Lipoteichoic Acid-Induced Nitric Oxide Production Depends on the Activation of Platelet-Activating Factor Receptor and Jak2

Seung Hyun Han, Je Hak Kim, Ho Seong Seo, Michael H. Martin, Gook-Hyun Chung, Suzanne M. Michalek and Moon H. Nahm

*J Immunol* 2006; 176:573-579; doi: 10.4049/jimmunol.176.1.573
http://www.jimmunol.org/content/176/1/573

**References**

This article **cites 62 articles**, 31 of which you can access for free at:
http://www.jimmunol.org/content/176/1/573.full#ref-list-1

**Subscription**

Information about subscribing to *The Journal of Immunology* is online at:
http://jimmunol.org/subscription

**Permissions**

Submit copyright permission requests at:
http://www.aai.org/About/Publications/JI/copyright.html

**Email Alerts**

Receive free email-alerts when new articles cite this article. Sign up at:
http://jimmunol.org/alerts
Lipoteichoic Acid-Induced Nitric Oxide Production Depends on the Activation of Platelet-Activating Factor Receptor and Jak2

Seung Hyun Han,* Je Hak Kim,* Ho Seong Seo,** Michael H. Martin,** Gook-Hyun Chung,* Suzanne M. Michalek,† and Moon H. Nahm2*†

NO production by macrophages in response to lipoteichoic acid (LTA) and a synthetic lipopeptide (Pam3CSK4) was investigated. LTA and Pam3CSK4 induced the production of both TNF-α and NO. Inhibitors of platelet-activating factor receptor (PAFR) blocked LTA- or Pam3CSK4-induced production of NO but not TNF-α. Jak2 tyrosine kinase inhibition blocked LTA-induced production of NO but not TNF-α. PAFR inhibition blocked phosphorylation of Jak2 and STAT1, a key factor for expressing inducible NO synthase. In addition, LTA did not induce IFN-β expression, and p38 mitogen-activated protein serine kinase was necessary for LTA-induced NO production but not for TNF-α production. These findings suggest that Gram-positive bacteria induce NO production using a PAFR signaling pathway to activate STAT1 via Jak2. This PAFR/Jak2/STAT1 signaling pathway resembles the IFN-β, type 1 IFN/Jak/STAT1 pathway described for LPS. Consequently, Gram-positive and Gram-negative bacteria appear to have different but analogous mechanisms for NO production. The Journal of Immunology, 2006, 176: 573–579.

During bacterial infections, bacterial constituents such as LPS can induce the production of various host factors (1), which can, in turn, cause multiorgan dysfunction. This medical condition often results in intractable hypertension, which is a significant cause of death for patients in intensive care units (2). Host factors associated with multiorgan dysfunction include increased levels of proinflammatory cytokines such as TNF-α, IL-1β, IL-6, and IL-8 (3), and glycerophospholipids (4) such as platelet-activating factor (PAF).3 TNF-α has been labeled “the hub of the cytokine network” (3) because it is sufficient to cause septic symptoms in animals (5) and because Abs to TNF-α protect animals in sepsis models (6, 7). PAF is elevated in patients with septic shock (8) and can cause hypotension in animals (9). It exerts its inflammatory properties by binding to a member of the 7-transmembrane receptor family (4) which then stimulates cells through mostly, but not always (10), G proteins.

NO has been implicated as another key host factor in sepsis for several reasons. First, excess amounts of NO are produced during sepsis (11). Second, NO can activate the myosin phosphatase and potassium channels in arterial smooth muscle cells, thereby causing vasodilation and hypotension (12). Third, inhibitors of NO synthesis have been shown to be beneficial for patients with severe sepsis (13) or for animals with various experimental infections (14–16). Lastly, animals do not become hypotensive after an LPS exposure if they are deficient in inducible NO synthase (iNOS) (17), an enzyme critical to the excessive production of NO.

Understanding the mechanisms for NO production during infections is an important goal for the prevention of sepsis. During Gram-negative sepsis, the LPS reacts with TLR4 and elicits the expression of IFN-β using a signaling pathway independent of the MyD88 gene (1, 18, 19). IFN-β then activates various transcription factors, including STAT1 (20–22) that is necessary for iNOS gene expression (23). Activation of STAT1 by IFN-β requires phosphorylation of both tyrosine and serine residues (21), with the phosphorylation being mediated by Tyk2/Jak kinases (20, 24), p38 MAPKs (25, 26), and various other kinases (27, 28).

The production of NO during Gram-positive bacterial infection is an enigma, even though half of the microbiologically confirmed cases of sepsis are due to infections by Gram-positive bacteria (29). Unlike Gram-negative bacteria, Gram-positive bacteria primarily stimulate innate immunity, not by TLR4 but by TLR2 (30). All TLR2 signaling is mediated by the MyD88 gene (19, 31), and various TLR2 ligands (such as peptidoglycan (PGN) and bacterial lipoproteins) have been reported not to elicit the production of IFN-β (1, 18) and NO (1, 32). Indeed, Gram-positive bacteria do not appear to elicit IFN-β production in mice (33) even though they can induce NO production (34). Because lipoteichoic acid (LTA) may induce NO production (34, 35), we have investigated NO production using LTA from pneumococci and staphylococci, which account for the majority of cases of Gram-positive sepsis (29).

Materials and Methods

Reagents and cells

Nov-nitro-L-arginine methyl ester hydrochloride (N-nitro-L-NAME), Nov-nitro-D-arginine methyl ester hydrochloride (D-nitro-NAME), E. coli LPS (055:B5) were obtained from Sigma-Aldrich. LPS was repurified by phenol extraction (36) before use. 2-Iminopiperidine hydrochloride, an iNOS-specific inhibitor (37), was purchased from Biorendt. Pertussis toxin, AG1478, AG490, SB203580, SP600125, and PD98059 were purchased from Calbiochem. PAF inhibitors (CV6209 and CV3988) and G-protein antagonist 2A were purchased from BIOMOL. All the reagents for RT-PCR were purchased from Promega, except for recombinant TagDNA Polymerase 5 from New England Biolabs. The Journal of Immunology, 2006, 176: 573–579.
polymerase (Taq) and dNTP, which were purchased from Takara Bio. Rabbit polyclonal Abs specific for p38, the phosphorylated forms of p38, Erk1/2, and stress-activated protein kinase (SAPK)/JNK were obtained from Cell Signaling Technology. Rabbit Abs to phosphorylated Jak2, STAT1 with phosphorylated serine at 727, and STAT1 with phosphorylated threonine at 701 along with HRP-conjugated anti-rabbit IgG were also obtained from Cell Signaling Technology. Mouse macrophage-like cell line RAW 264.7 (TIB-71) was purchased from the American Type Culture Collection.

Preparation of LTA

Highly purified and structurally intact pneumococcal LTA (PnLTA) and staphylococcal LTA (StLTA) were prepared from nonencapsulated pneumococci R36A and Staphylococcus aureus (ATCC 6538; American Type Culture Collection), respectively, by organic solvent extraction, Octyl-Sepharose and ion-exchange chromatography, as we have previously described (38–40). Our LTA preparations had <5 pg of endotoxin/mg LTA. Additional studies of the purity of our LTA preparations have been reported (40).

Culture of RAW 264.7 cells

RAW 264.7 cells were cultured with DMEM (Cellgro/Mediatech) supplemented with 10% FBS (HyClone), 2 mM L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin at 37°C in a 5% CO₂ humidified incubator. They were stimulated with various stimulants at 5 × 10⁵ cells/ml each.

Stimulation of bone marrow macrophages

C57BL/6 mice were obtained from The Jackson Laboratory, and TLR2-deficient mice on a C57BL/6 background were bred in our animal facility using the breeding pairs from Dr. S. Akira (Osaka University, Osaka, Japan) with an Institutional Review Board (IRB) approval. Bone marrow cells were harvested from the tibia of 6- to 8-wk-old C57BL/6- or the TLR2-deficient mice using an IRB-approved protocol. Bone marrow cells (10⁶ cells/ml) were suspended in DMEM supplemented with 10% FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml M-CSF (R&D Systems), and were cultured for 7 days. The adherent cells were harvested with trypsin-EDTA, washed with PBS, and suspended in DMEM containing 5% FCS. Two hundred microliters of the cell suspension (2 × 10⁶ cells/well) was placed in a 96-microwell plate (Corning Glass) and stimulated with various stimulants at 5 × 10⁵ cells/ml each.

Western blotting

RAW 264.7 cells (5 × 10⁵ cells/ml, 10 ml) were plated onto a 100-mm tissue-culture dish in serum-free DMEM supplemented with antibiotics (100 U/ml penicillin and 100 μg/ml streptomycin) for 3 h, washed, and resuspended in fresh serum-free DMEM supplemented with antibiotics for 1 h. The cells were exposed to an agonist (e.g., CV6209) for 1 h before being stimulated with 50 μg/ml PnLTA, 5 μg/ml StLTA, or 1 μg/ml LPS for 30 min (or the indicated time periods). Cells were then washed with PBS and lysed with radioimmunoprecipitation assay buffer (Upstate Biotechnology), as recommended by the manufacturer. Twenty micrograms of the whole-cell lysate were separated by 10% SDS-PAGE and electrotransferred to a polyvinylidene difluoride membrane (Millipore). The membrane was incubated with a blocking buffer (5% BSA/1× TBS/0.1% Tween 20) at room temperature for 1 h and then was kept on ice overnight with the same buffer containing rabbit polyclonal Abs for the MAPKs (or STAT1). After washing three times with TBST (1× TBS/0.1% Tween 20), the membrane was incubated with HRP-conjugated anti-rabbit IgG in the blocking buffer at room temperature for 1 h. Then, after washing three times with TBST, the immunoreactive bands were detected with ECL reagents (Amersham Biosciences).

Results

PnLTA induces NO production by murine macrophages

Cultures of the murine macrophage cell line RAW 264.7 were stimulated with various concentrations (1–100 μg/ml) of highly purified PnLTA for various time periods. The highly purified PnLTA was prepared using ion-exchange chromatography as well as hydrophobic interaction chromatography (38). Our LTA preparations had <5 pg of endotoxin/mg LTA and had undetectable amounts of other contaminants (e.g., DNA or protein) (40). Maximal NO production required 48 h (Fig. 1A), while maximal TNF-α production was observed in only 15 h (Fig. 1B). Consequently, in all subsequent experiments, NO production was measured after 48 h of stimulation and TNF-α after 15 h.

To examine the ability of LTAs from other Gram-positive bacteria to induce NO production, we stimulated RAW 264.7 cells with various concentrations of StLTA, PnLTA, and E. coli LPS and assessed NO production at 48 h (Fig. 1C). All three stimuli induced NO production but with different potencies. Half-maximal stimulations were achieved at ~0.01 μg/ml LPS, 2 μg/ml StLTA, and 50 μg/ml PnLTA. This indicates that, while both LTAs can stimulate NO production, PnLTA is less potent than StLTA, which in turn are less potent than LPS.

LTA does not induce IFN-β in murine macrophages

It has been reported that LPS stimulates NO production by inducing the expression of an autocrine stimulator, IFN-β (1), but many...
TLR2 stimulants such as a lipopeptide (Pam3CSK4) do not induce IFN-β (1). When we examined IFN-β mRNA expression, LPS induced the expression of IFN-β mRNA (Fig. 2A), but PnLTA and StLTA did not. However, all three stimuli (LPS, StLTA, and PnLTA) stimulated the production of iNOS mRNA (Fig. 2A). Furthermore, the PnLTA-induced NO production was almost completely suppressed by l-NAME but not by d-NAME (Fig. 2B). Also, NO production by StLTA, PnLTA, and LPS could be inhibited by iminopiperidine (Fig. 2C). l-NAME and iminopiperidine are inhibitors of iNOS; l-NAME can inhibit various isoforms of NOS but iminopiperidine is an iNOS-specific inhibitor (37). Thus, NO production is dependent on iNOS, and LTA, unlike LPS, can induce iNOS expression in the absence of IFN-β induction.

PAFR and TLR2 are involved in NO production by LTA
PnLTA resembles PAF in structure (42), and StLTA has been reported to stimulate PAFR to induce mucin gene expression by epithelial cells (43). In view of these observations, we investigated the involvement of PAFR in LTA-induced NO production. The PAFR inhibitor CV6209 did not inhibit NO production induced by LPS and CV3988 slightly reduced NO production by LPS (Fig. 3, C and F); however, they almost completely (>90%) suppressed NO production induced by either pneumococcal or staphylococcal LTA (Fig. 3, A, B, D, and E). In addition, the two PAFR inhibitors did not inhibit TNF-α production induced by any of the three stimulators (Fig. 3).

Although we found PAFR involvement in LTA-induced NO production, LTA is a TLR2 ligand; therefore, we used TLR2-deficient mice to determine whether TLR2 is necessary for NO production. We obtained bone marrow macrophages from C57BL/6 mice or TLR2-deficient mice and stimulated the marrow cells with StLTA, PnLTA, and LPS in two separate experiments (Table I). StLTA was able to induce normal macrophages but not TLR2-deficient macrophages to produce NO (*p < 0.02). Also, PnLTA induced NO production by normal macrophages but not by TLR2−/− mouse macrophages (*p = 0.0025). In contrast, LPS induced NO production in both normal and TLR2-deficient macrophages (*p = 0.39). These findings shown in Fig. 3 and Table I

![FIGURE 2.](http://www.jimmunol.org/)

**FIGURE 2.** A, LTA induces mRNA synthesis for iNOS and TNF-α but not for IFN-β. RAW 264.7 cells were stimulated with PnLTA, StLTA, or *E. coli* LPS for 3 h at the indicated concentrations. RT-PCR products of IFN-β, iNOS, TNF-α, and β-actin mRNA were separated in an agarose gel and visualized by ethidium bromide staining. B, NO production by the cells stimulated with PnLTA can be suppressed with a NO synthase inhibitor (l-NAME, ■) but not with an inert control molecule (d-NAME, □). **C**, Significant reduction (*p < 0.001) in nitrite levels by inhibitors. **C**, NO production by the cells stimulated with PnLTA can be suppressed with 2-IPD (iminopiperidine), an iNOS-specific inhibitor.

![FIGURE 3.](http://www.jimmunol.org/)

**FIGURE 3.** PAFR signaling is necessary for LTA induction of NO but not of TNF-α. RAW 264.7 cells were pretreated with varying concentrations of a PAFR antagonist for 1 h and then stimulated with (A and D) PnLTA, (B and E) StLTA, or (C and F) *E. coli* LPS for 16 or 48 h for assessment of TNF-α production or of NO production, respectively. PAFR antagonists were (A–C) CV6209 and (D–F) CV3988. At the end of stimulation, the amounts of nitrite (bar) and TNF-α (line) in the culture media were quantified. *, *p < 0.05 and ***, *p < 0.001 when the levels produced in the presence of an inhibitor are compared with those without an inhibitor.
indicate that TLR2 and PAFR stimulations are necessary for LTA-induced NO production. Also, these findings indicate that NO production is not due to LPS contaminating our LTA preparations.

A synthetic lipopeptide, Pam3CSK4, induces NO production just as LTA does.

To investigate whether another TLR2 ligand can induce NO production, we stimulated RAW 264.7 cells with Pam3CSK4, a chemically synthesized lipopeptide that mimics bacterial lipoprotein. Pam3CSK4 induced both TNF-α and NO production in a dose-dependent manner, with maximal responses seen at doses >40 ng/ml (Fig. 4A). To investigate the role of PAFR in this lipopeptide-induced NO production, RAW 264.7 cells were stimulated with Pam3CSK4 in the presence of one of two PAFR antagonists (CV6209 or ABT-491). ABT-491 was tested because it is not a PAF analog like CV6209 and its inhibition mechanism is different from CV6209 (44, 45). Production of NO, but not of TNF-α, was significantly reduced in the presence of CV6209 (Fig. 4B) or ABT-491 (Fig. 4C). The inhibition was achieved at inhibitor doses commonly used by others (44, 45). In summary, Pam3CSK4 stimulates RAW 264.7 cells to produce NO just as LTA does.

A different pathway of PAFR stimulation is used for NO production by LTA

StLTA has been shown to directly stimulate epithelial cells to produce mucin via PAFR, with epidermal growth factor receptor (EGFR) and G proteins shown to be the signaling intermediates (43). Furthermore, G proteins have been shown to mediate most of the known PAFR-signaling pathways (4). Therefore, we investigated the involvement of EGFR and G proteins in NO production. A G-protein inhibitor (pertussis toxin) and an EGFR inhibitor (AG1478) failed to block LTA-induced NO or TNF-α production (Fig. 5). In addition, two different G-protein inhibitors (GDP-βS and G-protein antagonist 2A) did not block NO or TNF-α production (data not shown). These findings suggest that LTA stimulates murine macrophages to produce NO using a PAFR signaling pathway that is different from the one previously suggested for inducing mucin expression (43).

Jak2 and p38 MAPK antagonists block production of NO but not of TNF-α

Without involving G proteins, PAFR has been shown to be able to activate Jak2 and Tyk2 tyrosine kinases directly and phosphorylate STAT1 (10, 46), which is a transcription factor important in iNOS gene expression (23). Thus, we examined the effect of a Jak2 inhibitor (AG490) on TNF-α and NO production. The inhibitor suppressed LTA-induced NO production without suppressing TNF-α.

### Table I. Effect of TLR2 on NO production

<table>
<thead>
<tr>
<th>Stimulus</th>
<th>Mice</th>
<th>p Value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>C57BL/6 mice</td>
<td>TLR2&lt;sup&gt;−/−&lt;/sup&gt; mice</td>
</tr>
<tr>
<td>Expt. 1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>0.31 (0.04)&lt;sup&gt;b&lt;/sup&gt;</td>
<td>0.27 (0.20)</td>
</tr>
<tr>
<td>StLTA (10 μg/ml)</td>
<td>0.76 (0.19)</td>
<td>0.24 (0.15)</td>
</tr>
<tr>
<td>Expt. 2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>&lt;0.49 (0.05)</td>
<td>&lt;0.49 (0.00)</td>
</tr>
<tr>
<td>PnLTA (50 μg/ml)</td>
<td>3.70 (0.32)</td>
<td>&lt;0.49 (0.00)</td>
</tr>
<tr>
<td>LPS (1 μg/ml)</td>
<td>13.89 (2.41)</td>
<td>12.29 (1.31)</td>
</tr>
</tbody>
</table>

<sup>a</sup> p values were obtained with nonpaired t test by comparing the normal mouse group with the TLR2-deficient mouse group.

<sup>b</sup> Average amount of nitrate (in micromoles) with standard deviation (in parenthesis) are shown. There are three samples per each test.

### FIGURE 4

A synthetic lipopeptide, Pam3CSK4, induces NO production, and NO production requires PAFR signaling. A, RAW 264.7 cells were stimulated with Pam3CSK4 at the indicated concentrations for 16 or 48 h for assessment of TNF-α production (C) or of NO production (■), respectively. B and C. The cells were pretreated with varying concentrations of CV6209 or ABT-491 for 1 h and then stimulated with the Pam3CSK4 for 16 or 48 h for assessment of TNF-α production (lines) or of NO production (bars), respectively. ***, Significant (p < 0.001) reduction in NO production by an inhibitor.

### FIGURE 5

LTA signaling for NO or TNF-α production does not involve G proteins or EGFR. RAW 264.7 cells were incubated with (A) pertussis toxin or (B) AG1478 for 1 h and then stimulated with StLTA, PnLTA, or E. coli LPS for 48 h for the NO assay and 15 h for the TNF-α assay. At the end of the culture period, the media were analyzed for the levels of TNF-α (lines) and nitrite (bars).
production (Fig. 6A). Similarly, it suppressed LPS-induced NO production without affecting TNF-α production (Fig. 6A). The suppression of NO production appears to be more marked for LTA than for LPS.

Because p38 MAPKs are important in autologous PAF production (47) and phosphorylating the serine residue of STAT1 (25, 26), we examined MAPKs for their involvement in NO or TNF-α production. RAW 264.7 cells were stimulated with PnLTA in the presence of inhibitors for p38, JNK, and Erk MAPK pathways (SB203580, SP600125, and PD98059, respectively). All three inhibitors could suppress NO production (>75%), whereas the inhibitors of JNK and Erk inhibited TNF-α production (Fig. 6B). Thus, p38 MAPK is involved in only the signaling pathway used for iNOS gene expression.

**PAFR inhibitor blocks LTA-induced phosphorylation of STAT1 and Jak2**

To further confirm the involvement of Jak2 and STAT1 downstream of PAFR, we examined the effect of a PAFR inhibitor on Jak2 and STAT1 phosphorylation. STAT1 from unstimulated RAW 264.7 cells was not phosphorylated (Fig. 7A, lane 1). When the cells were stimulated with either PnLTA (10 μg/ml) or StLTA (1 μg/ml), STAT1 was phosphorylated within 2 h at both serine (residue 727) and tyrosine (residue 701) (Fig. 7A, lanes 2 and 3). However, when the cells were stimulated with LTA in the presence of a PAFR antagonist (CV6209), tyrosine phosphorylation was almost completely abolished and serine phosphorylation was significantly reduced (Fig. 7A, lanes 4 and 5). This variation was observed even though comparable amounts of protein were in each lane, as shown by the even staining for “Total p38” (Fig. 7). Similar to STAT1, Jak2 from unstimulated cells was only weakly phosphorylated (Fig. 7B, lane 1), and StLTA (1 μg/ml) stimulation increased Jak2 phosphorylation (Fig. 7B, lanes 2–4). Jak2 phosphorylation was most intense after a 30-min incubation and became less afterward. However, when a PAF antagonist (CV6209) was present, LTA stimulation did not increase the Jak2 phosphorylation for any of the incubation periods (Fig. 7B, lanes 5–7). Thus, LTA-induced phosphorylation of Jak2 and STAT1, especially at the STAT1 tyrosine residue, depends on PAFR signaling.

**Discussion**

Because TLR2 ligands do not induce the expression of IFN-β that is important in LPS-induced NO production (1), it is possible that biologically active contaminants present among TLR2 ligands rather than the TLR2 ligands themselves are responsible for the observed induction of NO by TLR2 ligands. For example, LTA was widely reported to induce NO production (34, 48, 49), but many studies used a commercially available LTA preparation (34, 35, 48, 49), a preparation that is now known to be contaminated with LPS (50). We show here that highly purified pneumococcal and staphylococcal LTAs as well as Pam3CSK4 can induce NO production. Because chemically synthesized Pam3CSK4 was used, it should be free from biological contaminants. Also, our LTA preparations had undetectable amounts of LPS, did not induce IFN-β production, and induced NO production only when TLR2 was available. Taken together, our data indicate that some TLR2 ligands can induce NO production without inducing IFN-β production.

Our studies with TLR2-deficient mice also show that TLR2 stimulation is essential for LTA-induced NO production. The TLR2 stimulation should provide NF-κB activation, which was shown to be required for iNOS gene expression (48, 49, 51). In addition, we found that several PAFR inhibitors can block NO production, but not TNF-α production, in response to both

---

**FIGURE 6.** Intracellular signal transduction for LTA-induced NO vs TNF-α production. A, RAW 264.7 cells were stimulated with PnLTA (50 μg/ml), StLTA (1 μg/ml), or E. coli LPS (0.1 μg/ml) in the presence of varying concentration of a Jak2 inhibitor (AG490). Culture supernatants were harvested after 16 h for the TNF-α determination or after 48 h for the nitrite determination. B, RAW 264.7 cells were pretreated with specific inhibitors for p38 kinase (SB203580), SAPK/JNK (SP600125), or Erk (PD98059) for 1 h and then stimulated with PnLTA (50 μg/ml) for 16 h for the TNF-α determination or 48 h for the NO determination. At the end of the culture period, the media were analyzed for TNF-α and nitrite levels. *****, A significant (p < 0.001) change in TNF-α or nitrite levels due to an inhibitor.

**FIGURE 7.** Effect of PAFR inhibitor on LTA-induced phosphorylation of STAT1 and Jak2. A, RAW 264.7 cells were treated with (lane 1) none, (lanes 2, 4, and 6) PnLTA, or (lanes 3, 5 and 7) StLTA. Cells were treated with (lanes 4 and 5) a PAFR inhibitor or with (lanes 6 and 7) DMSO alone (0.01%). The PAFR inhibitor is CV6209 (1 μM) in DMSO (0.01%). Phosphorylated serine at position 727 is shown at the top, phosphorylated tyrosine at position 701 in the middle, and the “Total p38” at the bottom. B, RAW 264.7 cells were treated with (lanes 1) none, (lanes 2–4) StLTA (5 μg/ml), or (lanes 5–7) both StLTA (5 μg/ml) and CV6209 (1 μM) for the indicated time periods. Jak2 phosphorylation is shown at the top, and the “Total p38” is at the bottom.
Pam3CSK4 and LTA. These findings indicate that PAFR stimulation provides additional signaling necessary for iNOS gene expression. To investigate this possibility, we examined involvement of PAFR on the activation of STAT1, which is another transcription factor required for iNOS gene expression (51) and is often limiting (23). LTA stimulation results in rapid phosphorylation of Jak2 and STAT1, and inhibition of PAFR prevents their phosphorylation. Thus, our data suggest that LTA and Pam3CSK4 need both PAFR and TLR2 to elicit NO production and that PAFR stimulation activates STAT1 via Jak2.

It is likely that PAFR directly activates Jak2 without involving G proteins because LTA-induced NO production is blocked by a Jak2 inhibitor but not by several G-protein inhibitors. This possibility is greatly enhanced since recent studies of PAF-stimulated human monocytes have shown that PAFR can directly activate Jak2 without involving G proteins and that the activated Jak2 then leads to STAT activation (10, 46). Nevertheless, our studies are primarily based on chemical inhibitors of enzymes or receptors, which may have limitations in their specificities. Also, STAT1 activation requires phosphorylation at tyrosine (residue 701) and serine (residue 727), and serine kinase needs to be identified. p38 MAPKs are potential candidates because they participate in LPS stimulations (25, 26). Thus, further studies are needed to elucidate the mechanism of PAFR-mediated STAT1 activation.

The PAFR pathway may be analogous to the IFN-β, type I IFN pathway described in responses to LPS and may be widely used in inflammatory reactions to Gram-positive bacterial infections. For instance, this pathway may be involved in NO production during Gram-positive bacterial infections, which do not elicit IFN-β responses. Indeed, we observed that culture supernatants of various Gram-positive bacteria can induce NO production and that the NO production can be inhibited with various PAFR antagonists (our unpublished data). Also, this PAFR pathway may be involved in the expression of various inflammation-associated molecules (e.g., GARG16, CXCL10, and CXCL12), which were shown with LPS-stimulated cells to depend on endogenous IFN-β and STAT1 activation (52, 53). Since LPS stimulation has been shown to also induce PAF production (54, 55), the PAFR pathway may be operative in Gram-negative bacterial infections as well. However, the PAFR pathway may be more prominent in Gram-positive bacterial infections, particularly in Gram-positive infections, particularly in Gram-positive infections.

It has been proposed that PAFR directly stimulates the iNOS gene expression (51) and is often limiting (23). LTA stimulation results in rapid phosphorylation of Jak2 and STAT1, and inhibition of PAFR prevents their phosphorylation. Thus, our data suggest that LTA and Pam3CSK4 need both PAFR and TLR2 to elicit NO production and that PAFR stimulation activates STAT1 via Jak2.

References


