PKCα and novel PKCδ is equally important in caerulein-induced SP/NK1R up-regulation in the mouse pancreatic acinar cells.

Evidence from several models demonstrated the relationships between PKCs and MAPKs in the expression of genes. Some suggested that the PKC family, as a whole, activate the MAPK pathway (Lin et al., 2009); while many others attempted to identify the role of individual PKC isoforms in the activation of MAPKs (Yoshida et al., 2002; Cheng et al., 2009). There is also evidence that MAPKs is activated by

more than one PKC isoforms. Sun *et al*. suggested in a macrophage cell line that SP induced ERK phosphorylation via activation of both PKCα, PKCδ and PKCε (Sun et al., 2009). Therefore, there is still much unknown regarding the role of individual PKC isoforms in the activation of MAPKs. In this current study, we used the inhibitors that had been shown to inhibit SP and NK1R to investigate its effect on ERK and JNK activation. We found that pretreatment with Gö6976 or rottlerin significantly attenuated caerulein-induced JNK and ERK phosphorylation, showing that PKCα and PKCδ are up-stream of the MAPKs. Our results were consistent with the findings that rottlerin treatment affected the phosphorylation of JNK and ERK in SP-treated mouse pancreatic acinar cells (Ramnath et al., 2008). Gö6976 or rottlerin treatment was also found to inhibit renin receptor expression and osteoclastogenesis via the PKC-MAPK pathways (Huang and Siragy; Tiedemann et al., 2009). We also noticed that there is a consistent, but insignificant decrease of JNK phosphorylation with rottlerin pre-treatment. This could be due to baseline phosphatase activity, which dephosphorylates JNK when PKC kinase activity is inhibited by the drug.

In pancreatic acinar cells, it was suggested that stimulation of CCK receptors could modulate gene expression via activation of several transcription factors, such as NF-κB and AP-1 (Orlichenko et al.; Gukovsky et al., 2003). We have also shown that caerulein induced up-regulation of SP and NK1R expression was mediated via activation of ERK and JNK, followed by transcription factors NF-κB and AP-1 (Koh et al., 2010). In this study, we found that PKCα and PKCδ activation is necessary for the activation of MAPKs, and therefore it is logical to speculate that PKCs are located upstream of NF-κB and AP-1. Both Gö6976 and rottlerin has been shown frequently to inhibit the activation of NF-κB and AP-1 in various models (Bhatt et al.; Langlet et

al.; Kontny et al., 2000; Shin et al., 2007). However, there are reports suggesting PKC-independent activation of NF-κB (Meichle et al., 1990; Shames et al., 1999) . Results from a study from Holden *et al*. suggested that the conflicting results observed could be due to different stimulating agents used. In that report, PKC activation was not involved in tumor necrosis factor-α (TNFα) induced NF-κB activation, whereas phorbol 12-myristate 13-acetate (PMA) induced NF-κB activation was found to require the activation of novel PKCs in pulmonary A549 cells (Holden et al., 2008). Our data showed that cells pre-treated with Gö6976 or rottlerin significantly inhibited caerulein stimulated increase of NF-κB and AP-1 DNA binding activity. Due to the relatively small changes in NF-κB DNA binding activity observed, we verified the activation of NF-κB by checking the phosphorylation of IκB, which is an important step for removing the inhibition of NF-κB translocation into the nucleus. Western blot results of IκB phosphorylation showed significant results with a greater magnitude, while maintaining a similar response pattern with NF-κB DNA binding activity. Therefore, we suggest caerulein-induced PKCα and PKCδ activation is necessary for the activity of NF-κB and AP-1, which in turn required for SP and NK1R up-regulation.

Taken together, the results presented in this chapter demonstrated several key elements leading to the up-regulation of SP and NK1R in the mouse pancreatic acinar cells. Caerulein activates both PKCα and PKCδ, which in turn activated ERK and JNK, followed by an increased transcriptional activity of NF-κB and AP-1. These mechanisms eventually lead to the increased expression of SP and NK1R at both genetic and protein levels (Figure 3.14, 3.15). Overall, we did not find a difference in the role of conventional PKCα and novel PKCδ, as activation of both molecules are

necessary for the cellular responses observed. These results provide a clearer picture on the mechanisms that lead to up-regulation of SP and NK1R; thus providing the basis for a possible therapy for AP via controlling expression levels of SP or NK1R.

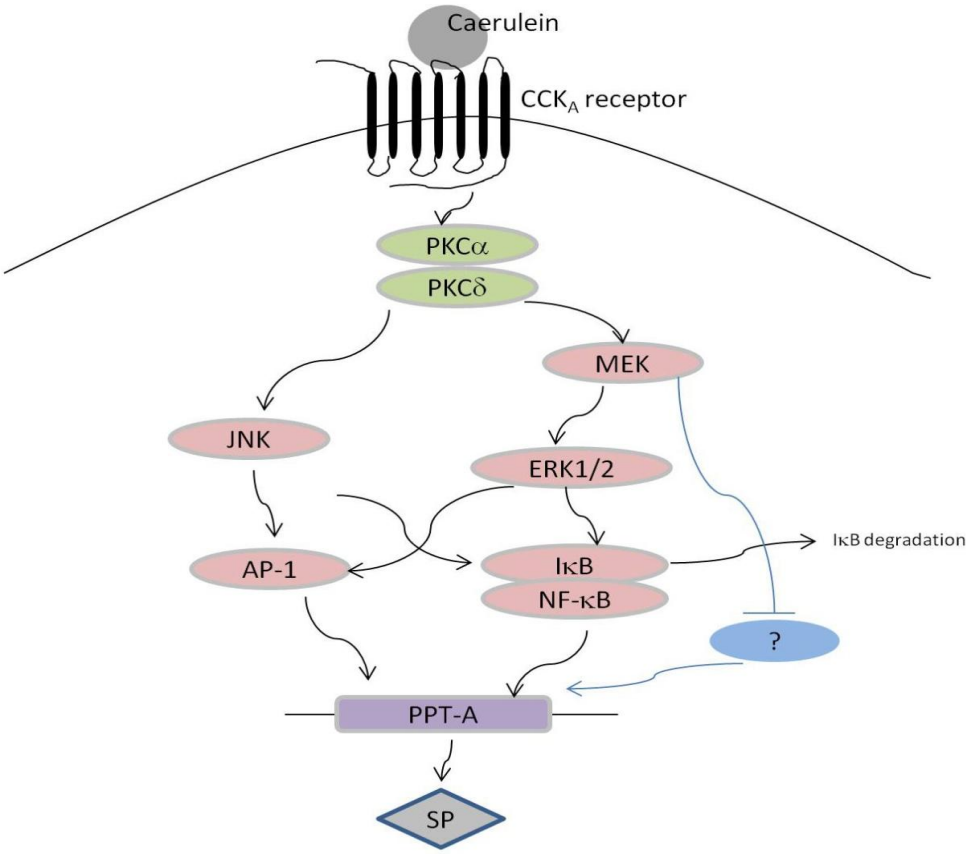


Figure 3.14 A schematic illustration of caerulein-induced up-regulation of SP in mouse pancreatic acinar cells.

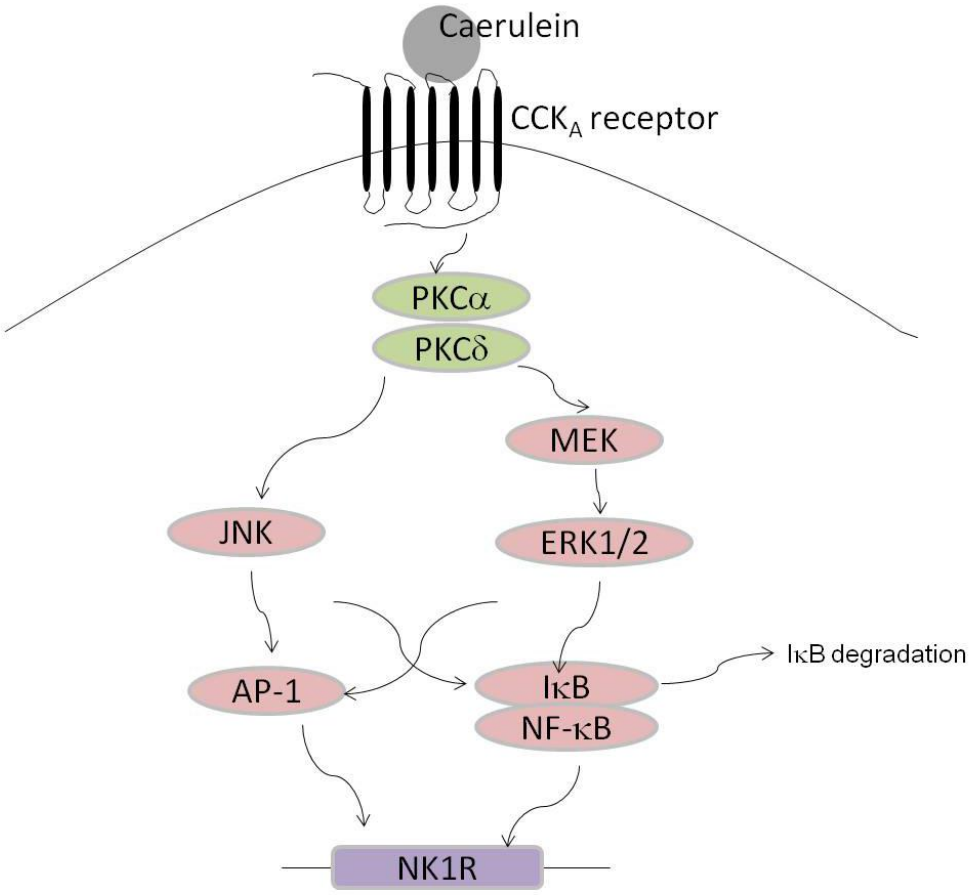


Figure 3.15 A schematic illustration of caerulein-induced up-regulation of NK1R in mouse pancreatic acinar cells.

CHAPTER 4: ACTIVATION OF NEUROKININ-1 RECEPTORS UP-REGULATES SUBSTANCE P AND NEUROKININ-1 RECEPTOR EXPRESSION IN MURINE PANCREATIC ACINAR CELLS

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4.1 INTRODUCTION

Although the importance of SP-NK1R interaction in AP and other inflammatory diseases has been brought into focus, mechanisms that regulate their expression were less explored. The expression of both SP and NK1R were shown to be up-regulated in the pancreas and lung in caerulein-induced AP (Bhatia et al., 1998). Similar up-regulation was observed when isolated mouse pancreatic acinar cells were incubated with caerulein (10^{-7}M). In a previous study, antagonism of NK1R with CP96,345 significantly reversed AP-induced *PPTA*/*NK1R* mRNA up-regulation in the pancreas (Lau and Bhatia, 2006). In other words, NK1R activation might be involved in SP/NK1R expression in mice. This observation hinted the possibility of an auto-regulatory mechanism in the pancreas, where activation of NK1R contributed to the overall elevated expression of SP and NK1R.

In chapter 2 and 3, we have recently demonstrated that treatment of mouse pancreatic acinar cells with caerulein induced SP/NK1R up-regulation via activation of CCK_A receptors. The response is rapid, with a significant up-regulation occurring within one hour of stimulation. Activation of CCK_A receptors causes subsequent PKC – MAPK – NF-κB/AP-1 activation, and leads to up-regulation of SP and NK1R expression. On the other hand, activation of NK1R is also known to activate similar pathways via its G-protein coupled downstream signaling (Ramnath et al., 2008). In mouse pancreatic acinar cells, it was shown that treatment of cells with SP up-regulated chemokine expression by a Src-Ca^{2+}-PKC-MAPK-NF-κB dependent pathway (Ramnath et al., 2009). Hence, there are similarities between NK1R and CCK_A receptors with regard to their downstream signaling pathways

In this chapter, we aim to examine the relationship between exposure to SP and the expression of SP and NK1R in isolated mouse pancreatic acinar cells. We assessed both protein and mRNA expression levels of SP and NK1R after pancreatic acinar cells were treated with SP or a selective NK1R agonist, GR73,632. In some experiments, we used CP96,345, a selective NK1R antagonist, or specific inhibitors of signaling molecules to elucidate the signaling pathway involved. Finally, we also compared the differences between SP-mediated responses with caerulein-mediated responses.

4.2 MATERIALS AND METHODS

4.2.1 Animals and chemicals.

Refer to section 2.2.1. Swiss mice (Male, 25-30 grams) were used for isolation of pancreatic acinar cells. PD98059 (Mitogen-activated protein kinase kinase (MEK) inhibitor), SP600125 (JNK inhibitor), Bay11-7082 (NF-κB inhibitor), Gö6976 (PKCα/β inhibitor) and rottlerin (PKCδ inhibitor), were purchased from Calbiochem (Darmstadt, Germany). CP96,345 (selective NK1R antagonist) and GR73,632 (selective NK1R agonist) was obtained from Sigma-Aldrich (Singapore). They were all dissolved in DMSO. SP and caerulein was purchased from Bachem (Torrance, CA) and dissolved in normal saline. All chemicals were purchased with the highest purity available.

4.2.2 Preparation and treatment of pancreatic acinar cells.

Refer to section 2.2.2. Freshly isolated pancreatic acinar cells were stimulated with caerulein (10^{-7}M), SP (10^{-6}M), or GR73,632 (10^{-6}M-10^{-10}M). The concentration of caerulein and SP used in this study was shown to activate NF-κB dependent gene

expression in pancreatic acinar cells (Ramnath and Bhatia, 2006; Koh et al., 2010). For time-dependent studies, cells were treated with caerulein or SP for 30, 60, 90, 120 minutes in a 37ºC water bath. For other experiments, cells were pre-treated with either Gö6976 (10nM), rottlerin (5μM), PD98059 (30μM), SP600125 (30μM), Bay11-7082 (30μM), or CP96,345 for 30 minutes before addition of caerulein, SP or GR73,632 (10^{-7}M). The concentration of these inhibitors was previously established and showed to be effective in caerulein-treated pancreatic acinar cells (Chapter 3). Gö6976, rottlerin, PD98059, SP600125, Bay11-7082, and CP96,345 were dissolved in dimethyl sulfoxide (DMSO) and diluted with ***buffer A***. The final concentration of DMSO in the cell culture is less than 1 percent. Other chemicals were dissolved in normal saline and diluted with ***buffer A***.

4.2.3 Substance P extraction and detection.

Refer to section 2.2.4.

4.2.4 RNA isolation and quantitative real time PCR analysis.

Refer to section 2.2.6 and 2.2.8

4.2.5 Whole cell lysate preparation and Western blot analysis.

Refer to section 2.2.9.

4.2.6 Statistical analysis.

Refer to section 2.2.10.

4.3 RESULTS

4.3.1 Substance P induces *PPTA* and *NK1R* mRNA expression in murine pancreatic acinar cells

In order to understand the role of SP-NK1R interaction in SP/NK1R expression, we first examine the effect of exogenous SP on *PPTA*/*NK1R* mRNA expression in pancreatic acinar cells. As we have showed that *PPTA* and *NK1R* mRNA expression could be induced by caerulein (Chapter 2), gene expression levels of caerulein-induced cells were compared alongside with SP induced-cells. To do this, freshly isolated pancreatic acinar cells were treated with either SP (10^{-6}M) or caerulein (10^{-7}M) for up to 120 minutes. After incubation of cells with SP, both *PPTA* and *NK1R* mRNA expression showed a time-dependant increase, as determined by real-time PCR assay. Expression levels start to rise significantly after 30 minutes, and peaked in 1-2 hours after treatment with SP (Figure 4.1A, 4.1B). In comparison, both SP-induced cells and caerulein-induced cells showed a similar 2-fold increase of *PPTA* mRNA expression (Figure 4.1A). On the other hand, *NK1R* expression in SP-treated cells (1.6 fold increase) was considerably lower when compared to caerulein-treated cells (2.5 fold increase) (Figure 4.1B). In our studies, mRNA expression of *β-actin*, which was used as a housekeeping gene, showed no significant changes in expression levels in all experiments groups (Data not shown).

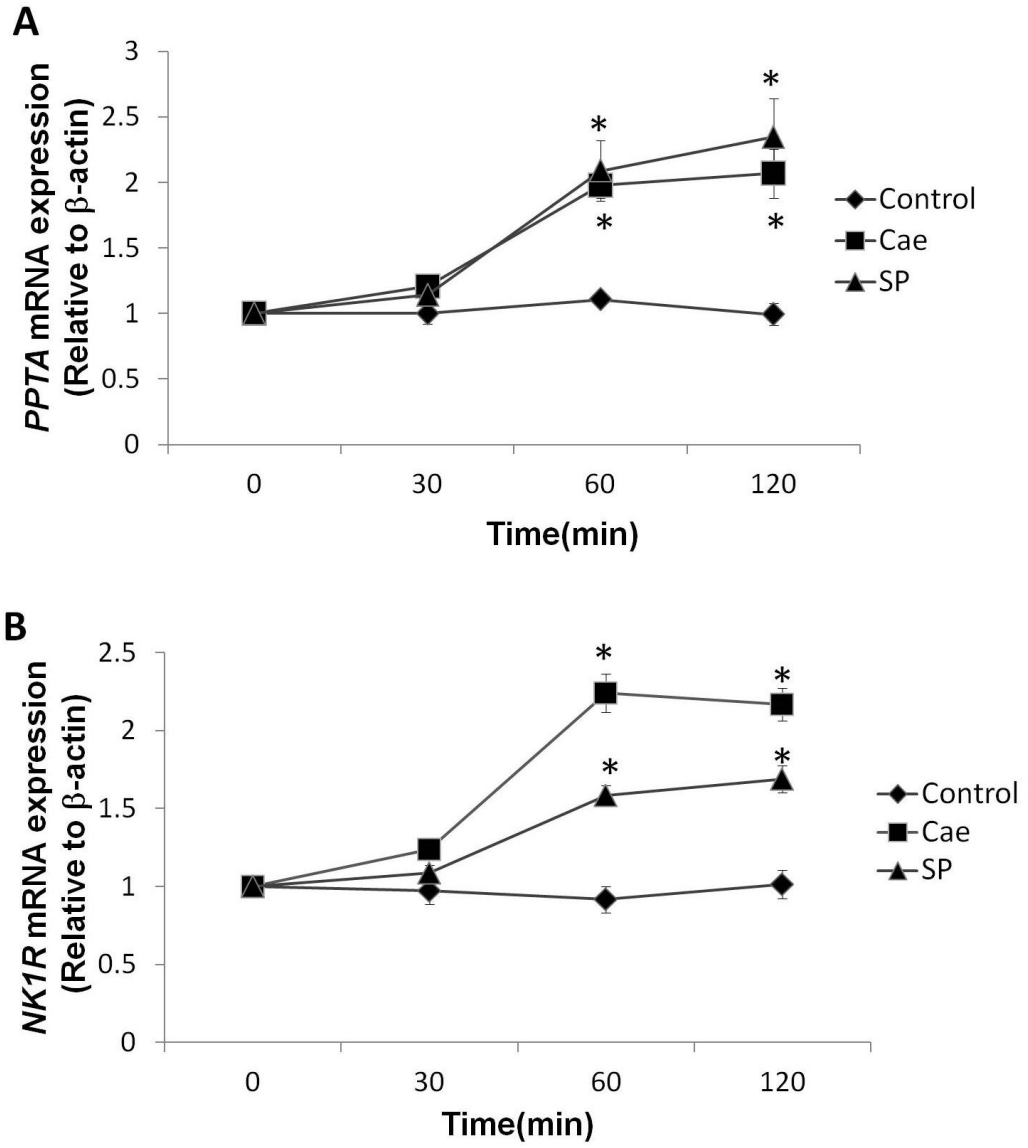

Figure 4.1 SP induced gene expression of PPTA and NK1R in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with either SP (10^{-6}M) or Cae (10^{-7}M) for 0-120 minutes. *PPTA*/*NK1R* mRNA expression was determined with real time PCR following TRIzol reagent extraction and normalized with *β-actin* expression in the samples. (A) Time course effect of SP and caerulein treatments on *PPTA* mRNA expression. (B) Time course effect of SP and caerulein treatments on *NK1R* mRNA expression. Results are expressed as means ± SEM from 4-6 independent experiments. *P<0.05, vs. control.

4.3.2 CP96,345 down-regulates exogenous SP-induced *PPTA* and *NK1R* mRNA expression

We have previously shown that SP up-regulated the expression of *PPTA* and *NK1R* in pancreatic acinar cells. To test whether this effect is due to SP-NK1R interaction, we pre-treated cells with CP96,345, a selective NK1R antagonist, before induction with SP (Snider et al., 1991). Pre-treatment of cells with CP96,345 inhibited *PPTA* and *NK1R* mRNA expression in a concentration dependent manner, when compared to their vehicle treated SP-induced cells. At a low concentration of 0.2μM, CP96,345 started to show minor inhibitory effect but at higher concentrations (5μM), it completely abolished SP-induced up-regulation of *PPTA*/*NK1R* mRNA expression (Figure 4.2A, 4.2B). Treatment of CP96,345 in the absence of SP did not alter expression levels when compared to the baseline control (data not shown), suggesting that NK1R activation is not involved in maintaining the basal expression level of *PPTA* and *NK1R*.

4.3.3 Caerulein induced *PPTA* and *NK1R* gene expression in murine pancreatic acinar cells does not involve the activation of NK1R

Previous results have demonstrated an increase in *PPTA* and *NK1R* mRNA expression in the pancreas when mice were induced with AP. On the other hand, antagonism of NK1R with CP96,345 significantly reduced their expression (Lau and Bhatia, 2006). This suggested that activation of NK1R partly attributed to the overall *PPTA*/*NK1R* up-regulation observed in caerulein-treated animals. We aim to reproduce this observation in our cellular model of isolated pancreatic acinar cells. To do this, cells were treated with CP96,345 (1μM) for 30 minutes before treatment with stimulatory concentration of caerulein (10^{-7}M). Caerulein treatment significantly increased the expression of *PPTA* and *NK1R*. However, contrary to the animal model of caerulein-induced AP, antagonism of NK1R did not reduce mRNA expression of *PPTA*/*NK1R* in isolated pancreatic acinar cells (Figure 4.2A, 4.2B).

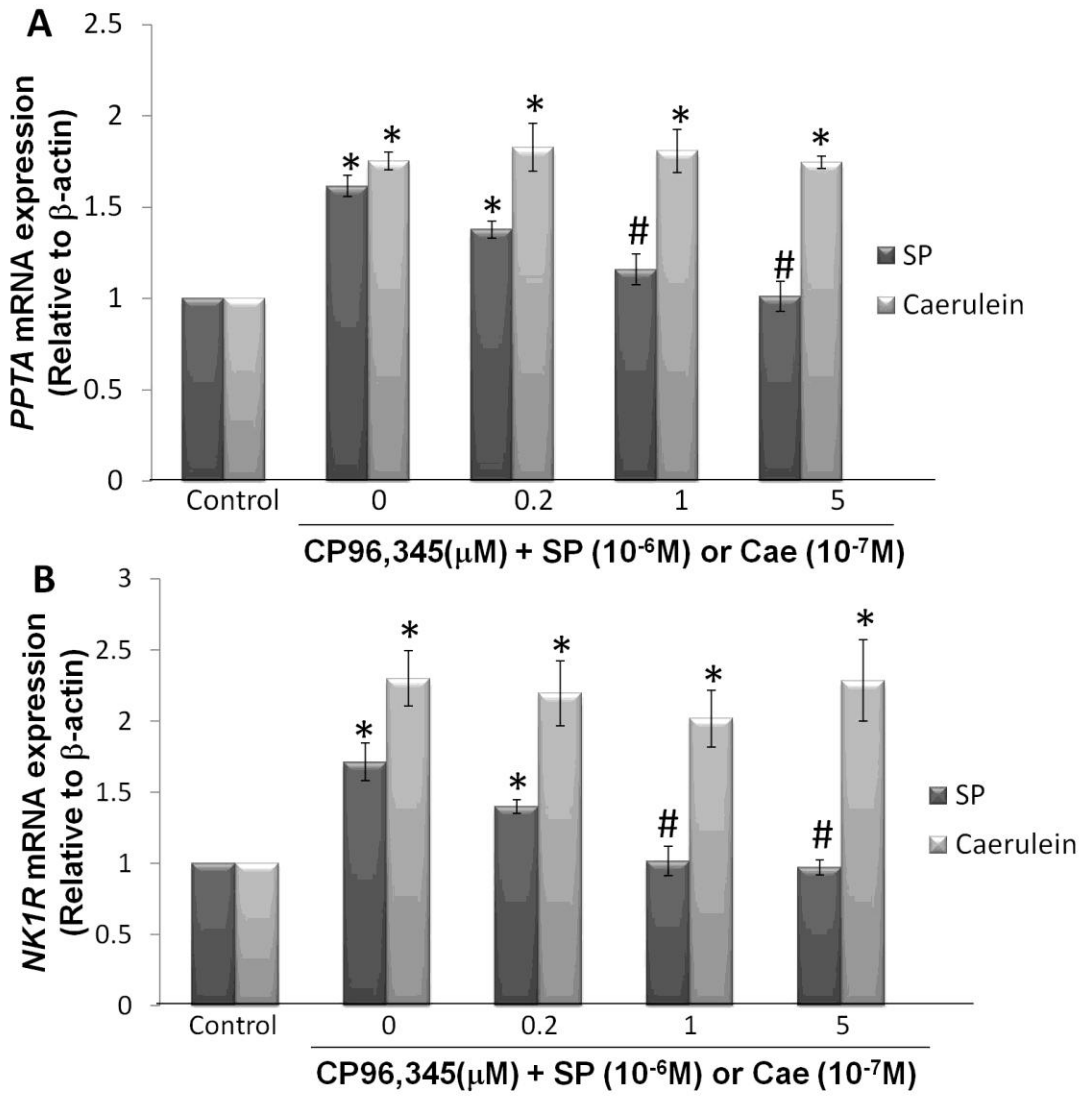

Figure 4.2 SP-induced, but not caerulein-induced PPTA/NK1R up-regulation is abolished by antagonism of NK1R. Pancreatic acinar cells were pre-treated with CP96,345 (0.2 – 5μM) for 30 minutes before treatment with caerulein (10^{-7}M) or SP (10^{-6}M) for 60 minutes. Subsequently, *PPTA* and *NK1R* mRNA expression was determined with real time PCR following TRIzol reagent extraction and normalized with *β-actin* expression in the samples. (A) Pre-treatment of CP96,345 reversed SP-induced, but not caerulein-induced *PPTA* upregulation. (B) Pre-treatment of CP96,345 reversed SP-induced, but not caerulein-induced NK1R up-regulation. Results are expressed as the means ± SE from 6 independent experiments. *P<0.05 vs. control, #P<0.05 vs. caerulein.

4.3.4 Activation of NK1R induces expression levels of SP peptides

Since *PPTA* and *NK1R* mRNA expression can be regulated through activation of NK1R, we further investigated SP peptide expression in pancreatic acinar cells. The presence of unlabelled exogenous SP was discovered to confound with endogenously produced SP using conventional ELISA assays (data not shown), and very likely with other protein assays. Therefore, we used a specific NK1R agonist, GR73,632, to investigate its effects on SP peptide expression. GR73,632 had no detectable reactivity in the ELISA assay at concentrations up to 10μg/ml. Isolated pancreatic acinar cells were cultured for 60 minutes in the presence of GR73,632 (10^{-10}M to 10^{-6}M). Our results showed that treatment of isolated murine pancreatic acinar cells with GR73,632 significantly increased SP peptide levels at concentrations larger than 10^{-8}M (Figure 4.3A). To further confirm that the observations were mediated through NK1R, we treated cells with GR73,632 (100nM) and CP96,345 (1μM). Pre-treatment of CP96,345 completely abolished the effects of GR73,632, indicating that the effect is specifically mediated by activation of NK1R (Figure 4.3B).

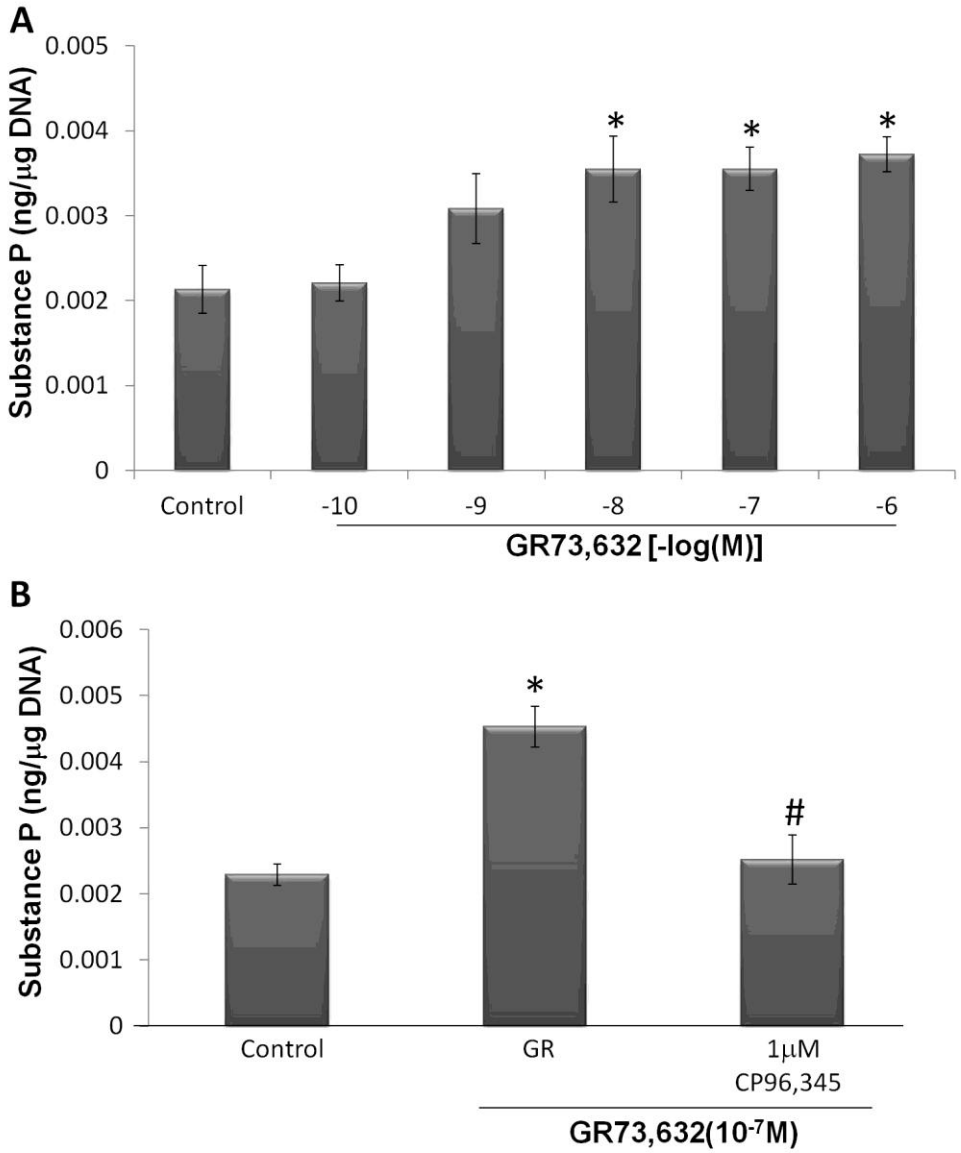

Figure 4.3 Activation of NK1R up-regulated SP peptide expression in isolated murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with GR73,632 (10^{-6}M-10^{-10}M) for 60 minutes. In some experiments, pancreatic acinar cells were pre-treated with CP96,345 (1μM) before treatment with GR73,632 (10^{-7}M). SP peptide levels were determined using a commercially available ELISA kit and normalized with the DNA content of the samples. (A) Time course effect of SP and caerulein treatment on *NK1R* mRNA expression. (B) Time course effect of SP on NK1R protein expression. Results are expressed as means ± SEM from 4-6 independent experiments. *P<0.05 vs. control. #P<0.05 vs GR73,632

4.3.5 Effect of substance P treatment on protein expression of NK1R

We also evaluated the effect of exogenous SP on NK1R protein expression. To do this, we performed a time course study of NK1R via Western blot. Using a specific antibody against NK1R, we found that cells cultured in control medium did not show a change in background expression of NK1R. On the other hand, a time-dependant up-regulation of NK1R protein expression was observed in whole cell lysates after treatment with SP (Figure 4.4). After 2 hours of SP stimulation, NK1R band intensity reached a peak of 2.5-fold increase when compared to the control.

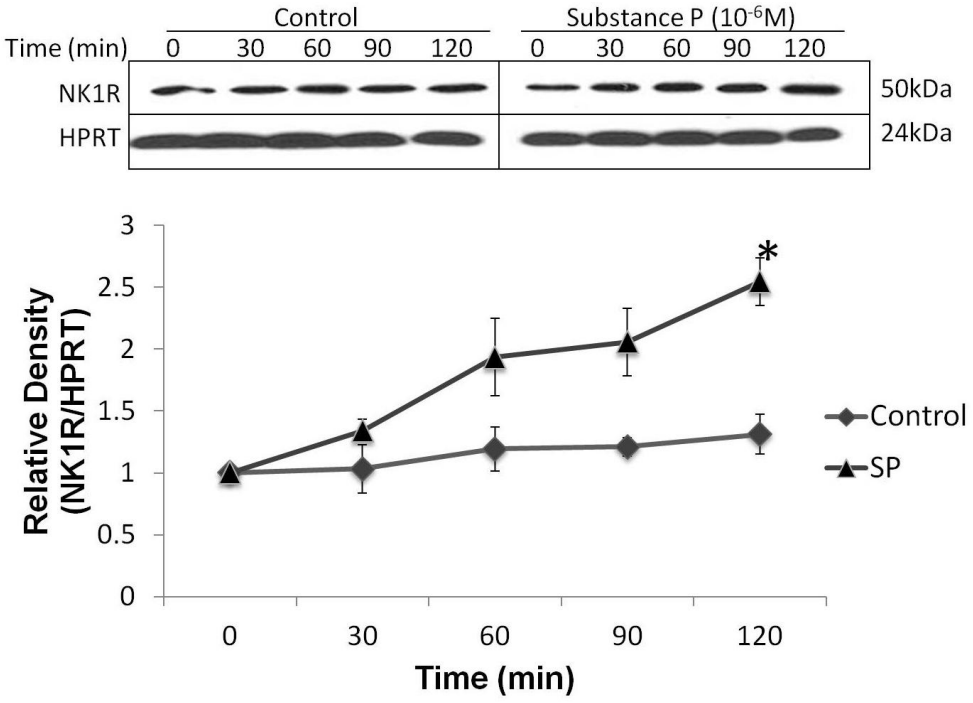

Figure 4.4 SP up-regulates NK1R protein expression in a time dependent manner. Freshly prepared pancreatic acinar cells were treated with SP for 0-120 minutes. After treatment, whole cell lysates were prepared and 50μg of protein were subjected to Western blot analysis. NK1R protein expression was normalized with HPRT expression in the samples. Results are expressed as means ± SEM from 4-6 independent experiments. *$p<0.05$, vs. control.

4.3.6 SP up-regulates SP and NK1R expression via PKC, MAPK, and NF-κB dependant pathways

It was previously demonstrated that SP induces the expression of chemokines via the Src family kinases (Sfk) – ERK/JNK – NF-κB pathway in pancreatic acinar cells (Ramnath et al., 2009). The PKC family was also found to be activated after cells were stimulated with SP (Ramnath et al., 2008). These activated signaling molecules share similarities with the PKC-MAPK-NF-κB pathway involved in caerulein-mediated responses (Chapter 2 and 3). To determine the signaling pathway in SP-induced expression of SP/NK1R, pancreatic acinar cells were pre-treated with specific inhibitors of PKCα (Gö6976, 10nM), PKCδ (Rottlerin, 5μM), MEK/ERK pathway (PD98059, 30μM), JNK (SP600125, 30μM), or NF-κB (Bay11-7082, 30μM) before induction with SP (10^{-6}M, 60 minutes). The addition of these inhibitors almost completely abolished SP-induced *PPTA*/*NK1R* mRNA up-regulation in the pancreatic acinar cells (Figure 4.5A, 4.6A). Protein expression of SP and NK1R was also abrogated by the addition of PKC, MAPK or NF-κB inhibitors (Figure 4.5B, 4.6B). These results confirm the similarity in downstream signaling pathways between caerulein-induced and SP-induced gene expression in the pancreatic acinar cells.

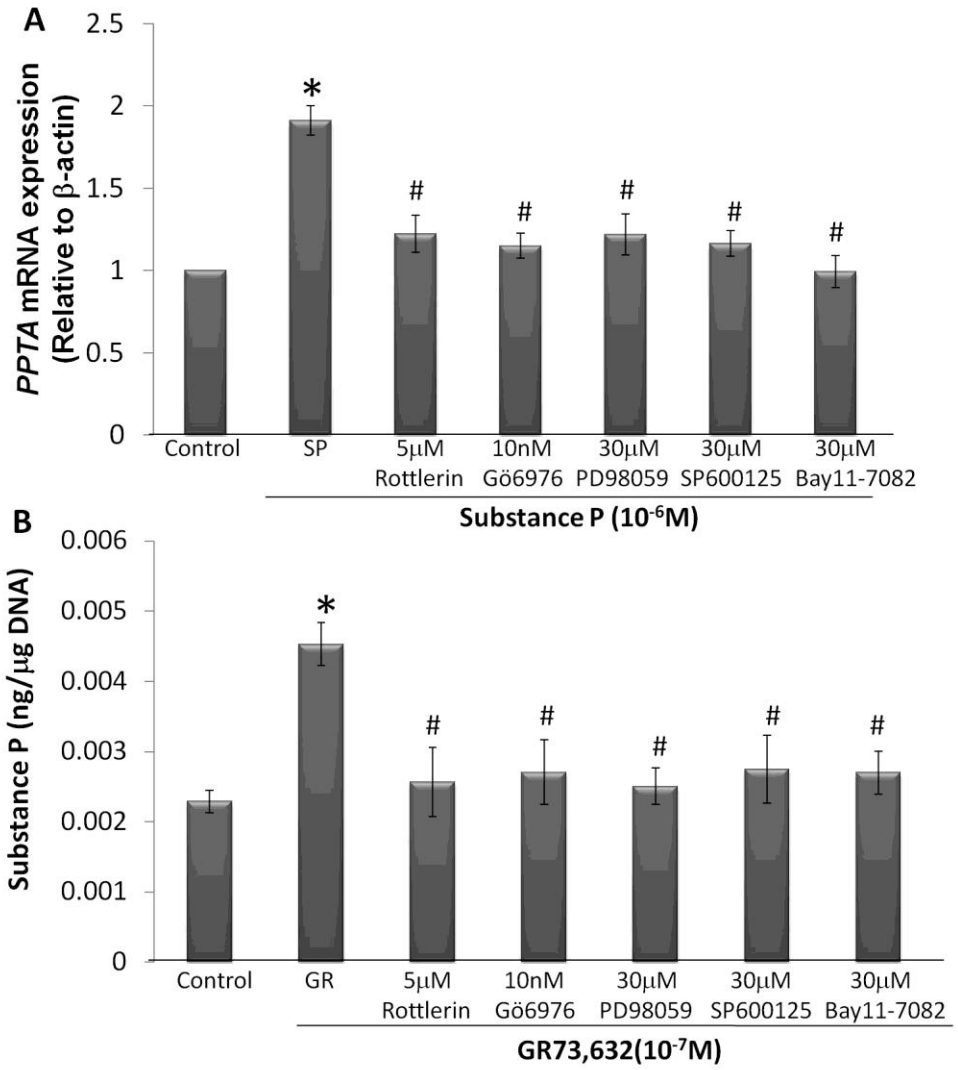

Figure 4.5 PKC, MAPK, and NF-κB are involved in SP induced SP up-regulation in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were pretreated with either Gö6976 (10nM), rottlerin (5μM), PD98059 (30μM), SP600125 (30μM), or Bay11-7082(30μM) for 30 minutes before stimulation with SP (10^{-6}M) or GR73,632(10^{-7}M) for 60 minutes. *PPTA* expression was determined with real time PCR and SP levels were determined by a commercially available ELISA kit. *PPTA* mRNA expression was normalized with *β-actin* expression and SP levels was normalized with DNA content in the samples. Results are expressed as means ± SEM from 4-6 independent experiments. *$p<0.05$ vs. control, #$p<0.05$ vs. SP/GR73,632 treated groups..

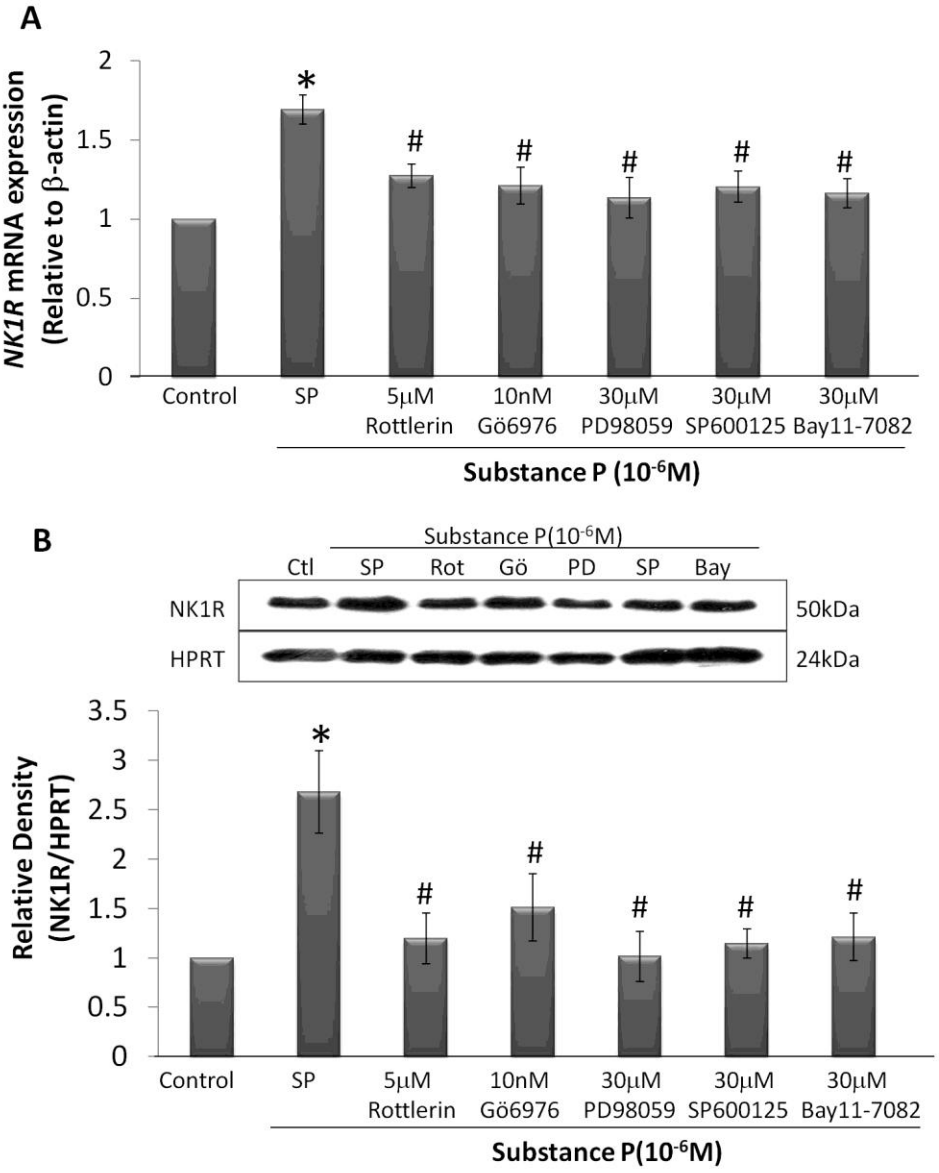

Figure 4.6 PKC, MAPK, and NF-κB are involved in SP induced NK1R up-regulation in murine pancreatic acinar cells. Freshly prepared pancreatic acinar cells were pretreated with either Gö6976 (10nM), rottlerin (5μM), PD98059 (30μM), SP600125 (30μM), or Bay11-7082(30μM) for 30 minutes before stimulation with SP (10^{-6}M for 60 minutes. *NK1R* mRNA expression was determined with real time PCR. Whole cell lysates were prepared and 50μg of protein subjected to Western blot analysis of NK1R and HPRT. *NK1R* mRNA expression was normalized with *β-actin* expression and NK1R protein expression was normalized with HPRT expression. *p<0.05 vs. control, #P<0.05 vs. SP treated groups.

4.4 DISCUSSION

SP-NK1R interaction was defined as a pro-inflammatory process that contributes to the progression of AP (Bhatia et al., 1998; Liddle and Nathan, 2004). Both pancreatic SP and NK1R expression are elevated in caerulein-induced AP models. We have previously reported caerulein-induced expression of SP and NK1R in isolated mouse pancreatic acinar cells and the mechanisms that led to their up-regulation (Koh et al., 2010; Koh et al., 2011). In the present study, we sought to investigate the effect of SP on the expression of SP and NK1R in isolated pancreatic acinar cells.

Herein we demonstrated that SP treatment increased *PPTA* and *NK1R* mRNA expression in isolated murine pancreatic acinar cells. We observed that the response was rapid, with significant up-regulations occurred within one hour after administration of SP. This is in agreement with previous studies, which also showed rapid increases in chemokine expression when pancreatic acinar cells were stimulated with SP (10^{-6}M) (Ramnath and Bhatia, 2006). SP treatment also induced a gradual increase in protein levels of NK1R. These rapid changes might enhance SP-NK1R dependent pro-inflammatory responses in early phase of AP. SP was widely reported to act through stimulation of NK1R, including isolated pancreatic acinar cells (Ramnath and Bhatia, 2006). Pre-treatment of cells with the selective NK1R antagonist CP96,345 completely reversed SP-induced up-regulation of *PPTA* and *NK1R* mRNA expression. Therefore, we suggest the observations were dependent on the activation of NK1R. These results proposed the presence of an "auto-regulatory" mechanism, in which activation of NK1R increases the expression of SP and NK1R,

thus potentially creating a positive feedback loop. However, the occurrence and extent of this feedback loop is currently unclear in physiological conditions.

GR73,632, a specific agonist of NK1R, induced a concentration-dependant increase of endogenous SP levels. Concurrent treatment of cells with an agonist and antagonist of NK1R completely abrogated the effects of NK1R activation. These observations confirmed that GR73,632 acts specifically through NK1R, and further demonstrated that NK1R activation is one possible mechanism that contribute to increased SP levels. The EC50 of GR73,632 was published as 2nM in guinea pig vas deferens, but information on pancreatic acinar cells was not available (Hall and Morton, 1991). In agreement with previously published EC_{50} concentrations, our results showed that SP levels were induced with low nanomolar concentrations of GR73,632 . On the other hand, previous work showed that the addition of exogenous SP (10^{-8}M) increased *PPTA* expression in cultured human normal fibroblasts (Bae et al., 2002). In light of these observations, the proposed self-perpetuating feedback loop might also occur at lower concentrations of SP depending on the cell type used.

Our results have suggested that caerulein stimulated CCK_A receptors and SP stimulated NK1R receptors in mouse pancreatic acinar cells. Current findings also suggested that CCK_A and NK1R downstream signaling may involve a similar pathway. Upon stimulation with caerulein, both *PPTA* and *NK1R* gene were up-regulated by a PKC-MAPK-NF-κB dependent pathway in the pancreatic acinar cells. Ramnath et al also showed SP-induced chemokine expression via the activation of PKC, MAPK and NF-κB pathway. These similarities are not surprising as both CCK_A receptors and SP receptors are both G-protein coupled receptors, acting via Gq/11 pathways (Khawaja and Rogers, 1996; Williams, 2001). In reference to previously

established effective concentrations, pre-treatment of cells with specific inhibitors of PKC, ERK/JNK, and NF-κB abolished SP-induced up-regulation of SP/NK1R in both protein and mRNA levels. In this case, activation of NK1R may lead to the activation of PKC and subsequent MAPK activation, followed by NF-κB transcription factor activation, which ultimately up-regulated expression of SP and NK1R (Figure 4.7). The role of MAPKs on the activation of NF-κB is poorly understood, but current evidence suggest that ERK/JNK could act upstream of NF-κB by modulating IκB or IκB kinase (Chen and Lin, 2001; Lin et al., 2005; Cheng et al., 2009). Despite these observations, the similarities in CCK_A signaling pathway and NK1R signaling pathway should not be extrapolated to other models, as they exhibit very different responses in physiological conditions.

Previous studies have hinted the presence of an auto-regulatory mechanism of SP/NK1R expression in AP. In the pancreas of caerulein-treated mice, up-regulation of *PPTA* and *NK1R* mRNA expression was partially abolished when they were pre-treated with a selective NK1R antagonist, CP96,345 (Lau and Bhatia, 2006). This is interesting because caerulein is not known to mediate through NK1R. Hence, we investigated whether similar observations could be reproduced in isolated pancreatic acinar cells. Unfortunately, CP96,345 neither reduced nor raised caerulein-induced up-regulation of *PPTA*/*NK1R* expression in these cells. Therefore, caerulein itself has no direct effects on the NK1R receptors. The difference between animal model and cellular model setting might be attributed to additional SP sources *in vivo*. SP can be released by nerve endings and also by infiltrating leukocytes, increasing its local levels so high that enabled the activation of NK1R to induce SP/NK1R up-regulation in pancreatic acinar cells. On the other hand, SP produced in pancreatic acinar cells

alone did not reach sufficient concentrations to trigger stimulatory effects on the NK1R in our design.

Autocrine regulation of SP and NK1R may have its significance in regulating the severity of AP and other inflammatory diseases. In immune cells, autocrine regulation of SP was suggested to be involved in T-lymphocyte proliferation (Lambrecht et al., 1999). During intestinal inflammation, it was suggested that autocrine/paracrine regulation of cytokine secretion by SP lead to a worsening of inflammation conditions (Castagliuolo et al., 1997). Besides involvement in inflammatory conditions, SP auto-regulation was also shown in other physiological functions. In one previous report, exogenously administered SP in sutured rat Achilles tendon rupture had a booster effect on endogenous SP for fibroblast proliferation via autocrine/paracrine stimulation (Steyaert et al., 2010). With SP currently known to promote chemokine production in macrophages and pancreatic acinar cells, this auto-regulatory mechanism might be of significance. Especially when pancreatic acinar cells consisted of the bulk of pancreatic mass, SP contributed by these cells cannot be discounted. Further work could be done to elucidate the occurrence and extent of this auto-regulatory effect during the course of AP.

The observation that activation of NK1R up-regulates the expression of SP and/or NK1R is very likely to depend on the cell type or model used. SP was found to cause a 13-fold increase in *PPTA* expression in human mononuclear phagocytes (Lai et al., 2002). Continuous administration of thiorphan, a specific neutral endopeptidase inhibitor, reduced SP degradation and enhanced rat thymocyte *PPTA* and *NK1R* mRNA expression as well as SP and NK1R protein levels in an NK1R-dependent manner (Amantini et al., 2008). It was also previously shown that stem cell factor treatment on bone marrow stroma induced *PPTA* and *NK1R* expression leading to

autocrine and/or paracrine cell activation (Qian et al., 2001). Previous studies has also shown direct evidence of an auto-regulatory role of SP/NK1R expression in human keratinocytes and human mononuclear phagocytes (Bae et al., 1999; Bae et al., 2002). The auto-regulatory mechanism could also be activated by a N-terminal metabolite of SP. In that study, only NK1R expression, but not SP expression, was up-regulated by treatment of a SP metabolite on rat spinal cord (Velazquez et al., 2002). Although there was evidence to suggest an auto-regulatory role of SP and NK1R expression, there were also several reports that showed otherwise. Hegde et al. showed that treatment of mice with SR140333, a selective NK1R antagonist, did not abolish sepsis-induced SP up-regulation in the lung (Hegde et al., 2010). Even in caerulein-induced AP model itself, it was shown that lung NK1R expression was further up-regulated when mice were treated with CP96,345, suggesting antagonism of NK1R further enhanced NK1R expression (Lau and Bhatia, 2006). Palmer *et al*. also reported that disruption of the NK1R gene does not prevent up-regulation of *PPTA* mRNA in the spinal cord of mice following peripheral inflammation, when SP levels was greatly up-regulated (Palmer et al., 1999). Therefore, much work needs to be done to elucidate the different mechanisms that regulate SP/NK1R expression in different cell types. Investigators should be cautious when extrapolating the results to all models.

Collectively, the results presented in this chapter describe a novel mechanism of the contribution of SP-NK1R interaction during AP. Auto-regulation of SP/NK1R expression was occasionally reported but it has not been brought to focus. Current knowledge suggests that the SP increase in the pancreas could be contributed to increased release from sensory nerve fibers, infiltrating leukocytes, and possibly from the pancreatic acinar cells. Together, these sources contribute to elevated SP levels

and up-regulate the expression of cytokines and chemokines via activating the NF-κB pathway. Further work could be done to investigate the contribution of each of these sources of SP and defining other mechanisms that lead to SP/NK1R up-regulation. It is also useful to study the expression of SP/NK1R in peripheral neurons following NK1R activation, as they were considered to be a major source of endogenously produced SP.

In conclusion, we report evidence for the auto-regulatory mechanisms of SP and NK1R expression. By activation of the NK1R receptors, it is possible to up-regulate the expression of SP and NK1R in a PKC-MAPK-NF-κB dependant pathway (Figure 4.7). With further understanding on the mechanisms that modulate physiological SP levels, new approaches may be achieved for the management of AP and the accompanying systemic inflammation.

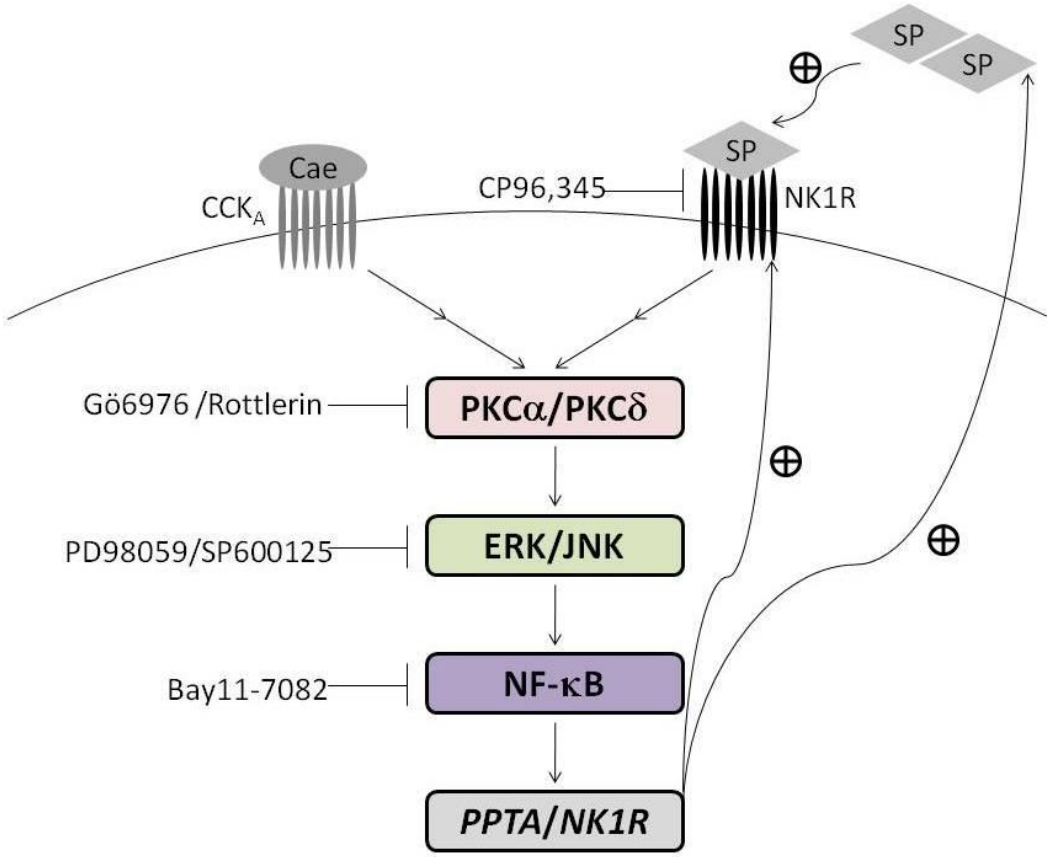

Figure 4.7 A schematic model summarizing the results of the chapter 4. The auto-regulatory effect of SP/NK1R expression could potentially elevate their expression to a much higher level.

CHAPTER 5: THE ROLE OF NEUTRAL ENDOPEPTIDASE IN CAERULEIN-INDUCED ACUTE PANCREATITIS

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5.1 INTRODUCTION

Increased expression of SP in SP-expressing cells and increased release of SP from nerve endings are two mechanisms that may contribute to elevated SP levels in experimental AP. In chapters 2 to 4, we have shown that both caerulein and SP are able to induce the expression of SP/NK1R in murine pancreatic acinar cells. However, inhibitory mechanisms that terminate the effect of SP were not clearly understood. Neutral endopeptidase (NEP) is a protease known to be capable of modulating inflammatory responses by degradation of SP (Sturiale et al., 1999; Scholzen et al., 2001). In a model of experimental colitis, knockout of the NEP gene increased SP levels in the colon and exacerbates inflammation (Sturiale et al., 1999). On the other hand, treatment of exogenous recombinant NEP protected animals against intestinal inflammation and burns (Neely et al., 1996; Sturiale et al., 1999; Kirkwood et al., 2001). These reports clearly show an anti-inflammatory role of NEP, possibly via the degradation of SP.

To date, the role of NEP in the pathogenesis of AP is not thoroughly understood. Genetic deletion of NEP exacerbated lung injury and greatly increased mortality rate in CDE-diet induced AP (Maa et al., 2000b). NEP is also a determinant of pancreatic elastase associated lung injury. Mice pre-treated with phosphoramidon, a NEP inhibitor, showed significant elevations in lung myeloperoxidase (MPO) levels and worsened lung histology compared to mice which did not receive phosphoramidon treatment (Day et al., 2005). In a similar model of pancreatic elastase-induced lung injury, treatment of mice with recombinant NEP ameliorates the detrimental effects of elastase (Lightner et al., 2002).

Despite an anti-inflammatory role of NEP in inflammation, physiological changes of NEP expression and activity in caerulein-induced AP are not known. Therefore, in the present study we investigated the potential regulatory role of caerulein on NEP activity and expression. Subsequently, NEP inhibitors and exogenous NEP were used to examine the effects of NEP on the outcome of AP. Although the primary focus of this chapter is on pancreatic injury, lung injury is often observed in severe AP (Maa et al., 2000b; Lau et al., 2005). Hence, lungs have also been investigated for pathological changes.

5.2 MATERIALS AND METHODS

5.2.1 Animals and chemicals.

All experimental procedures were approved by the Animal Ethics Committee of the National University of Singapore and carried out in accordance with established International Guiding Principles for Animal Research. Balb/C mice (Male, 20-25 grams) were acclimatized in a controlled environment with an ambient temperature of 23^{o}C and a 12:12-hour light-dark cycle. Caerulein was purchased from Bachem (Torrance, CA) and dissolved in normal saline. Phosphoramidon and thiorphan (NEP inhibitors) was purchased from Sigma-Aldrich and dissolved in normal saline and 100% ethanol respectively. Recombinant mouse neutral endopeptidase was purchased from Sino-Biological (Beijing, China) and diluted in normal saline. Caerulein was purchased from Bachem.

5.2.2 Preparation and treatment of pancreatic acinar cells.

Refer to section 2.2.2. Freshly isolated pancreatic acinar cells were treated with caerulein (10^{-7}M) for 0-120 minutes.

5.2.3 Induction of Acute pancreatitis.

Balb/c mice were randomly assigned to control or experimental groups by using ten animals for each group. Animals were given hourly intraperitoneal injections of saline containing caerulein (50μg/kg) for 3, 6, or 10 hours to induce mild, moderate, or severe AP respectively (in terms of relative severity of caerulein-induced models). Control mice received hourly normal saline injections. In order to investigate whether NEP inhibition exacerbates AP, mice received a pre-treatment of phosphoramidon (5mg/kg, i.v.) or thiorphan (10mg/kg, i.v.) 1 hour before 6 hourly caerulein injections. These concentrations were chosen in reference to previous studies (Buckner et al., 1991; Pollock et al., 1993). On the other hand, in order to investigate the protective effects of NEP, mice received 10 hourly caerulein injections and received post-treatment of recombinant mouse NEP (1mg/kg, i.v.) during the 2^{nd} injection of caerulein. One hour after the last caerulein injection, animals were killed by a lethal dose of sodium pentobarbitone (150mg/kg, i.p.). Samples of pancreas, lung and blood were collected. Plasma was prepared from anti-coagulated blood samples by centrifugation (10,000g, 5 minutes, 4^{o}C). Random cross sections of the head, body and tail of the pancreas and samples of the right lung were fixed in 10% neutral buffered formalin (Sigma-Aldrich). A small section of pancreas and lung was weighed and then dried for 72h at 55^{o}C and reweighed to determine pancreatic water content. Remaining samples were then stored at -80^{o}C for subsequent analysis.

5.2.4 Measurement of myeloperoxidase activity.

Neutrophil sequestration in the pancreas and lung was quantified by measuring tissue myeloperoxidase (MPO) activity. Tissue samples were thawed, homogenized in 20mM phosphate buffer (pH 7.4), centrifuged (13,000g, 10min, 4^{o}C), and the resulting pellet was resuspended in 50mM phosphate buffer (pH 6.0)

containing 0.5% (w/v) hexadecyltrimethulammonium bromide (Sigma-Aldrich). The suspension was subjected to four cycles of freezing and thawing and was further disrupted by sonication (40s). The sample was then centrifuged (13,000g, 5min, 4^{o}C) and the supernatant used for MPO assay. The reaction mixture consisted of the supernatant (50µl), 1.6mM tetramethylbenzidine (KPL, MD), 80mM sodium phosphate buffer (pH 5.4), and 0.3mM hydrogen peroxide (Reagent volume: 50µl). This mixture was incubated at 37^{o}C and terminated with 50µl of 0.18M H_2SO_4. The absorbance was measured at 450nm. This absorbance was then corrected for the DNA content of the tissue sample and results were expressed as fold change over control (Refer to section 2.2.5 for DNA assay).

5.2.5 Histopathological examination.

A small portion of pancreas was excised and fixed with 10% neutral buffered formalin (Sigma-Aldrich), dehydrated through a graded ethanol series, embedded in paraffin wax for routine histology. Sections of 5µm thickness were stained with hematoxylin and eosin and examined by light microscopy using Nikon Eclipse 80*i* microscope (objective lens magnification x20; eyepiece magnification x10).

5.2.6 ELISA analysis.

ELISA assays were performed for the measurement of cytokines (interleukin-1 (IL-1), interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)), chemokines (macrophage inflammatory protein-1α (MIP-1α), macrophage inflammatory protein-2 (MIP-2)) and adhesion molecules(intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion protein-1 (VCAM-1), p-selectin, e-selectin) in pancreas and lung tissue homogenates, according to manufacturer's instructions (R&D systems, Minneapolis, MN). Results were then corrected for the DNA content of the tissue

homogenates and were expressed as picograms per microgram of DNA (Refer to section 2.2.5 for DNA assay). In this study, tissue content of DNA was chosen over tissue weight or tissue protein content as a normalization factor in ELISA analysis. In the pancreas, it has been shown that total DNA content (milligrams DNA per pancreas) remained relatively stable, but total protein content (milligrams protein per pancreas) was decreased in caerulein-induced pancreatitis models (Tani et al., 1987). Pancreatic edema in AP also alters the proportion of water in the tissue, which might cause bias when total pancreatic weight was used as a normalization factor.

5.2.7 Measurement of NEP activity.

NEP enzymatic activity was determined spectrophotometrically from extracted protein samples as described previously (Sio et al., 2010). Briefly, treated pancreatic acinar cells, pancreas and lung tissue was homogenized in T-PER tissue protein extraction reagent (Pierce, Rockford, IL) and centrifuged (12,000g, 15 minutes, 4^{o}C). Cell free extracts (30μg protein) was incubated with 1mM succinyl-Ala-Ala-Phe-*p*-nitroanilide (Suc-Ala-Ala-Phe-pNA; Bachem) as a substrate in 0.1 M Tris–HCl (pH 7.6) in the presence of 1 unit of porcine kidney aminopeptidase (AP-N; Sigma). In this coupled activity assay, NEP cleaves Suc-Ala-Ala-Phe-pNA between Ala and Phe, yielding Phe-pNA. AP-N subsequently cleaves Phe-pNA, generating pNA as the final product. The kinetic change in absorbance at 405nm due to the accumulation of free pNA was determined each minute at 37 °C for 60 min using a micro-plate reader. Substrate alone and substrate with AP-N and Tris buffer blanks were run in parallel. The rate of absorbance change were normalized with the control and expressed as fold change to control. Protein concentrations were determined by Bradford protein assay (Bradford, 1976).

5.2.8 Substance P extraction and detection.

Refer to section 2.2.4.

5.2.9 RNA isolation and quantitative real time PCR analysis.

Refer to section 2.2.6 and 2.2.8

5.2.10 Whole cell lysate preparation and Western blot analysis.

Refer to section 2.2.9. Cells were lysed with RIPA lysis buffer and protein concentrations were determined by Bradford protein assay. 50-80 µg of total cell lysate from each sample were subjected to Western blot analysis. The blots were incubated overnight with primary antibody of NEP (1:2000 dilutions) or HPRT (1:1000 dilutions). After washing away the primary antibodies, the membranes were incubated for 2 hours with goat anti-rabbit HRP-conjugated secondary antibody at 1:2000 dilutions. Protein expression was then detected by adding SuperSignal™ West Pico chemiluminescent substrate before exposure to X-ray films.

5.2.11 Statistical analysis.

Refer to section 2.2.10.

5.3 RESULTS

5.3.1 Caerulein suppress NEP activity and mRNA expression in isolated pancreatic acinar cells

Pancreatic acinar cell NEP activity was significantly decreased as early as 30 minutes after treatment with caerulein (10^{-7}M), when compared to the control. A time-dependent decrease of NEP enzymatic activity was revealed, reaching a minimum after 2 hours of caerulein stimulation (Figure 5.1A). Moreover, *NEP* mRNA expression was significantly down-regulated in pancreatic acinar cells that were stimulated with caerulein for more than 60 minutes (Figure 5.1B). Cells that were incubated in control buffer did not show a significant change in both NEP activity and mRNA expression (Figure 5.1A, 5.1B).

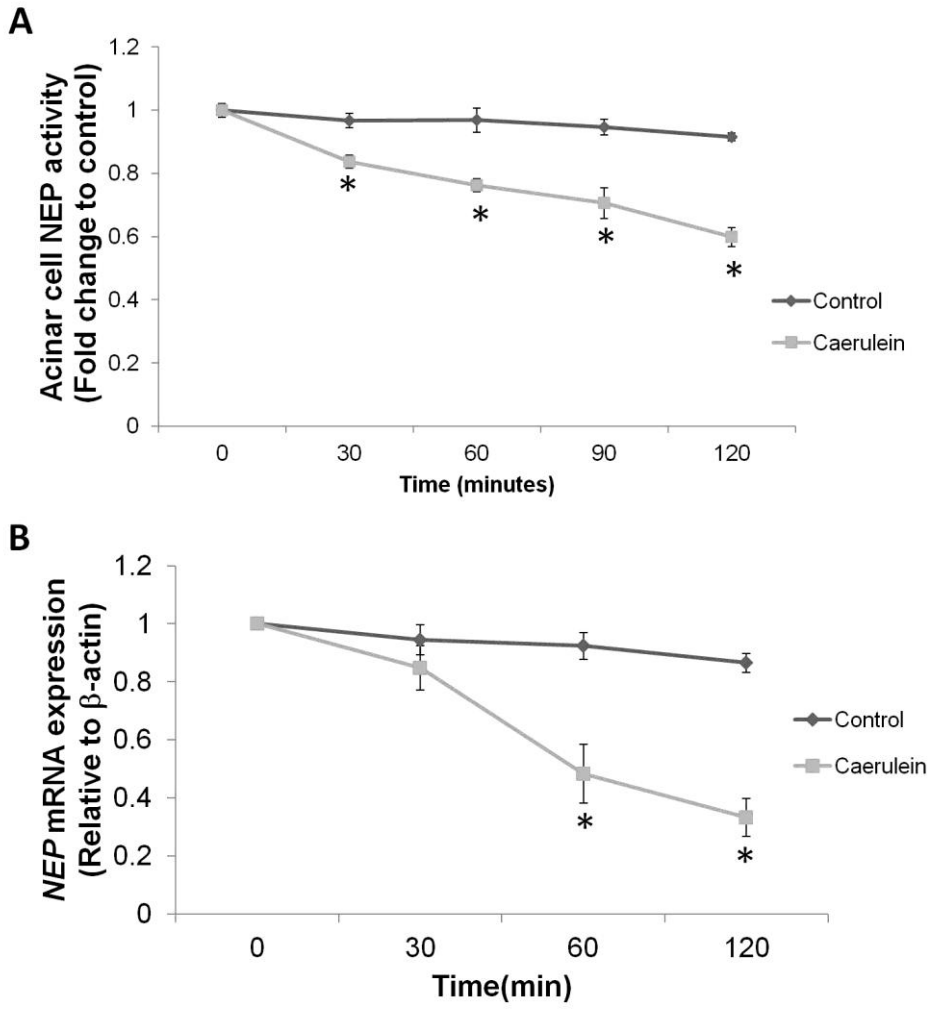
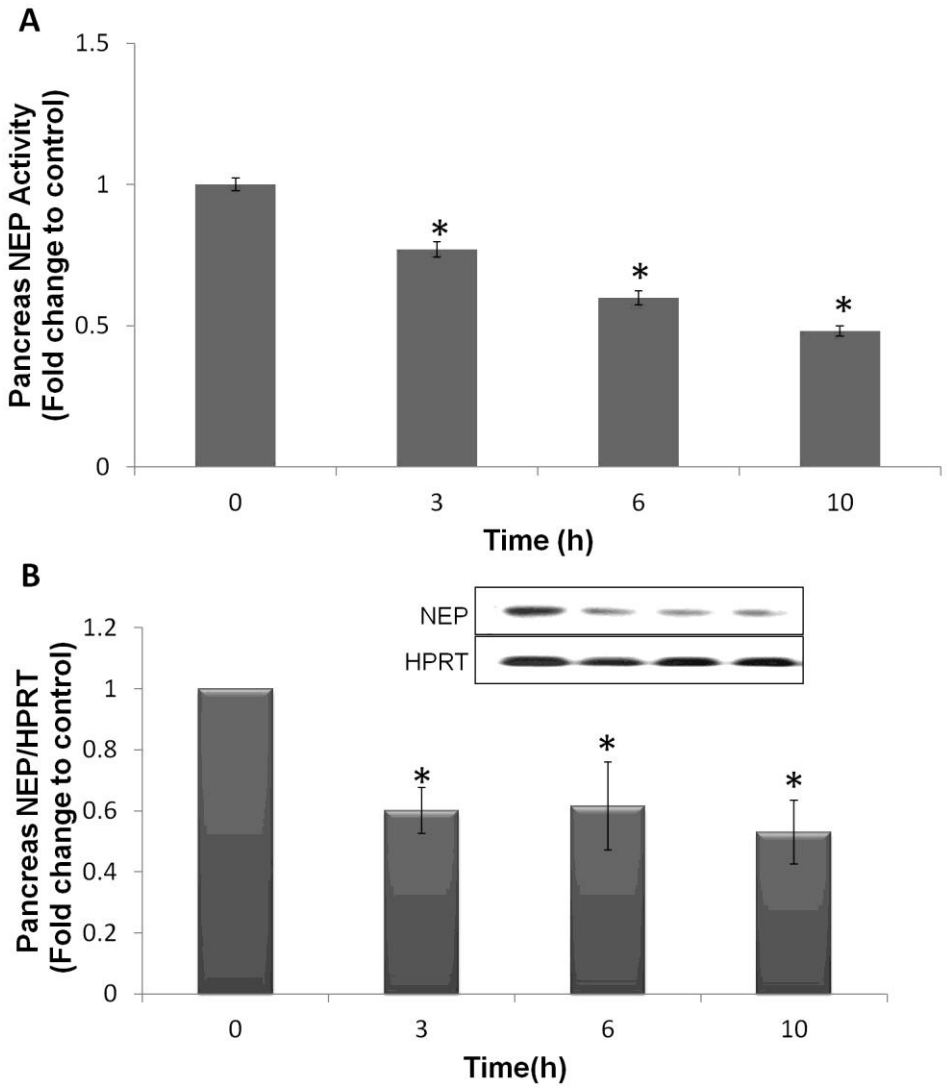


Figure 5.1 Administration of caerulein decreased NEP activity and expression in pancreatic acinar cells. Freshly prepared pancreatic acinar cells were treated with caerulein (10^{-7}M) for 0-120 minutes. (A) Time dependent decrease of NEP activity in pancreatic acinar cells. (B) *NEP* mRNA expression in pancreatic acinar cells. Values are means ± SEM; n=3-6 per time point. *P<0.05 vs. control.

5.3.2 Caerulein-induced AP suppress endogenous NEP activity

The suppression of NEP activity in caerulein-treated pancreatic acinar cells prompted us to investigate whether similar observations could be reproduced in animal models of AP. Balb/c mice were given hourly intraperitoneal injections of caerulein for 3, 6 or 10 hours to induce mild, moderate or severe AP respectively, in terms of relative severity. Caerulein-treated mice showed rapid decrease of pancreas NEP activity, with maximal suppression observed at severe AP conditions (Figure 5.2A). Consistent with NEP activity results, Western blot analysis confirmed early and sustained decrease of NEP protein expression in the pancreas (Figure 5.2B). In the lungs, similar decreases of NEP activity and protein expression were observed after induction of AP (Figure 5.2C, 5.2D). Notably, protein expression of NEP in the lung showed a large magnitude of decrease, when compared to the control (Figure 5.2D).


A
Pancreas NEP Activity
(Fold change to control)
1.5
1
0.5
0
*
*
*
0
3
6
10
Time (h)
B
Pancreas NEP/HPRT
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
NEP
HPRT
*
*
*
0
3
6
10
Time(h)

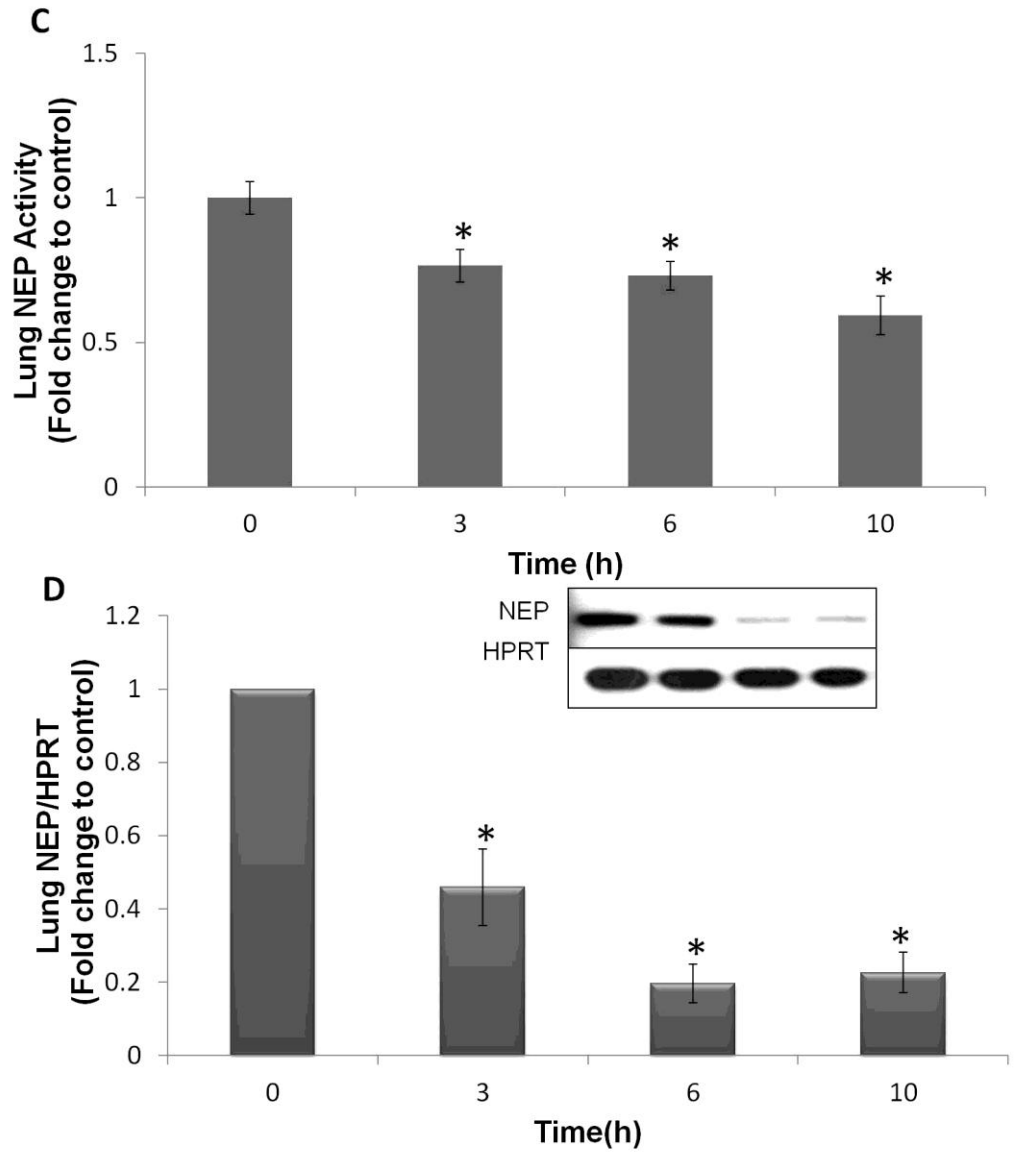
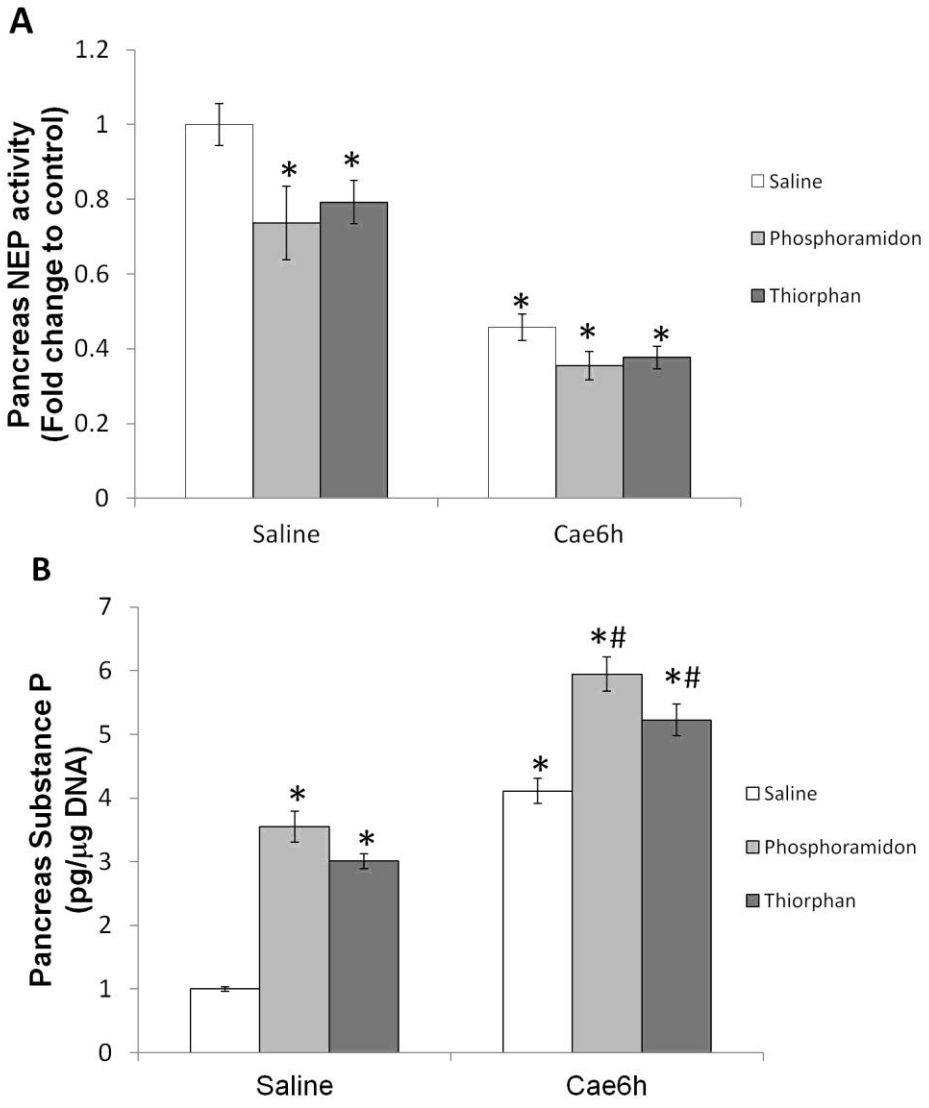
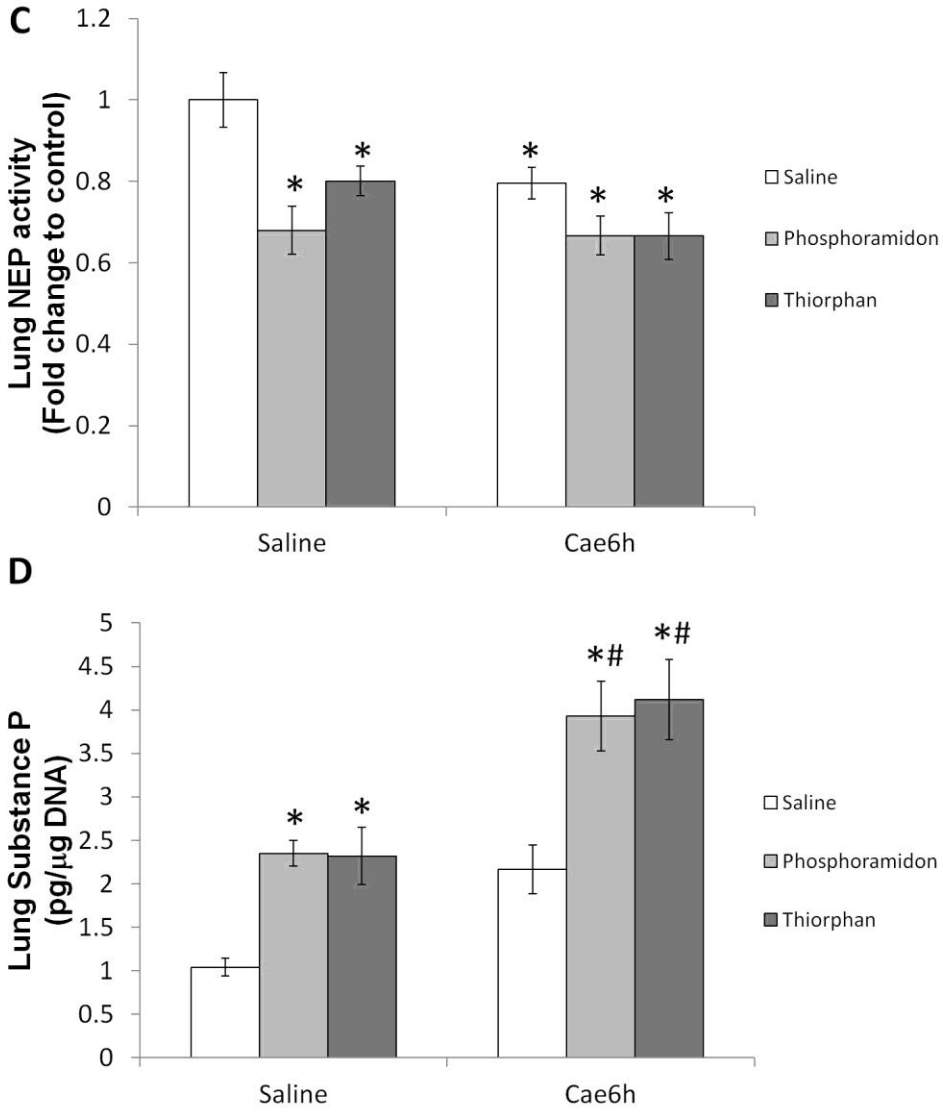


Figure 5.2 Administration of caerulein decreased NEP activity and expression in mice. Mice received hourly caerulein injections (50μg/kg) and assessed for tissue NEP activity and expression. (A) Pancreas NEP activity. (B) Pancreas NEP protein expression. (C) Lung NEP activity. (D) Lung NEP protein expression. Values are means ± SEM; n=10 mice per time point. *P<0.05 vs. control.

5.3.3 Phosphoramidon and thiorphan increase SP levels in the pancreas, lung, and plasma.

As NEP was previously shown to be protective against excessive inflammation, we anticipated that further suppression of NEP activity should exaggerate inflammatory conditions in AP. Therefore, we pre-inhibited NEP before induction of moderate AP, to investigate whether it would degenerate into a more severe form of AP. To do this, mice received a single dose of phosphoramidon (5mg/kg, i.v.) or thiorphan (10mg/kg, i.v.), followed by 6 hourly caerulein or saline injections. Both NEP inhibitors decreased NEP activity in the pancreas by approximately 70% one hour post-treatment (Data not shown). Control mice challenged with a single dose of phosphoramidon or thiorphan still showed a 20-30% reduction of NEP activity in the pancreas and lungs after 6 hourly saline injections, suggesting metabolism and drug clearance over time (Figure 5.3A, 5.3C). In contrast, SP levels rose significantly (Figure 5.3B, 5.3D). Induction of moderate AP suppressed NEP activity in both pancreas and lungs, coupled with a strong increase of SP in the pancreas and a modest but insignificant increase in the lungs ($0.10>p>0.05$). Interestingly, pre-treatment of NEP inhibitors followed by induction of AP provoked a further increase in SP levels (Figure 5.3B, 5.3D), but did not further reduce NEP activity (Figure 5.3A, 5.3C) in pancreas and lungs. Phosphoramidon or thiorphan administration did not alter background NEP activity in plasma, as NEP is a membrane-bound enzyme and not normally found in cell-free plasma (Figure 5.3E). Despite this, plasma SP concentrations were significantly elevated in all mice that received NEP inhibitor treatments, when compared to their respective untreated controls (Figure 5.3F).


A
Pancreas NEP activity
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
Saline
Cae6h
Saline
Phosphoramidon
Thiorphan
B
Pancreas Substance P
(pg/μg DNA)
7
6
5
4
3
2
1
0
Saline
Cae6h
Saline
Phosphoramidon
Thiorphan


C
Lung NEP activity
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
Saline
Cae6h
Saline
Phosphoramidon
Thiorphan
D
Lung Substance P
(pg/μg DNA)
5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
Saline
Cae6h
Saline
Phosphoramidon
Thiorphan

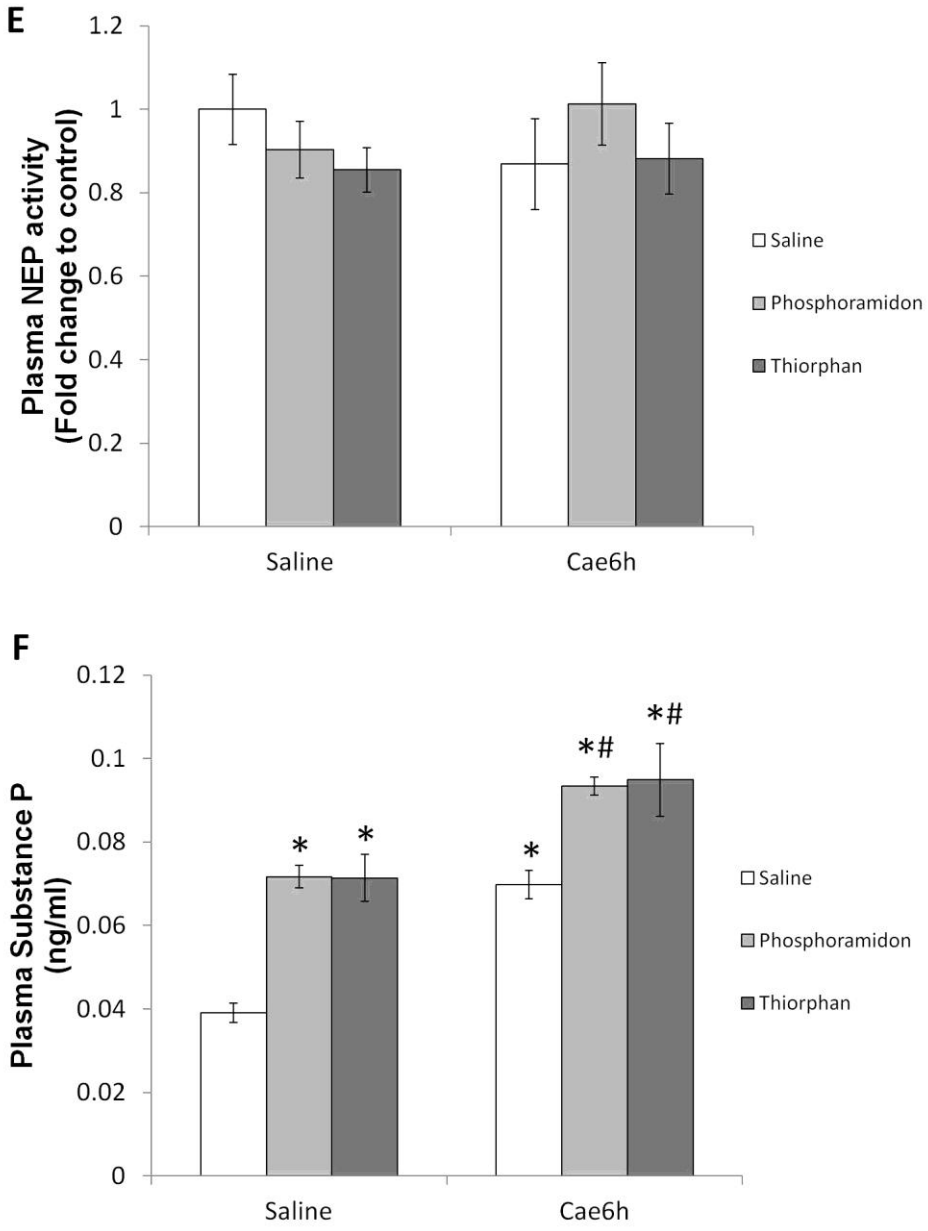
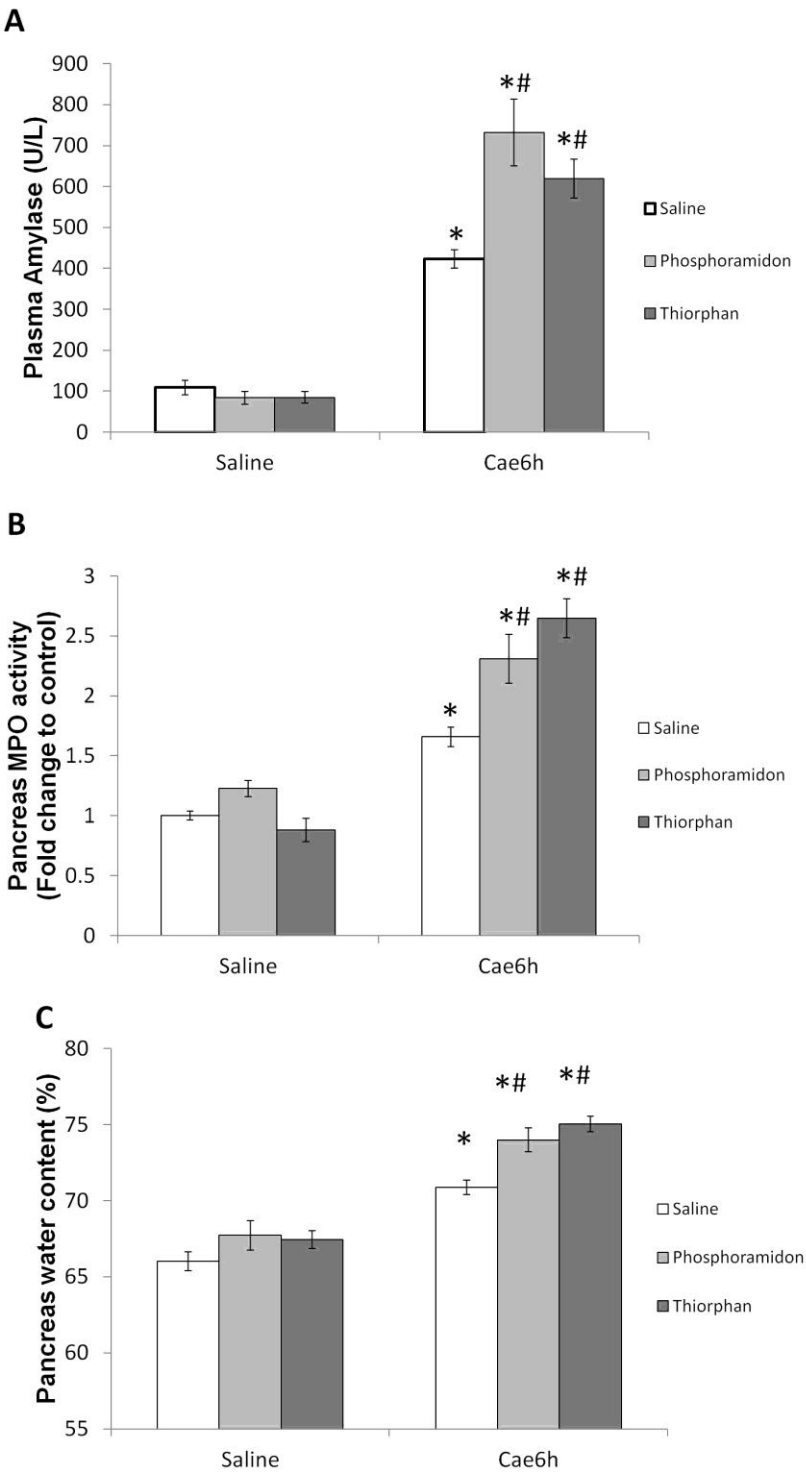


Figure 5.3 Inhibition of NEP by phosphoramidon and thiorphan decreased NEP activity and increased SP levels. Mice were randomly given phosphoramidon (5mg/kg. i.v.) or thiorphan (10mg/kg, i.v.) before 6 hourly injections of caerulein (50μg/kg, i.p.) or normal saline. One hour after the last caerulein injection, mice were sacrificed and NEP activity and SP ELISA assays were performed as described in Materials and Methods. (A) Pancreas NEP activity. (B) Pancreas SP levels. (C) Lung NEP activity. (D) Lung SP levels. (E) Plasma NEP activity. (F) Plasma SP levels. Values are means ± SEM; n=10 mice per time point. *P<0.05 vs. control.

5.3.4 Effect of NEP inhibition on plasma amylase activity, MPO activity, tissue water content and pancreatic histology

After confirming that phosphoramidon and thiorphan elevated SP levels in mice, we assessed how NEP inhibition influences the outcome of AP. Amylase is produced in the pancreatic acinar cells and released into the bloodstream when there is a pancreatic injury, therefore it is often used clinically as a marker for diagnosis of AP. Another important feature of AP is an elevated MPO activity in the pancreas and lungs, indicating infiltration of neutrophils into these tissues (Bhatia et al., 1998). Inhibition of NEP without induction of AP did not alter basal plasma amylase activity, tissue water content or tissue MPO activity, despite elevated SP levels (Figure 5.4A-E). On the other hand, NEP inhibitors further elevated plasma amylase activity, pancreas water content and pancreas MPO activity in AP-induced mice (Figure 5.4A-5.4C). The lungs were less affected; as NEP inhibitors did not alter AP-induced increases in lung MPO activity and water content (Figure 5.4D, 5.4E). Histological examination of the pancreas confirmed that inhibition of NEP worsen damage in AP-induced mice, characterized by increased pancreatic edema, neutrophil infiltration and pancreatic necrosis (Figure 5.5B, 5.5D, 5.5F). Normal pancreas architecture was observed in all control mice (Figure 5.5A, 5.5C, 5.5E).


A
Plasma Amylase (U/L)
900
800
700
600
500
400
300
200
100
0
*
*#
*#
Saline
Phosphoramidon
Thiorphan
Saline
Cae6h
B
Pancreas MPO activity
(Fold change to control)
3
2.5
2
1.5
1
0.5
0
*
*#
*#
Saline
Phosphoramidon
Thiorphan
Saline
Cae6h
C
Pancreas water content (%)
80
75
70
65
60
55
*
*#
*#
Saline
Phosphoramidon
Thiorphan
Saline
Cae6h

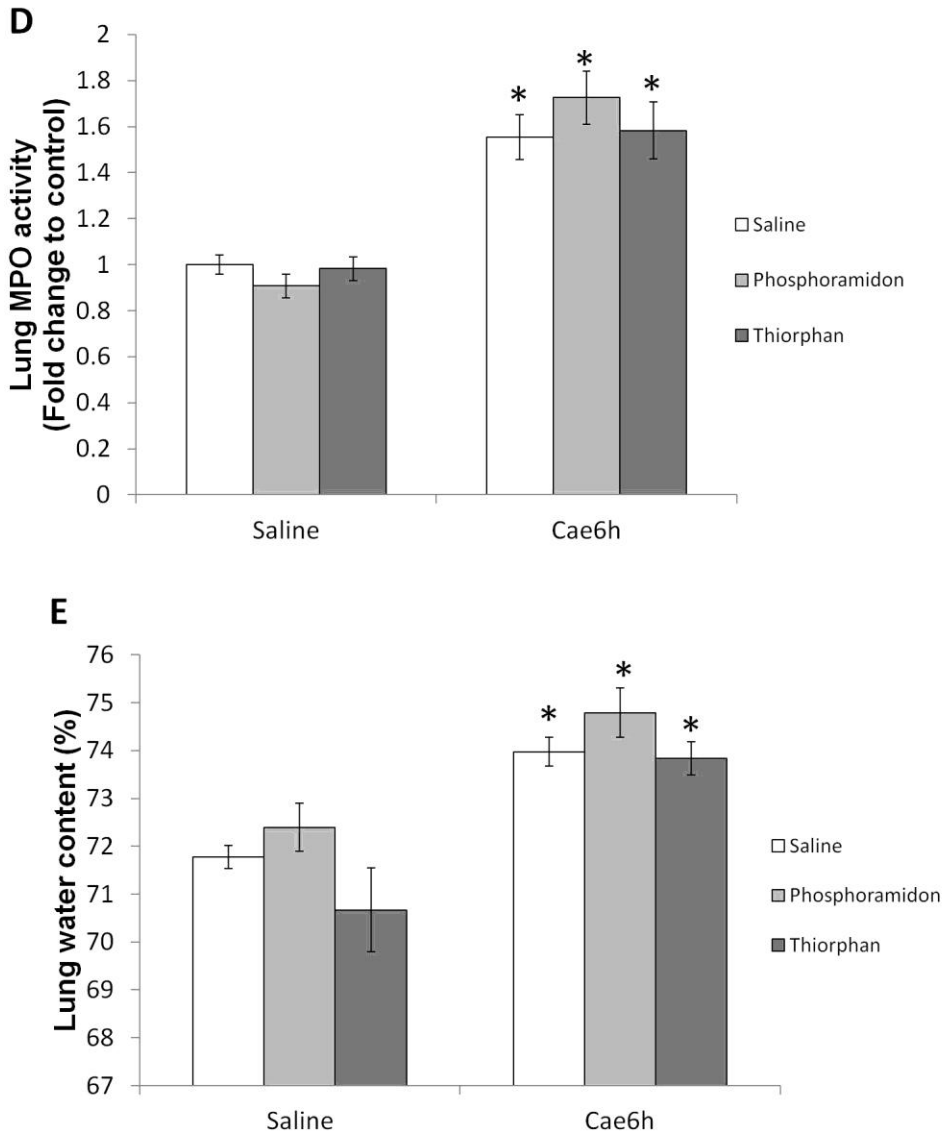


Figure 5.4 Effect of NEP inhibition on plasma amylase activity, tissue MPO activity and tissue water content. Mice were randomly given phosphoramidon (5mg/kg. i.v.) or thiorphan (10mg/kg, i.v.) before 6 hourly injections of caerulein (50μg/kg, i.p.) or normal saline. One hour after the last caerulein injection, mice were sacrificed and amylase activity, MPO activity, and water content measurement were performed as described in Materials and Methods. (A) Plasma amylase activity. (B) Pancreas MPO activity. (C) Pancreas water content. (D) Lung MPO activity. (E) Lung water content. Values are means ± SEM; n=10 mice per time point. *P<0.05 vs. control, #P<0.05 vs. caerulein group.

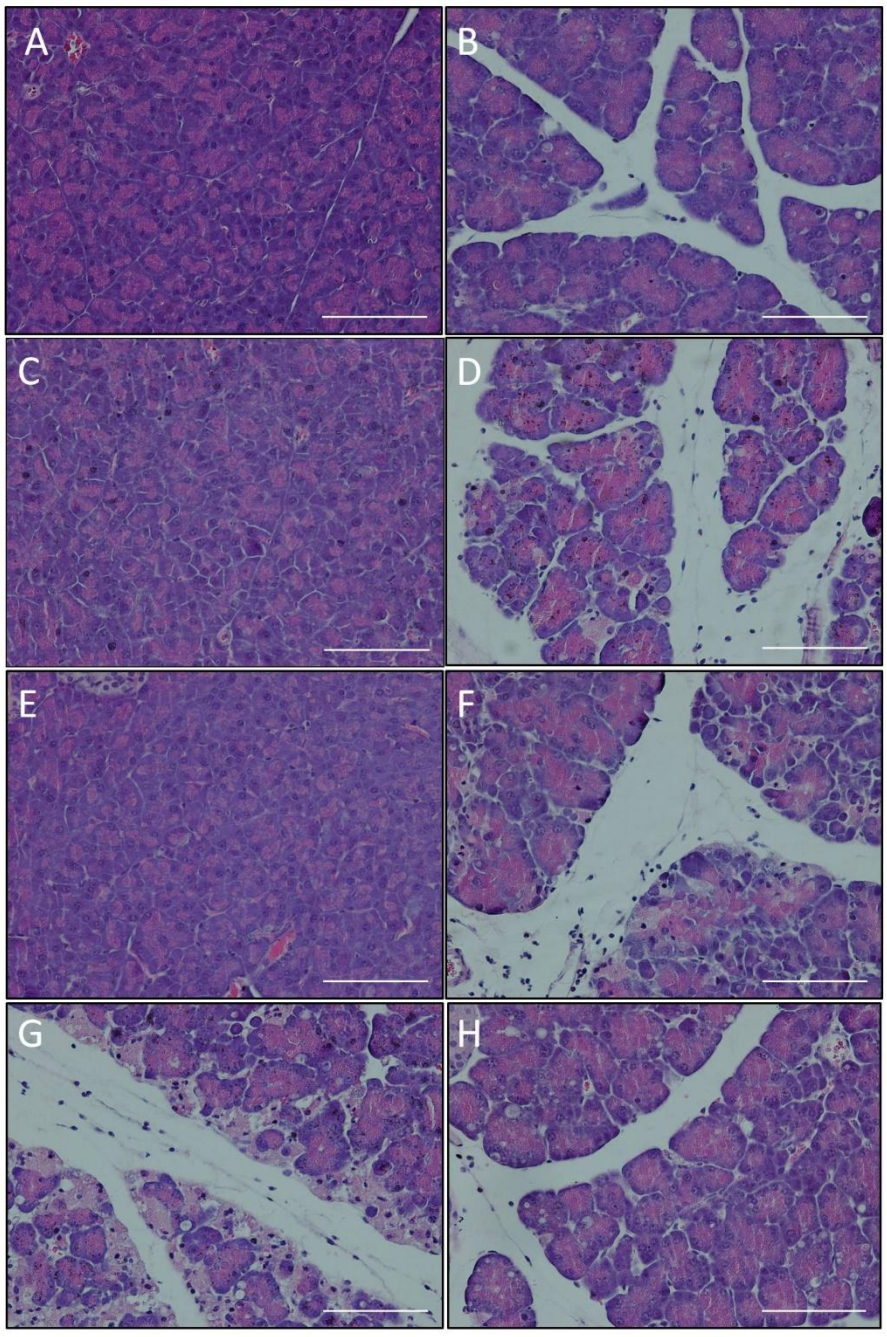


Figure 5.5 Histopathological evaluation (H&E staining) of pancreas polymorphonuclear leukocyte infiltration and injury. Formalin-fixed tissue sections were embedded in wax and cut into 5μM sections. (A) Control; (B) Mice received 6 hourly caerulein (50μg/kg) injections; (C) Mice received a single dose of phosphoramidon (5mg/kg) followed by saline injections. (D) Mice received a single dose of phosphoramidon (5mg/kg) followed by 6 hourly caerulein injections. (E) Mice received a single dose of thiorphan (10mg/kg) followed by saline injections. (F) Mice received a single dose of thiorphan (10mg/kg) followed by 6 hourly caerulein injections. (G) Mice received 10 hourly caerulein injections (H) Mice received a single dose of exogenous NEP (1mg/kg) and 10 hourly caerulein injections. Scale bars = 0.1 mm.

5.3.5 Effect of NEP inhibition on pro-inflammatory cytokine, chemokine, and adhesion molecule expression

Mice induced with moderate AP exhibited a significant elevation in pancreatic pro-inflammatory mediators which include cytokines (IL-1, IL-6, TNF-α) and chemokines (MIP-1α, MIP-2) (Table 5.1), compared with control mice which received saline injections. Adhesion molecule (ICAM-1, VCAM-1, p-selectin, e-selectin) expression also showed modest increases, but they were not significantly different from the control. In the lungs, a milder up-regulation of pro-inflammatory mediators was observed, with only IL-1 and ICAM-1 significantly increased from control mice (Table 5.1b). Inhibition of NEP in AP-induced mice generally showed a further increase of pro-inflammatory mediator expression in both pancreas and the lungs, when compared to the control groups (Table 5.1a, 5.1b). NEP inhibitors alone did not appear to raise tissue expression of any of the investigated molecules.

Table 5.1 Effect of NEP inhibition on expression of cytokine, chemokine and adhesion molecules. Mice were randomly given phosphoramidon (Phos, 5 mg/kg. i.v.) or thiorphan (Thior, 10 mg/kg, i.v.) before six hourly injections of caerulein (Cae6h, 50 mg/kg, i.p.) or normal saline. Tissue homogenates were subjected to ELISA assay and normalized with the DNA content of the homogenate and are expressed as picograms per microgram of DNA (n = 10 mice/group). *P <0.05 vs. control, #P<0.05 versus caerulein-treated mice.

(a) Expression in pancreas

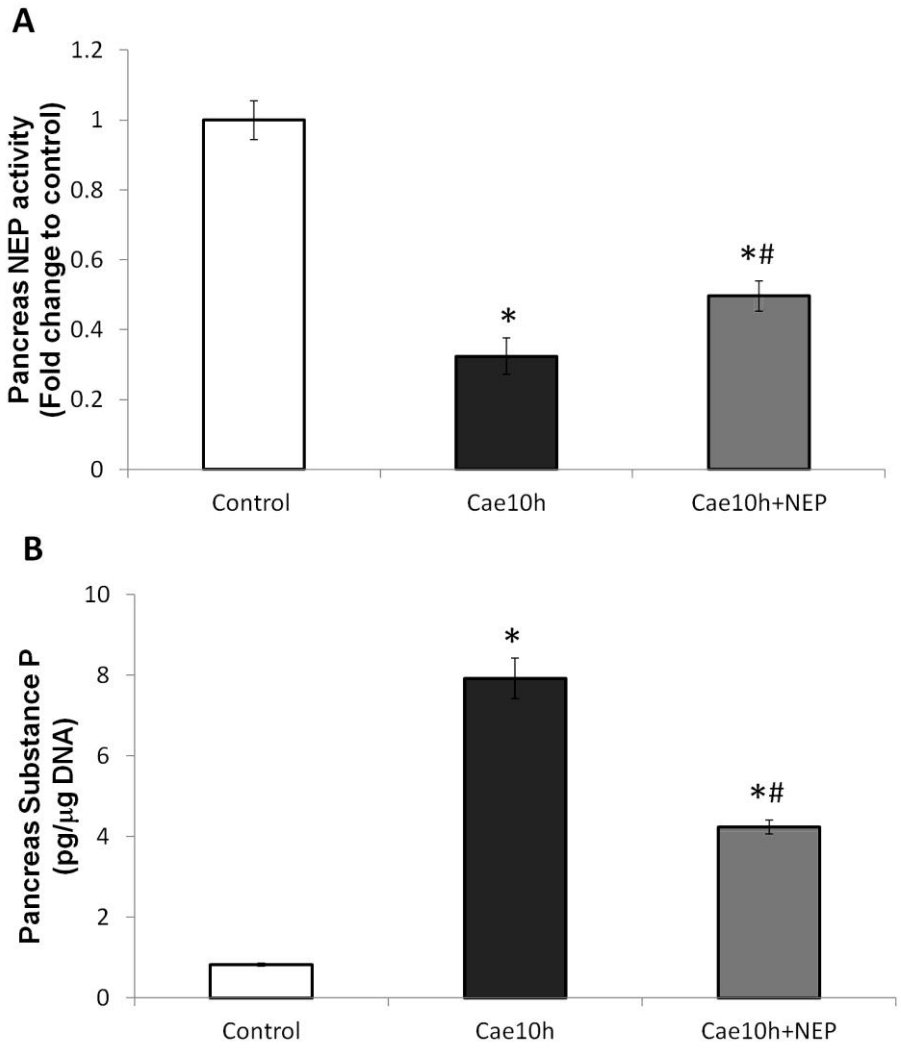
	Control	Cae6h	Phos+C6h	Thi+C6h	Phos	Thior
IL-1	3.15±0.22	5.79±0.41*	6.41±0.37*	7.38±0.41*,#	4.35±0.29	3.93±0.36
IL-6	4.08±0.27	5.98±0.61*	7.98±0.50*,#	7.74±0.63*	4.1±0.43	4.42±0.31
TNF-α	6.62±0.40	10.54±0.68*	12.1±0.66*,#	11.63±0.49*,#	7.38±0.45	7.70±0.69
MIP-1α	2.21±0.29	3.67±0.16*	6.00±0.39*,#	5.46±0.53*,#	2.85±0.27	2.47±0.27
MIP-2	4.11±0.24	6.36±0.58*	8.77±0.52*,#	6.68±0.50*	5.18±0.48	5.22±0.54
E-Selectin	8.10±0.57	9.33±0.81	12.25±1.20*	11.12±1.08	10.19±0.94	7.87±0.97
P-Selectin	26.36±1.99	31.15±3.52	43.55±4.63*	41.42±4.03*	33.42±2.28	26.60±3.19
ICAM-1	33.96±3.11	41.42±3.05	48.39±5.69	52.51±5.28*	42.51±4.98	40.31±4.71
VCAM-1	35.63±2.19	38.32±3.16	59.30±7.26*	48.49±5.49	35.49±2.46	38.07±4.48

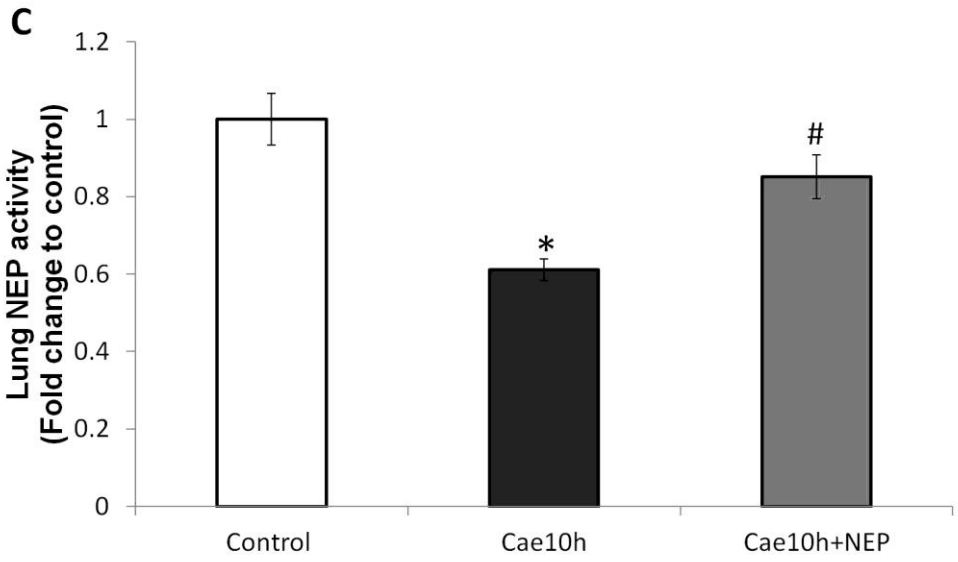
(b) Expression in lung

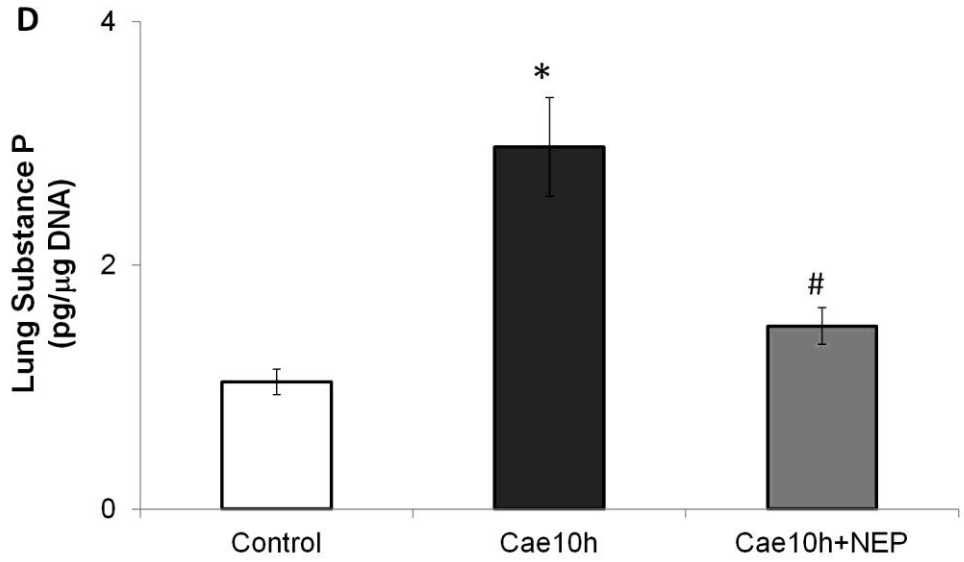
	Control	Cae6h	Phos+C6h	Thi+C6h	Phos	Thior
IL-1	13.52±1.12	21.54±2.25*	20.04±2.52	18.21±1.52	12.81±1.65	13.42±1.31
IL-6	11.91±0.53	13.57±2.05	14.07±1.40	12.34±1.36	9.78±1.44	11.21±1.49
TNF-α	10.00±0.94	16.34±2.49	20.94±1.84*	18.69±2.28*	13.17±2.28	15.50±2.75
MIP-1α	6.87±0.44	9.76±0.76	12.56±1.75*	10.57±1.44*	6.85±0.39	7.78±0.65
MIP-2	11.16±0.42	14.35±1.36	17.22±2.11*	15.77±1.45*	11.69±1.30	12.72±0.82
E-Selectin	21.92±1.35	26.39±2.88	29.60±4.63	25.02±2.94	18.43±1.93	21.78±2.02
P-Selectin	140.4±13.9	168.5±15.7	193.9±22.7	154.0±18.8	142.4±11.9	163.5±10.6
ICAM-1	218.3±12.5	366.0±21.6*	416.3±55.9*	369.7±44.5*	279.9±38.6	259.7±26.7
VCAM-1	199.0±16.3	220.0±9.7	249.0±32.6	184.2±14.3	199.7±28.6	177.8±13.7

5.3.6 Mouse recombinant NEP decreases SP levels in the pancreas, lung and plasma.

We have observed suppression of NEP activity in caerulein-induced AP. Therefore, we investigated whether mice would be protected against severe AP by treatment of exogenous NEP. Mice received 10 hourly caerulein injections develop severe AP and exhibited significantly suppressed NEP activity in the pancreas and lungs (Figure 5.6A, 5.6C). Administration of exogenous NEP markedly increase NEP activity as well as decreased SP levels in the lung and pancreas (Figure 5.6B, 5.6D). Induction of AP did not affect the background plasma NEP activity, but a 10-fold increase was observed after mice were treated with exogenous NEP (Figure 5.6E). Treatment of mice with exogenous NEP did not completely abolish pathological SP levels in the pancreas and plasma. SP levels were still significantly higher in NEP-treated AP mice, when compared to the control (Figure 5.6B, 5.6F).


A
Pancreas NEP activity
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
*#
Control
Cae10h
Cae10h+NEP
B
Pancreas Substance P
(pg/μg DNA)
10
8
6
4
2
0
*
*#
Control
Cae10h
Cae10h+NEP


C
Lung NEP activity
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
*
#
Control
Cae10h
Cae10h+NEP


D
Lung Substance P
(pg/μg DNA)
4
2
0
*
#
Control
Cae10h
Cae10h+NEP

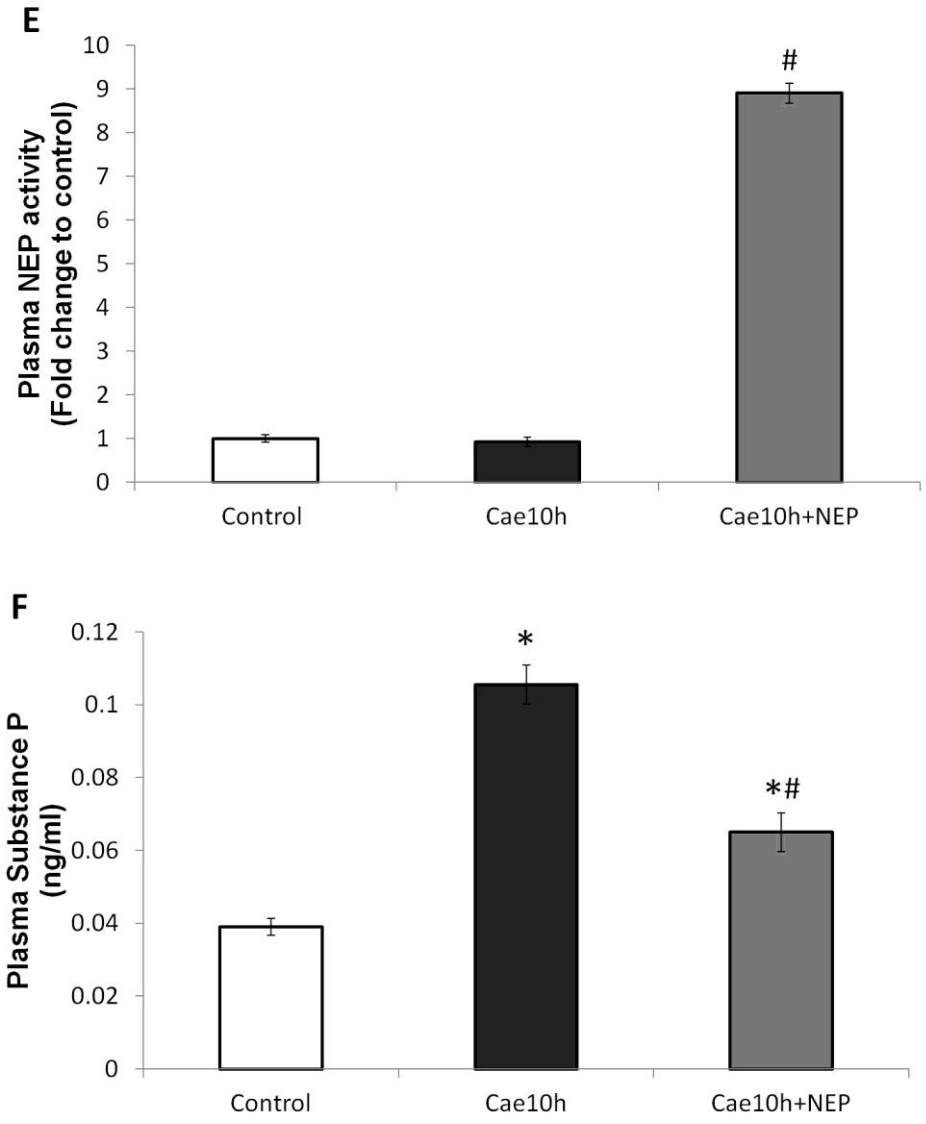
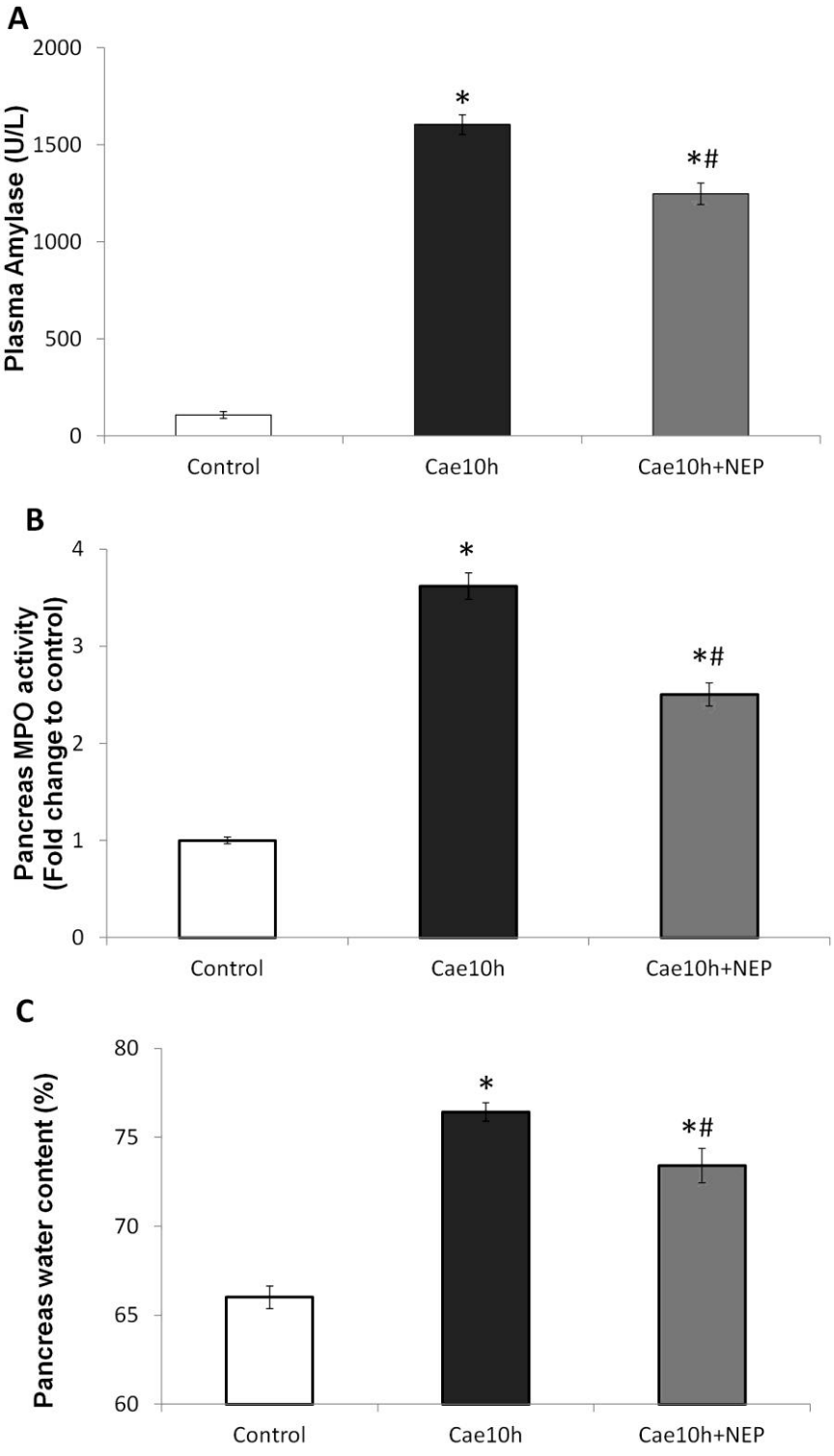


Figure 5.6 Effect of exogenous NEP on NEP activity and SP levels. Mice received a single dose of normal saline or exogenous NEP (1mg/kg) along with 10 hourly caerulein injections. One hour after the last caerulein injection, mice were sacrificed and NEP activity and SP ELISA assays were performed as described in Materials and Methods. (A) Pancreas NEP activity. (B) Pancreas SP levels. (C) Lung NEP activity. (D) Lung SP levels. (E) Plasma NEP activity. (F) Plasma SP levels. Values are means ± SEM; n=10 mice per time point. *P<0.05 vs. control.

5.3.7 Exogenous NEP protects mice against caerulein-induced pancreatic injury

Plasma amylase, as well as MPO activity and water content in the pancreas and lungs were strongly augmented after induction of severe AP (Figure 5.7A-5.7E). Mouse recombinant NEP (1mg/kg, i.v.), given during the second caerulein injection, significantly attenuated AP-induced MPO activity and water content (Figure 5.7B-5.7E). Exogenous NEP also protected mice against pancreatic injury, as shown by a decrease in plasma amylase activity (Figure 5.7A). Histological examination of the pancreas confirmed the protective effects, as demonstrated by reduced neutrophil infiltration, pancreatic edema and pancreatic necrosis (Figure 5.5G, 5.5H). Notably, the therapeutic effect of exogenous NEP correlated with a decrease of SP levels. However, a single dose of exogenous NEP treatment did not completely protect mice from severe AP, as pancreatic morphology, plasma amylase activity and tissue MPO activity were still significantly increased when compared to healthy control mice.


A
Plasma Amylase (U/L)
2000
1500
1000
500
0
*
*#
Control
Cae10h
Cae10h+NEP
B
Pancreas MPO activity
(Fold change to control)
4
3
2
1
0
*
*#
Control
Cae10h
Cae10h+NEP
C
Pancreas water content (%)
80
75
70
65
60
*
*#
Control
Cae10h
Cae10h+NEP

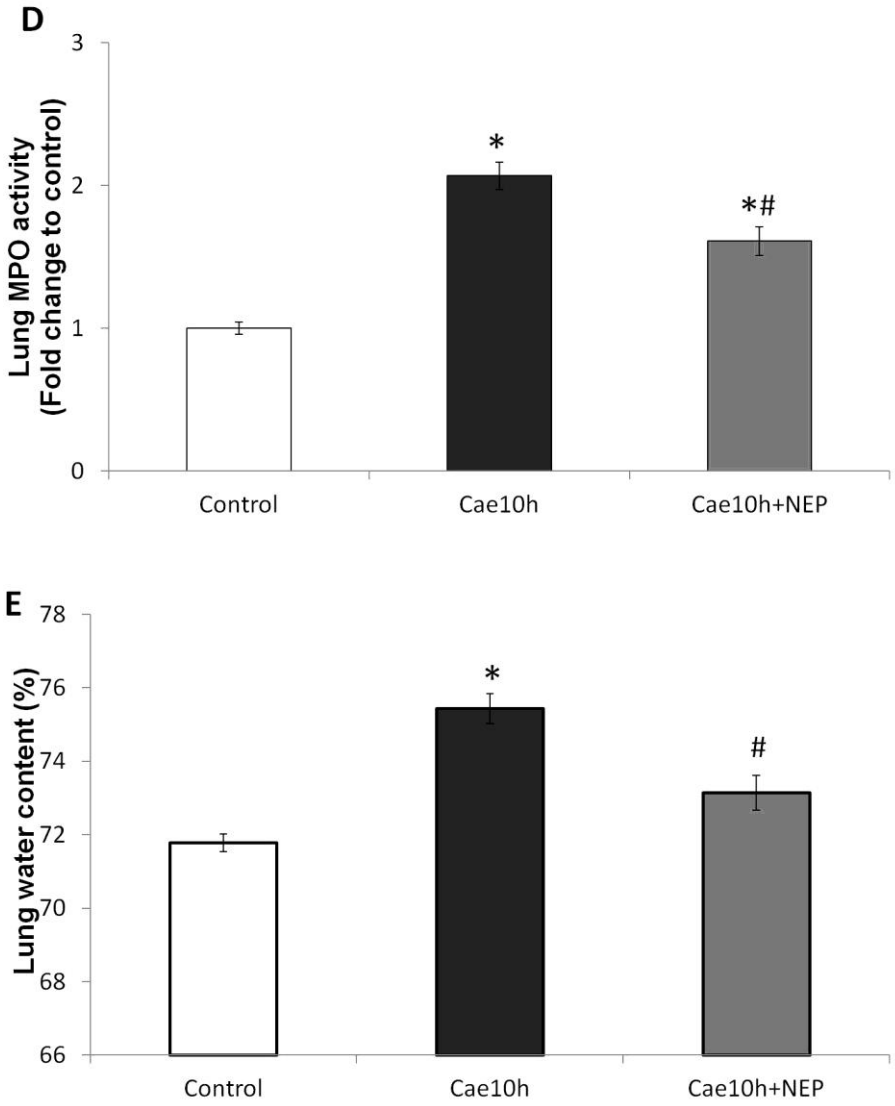


Figure 5.7 Effect of exogenous NEP on plasma amylase activity, tissue MPO activity and tissue water content. Mice received a single dose of normal saline or exogenous NEP (1mg/kg) along with 10 hourly caerulein injections. One hour after the last caerulein injection, mice were sacrificed and amylase activity, MPO activity, and water content measurement were performed as described in Materials and Methods. (A) Plasma amylase activity. (B) Pancreas MPO activity. (C) Pancreas water content. (D) Lung MPO activity. (E) Lung water content. Values are means ± SEM; n=10 mice per time point. *P<0.05 vs. control, #P<0.05 vs. caerulein group.

5.3.8 Effect of exogenous NEP treatment on pro-inflammatory cytokine, chemokine, and adhesion molecule expression

Mice received 10 hourly caerulein injections (severe AP) showed further increase of pro-inflammatory mediator levels in pancreas and lung tissue, when compared to mice that received 6 hourly caerulein injections (moderate AP) (Table 5.1a, 5.1b, 5.2a, 5.2b). Notably, NEP treatment decreased expression of pro-inflammatory cytokine (IL-1, IL-6, TNF-α), chemokine (MIP-1α, MIP-2), and adhesion molecule (ICAM-1, VCAM-1, e-selectin, p-selectin) in mice induced with severe AP (Table 5.2a, 5.2b). Consistent with MPO and histology results, exogenous NEP treatment did not completely protect mice from AP-induced up-regulation of cytokines, chemokines and adhesion molecules (Table 5.2a, 5.2b).

Table 5.2. Effect of NEP treatment on expression of cytokine, chemokine and adhesion molecules. Mice were randomly given 10 hourly caerulein (Cae10h; 50 mg/kg, i.p.) injections, and recombinant mouse NEP (1 mg/kg, i.v.) was given during the second caerulein injection. Tissue homogenates were subjected to ELISA assay and normalized with the DNA content of the homogenate and are expressed as picograms per microgram of DNA (n = 10 mice/group). *P<0.05 vs. control, #P<0.05 vs. caerulein-treated mice.

(a) In the pancreas

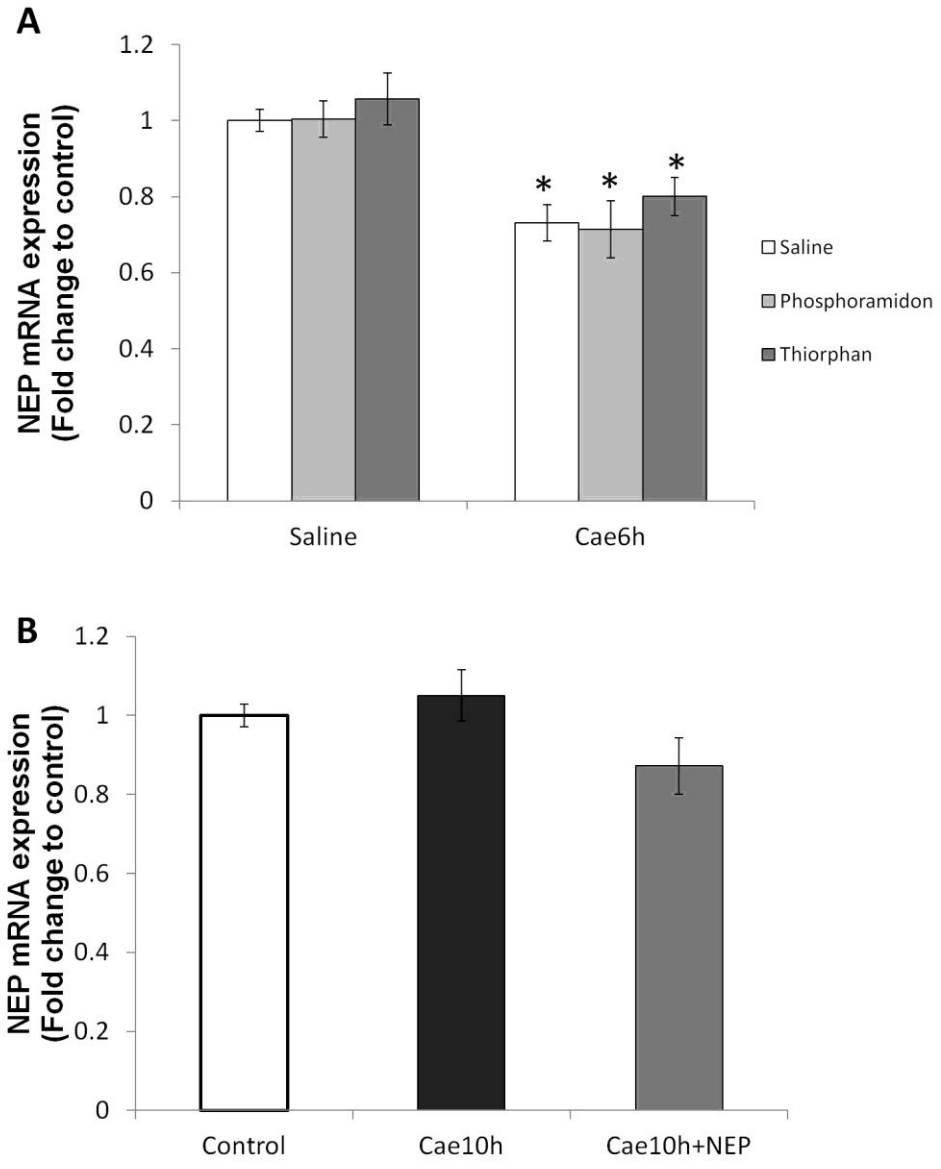
	Control	Cae10h	NEP+Cae10h
IL-1	3.15±0.22	7.93±0.69*	5.37±0.68*,#
IL-6	4.08±0.27	8.30±0.83 *	5.30±0.58#
TNF-α	6.62±0.40	19.02±2.08*	8.93±1.48#
MIP-1α	2.21±0.29	5.48±0.52*	3.56±0.46*,#
MIP-2	4.11±0.24	8.86±0.64*	6.46±0.53*,#
E-Selectin	8.10±0.57	12.77±0.96*	9.35±0.91#
P-Selectin	26.36±1.99	40.74±2.79*	27.96±2.58#
ICAM-1	33.96±3.11	55.71±5.97 *	41.31±3.14
VCAM-1	35.63±2.19	57.99±5.80*	42.82±4.86

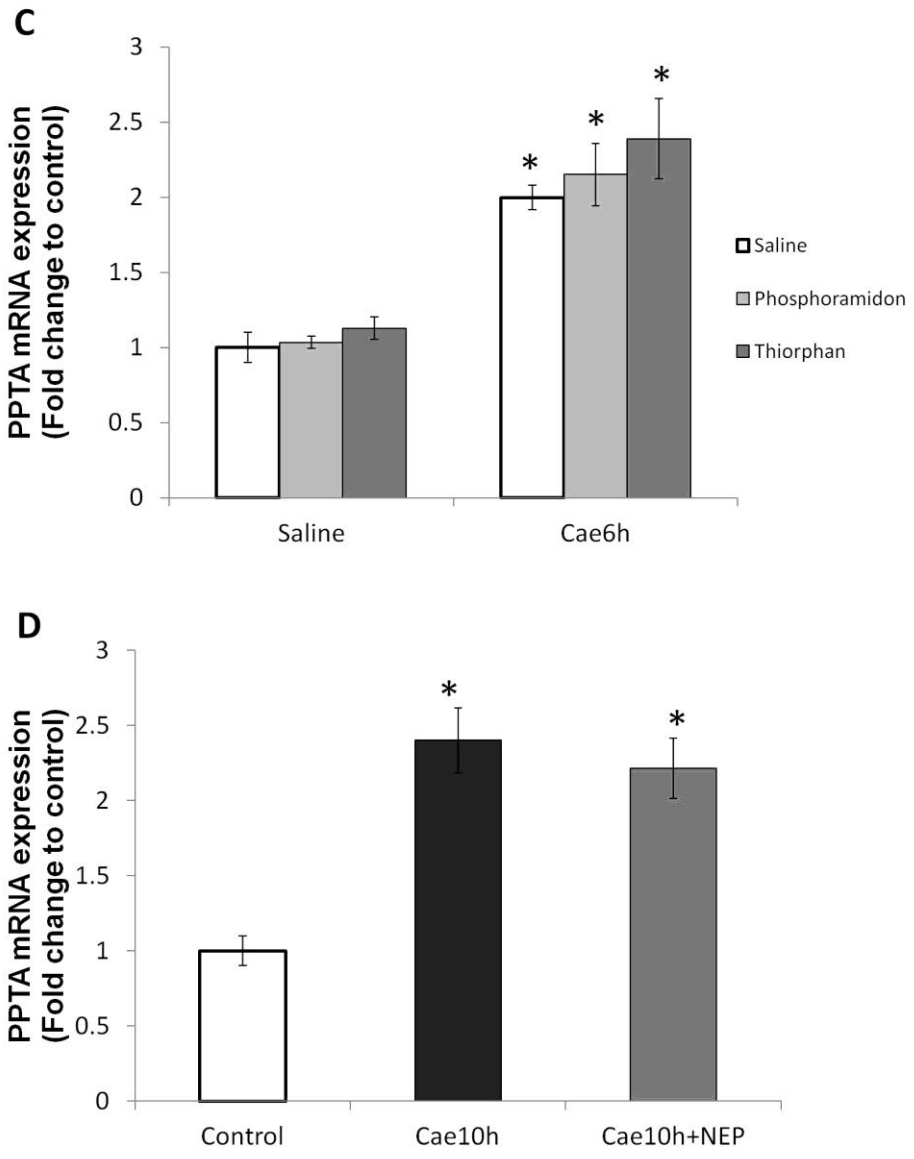
(b) In the lung

	Control	Cae10h	NEP+Cae10h
IL-1	13.52±1.12	24.75±1.88*	19.03±1.69#
IL-6	11.91±0.53	13.57±2.05	12.35±0.97
TNF-α	10.00±0.94	31.82±3.99*	18.07±1.94#
MIP-1α	6.87±0.44	11.71±0.83*	9.11±0.69 #
MIP-2	11.16±0.42	20.88±1.95*	15.37±1.27#
E-Selectin	21.92±1.35	32.98±3.83*	22.57±2.17#
P-Selectin	140.4±13.9	173.5±16.4	135.3±16.4
ICAM-1	218.3±12.5	473.5±30.8*	316.5±21.9*,#
VCAM-1	199.0±16.3	235.0±20.5	195.8±27.5

5.3.9 Exogenous NEP attenuates caerulein-induced *NK1R* mRNA up-regulation in the pancreas

It was previously reported that disruption of SP-NK1R interaction by CP96,345, a selective NK1R antagonist, decreased *PPTA* and *NK1R* mRNA expression in AP (Lau and Bhatia, 2006). Therefore in this study, we investigated the effect of NEP on mRNA expression of *PPTA*, *NK1R* and *NEP* in the pancreas. Expectedly, we observed up-regulation of *PPTA* and *NK1R* mRNA expression after induction of AP (Figure 5.8C-5.8F). However, we found only *NK1R* expression was down-regulated when mice with severe AP received exogenous NEP treatment (Figure 5.8F). Inhibition of NEP by phosphoramidon or thiorphan did not affect caerulein-induced *PPTA*/*NK1R* up-regulation (Figure 5.8C, 5.8E). Interestingly, *NEP* mRNA expression was significantly decreased in moderate AP, but recovered to normal levels in severe AP (Figure 5.8A, 5.8B).


A
NEP mRNA expression
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
Saline
Cae6h
Saline
Phosphoramidon
Thiorphan
B
NEP mRNA expression
(Fold change to control)
1.2
1
0.8
0.6
0.4
0.2
0
Control
Cae10h
Cae10h+NEP


C
PPTA mRNA expression
(Fold change to control)
3
2.5
2
1.5
1
0.5
0
*
*
*
Saline
Phosphoramidon
Thiorphan
Saline
Cae6h
D
PPTA mRNA expression
(Fold change to control)
3
2.5
2
1.5
1
0.5
0
*
*
Control
Cae10h
Cae10h+NEP

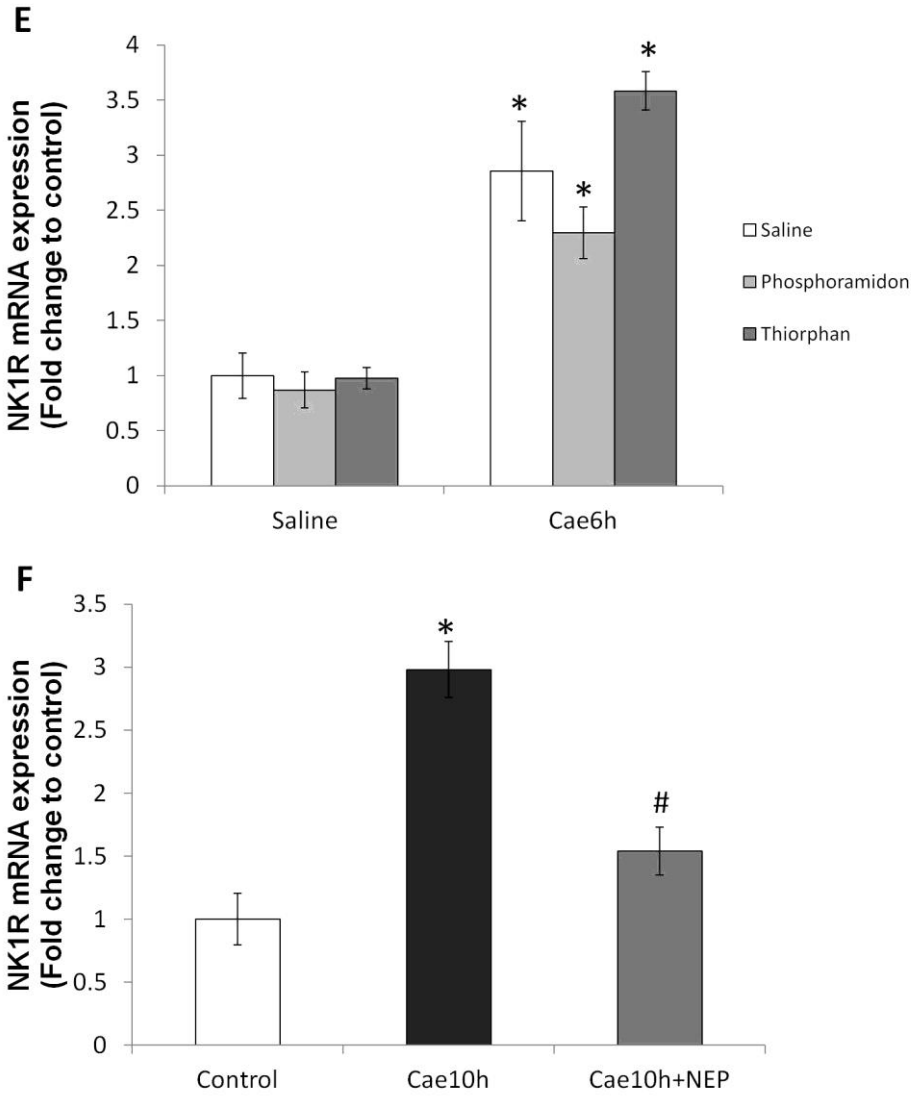


Figure 5.8 Effect of NEP on mRNA expression of NEP, NK1R and PPTA in the pancreas. NEP, *NK1R* and *PPTA* mRNA expression was determined with real time PCR. (A) Inhibition of NEP on *NEP* mRNA expression. (B) Exogenous NEP on *NEP* mRNA expression. (C) Inhibition of NEP on *PPTA* mRNA expression. (D) Exogenous NEP on *PPTA* mRNA expression. (E) Inhibition of NEP on *NK1R* expression. (F) Exogenous NEP on *NK1R* expression. Values are means ± SEM; n=8 mice per time point. *P<0.05 vs. control, #P<0.05 vs. caerulein group.

5.4 DISCUSSION

A great deal of preclinical data and some early clinical studies highlighted the importance of tachykinin interaction with their receptors for promoting inflammatory disorders (Sio et al.; Anichini et al., 1997; Sturiale et al., 1999; Scholzen et al., 2001; Tuncer et al., 2004). Thus, identifying the molecular mechanisms that modulate their expression is of crucial importance. In chapters 2 and 3, we had explained the mechanisms that lead to up-regulation of SP in the pancreatic acinar cells following caerulein stimulation (Koh et al., 2010; Koh et al., 2011). In this chapter, we report that caerulein-induced AP suppressed NEP activity in the pancreas and lungs, which could contribute to elevated SP levels in the system and promote subsequent inflammatory responses. We also report that inhibition of NEP with phosphoramidon or thiorphan exacerbated inflammation, and for the first time, treatment of AP-induced mice with exogenous NEP protected mice against severe AP.

Our findings identified that NEP was down-regulated by caerulein in mouse and isolated mouse pancreatic acinar cells. Time course studies revealed an early and rapid decrease of NEP activity after induction of AP, suggesting that NEP might be involved in early inflammatory responses. Besides, sustained down-regulation of NEP activity throughout the course of AP could contribute to uncontrolled inflammation. Interestingly, while pancreatic *NEP* mRNA expression remained down-regulated during moderate AP, its expression during severe AP recovered to a level comparable to normal, healthy mice. The effects of longer exposure to caerulein on pancreatic NEP expression might suggest activation of recovery mechanisms that help to reverse the ongoing inflammation.

Although we showed a down-regulation of NEP activity in caerulein-induced AP, but the mechanisms that regulate its expression remains unclear. Caerulein might directly inactivate transcription factors that regulate *NEP* mRNA expression. However, this did not adequately address why down-regulation of NEP activity occurred before down-regulation of *NEP* mRNA in the pancreatic acinar cells. Activation of proteases, necrosis of pancreatic tissue, or generation of reactive oxygen species might also indirectly reduce NEP activity in caerulein-induced models (Dabrowski et al., 1999). Oxidative stress was shown to inactivate NEP in the lungs (Dusser et al., 1989; Roisman and Dusser, 1995). Down-regulation of NEP was also observed in inflammatory diseases independent of caerulein stimulation, such as inflamed rat intestine and burns (Sio et al.; Hwang et al., 1993). Therefore, the regulatory mechanisms of NEP activity in vivo might also involve certain pro-inflammatory molecules, but it remains to be studied in greater detail.

SP is a major pro-inflammatory mediator in experimental AP (Maa et al., 2000b; Bhatia et al., 2003; Noble et al., 2006). NEP is of critical importance in modulating SP-induced inflammatory responses, as SP is mainly degraded and inactivated by NEP in vivo (Di Maria et al., 1998). In this study, we ascertained the role of NEP in two opposite ways: inhibition of NEP with phosphoramidon or thiorphan, or administration of exogenous recombinant mouse NEP to increase NEP activity. Treatment of mice with exogenous NEP significantly decreased SP levels in the mice, occurring in parallel with increased NEP activity. In contrast, NEP inhibitor-treated mice showed markedly increased SP levels when compared with vehicle-treated mice. Interestingly, exogenous NEP may be reasonably resilient to metabolism as markedly elevated activity in the pancreas, lungs and plasma was still present after ten hours post treatment. A previous study also suggested that the

stability of exogenous NEP was not compromised in the presence of oxidants or inflammatory fluids (Solan et al., 1998). On the other hand, the inhibitory effect of phosphoramidon and thiorphan decreased over time, showing evidence of drug metabolism. In normal healthy mice, NEP inhibitor treatment still decreased basal NEP activity at the end of the eight hour treatment protocol. On the contrary, in the AP-induced mice groups, pre-treatment of phosphoramidon or thiorphan showed no difference in NEP activity levels with mice which did not received the treatment. Hence, it appears that the effects of phosphoramidon and thiorphan were neutralized at a faster rate in AP-induced mice than normal, healthy mice.

The present study demonstrates that NEP is protective against the damaging effects of caerulein-induced AP by modulating physiological SP levels. Pancreatic edema, increased plasma amylase activity, and infiltration of neutrophils into the inflamed tissue are well known characteristics of caerulein-induced AP (Sandoval et al., 1996; Bhatia et al., 1998; Dabrowski et al., 1999; Grady et al., 2000). In AP, plasma amylase level is markedly increased due to this enzyme escaping into the blood from damaged pancreatic tissues (Frossard et al., 2008). Exogenous NEP protected mice against pancreatic damage and edema, as demonstrated by significantly lowered plasma amylase activity and tissue water content. On the contrary, mice pre-treated with NEP inhibitors exacerbated pancreatic damage. These results agreed with previous reports showing the protective role of NEP in diet-induced hemorrhagic pancreatitis in mice (Maa et al., 2000b). Our findings were also in line with previous reports showing potentiated pancreatitis when animals were challenged with exogenous SP. Exogenous SP elevated pancreatic microvascular permeability and interstitial space in caerulein-treated mice, which contribute to pancreatic microcirculatory dysfunction (Ito et al., 2007). Exogenous SP also

increased caerulein stimulated amylase output (Katoh et al., 1984). Despite this, the effects of exogenous SP administration on cytokine response in AP-induced animals remain to be explored in detail.

A major feature of AP is the infiltration of neutrophils into the pancreas. Induction of AP significantly increased MPO activity in the pancreas, indicating massive neutrophil infiltration. In AP-induced mice, MPO activity was abolished by exogenous NEP treatment but further increased by NEP inhibitor treatment. Various molecules are responsible for recruitment of inflammatory cells and propagation of inflammation. Clinically, the presence of endotoxins is linked to more severe cases of AP (Pierrakakis et al., 1990). Exogenous administration of endotoxins also strongly propagated inflammation and caused a much severe form of hemorrhagic AP in mice (Ding et al., 2003). However, the absence of detectable endotoxins in the plasma precludes its role as a pro-inflammatory mediator in our model of AP (Guice et al., 1991). Despite this, a shift in the balance towards up-regulation of pro-inflammatory mediators, which include cytokines (IL-1, IL-6 and TNF- α), chemokines (MIP-1α, MIP-2) and adhesion molecules (ICAM-1, VCAM-1, E-selectin, P-selectin) were responsible to propagate inflammation in caerulein-induced AP (Tsai et al.; Lau and Bhatia, 2006; Sun and Bhatia, 2007). In our study, a significant reduction of pro-inflammatory mediator expression was observed in NEP treated mice compared with mice without treatment. SP is able to up-regulate these mediators in leukocytes and pancreatic acinar cells mainly via activation of nuclear factor-κB (Ramnath et al., 2008; Sun et al., 2009). Thus, NEP reduces the pro-inflammatory effects of SP, and protects mice against AP.

Lung injury is a commonly associated with more severe cases of AP (Pastor et al., 2003). Uncontrolled inflammation in the pancreas elevates systemic cytokine levels and propagates damage to distant organs, causing SIRS. In our model of caerulein-induced AP, the lungs showed similar inflammatory responses with the pancreas, which were protected by exogenous NEP. Notably, pre-treatment of NEP inhibitors did not aggravate inflammation in the lung following moderate AP, despite an increase in lung SP levels. It is possible that in our model of moderate AP, inflammatory responses in the lungs is still in an early stage and the effects of SP were still insignificant. Nonetheless, the exact role of NEP in the lungs after induction of AP requires further investigation.

Diminished degradation of SP, in itself, did not appear to cause inflammation. Mice pre-treated with phosphoramidon or thiorphan without induction of AP raised systemic SP levels, but did not cause changes in tissue MPO activity, water content and histological appearance. These observations suggest that agents other than NEP substrates must first initiate pathological conditions, and then SP acts as a molecule to promote the progression of inflammation. In our case, caerulein acted as the initiating agent to cause AP. On the other hand, slightly increased cytokine/chemokine/adhesion molecule levels were observed in the pancreas and lung after inhibition of NEP, when compared to untreated controls. This is not surprising as previous studies have shown the expression of cytokines and chemokines can be directly up-regulated by NK1R activation via a NF-κB dependent mechanism (Ramnath and Bhatia, 2006; Sun et al., 2007).

Previous studies have shown disruption of SP-NK1R interaction by a NK1R antagonist, CP96,345, significantly reversed AP-induced *PPTA*/*NK1R* mRNA up-regulation in the pancreas (Lau and Bhatia, 2006). Therefore, it is necessary to test

how NEP affects mRNA expression of *PPTA* and *NK1R* after induction of AP. We found that only *NK1R* mRNA expression was abolished on NEP treated AP-induced mice, while all other treatment groups showed no significant changes when compared to their respective vehicle treated controls. Activation of NK1R may contribute, but not dictate, the expression of *PPTA*/*NK1R* in mice. Furthermore, in our experiments, exogenous NEP did not completely abolish physiological SP availability. An in depth study of SP on the expression of *PPTA*/*NK1R* is necessary to elucidate the mechanisms involved.

Preclinical experiments that involve disruption of SP-NK1R interaction, which include NK1R antagonism and blockade of SP release from sensory nerve endings, have successfully protected animals against AP. Results in this study describe targeting NEP as a novel mechanism to disrupt SP-NK1R interaction during AP, chiefly via degradation of SP. Administration of exogenous NEP was previously suggested as a safe and feasible method to protect animals against various inflammatory disorders (Neely et al., 1996; Solan et al., 1998; Sturiale et al., 1999; Kirkwood et al., 2001). Glucocorticoids, such as dexamethasone, are anti-inflammatory drugs that were found to increase NEP expression, making it a potential therapeutic option to target NEP in SP-mediated inflammatory responses (Borson and Gruenert, 1991; Lang and Murlas, 1992). One major limitation of our study is that the effects of NEP on other NEP substrates, such as bradykinins and amyloid beta, were not addressed. Besides this, SP can also be degraded by angiotensin converting enzyme (ACE) (Skidgel and Erdos, 2004). Future work can be done to address the role of ACE in SP-mediated responses in AP.

On the basis of this series of experiments, we have concluded that a high persistent level of SP during AP could be contributed by a disruption of NEP activity,

leading to a detrimental inflammatory condition that increases and perpetuates pancreatic and lung injury. Pharmacological inhibition of the SP-degrading enzyme NEP led to increased availability of SP and exacerbated AP. Conversely, treatment of mice with exogenous recombinant NEP protects mice against the detrimental effects of severe AP. Taken together, it is hoped that the results of these experiments and future studies will lead to new approaches for the prevention of inflammatory cascade in patients with AP.

CHAPTER 6: SUMMARY OF CONTRIBUTIONS AND FUTURE DIRECTIONS

6.1 SUMMARY OF CONTRIBUTIONS

SP-NK1R interaction has been identified as a key mediator of inflammation. Interruption of SP-NK1R interaction has shown promising results on managing the detrimental effects of experimental AP. On the other hand, the effects of SP-NK1R intereaction could be potentiated due to up-regulation of SP and NK1R. Even though many studies have reported elevated levels of SP and NK1R in the inflamed site, mechanisms that regulate their expression were not clear. Therefore, this study aimed to investigate different mechanisms that might lead to up-regulation of SP and its receptor, NK1R, in caerulein-induced AP.

In the first part of the study, we examined about how caerulein modulated SP/NK1R expression in isolated mouse pancreatic acinar cells. Our results showed that both gene and protein expression of SP/NK1R were induced by caerulein. The up-regulation of both ligand and receptor is interesting, as this might contribute to markedly increased SP-NK1R interaction during pathological conditions. It is also interesting to note that the expression levels of SP/NK1R were induced by supramaximal concentrations (10^{-7}M) of caerulein, but not at concentrations that stimulate physiological responses (10^{-10}M). The effects of caerulein were also rapid, with a significant up-regulation of SP/NK1R expression within 30-60 minutes, suggesting that increased SP/NK1R levels comes in the early stage of AP.

After confirming the effect of caerulein on SP/NK1R expression in pancreatic acinar cells, we proceeded to investigate the mechanisms involved. We first showed that caerulein-induced SP up-regulation was mediated through CCK_A receptors by

using a selective CCK_A antagonist. Then, we demonstrated that treatment of pancreatic acinar cells with caerulein (10^{-7}M) induced the phosphorylation of PKCs (PKCα and PKCδ) and MAPKs (ERK and JNK). We also showed that caerulein increased NF-κB and AP-1 DNA binding activity. By using specific inhibitors of these secondary messengers, we found that caerulein-induced SP/NK1R up-regulation was mediated through a PKC-MAPK-NF-κB/AP-1 dependent pathway. It is hoped that this finding could provide a hint on the regulation of SP/NK1R in other cell types.

In addition to caerulein-mediated responses, this thesis also explored other mechanisms that might regulate SP/NK1R expression in pancreatic acinar cells. In chapter 4, we demonstrated that activation of NK1R induced up-regulation of both SP and NK1R. By using specific inhibitors, we found that SP-induced up-regulation of SP/NK1R involved PKCs, MAPKs and NF-κB, which is similar to caerulein-mediated responses. Our findings also validated a previous study showing SP-induced activation of PKC and MAPKs in murine pancreatic acinar cells (Ramnath et al., 2007; Ramnath et al., 2008). Not surprisingly, we also found that caerulein-induced SP/NK1R up-regulation is independent of NK1R activation in isolated pancreatic acinar cells. This is interesting as it was previously showed that inhibition of NK1R ameliorated SP/NK1R expression in animal models of AP (Lau and Bhatia, 2006). Therefore, this auto-regulatory mechanism might be triggered only by a sufficiently high amount of SP, as happened during an inflammation. The positive feedback mechanism of SP/NK1R expression is significant, as this might cause a much higher availability of SP and NK1R, leading to a greater SP-NK1R interaction, and finally to a greater potentiation of inflammation.

In chapter 5, we investigated the role of NEP in caerulein-induced AP model. We found that NEP activity was significantly suppressed in both cellular and animal model of caerulein-induced AP. Furthermore, treatment of mice with NEP inhibitors raised systemic SP levels and worsened inflammation. On the other hand, treatment of mice with exogenous NEP offered partial protection against the detrimental effects of AP. Administration of exogenous NEP also decreased systemic SP levels. These interesting findings showed that elevated SP levels in AP were constituted from multiple mechanisms, which include a diminution of degratory mechanisms.

In conclusion, we have demonstrated three possible mechanisms that regulate SP/NK1R expression in a murine model of caerulein-induced AP (Figure 6.1). Firstly, caerulein could directly stimulate CCK_A receptors, and caused up-regulation via a PKC-MAPK-NF-κB dependant mechanism. Secondly, activation of NK1R itself could cause up-regulation of SP/NK1R, possibly via similar downstream mechanisms with caerulein. Lastly, caerulein might also cause inhibition and down-regulation of NEP activity. A diminished NEP activity will then increase SP bioavailability and thus potentiates SP-NK1R interaction and subsequent inflammation.

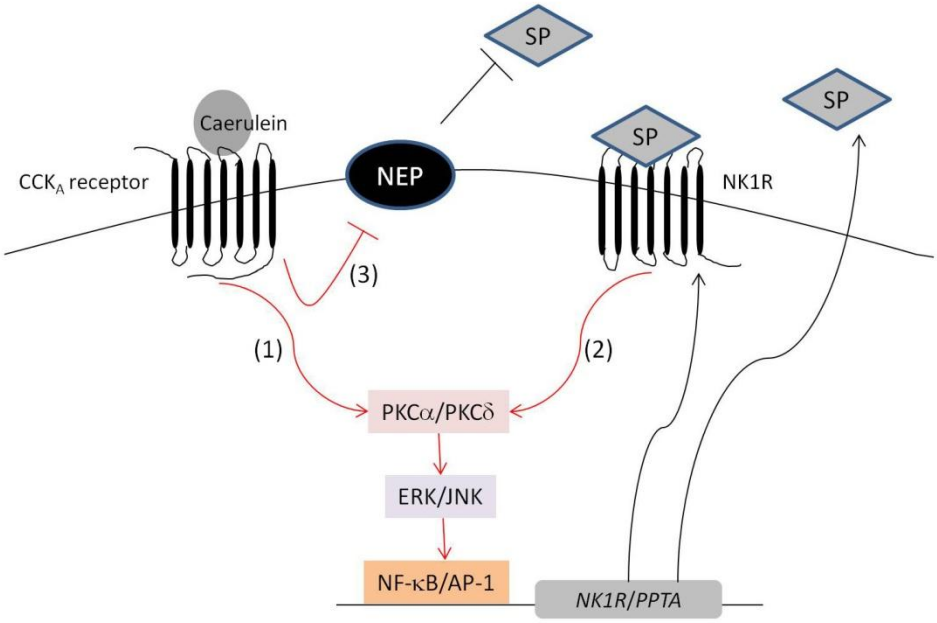


Figure 6.1 Schematic representation of proposed mechanisms that regulate SP/NK1R expression. (1) Activation of CCK_A receptors increased SP/NK1R expression. (2) Activation of NK1R increased SP/NK1R expression. (3) Caerulein down-regulated NEP activity and diminished SP degradation.

6.2 FUTURE DIRECTIONS

Understanding the mechanisms that mediate AP is crucial for the discovery and development of more effective treatment strategies. Currently, the focus of drug development is on the discovery of new NK1R antagonists. The findings of this thesis provided an alternate perspective. As we have shown in chapter 5, regulation of SP levels by manipulating NEP activity is one of the mechanisms that cause progression of inflammation. Therefore, new therapeutic options that enable regulation of SP levels might be a feasible way to manage diseases that involve the SP-NK1R pathway. This in turn would help to improve management of inflammatory diseases and improve treatment efficacy.

The research on SP/NK1R expression is still in its early stage. Therefore, several key aspects are recommended for future work:

1. As the neurons are considered as a major source of SP, it is crucial to understand how SP/NK1R expression is regulated in neuronal cells.

2. As angiotensin converting enzyme (ACE) was also shown to be involved in SP degradation, the role of ACE in AP could be investigated.

3. Elucidate the importance and role of SP derived from pancreatic acinar cells *in vivo*.

4. Further elucidate possible mechanisms that regulate SP/NK1R expression in pancreatic acinar cells.

REFERENCES

Adler G, Hupp T and Kern HF (1979) Course and spontaneous regression of acute pancreatitis in the rat. *Virchows Arch A Pathol Anat Histol* **382**:31-47.

Aho HJ, Koskensalo SM and Nevalainen TJ (1980) Experimental pancreatitis in the rat. Sodium taurocholate-induced acute haemorrhagic pancreatitis. *Scand J Gastroenterol* **15**:411-416.

Alexandre M, Pandol SJ, Gorelick FS and Thrower EC (2011) The emerging role of smoking in the development of pancreatitis. *Pancreatology* **11**:469-474.

Amantini C, Mosca M, Lucciarini R, Perfumi MC and Santoni G (2008) Thiorphan-induced survival and proliferation of rat thymocytes by activation of Akt/survivin pathway and inhibition of caspase-3 activity. *J Pharmacol Exp Ther* **327**:215-225.

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

Anichini M, Cesaretti S, Lepori M, Maddali Bongi S, Maresca M and Zoppi M (1997) Substance P in the serum of patients with rheumatoid arthritis. *Rev Rhum Engl Ed* **64**:18-21.

Appelros S and Borgstrom A (1999) Incidence, aetiology and mortality rate of acute pancreatitis over 10 years in a defined urban population in Sweden. *Br J Surg* **86**:465-470.

Bae S, Matsunaga Y, Tanaka Y and Katayama I (1999) Autocrine induction of substance P mRNA and peptide in cultured normal human keratinocytes. *Biochem Biophys Res Commun* **263**:327-333.

Bae SJ, Matsunaga Y, Takenaka M, Tanaka Y, Hamazaki Y, Shimizu K and Katayama I (2002) Substance P induced preprotachykinin-a mRNA, neutral endopeptidase mRNA and substance P in cultured normal fibroblasts. *Int Arch Allergy Immunol* **127**:316-321.

Bastani B, Yang L, Baldassare JJ, Pollo DA and Gardner JD (1995) Cellular distribution of isoforms of protein kinase C (PKC) in pancreatic acini. *Biochim Biophys Acta* **1269**:307-315.

Beer S, Weighardt H, Emmanuilidis K, Harzenetter MD, Matevossian E, Heidecke CD, Bartels H, Siewert JR and Holzmann B (2002) Systemic neuropeptide levels as predictive indicators for lethal outcome in patients with postoperative sepsis. *Crit Care Med* **30**:1794-1798.

Beger HG, Rau B, Mayer J and Pralle U (1997) Natural course of acute pancreatitis. *World J Surg* **21**:130-135.

Bhatia M, Saluja AK, Hofbauer B, Frossard JL, Lee HS, Castagliuolo I, Wang CC, Gerard N, Pothoulakis C and Steer ML (1998) Role of substance P and the neurokinin 1 receptor in acute pancreatitis and pancreatitis-associated lung injury. *Proc Natl Acad Sci U S A* **95**:4760-4765.

Bhatia M, Slavin J, Cao Y, Basbaum AI and Neoptolemos JP (2003) Preprotachykinin-A gene deletion protects mice against acute pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol* **284**:G830-836.

Bhatia M, Wong FL, Cao Y, Lau HY, Huang J, Puneet P and Chevali L (2005) Pathophysiology of acute pancreatitis. *Pancreatology* **5**:132-144.

Bhatt KH, Pandey RK, Dahiya Y and Sodhi A Protein kinase Cdelta and protein tyrosine kinase regulate peptidoglycan-induced nuclear factor-kappaB

activation and inducible nitric oxide synthase expression in mouse peritoneal macrophages in vitro. *Mol Immunol* **47**:861-870.
Bi Y and Williams JA (2004) Receptor biology and signal transduction in pancreatic acinar cells. *Curr Opin Gastroenterol* **20**:427-434.
Bladh LG, Johansson-Haque K, Rafter I, Nilsson S and Okret S (2009) Inhibition of extracellular signal-regulated kinase (ERK) signaling participates in repression of nuclear factor (NF)-kappaB activity by glucocorticoids. *Biochim Biophys Acta* **1793**:439-446.
Bondy B, Baghai TC, Minov C, Schule C, Schwarz MJ, Zwanzger P, Rupprecht R and Moller HJ (2003) Substance P serum levels are increased in major depression: preliminary results. *Biol Psychiatry* **53**:538-542.
Borson DB and Gruenert DC (1991) Glucocorticoids induce neutral endopeptidase in transformed human tracheal epithelial cells. *Am J Physiol* **260**:L83-89.
Boutros T, Chevet E and Metrakos P (2008) Mitogen-activated protein (MAP) kinase/MAP kinase phosphatase regulation: roles in cell growth, death, and cancer. *Pharmacol Rev* **60**:261-310.
Bradford MM (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem* **72**:248-254.
Brannon PM (1990) Adaptation of the exocrine pancreas to diet. *Annu Rev Nutr* **10**:85-105.
Browne GW and Pitchumoni CS (2006) Pathophysiology of pulmonary complications of acute pancreatitis. *World J Gastroenterol* **12**:7087-7096.
Bruzzone R, Regazzi R and Wollheim CB (1988) Caerulein causes translocation of protein kinase C in rat acini without increasing cytosolic free Ca2+. *Am J Physiol* **255**:G33-39.
Buckner CK, Dea D, Liberati N and Krell RD (1991) A pharmacologic examination of receptors mediating serotonin-induced bronchoconstriction in the anesthetized guinea pig. *J Pharmacol Exp Ther* **257**:26-34.
Castagliuolo I, Keates AC, Qiu B, Kelly CP, Nikulasson S, Leeman SE and Pothoulakis C (1997) Increased substance P responses in dorsal root ganglia and intestinal macrophages during Clostridium difficile toxin A enteritis in rats. *Proc Natl Acad Sci U S A* **94**:4788-4793.
Chang L and Karin M (2001) Mammalian MAP kinase signalling cascades. *Nature* **410**:37-40.
Chang MM, Leeman SE and Niall HD (1971) Amino-acid sequence of substance P. *Nat New Biol* **232**:86-87.
Chen BC and Lin WW (2001) PKC- and ERK-dependent activation of I kappa B kinase by lipopolysaccharide in macrophages: enhancement by P2Y receptor-mediated CaMK activation. *Br J Pharmacol* **134**:1055-1065.
Cheng CY, Hsieh HL, Sun CC, Lin CC, Luo SF and Yang CM (2009) IL-1 beta induces urokinase-plasminogen activator expression and cell migration through PKC alpha, JNK1/2, and NF-kappaB in A549 cells. *J Cell Physiol* **219**:183-193.
Chiwakata C, Brackmann B, Hunt N, Davidoff M, Schulze W and Ivell R (1991) Tachykinin (substance-P) gene expression in Leydig cells of the human and mouse testis. *Endocrinology* **128**:2441-2448.
Christophe J (1994) Pancreatic tumoral cell line AR42J: an amphicrine model. *Am J Physiol* **266**:G963-971.

Corcoran KE and Rameshwar P (2007) Nuclear factor-kappaB accounts for the repressor effects of high stromal cell-derived factor-1alpha levels on Tac1 expression in nontumorigenic breast cells. *Mol Cancer Res* **5**:373-381.

Cosen-Binker LI, Lam PP, Binker MG, Reeve J, Pandol S and Gaisano HY (2007) Alcohol/cholecystokinin-evoked pancreatic acinar basolateral exocytosis is mediated by protein kinase C alpha phosphorylation of Munc18c. *J Biol Chem* **282**:13047-13058.

Czako L, Takacs T, Varga IS, Tiszlavicz L, Hai DQ, Hegyi P, Matkovics B and Lonovics J (1998) Involvement of oxygen-derived free radicals in L-arginine-induced acute pancreatitis. *Dig Dis Sci* **43**:1770-1777.

Dabrowski A, Konturek SJ, Konturek JW and Gabryelewicz A (1999) Role of oxidative stress in the pathogenesis of caerulein-induced acute pancreatitis. *Eur J Pharmacol* **377**:1-11.

Day AL, Wick E, Jordan TH, Jaffray CE, Bunnett NW, Grady EF and Kirkwood KS (2005) Neutral endopeptidase determines the severity of pancreatitis-associated lung injury. *J Surg Res* **128**:21-27.

Delgado AV, McManus AT and Chambers JP (2003) Production of tumor necrosis factor-alpha, interleukin 1-beta, interleukin 2, and interleukin 6 by rat leukocyte subpopulations after exposure to substance P. *Neuropeptides* **37**:355-361.

Di Maria GU, Bellofiore S and Geppetti P (1998) Regulation of airway neurogenic inflammation by neutral endopeptidase. *Eur Respir J* **12**:1454-1462.

Ding SP, Li JC and Jin C (2003) A mouse model of severe acute pancreatitis induced with caerulein and lipopolysaccharide. *World J Gastroenterol* **9**:584-589.

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

Dusser DJ, Djokic TD, Borson DB and Nadel JA (1989) Cigarette smoke induces bronchoconstrictor hyperresponsiveness to substance P and inactivates airway neutral endopeptidase in the guinea pig. Possible role of free radicals. *J Clin Invest* **84**:900-906.

Esteban F, Gonzalez-Moles MA, Castro D, Martin-Jaen Mdel M, Redondo M, Ruiz-Avila I and Munoz M (2009) Expression of substance P and neurokinin-1-receptor in laryngeal cancer: linking chronic inflammation to cancer promotion and progression. *Histopathology* **54**:258-260.

Estival A, Pradel P, Wicker-Planquart C, Vaysse N, Puigserver A and Clemente F (1991) Regulation of amylase and chymotrypsinogen expression by dexamethasone and caerulein in serum-free-cultured pancreatic acinar AR4-2J cells. Influence of glucose. *Biochem J* **279 (Pt 1)**:197-201.

Fagenholz PJ, Fernandez-del Castillo C, Harris NS, Pelletier AJ and Camargo CA, Jr. (2007) Direct medical costs of acute pancreatitis hospitalizations in the United States. *Pancreas* **35**:302-307.

Figini M, Emanueli C, Grady EF, Kirkwood K, Payan DG, Ansel J, Gerard C, Geppetti P and Bunnett N (1997) Substance P and bradykinin stimulate plasma extravasation in the mouse gastrointestinal tract and pancreas. *Am J Physiol* **272**:G785-793.

Fiskerstrand CF, Newey P, Millan F and Quinn JP (1997) The role of the octamer binding proteins and the NF-kB complex in neuropeptide gene expression. *Biochem Soc Trans* **25**:S573.

Frossard JL, Steer ML and Pastor CM (2008) Acute pancreatitis. *Lancet* **371**:143-152.

Ghosh S and Hayden MS (2008) New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* **8**:837-848.
Gonzalez-Moles MA, Brener S, Ruiz-Avila I, Gil-Montoya JA, Tostes D, Bravo M and Esteban F (2009) Substance P and NK-1R expression in oral precancerous epithelium. *Oncol Rep* **22**:1325-1331.
Goode T, O'Connell J, Anton P, Wong H, Reeve J, O'Sullivan GC, Collins JK and Shanahan F (2000) Neurokinin-1 receptor expression in inflammatory bowel disease: molecular quantitation and localisation. *Gut* **47**:387-396.
Gorelick FS and Otani T (1999) Mechanisms of intracellular zymogen activation. *Baillieres Best Pract Res Clin Gastroenterol* **13**:227-240.
Gorr SU and Tseng SY (1995) Aggregation and concentration-dependent sorting of exocrine regulated secretory proteins. *Biochem Biophys Res Commun* **215**:82-88.
Grady EF, Yoshimi SK, Maa J, Valeroso D, Vartanian RK, Rahim S, Kim EH, Gerard C, Gerard N, Bunnett NW and Kirkwood KS (2000) Substance P mediates inflammatory oedema in acute pancreatitis via activation of the neurokinin-1 receptor in rats and mice. *Br J Pharmacol* **130**:505-512.
Grady T, Mah'Moud M, Otani T, Rhee S, Lerch MM and Gorelick FS (1998) Zymogen proteolysis within the pancreatic acinar cell is associated with cellular injury. *Am J Physiol* **275**:G1010-1017.
Gravante G, Garcea G, Ong SL, Metcalfe MS, Berry DP, Lloyd DM and Dennison AR (2009) Prediction of mortality in acute pancreatitis: a systematic review of the published evidence. *Pancreatology* **9**:601-614.
Gschwendt M, Muller HJ, Kielbassa K, Zang R, Kittstein W, Rincke G and Marks F (1994) Rottlerin, a novel protein kinase inhibitor. *Biochem Biophys Res Commun* **199**:93-98.
Guedea AL, Schrick C, Guzman YF, Leaderbrand K, Jovasevic V, Corcoran KA, Tronson NC and Radulovic J (2011) ERK-associated changes of AP-1 proteins during fear extinction. *Mol Cell Neurosci* **47**:137-144.
Guice KS, Oldham KT, Remick DG, Kunkel SL and Ward PA (1991) Anti-tumor necrosis factor antibody augments edema formation in caerulein-induced acute pancreatitis. *J Surg Res* **51**:495-499.
Gukovsky I, Reyes CN, Vaquero EC, Gukovskaya AS and Pandol SJ (2003) Curcumin ameliorates ethanol and nonethanol experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* **284**:G85-95.
Gullo L, Migliori M, Olah A, Farkas G, Levy P, Arvanitakis C, Lankisch P and Beger H (2002) Acute pancreatitis in five European countries: etiology and mortality. *Pancreas* **24**:223-227.
Guo CJ, Douglas SD, Gao Z, Wolf BA, Grinspan J, Lai JP, Riedel E and Ho WZ (2004) Interleukin-1beta upregulates functional expression of neurokinin-1 receptor (NK-1R) via NF-kappaB in astrocytes. *Glia* **48**:259-266.
Halangk W, Lerch MM, Brandt-Nedelev B, Roth W, Ruthenbuerger M, Reinheckel T, Domschke W, Lippert H, Peters C and Deussing J (2000) Role of cathepsin B in intracellular trypsinogen activation and the onset of acute pancreatitis. *J Clin Invest* **106**:773-781.
Hall JM and Morton IK (1991) Novel ligands confirm NK-1 receptor-mediated modulation of neurotransmission in the guinea pig vas deferens preparation. *Ann N Y Acad Sci* **632**:404-406.
Harrison S and Geppetti P (2001) Substance p. *Int J Biochem Cell Biol* **33**:555-576.

Hegde A, Koh YH, Moochhala SM and Bhatia M (2010) Neurokinin-1 receptor antagonist treatment in polymicrobial sepsis: molecular insights. *Int J Inflam* **2010**:601098.

Hegyi P, Gray MA and Argent BE (2003) Substance P inhibits bicarbonate secretion from guinea pig pancreatic ducts by modulating an anion exchanger. *Am J Physiol Cell Physiol* **285**:C268-276.

Hegyi P, Rakonczay Z, Jr., Sari R, Gog C, Lonovics J, Takacs T and Czako L (2004) L-arginine-induced experimental pancreatitis. *World J Gastroenterol* **10**:2003-2009.

Hirota M, Ohmuraya M and Baba H (2006) The role of trypsin, trypsin inhibitor, and trypsin receptor in the onset and aggravation of pancreatitis. *J Gastroenterol* **41**:832-836.

Ho WZ, Lai JP, Zhu XH, Uvaydova M and Douglas SD (1997) Human monocytes and macrophages express substance P and neurokinin-1 receptor. *J Immunol* **159**:5654-5660.

Hofbauer B, Saluja AK, Lerch MM, Bhagat L, Bhatia M, Lee HS, Frossard JL, Adler G and Steer ML (1998) Intra-acinar cell activation of trypsinogen during caerulein-induced pancreatitis in rats. *Am J Physiol* **275**:G352-362.

Hokfelt T and Kuteeva E (2006) Substance P: a neuropeptide. *Am J Psychiatry* **163**:578.

Holden NS, Squires PE, Kaur M, Bland R, Jones CE and Newton R (2008) Phorbol ester-stimulated NF-kappaB-dependent transcription: roles for isoforms of novel protein kinase C. *Cell Signal* **20**:1338-1348.

Holzer P and Holzer-Petsche U (2001) Tachykinin receptors in the gut: physiological and pathological implications. *Curr Opin Pharmacol* **1**:583-590.

Huang J and Siragy HM Regulation of (pro)renin receptor expression by glucose-induced mitogen-activated protein kinase, nuclear factor-kappaB, and activator protein-1 signaling pathways. *Endocrinology* **151**:3317-3325.

Hutter MM, Wick EC, Day AL, Maa J, Zerega EC, Richmond AC, Jordan TH, Grady EF, Mulvihill SJ, Bunnett NW and Kirkwood KS (2005) Transient receptor potential vanilloid (TRPV-1) promotes neurogenic inflammation in the pancreas via activation of the neurokinin-1 receptor (NK-1R). *Pancreas* **30**:260-265.

Hwang L, Leichter R, Okamoto A, Payan D, Collins SM and Bunnett NW (1993) Downregulation of neutral endopeptidase (EC 3.4.24.11) in the inflamed rat intestine. *Am J Physiol* **264**:G735-743.

Ito Y, Lugea A, Pandol SJ and McCuskey RS (2007) Substance P mediates caerulein-induced pancreatic microcirculatory dysfunction in mice. *Pancreas* **34**:138-143.

Jiang B, Xu S, Hou X, Pimentel DR, Brecher P and Cohen RA (2004) Temporal control of NF-kappaB activation by ERK differentially regulates interleukin-1beta-induced gene expression. *J Biol Chem* **279**:1323-1329.

Kage R, McGregor GP, Thim L and Conlon JM (1988) Neuropeptide-gamma: a peptide isolated from rabbit intestine that is derived from gamma-preprotachykinin. *J Neurochem* **50**:1412-1417.

Kanazawa I, Ogawa T, Kimura S and Munekata E (1984) Regional distribution of substance P, neurokinin alpha and neurokinin beta in rat central nervous system. *Neurosci Res* **2**:111-120.

Kangawa K, Minamino N, Fukuda A and Matsuo H (1983) Neuromedin K: a novel mammalian tachykinin identified in porcine spinal cord. *Biochem Biophys Res Commun* **114**:533-540.
Karin M and Gallagher E (2005) From JNK to pay dirt: jun kinases, their biochemistry, physiology and clinical importance. *IUBMB Life* **57**:283-295.
Katoh K, Murai K and Nonoyama T (1984) Effects of substance P on fluid and amylase secretion in exocrine pancreas of rat and mouse. *Res Vet Sci* **36**:147-152.
Khawaja AM and Rogers DF (1996) Tachykinins: receptor to effector. *Int J Biochem Cell Biol* **28**:721-738.
Kimura S, Goto K, Ogawa T, Sugita Y and Kanazawa I (1984) Pharmacological characterization of novel mammalian tachykinins, neurokinin alpha and neurokinin beta. *Neurosci Res* **2**:97-104.
Kirkwood KS, Bunnett NW, Maa J, Castagliolo I, Liu B, Gerard N, Zacks J, Pothoulakis C and Grady EF (2001) Deletion of neutral endopeptidase exacerbates intestinal inflammation induced by Clostridium difficile toxin A. *Am J Physiol Gastrointest Liver Physiol* **281**:G544-551.
Klar E, Schratt W, Foitzik T, Buhr H, Herfarth C and Messmer K (1994) Impact of microcirculatory flow pattern changes on the development of acute edematous and necrotizing pancreatitis in rabbit pancreas. *Dig Dis Sci* **39**:2639-2644.
Koh YH, Tamizhselvi R and Bhatia M (2010) Extracellular signal-regulated kinase 1/2 and c-Jun NH2-terminal kinase, through nuclear factor-kappaB and activator protein-1, contribute to caerulein-induced expression of substance P and neurokinin-1 receptors in pancreatic acinar cells. *J Pharmacol Exp Ther* **332**:940-948.
Koh YH, Tamizhselvi R, Moochhala S, Bian JS and Bhatia M (2011) Role of protein kinase C in caerulein induced expression of substance P and neurokinin-1-receptors in murine pancreatic acinar cells. *J Cell Mol Med* **15**:2139-2149.
Kondoh K and Nishida E (2007) Regulation of MAP kinases by MAP kinase phosphatases. *Biochim Biophys Acta* **1773**:1227-1237.
Kontny E, Kurowska M, Szczepanska K and Maslinski W (2000) Rottlerin, a PKC isozyme-selective inhibitor, affects signaling events and cytokine production in human monocytes. *J Leukoc Biol* **67**:249-258.
Kook SH, Hwang JM, Park JS, Kim EM, Heo JS, Jeon YM and Lee JC (2009) Mechanical force induces type I collagen expression in human periodontal ligament fibroblasts through activation of ERK/JNK and AP-1. *J Cell Biochem* **106**:1060-1067.
Krishna M and Narang H (2008) The complexity of mitogen-activated protein kinases (MAPKs) made simple. *Cell Mol Life Sci* **65**:3525-3544.
Kundu JK, Shin YK and Surh YJ (2006) Resveratrol modulates phorbol ester-induced pro-inflammatory signal transduction pathways in mouse skin in vivo: NF-kappaB and AP-1 as prime targets. *Biochem Pharmacol* **72**:1506-1515.
Labarca C and Paigen K (1980) A simple, rapid, and sensitive DNA assay procedure. *Anal Biochem* **102**:344-352.
Lai JP, Douglas SD and Ho WZ (1998) Human lymphocytes express substance P and its receptor. *J Neuroimmunol* **86**:80-86.
Lai JP, Ho WZ, Yang JH, Wang X, Song L and Douglas SD (2002) A non-peptide substance P antagonist down-regulates SP mRNA expression in human mononuclear phagocytes. *J Neuroimmunol* **128**:101-108.

Lambrecht BN, Germonpre PR, Everaert EG, Carro-Muino I, De Veerman M, de Felipe C, Hunt SP, Thielemans K, Joos GF and Pauwels RA (1999) Endogenously produced substance P contributes to lymphocyte proliferation induced by dendritic cells and direct TCR ligation. *Eur J Immunol* **29**:3815-3825.

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

Lang Z and Murlas CG (1992) Neutral endopeptidase of a human airway epithelial cell line recovers after hypochlorous acid exposure: dexamethasone accelerates this by stimulating neutral endopeptidase mRNA synthesis. *Am J Respir Cell Mol Biol* **7**:300-306.

Langlet C, Springael C, Johnson J, Thomas S, Flamand V, Leitges M, Goldman M, Aksoy E and Willems F PKC-alpha controls MYD88-dependent TLR/IL-1R signaling and cytokine production in mouse and human dendritic cells. *Eur J Immunol* **40**:505-515.

Lankisch PG, Burchard-Reckert S and Lehnick D (1999) Underestimation of acute pancreatitis: patients with only a small increase in amylase/lipase levels can also have or develop severe acute pancreatitis. *Gut* **44**:542-544.

Larvin M and McMahon MJ (1989) APACHE-II score for assessment and monitoring of acute pancreatitis. *Lancet* **2**:201-205.

Lau HY and Bhatia M (2006) The effect of CP96,345 on the expression of tachykinins and neurokinin receptors in acute pancreatitis. *J Pathol* **208**:364-371.

Lau HY, Wong FL and Bhatia M (2005) A key role of neurokinin 1 receptors in acute pancreatitis and associated lung injury. *Biochem Biophys Res Commun* **327**:509-515.

Lay JM, Jenkins C, Friis-Hansen L and Samuelson LC (2000) Structure and developmental expression of the mouse CCK-B receptor gene. *Biochem Biophys Res Commun* **272**:837-842.

Lee FY, Lin HC, Tsai YT, Chang FY, Lu RH, Hou MC, Li CP, Chu CJ, Wang SS and Lee SD (1997) Plasma substance P levels in patients with liver cirrhosis: relationship to systemic and portal hemodynamics. *Am J Gastroenterol* **92**:2080-2084.

Lerch MM, Saluja AK, Runzi M, Dawra R, Saluja M and Steer ML (1993) Pancreatic duct obstruction triggers acute necrotizing pancreatitis in the opossum. *Gastroenterology* **104**:853-861.

Li C, Chen X and Williams JA (2004) Regulation of CCK-induced amylase release by PKC-delta in rat pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* **287**:G764-771.

Liddle RA and Nathan JD (2004) Neurogenic inflammation and pancreatitis. *Pancreatology* **4**:551-559; discussion 559-560.

Lieb K, Fiebich BL, Berger M, Bauer J and Schulze-Osthoff K (1997) The neuropeptide substance P activates transcription factor NF-kappa B and kappa B-dependent gene expression in human astrocytoma cells. *J Immunol* **159**:4952-4958.

Lightner AM, Jordan TH, Bunnett NW, Grady EF and Kirkwood KS (2002) Recombinant human neutral endopeptidase ameliorates pancreatic elastase-induced lung injury. *Surgery* **132**:193-199.

Lin FS, Lin CC, Chien CS, Luo SF and Yang CM (2005) Involvement of p42/p44 MAPK, JNK, and NF-kappaB in IL-1beta-induced ICAM-1 expression in human pulmonary epithelial cells. *J Cell Physiol* **202**:464-473.

Lin WN, Luo SF, Lin CC, Hsiao LD and Yang CM (2009) Differential involvement of PKC-dependent MAPKs activation in lipopolysaccharide-induced AP-1 expression in human tracheal smooth muscle cells. *Cell Signal* **21**:1385-1395.

Lombardi B, Estes LW and Longnecker DS (1975) Acute hemorrhagic pancreatitis (massive necrosis) with fat necrosis induced in mice by DL-ethionine fed with a choline-deficient diet. *Am J Pathol* **79**:465-480.

Lombardi B and Rao NK (1975) Acute hemorrhagic pancreatic necrosis in mice. Influence of the age and sex of the animals and of dietary ethionine, choline, methionine, and adenine sulfate. *Am J Pathol* **81**:87-100.

Lu Z, Kolodecik TR, Karne S, Nyce M and Gorelick F (2003) Effect of ligands that increase cAMP on caerulein-induced zymogen activation in pancreatic acini. *Am J Physiol Gastrointest Liver Physiol* **285**:G822-828.

Maa J, Grady EF, Kim EH, Yoshimi SK, Hutter MM, Bunnett NW and Kirkwood KS (2000a) NK-1 receptor desensitization and neutral endopeptidase terminate SP-induced pancreatic plasma extravasation. *Am J Physiol Gastrointest Liver Physiol* **279**:G726-732.

Maa J, Grady EF, Yoshimi SK, Drasin TE, Kim EH, Hutter MM, Bunnett NW and Kirkwood KS (2000b) Substance P is a determinant of lethality in diet-induced hemorrhagic pancreatitis in mice. *Surgery* **128**:232-239.

Malcangio M and Bowery NG (1999) Peptide autoreceptors: does an autoreceptor for substance P exist? *Trends Pharmacol Sci* **20**:405-407.

Mantyh CR, Maggio JE, Mantyh PW, Vigna SR and Pappas TN (1996) Increased substance P receptor expression by blood vessels and lymphoid aggregates in Clostridium difficile-induced pseudomembranous colitis. *Dig Dis Sci* **41**:614-620.

Mantyh CR, Vigna SR, Bollinger RR, Mantyh PW, Maggio JE and Pappas TN (1995) Differential expression of substance P receptors in patients with Crohn's disease and ulcerative colitis. *Gastroenterology* **109**:850-860.

Martiny-Baron G, Kazanietz MG, Mischak H, Blumberg PM, Kochs G, Hug H, Marme D and Schachtele C (1993) Selective inhibition of protein kinase C isozymes by the indolocarbazole Go 6976. *J Biol Chem* **268**:9194-9197.

McCarthy PW and Lawson SN (1989) Cell type and conduction velocity of rat primary sensory neurons with substance P-like immunoreactivity. *Neuroscience* **28**:745-753.

Meichle A, Schutze S, Hensel G, Brunsing D and Kronke M (1990) Protein kinase C-independent activation of nuclear factor kappa B by tumor necrosis factor. *J Biol Chem* **265**:8339-8343.

Mellor H and Parker PJ (1998) The extended protein kinase C superfamily. *Biochem J* **332 (Pt 2)**:281-292.

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

Morita Y, Takiguchi M, Yasuda J, Kitamura T, Syakalima M and Hashimoto A (1998) Endoscopic and transcutaneous ultrasonographic findings and grey-scale histogram analysis in dogs with caerulein-induced pancreatitis. *Vet Q* **20**:89-92.

Munoz M, Rosso M and Covenas R (2010) A new frontier in the treatment of cancer: NK-1 receptor antagonists. *Curr Med Chem* **17**:504-516.

Namkung W, Yoon JS, Kim KH and Lee MG (2008) PAR2 exerts local protection against acute pancreatitis via modulation of MAP kinase and MAP kinase phosphatase signaling. *Am J Physiol Gastrointest Liver Physiol* **295**:G886-894.

Nathan JD, Patel AA, McVey DC, Thomas JE, Prpic V, Vigna SR and Liddle RA (2001) Capsaicin vanilloid receptor-1 mediates substance P release in experimental pancreatitis. *Am J Physiol Gastrointest Liver Physiol* **281**:G1322-1328.

Navina S, Acharya C, DeLany JP, Orlichenko LS, Baty CJ, Shiva SS, Durgampudi C, Karlsson JM, Lee K, Bae KT, Furlan A, Behari J, Liu S, McHale T, Nichols L, Papachristou GI, Yadav D and Singh VP (2011) Lipotoxicity causes multisystem organ failure and exacerbates acute pancreatitis in obesity. *Sci Transl Med* **3**:107ra110.

Nawa H, Doteuchi M, Igano K, Inouye K and Nakanishi S (1984) Substance K: a novel mammalian tachykinin that differs from substance P in its pharmacological profile. *Life Sci* **34**:1153-1160.

Neely AN, Imwalle AR and Holder IA (1996) Recombinant neutral endopeptidase decreases oedema in the skin of burned guinea-pigs. *Burns* **22**:520-523.

Niederau C, Fronhoffs K, Klonowski H and Schulz HU (1995) Active pancreatic digestive enzymes show striking differences in their potential to damage isolated rat pancreatic acinar cells. *J Lab Clin Med* **125**:265-275.

Nishimura K, Frederick J and Kwatra MM (1998) Human substance P receptor expressed in Sf9 cells couples with multiple endogenous G proteins. *J Recept Signal Transduct Res* **18**:51-65.

Noble F, Wank SA, Crawley JN, Bradwejn J, Seroogy KB, Hamon M and Roques BP (1999) International Union of Pharmacology. XXI. Structure, distribution, and functions of cholecystokinin receptors. *Pharmacol Rev* **51**:745-781.

Noble MD, Romac J, Wang Y, Hsu J, Humphrey JE and Liddle RA (2006) Local disruption of the celiac ganglion inhibits substance P release and ameliorates caerulein-induced pancreatitis in rats. *Am J Physiol Gastrointest Liver Physiol* **291**:G128-134.

O'Connor TM, O'Connell J, O'Brien DI, Goode T, Bredin CP and Shanahan F (2004) The role of substance P in inflammatory disease. *J Cell Physiol* **201**:167-180.

Okada N, Ohshio G, Tanaka T, Wang ZH, Doi R and Imamura M (1998) Intracellular Ca2+ response of pancreatic acini in cerulein-induced acute pancreatitis in rats. *Hepatogastroenterology* **45**:840-845.

Orebaugh SL (1994) Normal amylase levels in the presentation of acute pancreatitis. *Am J Emerg Med* **12**:21-24.

Orlichenko LS, Behari J, Yeh TH, Liu S, Stolz DB, Saluja AK and Singh VP Transcriptional regulation of CXC-ELR chemokines KC and MIP-2 in mouse pancreatic acini. *Am J Physiol Gastrointest Liver Physiol* **299**:G867-876.

Palmer JA, De Felipe C, O'Brien JA and Hunt SP (1999) Disruption of the substance P receptor (neurokinin-1) gene does not prevent upregulation of preprotachykinin-A mRNA in the spinal cord of mice following peripheral inflammation. *Eur J Neurosci* **11**:3531-3538.

Pandol SJ, Gukovsky I, Satoh A, Lugea A and Gukovskaya AS (2003) Animal and in vitro models of alcoholic pancreatitis: role of cholecystokinin. *Pancreas* **27**:297-300.

Pastor CM, Matthay MA and Frossard JL (2003) Pancreatitis-associated acute lung injury: new insights. *Chest* **124**:2341-2351.

Patel L and Lindley C (2003) Aprepitant--a novel NK1-receptor antagonist. *Expert Opin Pharmacother* **4**:2279-2296.

Paterson JM, Mendelson SC, McAllister J, Morrison CF, Dobson S, Grace C and Quinn JP (1995) Three immediate early gene response elements in the proximal preprotachykinin-A promoter in two functionally distinct domains. *Neuroscience* **66**:921-932.

Patto RJ, Vinayek R, Jensen RT and Gardner JD (1992) Carbachol does not down-regulate substance P receptors in pancreatic acini. *Pancreas* **7**:447-452.

Pickel VM, Miller R, Chan J and Sumal KK (1983) Substance P and enkephalin in transected axons of medulla and spinal cord. *Regul Pept* **6**:121-135.

Pierrakakis S, Xepapadakis G, Mega AM, Megas T, Katergiannakis VG, Perpirakis G and Filippakis M (1990) [Blood endotoxin in acute pancreatitis using limulus amebocyte lysate test (LAL test)]. *Minerva Chir* **45**:295-301.

Pollock DM, Divish BJ, Milicic I, Novosad EI, Burres NS and Opgenorth TJ (1993) In vivo characterization of a phosphoramidon-sensitive endothelin-converting enzyme in the rat. *Eur J Pharmacol* **231**:459-464.

Qian J, Yehia G, Molina C, Fernandes A, Donnelly R, Anjaria D, Gascon P and Rameshwar P (2001) Cloning of human preprotachykinin-I promoter and the role of cyclic adenosine 5'-monophosphate response elements in its expression by IL-1 and stem cell factor. *J Immunol* **166**:2553-2561.

Quartara L and Altamura M (2006) Tachykinin receptors antagonists: from research to clinic. *Curr Drug Targets* **7**:975-992.

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

Rakonczay Z, Jr., Hegyi P, Takacs T, McCarroll J and Saluja AK (2008b) The role of NF-kappaB activation in the pathogenesis of acute pancreatitis. *Gut* **57**:259-267.

Ramnath RD and Bhatia M (2006) Substance P treatment stimulates chemokine synthesis in pancreatic acinar cells via the activation of NF-kappaB. *Am J Physiol Gastrointest Liver Physiol* **291**:G1113-1119.

Ramnath RD, Sun J, Adhikari S and Bhatia M (2007) Effect of mitogen-activated protein kinases on chemokine synthesis induced by substance P in mouse pancreatic acinar cells. *J Cell Mol Med* **11**:1326-1341.

Ramnath RD, Sun J, Adhikari S, Zhi L and Bhatia M (2008) Role of PKC-delta on substance P-induced chemokine synthesis in pancreatic acinar cells. *Am J Physiol Cell Physiol* **294**:C683-692.

Ramnath RD, Sun J and Bhatia M (2009) Involvement of SRC family kinases in substance P-induced chemokine production in mouse pancreatic acinar cells and its significance in acute pancreatitis. *J Pharmacol Exp Ther* **329**:418-428.

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

Reed KL, Fruin AB, Gower AC, Gonzales KD, Stucchi AF, Andry CD, O'Brien M and Becker JM (2005) NF-kappaB activation precedes increases in mRNA encoding neurokinin-1 receptor, proinflammatory cytokines, and adhesion molecules in dextran sulfate sodium-induced colitis in rats. *Dig Dis Sci* **50**:2366-2378.

Reynolds WJ, Chiu B and Inman RD (1988) Plasma substance P levels in fibrositis. *J Rheumatol* **15**:1802-1803.
Roisman GL and Dusser DJ (1995) Hydrogen peroxide inhibits lung neutral endopeptidase. *Exp Lung Res* **21**:215-225.
Roush ED and Kwatra MM (1998) Human substance P receptor expressed in Chinese hamster ovary cells directly activates G(alpha q/11), G(alpha s), G(alpha o). *FEBS Lett* **428**:291-294.
Saito I, Hashimoto S, Saluja A, Steer ML and Meldolesi J (1987) Intracellular transport of pancreatic zymogens during caerulein supramaximal stimulation. *Am J Physiol* **253**:G517-526.
Sakorafas GH and Tsiotou AG (2000) Etiology and pathogenesis of acute pancreatitis: current concepts. *J Clin Gastroenterol* **30**:343-356.
Saluja A, Saito I, Saluja M, Houlihan MJ, Powers RE, Meldolesi J and Steer M (1985) In vivo rat pancreatic acinar cell function during supramaximal stimulation with caerulein. *Am J Physiol* **249**:G702-710.
Saluja AK, Saluja M, Printz H, Zavertnik A, Sengupta A and Steer ML (1989) Experimental pancreatitis is mediated by low-affinity cholecystokinin receptors that inhibit digestive enzyme secretion. *Proc Natl Acad Sci U S A* **86**:8968-8971.
Samuel I, Tephly L, Williard DE and Carter AB (2008) Enteral exclusion increases MAP kinase activation and cytokine production in a model of gallstone pancreatitis. *Pancreatology* **8**:6-14.
Samuel I, Yuan Z, Meyerholz DK, Twait E, Williard DE and Kempuraj D A novel model of severe gallstone pancreatitis: murine pancreatic duct ligation results in systemic inflammation and substantial mortality. *Pancreatology* **10**:536-544.
Samuel I, Zaheer A and Fisher RA (2006) In vitro evidence for role of ERK, p38, and JNK in exocrine pancreatic cytokine production. *J Gastrointest Surg* **10**:1376-1383.
Sandoval D, Gukovskaya A, Reavey P, Gukovsky S, Sisk A, Braquet P, Pandol SJ and Poucell-Hatton S (1996) The role of neutrophils and platelet-activating factor in mediating experimental pancreatitis. *Gastroenterology* **111**:1081-1091.
Sans MD, DiMagno MJ, D'Alecy LG and Williams JA (2003) Caerulein-induced acute pancreatitis inhibits protein synthesis through effects on eIF2B and eIF4F. *Am J Physiol Gastrointest Liver Physiol* **285**:G517-528.
Satoh A, Gukovskaya AS, Nieto JM, Cheng JH, Gukovsky I, Reeve JR, Jr., Shimosegawa T and Pandol SJ (2004) PKC-delta and -epsilon regulate NF-kappaB activation induced by cholecystokinin and TNF-alpha in pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* **287**:G582-591.
Schmitz ML and Baeuerle PA (1991) The p65 subunit is responsible for the strong transcription activating potential of NF-kappa B. *EMBO J* **10**:3805-3817.
Schneider A, Whitcomb DC and Singer MV (2002) Animal models in alcoholic pancreatitis--what can we learn? *Pancreatology* **2**:189-203.
Scholzen TE, Stander S, Riemann H, Brzoska T and Luger TA (2003) Modulation of cutaneous inflammation by angiotensin-converting enzyme. *J Immunol* **170**:3866-3873.
Scholzen TE, Steinhoff M, Bonaccorsi P, Klein R, Amadesi S, Geppetti P, Lu B, Gerard NP, Olerud JE, Luger TA, Bunnett NW, Grady EF, Armstrong CA and

Ansel JC (2001) Neutral endopeptidase terminates substance P-induced inflammation in allergic contact dermatitis. *J Immunol* **166**:1285-1291.
Severini C, Improta G, Falconieri-Erspamer G, Salvadori S and Erspamer V (2002) The tachykinin peptide family. *Pharmacol Rev* **54**:285-322.
Shames BD, Selzman CH, Pulido EJ, Meng X, Meldrum DR, McIntyre RC, Jr., Harken AH and Banerjee A (1999) LPS-Induced NF-kappaB activation and TNF-alpha release in human monocytes are protein tyrosine kinase dependent and protein kinase C independent. *J Surg Res* **83**:69-74.
Shin Y, Yoon SH, Choe EY, Cho SH, Woo CH, Rho JY and Kim JH (2007) PMA-induced up-regulation of MMP-9 is regulated by a PKCalpha-NF-kappaB cascade in human lung epithelial cells. *Exp Mol Med* **39**:97-105.
Shirai Y and Saito N (2002) Activation mechanisms of protein kinase C: maturation, catalytic activation, and targeting. *J Biochem* **132**:663-668.
Sio SW, Moochhala S, Lu J and Bhatia M Early protection from burn-induced acute lung injury by deletion of preprotachykinin-A gene. *Am J Respir Crit Care Med* **181**:36-46.
Sio SW, Moochhala S, Lu J and Bhatia M (2010) Early protection from burn-induced acute lung injury by deletion of preprotachykinin-A gene. *Am J Respir Crit Care Med* **181**:36-46.
Skidgel RA, Engelbrecht S, Johnson AR and Erdos EG (1984) Hydrolysis of substance p and neurotensin by converting enzyme and neutral endopeptidase. *Peptides* **5**:769-776.
Skidgel RA and Erdos EG (2004) Angiotensin converting enzyme (ACE) and neprilysin hydrolyze neuropeptides: a brief history, the beginning and follow-ups to early studies. *Peptides* **25**:521-525.
Smith RC, Southwell-Keely J and Chesher D (2005) Should serum pancreatic lipase replace serum amylase as a biomarker of acute pancreatitis? *ANZ J Surg* **75**:399-404.
Smotkin J and Tenner S (2002) Laboratory diagnostic tests in acute pancreatitis. *J Clin Gastroenterol* **34**:459-462.
Snider RM, Constantine JW, Lowe JA, 3rd, Longo KP, Lebel WS, Woody HA, Drozda SE, Desai MC, Vinick FJ, Spencer RW and et al. (1991) A potent nonpeptide antagonist of the substance P (NK1) receptor. *Science* **251**:435-437.
Solan NJ, Ward PE, Sanders SP, Towns MC and Bathon JM (1998) Soluble recombinant neutral endopeptidase (CD10) as a potential antiinflammatory agent. *Inflammation* **22**:107-121.
Soltoff SP (2001) Rottlerin is a mitochondrial uncoupler that decreases cellular ATP levels and indirectly blocks protein kinase Cdelta tyrosine phosphorylation. *J Biol Chem* **276**:37986-37992.
Steyaert A, Burssens P, Forsyth R and Vanderstraeten G (2010) Qualitative analysis of substance P, NK1-receptor and nerve ingrowth in substance P-treated ruptured rat Achilles tendon. *Acta Orthop Belg* **76**:387-395.
Sturiale S, Barbara G, Qiu B, Figini M, Geppetti P, Gerard N, Gerard C, Grady EF, Bunnett NW and Collins SM (1999) Neutral endopeptidase (EC 3.4.24.11) terminates colitis by degrading substance P. *Proc Natl Acad Sci U S A* **96**:11653-11658.
Subissi A, Guelfi M and Criscuoli M (1990) Angiotensin converting enzyme inhibitors potentiate the bronchoconstriction induced by substance P in the guinea-pig. *Br J Pharmacol* **100**:502-506.

Sun J and Bhatia M (2007) Blockade of neurokinin-1 receptor attenuates CC and CXC chemokine production in experimental acute pancreatitis and associated lung injury. *Am J Physiol Gastrointest Liver Physiol* **292**:G143-153.

Sun J, Ramnath RD and Bhatia M (2007) Neuropeptide substance P upregulates chemokine and chemokine receptor expression in primary mouse neutrophils. *Am J Physiol Cell Physiol* **293**:C696-704.

Sun J, Ramnath RD, Tamizhselvi R and Bhatia M (2009) Role of protein kinase C and phosphoinositide 3-kinase-Akt in substance P-induced proinflammatory pathways in mouse macrophages. *Faseb J* **23**:997-1010.

Sun J, Ramnath RD, Zhi L, Tamizhselvi R and Bhatia M (2008) Substance P enhances NF-kappaB transactivation and chemokine response in murine macrophages via ERK1/2 and p38 MAPK signaling pathways. *Am J Physiol Cell Physiol* **294**:C1586-1596.

Takacs T, Czako L, Morschl E, Laszlo F, Tiszlavicz L, Rakonczay Z, Jr. and Lonovics J (2002) The role of nitric oxide in edema formation in L-arginine-induced acute pancreatitis. *Pancreas* **25**:277-282.

Tamizhselvi R, Moore PK and Bhatia M (2007) Hydrogen sulfide acts as a mediator of inflammation in acute pancreatitis: in vitro studies using isolated mouse pancreatic acinar cells. *J Cell Mol Med* **11**:315-326.

Tando Y, Algul H, Wagner M, Weidenbach H, Adler G and Schmid RM (1999) Caerulein-induced NF-kappaB/Rel activation requires both Ca2+ and protein kinase C as messengers. *Am J Physiol* **277**:G678-686.

Tani S, Otsuki M, Itoh H, Fujii M, Nakamura T, Oka T and Baba S (1987) Histologic and biochemical alterations in experimental acute pancreatitis induced by supramaximal caerulein stimulation. *Int J Pancreatol* **2**:337-348.

Tapia JA, Jensen RT and Garcia-Marin LJ (2006) Rottlerin inhibits stimulated enzymatic secretion and several intracellular signaling transduction pathways in pancreatic acinar cells by a non-PKC-delta-dependent mechanism. *Biochim Biophys Acta* **1763**:25-38.

Tatemoto K, Lundberg JM, Jornvall H and Mutt V (1985) Neuropeptide K: isolation, structure and biological activities of a novel brain tachykinin. *Biochem Biophys Res Commun* **128**:947-953.

Thrower EC, Osgood S, Shugrue CA, Kolodecik TR, Chaudhuri AM, Reeve JR, Jr., Pandol SJ and Gorelick FS (2008) The novel protein kinase C isoforms -delta and -epsilon modulate caerulein-induced zymogen activation in pancreatic acinar cells. *Am J Physiol Gastrointest Liver Physiol* **294**:G1344-1353.

Thrower EC, Wang J, Cheriyan S, Lugea A, Kolodecik TR, Yuan J, Reeve JR, Jr., Gorelick FS and Pandol SJ (2009) Protein kinase C delta-mediated processes in cholecystokinin-8-stimulated pancreatic acini. *Pancreas* **38**:930-935.

Tiedemann K, Hussein O, Sadvakassova G, Guo Y, Siegel PM and Komarova SV (2009) Breast cancer-derived factors stimulate osteoclastogenesis through the Ca2+/protein kinase C and transforming growth factor-beta/MAPK signaling pathways. *J Biol Chem* **284**:33662-33670.

Tregear GW, Niall HD, Potts JT, Jr., Leeman SE and Chang MM (1971) Synthesis of substance P. *Nat New Biol* **232**:87-89.

Tsai MJ, Chen C, Chen SH, Huang YT and Chiu TH Pomalidomide suppresses cerulein-induced acute pancreatitis in mice. *J Gastroenterol* **46**:822-833.

Tsui NC, Zhao E, Li Z, Miao B, Cui Y, Shen Y and Qu P (2009) Microbiological findings in secondary infection of severe acute pancreatitis: a retrospective clinical study. *Pancreas* **38**:499-502.

Tuncer LI, Alacam T and Oral B (2004) Substance P expression is elevated in inflamed human periradicular tissue. *J Endod* **30**:329-332.
US VE and Gaddum JH (1931) An unidentified depressor substance in certain tissue extracts. *J Physiol* **72**:74-87.
Velazquez RA, McCarson KE, Cai Y, Kovacs KJ, Shi Q, Evensjo M and Larson AA (2002) Upregulation of neurokinin-1 receptor expression in rat spinal cord by an N-terminal metabolite of substance P. *Eur J Neurosci* **16**:229-241.
Vera-Portocarrero LP and Westlund KN (2004) Attenuation of nociception in a model of acute pancreatitis by an NK-1 antagonist. *Pharmacol Biochem Behav* **77**:631-640.
Wagner AC, Mazzucchelli L, Miller M, Camoratto AM and Goke B (2000) CEP-1347 inhibits caerulein-induced rat pancreatic JNK activation and ameliorates caerulein pancreatitis. *Am J Physiol Gastrointest Liver Physiol* **278**:G165-172.
Walker NI (1987) Ultrastructure of the rat pancreas after experimental duct ligation. I. The role of apoptosis and intraepithelial macrophages in acinar cell deletion. *Am J Pathol* **126**:439-451.
Weaver C, Bishop AE and Polak JM (1994) Pancreatic changes elicited by chronic administration of excess L-arginine. *Exp Mol Pathol* **60**:71-87.
Wick EC, Hoge SG, Grahn SW, Kim E, Divino LA, Grady EF, Bunnett NW and Kirkwood KS (2006) Transient receptor potential vanilloid 1, calcitonin gene-related peptide, and substance P mediate nociception in acute pancreatitis. *Am J Physiol Gastrointest Liver Physiol* **290**:G959-969.
Williams JA (2001) Intracellular signaling mechanisms activated by cholecystokinin-regulating synthesis and secretion of digestive enzymes in pancreatic acinar cells. *Annu Rev Physiol* **63**:77-97.
Williams M and Simms HH (1999) Prognostic usefulness of scoring systems in critically ill patients with severe acute pancreatitis. *Crit Care Med* **27**:901-907.
Wisner JR and Renner IG (1988) Allopurinol attenuates caerulein induced acute pancreatitis in the rat. *Gut* **29**:926-929.
Wittel UA, Wiech T, Chakraborty S, Boss B, Lauch R, Batra SK and Hopt UT (2008) Taurocholate-induced pancreatitis: a model of severe necrotizing pancreatitis in mice. *Pancreas* **36**:e9-21.
Xu C, Shen G, Yuan X, Kim JH, Gopalkrishnan A, Keum YS, Nair S and Kong AN (2006) ERK and JNK signaling pathways are involved in the regulation of activator protein 1 and cell death elicited by three isothiocyanates in human prostate cancer PC-3 cells. *Carcinogenesis* **27**:437-445.
Yadav D and Lowenfels AB (2006) Trends in the epidemiology of the first attack of acute pancreatitis: a systematic review. *Pancreas* **33**:323-330.
Yeung YP, Lam BY and Yip AW (2006) APACHE system is better than Ranson system in the prediction of severity of acute pancreatitis. *Hepatobiliary Pancreat Dis Int* **5**:294-299.
Yoshida K, Miki Y and Kufe D (2002) Activation of SAPK/JNK signaling by protein kinase Cdelta in response to DNA damage. *J Biol Chem* **277**:48372-48378.
Yu JH, Lim JW, Namkung W, Kim H and Kim KH (2002) Suppression of caerulein-induced cytokine expression by antioxidants in pancreatic acinar cells. *Lab Invest* **82**:1359-1368.
Zakko S, Barton G, Weber E, Dunger-Baldauf C and Ruhl A (2011) Randomised clinical trial: the clinical effects of a novel neurokinin receptor antagonist, DNK333, in women with diarrhoea-predominant irritable bowel syndrome. *Aliment Pharmacol Ther* **33**:1311-1321.

Zenz R, Eferl R, Scheinecker C, Redlich K, Smolen J, Schonthaler HB, Kenner L, Tschachler E and Wagner EF (2008) Activator protein 1 (Fos/Jun) functions in inflammatory bone and skin disease. *Arthritis Res Ther* **10**:201.