Priming Interleukin 8 Production
Role of Platelet-Activating Factor and p38

Saman Arbabi, MD; Matthew R. Rosengart, MD; Iris Garcia; Sandra Jelacic; Ronald V. Maier, MD

Hypothesis: Platelet-activating factor (PAF) activates p38, an important intracellular signal transduction kinase, and primes human mononuclear cells for the production of interleukin 8 (IL-8), a potent chemoattractant and activator of neutrophils.

Methods: Human mononuclear cells were isolated from healthy adults by Ficoll-paque density-gradient centrifugation. Interleukin-8 in the supernatant was measured by enzyme-linked immunosorbent assay. Dual phosphospecific p38 antibody was used to detect activated p38 by Western blotting.

Results: Lipopolysaccharide (LPS) and PAF activated p38. There was a shorter latency to peak p38 activation with PAF vs LPS stimulation, 5 vs 30 minutes. Platelet-activating factor–induced p38 activation was calcium dependent because it was inhibited by ethyleneglycoltetraacetic acid. Lipopolysaccharide, 0.01 to 1.00 ng/mL, induced significant IL-8 production. Although PAF did not induce significant IL-8 production, it potentiated LPS-induced IL-8 production. Production of IL-8, in response to LPS alone or in combination with PAF, was inhibited by SB202190, a specific p38 inhibitor.

Conclusions: Although LPS and PAF activated p38, only LPS induced IL-8 production; PAF acted as a priming agent. It seems that p38 activation is necessary but not sufficient for IL-8 production by human mononuclear cells. Identifying and evaluating the activation state of inflammatory signal transduction pathways might lead to methods for controlling and preventing neutrophil-induced tissue injury without interfering with the normal host immune response.

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The objective of the innate immune system is to produce an aggressive, focused inflammatory response by delivering and activating effector cells to the area of bacterial infection or other inflammatory stimuli. Ideally, this response is prompt, focused, and turned off after resolution of the infectious challenge. A delayed or inadequate host response might lead to dissemination of the infectious source, resulting in sepsis. However, on the other side of the balance, an aggressive, nonfocused activation of the effector cells can also cause nondiscriminatory significant injury to the host. The generalized release of cytotoxic mediators by host immune cells can cause diffuse organ injury, and lead to multiple organ dysfunction syndrome (MODS) and acute adult respiratory distress syndrome. Understanding the mechanisms underlying the immune responses, including intracellular signal transduction, may be necessary to focus therapeutic interventions for restoring balance in the critically ill patient who faces multiple complications.

Gram-negative bacteria are a major source of infectious complications in the surgical intensive care unit. In the United States, more than 20,000 people die each year of septic shock brought on by Gram-negative infections. Endotoxin, or lipopolysaccharide (LPS), the outer component of the Gram-negative bacterial cell wall, is the principal constituent recognized by the innate immune response. Platelet-activating factor (PAF), an ether phospholipid, is a potent inflammatory mediator secreted by multiple cells that might play an important role in endotoxic shock and MODS. Specific PAF receptors, which are G-coupled protein receptors, have been identified in multiple cells involved in the inflammatory response, such as monocytes, macrophages, neutrophils, and platelets.

Monocytes and macrophages play an orchestrating role in the inflammatory response partly by producing inflammatory cytokines. Several researchers have studied the effect of LPS and PAF on monocyte and macrophage cytokine production, and have demonstrated a synergistic effect on the production of interleukin 1 (IL-1), tumor necrosis factor α, and tissue factor activity. In contrast to LPS, PAF alone had minimal effects on cytokine production, and mainly contributed...
MATERIALS AND METHODS

REAGENTS AND CELL CULTURES

Human blood samples from healthy male volunteers were drawn into polypropylene syringes containing sodium citrate. Human mononuclear cells were isolated from theuffy coat layer of a Ficoll-paque (Amersham Pharmacia Biotech, Piscataway, NJ) density centrifugation gradient. Cells were washed with normal isotonic sodium chloride solution, resuspended in RPMI 1640 (BioWhittaker, Walkersville, Md) with gentamicin sulfate (100 µg/mL) and 5% heat-inactivated adult bovine serum at 1 × 10^6 cells/mL, and placed in round-bottom polypropylene tubes.

EXPERIMENTAL DESIGN

Cultures were treated at various times with either PAF [L-α-phosphatidylcholine, β-acetyl-g-O-(octadec-9-cis-ethyl)] or LPS (Escherichia coli 0111:B4), both from Sigma-Aldrich Corp (St Louis, Mo). For PAF pretreatment, cell cultures were treated with PAF (10^−6 to 10^−3 mol/L) for 15 minutes before LPS stimulation. Some cells were pretreated with p38 inhibitors (SB202190 or SB203580; Calbiochem, La Jolla, Calif) for 1 hour before stimulation. Cell viability was confirmed by trypan blue exclusion at the conclusion of the experiments. Cell supernatants were harvested and frozen at −70°C for later cytokine analysis.

IL-8 ASSAY

Interleukin-8 was quantitated by an enzyme immunoassay kit (Titerzyme; PerSeptive Biosystems, Framingham, Mass) that is based on a coated-well, sandwich enzyme immunoassay.

WESTERN BLOTS

Human mononuclear cells were placed in 50-mL polypropylene tubes (10 × 10^6 cells per tube). Cell cultures were treated with varying doses of LPS and PAF for different time courses. In some cultures, the calcium chelator ethyleneglycoltetracetic acid, 3 mmol/L (Sigma-Aldrich Corp), was added to the media 15 minutes before PAF stimulation.12 In a separate set of experiments, cells were treated with a calcium ionophore (A23187; Sigma-Aldrich Corp) for different time courses. Total cellular protein was extracted by lysing the cells in 1 mL of lysis buffer (20 mmol/L Tris, 137 mmol/L sodium chloride, 2 mmol/L EDTA, 10% glycerol, 1% Triton X-100, 1 µmol/L sodium orthovanadate, 100 µmol/L dithiothreitol, 200 µmol/L phenylmethanesulfonyl fluoride, leupeptin [10 µg/mL], and aprotonin [0.15 U/mL]) at 4°C. Protein concentration was determined by a bicinchoninic acid protein assay (Pierce, Rockford, Ill). Twenty micrograms of protein was electrophoresed in a 10% sodium dodecyl sulfate-polyacrylamide electrophoreses gel and transferred to a nitrocellulose membrane (Hybond-ECL; Amersham Pharmacia Biotech). The membrane was blocked for 1 hour at room temperature with 5% milk, then incubated overnight at 4°C with a phospho-specific p38 activated protein kinase antibody (Thr180/Tyr182; New England Biolabs, Beverly, Mass), which detects p38 only when activated by dual phosphorylation. The membrane was then incubated with a horseradish peroxidase-conjugated secondary antibody for 1 hour at room temperature. The blot was developed using a chemiluminescent substrate (SuperSignal; Pierce) and exposed to x-ray film (XAR-5; Eastman Kodak, Rochester, NY). Densitometry was performed with an image software program of the National Institutes of Health, Bethesda, Md, to quantitate the optical density.

STATISTICAL ANALYSIS

Values are expressed as mean ± SE. Data were analyzed using analysis of variance with post-hoc testing using the Fisher least significant difference test. P<.05 was considered significant.

RESULTS

IL-8 PRODUCTION IN RESPONSE TO PAF AND LPS

Platelet-activating factor–induced IL-8 production, over the dose range of 10−8 to 10−3 mol/L, did not attain statistical significance (P=.15) (Figure 1). The dose range for PAF was similar to that in previously published studies.12,13,14,15,16 In contrast, LPS at a dose range of 0.01 to 1.00 ng/mL induced significant IL-8 production. Lipopolysaccharide at 0.10 ng/mL induced a 25-fold increase in IL-8 production over the control.

Platelet-activating factor pretreatment of HMOs potentiated LPS-induced IL-8 production (Figure 2). Platelet-activating factor at 10−6 mol/L increased 0.01 ng/mL of LPS-induced IL-8 production by a factor of 2, which reached statistical significance compared with LPS alone (P=.005). There was a priming effect on IL-8 production with PAF pretreatment of 15 min-

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utes to 1 hour (data not shown). Lipopolysaccharide-induced IL-8 production, in either PAF-primed or LPS-alone HMOs, was inhibited by SB202190, a specific p38 inhibitor (Figure 2).27,28

**PAF- AND LPS-ACTIVATED p38 KINASE**

Western blot analysis with dual phospho-specific p38 antibody demonstrated that PAF and LPS activated p38 kinase in HMOs. Platelet-activating factor induced a peak p38 activation within 5 minutes, with a subsequent rapid decline (Figure 3). Lipopolysaccharide induced p38 activation in 30 minutes, with a return to baseline in 2 hours (data not shown). Comparing the maximum intensity of PAF-induced p38 activation with that of LPS demonstrated that PAF at 10−5 mol/L activated p38 kinase slightly more than LPS at 0.10 ng/mL (Figure 4).

**PAF-INDUCED p38 ACTIVATION IS CALCIUM DEPENDENT**

Platelet-activating factor induces a rapid increase in intracellular calcium concentration.13,29,30 We investigated whether this increase plays a role in p38 phosphorylation and activation. First, the addition of 2 µmol/L of A23187, a calcium ionophore, to unstimulated HMOs induced rapid p38 phosphorylation and activation (data not shown). Level of activation was by a factor of 4 over the control and, similar to PAF, occurred within 5 minutes. Second, PAF-induced p38 activation was inhibited with 3 mmol/L of ethyleneglycoltetraacetic acid, an extracellular calcium chelator (Figure 5).

**COMMENT**

The present study suggests that, although PAF alone induced only a minimal increase in IL-8 production, it primed the HMO proinflammatory phenotype for significant enhanced production in response to LPS. Similar patterns have been shown to apply to LPS-induced IL-1, tumor necrosis factor α, prostaglandin production by monocytes and macrophages, and superoxide production by neutrophils.13-15,25,31 Platelet-activating factor seems to prime or reprogram the innate immune system to produce enhanced amounts of proinflammatory mediators in response to a second inflammatory stimulus. However, PAF as the sole stimulus is only a weak inducer of cytokine production. Because PAF is an autocrine and paracrine mediator produced by a variety of cells, it may act as a locoregional “alarm” for the innate immune system. In other words, production of PAF at the site...
of inflammation may be a means to alert the inflammatory cells in the vicinity without fully activating them. These cells are now primed or “sensitized” for enhanced production of inflammatory mediators in response to a second stimulus that otherwise would have been insufficient to trigger an inflammatory response. In this manner, even if a small amount of the offending agent, such as bacteria, is able to evade the host defense at the site of inflammation, neighboring cells such as macrophages would readily respond to amplify the host response, contain the infection, and decrease the chance of dissemination. Beneficial effects of priming are apparent as a local phenomenon. However, if generalized, and if inflammatory cells in multiple organ systems are primed, it may become harmful. In such a case, a second stimulus, which would be considered minor by the “unprimed” innate immune system, would induce an aggressive, diffuse, and nonfocused release of inflammatory mediators, and lead to MODS. In fact, results of several animal studies using PAF and various PAF receptor antagonists suggest a role for PAF in sepsis, circulatory shock, and ischemic bowel necrosis. This role might partly be as a priming agent of the innate immune system to subsequent stimuli.

The intracellular signal transduction required for IL-8 production by HMOs is ill-defined. Interleukin-8 is a potent neutrophil chemoattractant, and is reportedly25-26 involved in the pathogenesis of several diseases, including sepsis and acute adult respiratory distress syndrome. Inhibition of IL-8 production by SB202190, a p38 inhibitor, suggests that p38 activation is necessary for IL-8 production by HMOs.21,28,37 Platelet-activating factor, 10−5 mol/L, activated p38 with the same peak intensity as that of LPS, 0.10 ng/mL. However, whereas LPS, 0.10 ng/mL, induced a 25-fold increase in IL-8 production, PAF, 10−5 mol/L, induced minimal IL-8 production. A likely explanation is that, although p38 activation is necessary for IL-8 production, it is not sufficient. Nick et al25 reached the same conclusion for FMLP-induced superoxide production by neutrophils. Lipopolysaccharide seems to activate other pathways necessary for IL-8 production that PAF might not. One such pathway might be through increasing oxygen radical intermediates; antioxidants have been shown30 to inhibit LPS-induced IL-8 production by peripheral blood mononuclear cells. Further study in this area is required.

Another possible explanation for the lack of significant PAF-induced IL-8 production is that, although the peak intensities in p38 activation are similar for PAF, 10−5 mol/L, and LPS, 0.10 ng/mL, the duration and pattern of activation are different. Platelet-activating factor activation of p38 is early, with a rapid decline. For LPS, latency to peak p38 activation was longer, with a slower decline. A specific pattern of p38 activation might be necessary for optimal IL-8 production by HMOs.

Can an increase in p38 activation by PAF explain the priming of HMOs for IL-8 production? We demonstrate that PAF induces p38 activation in HMOs; it has also been shown to induce p38 activation in neutrophils, epithelial cells, and neurons.25,26,39,40 Inhibitors of p38 such as SB202190 and SB203580 inhibit LPS-induced IL-8 production by HMOs efficiently in PAF-primed and PAF-unprimed cells. Because it seems that p38 activation is necessary for LPS-induced IL-8 production, the data are supportive but not conclusive regarding the involvement of this pathway in the priming of the HMO.

The calcium-dependent messenger system might play an important role in PAF-induced p38 activation in HMOs. Platelet-activating factor induces a rapid increase in calcium influx from the extracellular space in monocytes and macrophages.13,29,30 Ethylenglycoltetraacetate acid, an extracellular calcium chelator, inhibited PAF-induced p38 activation in HMOs. Moreover, using a calcium ionophore, we demonstrated that nonmediator increases in the intracellular calcium can induce p38 activation in the HMO. It seems that the increase in free cytosolic calcium concentration is essential for PAF-induced p38 activation.
The generalized release of cytotoxic mediators by host immune cells can cause diffuse organ injury, and lead to MODS and acute adult respiratory distress syndrome. Understanding the mechanisms underlying the immune responses, including intracellular signal transduction, might be necessary to focus therapeutic interventions for restoring balance in the critically ill patient who faces multiple complications. Interleukin 8 is a potent neutrophil chemoattractant, and is reportedly involved in the pathogenesis of sepsis and acute adult respiratory distress syndrome. We demonstrate that, although p38 activation—an important intracellular signal transduction pathway—is necessary for IL-8 production, it is not sufficient. We also demonstrate the importance of PAF in priming monocytes for enhanced IL-8 production, and discuss the possible role of calcium-dependent p38 activation in priming. It is important to identify the “primed” patient who is at greatest risk for MODS. However, in order for therapeutic intervention with anti-inflammatory agents to be effective, treatment should be administered before the patient manifests the clinical symptoms of MODS. We propose evaluating the activation state of signal transduction pathways involved in the inflammatory response, such as p38, as a method for early selection of patients who will benefit optimally from treatments with anti-inflammatory agents.

Anti-inflammatory agents, including PAF receptor antagonists, anti-tumor necrosis factor antibodies, and IL-1 receptor antagonists, have not demonstrated an improvement in survival in patients with MODS and sepsis. Considering a model in which extensive priming of the host inflammatory response has occurred, 2 major issues become apparent: the timing and selection criteria for the treatment of patients. In surgical patients, it is neither practical nor, hopefully, essential to deliver anti-inflammatory agents before the priming stimulus; to be effective, these agents must be administered before the second stimulus. Because host immune cells do not produce inflammatory mediators before the second stimulus, patients at risk for MODS or sepsis might not demonstrate any physiologic symptoms. Therefore, using physiological variables or serum inflammatory mediator levels as selection criteria for treatment might not be appropriate. Also, because inflammation is necessary for many processes, including healing, treating all patients in the intensive care unit with anti-inflammatory treatment might not be appropriate. Measures to identify patients at greatest risk for MODS and sepsis. Elucidating and evaluating the activation state of signal transduction pathways involved in the inflammatory response such as p38 might prove to be a valid method to select patients who will benefit optimally from treatment with anti-inflammatory agents.

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Corresponding author: Saman Arbabi, MD, Department of Surgery, University of Washington, 1959 NE Pacific St, Campus Box 356410, Seattle, WA 98195 (e-mail: sarbabi@u.washington.edu).

REFERENCES

DISCUSSION

Jorge L. Rodriguez, MD, Minneapolis, Minn: This study suggests that, in normal donor macrophages or monocytes, PAF alone induces a minimal increase in IL-8 production, yet it acted as a priming agent in human monocytes for a significant, enhanced LPS response. I think this pattern has been demonstrated by the authors before, but also has been demonstrated for IL-1 and prostaglandins for human monocytes, and also superoxide production of neutrophils. Second, this priming PAF effect on LPS IL-8 production was inhibited by a specific p38 inhibitor. Third is that the priming event is related to p38 activation. The one West

Dr Arbabi: I agree with you completely.

Carl Hauser, MD, Newark, NJ: There are many ways that you can prime cells, and it is vitally important to look at the system as a whole. It is not only the production of IL-8 that is important, it is its effect at the end cell, which in this case, is the neutrophil. And I would point out that some of the work we are doing right now suggests that PAF also acts to prime the response of neutrophils to IL-8. So we’re dealing with more than just a simple 1-way priming phenomenon here: this is an interdependent system involving 2 cell types.

Dr Arbabi: I agree with you 100%. This is a wide variety. Multiple studies have shown that PAF does different things and, in most cases, acts as a priming agent for neutrophils, monocytes, macrophages, and even bronchial epithelial cells and other cell lines. So I agree with you completely.

Ori D. Rotstein, MD, Toronto, Ontario: You suggest here that the priming event is related to p38 activation. The one West

Dr Arbabi: First, let me comment on your last point, that that is definitely a possibility, that PAF may do this. I can tell you that it doesn’t do it through other mitogen-activated protein kinases, ERK, and JNK, but that is definitely a possibility. It really depends on the timing of PAF and LPS, but generally does potentiate it, and we are currently doing more study in that area.

need more than 1 pathway in the mitogen-activated protein kinase family to get maximal production of cytokines. There are many papers, including some from our lab, demonstrating that you would require other pathways, such as extracellular signal-regulated kinase (ERK) and c-jun NH2-terminal kinase (JNK). We did look at ERK activation, and neither LPS nor PAF activated ERK in the nonadherent mononuclear cells that we used. When you adhere them, the story becomes different. So that was not one of the other pathways.

We believe the pathway that may be responsible is the p38 pathway. Lipopolysaccharide is known to produce significant radical oxidants, and it has been shown by Remick et al that, in using antioxidants, you may inhibit IL-8 production in whole blood. So that pathway may be one of the pathways, and we have to investigate that further.

For your second question, the latency period was very interesting. p38 activation with PAF was within 5 minutes. It probably was even less, but it is hard to measure when it is less than 5 minutes with Western blot. And this really depends on calcium; without calcium, this does not happen. Lipopolysaccharide takes 30 minutes, and it takes longer to come down. For PAF, it peaks up and comes down very fast. So maybe the difference is in the pattern of activation for IL-8 production and not that other pathways are necessary.

Regarding looking at other monocytes in injured patients, no, we did not look at monocytes in injured patients to see if there is any different response. Interestingly, we have seen different responses with different populations, although this is just a preliminary study and we haven’t done extensive study on it. Of interest to this group would be that, in young women, PAF hardly activated p38, whereas in males and in an older population, it did. So there is a different response in a different population, even the ones that are not injured.

Carol L. Miller-Graziano, MD, Worcester, Mass: There is quite a bit of evidence in the literature, also in regard to IL-8, that the JNK pathway actually increases translation of a number of cytokines, and so I am wondering if you noticed if there was any activation of phosphor JNK?

Dr Arbabi: With the nonadherent cells, we don’t get any activation of JNK. We have not looked at it with adherent cells, and they do differ. That is why I emphasize they become different when they become adherent; but no, the answer is no.

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Dr Arbabi: First, let me comment on your last point, that that is definitely a possibility, that PAF may do this. I can tell you that it doesn’t do it through other mitogen-activated protein kinases, ERK, and JNK, but that is definitely a possibility. It really depends on the timing of PAF and LPS, but generally does potentiate it, and we are currently doing more study in that area.

In your manuscript, you elucidated that LPS appears to activate other pathways necessary for IL-8 production. Could you expand on this and their relationship to your PAF production and p38 activation?

Second, you demonstrated a difference in peak intensity and length of time of p38 activation between PAF and LPS. Could you comment on the significance of these distinct patterns, and what are the regulatory molecular implications of this process?

And last, in 1996, our group reported that, in injured patients utilizing a whole blood model, the production of tumor necrosis factor, IL-1, and IL-8 were significantly greater to an external stimulus—FMLP, LPS, or immune complexes—when compared with a control group. Have you evaluated the monocytes from injured patients in regards to PAF and priming of IL-8 and the role of p38? I think this is a nice piece of work.

Dr Arbabi: For your first question regarding the other pathways that LPS may activate, there are suggestions that you may