

Effect of phospholipase A2 silencing on acute experimental pancreatitis

K.-J. ZHANG, D.-L. ZHANG, X.-L. JIAO, C. DONG

General Surgery, The Affiliated Hospital of Medical College, Qingdao University, Qingdao, Shangdong, China

Abstract. – BACKGROUND AND OBJECTIVES: The group II phospholipase A2 (PLA2 II) in blood has been reported to increase in acute pancreatitis and to reflect the severity of pancreatitis. Here we investigated the effect of inhibition of PLA2 using siRNA gene knockdown *in vitro* and in an *in vivo* model of experimental pancreatitis.

MATERIALS AND METHODS: Pancreatic acinar cell line AR42J *in vitro* was cultured with lysophosphatidylcholine (lyso-PC) (50 μ M) to induce expression of PLA2 with subsequent transfection of siRNA into stimulated AR42J cells. Acute pancreatitis *in vivo* was induced in Sprague Dawley rats by retrograde infusion of 4% sodium taurocholate (NaT) into the pancreatic duct. PLA2 II -specific siRNA was subsequently administered, subcapsularly, after infusion of NaT. Controls included administration of scrambled siRNA (SC-RNA) or vehicle alone. After 24hr, pancreata were harvested and assessed for worsening pancreatitis by histopathology; The serum levels of PLA2 II and inflammatory mediators were analyzed. In both models endogenous PLA2 II gene expression was assessed at 24 hrs using real time RT-PCR.

RESULTS: *In vitro*, PLA2 II protein and mRNA levels were reduced in cells treated with PLA2-II siRNA when compared with control treatment. *In vivo*, induction of pancreatitis was confirmed by histopathology, inflammatory mediators such as the tumor necrosis factor-alpha, interleukin (IL)-1beta, IL-6 and IL-8. PLA2 expression were reduced 69% in siRNA treated rats, compared with controls. Serum inflammatory mediators levels decreased after administration of siRNA compared with vehicle control ($p < 0.05$, respectively).

CONCLUSIONS: siRNA mediated gene knockdown of PLA2-II appeared to relieve pancreatitis severity. PLA2-II may serve as a novel and effective therapeutic target for acute pancreatitis.

Key Words:

Pancreatitis; Phospholipase A2; siRNA; Gene therapy.

with an uneventful recovery and a mortality of less than 2%¹. In about 20% of the attacks, however, severe acute pancreatitis develops. Despite considerable improvements in the treatment, the mortality remains between 15 and 25%²⁻⁴. In about 80% of the cases acute pancreatitis is caused either by biliary stones or by ethanol abuse⁵. The pathophysiology of acute pancreatitis is still only partly understood.

An important reorganization of the gene expression pattern occurs during inflammation of the pancreas as part of a well-structured response to exogenous or endogenous damage. Phospholipase A2 (PLA2) is an enzyme that cleaves fatty acids from phospholipids, leading to the generation of inflammatory eicosanoids⁶. Of the different phospholipase A2 (PLA2-II) isoenzymes, group II PLA2 shows correlation with disease severity in acute pancreatitis⁷. Group II PLA2 concentrations in serum have been shown to be higher in patients with infected pancreatic necrosis than in those with sterile necrosis⁸. Additionally, group II PLA2 correlated with the appearance of systemic inflammatory response syndrome (SIRS) in severe acute pancreatitis⁹. Tomita et al¹⁰ has found inhibition of PLA2 activities by prophylactic intravenous treatment with S-5920/LY315920Na significantly reduced mortality at 7 days, and significant reduction of lipase activity, amylase, aspartate aminotransferase, and hemorrhage at 6 h. It also significantly reduced histological damage such as edema and parenchymal and fat necroses of the pancreatic tissue. It is suggested sPLA2 inhibitor could become an effective agent for the treatment of severe acute pancreatitis.

To further elucidate the function of PLA2 II, siRNA gene knockdown was applied in the present study. Small interference RNA (siRNA) knockdown technology represents a new powerful approach to studying protein function by a mechanism distinct from antisense technology and has been shown to be effective *in vitro* and *in vivo*¹¹.

Introduction

Acute pancreatitis is a commonly encountered disease that in the majority of cases is self-limiting

Materials and Methods

Cell Culture

The AR42J rat pancreatic acinar cell line (ATCC Inc., Shanghai, China) were routinely cultivated in a 5% CO₂, 95% air atmosphere in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (Gibco Inc., Carlsbad, CA, USA), 4 mM L-glutamine, 50 units/ml penicillin, and 50 µg/ml streptomycin. Cells were incubated at 80 to 90% confluence with siRNA (100 µM) along in cultures with or without cerulein (0.02 µM). After different length of treatment, cells were lysed for RNA extraction using the Trizol reagent as recommended by the supplier.

Animals

Female Sprague Dawley rats were obtained from Animal Center (Shanghai, China), and weighing 175-200 g at onset of the studies, served as subjects. Animals were fed standard laboratory rats chow, given water *ad libitum*, and randomly assigned to control or experimental groups. All animal studies have been approved by the Animal and Laboratory Resources, the Affiliated Hospital of Medical College.

Treatment of AR42J with siRNA

PLA2-II siRNA and scrambled control siRNA (100 µM) was transfected into the AR42J for 48 hr as the manufacture's instruction. The silencing of PLA2-II in the cell line was confirmed by Western blot.

Pancreatitis Induction

Acute pancreatitis was induced with 4% sodium taurocholate (NaT). Briefly, under pentobarbital anesthesia, a midline incision was performed. The common bile duct was identified and cannulated in an antegrade direction with PE-10 tubing such that the proximal end of the tube was beyond the ampulla of Vater in the duodenum. The bile duct was then ligated to prevent the flow of bile and 4% NaT in sterile saline was consistently infused into the pancreatic duct at a rate of 1 mL/kg over 10 min.

siRNA Administration

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 µL total volume) 1 h after the administration of NaT for induction of pancreatitis. Twenty-four h after pancreatitis induction, the rats were killed. Pan-

creas and blood samples were harvested immediately afterwards; pancreata were snap frozen in liquid nitrogen and sera were frozen at -80°C. The integrity of RNA obtained from pancreas was ascertained by visualization of β-actin. Six rats were included in each of the three groups (saline, SC-siRNA, siRNA- PLA2-II).

Preparation of Serum and Tissue Samples

Mice were sacrificed at 24 hours after the first injection of NaT. Whole blood samples were centrifuged at 4°C, and serum was stored at -80°C for further studies. Serum was used for the PLA2, TNF-α, IL-1β, IL-6 and IL-8 analysis were measured by ELISA according to standard procedures. Tissues from the pancreas was removed, placed on ice, immediately frozen in liquid nitrogen, and stored at -80°C. PLA2 mRNA was detected by RT-PCR.

Western Analysis

siRNA treated and control AR42J cells were homogenized and lysate was run on 15% acrylamide gel and subsequently blotted with appropriately diluted anti-PLA2-II antibody (1:200) as the manufacture's instruction.

Real-time PCR

Total tissue RNA was extracted using the RNeasy kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. Total RNA was quantified by ultraviolet absorption and reversibly transcribed into cDNA using SuperScript II reverse transcriptase (Invitrogen, Carlsbad, CA, USA) and OligodT primers (Invitrogen) according to the manufacturer's instructions. Transcribed cDNA was further analyzed by real-time PCR. All values were normalized to the level of cyclophilin cDNA.

Statistical Analysis

Data are expressed as mean ± SD. Differences were analyzed by Student's *t* test. A *p* value of 0.05 or less was considered significant.

Results

In vitro siRNA Knockdown of PLA2-II Protein and mRNA Expression

When AR42J cells were stimulated with lysophosphatidylcholine (lyso-PC) (50 µM), expression of PLA2-II protein and mRNA were increased when compared with vehicle control cells,

respectively (Figure 1A-B). In contrast, when cerulein stimulated AR42J cells were treated with siRNA-PLA2-II, PLA2-II gene expression significantly decreased when compared with vehicle treated controls. Furthermore, western blot analysis of cellular lysates revealed decreased protein levels of PLA2-II when treated with siRNA when compared with vehicle control (Figure 1A-B). RNAi transfection efficiency was assessed on stimulated AR42J cells which were treated with GFP-conjugated scrambled siRNA (data not shown).

In vivo siRNA Knockdown of PLA2-II Protein and mRNA Expression

As in previous studies, animals injected with NaT through the pancreatic duct demonstrated evidence of pancreatitis including increased leukocytic infiltration and necrosis¹⁰⁻¹². In the present study, pancreata obtained from animals induced with NaT pancreatitis had increased expression of PLA2-II (Figure 2A-B). In contrast, pancreata obtained from PLA2-II siRNA treated animals had significantly inhibited expression of PLA2-II.

Effect of PLA2-II siRNA on Pancreatitis Severity

To clarify whether the extent of tissue damage during AP is regulated by overexpression of PLA2-II, we used siRNA to knock down PLA2-

II expression during inflammation. When mixtures of PLA2-II specific siRNA was injected subcapsularly into the pancreas of NaT induced pancreatitis rats, pancreatitis severity was relieved as evidenced by decreased leukocytic infiltration, and necrosis (Figure 3). However, no differences in edema was observed when compared with scrambled siRNA treated tissue (data not shown).

Effect of PLA2-II siRNA on Proinflammatory Cytokines

It has demonstrated that serum obtained from animals which were induced with NaT pancreatitis contained high levels of proinflammatory cytokine IL-1 β , IL-6, IL-8, TNF- α and PLA2-II. Interestingly, when these animals were treated with PLA2-II siRNA serum levels of the proinflammatory cytokines were significantly decreased (Figure 4).

Discussion

Acute pancreatitis is classified clinically into mild and severe forms¹³. The majority of patients suffer from mild acute pancreatitis, a self-limiting disease which responds well to conservative treatment. Up to 10 per cent of patients with acute

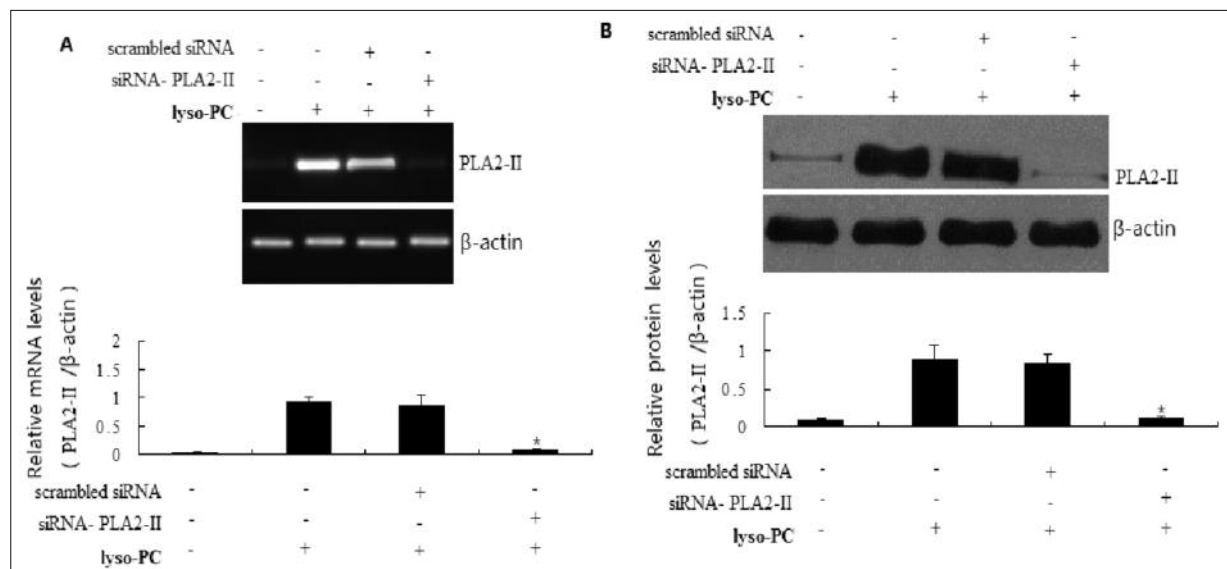


Figure 1. siRNA gene inhibition of cerulein-treated AR42J cells. Cells were transfected with siRNA (100 μ M) 24 hours prior to treatment with lysophosphatidylcholine (lyso-PC) (50 μ M). After 24 hours of stimulation, cells were harvested and total mRNA was isolated and assessed for PLA2-II expression (real time PCR) **(A)**. Western blot assay of PLA2-II expression in lyso-PC-stimulated AR42J cells and PLA2-II siRNA/lyso-PC-stimulated AR42J cells **(B)**. Data are presented as mean fold change mRNA/protein expression $\pm$ SD; controls consisted of untreated and vehicle treatment alone. β -actin served as protein control. *Significance $p \leq 0.05$.

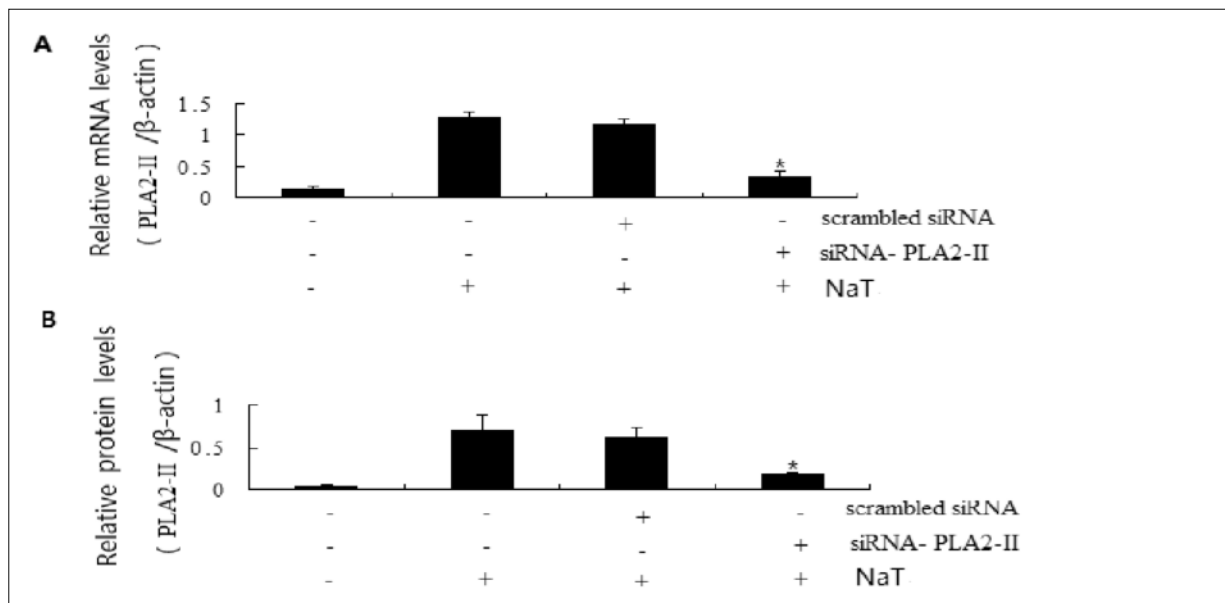


Figure 2. Effect of siRNA on PLA2-II expression *in vivo*. Rats were injected with control siRNA (SC siRNA) or PLA2-II siRNA after ductal injection of 4%NaT. Pancreatic tissue was harvested 24 hrs later, total mRNA **(A)** and protein **(B)** was isolated and PLA2-II were determined (real time PCR) and compared with rats induced with NaT pancreatitis. Data represent mean fold change PLA2-II siRNA expression $\pm$ SD. controls consisted of untreated and vehicle treatment alone. β -actin served as protein control. *Significance $p \leq 0.05$.

pancreatitis, however, proceed to a clinically severe form involving both pancreatic and extra-pancreatic necrosis, with SIRS 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^{14,15}.

Phospholipase A (PLA, EC 3. 1. 1.4) hydrolyzes the sn-2 fatty acyl ester bond of phosphoglycerides, which liberates arachidonic acid and lysophospholipid. PLA, in mammals can be classified into three types, type I (pancreatic), type II (membranous), and type IV (cytosolic), based on their primary structures¹⁶. There is evidence that phospholipase A2 (PLA2) plays an important pathophysiological role in various in-

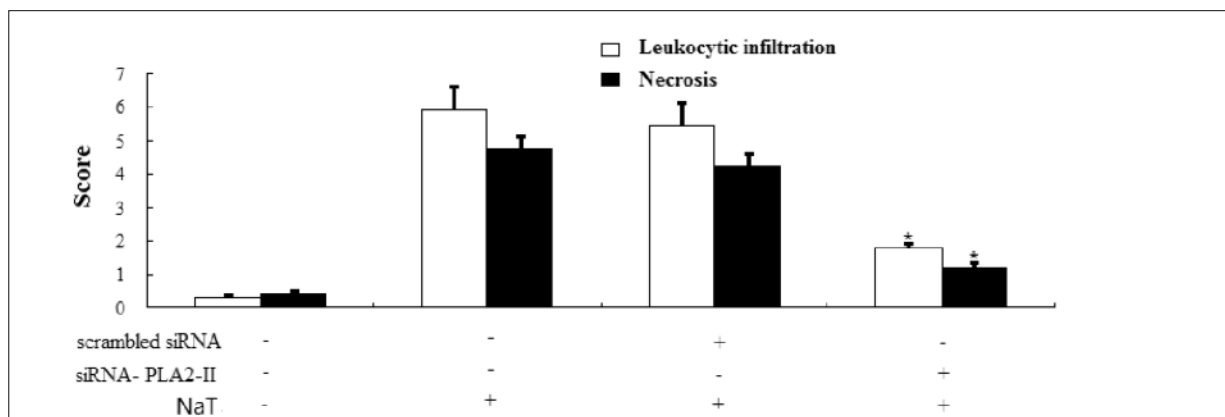


Figure 3. Effect of siRNA on pancreatitis severity. Pancreas tissue from NaT induced pancreatic rats were injected with PLA2-II specific siRNA and control (scrambled) siRNA. Pancreas was harvested 24 hours post RNAi administration and assessed for severity (leukocytic infiltration and necrosis). Histopathological scoring of leukocytic infiltration and necrosis. Data represent mean scoring ($\pm$ SD), *Significance $p \leq 0.05$.

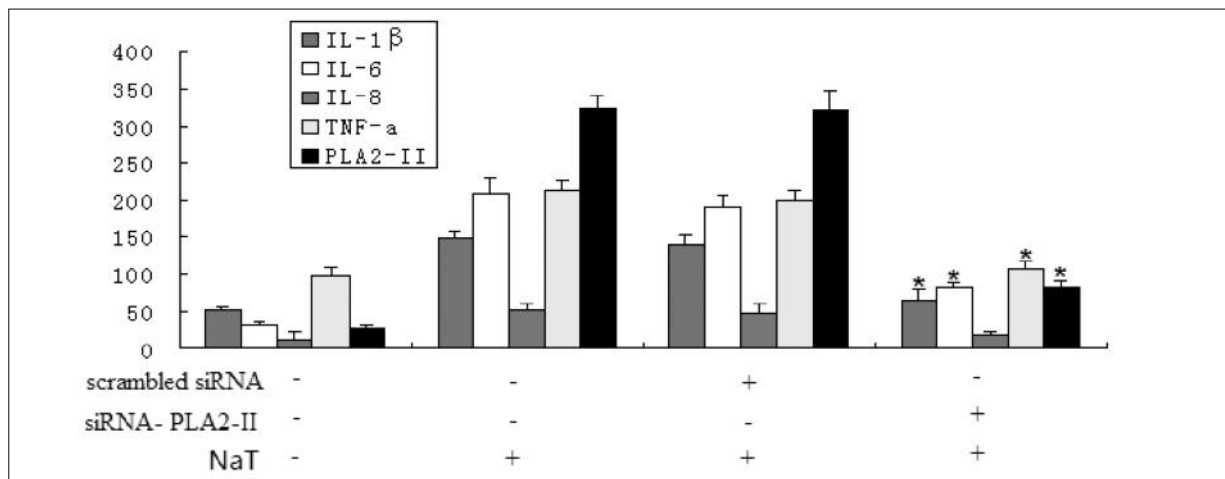


Figure 4. Effect of siRNA on inflammatory cytokines. Serum obtained from pancreatic rats treated with scrambled siRNA (SC siRNA) or PLA2-II specific siRNA (siRNA) was collected 24 hrs after induction of acute pancreatitis. Serum cytokine levels were measured by ELISA as described in Materials and Methods. Data represented mean serum levels of respective cytokines $\pm$ SD. Compared with scrambled siRNA treated tissue, *Significance $p \leq 0.05$.

flammatory diseases, such as septic shock¹⁷, adult respiratory distress syndrome (ARDS)¹⁸, multiple injuries¹⁹, diffuse peritonitis^{20,21}, and acute pancreatitis²²⁻²⁴. In human acute pancreatitis, highly significant increases in serum catalytic PLA2 activity have been found which correlate well with the severity of the disease.

Group II PLA2 (PLA2-II) seems to be the major enzyme in acute pancreatitis (AP) responsible for the systemic inflammatory process^{25,26}. It has reported previously, PLA2-II expression can be detected in pancreas and in serum within 24 hours of induction of acute pancreatitis²⁷. The AR42J cell line can be induced to express PLA2-II with stimulation of lysophosphatidylcholine (lyso-PC)²⁸.

In this work, we demonstrated that PLA2-II siRNA was able to decrease PLA2-II expression in rat acute pancreatitis and AR42J cell line. The present findings showed marked changes in PLA2-II expression 24 h after administration of 4 per cent sodium taurocholate stimulating severe acute pancreatitis. Administration of PLA2-II specific siRNA *in vivo* significantly decreased expression of PLA2-II. Bradley et al¹¹ demonstrated siRNA mediated gene knockdown of the Ins2 mRNA when administered IV through the tail vein. In those studies animals received a 100 μ g of siRNA which facilitated a 33% reduction of the targeted gene mRNA expression. However, the effect of intra-pancreatic administration and siRNA turnover were not studied. Our subcapsular administration of PLA2-II specific siRNA decreased specific PLA2-II mRNA expression up to 73%. In agree-

ment with PLA2-II mRNA expression, serum levels of PLA2-II was also decreased significantly.

Severe necrotizing pancreatitis is characterized by a systemic inflammatory response that mostly targets the lung and causes ARDS in humans. In order to determine the systemic inflammatory response following pancreatitis in our mouse model, we studied serum levels of IL-6, IL-8, TNF- α and IL-1 β and the degree of leukocyte infiltration. Retrograde infusion of 4% sodium taurocholate (NaT) increased the serum levels of IL-6, IL-8, TNF- α , IL-1 β and PLA2-II, and displayed neutrophil infiltration of the interalveolar tissue. In contrast, PLA2-II specific siRNA treatment significantly decreased the inflammatory mediators, and alleviated tissues damage and neutrophil infiltration.

Conclusions

Our data suggest that deletion of PLA2-II signaling may serve as a novel and effective therapeutic target for acute pancreatitis. Gene knockdown technology represents a powerful tool to study PLA2-II function.

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Conflict of Interest

The Authors declare that there are no conflicts of interest.

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