

# Phospholipases A-II (PLA2-II) induces acute pancreatitis through activation of the transcription factor NF-kappaB

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**Abstract. – OBJECTIVES:** Acute pancreatitis (AP) is an inflammatory disease of the pancreas characterized by local inflammation. Secretory phospholipases A-II (sPLA2-II) have been implicated in triggering AP, but their exact role to evoke AP is largely unknown. NF-κB activation has previously been shown to induce acute pancreatitis. The aim of this study is to explore that PLA2-II triggers AP by activation of NF-κB and the expression of inducible inflammatory mediators.

**MATERIALS AND METHODS:** Acute pancreatitis in vivo was induced in Wistar rats by retrograde infusion of 4% sodium taurocholate (TAC) into the pancreatic duct. Then the Wistar rats were divided into 2 groups: (1) PLA2 II-specific siRNA was subsequently administrated subcapsularly after infusion of TAC. (2) One hour before the intraductal injection of TCA, the rats were treated with PDTC 100 mg/kg twice i.p. in 1 h interval. Induction of pancreatitis was confirmed by histopathology, NF-κB activity and expression in pancreas was detected by EMSA and immunohistochemistry. Inflammatory mediators such as the tumor necrosis factor-alpha (TNF-α), interleukin (IL)-1beta, intercellular adhesion molecule-1 (ICAM-1), IL-6 and IL-8 in blood was detected by ELISA. The severity of the disease and the mortality were observed.

**RESULTS:** We demonstrated that TAC specifically induces pancreatitis, induces PLA2-II expression and activates NF-kappaB and proinflammatory cytokine synthesis in the pancreas of rats. sPLA2-II siRNA transfection blocked NF-kappaB activation and proinflammatory cytokine expression and relieved pancreatitis severity. PDTC treatment blocked NF-kappaB activation and proinflammatory cytokine expression. Pretreatment with PDTC or PLA2 II-specific siRNA transfection improved the survival of the rats.

**CONCLUSIONS:** These findings suggest that PLA2-II induces acute pancreatitis through activation of the transcription factor NF-kappaB. siRNA mediated gene knockdown of PLA2-II relieves pancreatitis severity at least partly mediated by the inhibition of NF-kappaB activation and proinflammatory cytokine synthesis.

*Key Words:*

Acute pancreatitis. Phospholipases A-II, NF-κB.

## Abbreviations

Phospholipases A-II, PLA2-II; EMSA = electrophoretic mobility shift assay; IL = interleukin; ICAM-1 = intercellular adhesion molecule-1; NF-κB = nuclear factor-κB; TCA = taurocholate; PDTC = pyrrolidine dithiocarbamate; PBS = phosphate buffered saline.

## Introduction

Acute pancreatitis is an inflammatory disease of the pancreas which, in its most severe form, is associated with multi-organ failure and death. Recently, signaling molecules and pathways which are responsible for the initiation and progression of this disease have been under intense scrutiny<sup>1</sup>.

Phospholipase A2 (PLA2), an enzyme involved in lipid metabolism, hydrolyzes phospholipids, which liberate arachidonic acid and lysophospholipids. Different types of PLA2 have been identified, which play a role in several neoplastic and inflammatory conditions. In acute pancreatitis (AP) two types of PLA2 have been described as playing important pathophysiological roles: (1) pancreatic group I PLA2, secreted by acinar cells, and (2) nonpancreatic group II PLA2 (PLA2-II)<sup>1</sup>. The group IIA secretory phospholipase A<sup>2</sup> (PLA2-II) is believed to play an important role in inflammation and cell injury. PLA2-II seems to be the major enzyme in AP responsible for the systemic inflammatory process<sup>3</sup>. Previous study showed PLA2-II expression was increased in the pancreas after 4% sodium taurocholate (TAC) into the pancreatic duct to induce acute pancreatitis, and siRNA mediated

gene knockdown of PLA2-II relieves pancreatitis severity<sup>4</sup>. This suggested PLA2-II plays an important role in the AP. But the exact role of PLA2-II to evoke AP is largely unknown.

One important signaling molecule, nuclear factor kappaB (NF-kappaB), has been shown to play a critical role in the development of acute pancreatitis. Activation of the transcription factor NF-kB/Rel is detectable very early in the course of experimental pancreatitis<sup>5,6</sup>. Under resting conditions, NF-kB/Rel consists of a heterodimer of NF-kBp50 and RelA/p65, which forms a complex with inhibitor of NF-kB  $\alpha$  (I $\kappa$ B- $\alpha$ ) as a negative regulator of the complex that keeps NF-kB/Rel inactivated in the cytoplasm. Stimulatory factors including physical trauma, UV irradiation, mitogens, and cytokines such as TNF- $\alpha$  and IL-1 $\beta$  mediate hyperphosphorylation of the inhibitor protein I $\kappa$ B $\alpha$  via the kinase complex I $\kappa$ B kinase (IKK), which is followed by ubiquitination and subsequent proteasome-mediated degradation of the inhibitory protein<sup>7</sup>. Subsequently, the NF-kB/Rel dimers translocate into the nucleus and bind specific  $\kappa$ B binding sites. In contrast to NF-kBp50, RelA/p65 contains C-terminal transactivation domains, which induce the transcription of genes encoding cytokines, chemokines, growth factors, and antiapoptotic factors<sup>8</sup>. Many of these genes have been implicated as central players in the development and progression of acute pancreatitis<sup>9</sup>.

Baek et al<sup>10</sup> has early found stimulation of cells with PLA2-II caused NF-kappaB activation, and PLA2-II-induced NF-kappaB activation was prevented in the presence of rho-BPB. Furthermore, the NF-kappaB inhibitor PDTC suppressed PLA2-II-induced nitrite production and iNOS expression as well as I $\kappa$ B $\alpha$  degradation, suggested NF-kappaB signals was regulated by PLA2-II. Recent studies in literature have shown NF-kappaB was positively regulated by PLA2-II<sup>11-13</sup>.

We hypothesize that secretory phospholipases A-II triggers AP by NF-kB activation. And knockdown of PLA2-II reduces pancreatitis severity and that this effect will correlate with decreased NF-kB activity.

## Materials and Methods

### *Taurocholate (TAC) Pancreatitis Induction*

Male Wistar rats weighing 200-250 g were obtained from Animal Center (Shanghai, China), and maintained at 23°C on a 12 hour light/dark

cycle and allowed free access to water and standard laboratory chow. From 12 hours before the start of the experiments, the animals were deprived of food but were allowed access to water. This study was conducted with the consent of the Ethics Committee for the use of experimental animals of the Affiliated Hospital of Qingdao University. TCA pancreatitis was produced by the method of Aho et al<sup>13</sup>. Briefly, under pentobarbital anaesthesia (50 mg/kg body weight), a laparotomy was performed and 5% sodium taurocholate (1 ml/kg body weight) was injected into the biliopancreatic duct at a rate of 0.2 ml/min by a microinfusion pump. Controls received an intraductal infusion of saline (0.2 ml/min). The rats recovered from the anaesthesia and were allowed access to water. Then at 24 hours after the operation, the rats were killed by an overdose of pentobarbital sodium and blood was collected by cardiac puncture. A segment of the pancreas close to the spleen were removed 24 hours after the induction of TCA pancreatitis. Part of the tissues were fixed by immersion in 4% paraformaldehyde and embedded in paraffin wax. Sections (3  $\mu$ m thick) were cut, deparaffinized, and stained with haematoxylin and eosin for histological examination. Part of the tissues were immediately frozen in liquid nitrogen and stored at -80°C until use.

### *Pancreatitis Treatment with siRNA- PLA2-II*

PLA2-II specific (siRNA-PLA2-II) or scrambled control siRNA (SC- siRNA) was suspended in lipofectamine 2000 and administered via intrapancreatic subcapsular injection (800 nm, 300  $\mu$ L total volume) 1 h after the administration of TAC for induction of pancreatitis by the method of ours before<sup>4</sup>. Twenty-four after pancreatitis induction, the rats were killed. Pancreas and blood samples were collected and harvested immediately as the methods above. Ten rats were included in each of the three groups (saline, SC-siRNA, siRNA- PLA2-II). The effects of siRNA-PLA2-II on the mortality of the rats with TCA pancreatitis were assessed for seven days.

### *Pancreatitis Treatment with PDTC*

To evaluate the effect of PDTC, 100 mg/kg were injected intraperitoneally one hour before the induction of TCA pancreatitis. Twenty-fours after pancreatitis induction, the rats were killed. Pancreas and blood samples were collected and harvested immediately as the methods above.

The effects of PDTC on the mortality of the rats with TCA pancreatitis were assessed for seven days.

#### **Western Blotting for Detection of RelA/p65 and PLA2-II Expression**

Tissues frozen in liquid nitrogen was used for RelA/p65 and PLA2-II protein detection as the manufacture's method. Briefly, Eighty mg of each collected pancreas tissue was homogenized in RIPA lysis buffer to extract the total protein. The protein concentration was measured using the BCA (bicinchoninic acid) method. After a 12% S-PAGE gel was cast, 40  $\mu$ g of total protein was loaded into each lane, and the electrophoresis separation was performed. The proteins in the gel were then transferred to a polyvinylidene fluoride (PVDF) membrane in a semi-dry blotting system. The PVDF membrane was blocked with TBST (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.1% Tween-20) solution containing 5% skim milk at room temperature for 1 hr. Then, the anti-RelA/p65 and anti-PLA2-II polyclonal antibody (1:1,000 dilution) and rabbit anti- $\beta$ -actin polyclonal antibody (1:2,000 dilution) were added, with overnight incubation at 4°C. Subsequently, the horse-radish peroxidase (HRP)-conjugated goat anti-rabbit IgG polyclonal antibody (1:2,000 dilution) was added, with incubation at 37°C for 1 hr. After TBST washing, autoradiography was conducted with ECL chemiluminescence reagents. The results were analyzed with the QuantityOne software. With  $\beta$ -actin set as an internal reference, the relative expression of the RelA/p65 and PLA2-II protein content was presented as the gray value ratio of RelA/p65 and PLA2-II and  $\beta$ -actin.

#### **Electrophoretic Mobility Shift Assay (EMSA)**

To detect the DNA binding activity of NF- $\kappa$ B, electrophoretic mobility shift assay was done according to the manufacturer's instructions. Briefly, nuclear proteins were prepared in frozen pancreas using a nuclear extraction kit and their concentrations determined by protein estimation procedure. A biotin-labeled NF- $\kappa$ B probe with a 5'-AGTTGAGGGGACTTTCCAGGC-3' sequence or an unlabeled cold probe was used to bind nuclear proteins at 15°C to 20°C for 30 min. Products were run on a 6% nondenaturing polyacrylamide gel in 0.5 $\times$  Tris-borate EDTA at 120 V for 60 min at 4°C; the shifted bands corresponding to the protein/DNA complexes were separated relative to the unbound dsDNA. The

gel was then transferred onto a presoaked membrane at 300 mA for 30 min at 4°C. Following the immobilization of bound oligonucleotides in the membrane by a UV-cross-linking oven for 5 min, the shifted bands were visualized after exposure to film.

#### **ELISA for Detection of Serum levels of Inflammatory Mediators**

Rat serum was collected as described above. ELISA was used to detect the serum levels of ICAM-1, TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8 by using a commercial kit (R&D Systems Inc., Minneapolis, MN, USA) in strict accordance with the manufacturer's instructions. A Synergy HT Microplate Reader was used to read the optical density at 450 nm, and the concentration of the sample was determined using a standard curve.

#### **Histopathologic Examination**

For the histologic evaluation, the pancreas was removed at 24 h after the induction of pancreatitis, fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. Hemorrhage and necrosis in the pancreas were evaluated by using a modified scoring system by Widdison et al<sup>14</sup>, according to the extent of the lesion as follows: 0, none; 1, minimal; 2, mild; 3, moderate; and 4, severe.

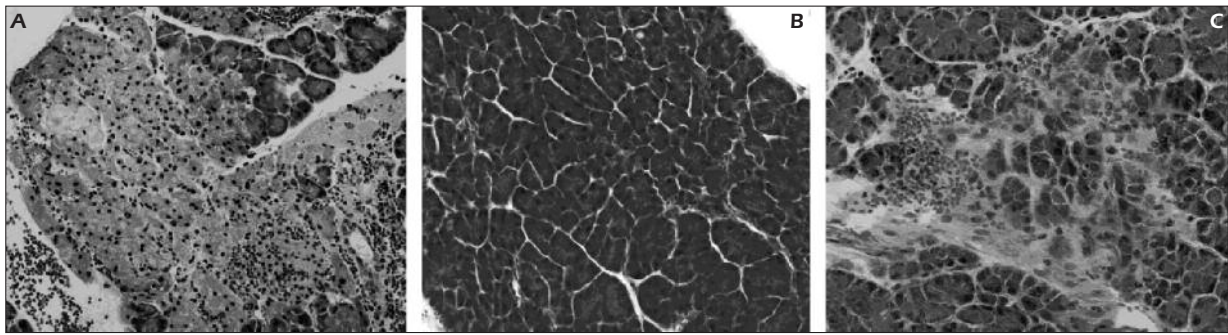
#### **Statistical Analysis**

The results reported represent mean  $\pm$  SD values obtained from multiple determinations in three or more separate experiments. In any one experiment, there were ten animals in each group. Statistical analysis for the difference were analyzed by Student's *t* test or  $\chi^2$  test. The Wilcoxon rank-sum test was applied to compare survival rates. The Mann-Whitney test was applied to compare the histologic scores. A *p* value of  $< 0.05$  was considered to be significant.

## **Results**

### **TAC Induces Acute Pancreatitis**

An injection of TAC into the pancreatic duct caused hemorrhage and edema in the pancreases. The animals had hemorrhagic ascitic fluid and fat necrosis in the abdominal cavity at 24 hr. Large necrosis in the abdominal cavity leukocytes surrounding the pancreatic ducts were seen by light microscopy (Figure 1A). The TAC injected animals had edematous pancreases with occasional

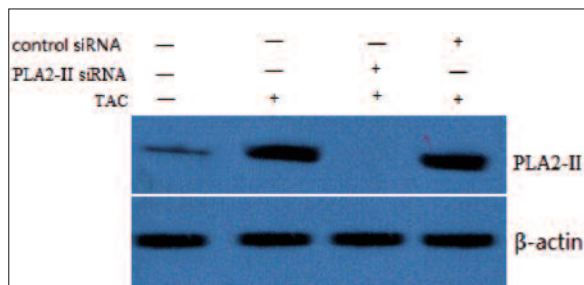


**Figure 1.** Histological findings for the pancreas. **A**, 24 hours after the induction of taurocholate (TCA) pancreatitis. **B**, 24 hours after the induction of the sham-operated pancreas. **C**, PLA2-II specific siRNA was injected subcapsularly into the pancreas of TAC induced pancreatitis rats. Original magnification  $\times 40$ .

necrotic areas in the periphery of pancreatic lobules. Polymorphonuclear leukocytes were sparse. The pancreases of sham-operated animals appeared normal (Figure 1B).

#### ***PLA2-II Specific siRNA Inhibits TAC Induced PLA2-II Expression and Inflammatory Mediators in Acute Experimental Pancreatitis***

In the TAC-induced pancreatitis, PLA2-II protein was significantly increased in the tissue of pancreas compared with the sham-operated animals by western blot assay (Figure 2). The inflammatory mediators of the TNF- $\alpha$ , IL-1 $\beta$ , ICAM-1, IL-6 and IL-8 was also significantly increased in the plasma of TAC-induced pancreatitis compared with the sham-operated by ELISA assay (data not shown). When mixtures of PLA2-II specific siRNA was injected subcapsularly into the pancreas of TAC induced pancreatitis rats, the plasma inflammatory mediators of the TNF- $\alpha$ , IL-1 $\beta$ , ICAM-1, IL-6 and IL-8 was significantly decreased in the plasma of TAC/PLA2-II siRNA-induced pancreatitis compared with the TAC/sham-operated by ELISA assay (data not



**Figure 2.** Western blot assay for PLA2-II expression in pancreas when mice were treated with TAC or/and transfected with PLA2-II specific siRNA.

shown), the PLA2-II was also decreased when treated with PLA2-II specific siRNA (Figure 2).

#### ***PLA2-II siRNA Treatment Relieves Pancreatitis Severity***

When mixtures of PLA2-II specific siRNA was injected subcapsularly into the pancreas of TAC induced pancreatitis rats, pancreatitis severity was significantly relieved as evidenced by decreased leukocytic infiltration, and necrosis compared with scrambled siRNA treated tissues and TAC injected tissues (data not shown); macroscopic examination of the pancreas revealed no significant differences between controls and rats treated with scrambled siRNA. Histologic findings of the pancreas in PLA2-II siRNA treated rats were milder than those in control rats. The necrosis score in rats treated with PLA2-II siRNA was significantly lower than that in control rats, but there was no significant difference in the hemorrhagic score (Table I).

#### ***PDTC Inhibits TAC-induced NF- $\kappa$ B Activation and Inflammatory Mediators in Acute Experimental Pancreatitis***

Translocation of NF- $\kappa$ B to the nuclei, which indicated activation of NF- $\kappa$ B, had significantly increased 24 hours after the induction of TCA pancreatitis compared with the sham-operated animals by western blot assay (Figure 3A). In addition, the NF- $\kappa$ B activity was also significantly increased by EMSA assay (Figure 3B).

When mixtures of PLA2-II siRNA was injected subcapsularly into the pancreas of TAC induced pancreatitis rats, the RelA/p65 protein and NF- $\kappa$ B activity was significantly decreased in the tissue of pancreas compared with the sham-operated animals by western blot assay (Figure 3A), and EMSA assay (Figure 3B).

**Table II.** Effects of PLA2-II siRNA and PDTC on histologic findings/hemorrhage and necrosis in TAC induced pancreatitis in rats.

Grade (score)	TAC	TAC/scrambled siRNA	TAC/PLA2-II siRNA	TAC/PDTC
<b>Hemorrhage</b>				
None (0)	0	0	0	0
Minimal (1)	1	0	0	1
Mild (2)	2	2	3	2
Moderate (3)	2	2	3	3
Severe (4)	5	6	5	4
<b>Necrosis</b>				
None (0)	0	0	0	0
Minimal (1)	0	0	1	1
Mild (2)	1	2	4	4
Moderate (3)	2	2	3	4
Severe (4)	7	6	2	1

In each group, n=10.

PDTC is a potent inhibitor of NF-κB<sup>15-16</sup>. To evaluate the effect of PDTC (100 mg/kg), PDTC were injected intraperitoneally one hour before the induction of TCA pancreatitis, and NF-κB activation and expression in the pancreas was studied. Rats were treated with 100 mg/kg of PDTC one hour before the intraductal injection of TCA. NF-κB activation, NF-κB expression and plasma PLA2-II and inflammatory mediators were examined 24 hours after the induction of pancreatitis. The results showed the NF-κB activation (Figure 3B) and expression (Figure 3A) in the pancreas was significantly inhibited by PDTC. Furthermore,

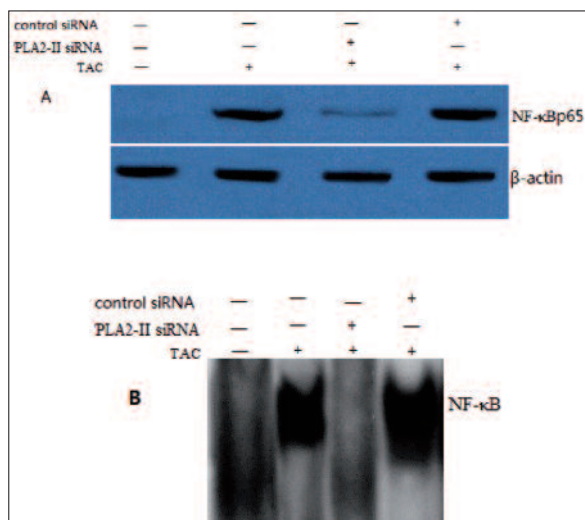
the plasma inflammatory mediators of the TNF-α, IL-1β, ICAM-1, IL-6 and IL-8 was also significantly decreased in the plasma of PDTC/TAC-pancreatitis compared with the TAC alone by ELISA assay (data not shown).

**PDTC Treatment Relieves TAC-induced Pancreatitis Severity**

To evaluate the effect of PDTC (100 mg/kg) on TAC-induced pancreatitis, PDTC were injected intraperitoneally one hour before the induction of TCA pancreatitis. The pancreatitis severity was significantly relieved as evidenced by decreased leukocytic infiltration and necrosis compared with TAC-induced pancreatitis alone (Figure 1E); histologic findings of the pancreas in PDTC treated rats were milder than those in control rats. The necrosis score in rats treated with PDTC was significantly lower than that in control rats, but there was no significant difference in the hemorrhagic score (Table I).

**Survival Rate**

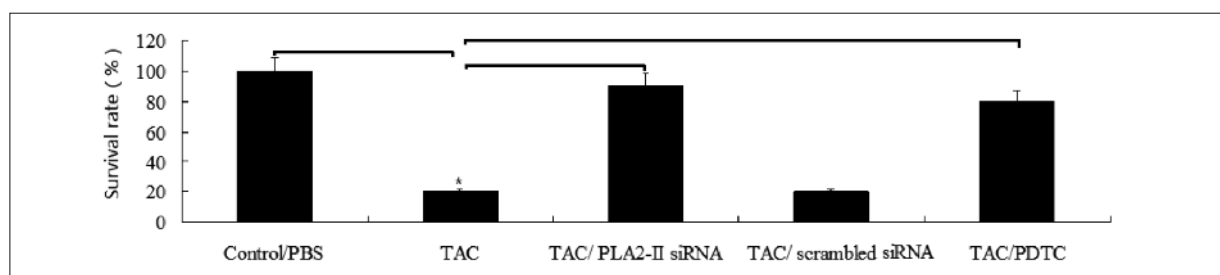
Survival rate for 24 h in the control group (n = 10) was 20%, the scrambled siRNA groups (n = 10) was 20%, PLA2-II siRNA groups (n = 10) was 90% and PDTC groups (n = 10) was 80%. Survival rate was significantly higher in the PLA2-II siRNA groups and PDTC groups compared to the controls (p < 0.05, respectively) (Figure 4).



**Figure 3. A,** Effect of TAC or/and transfected with PLA2-II specific siRNA on NF-Kbp65 expression in rat pancreas by western blot assay. **B,** Effect of TAC or/and transfected with PLA2-II specific siRNA on NF-κB activity in rat pancreas by EMSA assay.

**Discussion**

Acute pancreatitis is classified clinically into mild and severe forms. The majority of patients



**Figure 4.** Survival rate of rats treated with PLA2-II siRNA or PDTC in TAC-induced pancreatitis.  $n = 10$ . \* $p < 0.05$ .

suffer from mild acute pancreatitis, a self-limiting disease which responds well to conservative treatment. Up to 20 per cent of patients with acute pancreatitis, however, proceed to a clinically severe form involving both pancreatic and extrapancreatic necrosis, with systemic inflammatory response syndrome and organ complications. Although the initial steps in the pathogenesis of acute pancreatitis are not fully understood, the term ‘autodigestion’, introduced by Chiari in 1896, summarizes the prevailing concept that the acute inflammation is triggered by the activation of digestive enzymes<sup>17,18</sup>. Acute pancreatitis is acute inflammatory disease of the pancreas, and it is the most promising nonspecific treatment modality to date<sup>19</sup>. Patients with acute pancreatitis also pose a considerable financial burden to health care systems<sup>20</sup>. However, the cellular events leading to acute pancreatitis are not well defined and the mechanism by which known aetiological factors initiate the disease process remains to be established.

We have recently found PLA2-II upregulation was needed in TAC induced rat acute pancreatitis<sup>4</sup>. SiRNA mediated gene knockdown of PLA2-II appeared to relieve pancreatitis severity. However, the mechanism of how PLA2-II functioned is not known.

Jessica et al<sup>21</sup> has found knockdown of PLA2 II suppresses lung cancer growth in part by attenuating NF- $\kappa$ B activity. Targeting PLA2 II may exploit its relationship with NF- $\kappa$ B because NF- $\kappa$ B target genes include transcription factors for a number of growth factors such as vascular endothelial growth factor, fibroblast growth factor, and platelet-derived growth factor, which have been linked to promoting cancer cell growth, tumor stroma development, and angiogenesis<sup>22-24</sup>, suggested significant relationship between PLA2 II and NF- $\kappa$ B.

In the present study, we found an injection of TAC into the pancreatic duct caused hemorrhage and large necrosis in the pancreas. The animals had

hemorrhagic ascitic fluid and fat necrosis in the abdominal cavity at 24 hr. In the TAC-induced pancreatitis, PLA2-II protein was significantly increased in the tissue of pancreas by western blot assay. Furthermore, NF- $\kappa$ B activity and expression was also increased 24 hours after the induction of TCA pancreatitis. The inflammatory mediators of the TNF- $\alpha$ , IL-1 $\beta$ , ICAM-1, IL-6 and IL-8 was also significantly increased in the plasma of TAC-induced pancreatitis. After administration of PLA2-II specific siRNA *in vivo* decreased expression of PLA2-II expression in the pancreas of TAC induced pancreatitis rats, pancreatitis severity was significantly relieved as evidenced by decreased leukocytic infiltration and necrosis. Histologic findings of the pancreas in PLA2-II siRNA treated rats were milder, and the necrosis score was significantly lower. With the decreased PLA2-II expression in PLA2-II siRNA treated rats, NF- $\kappa$ B activity and expression was also decreased 24 hours after the induction of TCA pancreatitis. The inflammatory mediators of the TNF- $\alpha$ , IL-1 $\beta$ , ICAM-1, IL-6 and IL-8 were also significantly decreased in the plasma of TAC-induced pancreatitis.

Inhibition of NF- $\kappa$ B by PDTC was also accompanied by significant improvements in mortality, decreased leukocytic infiltration and necrosis and decreased inflammatory mediators. Furthermore, PDTC treatment did not decrease the PLA2-II expression in the pancreas of TAC-induced pancreatitis, suggested that NF- $\kappa$ B might be regulated by PLA2-II. The important inference from our results is that the beneficial effects of PDTC in the normally lethal experimental pancreatitis may be the result of improvement in the inflammatory processes of the pancreas.

## Conclusions

In, PLA2 II plays a significant role in TAC-induced pancreatitis. TAC-induced PLA2 II-depend-

dent NF-κB activation and inflammatory mediators release are available in TAC-induced pancreatitis.

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### Conflict of Interest

The Authors declare that there are no conflicts of interest.

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