Role of gut flora on intestinal group II phospholipase A2 activity and intestinal injury in shock

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Rozenfeld, Ranna A., Xueli Liu, Isabelle DePlaen, and Wei Hsueh. Role of gut flora on intestinal group II phospholipase A2 activity and intestinal injury in shock. Am J Physiol Gastrointest Liver Physiol 281: G957–G963, 2001.—We previously showed that group II phospholipase A2 (PLA2-II), a secretory, bactericidal, and proinflammatory protein in intestinal crypts, is upregulated after lipopolysaccharide (LPS) and platelet-activating factor (PAF) challenge. Here we examined whether germ-free environment (GF) or antibiotic treatment (ABX) affects the pathophysiological responses and intestinal PLA2-II activity after PAF (1.5 mg/kg) or LPS (8 mg/kg) injection. We found that LPS and PAF induced hypotension and mild intestinal injury in conventionally fed (CN) rats; these changes were milder in ABX rats, whereas GF rats showed no intestinal injury. PLA2-II enzyme activity was detected in normal rat small intestine; the basal level was not diminished in ABX or GF rats. PAF and LPS caused an increase in PLA2-II activity, which was abrogated in GF and ABX rats. Recolonization of GF rats by enteral contamination reconstituted their PLA2-II response to PAF and LPS and susceptibility to bowel injury. We conclude that PAF- and LPS-induced increases in PLA2-II activity are dependent on gut bacteria, and ABX and GF rats are less susceptible to LPS-induced injury than CN rats.

Lipopolysaccharide (LPS) induces PLA2-II mRNA expression in rat aorta, spleen, lungs, thymus (32), liver (12), and kidneys (18) and activates enzyme activity in the lungs (3, 26). Proinflammatory cytokines such as interleukin-1 and tumor necrosis factor up-regulate the mRNA synthesis and secretion of PLA2-II in vitro (in mesangial cells; Refs. 42, 48). Under intestinal inflammatory conditions, such as ulcerative colitis and Crohn’s disease (15, 16, 29), PLA2-II expression is increased. PLA2-II has been recently subdivided into groups IIA, IIB, and IIC (10, 31). Studies (25, 41) have shown that the PLA2-IIA gene is constitutively expressed in rat intestine and markedly elevated 24 h after LPS injection.

Platelet-activating factor (PAF) is an endogenous mediator of intestinal injury in endotoxin shock (20, 43, 49). Direct injection of PAF into animals induces systemic pathophysiological responses, including shock, capillary leak, thrombocytopenia, neutropenia, pulmonary hypertension, bronchoconstriction (4, 5, 17), and intestinal injury (49). Previous studies (45) showed that PAF also induces gene transcription and enzyme activation of PLA2-II in the small intestine. Much of the in vivo effect of LPS, including shock and bowel injury (20, 49), could be largely abolished by PAF antagonists, indicating that PAF is the endogenous mediator of LPS, and the two agents may share a common final pathway. However, a previous study (45) suggests that different pathways may exist for PAF and LPS in the upregulation of intestinal PLA2-II, because the LPS effect is not blocked by the administration of a PAF antagonist. PAF-induced intestinal injury has been shown (43) to be largely dependent on the presence of intestinal flora (or their products), since germ-free environment (GF) and antibiotic-treated (ABX) rats are protected from PAF-induced injury. It is unclear whether PAF- or LPS-induced intestinal PLA2-II activation depends on intestinal flora and/or their products. The purpose of this study is to examine the effect of GF or ABX on 1) basal PLA2-II activity in the intestine, 2) intestinal PLA2-II activity after PAF or LPS challenge, and 3) the pathophysiological responses to PAF or LPS challenge.

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MATERIALS AND METHODS

The stock solution of PAF (2 mg/ml 1-O-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, Sigma Chemical, St. Louis, MO) in saline albumin (5 mg/ml) was stored frozen in aliquots. The working solution was made fresh daily. LPS (Salmonella typhosa) was purchased from Sigma Chemical. Male GF Sprague-Dawley rats (80–120 g) were purchased from Taconic (Germantown, NY); male normal Sprague-Dawley rats (80–120 g) were purchased from Harlan Sprague Dawley (Indianapolis, IN). GF rats were kept in a sterile institutional animal facility for no more than 2 days before use. Neomycin, polymyxin B and metronidazole were obtained from the local pharmacy. The working solution was made fresh daily. LPS (Salmonella typhosa) was stored frozen in aliquots.

Animal experiments. Animals were anesthetized with Nembutal (65 mg/kg ip, Abbott Laboratories, North Chicago, IL) and placed under warming lights. After tracheotomy, the carotid artery and jugular vein were catheterized for continuous blood pressure recording, blood sampling, and drug injection. The first part of the experiment consisted of two groups of animals: conventionally fed (CN) and ABX rats. Each group was divided into three subgroups: sham operated, PAF (1.5 μg/kg iv), and LPS (8 mg/kg iv). Each animal that was experimentally treated (PAF or LPS) was always paired with a sham control. Preliminary experiments were done to select doses of PAF and LPS that would avoid profound shock and gross intestinal necrosis and thus loss of enzyme activity. The combined antibiotic regimen (given in drinking water) included a mixture of neomycin (250 mg·kg·day⁻¹), polymyxin B (9 mg·kg·day⁻¹), and metronidazole (50 mg·kg·day⁻¹) for 3 days before the experiment. This choice of ABX is based on previous findings (43) showing protection against PAF-induced injury. However, in the current study, animals were treated for 3 days instead of 7 days (43), because preliminary data showed that rats treated for 3 days have the lowest number of intestinal bacterial colonies. The second part of the experiments consisted of GF rats with or without recolonization by enteral flora.

Gross and microscopic injury score. The severity score of gross injury was defined as follows: 1, mild (slight reddish discoloration); 2, moderate (red discoloration often with hemorrhage); and 3, severe (grossly necrotic, blackish red, friable, and lusterless) (44). Sections were taken from the most severely affected areas and processed for paraffin embedding and subsequent sectioning and staining for histological confirmation of necrosis. Multiple random sections were taken if the bowel appeared normal. A pathologist recorded the histological changes in a blinded fashion. The severity score for microscopic injury was defined as follows: 0, no injury with intact surface epithelium; 0.5, minimal injury involving epithelial cells at villus tips; 1, mild mucosal injury confined to the top of the villi; 2, moderate injury involving nearly one-half of the villi; and 3, severe injury with complete loss of villi or extending to submucosa. The length of injured intestinal segment was measured. Gross and histological scores were calculated by multiplying the percentage of intestinal involvement (length of abnormal intestine/total bowel length) by the severity score (44).

Intestinal PLA₂-II assay. Intestinal tissue was homogenized in buffer solution containing HEPES, sucrose, EDTA, EGTA, leupeptin, pepstatin, and phenylmethylsulfonyl fluoride. The debris and nuclei were removed by centrifugation at 1,000 g for 10 min (33), and the supernatant was then centrifuged at 200,000 g for 1 h. The PLA₂-II assay was performed following method A (33) or B (7, 13) after preliminary kinetic study. In method A, the enzyme (100 μg protein) was added to the sonicated substrate, 1-palmitoyl, 2-[¹⁴C]arachidonyl-sn-3-glycerophosphocholine, and incubated at 37°C for 30 min. The reaction was terminated with ethanol, developed containing arachidonate acid and acetic acid (−20°C) and then centrifuged. Lipids were extracted, separated by TLC, developed in benzene-diethyl ether-ethyl acetate-glacial acetic acid (80:10:10:2), scraped, and counted in a scintillation counter (33). In method B, the enzyme and substrate, autoclaved [¹⁴C]labeled Escherichia coli, were incubated at 37°C for 10 min. The reaction was terminated with chloroform-methanol (2:1) followed by acidified water. Phospholipids were extracted by Folch’s method (7, 13); TLC was performed as described for method A above. Method A gives an absolute quantity of PLA₂-II activity. Method B is simpler and more rapid than method A. PAF experiments were performed using both method A and method B, which yielded similar results. Thus only method B was used in the LPS experiments. Statistical analysis. Statistical analyses were performed using Wilcoxon’s multivariate analysis. Data are presented as means ± SE. P < 0.05 was considered significant.

RESULTS

Bacterial cultures of intestinal content from ABX rats showed results similar to previous findings (43), namely, complete elimination of E. coli and marked reduction of other gram-negative bacteria. Lactobacillus was the predominant bacteria after 3 days of treatment, and this was only present in one-third of the colony-forming units of sham controls. Bacterial cultures of GF rats confirmed the sterile state of the animals. RGF animals had similar intestinal flora to CN rats, as shown by bacterial culture.

PAF, at the dose used, induced immediate, marked hypotension with the nadir reached by 5 min (Fig. 1) in all groups of rats (CN, ABX, GF, and RGF), which slowly recovered within 30 min in all except RGF rats. These rats showed a persistent significant hypotension at 30 min, significantly more severe than in other groups. (Fig. 1B). PAF induced peripheral leukocytosis in all groups of rats, but not significantly so in GF and...

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and hemoconcentration (increase in hematocrit) (Fig. 4B) in all four groups of rats, but not significantly so in GF rats. Antibiotic pretreatment partially ameliorated LPS-induced changes in the white blood cell count and hematocrit (Figs. 3B and 4B).

LPS induced mild gross and microscopic injury of the intestine in CN rats (Table 1). GF rats had minimal gross (congestion) and no microscopic injury after LPS. ABX rats had statistically less microscopic injury after LPS than CN rats. RGF rats had mild gross and microscopic injury. LPS caused no mortality at the dose used.

PLA2-II enzyme activity was detected in the small intestine of unstimulated normal animals (Fig. 5). Neither ABX nor GF diminished the basal level of PLA2-II. Both PAF and LPS increased PLA2-II activity (to 2-fold baseline) in CN rats. The effect of PAF peaked at 30 min. The LPS effect was slower than PAF, just beginning to plateau at 2 h. Thus 30 min was chosen as the end point for PAF and 2 h for LPS experiments. The increase in PLA2-II activity in response to PAF (Fig. 5, A and B) and LPS (Fig. 5C) was abrogated in GF and ABX rats. PAF and LPS did not significantly cause an increase in the activity of PLA2-II in ABX and GF groups. The responses of RGF rats were similar to those of CN rats to PAF and LPS.

A specific PLA2-II inhibitor (LY-311727) was given to CN and GF rats before LPS injection to evaluate the effect on pathophysiological changes. The PLA2-II inhibitor had minimal protection against hypotension in GF rats (not statistically significant) but none in CN rats (data not shown). The inhibitor had no effect on hematocrit or white blood cell count. There was no mortality in the inhibitor-treated groups. The CN animals had similar to CN animals. Lipopolysaccharide (LPS) induced bowel injury in all 4 groups. LPS CN animals had mild gross and microscopic injury, and GF rats had mild gross but no microscopic injury. There was no mortality in any of the groups. See Gross and microscopic injury score for descriptions of gross and microscopic injury scores.

Table 1. Gross and microscopic injury

<table>
<thead>
<tr>
<th>Animal Type</th>
<th>Treatment</th>
<th>$n$</th>
<th>Gross Injury Score</th>
<th>Histological Injury Score</th>
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<tr>
<td>CN</td>
<td>Sham</td>
<td>21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CN</td>
<td>PAF</td>
<td>14</td>
<td>0.22 ± 0.05</td>
<td>0.18 ± 0.12</td>
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<td>CN</td>
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<td>0.78 ± 0.17</td>
<td>0.6 ± 0.37</td>
</tr>
<tr>
<td>ABX</td>
<td>Sham</td>
<td>12</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>ABX</td>
<td>PAF</td>
<td>11</td>
<td>0.09 ± 0.04</td>
<td>0.2 ± 0.12</td>
</tr>
<tr>
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<td>0.125 ± 0.125</td>
</tr>
<tr>
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<td>0</td>
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<td>0</td>
</tr>
<tr>
<td>GF</td>
<td>LPS</td>
<td>9</td>
<td>0.16 ± 0.11</td>
<td>0</td>
</tr>
<tr>
<td>RGF</td>
<td>Sham</td>
<td>5</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>RGF</td>
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<tr>
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<td>LPS</td>
<td>5</td>
<td>0.44 ± 0.15</td>
<td>0.2 ± 0.2</td>
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</table>

Values are means ± SE for gross and microscopic injury; $n$ = no. of animals. Platelet-activating factor (PAF)-treated conventionally fed (CN) animals had minimal gross and microscopic injury. PAF germ-free environment (GF) animals had no injury, and antibiotic-treated (ABX) animals had minimal microscopic injury. PAF reconstituted (RGF) animals had minimal gross and microscopic injury, similar to CN animals. Lipopolysaccharide (LPS) induced bowel injury in all 4 groups. LPS CN animals had mild gross and microscopic injury. ABX and RGF animals had minimal gross and microscopic injury, and GF rats had mild gross but no microscopic injury. There was no mortality in any of the groups. See Gross and microscopic injury score for descriptions of gross and microscopic injury scores.

Fig. 1. Systemic blood pressure (in mmHg) over time after injection of platelet-activating factor (PAF; 1.5 μg/kg iv) in conventionally fed (CN) and antibiotic-treated (ABX) rats (A) or germ-free (GF) and reconstituted GF (RGF) rats (B). Values are means ± SE. All 4 groups had significant hypotension at 5 min compared with sham controls. RGF still had significant hypotension at 30 min compared with other groups. *$P < 0.05$, compared with sham rats; **$P < 0.05$, compared with GF rats.

RGF groups (see Fig. 3A). PAF also induced hemoconcentration (indicated by increased hematocrit) in all groups of rats (see Fig. 4A). GF (see Fig. 4A) partially ameliorated the hemoconcentration. PAF, at the dose used, did not cause lethality and only resulted in minimal gross and microscopic injury (Table 1) of the intestine in CN rats and minimal microscopic injury in ABX rats. GF rats had no gross or microscopic injury. RGF rats had minimal gross and microscopic injury, similar to CN rats.

The blood pressure slowly decreased after LPS administration with the first nadir reached at 15–20 min (Fig. 2). In all groups of animals, there appeared to be some recovery of blood pressure, with a second nadir reached at 90 min. At 2 h, all groups of rats remained hypotensive (Fig. 2). LPS-induced shock was partially abrogated by ABX pretreatment (Fig. 2A). RGF rats showed more severe hypotension than CN and GF rats (Fig. 2B). LPS caused peripheral leukopenia (Fig. 3B)
role in the development of these inflammatory disorders. The concentration of PLA2-II is increased (up to 100- to 150-fold) in the sera of patients suffering from inflammatory diseases (34). The role of PLA2-II in the pathogenesis of sepsis is further supported by the observation that administration of PLA2-II inhibitors prolonged survival in a murine model of LPS shock (27).

PAF, an endogenous mediator of endotoxin shock (20, 49), induces intestinal necrosis (49), which may augment the development of MODS. A previous study (43) showed that PAF causes elevation of serum LPS levels (indicating bacterial translocation), and PAF-induced intestinal injury and lethality are reduced in GF and ABX rats. PAF also upregulates the gene expression and enzyme activity of intestinal PLA2-II, even at doses insufficient to cause prolonged shock and intestinal necrosis (45). The PAF-induced increase in PLA2-II activity was abrogated in GF and ABX rats,

Fig. 3. Change in peripheral white blood cell (WBC) count (expressed as % of values at time 0) after PAF (A) or LPS injection (B). Values are means ± SE. PAF caused leukocytosis in CN and ABX rats; LPS caused leukopenia in CN and ABX rats. After LPS, ABX and GF rats had less severe leukopenia compared with CN rats. *P < 0.05, compared with respective control; #P < 0.05 compared with CN-LPS-treated rats.

Fig. 2. Systemic blood pressure (in mmHg) over time after injection of lipopolysaccharide (LPS; 8 mg/kg iv) in CN and ABX rats (A) or GF and RGF rats (B). Values are means ± SE. Compared with sham controls, all 4 groups showed significant hypotension at 15 min and also at 2 h. LPS-induced hypotension was partially abrogated in ABX rats. RGF rats had more severe hypotension compared with CN or GF rats. *P < 0.05, compared with sham controls; #P < 0.05, compared with CN-LPS rats.

similar gross and microscopic injury compared with controls.

DISCUSSION

Intestinal injury is important in the perpetuation of septic shock, multiple organ dysfunction syndrome (MODS), and acute respiratory distress syndrome (ARDS). Circulating PLA2 has been recognized as a mediator of cardiovascular collapse in septic shock (46). Circulating PLA2 is vasoactive, causing hypotension, increased vascular permeability, and acute lung injury (14). Excessive release and/or activation of PLA2 appears to be a pivotal event in the development of sepsis, septic shock, and MODS (14). Release of PLA2 into the circulation occurs in states of profound illness, including sepsis, shock, severe injury, and pancreatitis, all of which are linked to the development of ARDS and MODS. Experimental and clinical evidence (1) suggests that PLA2 may serve a primary regulatory
suggesting that the effect of PAF on this enzyme is largely mediated by "indigenous" intestinal bacteria or their products.

Previous studies (41) have shown that intravenous injection of LPS induces PLA2-II mRNA expression in various organs, including the intestine. In the present study, we have shown that the increase in PLA2-II activity induced by LPS was abrogated in GF and ABX rats. This observation suggests that LPS activation of this enzyme is also largely mediated by indigenous intestinal bacteria and/or their products. Indeed, endotoxin has been shown to cause bacterial translocation in mice (8, 9). However, our preliminary experiments showed that bacterial translocation does not occur up to 6 h after the injection of PAF or LPS, much longer than our experimental periods, suggesting that bacterial prod-

Fig. 4. Change in hematocrit value (expressed as % of values at time 0) after PAF (A) or LPS injection (B). Values are means ± SE. PAF caused hemoconcentration in all groups. LPS caused hemoconcentration in CN, ABX, and RGF groups. After PAF, GF rats had less severe hemoconcentration compared with CN rats. RGF rats had more severe hemoconcentration compared with GF rats. After LPS, ABX and GF rats had less severe hemoconcentration compared with CN rats. *P < 0.05, compared with respective control; #P < 0.05, compared with CN PAF- or LPS-treated rats; $P < 0.05, compared with GF PAF-treated rats.

Fig. 5. Group II phospholipase A2 (PLA2-II) activity in the small intestine. A: activity expressed in nmol·mg protein^{-1}·h^{-1} using method A (see MATERIALS AND METHODS) for PLA2-II assay after PAF injection. B: PLA2-II activity expressed as %conversion/min, as assayed by method B (see MATERIALS AND METHODS) after PAF injection. C: enzyme activity expressed as %conversion/min after LPS injection. Values are means ± SE. PAF increases PLA2-II activity. The effect of PAF is abrogated in GF and ABX rats. RGF rats have a normal PLA2-II response to PAF. LPS increases PLA2-II activity. The effect of LPS is abrogated in GF and ABX rats. RGF rats have a normal PLA2-II response to LPS. *P < 0.05, compared with respective control; #P < 0.05, compared with CN PAF- or LPS-treated rats; $P < 0.05, compared with GF PAF- or LPS-treated rats.
products such as LPS, rather than bacteria themselves, are responsible for the injurious effects observed in CN rats. Although enterocytes do not express the LPS receptor CD14 on their cell surface, they express Toll-like receptors (TLR) (6) and could be directly stimulated by LPS in vitro. It is possible that LPS binds to TLR, resulting in a change in mucosal permeability and loss of barrier function. The role of enteral bacteria or their products in this model is further supported by the observation that GF and ABX reduce LPS-induced hemoconcentration and bowel injury. ABX also ameliorates LPS-induced hypotension. The impaired response in GF animals may be partly explained by a deficient inflammatory response, because GF animals have fewer intestinal mast cells (28) and impaired production of cytokines (36) and superoxide (37). Furthermore, because ABX animals are also protected from the adverse effects of PAF and LPS, an abnormal inflammatory response need not be invoked as the sole cause of the observed protection. Thus it appears that gut flora and/or their products are necessary for the injurious effect of PAF and LPS. Hence, the important clinical implication that ABX, by reducing gut bacteria and their products, may ameliorate local as well as systemic inflammation and therefore prevent the development of shock and bowel necrosis in septic patients.

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