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Effect of a Selective Inhibitor of Secretory Phospholipase A₂, S-5920/LY315920Na, on Experimental Acute Pancreatitis in Rats

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Abstract. We investigated the efficacy of a potent inhibitor of secretory phospholipase A₂ (sPLA₂), S-5920/LY315920Na, in an experimental model of acute pancreatitis in rats. Combined intraductal injection of sodium taurocholate (5 mg/rat) and porcine pancreatic sPLA₂-IB (300 µg/rat) caused severe hemorrhagic necrotizing pancreatitis resulting in high mortality, along with rapid increases of catalytic PLA₂ and lipase activities in plasma and ascites and with gradual increases of plasma amylase and aspartate aminotransferase levels over 9 h after the pancreatitis. Prophylactic intravenous treatment with S-5920/LY315920Na significantly reduced mortality at 7 days, and strongly abrogated PLA₂ activities in both plasma and ascites along with 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. This sPLA₂ inhibitor could become an effective agent for the treatment of severe acute pancreatitis.

Keywords: phospholipase A₂, pancreatitis, parenchymal necrosis, S-5920/LY315920Na

Introduction

Phospholipase A₂ (PLA₂) comprises a diverse family of lipolytic enzymes that hydrolyze the *sn*-2 fatty acid ester bond of glycerophospholipids to produce free fatty acid and lysophospholipids (1, 2). Over the past two decades, a number of PLA₂s have been identified and characterized. From their structural and biochemical features, these PLA₂s are classified into several families, including secretory PLA₂ (sPLA₂), arachidonoyl-specific cytosolic PLA₂ (cPLA₂), and Ca²⁺-independent PLA₂ (3). Among these, the non-pancreatic group IIA-sPLA₂ (sPLA₂-IIA) has been postulated to play important roles in the pathophysiology of various diseases such as acute pancreatitis, septic shock, acute lung injury, and rheumatoid arthritis (4, 5).

Acute pancreatitis is diagnosed by the sudden onset of

acute abdominal pain accompanied by increases of digestive enzymes such as amylase and lipase (6). In severe necrotizing acute pancreatitis, changes of serum levels of both pancreatic group IB (sPLA₂-IB) and non-pancreatic sPLA₂-IIA have been documented; the former increased at the early stages (1 to 2 days after admission) while the latter increased during 2 to 9 days of the disease (7, 8). Increased serum catalytic PLA₂ activity has been implicated in the pathogenesis of remote organ failure such as pulmonary insufficiency arising from necrotizing acute pancreatitis (7 – 9).

Recently, highly potent inhibitors of sPLA₂-IIA have been found by using computer-aided drug design and chemical modification (10). Among them, S-5920/LY315920Na was most potent and specific for sPLA₂-IIA (10, 11) and has been already found to show efficacy against models of acute lung injury (12, 13) and trypsin-taurocholate (TCA)-induced pancreatitis in rats (14). Another sPLA₂-IIA inhibitor, BM 16.2056, also showed a beneficial effect on taurocholate-induced acute pancreatitis in rats (15), suggesting that sPLA₂-IIA

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plays pathophysiological roles in these models.

Acute pancreatitis induced by a mixture of pancreatic sPLA₂-IB and bile acids has been used as a model related to lipolytic pancreatic enzyme because sPLA₂-IB has been postulated to be responsible for increased serum catalytic sPLA₂ activity and the pathogenesis of necrosis or hemorrhage in the pancreas (7, 16, 17). Indeed, when combined with bile acid and injected into the pancreatic duct, this enzyme causes various degrees of acute pancreatitis in animals depending on its dose (16). Although the IC₅₀ value of S-5920/LY315920 for sPLA₂-IB is 20- to 90-fold higher than that for sPLA₂-IIA (18), sPLA₂-IB should be also inhibited by this compound when the concentration of this compound in plasma is high enough. Thus, we evaluated the effect of S-5920/LY315920Na on the lipolytic enzyme-related severe acute pancreatitis in rats to find whether an sPLA₂ inhibitor could show beneficial effects against mortality and biochemical and histological changes arising from the development of pancreatitis.

Materials and Methods

Drugs and chemicals

S-5920/LY315920Na (sodium[[3-(aminooxoacetyl)-2-ethyl-1-(phenylmethyl)-1*H*-indol-4-yl]oxy] acetate; Lot. 268SB4) was synthesized at Eli Lilly and Company (Indianapolis, IN, USA) (18). Pentobarbital sodium (Nembutal® injection) was purchased from Dainippon Pharmaceutical Co. (Osaka). Sodium taurocholate (TCA), sodium deoxycholate, and bovine serum albumin (BSA) were from Sigma Chemical Co. (St. Louis, MO, USA). 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine was from Avanti Polar Lipid, Inc. (Alabaster, AL, USA). Triolein was from Doosan Serdary Research Laboratories (Ontario, Canada). Porcine pancreatic PLA₂-IB from Boehringer Mannheim GmbH (Mannheim, Germany) was dialyzed, concentrated in phosphate-buffered saline (PBS), and stocked as aliquots at -80°C until use. All other reagents were of analytical grade or better.

Acute pancreatitis in the rat

These animal experiments were approved by the Animal Care and Use Committee of Shionogi Research Laboratories, Osaka. Acute experimental pancreatitis was induced in 9- to 10-week-old male Wistar rats (Shizuoka Laboratory Center, Shizuoka) with a body weight of 220–280 g (19). After fasting overnight, the animals were anesthetized with an intraperitoneal injection of pentobarbital (35 mg/kg), and a freely-moving catheter (DS-10 Disposal Swivel Kit; BRC Bioresearch Center, Nagoya) was placed in the jugular vein for the

administration of drugs. After laparotomy, the duodenal loop was pulled out, and a polypropylene catheter (internal diameter, 0.5 mm; external diameter, 0.8 mm) was introduced through the intact wall of the duodenum via the papilla of Vater into the common biliopancreatic duct. After obstruction of the common bile duct at the porta hepatis with a clamp, pancreatitis was induced by retrograde infusion of 0.2 ml of a mixture of 2.5% TCA (5 mg/animal) and porcine PLA₂-IB (300 µg/animal) into the common biliopancreatic duct over 10 s. The clamp was removed 10 s after the infusion, the catheter was withdrawn, and the duodenotomy was closed with surgical adhesive (Aron Alpha A; Sankyo, Tokyo). The abdomen was then closed with Michel's clips. Animals in the pancreatitis sham group received only cannulation of the biliopancreatic duct without infusion. After the operation, each rat was allowed to wake up, was kept in an individual cage, and had free access to water but received no solid food for 24 h.

Characteristics of PLA₂ inhibitor and treatment protocol

S-5920/LY315920Na inhibits human sPLA₂-IIA activity stoichiometrically through optimized tight-binding interactions at the enzyme catalytic site (10, 11). Its median inhibitory concentration (IC₅₀) values for human non-pancreatic sPLA₂-IIA, human pancreatic sPLA₂-IB, and porcine pancreatic sPLA₂-IB were 0.009, 0.228, and 0.048 µM, respectively (18). It also inhibits rat non-pancreatic sPLA₂-IIA and pancreatic sPLA₂-IB with IC₅₀ values of 0.002 and 0.027 µM, respectively (13).

Prophylactic treatment: S-5920/LY315920Na was dissolved in 5% glucose and injected i.v. as a bolus (2 ml/kg) 5 min before the induction of pancreatitis and then infused (0.44 ml/h per animal) for 24 h in the mortality study or for 6 h in the biochemical and histological study. In the mortality study, the S-5920/LY315920Na groups consisted of high-, medium-, and low-dose groups. The high-dose group received 3 mg/kg, i.v. bolus followed by sustaining infusion at 0.5 mg/(kg·h) for 24 h; the medium-dose group was given 1 mg/kg bolus and 0.15 mg/(kg·h) infusion; and the low-dose group was given 0.3 mg/kg bolus and 0.05 mg/(kg·h) infusion. The vehicle control group received bolus injection and infusion of a similar volume of the 5% glucose solution instead of S-5920/LY315920Na.

In the biochemical and histology study, the S-5920/LY315920Na group received 3 mg/kg, i.v. bolus and 0.5 mg/(kg·h) for 6 h, and then the samples were harvested under pentobarbital anesthesia (100 mg/kg, s.c.). The vehicle control group and the sham-operated group were given 5% glucose solution.

Therapeutic treatment: S-5920/LY315920Na (3 mg

/kg, i.v.) was injected as a bolus 1 min after or 1 h after the induction of pancreatitis and then infused for 24 or 23 h, respectively. The mortality was assessed for 7 days. The vehicle control group was given 5% glucose solution.

Preparation of samples

At given intervals, the animals were anesthetized subcutaneously with 100 mg/kg pentobarbital. After performing relaparotomy, ascites samples were collected with a disposable tube containing EDTA Na (7.7 mM, pH 7.4) and prostaglandin E₁ (PGE₁) (1.5 µg/ml). Thereafter, plasma samples were obtained from the abdominal aorta using a 21-gauge needle and a trigonal stopcock. Plasma samples (1 ml) for PLA₂ and lipase activities were collected with a syringe containing 0.1 ml of EDTA Na (7.7 mM, pH 7.4) and PGE₁ (15 µg/ml). Plasma samples (2.5 ml) for the determination of amylase, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and creatinine were then collected via a heparinized syringe. Ascites and plasma samples were centrifuged at 2000 × g for 15 min to remove the cells, and the supernatants were stored at -40°C until analysis.

Biochemical analysis of plasma and ascites

Amylase was determined with Neo-Amylase Test Daiichi (Lot. 048RDQ; Daiichi Chemicals, Tokyo). ALT and AST were determined with Transaminase CII-Test Wako, and creatinine was with Creatinine Test Wako (Wako Pure Chemicals, Osaka). The hemoglobin content in the ascites was measured with Hemoglobin Test Wako (Wako Pure Chemicals).

PLA₂ and lipase assay

The catalytic activity of PLA₂ in plasma and ascites was measured as described previously (14). Briefly, the substrate was used in the form of mixed micelles of 1 mM 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine and 3 mM sodium deoxycholate. The assay mixture contained 5 mM CaCl₂, 1 mg/ml fatty acid free BSA, 150 mM NaCl, and 100 mM Tris-HCl (pH 8.0). The reactions were initiated by addition of plasma or ascites samples to the assay mixture. The reaction was carried out at 40°C for 30 min and then stopped, and the mixture was subjected to extraction according to Dole's extraction system followed by silicic acid treatment (20). PLA₂ activity was determined by measuring 9-anthryldiazomethane (ADAM)-labeled free fatty acid with HPLC (20).

Lipase activity was measured using mixed micelles of 0.5 mM triolein (Doosan Serdary Research Laboratories, Ontario, Canada) and 3 mM sodium deoxycholate. The

assay mixture contained 5 mM CaCl₂, 1 mg/ml BSA, 150 mM NaCl, and 100 mM Tris-HCl (pH 8.0). After the incubation at 37°C for 30 min, the reaction was stopped by addition of Dole's reagent, and the released fatty acids were quantified as described above for the assay of PLA₂ activity.

Histology

For histologic evaluation, the pancreas was removed at 6 h after the induction of pancreatitis, fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin. Edema, inflammatory infiltration, fat necrosis, parenchymal necrosis, and hemorrhage were evaluated according to the scoring system of Nevalainen and Aho (21) as indicated in Table 1.

Also, gross observation of hemorrhage of the whole pancreas was performed just after removal of the pancreas and graded as follows (hemorrhage area was edematous and reddish): grade 0, no hemorrhage; grade 1, hemorrhage in less than 50% of area adjacent to the duodenum; grade 2, hemorrhage in more than 50% of area adjacent to the duodenum; grade 3, grade 2 plus hemorrhage in less than 50% of area adjacent to the spleen; grade 4, hemorrhage in whole pancreas including area adjacent to the retroperitoneal space.

Statistical analyses

Data are expressed as the mean or mean ± S.E.M. Data were statistically analyzed using Welch's *t*-test (biochemical parameters), Mann-Whitney's *U*-test (histological grade), or χ^2 -test (mortality).

Table 1. Criteria for the microscopic assessment of the severity of experimental acute pancreatitis

Histological patterns	Assessment	Score
Edema	mild	1
	moderate	2
	severe	3
Inflammatory infiltration	mild	1
	moderate	2
	severe	3
Fat necrosis	<2/section	3
	3-5/section	5
	>5/section	7
Parenchymal necrosis	focal (<5%)	3
	and/or sublobular (<20%)	5
	and/or lobular (>20%)	7
Hemorrhages	mild	3
	moderate	5
	severe	7

Results

Enhancement of mortality by porcine pancreatic sPLA₂-IB

Different amounts (30, 100, or 300 $\mu\text{g}/\text{animal}$) of porcine pancreatic sPLA₂-IB were infused into the common biliopancreatic duct together with a fixed amount of TCA (5 mg/animal). The survival rate decreased with an increasing amount of sPLA₂-IB, reaching 0% within 3 days when 300 μg sPLA₂-IB was combined with 5 mg TCA, while each alone resulted in a survival rate of more than 80% at 7 days (Fig. 1). Thus, a mixture of 300 μg sPLA₂-IB and 5 mg TCA in 0.2 ml was used to induce severe hemorrhagic pancreatitis thereafter.

Effect of S-5920/LY315920Na on mortality

The survival rate of the vehicle control group decreased rapidly with time after the induction and reached 0% within 48 h. The prophylactic treatment with S-5920/LY315920Na significantly improved the survival rate for 48 h with the all doses used, and this beneficial effect was sustained for 7 days with medium (1 mg/kg bolus and 0.15 mg/(kg·h) infusion) and high (3 mg/kg bolus and 0.5 mg/(kg·h) infusion) doses of the inhibitor with a survival rate of 50% and 42%, respectively (Fig. 2). Among the surviving animals, 10 out of 11 survived for at least 2 months after the pancreatitis. By contrast, therapeutic treatment with the inhibitor (high dose, at 1 min after or 1 h after the induction of the pancreatitis) tended to increase the survival rate for 24 h but failed to improve it at 7 days afterward (Fig. 3: A and B).

Fig. 1. Survival rate in phospholipase A₂ (PLA₂)-taurocholate (TCA)-induced acute pancreatitis in rats. Pancreatitis was induced by infusion of a mixture of various amounts of porcine pancreatic group IB PLA₂ and TCA (5 mg/rat) into the common bile duct in a volume of 0.2 ml/rat.

Changes of PLA₂ activity and other parameters after the pancreatitis

To obtain the optimal time-frame for assessing the effect of S-5920/LY315920Na on biochemical parameters, the time course changes of PLA₂ activity and other parameters in plasma and/or ascites were determined at 1, 3, 6, and 9 h after the induction of pancreatitis (Table 2). The plasma PLA₂ activity increased from 1 to 9 h after the induction of pancreatitis with a peak at 3 h, while the ascites PLA₂ activity peaked at 1 h and rapidly decreased thereafter. The changes of lipase activity in plasma and ascites were similar to those of PLA₂ activity though the ascites lipase activity remained relatively high compared to the rapid decline of the ascites PLA₂ activity. Plasma amylase gradually increased with a peak at 6 h, while both AST activity and creatinine level in plasma gradually increased from 1 to 9 h.

Table 3 shows the changes of ascites volume and the gross hemorrhage grade of the pancreatic tissue. The ascites volume increased with time with a peak at 6 h. In contrast, hemorrhage in the pancreatic tissue was already severe at 1 h and remained almost maximum until 9 h.

To elucidate the source of PLA₂ activity in plasma and ascites, the samples collected 1 and 6 h after the pancreatitis were incubated with a rabbit antibody to porcine sPLA₂-IB in vitro. It appeared that about 90% of the PLA₂ activity in the samples was absorbed by the above antibody (Table 4), clearly indicating that PLA₂ activity in the plasma and ascites were mainly due to

Fig. 2. Prophylactic effect of S-5920/LY315920Na on survival rate in phospholipase A₂ (PLA₂)-taurocholate (TCA)-induced acute pancreatitis in rats. Pancreatitis was induced by infusion of a mixture of porcine pancreatic group IB PLA₂ (300 $\mu\text{g}/\text{rat}$) and TCA (5 mg/rat) into the common bile duct in a volume of 0.2 ml/rat. S-5920/LY315920Na was injected as a bolus 5 min before the induction of pancreatitis and then infused for 24 h. * $P < 0.05$, ** $P < 0.01$ vs vehicle group (χ^2 -test).

Fig. 3. Therapeutic effect of S-5920/LY315920Na on survival rate in phospholipase A₂ (PLA₂)-taurocholate (TCA)-induced acute pancreatitis in rats. Pancreatitis was induced as described for Fig. 2. S-5920/LY315920Na was injected as a bolus 1 min after (A) or 1 h after (B) the induction of pancreatitis and then infused for 24 (A) or 23 h (B), respectively. * $P < 0.05$ vs vehicle group (χ^2 -test).

the porcine sPLA₂-IB that had leaked into the systemic circulation and to the peritoneal cavity after being injected with taurocholate to induce pancreatitis.

Effect of S-5920/LY315920Na on PLA₂ activity and other parameters

The effect of S-5920/LY315920Na on the parameters at 6 h after the pancreatitis was examined. The inhibitor was administered 3 mg/kg, i.v. as a bolus 5 min before the pancreatitis and then infused 0.5 mg/(kg·h), i.v. for 6 h. As shown in Fig. 4, the increase of PLA₂ activity in both plasma and ascites was almost completely inhibited by 96% and 89%, respectively, with S-5920/LY315920Na treatment. The concentrations of the inhibitor in these plasma and ascites samples were 4.78 ± 1.13 and 23.55 ± 3.92 μ M ($n = 7$), respectively. Also, the increase of lipase activity in plasma and

ascites was significantly reduced by more than 50% with the inhibitor. Increases of other plasma biochemical parameters such as amylase, ALT and AST were significantly suppressed by the inhibitor to the levels of those in the sham-operated group, while the increase of creatinine was not affected (Fig. 5). In addition, the inhibitor treatment led to a slight decrease (by 22%) of the ascites volume ($P = 0.052$) and to moderate and significant reduction (by 44%) of the hemorrhage in the pancreatic tissue on gross observation (Fig. 6). The latter finding was further confirmed by the significant decrease (by 59%) of the hemoglobin levels in ascites by the inhibitor.

Effect of S-5920/LY315920Na on histological changes

The typical features of severe acute pancreatitis noted at 6 h in the vehicle control group included parenchymal necrosis with hemorrhage, interstitial edema, inflammatory cell infiltration, and necrosis of the adipose tissue (Fig. 7: A and B). There was mild infiltration of polymorphonuclear leukocytes in and around the necrotic lobules (Fig. 7C).

Prophylactic treatment with S-5920/LY315920Na clearly abrogated parenchymal necrosis and to a lesser degree, fat necrosis (Fig. 7D). Figure 8 shows the quantitative histological analysis revealing that the inhibitor significantly lessened the severity of interstitial edema (vehicle vs S-5920/LY315920Na; 1.71 ± 0.18 vs 0.57 ± 0.20 , $P < 0.01$); parenchymal necrosis (5.57 ± 0.37 vs 2.57 ± 0.43 , $P < 0.01$); and fat necrosis (5.57 ± 0.72 vs 3.57 ± 0.37 , $P < 0.05$), but not that of inflammatory cell infiltration and hemorrhage.

Discussion

Acute pancreatitis is considered to develop by auto-digestion following leakage of already activated pancreatic enzymes into the interstitium. Several protease inhibitors have been developed to treat acute pancreatitis, but there was no low molecular inhibitor of lipolytic enzymes such as PLA₂ until the discovery of S-5920/LY315920Na (18). In the present study, the systemic administration of this novel sPLA₂ inhibitor showed apparent efficacy against the severe hemorrhagic necrotizing acute pancreatitis induced by the intraductal injection of porcine pancreatic sPLA₂-IB plus taurocholate.

Although a large amount of sPLA₂-IB was needed, the mortality increased in proportion to the amount of sPLA₂-IB with the fixed dose of taurocholate, in which neither agent alone caused high mortality, clearly indicating that when coexisting with bile acid, this enzyme is capable of inducing severe hemorrhage into the

Table 2. Changes of PLA₂ activity and other parameters in plasma and/or ascites after induction of PLA₂-taurocholate pancreatitis in rats

	Time (h)				
	0 (Normal)	1	3	6	9
PLA ₂ activity in plasma (nmol/(min · ml))					
Sham	0.5 ± 0.2	2.2 ± 1.2	0.5 ± 0.1	0.6 ± 0.1	0.2 ± 0.1
Pancreatitis		104.8 ± 1.8**	118.1 ± 18.1**	89.5 ± 2.0**	46.9 ± 6.3**
PLA ₂ activity in ascites (nmol/(min · ml))					
Sham	0.1 ± 0.0	1.6 ± 0.6	1.2 ± 0.8	0.8 ± 0.3	0.4 ± 0.0 ^{##}
Pancreatitis		7549 ± 168**	3174 ± 121**	1985 ± 385*	918 ± 99**
Lipase activity in plasma (nmol/(min · ml))					
Sham	0.4 ± 0.1	4.4 ± 3.0	1.6 ± 1.2	0.7 ± 0.3	0.7 ± 0.1 [#]
Pancreatitis		148.8 ± 42.4*	483.0 ± 150.1*	478.5 ± 75.1**	269.2 ± 56.7**
Lipase activity in ascites (nmol/(min · ml))					
Sham	0.3 ± 0.0	76.7 ± 11.0 ^{##}	53.2 ± 30.9	28.5 ± 14.5	2.0 ± 0.6
Pancreatitis		79513 ± 5518**	76697 ± 14189**	30575 ± 4981**	19286 ± 5602*
Amylase activity in plasma (IU/1 × 1000)					
Sham	5.9 ± 0.1	8.3 ± 1.3	8.9 ± 2.3	7.2 ± 0.9	4.1 ± 0.5 [#]
Pancreatitis		16.1 ± 2.5*	36.6 ± 7.2*	70.1 ± 15.5*	41.5 ± 10.8*
ALT activity in plasma (Karmen Unit)					
Sham	13.7 ± 0.4	73.0 ± 28.9	79.1 ± 45.8	100.2 ± 16.8 ^{##}	92.2 ± 35.7
Pancreatitis		9.6 ± 9.8	52.9 ± 7.7	194.0 ± 72.5	218.2 ± 71.8
AST activity in plasma (Karmen Unit)					
Sham	52 ± 2	359 ± 153	249 ± 141	306 ± 48 ^{##}	273 ± 67 [#]
Pancreatitis		104 ± 34	296 ± 48	1096 ± 238*	1111 ± 257*
Creatinine in plasma (mg/dl)					
Sham	0.96 ± 0.05	0.95 ± 0.04	0.96 ± 0.06	0.98 ± 0.07	1.05 ± 0.07
Pancreatitis		1.10 ± 0.09	1.47 ± 0.10**	1.76 ± 0.10**	1.77 ± 0.15**

Each value represents the mean ± S.E.M. of 4 or 5 animals. * $P < 0.05$, ** $P < 0.01$ vs Sham group; [#] $P < 0.05$, ^{##} $P < 0.01$ vs Time 0 (Normal) group (Welch's *t*-test).

Table 3. Changes of ascites volume and hemorrhage grade after induction of PLA₂-taurocholate pancreatitis in rats

	Time (h)				
	0 (Normal)	1	3	6	9
Ascites volume (ml)					
Sham	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
Pancreatitis		2.4 ± 0.3**	5.3 ± 0.5**	7.9 ± 0.4**	5.7 ± 0.4**
Hemorrhage grade [†]					
Sham	0.0 ± 0.0	0.3 ± 0.3	0.3 ± 0.3	0.4 ± 0.2	0.5 ± 0.3
Pancreatitis		3.0 ± 0.4*	3.8 ± 0.2*	4.0 ± 0.0*	3.8 ± 0.3*

Each value represents the mean ± S.E.M. of 4 or 5 animals. * $P < 0.05$, ** $P < 0.01$ vs Sham group (Welch's *t*-test for ascites volume, and Mann-Whitney's *U*-test for hemorrhage grade). [†]Grade 0 = no hemorrhage, 1 = hemorrhage in less than 50% of area adjacent to duodenum, 2 = hemorrhage in more than 50% of area adjacent to duodenum, 3 = grade 2 plus hemorrhage in less than 50% of area adjacent to spleen, 4 = hemorrhage in whole pancreas including area adjacent to retroperitoneal space.

ascites, parenchyma, and fat necrosis of the pancreas and that the blood biochemical changes are indicative of multiple organ failure such as of the kidney (creatinine)

and the liver (AST). Thus, although this model is a pharmacological rather than a physiopathological model of severe acute pancreatitis, it is useful for the evaluation

Table 4. Effect of anti-porcine pancreatic PLA₂-IB antibody on PLA₂ activity in plasma and ascites in vitro in PLA₂-taurocholate pancreatitis in rats

	Hours after pancreatitis	No. of samples	PLA ₂ activity (nmol/min/ml)		% inhibition
			Normal rabbit IgG	Anti-porcine sPLA ₂ -IB IgG	
Plasma					
	1 h	4	43.1 ± 4.2	4.8 ± 0.3	89
	6 h	4	45.2 ± 0.8	4.3 ± 1.4	90
Ascites					
	1 h	4	3231 ± 359	227 ± 29	93
	6 h	4	496 ± 38	53 ± 3	89

Each value represents the mean ± S.E.M.

Fig. 4. Effect of S-5920 /LY315920Na on PLA₂ (A, B) and lipase (C, D) activities in plasma and ascites at 6 h after the induction of pancreatitis. S-5920/LY315920Na (3 mg/kg, i.v.) was injected as a bolus 5 min before the induction of pancreatitis and then infused (0.5 mg/(kg · h), i.v.) for 6 h. Each bar represents the mean ± S.E.M. of 5 animals for the sham group and 7 animals for the vehicle and S-5920 groups. †*P*<0.01 vs sham group, ***P*<0.01 vs vehicle group (Welch's test).

of sPLA₂ inhibitors to see if they could impact on the various parameters of acute pancreatitis by systemic administration.

In the present study, catalytic PLA₂ activity in plasma changed differently from that in the ascites; the former peaked at 3 h and decreased gradually while the latter peaked at 1 h and decreased rapidly thereafter. Since the PLA₂ activity in ascites is 20- to 70-fold higher than that in plasma, and 90% of the PLA₂ activity in both plasma and ascites were absorbed by the antibody to porcine pancreatic sPLA₂-IB (Table 4), it is highly probable that the injected enzyme destroyed the paren-

chymal cells of the pancreas and leaked out into the peritoneal cavity as ascites and to the systemic circulation mainly by peritoneal absorption and partly by lymphatic drainage pathways (22–25). The changes of plasma lipase and amylase and that of ascites lipase were similar to those of the corresponding sPLA₂ activity, indicating that these enzymes leaked out from the pancreas and were distributed through the above-mentioned pathways. In contrast, the plasma AST and creatinine increased gradually with time, suggesting that failure of the liver and the kidney is caused by progressive systemic inflammatory responses due to

Fig. 5. Effect of S-5920/LY315920Na on plasma amylase (A), creatinine (B), ALT (C), and AST (D) levels at 6 h after the induction of pancreatitis. Each column represents the mean $\pm$ S.E.M. of 5 animals for the sham group and 7 animals for the vehicle and S-5920 groups. [†] $P < 0.01$ vs sham group, ^{**} $P < 0.01$ vs vehicle group (Welch's test).

Fig. 6. Effect of S-5920/LY315920Na on ascites volume (A), gross hemorrhage in the pancreas (B), and hemoglobin content in ascites (C) at 6 h after the induction of pancreatitis. Each column represents the mean $\pm$ S.E.M. of 5 animals for the sham group and 7 animals for the vehicle and S-5920 groups. [†] $P < 0.01$ vs sham group, ^{**} $P < 0.01$ vs vehicle group (Welch's test).

severe acute pancreatitis.

The prophylactic (5 min before) treatment with S-5920/LY315920Na significantly improved the survival rate for more than 7 days, while the therapeutic (1 min or 1 h after) treatment only tended to improve the rate for 24 h. These findings clearly indicate that major destruction of the pancreatic tissue was induced just after the intraductal injection of pancreatic sPLA₂-IB plus taurocholate as evidenced by the finding that the gross hemorrhage in the pancreas had already reached maximum 1 h after the induction of pancreatitis

(Table 3). Similarly, very early (5 min after) biochemical and morphological changes were reported for taurocholate-induced pancreatitis in rats (22). Therefore, long lasting improvement of the mortality rate by prophylactic S-5920/LY315920Na could be ascribed mainly to the prevention of hemorrhage and necrosis of the pancreatic tissue induced by sPLA₂-IB plus taurocholate, which consequently led to decrease of the systemic inflammatory reaction and multiple organ failure. Although S-5920/LY315920 almost completely abolished PLA₂ activity in the plasma and ascites, prophy-

Fig. 7. Representative photomicrographs showing hematoxylin and eosin staining of the pancreatic tissue at 6 h after the induction of pancreatitis in sham group (A), vehicle group (B, C), and S-5920 group (D). Bar represents 200 μm in A, B, D, and 50 μm in C. There was mild infiltration of polymorphonuclear leukocytes (arrows) in and around the necrotic lobules in vehicle group (C). Note that parenchymal necrosis was markedly reduced in the S-5920 group (D) compared to the vehicle group (B).

Fig. 8. Quantitative histological analysis of the effect of S-5920/LY315920Na on the pancreas stained with hematoxylin and eosin. † $P<0.01$ vs sham group; * $P<0.05$, ** $P<0.01$ vs vehicle group (Mann-Whitney's *U*-test).

lactic treatment with this compound did not completely reverse survival rate in this pancreatitis model. We also examined the efficacy of the potent proteinase inhibitor nafamostat methyleate (Futhane) in this model, but the

inhibitor did not show any prophylactic effect on survival rate (Y. Hori, unpublished data). These findings clearly indicate that exogenous sPLA₂-IB rather than endogenous sPLA₂s is the key enzyme involved in the

pathogenesis in the present model. Thus one possible explanation is that local concentration of sPLA₂-IB injected to the pancreatic duct could be so high that S-5920/LY315920 might not be able to completely inhibit its activity just after the injection of the enzyme plus taurocholate, which subsequently resulted in moderate prevention of the tissue destruction and the survival rate.

Previously S-5920/LY315920 showed prophylactic but not therapeutic efficacy in pancreatitis induced by trypsin-taurocholate in rats (14). In this model, the activity of sPLA₂-IB would also be high in the pancreas because the pro-sPLA₂-IB should be activated by digestion with trypsin. Though the type of PLA₂ was not determined in trypsin-taurocholate pancreatitis model, the PLA₂ activity in plasma seems to be due to type II sPLA₂ as we have already reported on deoxycholate-induced pancreatitis in rats (19). However, the peak plasma PLA₂ activity of 118.1 nmol/(min·ml) at 3 h in the current PLA₂-taurocholate model was more than 6-fold higher than that in the trypsin-taurocholate model, that is, 18.2 nmol/(min·ml) at 3 h (14), suggesting that more than 80% of PLA₂ activity in plasma should be due to exogenous pancreatic sPLA₂-IB that leaked from the pancreatic tissue into the systemic circulation after being injected to the biliopancreatic duct. Thus, the changes of endogenous sPLA₂s could be similar in the two models, but the level of exogenous sPLA₂-IB in the present sPLA₂-taurocholate model was so high that it overwhelmed the changes of endogenous sPLA₂s. In contrast, endogenous sPLA₂s such as pancreatic sPLA₂-IB in the pancreas and non-pancreatic sPLA₂-IIA in plasma play roles in the trypsin-taurocholate model. The lack of therapeutic efficacy of S-5920/LY315920 in the above two models strongly suggest that once the pancreatic tissue suffers severe destruction, therapeutic drugs hardly reach the afflicted tissues and consequently fail to improve survival rate. It is well known that severe acute pancreatitis in animals is a disease of very high mortality regardless of the model or species used and that animals are highly likely to have long-term survival when they survive the first 48–72 h, as shown in the present study (26).

S-5920/LY315920Na significantly prevented increases of the levels of plasma and ascites lipase and the levels of amylase, ALT and AST in plasma at 6 h after the pancreatitis. Since the inhibitor has no direct inhibitory action against lipase (Y. Hori, unpublished data), improvement of these biochemical parameters should be due to its preventive effect on the destruction of the pancreas, as evidenced by the significant decrease of the severe hemorrhage into the ascites, and the parenchymal and fat necrosis of the pancreas caused by

the inhibitor.

Recent studies on human and experimental severe acute pancreatitis suggest that sPLA₂-IIA rather than sPLA₂-IB plays a central role in the development of local and systemic complications (7, 8, 14, 15, 19, 27). In human patients with necrotizing pancreatitis, however, serum catalytic PLA₂ activity in one or two days after the onset is ascribable to pancreatic sPLA₂-IB and corresponds well with the lung insufficiency observed in this early stage, which is thereafter replaced by non-pancreatic sPLA₂-IIA (7, 8). Therefore, the present model seems to partly mimic the early phase (the first few days) of human necrotizing pancreatitis in terms of changes of catalytic sPLA₂ activity in the systemic circulation. Furthermore, we previously reported that although sPLA₂-IIA was a major sPLA₂ in plasma, more than 50% of catalytic PLA₂ activity in ascites was due to pancreatic sPLA₂-IB which might contribute to peritoneal permeability increase in rat severe hemorrhagic pancreatitis induced by 10% sodium deoxycholate (19). Thus, although S-5920/LY315920Na is a strong inhibitor of sPLA₂-IIA, the present study clearly demonstrates that this agent is capable of inhibiting sPLA₂-IB in plasma and ascites when a sufficient amount is systemically administered, suggesting that this agent could be of clinical value for inhibiting catalytic PLA₂ activity arising from sPLA₂-IIA or sPLA₂-IB in patients with necrotizing acute pancreatitis. In these patients, increased serum catalytic sPLA₂ activity has been implicated in the pathogenesis of remote organ failure such as pulmonary insufficiency (7–9), and both types of sPLA₂ have been suggested to be involved in clinical acute lung injury such as adult respiratory distress syndrome (28, 29), as supported by the effective degradation of lung surfactant phospholipids by both types of sPLA₂ (30, 31). In this connection, since S-5920/LY315920Na showed a beneficial effect on the experimental acute lung injury induced by oleic acid (12) or intestinal ischemia-reperfusion (13), this inhibitor could be useful for preventing not only further destruction of the pancreas but also acute lung injury complicated by necrotizing pancreatitis.

In conclusion, the novel sPLA₂ inhibitor strongly reduced catalytic PLA₂ activity in both plasma and ascites and showed prophylactic effects on the high mortality, severe pancreas tissue damage, and blood biochemical changes in a lipolytic enzyme-related severe pancreatitis model. This sPLA₂ inhibitor could become an effective agent for the treatment of life-threatening severe acute pancreatitis.

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