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# Characterization of the acute pancreatitis induced by secretory phospholipases $A_2$ in rats

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**Abstract**

Acute pancreatitis (AP) is an inflammatory disease of the pancreas characterized by local inflammation and extrapancreatic effects such as lung injury. Secretory phospholipases $A_2$ ($PLA_2$s) have been implicated in triggering AP, but their exact role to evoke AP is largely unknown. Therefore, we have tested the ability of $sPLA_2$s to induce AP in rats, using venom $sPLA_2$s with residual or high enzymatic activity (bothropstoxin-II and *Naja mocambique mocambique* venom $PLA_2$, respectively), as well as $sPLA_2$ devoid of catalytic activity (piratoxin-I). The injection of *Naja m. mocambique* venom $PLA_2$, bothropstoxin-II or piratoxin-I (300 μg/kg each) into the common bile duct increased significantly the pancreatic plasma extravasation and myeloperoxidase activity. The lung myeloperoxidase and serum amylase were also increased for all groups, although the *Naja mocambique mocambique* venom $PLA_2$ induced higher lung myeloperoxidase and serum amylase values, compared with piratoxin-I and/or bothropstoxin-II. Histopathology of pancreas and lungs in piratoxin-I-injected rats showed interstitial oedema in both tissues, and neutrophil infiltration with acinar cell necrosis in pancreas. In conclusion, $sPLA_2$s induce AP in rats and the catalytic activity is not essential to induce the local effects in pancreas, although it appears to contribute partly to the remote lung injury.


*Keywords:* Phospholipases $A_2$; Acute pancreatitis; Catalytic activity; Sodium taurocholate

## 1. Introduction

Acute pancreatitis is a disease characterized by premature activation of pancreatic enzymes, causing a local inflammatory reaction that can progress to a systemic response and subsequent multiorgan failure (Bhatia, 2002). The severity of acute pancreatitis ranges from mild, self-limited type of edematous pancreatitis to severe necrotizing form. In the most severe forms of the disease, the pancreatic and extrapancreatic tissue damage cause high mortality, mainly due to the impairment of the lung function, which closely resembles the adult respiratory distress syndrome associated with other process such as shock and ischemia/reperfusion (Steer, 2001). The mechanisms underlying the

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lung injury induced by acute pancreatitis are not clearly understood, but it is known that activated enzymes and diverse proinflammatory mediators generated in the pancreas and activated leukocytes contribute to the lung complications (Bhatia et al., 2000; Pastor et al., 2003).

Phospholipases $A_2$ ($PLA_2$s) are enzymes that catalyses the hydrolysis of the cell membrane phospholipids, generating arachidonic acid, which is the common precursor for the formation of arachidonic acid metabolites and the platelet-activating factor (PAF). Generally, the $PLA_2$s can be divided into cytosolic $Ca^{2+}$-dependent $PLA_2$ ($cPLA_2$), intracellular $Ca^{2+}$-independent $PLA_2$ ($iPLA_2$) and secretory ($sPLA_2$) enzymes. The $cPLA_2$s are high molecular mass (85 kDa) enzymes found in the cytosolic fraction of nearly all cell types, and are able to translocate to membranes in response to increases in intracellular $Ca^{2+}$. The $iPLA_2$s are also intracellular, present high molecular mass and show absence of $Ca^{2+}$ requirement for their catalytic activity. The $sPLA_2$s are low molecular mass (14 kDa) extracellular enzymes that have many sources, such as snake venoms, mammalian pancreatic juice and synovial fluid (Dennis, 1994; Balsinde et al., 1999). An interesting subgroup of venom $PLA_2$s includes homologue enzymes presenting variable catalytic activity due to the critical amino acid substitutions at the calcium-binding loop. The Asp-49 $PLA_2$s present variable enzymatic activity; however, when the aspartate is substituted by lysine at the position 49, the resulting Lys-49 $PLA_2$s show a lack of catalytic activity. These $PLA_2$s can be found in various snake venoms, mainly in the *Bothrops* genus (Van den Bergh et al., 1989; Gutiérrez and Lomonte, 1995). A considerable number of works have reported an important role for $PLA_2$s in triggering various inflammatory diseases, including acute pancreatitis (Buchler et al., 1989a; Nevalainen, 1993; Uhl et al., 1997; Makela et al., 1999). Increased serum catalytic $PLA_2$ activity has been implicated in the pathogenesis of remote organ failure such as the pulmonary insufficiency arising from necrotizing acute pancreatitis (Buchler et al., 1989b).

Some experimental models have been developed to study acute pancreatitis in laboratory animals, such as the retrograde injection of biliary salts, bile or activated enzymes into the common bile duct (Aho et al., 1980; Steer, 1999). However, no study has attempted to investigate whether $PLA_2$s itself are able to trigger acute pancreatitis in the rat. Therefore, we have used the pancreatic duct injection of different $PLA_2$s as a model of induction of pancreatitis in rats, analyzing the pancreatic and lung myeloperoxidase activity (as a marker of neutrophil influx), pancreatic plasma extravasation, serum amylase levels determination, and the histopathology of pancreas and lungs. To achieve this, the $sPLA_2$ homologues bothropstoxin-II, piratoxin-I and *Naja mocambique mocambique* venom $PLA_2$ were used. Bothropstoxin-II is an Asp-49 $PLA_2$ isolated from *Bothrops jararacussu* snake venom, which contains residual enzymatic activity (Homsi-Brandeburgo et al., 1988; Pereira et al., 1998), whereas Piratoxin-I is a Lys-49 $PLA_2$ isolated from *Bothrops pirajai* snake venom, which is completely devoid of enzymatic activity (Mancuso et al., 1995; Holzer and Mackessy, 1996). In contrast, the $PLA_2$ isolated from *Naja mocambique mocambique* venom present high catalytic activity.

## 2. Experimental procedures

### 2.1. Materials and drugs

Bothropstoxin-II and piratoxin-I were isolated and purified according to previous studies (Homsi-Brandeburgo et al., 1988; Mancuso et al., 1995). *Naja mocambique mocambique* venom $PLA_2$, sodium taurocholate (taurocholic acid and sodium salt), hexadecyltrimethylammonium bromide and *o*-dianisidine dihydrochoride were obtained from Sigma Chemical Co. (St Louis, MO, USA). Hydrogen peroxide, paraformaldehyde and ParaplastTM resin were obtained from Merck SA (Rio de Janeiro, Brazil). $^{125}$I-Human serum albumin was radio-labelled in the Biotechnology Department at Biological Sciences of University of São Paulo (IPEN/CENEN-USP, São Paulo, Brazil).

### 2.2. Acute pancreatitis induction

Experiments were performed in male Wistar rats (220–250 g). All experiments were carried out in accordance with the guidelines for animal care of the State University of Campinas (UNICAMP). The animals were anaesthetized with sodium pentobarbital (50 mg/kg) and a medium laparotomy was performed. The common bile duct was cannulated transduodenally with a polyethylene tube according to the method described by Storck (1971). Saline (0.9%), *Naja mocambique mocambique* venom $PLA_2$, bothropstoxin-II or piratoxin-I (300 μg/kg, each) were injected into the duct in a final volume of 0.3 ml, with a constant flow over a 1-min period. The bile salt sodium taurocholate (5%, 0.3 ml) was also injected into the duct as a positive control. The hepatic portion of the common bile duct was clipped before injecting the solutions. After the procedure, the abdomen was closed in two layers, and the animals were allowed to recover from anaesthesia. After selected time post-injection, the animals were sacrificed and the pancreas, lung and blood samples were collected.

### 2.3. Pancreatic plasma extravasation

Pancreatic plasma protein extravasation was measured by the accumulation of intravenously injected $^{125I}$-human serum albumin (2.5 μCi/rat). A blood sample was collected, centrifuged at 8000 *g* for 10 min to obtain a plasma sample. The pancreas and plasma samples were counted for radioactivity (γ-counter). Plasma extravasation was expressed as the volume (μl) of plasma accumulated at

each pancreas compared to total counts in 1 ml of plasma (Brain and Williams, 1985).

### 2.4. Pancreatic and lung myeloperoxidase activity

The pancreas and lung were collected and placed in a test tube in the presence of 0.5% of hexadecyltrimethylammonium bromide in 50 mM potassium phosphate buffer, pH 6.0. Each tissue sample was homogenized and 1-ml aliquots of the homogenate were decanted into Eppendorf tubes. These tubes were then centrifuged at 14.000 rpm for 2 min, and the supernatants were collected. A myeloperoxidase assay was performed using a microliter plate scanner (Spectra Max 34, Molecular Devices, USA). This consisted of mixing 5 μl of sample with 200 μl of *o*-dianisidine solution (0.167 mg/ml of *o*-dianisidine dihydrochoride and 0.0005% hydrogen peroxide) prior to reading the plate. The changes in absorbance were measured at 460 nm for 15 s over a period of 5 min. The myeloperoxidase activity was expressed as myeloperoxidase units/mg of tissue. One unit of myeloperoxidase activity was defined as that degrading 1 μmol of peroxide/min at 25 °C (Bradley et al., 1982).

### 2.5. Serum α-amylase levels determination

The serum α-amylase levels were measured using a commercial kit (LaborLab, São Paulo, Brazil). The values were expressed as units of enzyme (U)/l. This method is based in the hydrolysis of the synthetic specific substrate (2-cloro-4-phenyl-galactate piranosil maltodiose) by the α-amylase, generating the cloronitrophenol, which absorbance is detected at 405 nm.

### 2.6. Histopathology of pancreas and lungs

The animals were submitted to injection of saline or piratoxin-I (300 μg/kg) were anesthetized and exsanguinated from the abdominal aorta. The pancreas and lung were collected, fixed by immersion in Millonig buffer content 4% paraformaldehyde and processed to embed in ParaplastTM resin. Sections of 5 μm were obtained and stained by hematoxilin-eosin (HE) and Toluidine blue methods. The tissues were evaluated for the tissue necrosis, parenchyma abscess, leukocyte infiltration and interstitial oedema.

### 2.7. Statistical analysis

Results were expressed as mean values ± SEM for $n$ animals. The values were analysed by Student's unpaired $t$-test or analysis of variance (ANOVA) for multiple comparisons followed by Bonferroni's modified $t$-test. $P < 0.05$ was taken as significant.

## 3. Results

### 3.1. Local pancreatic inflammatory reaction induced by $sPLA_2s$

The injection of *Naja m. mocambique* venom $PLA_2$, bothropstoxin-II or piratoxin-I (300 μg/kg each, 0.3 ml) into the common bile duct increased the pancreatic plasma extravasation, in comparison with the saline group, as observed at 4 h after duct injection (Fig. 1(A)). Injection of 0.3 ml of sodium taurocholate solution (5%) into the pancreatic duct also caused a significant increase in pancreatic plasma extravasation (Fig. 1(A)). No statistical differences among all tested groups were found. The injection of *Naja m. mocambique* venom $PLA_2$, bothropstoxin-II, piratoxin-I (300 μg/kg each, 0.3 ml) and sodium taurocholate (0.3 ml of a 5% solution) also markedly increased neutrophil sequestration to the pancreas, compared with saline group (Fig. 1(B)). No statistical differences among all tested groups were found.

### 3.2. Remote lung inflammatory reaction induced by $sPLA_2s$

To investigate whether a local inflammation by $sPLA_2s$ could lead to a remote lung injury, we evaluated the myeloperoxidase activity in the lung tissues. The injection of piratoxin-I, bothropstoxin-II and *Naja m. mocambique* venom $PLA_2$ (300 μg/kg each) enhanced significantly the myeloperoxidase activity, but the increases induced by bothropstoxin-II and *Naja m. mocambique* venom $PLA_2$ were higher ($P < 0.05$) compared with piratoxin-I. The increased lung myeloperoxidase activity induced by sodium taurocholate was significantly higher than the $sPLA_2s$ (Fig. 1(C)).

### 3.3. Effect of $sPLA_2s$ on the serum amylase levels

Fig. 1(D) shows that injection of *Naja m. mocambique* $PLA_2$, bothropstoxin-II or piratoxin-I (300 μg/kg each, 0.3 ml) into the common bile duct significantly increased the serum amylase levels in comparison with the saline group. The increases induced by *Naja m. mocambique* venom $PLA_2$ were higher ($P < 0.05$) compared with piratoxin-I or bothrosptoxin-II. The increased serum amylase levels in response to sodium taurocholate were significantly higher than the all $sPLA_2s$ tested.

### 3.4. Time course of piratoxin-I-induced pancreatitis

We have further evaluated the time-course of piratoxin-I (300 μg/kg)-induced pancreatitis. The higher pancreatic plasma extravasation was observed at 4 h after the induction, decreasing at 8 and 12 h, but still significant compared with the saline group ($n = 5$; Table 1). The basal

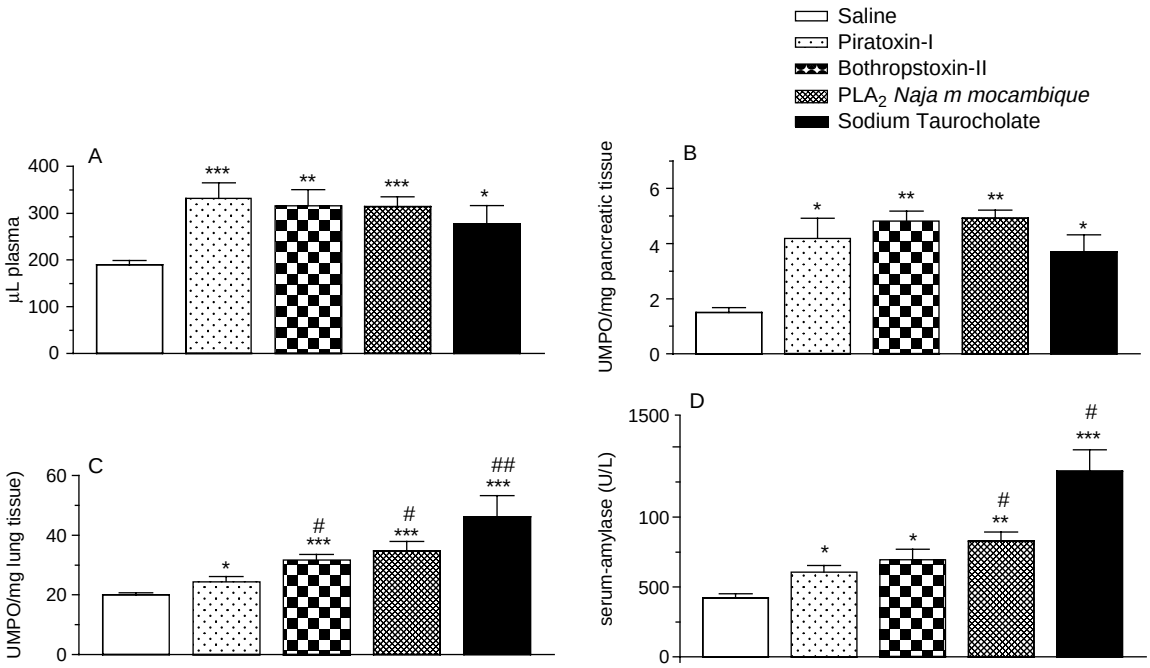


Fig. 1. Acute pancreatitis induced by injection of secretory phospholipases $A_2$ into the common bile duct of rats. Saline (vehicle), piratoxin-I (300 μg/kg), bothropstoxin-II (300 μg/kg), *Naja m. mocambique* venom $PLA_2$ (300 μg/kg), or sodium taurocholate (5%) were injected into the common bile duct in a final volume of 0.3 ml. Pancreatic plasma extravasation (A), pancreatic myeloperoxidase activity (B), lung myeloperoxidase activity (C) and serum amylase (D) were measured 4 h after duct injection. Each column represents the mean ± SEM of 6–8 rats; $*P<0.05$, $**P<0.01$ and $***P<0.001$ compared to saline group; $P<0.05$ and $P<0.01$ compared to piratoxin-I group.

values for the pancreatic plasma extravasation were reached at 48 and 96 h. The lung myeloperoxidase activity showed significant increases at 4 and 8 h after the induction, returning to basal values at 12–96 h post-induction. The amylase levels also peaked at 4 h, decreasing significantly thereafter (Table 1).

### 3.5. Histological analysis of piratoxin-I-induced pancreatitis

Histological examination of the lungs from control rats (injected intraductally with saline) showed normal tissue ($n=3$). The histological analysis of the lungs of animals submitted to the intraductal injection of piratoxin-I (300 μg/kg; $n=3$) showed areas of interstitial oedema with concomitant diminution of the alveolar space, as observed from 4 to 96 h after the pancreatitis induction, which peaked at 8–12 h. In the pancreatic tissue, neutrophil infiltration with areas of acinar cell necrosis and interstitial edema was seen at 4 h, peaking at 12 h post-piratoxin-I injection. After 48 and 96 h, pancreatic parenquima regeneration was observed. Thus, the acinar cell necrosis, the increased vascular permeability and the inflammatory cells infiltration probably represent early events in the pancreatic tissue damage.

Table 1
Time course of rat pancreatitis induced by piratoxin-I (PRTX)

| | Group | Time after induction | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4 h | 8 h | 12 h | 24 h | 48 h | 96 h |
| Pancreatic plasma extravasation (μl plasma) | Saline | 189±9 | 217±13 | 204±7 | 132±12 | 92±7 | 90±4 |
| | PRTX | 332±34* | 273±24* | 264±6* | 154±8 | 98±3 | 96±3 |
| Lung MPO activity (UMPO/mg tissue) | Saline | 20±1 | 19±1 | 15±1 | 14±2 | 15±1 | 11±1 |
| | PRTX | 26±2* | 23±1* | 17±2 | 13±2 | 16±1.5 | 12±2 |
| Serum amylase levels (U/L) | Saline | 422±31 | 521±49 | 428±80 | 346±21 | 164±6 | 174±24 |
| | PRTX | 607±43* | 649±78 | 434±29 | 342±21 | 201±±4 | 188±7 |

Different groups of animals were killed at the indicated time after the injection of sterile saline or piratoxin-I (300 μg/kg) into the common bile duct. Measurement of pancreatic plasma extravasation (μl plasma), lung myeloperoxidase activity (MPO; UMPO/mg tissue) and serum amylase (U/L) were performed. Each value represents the mean values ± SEM of five rats; $*P<0.05$ compared to the respective saline group.

## 4. Discussion

In the present study, we have showed that common bile duct injection of *Naja m. mocambique* venom $PLA_2$, bothropstoxin-II and piratoxin-I significantly increase the vascular permeability and neutrophil influx in the pancreas, which is accompanied by elevated serum amylase levels and neutrophil accumulation into the lungs. The histopathogical study in the pancreatic tissue confirmed the presence of neutrophil infiltration with areas of acinar cell necrosis and interstitial edema, which are signs of early events in the pancreatic tissue damage.

Previous studies have demonstrated that snake venom $PLA_2$s evoke local inflammatory effects characterized by increases in vascular permeability and oedema formation, leukocyte recruitment into tissues and release of inflammatory mediators, that take place by mechanisms dependent (Cirino et al., 1989; Moreno et al., 1992; Lloret and Moreno, 1993) or independent of their catalytic activity (Landucci et al., 1998; 2000; Castro et al., 2000; see also Teixeira et al., 2003 for review). In our present study, the local inflammatory reactions in the pancreas (oedema and neutrophil influx) were of the same magnitude for piratoxin-I (Lys-49 $PLA_2$ devoid of catalytic activity), bothropstoxin-II (Asp-49 $PLA_2$ with small catalytic activity) and *Naja mocambique mocambique* venom $PLA_2$ (enzyme with high catalytic activity), indicating that catalytic activity does not play a role in triggering such local pancreatic effects. On the other hand, both the neutrophil influx into the lung tissue and serum amylase levels were higher for bothropstoxin-II and *Naja mocambique mocambique* venom $PLA_2$ in comparison with piratoxin-I, suggesting that remote pulmonary inflammation depends, at least partly, on their catalytic activity. Considering that bothropstoxin-II evokes pulmonary neutrophil accumulation at the same extent as *Naja mocambique mocambique* venom $PLA_2$, it is likely that a low catalytic activity is already sufficient to trigger the remote lung inflammation. Additionally, *Naja mocambique mocambique* venom $PLA_2$ induced higher serum amylase levels than piratoxin-I, further suggesting that catalytic activity of $sPLA_2$ may play a role in initiating the systemic and remote inflammation in response to a local pancreatic lesion. Interestingly, interactions of snake venom $sPLA_2$s with membrane glycosaminoglycans in neutrophil membranes have been shown to trigger a cascade of biochemical events consisting of activation of G-protein, PKC and intracellular $Ca^{2+}$-independent $PLA_2$, the latter of which is supposed to regulate the release of arachidonic acid and hence the generation of the lipid chemoattractants leukotriene $B_4$ and PAF, leading in turn to neutrophil activation and locomotion (Gambero et al., 2002; 2004).

Sodium taurocholate was first described to induce haemorragic necrotizing pancreatitis in rats (Aho et al., 1980). Several works have demonstrated that in pancreatitis induced by sodium taurocholate, the lung myeloperoxidase, serum amylase and other inflammatory mediators are markedly increased (Marton et al., 1998; Vaquero et al., 2001; Cosen-Binker et al., 2003; Pereda et al., 2004). Accordingly, in our study, sodium taurocholate increased the pancreatic protein plasma extravasation and neutrophil influx in a similar extent to the three $PLA_2$ homologues used, but the effects on lung neutrophil influx and serum amylase levels were markedly higher than those of the $PLA_2$s. Interestingly, the pancreatitis induced by sodium taurocholate has recently been shown to involve the activation of an endogenous $sPLA_2$, since a selective inhibitor of $sPLA_2$ (S-5920/LY315920Na) strongly reduced the mortality, the pancreas tissue damage, and the blood biochemical changes, including amylase levels (Tomita et al., 2004).

In our study, time-course experiments were performed with piratoxin-I. For this purpose, the animals were sacrificed at 4, 8, 12, 48 and 96 h after the induction of pancreatitis. The higher differences between piratoxin-I and saline groups were observed at 4 h after the intraductal injection of piratoxin-I, the time by which myeloperoxidase activity, plasma extravasation and serum amylase levels are higher. At 4 h, the histological analysis of piratoxin-I-injected rats showed acinar cell necrosis, oedema formation and cell infiltration, strongly suggesting that they are early events in the pancreatic tissue damage.

In summary, our study demonstrated that the injection of $sPLA_2$s into the common bile duct causes acute pancreatitis in rats. We also showed that the catalytic activity is not essential for inducing the local effects in the pancreas, although it appears to contribute at least in part to the remote lung neutrophil accumulation observed during this process. We also suggest that the common bile duct injection of $PLA_2$s consist of a useful experimental model to reproduce some of the changes observed in the human pancreatitis, representing a new perspective to study the role of $PLA_2$ in acute pancreatitis.

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