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A Direct Role for Secretory Phospholipase A_2 and Lyso-Phosphatidylcholine in the Mediation of Lipopolysaccharide-induced Gastric Injury

Elizabeth J. Dial, **Duy M. Tran**, **Jimmy J. Romero**, **Mayssa Zayat**, and **Lenard M. Lichtenberger**
Department of Integrative Biology and Pharmacology, The University of Texas Medical School at Houston

Abstract

Background & Aims—Endotoxemia from sepsis can injure the GI tract through mechanisms that have not been fully elucidated. We have shown that lipopolysaccharide (LPS) induces an increase in gastric permeability in parallel with the luminal appearance of secretory phospholipase A_2 ($sPLA_2$) and its product, lyso-phosphatidylcholine (lyso-PC). We proposed that $sPLA_2$ acted on the gastric hydrophobic barrier, composed primarily of PC, to degrade it and produce lyso-PC, an agent that is damaging to the mucosa. In the present study we have tested whether lyso-PC and/or $sPLA_2$ have direct damaging effects on the hydrophobic barriers of synthetic and mucosal surfaces.

Methods—Rats were administered LPS (5 mg/kg, ip), and gastric contents were collected 5 h later for analysis of $sPLA_2$ and lyso-PC content. Using these measured concentrations, direct effects of $sPLA_2$ and lyso-PC were determined on: 1) surface hydrophobicity as detected with an artificial PC surface and with intact gastric mucosa (contact angle analysis); and 2) cell membrane disruption of gastric epithelial cells (AGS).

Results—Both lyso-PC and $sPLA_2$ increased significantly in the collected gastric juice of LPS-treated rats. Using similar concentrations to the levels in gastric juice, the contact angle of PC-coated slides declined after incubation with either pancreatic $sPLA_2$ or lyso-PC. Similarly, gastric contact angles seen in control rats were significantly decreased in $sPLA_2$ and lyso-PC treated rats. Additionally, we observed dose-dependent injurious effects of both lyso-PC and $sPLA_2$ in gastric AGS cells.

Conclusions—An LPS-induced increase in $sPLA_2$ activity in the gastric lumen, and its product lyso-PC, are capable of directly disrupting the gastric hydrophobic layer and may contribute to gastric barrier disruption and subsequent inflammation.

Keywords

phosphatidylcholine; endotoxin; hydrophobicity; gastric mucosa; epithelium

INTRODUCTION

Acute endotoxemia is associated with shock and injury to the gastrointestinal (GI) tract. The occurrence of ileus due to endotoxic shock is accompanied by production of inflammatory mediators such as TNFα and other cytokines or chemokines that are thought to be responsible for much of the injury (1–3). However, other mechanisms could also be a part of the process.

Correspondence: Elizabeth Dial, Ph.D. Dept. of Integrative Biology and Pharmacology University of Texas Medical School 6431 Fannin Houston, TX 77030 Phone: 713-500-6318 Fax: 713-500-7444 Elizabeth.J.Dial@uth.tmc.edu.

The use of bacterial lipopolysaccharide (LPS) in rodents as a model of endotoxemia has allowed a closer examination of other factors that may be involved in this shock-induced GI injury and dysfunction.

LPS has been shown to inhibit gastric acid secretion and also produce a gastroparesis, allowing the stomach to fill with an alkaline fluid (4–5). In addition, our group has reported that LPS induces a duodenogastric reflux of intestinal contents, including bile acid, into the gastric lumen, which may also contribute to gastric alkalinization (6). We have further shown that LPS induces an increase in gastric permeability to a fluorescent probe of substantial size (4000 molecular weight), without overt disruption to the mucosal architecture (7). Concurrently, there is an increase in the gastric luminal appearance of secretory phospholipase A_2 ($sPLA_2$), as well as the product of this enzyme, lyso-phosphatidylcholine (lyso-PC). The source of this gastric luminal $sPLA_2$ is unclear, but at least part of it is likely to have derived from intestinal reflux material of pancreatic origins. Regardless of its source, $sPLA_2$ within the gastric lumen, at an appropriate pH, may be capable of degrading its primary substrate, phosphatidylcholine (PC).

Our group and several others have shown that a portion of the gastric barrier to acid is due to the presence of a hydrophobic layer of phospholipid, mainly PC, which overlies the mucus gel layer on the mucosa (8–10). Disruption of the hydrophobic layer is associated with injury and disease states such as ulcer disease (11–12), colitis (13–14) and NSAID toxicity (15–16). LPS was shown to reduce surface hydrophobicity in a time and dose-dependent manner (6). We have proposed that LPS acts indirectly on this barrier by increasing bile acid content and $sPLA_2$ activity in the gastric lumen, and these agents are capable of degrading the PC layer. In the current paper, we have focused on $sPLA_2$ activity and how it may act to disrupt the hydrophobic barrier.

METHODS

Animals

Male Sprague-Dawley rats (175–250 g) were obtained from Harlan Sprague-Dawley (Indianapolis, IN). All animals were fasted overnight before use to ensure an empty stomach. Institutional Animal Welfare Committee approval was obtained prior to all studies.

LPS effect on gastric contents

Saline (control) or LPS (5 mg/kg, ip) were administered to rats and gastric contents were collected five hours later after euthanasia. The determination of $sPLA_2$ activity, as well as lipid extraction and thin layer chromatography to separate lyso-PC was performed on the gastric fluid as previously described (7).

PC coated slides

Well-cleaned glass slides were coated with a monolayer of PC (dipalmitoyl phosphatidylcholine) using a Langmuir trough and a modification of the Blodgett and Gaines techniques as previously described (17). The slides were then incubated in normal saline or saline containing various test agents, including pancreatic $sPLA_2$ (10 units) or lyso-PC (0.1, 0.5, 1, or 5 mM) at 37 °C for two hours with gentle stirring. The slides were rinsed, air-dried and contact angle analysis (17) was performed with a Rame-Hart goniometer (Hicksville, NY).

Effect of $sPLA_2$ and lyso-PC on gastric hydrophobicity

Rats were anesthetized with isoflurane and underwent a pylorus ligation. The animals were then dosed by oral gavage with 1 ml of either vehicle, $sPLA_2$ (10 units), or lyso-PC (2 mM). The agents were dissolved in 100 mM HEPES buffer at pH 7.4 to maintain gastric pH above

6.5 for the duration of the experiment. After 60 minutes, the rats were euthanized and gastric tissue was collected for contact angle analysis as previously described (17).

Cell culture

AGS cells are human gastric epithelial cells originally derived from an adenocarcinoma (18). They were maintained in Ham's F12K media plus 10% fetal bovine serum and penicillin/streptomycin. Cells were plated into 24-well culture plates at 2×10^5 cells per well, and were exposed to test agents lyso-PC (0.01 to 0.2 mM for 3h) or bovine pancreatic PLA_2 (0.1 to 10 U/ml for 24h), after which the media was collected for lactate dehydrogenase assay (LDH) as a measure of cell membrane injury and the cell number was analyzed by the thiazolyl blue tetrazolium bromide (MTT) assay as previously described (19). For comparison to other forms of phospholipase, $sPLA_2$ from bee venom and snake venom (*Naja naja*), and phospholipase C (PLC) from *Clostridium perfringens* were also tested. For comparison to another epithelial cell type, selected phospholipase studies were performed on IEC-6 cells which are derived from normal rat intestinal crypt epithelial cells (20). IEC-6 cells were maintained in Dulbecco's modified Eagle medium plus 10% fetal bovine serum, penicillin/streptomycin, and insulin (0.1 units/ml).

Materials

LPS (E. coli 0111:B4), all phospholipases, all lipids and a kit (TOX-7) for LDH assay were obtained from Sigma Chemical Co. (St. Louis, MO). The PC used in the monolayer slide studies was dipalmitoyl phosphatidylcholine. AGS cells were obtained from the Texas Medical Center Digestive Diseases Center (Houston, TX). IEC-6 cells were obtained from The University of Texas Trauma Research Center (Houston, TX).

Statistics

Differences among treatment groups were analyzed by analysis of variance and the Fisher LSD test. The Student t-test was used to compare differences between two groups. The level for significance was set at $p<0.05$.

RESULTS

Lyso-PC and $sPLA_2$ after LPS

The gastric contents of control rats contained small, but detectable amounts of lyso-PC measured at 0.08 ± 0.02 mM. At five hours after the systemic administration of LPS at a dose of 5 mg/kg, that value increased significantly at least 60-fold to 1.29 ± 0.17 mM ($p<0.001$). Similarly, the gastric contents of control animals had a total of 0.6 ± 0.06 units of $sPLA_2$ activity that increased ~5 fold to 3.2 ± 0.56 units in LPS-treated rats ($p<0.05$). A unit of $sPLA_2$ activity is the amount that will hydrolyze 1.0 μmole of PC to lyso-PC producing a free fatty acid per minute at pH 9 and 37°C.

PC coated slides

The mean contact angle for PC-coated slides incubated for 2 h in saline was 58° (n=9). PC coated slides incubated in either 10 units/ml of pancreatic $sPLA_2$ or 0.1 to 5 mM lyso-PC showed significant reductions in contact angles, as shown in Figure 1.

Effect of $sPLA_2$ and lyso-PC on rat gastric hydrophobicity

Separate *in vivo* studies were performed with each test agent as seen in Figure 2A and B. In the study testing $sPLA_2$, the treated rats showed a significant 7% reduction in gastric hydrophobicity over control animals ($p<0.05$). In the study testing lyso-PC, treated rats had a significant 18% reduction in gastric hydrophobicity over control values ($p<0.02$).

Effect of lyso-PC and $sPLA_2$ on cultured cells

Gastric cells incubated for 3 h with lyso-PC showed a dose-dependent increase in LDH release, indicative of membrane injury (Figure 3). At the same time, the number of viable cells, as detected by the MTT assay, was correspondingly decreased. The ED50 for both measures was estimated to be about 30 to 40 μM.

When AGS cells were initially incubated with pancreatic $sPLA_2$ for 3 h, there were no observable changes in cell number, so the incubation time was lengthened to 24 h. At 24 h (Figure 4), it was found that LDH was not increased by $sPLA_2$ treatment, while MTT indicated a slight, but significant, dose-dependent $sPLA_2$-induced reduction in cell numbers. It should be noted that all cell assays of LDH activity were accompanied by a positive control (cells treated with deoxycholate for complete cell lysis) to verify that the assay was working properly.

To determine the specificity of the $sPLA_2$ effect, two other types of $sPLA_2$ were tested on AGS cells. Neither bee venom nor snake venom $sPLA_2$ (Table 1) had an effect on LDH or MTT of AGS cells after a 24 h incubation. In contrast, phospholipase C (PLC) was clearly injurious to the cells at ≥0.1 U/ml. Another epithelial cell type, IEC-6 cells, showed a similar lack of sensitivity to the $sPLA_2$ enzymes (Table 1), and were slightly responsive to pancreatic $sPLA_2$ (10 U/ml = 92 ± 1% of control for MTT (not significant) and 150 ± 4% of control for LDH ($p<0.05$ vs control)). IEC-6 cells were equally injured by PLC as the AGS cells.

DISCUSSION

Using a combination of *in vitro* and *in vivo* techniques, we have shown in the current studies that LPS induces the appearance of $sPLA_2$ and lyso-PC in the gastric lumen, and these factors are capable of directly disrupting the gastric hydrophobic barrier.

The dose of LPS is one that we have used previously (6). Internally, the 5 mg/kg dose of LPS induces gastroparesis and accumulation of alkaline fluid within the stomach. At five hours after dosing with LPS, the accumulated load of lyso-PC within the gastric lumen was clearly elevated by at least 60-fold and measured about 1.3 mM. Similarly, we measured the accumulated level of $sPLA_2$ activity and found an average total of about 3.2 units per stomach in LPS-treated rats, which was 5-fold higher than observed in control stomachs.

Concentrations of lyso-PC and $sPLA_2$ similar to those obtained experimentally in rats were then tested in an *in vitro* monolayer system. Slides that had been coated with PC to mimic the stomach's hydrophobic barrier were exposed to test agents for only two hours, in comparison to the five hours of the *in vivo* study. This time was a conservative estimate since in the *in vivo* study, the rat stomach was exposed to an increasing amount of agent over a five hour period. We have previously shown that both $sPLA_2$ and lyso-PC are beginning to be elevated intraluminally in the stomach by 3 h after a dose of LPS, and this amount is further increased by 5 h (7). Both $sPLA_2$ and lyso-PC reduced the slide contact angle in confirmation of their ability to degrade or solubilize PC, respectively.

To verify that $sPLA_2$ and lyso-PC were capable of causing a loss of gastric barrier hydrophobicity in an animal, these agents were incubated in a closed stomach preparation at levels used *in vitro* and measured *in vivo* after LPS. In fact, these *in vivo* data on lyso-PC agree with numerous reports on the toxicity of lyso-PC in the stomach (21–24) and the concentrations which induce injury (1–10 mM). In addition, we were careful to maintain the intragastric pH with buffer at no lower than pH 6.8 in these studies, as that is similar to the gastric pH measured following LPS administration (38), and it is also needed for enzymatic activity of $sPLA_2$, which is most active in an alkaline range (pH 8–9). At pH 6–7, $sPLA_2$ will still be active. Both agents caused a significant reduction in surface hydrophobicity, confirming their *in vivo* activity. The

extent of contact angle reduction in these studies was relatively small (7% and 18%), compared to other animal studies where reported reductions due to disease (25) or chemical insults (26–28) could be 50% or more. However, the present studies were performed in pylorus-ligated rats where a single agent at a physiologically relevant concentration was exposed to the gastric surface. In our previous studies involving contact angle measurements, the studies were performed in conscious, intact animals and were the net result of multiple factors such as acid, bile/enzyme reflux, motility dysfunction, or high dose of damaging agent, as opposed to the single agent tested here. It also should be noted that our relative reductions in contact angle in the current study are comparable to changes seen in clinical studies involving gastritis or infection with the pathogen *Helicobacter pylori* where reductions of 8 to 19% were consistently reported (29–30). We have also shown previously in isolated canine gastric mucosa that there is a strong relationship between extent of reduction in gastric contact angle and reduction in electrical potential difference which is inversely related to gastric injury (26). Thus it is reasonable to conclude from our data that these individual agents ($sPLA_2$ and lyso-PC) have the ability to directly affect a meaningful reduction in contact angle.

Finally, cultured gastric cells were exposed to concentrations of lyso-PC and $sPLA_2$ at or below those that had been used in the studies above. The effect of lyso-PC on AGS cells was to induce cell membrane damage and release the cytosolic enzyme, LDH. Results with the MTT assay were consistent with that interpretation of loss of cell number due to injury. It was further noted that the gastric cells were quite sensitive to lyso-PC, with toxicity found at 10 to100-fold lower concentrations than were observed to be injurious in intact animals or the other *in vitro* system. Clearly, lyso-PC is particularly damaging to gastric epithelial cells, and the levels of lyso-PC that were detected *in vivo* after LPS treatment are capable of directly disrupting both the extracellular hydrophobic layer and mucosal epithelial cells.

In contrast to lyso-PC, the inability of pancreatic $sPLA_2$ to induce cell membrane damage, as seen by no LDH release, was unexpected. One explanation for the lack of an injurious effect may be related to the absence or more limited nature of an extracellular hydrophobic PC layer on these cultured cells, and the related inability of extracellular $sPLA_2$ to act on the extracellular PC to produce lyso-PC. In that regard, we attempted to add extracellular PC to cells incubated with $sPLA_2$ in order to promote lyso-PC formation, but no injury was detected (not shown). It is likely that any PC added to this cell system will be protective, as we have shown for other cell cultures (31). Yet we would have expected that $sPLA_2$ would attack membrane PC and it apparently did not. There was a small effect of $sPLA_2$ to reduce numbers of AGS cells as seen by a decreased MTT assay, an effect that was apparently independent of cell membrane injury. To investigate whether the pancreatic $sPLA_2$ effect was peculiar to AGS cells, we also tested another epithelial cell line, that of IEC-6 cells. This culture, too, was unresponsive by LDH and also MTT assay, to the $sPLA_2$, supporting the possibility of a general effect. Although we cannot locate any references that report on effects of pancreatic $sPLA_2$ on AGS or other human gastric cells, there is one report of damaging actions of $sPLA_2$ on primary isolated rat gastric cells (32) in which bee venom PLA_2 was used. This latter $sPLA_2$ has similar substrate preferences to pancreatic $sPLA_2$, but is associated with a different gene (Pla2 versus PLA2G1B) and has a different pH optimum. Those investigators found injury after short-term incubation of bee venom PLA_2 with rat cells and also found a similar injurious sensitivity to lyso-PC as us (10–100 μM). Whether a difference in cell isolation or species of origin may explain these differences in sensitivity to $sPLA_2$ is not known. Another explanation for our lack of effect with $sPLA_2$ is that, in contrast to other types of $sPLA_2$, the pancreatic form (1B) which we used is reported to also act through specific receptors (33–37), although its physiological role is unclear. Thus, there may be non-enzymatic actions of pancreatic $sPLA_2$ on the gastric mucosa that explain our results.

To investigate the specificity of the pancreatic $sPLA_2$ effect, we tested $sPLA_2$ from bee venom and snake venom. Neither of these latter $sPLA_2$s were active against AGS or IEC-6 cells by the LDH or MTT assays. This would support the uniqueness of the limited pancreatic $sPLA_2$ effect on gastric cells. In addition, PLC clearly had a toxic effect on the AGS and IEC-6 cells, as would be expected from this membrane surface-acting enzyme. Further studies will be necessary to fully elucidate the effects of pancreatic $sPLA_2$ on AGS cells.

Regardless of the direct effects of $sPLA_2$ on gastric cells, our other results are fully consistent with the ability of $sPLA_2$ to degrade the extracellular protective PC surface layer, and along with lyso-PC, to disrupt the PC layer, as well as cell membranes. Thus, circumstances under which $sPLA_2$ appears in the gastric lumen, such as during endotoxic shock produced here or other duodenogastric reflux-inducing situations, can lead to disruption of the protective PC barrier and exposure of the underlying epithelium to potentially injurious luminal contents such as lyso-PC. Methods to counter the loss of surface PC, such as addition of exogenous PC which we have previously shown to be effective at preventing an increase in LPS-induced gastric permeability (38), offer a means to prevent local, as well as systemic inflammatory conditions. We hypothesize that the additional PC may form non-injurious liposomes with lyso-PC, lessening the free lyso-PC concentration.

Our preclinical findings reported here have potential clinical relevance. We have recently reported that the gastric fluid of patients following a traumatic shock is alkaline and contains intestinal reflux material such as bile acid (39). Other critically ill patients have also been reported to acquire bile and reflux material in their stomachs (40–41). Thus, among critically ill patients, there are circumstances where bile acids and $sPLA_2$ from the intestines may be present in the stomach and could contribute to gastric barrier disruption by PC degradation. Based on our findings in rats reported here, we speculate that some of the altered GI permeability in critically ill patients may be due to exposure of the gastric lumen to $sPLA_2$ and appearance of the potent damaging agent lyso-PC. Future clinical studies are warranted to address this possibility.

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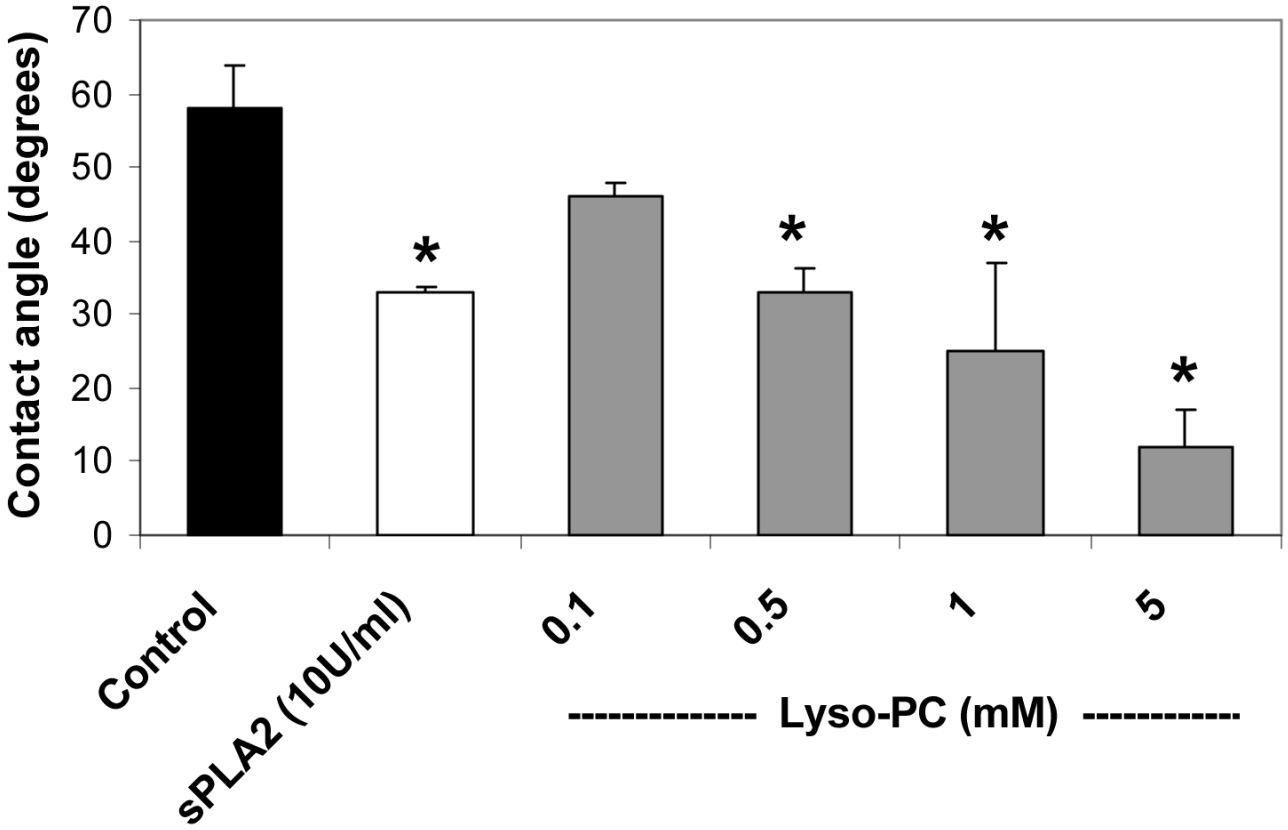

Figure 1.
Effect of $sPLA_2$ and lyso-PC *in vitro*. PC coated slides were incubated for 2 h with $sPLA_2$ (10 U/ml) or lyso-PC (0.1 to 5 mM), followed by contact angle readings. Values are expressed as the mean contact angle in degrees ± standard deviation for N = 4/group. * $p<0.05$ versus Control.

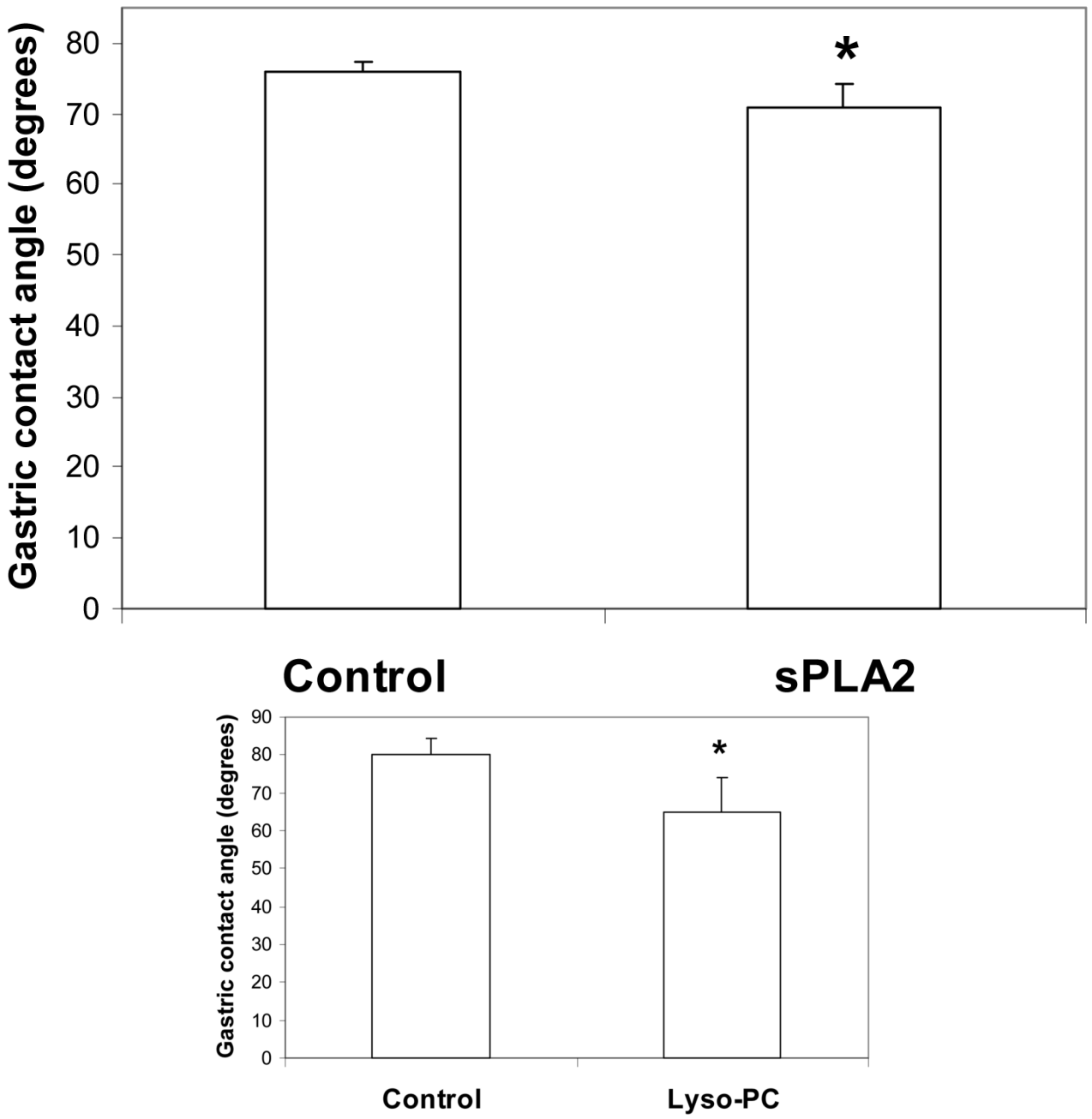

Figure 2.

Effect of $sPLA_2$ and lyso-PC *in vivo*. Rat stomachs were exposed to: A) pancreatic $sPLA_2$ or B) lyso-PC, for 1 h and the gastric contact angles were then measured. Values are expressed as the mean contact angle in degrees ± standard deviation for N = 4–6/group. * $p<0.05$ versus Control.

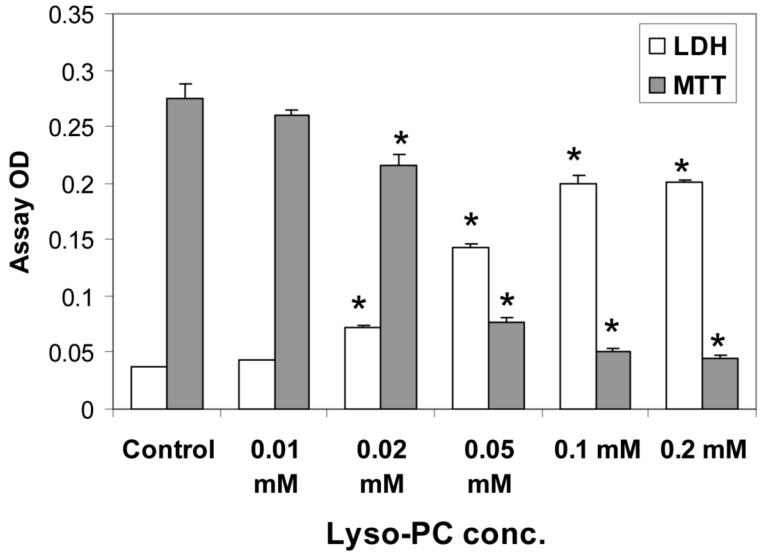

Figure 3.

Effect of lyso-PC on gastric AGS cells. Cells were incubated for 3 h with varying concentrations of lyso-PC. LDH was measured in the media and MTT was assayed in the cells. Values are expressed as the mean optical density (OD) of the assay ± standard error of the mean. The study was performed three times. * $p<0.05$ versus Control.

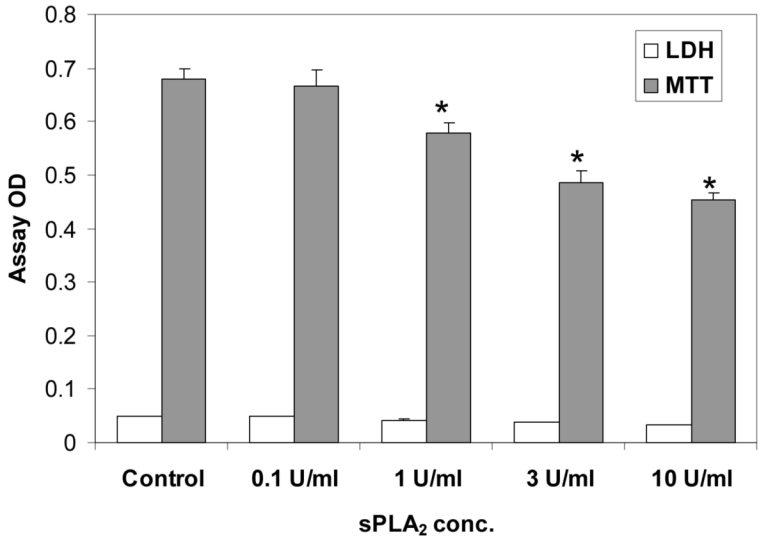

Figure 4.

Effect of $sPLA_2$ on gastric AGS cells. Cells were incubated for 24 h with varying concentrations of pancreatic $sPLA_2$. LDH was measured in the media and MTT was assayed in the cells. Values are expressed as the mean OD of the assay ± standard error of the mean. The study was performed three times. * $p<0.05$ versus Control.

Table 1

Phospholipase-induced injury to gastrointestinal cells.

	AGS cells		IEC-6 cells	
Phospholipase	**LDH**	**MTT**	**LDH**	**MTT**
Bee venom PLA_2				
0.1 U/ml	100 ± 3	106 ± 2	104 ± 3	111 ± 2
1 U/ml	107 ± 3	113 ± 2	97 ± 8	115 ± 3
10 U/ml	100 ± 1	106 ± 2	100 ± 2	111 ± 1
Snake venom PLA_2				
0.1 U/ml	98 ± 1	97 ± 3	100 ± 3	109 ± 5
1 U/ml	102 ± 2	101 ± 2	95 ± 10	121 ± 7
10 U/ml	102 ± 5	111 ± 2	140 ± 26	94 ± 7
PLC				
0.1 U/ml	149 ± 5*	79 ± 3*	292 ± 20*	69 ± 1
1 U/ml	423 ± 5*	12 ± 0.1*	464 ± 2*	17 ± 1*
10 U/ml	446 ± 44*	12 ± 0.1*	473 ± 7*	15 ± 0.1*

AGS or IEC-6 cells were incubated for 24 h with 0.1, 1 or 10 U/ml of the indicated phospholipases. LDH and MTT were measured as described in the Methods. Values are expressed as the percent of control ± standard error of the mean for four separate studies.

* = $p<0.05$ versus Control