

## Effect of p38 MAPK Inhibitor on Adhesion Molecule Expression and Microvascular Permeability of Renal Injury in a Rat Model of Acute Necrotizing Pancreatitis

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**Abstract:** To investigate effect of p38 MAPK inhibitor on adhesion molecule expression and microvascular permeability of renal injury in a rat model of acute necrotizing pancreatitis. Sixty pathogen free male Sprague-Dawley rats were randomly divided into the sham group, acute necrotizing pancreatitis group (ANP group) and ANP group of rats with treatment of SB203580 (SB203580, 0.5 mg kg<sup>-1</sup>, iv). The rats were sacrificed at 1, 3, 6 and 12 h after operation. The serum of BUN, Cr,  $\beta$ 2-MG, serum and renal tissue of TNF- $\alpha$  were determined. The expressions of p38 MAPK in the pancreas and renal tissue of SAP rat were determined by immunohistochemical technique and the expression of ICAM-1 mRNA was detected through RT-PCR. The pancreas and renal tissue samples were stained with HE for histopathological evaluation. The Serum of BUN, Cr and  $\beta$ 2-MG, serum and renal tissue of TNF- $\alpha$ , the expression and p38 MAPK and ICAM-1 mRNA of pancreas and renal tissue in ANP group and SB group were more significantly increased than those of the sham group ( $p < 0.05$ ). Moreover, those of SB group were more significantly reduced than those of SAP group ( $p < 0.05$ ). The renal sections from SB203580-treated groups displayed significantly less proximal tubule cell necrosis than those of ANP group. The activation and over expression of p38 MAPK and ICAM-1 mRNA of renal tissue may be one of the reasons for renal injury in ANP and SB203580 can be used to treat the ANP associated renal injury through down regulating the expression of p38 MAPK and ICAM-1 mRNA in renal tissue.

**Key words:** Acute pancreatitis, p38 Mitogen-activated protein kinases, renal injury, adhesion molecule

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### INTRODUCTION

Acute Pancreatitis (AP) is a life threatening illness with an annual incidence of 30-50 attacks per 100,000 inhabitants (McKay and Imrie, 2004). The clinical presentation ranges from a mild edematous, self-limiting disease with good prognosis to severe necrotizing inflammation, fatal in about 15-20% of instances. Death from acute pancreatitis occurs biphasically from two different causes. Early death (within the first 2 weeks) results from acute consequences of the pancreatic inflammatory process and the systemic inflammatory response with subsequent multiorgan dysfunction; late death is mainly caused by sepsis. Factors contributing to early multiorgan dysfunction in acute pancreatitis are believed to involve proinflammatory cytokines and other still unknown immunomodulators and vasoactive substances (Mann *et al.*, 1994; Debeaux *et al.*, 1996).

Although, the precise mechanism by which such local inflammation in the pancreas progresses to systemic illness is still unclear. Recently, this Systemic Inflammatory Response Syndrome (SIRS) has become a widely accepted disease state (Debeaux *et al.*, 1996) which could lead to the failure of distant organ systems such as the lungs, intestine, stomach and kidneys (Huang *et al.*, 2005). Acute Pancreatitis (AP) is often complicated by renal injury. However, its pathogenesis remains unclear. The p38 Mitogen-Activated Protein Kinase (MAPK) is one of three major MAPK signaling pathways which is triggered by a wide range of extracellular ligands and stresses such as proinflammatory cytokines (IL-1, TNF- $\alpha$ ), growth factors (TGF- $\beta$ 1, PDGF), reactive oxygen species, stretch, osmotic stress and UV irradiation. There are four isoforms of the p38 kinase of which p38 $\alpha$ ,  $\beta$  and  $\delta$  are expressed in the kidney. The p38 kinase is activated by phosphorylation of a conserved Thr-Gly-Tyr motif in the

activation loop. The activated p38 is then able to phosphorylate a wide variety of targets in the cytoplasm and nucleus, resulting in cellular responses such as apoptosis, inflammation and fibrosis (Kyriakis and Avruch, 2001). The activation loop of the p38 kinase is phosphorylated through the action of the upstream kinases MAPKK3 (MKK3) and MAPKK6 (MKK6) although, other mechanisms of p38 activation have been described in response to specific stimuli. Components of the p38 signaling pathway are present in many cell types. However, activation of this pathway can lead to different outcomes depending on the nature of the activating signal and the cell type involved. For example, p38 signaling can either enhance or suppress apoptosis of cultured tubular epithelial cells depending on the nature of the stimulus.

Signaling via the p38 MAPK plays an important role in the inflammatory response. P38 blockade has been shown to provide protection from inflammatory renal injury in rat models of anti-GBM glomerulonephritis which has been attributed to a reduction in neutrophil and macrophage infiltration and reduced production of proinflammatory cytokines (Stambe *et al.*, 2003; Wada *et al.*, 2001). Therefore, p38 MAPK inhibitors are expected to inhibit not only the production of proinflammatory cytokines but also their downstream effects, thereby potentially ameliorating inflammatory and immune-mediated diseases. SB 203580, a widely used p38 MAPK inhibitor has been investigated *in vivo* in several models of disease and has shown activity in TNF- $\alpha$  mediated inflammation.

However, the contribution of p38 MAPK signaling in the development of kidney disease in ANP rats is unknown. This study was to investigate the biosynthesis mechanisms of cytokine production and block them with special inhibitor and effect of p38 MAPK inhibitor on adhesion molecule expression and microvascular permeability of renal injury in a rat model of severe acute pancreatitis so as to improve ANP and associated renal injury.

## MATERIALS AND METHODS

About 60 pathogen free male Sprague-Dawley (SD) rats (Animal Center, Xi'an Jiaotong University), weighing 250-300 g were used in this experiment. The animals were kept at a constant room temperature of 25°C with a 12 h light-dark cycle and were allowed free access to water. Animals were housed in the animal facility for at least 7 days prior to use in order to stabilize their intestinal flora. All procedures including the rats receiving slight

methoxyflurane for induction and pentobarbital intraperitoneal injection (50 mg kg<sup>-1</sup>) for anesthesia performed under sterile condition. SD rats were randomly divided into the sham group, acute necrotizing pancreatitis group (ANP group) and ANP of rats with treatment of SB203580 (SB203580, 0.5 mg kg<sup>-1</sup>, iv) (Sigma). Taurocholate (5%, Sigma) at doses of 1 mL kg<sup>-1</sup> was administered into the biliopancreatic duct of the rats to establish Acute Necrotizing Pancreatitis (ANP). In the sham group, the mice were only operated and not infused anything. All values reported are from animals that were sacrificed at 1, 3, 6 and 12 h.

**Microscopic assessment of the pancreas and renal tissue:** Pancreas tissue and renal tissue samples were prepared for routine light microscopy, using Hematoxylin and Eosin (HE) staining and examined by a pathologist who was kept unaware of the source of specimens. HE-stained pancreas sections were observed with standard light microscope to evaluate morphologic alterations following ANP. edema, hemorrhage and Necrosis of the pancreas were each graded from 0-3.

**Serum and renal tissue of TNF- $\alpha$ :** Blood was collected and centrifuged (3000 rpm min<sup>-1</sup> for 5 min). The serum was captured and stored at -80°C refrigerator. The equal renal tissues were collected, triturated and centrifuged (3000 rpm min<sup>-1</sup> for 5 min). Collected liquids were stored at -80°C refrigerator. The pro-inflammatory TNF- $\alpha$  in serum and renal tissue were measured by Enzyme-Linked Immunosorbent Assay (ELISA).

**Detection of serum BUN, Cr and  $\beta$ 2-Microglobulin ( $\beta$ 2-MG):** The blood from the superior mesenteric vein was collected into a tube. The tube was immediately centrifuged at 3500 r min<sup>-1</sup> for 15 min. Collected plasma was stored at -40°C until use. BUN and Cr levels were assayed using an automatic biochemistry analyzer (CL-7300; SHIMADZU Corporation, Kyoto, Japan) with assay kits.  $\beta$ 2-microglobulin contents of serum and urine were measured by radioimmunoassay (FJ-2008PS, 262 factory, Xian, China) and radioimmunoassay kit was purchased from northern institute of industrial biotechnology, Chinese Academy of Sciences.

**Detection of renal tissue ICAM-1 mRNA (RT-PCR):** The same renal tissues were collected and stored at -80°C refrigerator. The expressions of renal tissue ICAM-1 mRNA were measured by RT-PCR after 6 and 12 h, respectively. The total cellular RNA was extracted from the MECs using an RNA-zol kit according to the

manufacturer's instructions (MRC Company). The RNA (2 µg) was reverse transcribed and the complementary DNA was amplified in a 50 µL reaction by Perkin Elmer thermocycler for 30 cycles with 15 µL of commercially available PCR primers for ICAM. The sequence for the 5' primer was CGGTAGACACAAGCAAGAGA and the 3' primer was GCAGGGATTGACCATAATT. The PCR conditions were as follows: 35 cycles of denaturation at 94.5°C for 30 sec, annealing at 60°C for 30 sec and extension at 72°C for 1 min. Initial heating at 95°C for 9 min and final extension at 72°C for 7 min were performed for all PCRs. The PCR products were determined by electrophoresis on 1.8% (w/v) agarose gel containing 0.05 µg mL<sup>-1</sup> ethidium bromide and the PCR products were quantified by Kodak software.

**Immunohistochemical studies:** Pancreas and kidney tissues were immediately snap-frozen in liquid nitrogen after scission and stored at -80°C until further use. Frozen and paraffin-embedded kidney biopsy sections (5 µm) were subjected to indirect immunohistochemistry using standardized avidin-biotin peroxidase methodology. Before immunohistochemical labeling, paraffin sections were deparaffinized, rehydrated and boiled in a pressure cooker containing citrate buffer (pH 6) for 2 min. The sections were then cooled, rinsed in PBS and processed for immunohistochemistry. All sections were examined and photographed under blind conditions.

**RESULTS AND DISCUSSION**

**Serum levels of BUN, Cr and β2-MG:** Compared with the sham group, the BUN, Cr and β2-MG levels in the ANP group and Sb203580 group were elevated significantly (p<0.01 or p<0.05) at 1, 3, 6 and 12 h (p<0.05). Compared with the ANP group, the BUN, Cr and levels β2-MG were decreased significantly (p<0.01 or p<0.05), at 3, 6 and 12 h in the SB203580 group (p<0.01 or p<0.05) (Table 1-3).

**Serum levels and renal tissue of TNF-α:** The serum and renal tissue levels of TNF-α in ANP group were significantly higher than those in sham group at 3 and 6 h (p<0.01). The serum and renal tissue levels of TNF-α at 6 h were significantly higher than those at 3 h in ANP group and SB203580 group (p<0.05). Compared with the ANP group, The serum and renal tissue levels of TNF-α were decreased significantly at 3 and 6 h in the SB203580 group (p<0.01 or p<0.05) (Table 4).

**Pathological examination:** After induction of ANP model, the pancreas showed mild edema and congestion. At 1, 3,

Table 1: Serum BUN level in groups sham, ANP and SB203580 (mean±SD, mmol L<sup>-1</sup>)

Group	1 h	3 h	6 h	12 h
Sham	10.10±1.24	10.21±1.41	10.30±1.25	10.31±1.08
ANP	11.87±1.45 <sup>a</sup>	12.81±1.69 <sup>a</sup>	14.83±1.79 <sup>b</sup>	15.68±1.85 <sup>b</sup>
SB203580	10.52±1.17 <sup>c</sup>	11.76±1.85 <sup>c</sup>	11.52±1.87 <sup>d</sup>	11.93±1.77 <sup>d</sup>

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01 vs. sham group; <sup>c</sup>p<0.05, <sup>d</sup>p<0.01 vs. ANP group

Table 2: Serum Cr level in groups C, P and T (mean±SD, mmol L<sup>-1</sup>)

Group	1 h	3 h	6 h	12 h
Sham	21.12±1.67	21.06±1.58	21.50±1.78	21.62±1.75
ANP	24.97±3.40 <sup>a</sup>	25.36±3.11 <sup>b</sup>	30.40±1.93 <sup>b</sup>	30.60±2.04 <sup>b</sup>
SB203580	22.13±1.57 <sup>c</sup>	23.5±2.61 <sup>a,c</sup>	23.58±2.61 <sup>a,c</sup>	24.73±1.01 <sup>b,d</sup>

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01 vs. sham group; <sup>c</sup>p<0.05, <sup>d</sup>p<0.01 vs. ANP group

Table 3: Serum β2-MG level in groups sham, ANP and SB203580 (mean±SD, mmol L<sup>-1</sup>)

Group	1 h	3 h	6 h	12 h
Sham	20.13±1.56	20.86±1.59	21.06±1.612	1.12±1.65
ANP	25.36±2.52 <sup>a</sup>	25.89±3.08 <sup>b</sup>	31.40±1.93 <sup>b</sup>	30.60±2.04 <sup>b</sup>
SB203580	23.13±1.57 <sup>a,c</sup>	23.51±2.51 <sup>a,c</sup>	25.55±2.62 <sup>a,d</sup>	25.81±2.21 <sup>b,d</sup>

<sup>a</sup>p<0.05, <sup>b</sup>p<0.01 vs. sham group; <sup>c</sup>p<0.05, <sup>d</sup>p<0.01 vs. ANP group

Table 4: Serum and renal tissue levels of TNF-α in ANP (mean±SD, pg mL<sup>-1</sup>)

Group	Serum		Renal tissue	
	3 h	6 h	3 h	6 h
Sham	36.62±0.74	36.90±0.52	30.79±0.89	31.08±1.12
ANP	178.56±1.00 <sup>a</sup>	257.40±4.52 <sup>b</sup>	163.02±4.71 <sup>a</sup>	203.01±5.38 <sup>b</sup>
SB203580	158.53±9.29 <sup>c</sup>	199.73±8.23 <sup>b</sup>	151.53±5.32 <sup>b</sup>	173.06±4.89 <sup>b</sup>

<sup>a</sup>p< 0.01 vs. sham group; <sup>b</sup>p<0.05 6 h group vs. 3 h group; <sup>c</sup>p<0.05 vs. ANP group

6 and 12 h, typical pathological changes of ANP were found such as a large number of inflammatory cells, necrosis of the adjacent fat tissues, interstitial edema, parenchyma hemorrhage and necrosis, large amount of ascites (Fig. 1).

The pancreas tissues of SAP rat at 6 h after SB2 administered were observed more mildly edematous, hemorrhagic and then necrotic change, lots of monocytes infiltration (Fig. 2). The different swelling denaturation and necrosis of renal tubular epithelial cells were also observed in ANP group and Alterations in cell morphology, including loss of brush border, tubular necrosis, mitochondrial damage, interstitial congestion, edema and infiltration of inflammatory cells were also observed (Fig. 3 and 4). The changes became severer with the prolongation of time.

The renal pathological changes were aggravated significantly in the ANP group. Renal sections from SB203580-treated groups displayed significantly less proximal tubule cell necrosis (Fig. 5) Histopathologic scores were higher in the ANP group than in the sham group throughout the experiment (p<0.05) and lower in the SB203580 group than in the ANP group.

**Detection of renal tissue ICAM-1 mRNA:** The renal tissue ICAM-1 mRNA in ANP group were significantly higher than those in sham group at 3, 6 and 12 h (p<0.05).

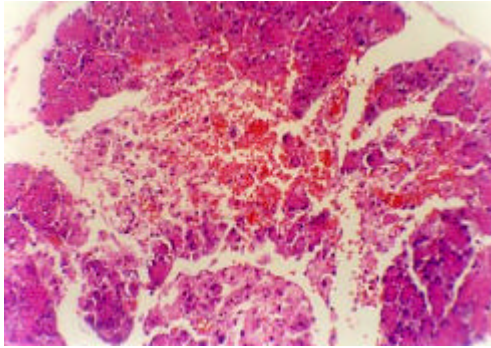


Fig. 1: Edematous, hemorrhagic and then necrotic change, lots of monocytes infiltration in the pancreas tissue of SAP rat at 6 h (10×20)

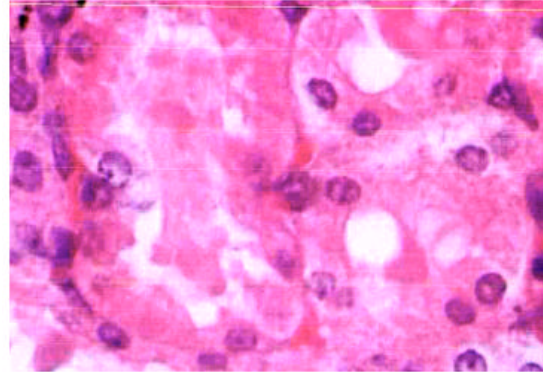


Fig. 4: Renal tubular epithelial apparent degeneration and cellular necrosis of nephric tubule in SAP rats at 6 h (level 111) (10×40)

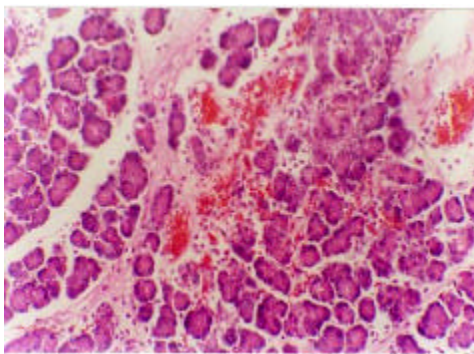


Fig. 2: Mildly edematous, hemorrhagic and then necrotic change, lots of monocytes infiltration in the pancreas tissue of SAP rat at 6 h after SB2 administered (10×40)

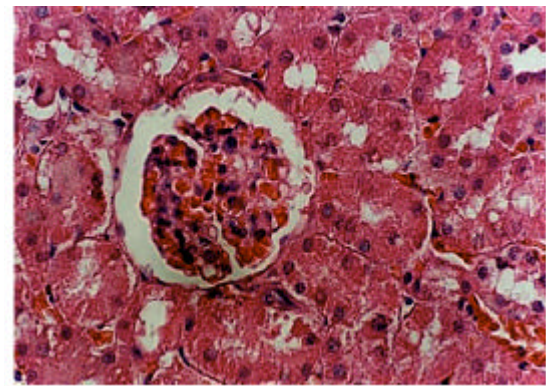


Fig. 5: SB203580 alleviating and improving oedema and congestion of glomerulus in SAP rats at 6 h (level 1) (10×40)



Fig. 3: Oedema and congestion of glomerulus in SAP rats at 6h (level 111) (10×40)

ICAM-1 mRNA in ANP group were decreased significantly at 3, 6 and 12 h in the SB203580 group ( $p < 0.01$  or  $p < 0.05$ ) (Table 5).

**The effect of p38MAPK inhibitor SB203580 on the expression of phosphorylated p38MAPK in the pancreas and renal tissues:** Moderately positive expression of the pancreas and renal tissues in sham rats could be examined by immunohistochemistry. Moreover, there were strongly positive expressions of phosphorylated p38MAPK in the pancreas and renal tissues of SAP rats and got a peak at 6 h after operation (Fig. 6 and 8). Positive expressions of phosphorylated p38 MAPK were significantly decreased after SB203580 administered ( $p < 0.01$ ) (Fig. 7 and 9).

The renal tissue ICAM-1 mRNA at 6 and 12 h were significantly higher than those at 3 h in ANP group ( $p < 0.05$ ). Compared with the ANP group, the renal tissue

**Correlated analysis:** Correlated analysis showed that there was a positive correlation between renal tissue

Table 5: Renal tissue ICAM-1 mRNA in groups sham, ANP and SB203580

Groups	3 h	6 h	12 h
Sham	0.58±0.12	0.56±0.32	0.61±0.56
ANP	1.57±0.40 <sup>a</sup>	2.36±0.51 <sup>a,b</sup>	2.60±0.95 <sup>a,b</sup>
SB203580	0.95±0.51 <sup>c</sup>	1.15±0.64 <sup>c</sup>	1.28±0.62 <sup>c</sup>

<sup>a</sup>p<0.05 vs. sham group; <sup>b</sup>p<0.05 6 and 12 h group vs. 3 group; <sup>c</sup>p<0.05 vs. ANP group

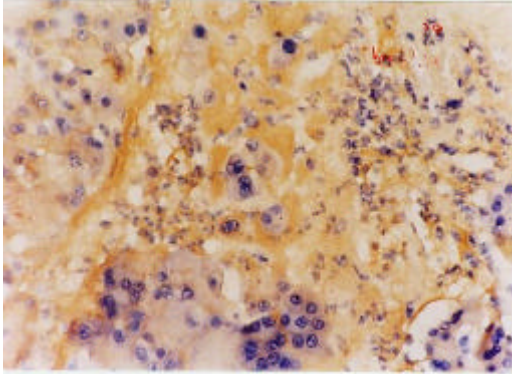


Fig. 6: Strongly positive expression of phospho-p38 MAPK in the pancreas tissue of SAP rat at 6h (SABC×40)

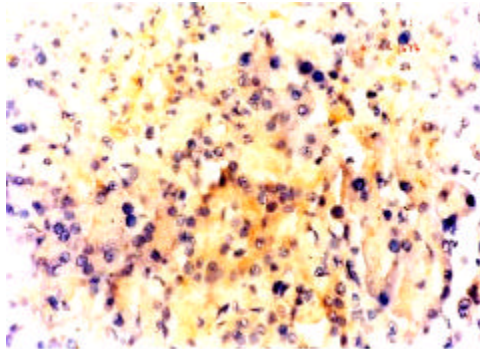


Fig. 7: Moderately positive expression of phospho-p38 MAPK in the pancreas tissue of SAP rat at 6h after SB2 administered (SABC×40)

ICAM-1 mRNA and serum BUN, Cr and serum TNF- $\alpha$  and renal histopathologic scores ( $r = 0.742$ ,  $-p < 0.01$ ,  $r = 0.781$ ,  $p < 0.01$ ,  $r = 0.775$ ,  $p < 0.01$ ).

Acute pancreatitis is a nonbacterial inflammatory disease that results from intrapancreatic activation release and digestion of the organ by its own enzymes but the mechanism by which enzyme activation occurs in humans remains obscure. Severe Acute Pancreatitis (SAP) is often associated with life-threatening distant organ dysfunction including Acute Lung Injury (ALI) or Acute Respiratory Distress Syndrome (ARDS) and renal failure at the early stage. SAP may combine with renal damage and even renal failure, cause pancreatic nephropathy. In the pathogenesis factors role of the digestion enzyme of

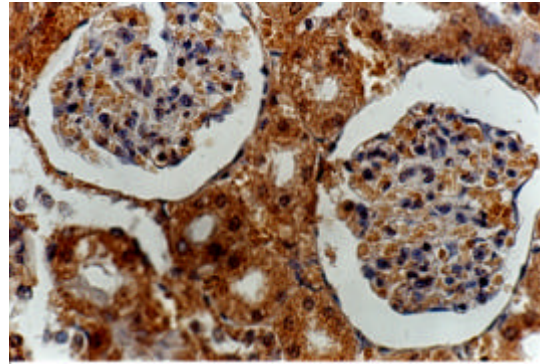


Fig. 8: Strongly positive expression of phospho-p38 MAPK in the renal tissue of SAP rat at 6h (SABC×40)

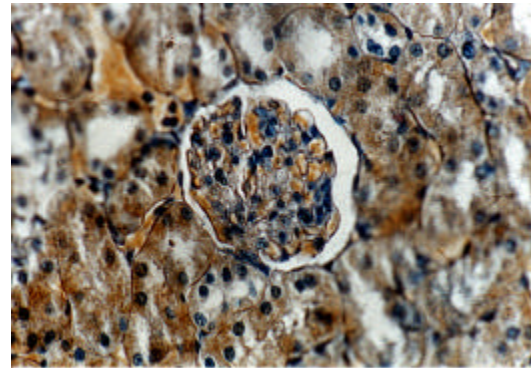


Fig. 9: Moderately positive expression of phospho-p38 MAPK/Hsp27 in the renal tissue of SAP rat at 6h after SB2 administered (SABC×40)

pancreas is activated and caused the abnormality of blood coagulation. Those come forth highly congeal state and cause severe microcirculatory disorder and conduce to be short of blood and oxygen in kidney.

The mechanisms of renal damage and even renal failure in severe acute pancreatitis are unknown. High serum concentrations of pancreatic enzymes may contribute to renal damage and even renal failure. Cytokines released by the pancreas or liver have also been implicated. It has gone through three theories: the theory of abnormal enzyme activation, the theory of disorder of pancreatic microcirculation and the theory of cytokine which a host inflammatory response is evoked in the pancreas and this locally limited inflammation is then manifested and amplified by the actions of diverse inflammatory mediators such as cytokines TNF- $\alpha$ , IL-1 and IL-6, ROS, proteolytic enzymes as well as gaseous mediators released by PMNs and monocytes/macrophages and so on (Dambrauskas *et al.*, 2010). Moreover, leukocytes infiltrate and these infiltrating cells can accelerate further production and

secretion of cytokines as well as other inflammatory mediators. As a result, leukocytes in the circulation as well as endothelial cells in the pancreas and in specific distant organ are activated, eliciting further microcirculatory derangements such as increased vascular permeability and accelerated leukocyte transmigration which were mediated by ICAM-1, resulting in the induction of SIRS, ARDS and renal failure.

Recent experimental studies have shed some light on the intracellular signaling pathway in the inflammatory cascade in SAP. It was reported that LPS-mediated responses on cytokine transcription and biosynthesis bifurcate at the level of Ras/Raf G-coupled proteins into major signaling pathways: One that runs through the nuclear factor- $\kappa$ B(NF- $\kappa$ B)/inhibitory- $\kappa$ B(I $\kappa$ B) which activates I $\kappa$ B kinase (IKK) leading to I $\kappa$ B phosphorylation/degradation and subsequently allowing NF- $\kappa$ B complex to translocate to the nucleus and promoting gene expression and another which is mediated through the ERK and p38 MAP kinase which involves the phosphorylation of MAPK Kinase (MKK) which phosphorylates and activates p38 MAPK. This pathway is selectively blocked by the pyridinyl-imidazole SB203580 (Ellen and Zarin, 1999). The activation of p38 MAPK pathway regulates the downstream activation of MAPKAP which phosphorylates the Hsp27 and activates the stability of transcripts of cytokines bearing the ARE. Increased understanding of the signal transduction pathways involved in the regulation of cytokine production and cytokine signaling in inflammatory cells has opened the door for the discovery of novel therapeutics useful for treating a variety of inflammatory diseases in which cytokine production or signaling is implicated. Some studies have confirmed that the stress-activated protein kinases, p38 and JNK are promising candidates for therapeutic intervention in human renal diseases (Ma *et al.*, 2007, 2009; Prakash *et al.*, 2006).

### CONCLUSION

In this study, there were strongly positive expressions of phosphorylated p38MAPK in the pancreas and renal tissue of SAP and associated renal injury rats. Moreover, these positive expressions could be prevented after the p38 MAP kinase inhibitor SB203580 were used in SAP rats. The p38 MAP kinase inhibitor SB203580 was effective in treatment of SAP and associated renal injury, whose mechanism might inhibit the positive expression of phosphorylated p38MAPK in the pancreas and renal

tissue to decrease the serum levels of TNF- $\alpha$  thus primary injury in local pancreas decreased and renal injury indirectly ameliorated; Moreover, SB203580 could also decrease the positive expression of ICAM-1 in the renal tissue to decrease the serum levels of TNF- $\alpha$ , PMN-endothelial cell adhesion, macrophages and PMNs infiltration in the renal tissue and renal microvascular permeability. The different swelling denaturation and necrosis of renal tubular epithelial cells were also observed in ANP group and Alterations in cell morphology, including loss of brush border, tubular necrosis, mitochondrial damage, interstitial congestion, edema and infiltration of inflammatory cells were also observed. The changes became severer with the prolongation of time. The renal pathological changes were aggravated significantly in the ANP group. Renal sections from SB203580-treated groups displayed significantly less proximal tubule cell necrosis. Acute renal injury could be directly ameliorated by the p38 MAP kinase inhibitor SB203580.

Thus it is very important for improving SAP and associated renal injury to learn and block the biosynthesis mechanisms of cytokine production and to investigate Effect of p38 MAPK inhibitor on adhesion molecule expression and microvascular permeability of renal injury in a rat model of severe acute pancreatitis.

### ACKNOWLEDGEMENTS

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