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Tumour necrosis factor and endotoxin synergistically activate intestinal phospholipase A_2 in mice. Role of endogenous platelet activating factor and effect of exogenous platelet activating factor

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Abstract

Previous studies have shown that: (a) platelet activating factor induces shock and intestinal injury, (b) exogenous platelet activating factor stimulates synthesis of endogenous platelet activating factor, and (c) tumour necrosis factor α and endotoxin synergise to induce shock and bowel injury in animals. These last two effects are largely mediated by platelet activating factor forming phospholipase A_2 A_2, a key enzyme for platelet activating factor synthesis, was examined in mouse intestine. It was found that tumour necrosis factor α and endotoxin synergise to stimulate platelet activating factor forming phospholipase A_2 activity in the intestine, as well as platelet activating factor production, and these effects were blocked by pretreatment with platelet activating factor antagonists, SRI-63-441 and WEB 2086. In addition, exogenous platelet activating factor stimulates intestinal phospholipase A_2 activity. These results show that tumour necrosis factor α and lipopolysaccharide synergistically activate the phospholipase A_2 that participates in platelet activating factor formation, and this activation is largely mediated by endogenous platelet activating factor. Furthermore, platelet activating factor itself increases phospholipase A_2 activity, suggesting that platelet activating factor induces its own synthesis, probably by phospholipase A_2 activation.

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The importance of platelet activating factor (or paf acether), tumour necrosis factor α, and bacterial endotoxin (LPS) in inflammatory diseases has been well recognised. Tumour necrosis factor α is a polypeptide cytokine produced mainly by macrophages in response to lipopolysaccharide and other stimuli,[1 2] and is thought to be one of the main mediators in septic shock.[3 4] Platelet activating factor, a phospholipid with potent biological effects, has been implicated as an important mediator in inflammation and shock.[5] We have previously shown that tumour necrosis factor α and lipopolysaccharide synergise to induce bowel injury in rats[6] and mice,[7] and these effects are largely mediated by platelet activating factor.[6 8] Thus, platelet activating factor seems to play a central part in mediating intestinal injury. Furthermore, platelet activating factor induces prolonged hypotension and intestinal necrosis in rats[9] and mice.[8] One possible cause of the longacting effect of platelet activating factor is that platelet activating factor induces its own formation. Platelet activating factor synthesis in neutrophils is stimulated by exogenous platelet activating factor or platelet activating factor analogue.[10] We have also shown that the intestinal level of platelet activating factor was considerably increased two hours after exogenous platelet activating factor was given, and this increase was prevented by platelet activating factor antagonist.[9] Thus, at least in the intestinal tissue, platelet activating factor induces its own synthesis by a positive feedback loop.

The platelet activating factor level in normal intestinal tissue is very low.[6 9] After inflammatory stimuli such as lipopolysaccharide, the tissue platelet activating factor level is considerably increased.[11] Platelet activating factor can be synthesised by two biochemical pathways.[12] The predominant pathway in the inflammatory cells entails the activation of phospholipase A_2, which cleaves arachidonic acid from the second position of the precursor, 1-alkyl-2-arachidonoyl-3-phosphocholine, to form lyso-platelet activating factor.[12] Platelet activating factor is then formed by acetylation of lyso-platelet activating factor by an acetyltransferase.[12]

In this study, phospholipase A_2, a key enzyme for platelet activating factor synthesis in the murine small intestine is examined. Because exogenous platelet activating factor,[9] as well as tumour necrosis factor α with lipopolysaccharide together[6] stimulate endogenous platelet activating factor synthesis in vivo, we hypothesised that these stimuli cause phospholipase A_2 activation leading to platelet activating factor production. Therefore we sought to find out if: (a) tumour necrosis factor α and lipopolysaccharide activate phospholipase A_2 in vivo, and if their effects are synergistic; (b) platelet activating factor itself activates phospholipase A_2; (c) tumour necrosis factor α/lipopolysaccharide-induced phospholipase A_2 activation is mediated by the action of platelet activating factor; and (d) platelet activating factor release correlates phospholipase A_2 activation.

Materials and methods

Platelet activating factor (1-0-hexadecyl-2-acetyl-sn-glycero-3-phosphocholine, Sigma Chemical Co, St Louis, Mo, USA) (2 mg/ml) was dissolved in albumin-saline (5 mg/ml) solution and stored frozen in 10–20 μl aliquots. Working solution was made fresh daily in albumin-saline. 1-0-hexadecyl-2-[^{3}H] arachidonoyl-3-phosphocho-

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line (74·3 Ci/mmol) was purchased from New England Nuclear, Boston, MA, USA. 1-0-hexadecyl - 2 - arachidonoyl - 3 - phosphorylcholine was obtained from Biomol Research Laboratories Plymouth Meeting, PA, USA. [^{3}H] platelet activating factor (80 Ci/mmol) was purchased from Amersham, Arlington Heights, IL, USA. Lipase from *R asshizus* was purchased from Sigma Co, recombinant hTNF-α was a generous gift from Genentec Inc, South San Francisco, CA, USA. Lipopolysaccharide (Salmonella typhosa) was purchased from Difco, Detroit, MI, USA. Two structurally unrelated platelet activating factor antagonists, SRI 63-441, and WEB 2086, were gifts provided respectively by Dr D Handley, Sandoz Research Institute, East Hanover, NJ, USA and Dr H Heuer, Boehringer Ingelheim, Mainz, Germany.

ANIMAL EXPERIMENT

Six to 10 week old male C3H/HeN mice (Harlan Sprague Dawley, Indianapolis, IN, USA), fasted overnight, were anaesthetised with nembutal (65 μg/g, intraperitoneally) and tracheotomised The carotid artery was catheterised for continous blood pressure recording, blood sampling, and drugs injection. The animals were divided into six groups: sham operated; human tumour necrosis factor α (1 mg/kg, intra-arterially) only; lipopolysaccharide (1 mg/kg, intra-arterially) only, tumour necrosis factor α followed 30 minutes later by lipopolysaccharide; and SRI 63-441 (5 mg/kg), divided into two doses, injected 15 minutes before tumour necrosis factor α and five minutes before lipopolysaccharide; WEB 2086 (1 mg/kg, 30 minutes before tumour necrosis factor α)+tumour necrosis factor α+lipopolysaccharide. In addition, experiments consisting of sham operated mice and two groups of mice receiving platelet activating factor (2 and 2·5 μg/kg, intra-arterially) were also performed. All drugs were injected into the cannulated carotid artery. At the end of the experiment (1·5 hours after tumour necrosis factor α or immediately before death in the platelet activating factor group), blood samples were collected for packed cell volume measurement and platelet activating factor assay, and the entire small intestine was removed, washed with cold saline, weighed, and stored in −70°C. Frozen intestines were subsequently thawed, homogenised in 10 volumes of hydroxyethylpiperazine-ethanesulphonic acid (HEPES) buffer (pH 7·2) for 1·5 minutes (with one break in between), and centrifuged at 1000 rpm for 10 minutes. The supernatants were aliquoted and stored at −70°C for future phospholipase A_2 assay. The study was approved by the Institutional Animal Care and Use committee.

PHOSPHOLIPASE A_2 ASSAY

Platelet activating factor-forming phospholipase A_2 activity was assayed by using platelet activating factor precursor, 1-0-alkyl-2-arachidonoyl-3-phosphocholine, as the substrate, according to a method modified from that of Diaz and Mong.[13] The assay mixture (total volume 150 μl) contained 0·1 M TRIS buffer (pH 7·4) with 2 mM $CaCl_2$, 5 mg/ml bovine serum albumin (BSA), 0·1 mg/ml sodium deoxycholate, 2×10^4 cpm of 1-0-hexadecyl-2-[^{3}H] arachidonoyl-3-phosphocholine, and 0·15 μg of 1-0-hexadecyl-2-arachidonoyl-3-phosphocholine as carrier, and 50 μl of tissue supernatant (1:3 dilution). The substrate was prepared by sonication in the assay buffer until the phospholipid suspension was clear (five minutes). The reaction was started by adding tissue enzyme. After incubation at 37°C for 30 minutes, the reaction was stopped by adding 3 ml of chloroform:methanol (2:1). (Preliminary experiments were performed to find out if the reaction was linear at 30 minutes). Total lipids were extracted by Folch's method,[14] and separated by thin layer chromatography on silica gel G plates, by using a solvent system chloroform:methanol:ammonium hydroxide (65:35:5). Zones comigrating with fatty acids and other phospholipid standards were scraped and counted. Blank and control (sham operated) samples were included in each assay. Although there is no currently available method of measuring platelet activating factor-specific phospholipase A_2, this method, by using the specific substrate, measures the activity of the phospholipase A_2 that participates in platelet activating factor formation.

QUANTITATION OF PLASMA PLATELET ACTIVATING FACTOR

At the end of the experiment, blood was drawn from the catheter into 0·1 volumes of 0·4 M citric acid and was centrifuged. (Citric acid was used to prevent clotting and to inactivate acetylhydrolase). The plasma was stored in 20 volumes of chloroform:methanol (2:1) at −70°C until assayed.

Platelet activating factor was extracted with Folch's method as previously described.[14] [^{3}H] platelet activating factor (1×10^4 cpm) was added to each sample before extraction for calculation of extraction efficiency. Extracted lipids were dried under a stream of N_2, and reconstituted in 1 ml of saline containing BSA, 5 mg/ml. An equal volume of 20% acetic acid was then added, and the sample was loaded to an activated C18 column (Varian Associates, Harbor City, CA). After removing unwanted materials with two washes of 10% acetic acid (1 ml each), and three washes of ethyl acetate (2 ml each), platelet activating factor was eluted with 6 ml of methanol. The extraction efficiency of column chromatography was 65–70%. To further purify the platelet activating factor fraction, the eluate was mixed with 2·4 ml of water and 3 ml of chloroform (chloroform:methanol:water, 1:2:0·8), and 0·1 g of DEAE cellulose, and was vortexed. Additional chloroform (3 ml) and water (3 ml) were then added, and mixed (chloroform:methanol:water, 1:1:0·9). After centrifugation, the lower (chloroform) phase was transferred to another tube, dried under N_2, and reconstituted in 0·3 ml of phosphate buffered saline containing 5 mg/ml albumin. Platelet activating factor activity was determined by using the rabbit platelet serotonin release method as previously described.[9] A platelet activating factor-

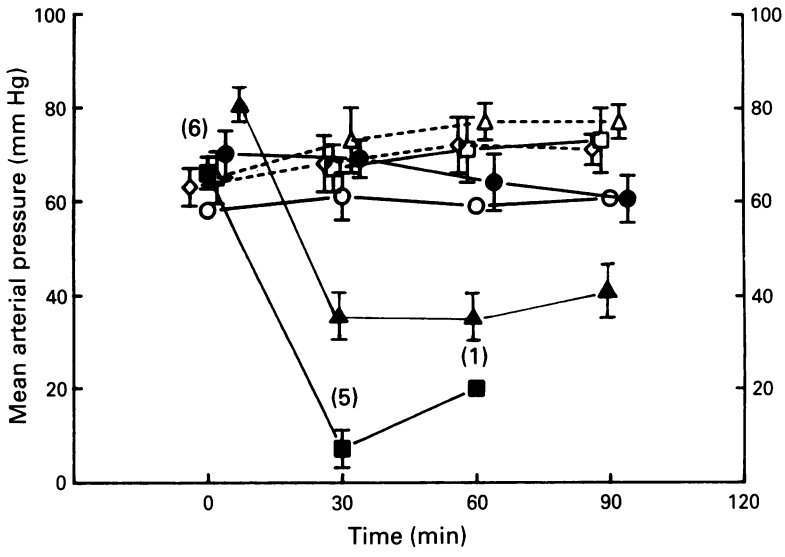

Figure 1: Changes of mean blood pressure after various treatments. —○—, sham operated (n=3); —△—, tumour necrosis factor α (1 mg/kg, at time 0, n=5); —□—, lipopolysaccharide (1 mg/kg, at time 0, n=3); —●—, tumour necrosis factor α (1 mg/kg at time 0)+ lipopolysaccharide (1 mg/kg, at time 30 minutes) (n=8); —◇—, SRI 63-441 at time −15 minutes and 15 minutes, 2·5 mg/kg each)+tumour necrosis factor α (at time 0)+ lipopolysaccharide (at time 30 minutes) (n=5); —▲—, platelet activating factor (2 μg/kg, n=3); and —■—, platelet activating factor (2·5 μg/kg, at time 0, n=6). All high dose (2·5 μg/kg) – platelet activating factor treated mice died within one hour. Mean (SEM).

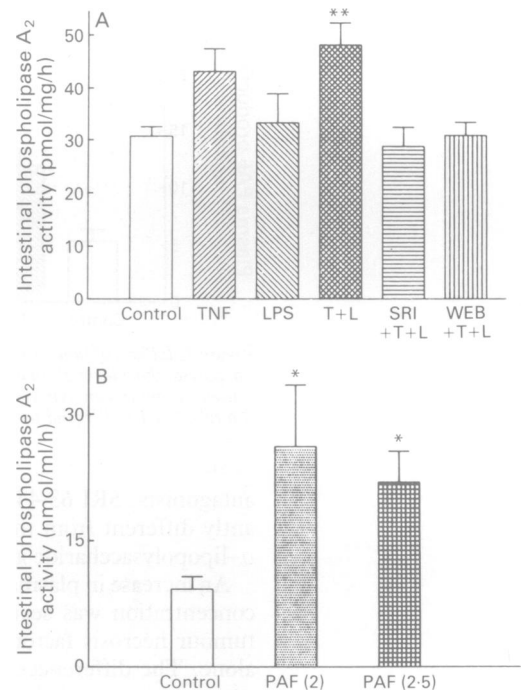

*Figure 3: (A) Effects of tumour necrosis factor α and lipopolysaccharide on intestinal phospholipase A_2 activity, and its inhibition by platelet activating factor antagonist (mean (SEM)). See Figs 1 and 2 legends for doses and abbreviations. **p<0·01 (compared with control). There is also a significant difference between the group pretreated with SRI and the group receiving tumour necrosis factor α and lipopolysaccharide only (p<0·05). (B) Effect of platelet activating factor on intestinal phospholipase A_2 activity. *p<0·05.*

antagonist, SRI 63-441 (10 μg/ml) was used in each sample to confirm that the active compound present was platelet activating factor. In addition, some samples were treated with lipase from *R azzhizus* (100 μg/ml, room temperature, five minutes) for platelet activating factor verification. Teflon tubes or siliconised tubes were used throughout the experiment.

STATISTICAL ANALYSIS

Data were analysed by analysis of variance. All data are presented as means (SEM).

Results

In the first set of experiments, we examined the changes of systemic blood pressure (Fig 1) and packed cell volume (PCV) (Fig 2) after injection of tumour necrosis factor α, lipopolysaccharide, tumour necrosis factor α+lipopolysaccharide, and the preventive effects of platelet activating factor antagonists, SRI 63-441 and WEB 2086 on the combined treatment of tumour necrosis factor α and lipopolysaccharide. Tumour necrosis factor α, lipopolysaccharide, or combined treatment of tumour necrosis factor α and lipopolysaccharide did not cause hypotension or death. Mice pretreated with SRI-63-441 and WEB 2086 also showed no change in blood pressure (data not presented).

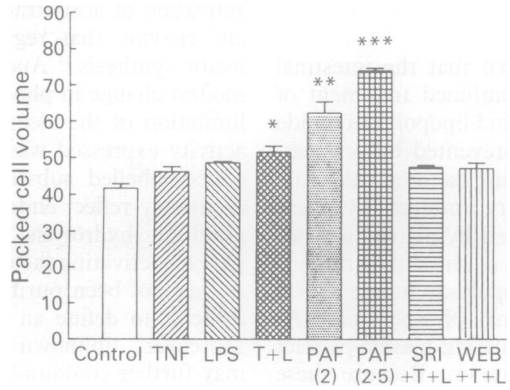

*Figure 2: Effects of tumour necrosis factor α, lipopolysaccharide, and platelet activating factor on packed cell volume (mean (SEM)). See Fig 1 legend for doses and numbers of animals in each group. T=tumour necrosis factor α, L=lipopolysaccharide, and SRI=SRI 63-441. *p<0·05 (compared with control), **p<0·01 (compared with control), ***p<0·001.*

Figure 2 shows that mice receiving tumour necrosis factor α+lipopolysaccharide developed haemoconcentration (PCV:51·6 (1·6), significantly higher than that of control (sham operated) mice (41·7 (1·2), p<0·05). This response was partially blocked by pretreatment with SRI 63-441 (PCV: 47·6(0·5) or WEB 2086 (PCV: 47·3 (1·4)). Mice receiving tumour necrosis factor α or lipopolysaccharide alone showed only a mild increase in PCV (46·2 (1·4) and 48·7 (0·3) respectively, p=NS). Tumour necrosis factor α, lipopolysaccharide or combined tumour necrosis factor α and lipopolysaccharide did not cause grossly evident intestinal injury.

Lipopolysaccharide, at the dose used, did not induce phospholipase A_2 activation in the small bowel (Fig 3A). Tumour necrosis factor α alone caused mild phospholipase A_2 activation. Tumour necrosis factor α and lipopolysaccharide together significantly increased intestinal phospholipase A_2 activity above controls (p<0·01, Fig 3A). This increase was completely inhibited by pretreatment with platelet activating factor

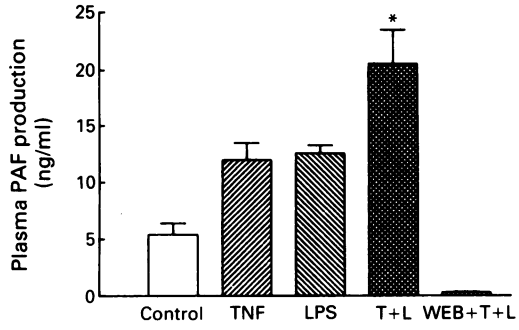

*Figure 4: Effects of tumour necrosis factor α and lipopolysaccharide on plasma platelet activating factor concentrations (mean (SEM)). *p<0·01 (compared with control). See Fig 1 legend for doses.*

antagonists, SRI 63-441 or WEB 2086 (significantly different from the tumour necrosis factor α–lipopolysaccharide group, p<0·05, Fig 3A).

An increase in plasma platelet activating factor concentration was seen after administration of tumour necrosis factor α or lipopolysaccharide alone. The differences from the controls, however, were not statistically significant. Combined tumour necrosis factor α and lipopolysaccharide treatment resulted in an almost fourfold increase in plasma platelet activating factor (p<0·01, Fig 4).

In the second set of experiments, we examined the effect of platelet activating factor on blood pressure (Fig 1), PCV (Fig 2), and intestinal phospholipase A_2 (Fig 3B). A lethal dose of platelet activating factor (2·5 μg/kg) induced severe hypotension (7·2 (4) mm Hg at 30 minutes Fig 1), severe haemoconcentration (74 (0·7), p<0·01 compared with controls) (Fig 2), and death by one hour in all animals. All animals had evidence of gross intestinal necrosis, as shown by multifocal, segmental areas of violaceous, dark red and lusterless bowel. A sublethal dose (2 μg/kg) of platelet activating factor caused hypotension (Fig 1) and haemoconcentration (62 (2·6), p<0·05, Fig 2) at 1·5 hours after platelet activating factor injection. Similar to the tumour necrosis factor α and lipopolysaccharide treated group, exogenous platelet activating factor, at both doses, significantly increased intestinal phospholipase A_2 activity (p<0·05, Fig 3B).

Discussion

We have previously shown that the intestinal necrosis induced by a combined treatment of tumour necrosis factor α and lipopolysaccharide in rats[6] and mice[7 8] was prevented by pretreatment with platelet activating factor antagonists, suggesting that the injury induced by these agents was largely mediated by platelet activating factor. Our results here show that tumour necrosis factor α and lipopolysaccharide synergistically increase intestinal phospholipase A_2 activity and increase circulating plasma platelet activating factor concentration. Because these effects were prevented by two structurally different platelet activating factor receptor antagonists, our data clearly support the role of phospholipase A_2 activation and subsequent platelet activating factor synthesis in this model of intestinal injury.

Phospholipase A_2 is the key enzyme in platelet activating factor synthesis in inflammatory cells.[12] As 1-alkyl-2-arachidonoyl-sn-GPC may be an obligatory precursor of platelet activating factor in platelets and inflammatory cells,[15] activation of phospholipase A_2 results in lysoplatelet activating factor formation as well as arachidonic acid release,[16] which is further metabolised to prostaglandin and leukotrienes to perpetuate the inflammatory response.[17] Many stimuli can serve as signals to activate phospholipase A_2, including lipopolysaccharide,[18 19] zymosan,[20] and products of Gram positive bacteria.[19] It has been reported that cytokines such as tumour necrosis factor α and interleukin 1[21–24] both stimulate the synthesis and secretion of phospholipase A_2 in vitro, and the combination of these two agents is strongly synergistic.[23] In fact, the mechanism of tumour cell killing by tumour necrosis factor α has been proposed to be by phospholipase A_2 activation.[25] The effect of tumour necrosis factor α on non-tumour cells is, however, less well defined; several investigators claim that tumour necrosis factor α alone at any level cannot cause phospholipase A_2 activation in polymorphonuclear neutrophils.[17 26–28] As a priming agent, however, tumour necrosis factor α synergistically enhances phospholipase A_2 activity induced by N-formyl-methionyl-leucyl-phenylalanine.[17] This study shows that tumour necrosis factor α and lipopolysaccharide synergistically activate tissue phospholipase A_2 in vivo, whereas tumour necrosis factor α or lipopolysaccharide alone, at the dose used, had no significant effect. Moreover, our data show that intestinal phospholipase A_2 was activated by low doses of tumour necrosis factor α and lipopolysaccharide, which are below the threshold for induction of shock and bowel necrosis. That phospholipase A_2 activation occurs before any changes in systemic blood pressure and intestinal injury is in accordance with our hypothesis that phospholipase A_2 activation may be one of the initial steps of tumour necrosis factor α and lipopolysaccharide-induced shock and intestinal injury.

The increase seen in phospholipase A_2 activity is modest when compared with the increase of platelet activating factor production. This discrepancy may be because of a simultaneous activation of acetyltransferase, another important enzyme that regulates platelet activating factor synthesis.[12] Another explanation for the modest change in phospholipase A_2 may be the limitation of the assay. The phospholipase A_2 activity expressed was calculated based on the added labelled substrate only, thus may not accurately reflect endogenous membrane phospholipid hydrolysis. Furthermore, because platelet activating factor-forming phospholipase A_2 has not been purified or characterised, it is difficult to define an optimal assay condition. Moreover, unknown endogenous inhibitor(s) may further confound the interpretation of the assay.

Exogenous platelet activating factor administration induces prolonged shock and bowel necrosis in animals.[8 9] Our previous studies have suggested that part of the reason for the long in vivo response to platelet activating factor may

be the production of other mediators such as peptide leukotrienes,[29 30] catecholamines,[31] and activated complement.[8] Another cause of the longacting effect of platelet activating factor is the ability of exogenous platelet activating factor to stimulate endogenous platelet activating factor formation,[9] which in turn may promote sustained shock and intestinal injury.[9] Results presented in this report confirm that exogenous platelet activating factor activates intestinal phospholipase A_2 leading to enhanced concentrations of endogenous platelet activating factor, which may mediate tissue injury.

In this study we also found that the effect of tumour necrosis factor α/lipopolysaccharide on intestinal phospholipase A_2 activation was efficiently blocked by pretreating mice with platelet activating factor antagonists. This finding suggests that although tumour necrosis factor α-lipopolysaccharide activates phospholipase A_2 to produce platelet activating factor, this effect may be transient. It is probably the endogenous platelet activating factor production that sustains the phospholipase A_2 activation. Both platelet activating factor[32 33] and lipoxygenase products of arachidonic acid[34] have been shown to activate phospholipase A_2 in vitro. Our previous investigations,[29 30] as well as the in vitro studies of others[35 36] have shown the formation of lipoxygenase products (including leukotrienes) in response to platelet activating factor. Whether sustained phospholipase A_2 activation in vivo is due to platelet activating factor itself or lipoxygenase products remains unclear. Whichever is the case, the ultimate result is prolonged phospholipase A_2 activation and release of lipid mediators in vivo. The interaction of these mediators with cytokines and lipopolysaccharide may eventually lead to irreversible tissue injury.

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