Platelet-activating factor (PAF), a phospholipid-derived messenger molecule, is now recognized as the most proximal mediator of cellular events triggered by bacterial lipopolysaccharide (LPS) stimulation. In this study, we assessed the role of PAF in the disturbances in salivary mucin synthesis evoked by LPS of periodontopathic bacterium, Porphyromonas gingivalis. Using primary culture of mucous acinar cells of sublingual salivary gland, we show that a specific PAF antagonist, BN52020, prevents in a dose-dependent fashion (up to 83.7%) the LPS-induced reduction in mucin synthesis, and the effect is reflected in a marked decrease in the LPS-induced apoptosis (74.8%), NO generation (82.6%), and the expression of TNF-α (76.1%). The impedance by BN52020 of the LPS inhibitory effect on mucin synthesis was blocked by wortmannin, an inhibitor of phosphatidylinositol 3-kinase (PI3K), which also obviated the inhibitory effect of BN52020 on the LPS-induced upregulation in apoptosis, TNF-α, and NO. A potentiation in the impedance by BN52020 of the LPS detrimental effect on mucin synthesis was however attained with NOS-2 inhibitor, 1400W, while cNOS inhibitor, L-NNA caused a reduction in the impedance effect of BN52020. However, while 1400W and BN52020 countered the potentiating effect of wortmannin on the LPS-induced decrease in mucin synthesis, a further exacerbation of the effect of wortmannin occurred in the presence of L-NNA. The findings implicate PAF as a pivotal factor affecting the extent of pathological consequences of P. gingivalis infection on salivary glands capacity for mucin production, and suggest that its release in response to the LPS serves as a negative regulator of PI3K controlling the pathway of cNOS activation.

**Key words:** P. gingivalis LPS; salivary mucin synthesis; PAF; PI3K; cNOS.
INTRODUCTION

Porphyromonas gingivalis is a Gram-negative bacterium found in periodontal packets of patients with periodontitis, a chronic inflammatory disease that affects 10 - 15% of adult population (1, 2). Moreover, implantation of the bacterium into periodontal microbiota leads to the formation of periodontal lesions, and its cell wall lipopolysaccharide (LPS) has been implicated as a key factor in the development of inflammatory responses that characterize periodontitis (3 - 5). Indeed, the pathogenic effects of P. gingivalis LPS on soft oral tissue integrity are manifested by the increase in proinflammatory interleukin (IL-1, IL-6, and IL-8) production, upregulation in TNF-α, excessive NO generation, and a massive rise in epithelial cell apoptosis (4, 5). Furthermore, P. gingivalis LPS, through the induction in TNF-α and apoptosis, has been shown to exert the inhibitory effect on the synthesis of salivary mucin, thus weakening the protective performance of the saliva-derived mucus coat that constitutes pre-epithelial element of oral mucosal defense (6 - 8).

Although the induction in TNF-α and subsequent activation of the transcriptional factor NF-κB are well documented features of LPS-induced inflammatory responses, the emerging new insight into the pathways of NF-κB activation suggests that the most proximal mediator released in response to LPS and bacterial infection is a phospholipid-derived messenger, platelet-activating factor (PAF) (9, 10). Once released, PAF is known to elicit a rapid induction of TNF-α expression through the activation of NF-κB target genes encoding proinflammatory cytokines, chemokines, growth factors as well as inducible enzymes, including that of NOS-2 (10, 11). Interestingly, the stimulation of NO production, through NOS-2 induction or the exogenous NO donors, has been shown to lead to the enhancement in apoptosis, loss of extracellular matrix proteoglycans, and the inhibition of glycoprotein synthesis (12, 13). The literature data, furthermore, indicate that LPS-induced upregulation in NO production through NOS-2 induction is subject to inhibition by phosphatidylinositol 3-kinase (PI3K) activation, and that pretreatment with wortmannin, a specific inhibitor of PI3K, leads to the enhancement of LPS-induced NOS-2 expression (14, 15). The role of PI3K in mediation of the events that affect glycoprotein synthesis is supported by the finding showing that inhibition of PI3K leads to the induction in NOS-2 activity, enhancement in NO generation, and potentiation of the detrimental effect of P. gingivalis LPS on salivary mucin synthesis (8).

Recently, using an animal model of experimentally induced buccal mucosal ulcer, we have shown that inhibition of PAF activation leads to the suppression of mucosal inflammatory reaction as reflected in a marked decline in apoptosis, NOS-2 activity and TNF-α (16). In this study, using a specific PAF antagonist, BN52020, and primary culture of rat sublingual gland acinar cells, we investigated the interplay of factors involved in the disturbances in salivary mucin synthesis elicited by P. gingivalis LPS.
MATERIALS AND METHODS

Sublingual gland acinar cell incubation

The study was conducted with Sprague-Dawley rats in compliance with the institutional Animal Care and Use Committee. The dissected sublingual salivary glands were trimmed of fat and connective tissue, and minced by passage through a metal grid (13). The minced tissue was suspended in 5 volumes of ice-cold Dulbecco’s modified Eagle’s minimal essential medium (DMEM), supplemented with fungizone (50 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum, and dispersed into single cells and cell clusters by trituration with a glass homogenizer, and settled by centrifugation (13). Following three consecutive rinses with DMEM, the cells were resuspended in the medium to a concentration of 2 x 10^7 cell/ml, transferred in 1 ml aliquots to DMEM in culture dishes containing [3H] glucosamine (100 µCi) (New England Nuclear, Boston, MA), and incubated under 95% O_2-5% CO_2 atmosphere at 37°C for various periods of time up to 20 h in the presence of 0 - 700 ng/ml of P. gingivalis LPS (7). In the experiments on the effect of a specific PAF antagonist, BN52020 (Calbiochem, La Jolla, CA), PI3K inhibitor, wortmannin (Sigma, St. Louis, MO),NOS-2 inhibitor, 1400W (17, 18), (N-(3-Aminomethyl)benzylacetamidine, Calbiochem), and cNOS inhibitor L-NNA (N^G-Nitroso-L-arginine, Calbiochem), the cells prior to the addition of LPS were first incubated for 30 min with the indicated dose of the agent. At the end of the specified incubation period, the cells were centrifuged at 300 g for 5 min, washed three times with phosphate-buffered saline, and the combined supernatants used for [3H] glucosamine labeled mucus glycoprotein assay (19).

Cell viability

Cell preparations before and during the experimentation were assessed for viability and cellular integrity using Trypan blue exclusion assay and the determination of lactate dehydrogenase released into the medium (7).

Mucin analysis

The combined cell wash and incubation medium containing [3H]-labeled mucin were treated at 4°C with 10 volumes of 2% phosphotungstic acid in 20% trichloroacetic acid for 4 h and the formed precipitates were collected by centrifugation (19). The glycoprotein precipitates were dissolved in 6 M urea and chromatographed on Bio-Gel (Bio-Rad, Richmond, CA) A-1.5 column (0.9 x 100 cm). The eluted fractions were monitored spectrophotometrically for protein and for radioactivity by liquid scintillation spectrometry. The mucus glycoprotein fractions eluted in the excluded volume were pooled, dialyzed, lyophilized, and subjected to analysis for total incorporation of radiolabel and for protein content (13, 19).

Porphyromonas gingivalis LPS

P. gingivalis used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 (Rockville, MD) (20). The bacterium was washed with water, treated with ethanol and acetone, dried and homogenized with liquid phenol-chloroform-petroleum ether (8). The suspension was centrifuged, and the lipopolysaccharide contained in the supernatant was precipitated with water, washed with 80% phenol solution and dried with ether. The dry residue was dissolved in a small volume of water at 45°C, centrifuged at 100,000 g for 4 h, and the resulting
LPS sediment subjected to lyophilization. The preparation was essentially free of nucleic acids, and its protein content was less than 0.2% (8).

**Apoptosis and TNF-α assays**

The acinar cells were settled by centrifugation, rinsed with phosphate buffered saline, and incubated in the lysis buffer in accordance with the manufacturer's (Boehringer Mannheim, Indianapolis, IN) instructions. Following centrifugation, the supernatant containing the cytoplasmic histone-associated DNA fragments was diluted and reacted in the microtitrator wells with immobilized anti-histone antibody. The retained complex was then reacted with anti-DNA peroxidase and probed with ABTS reagent for spectrophotometric quantification (7). TNF-α was quantitated with an enzyme-linked immunosorbent assay (Genzyme, Cambridge, MA) using 100 µl aliquots of the culture supernatant and horseradish peroxidase-conjugated anti-TNF-α. The complex was then probed with TMB reagent for spectrophotometric TNF-α quantification (21).

**Nitrite assay**

NO production was determined by measuring the stable NO metabolite, nitrite, accumulation in the culture medium using the Griess reaction (22). A 100 µl aliquot of spent culture medium was incubated for 10 min at room temperature with 100 µl of Griess reagent (Sigma, St. Louis, MO), (0.1% naphthyl ethylenediamine dihydrochloride, 1% sulfanilamide, and 2.5% phosphoric acid), and the absorbance was measured at 570 nm. The concentration of nitrite was calculated with sodium nitrite as a standard (8).

**Data analysis**

All experiments were carried out in duplicate, and the results are expressed as means ± SD. Analysis of variance (ANOVA) was used to determine significance, and the significance level was set at P < 0.05. The protein content of samples was measured with BCA protein assay kit (Pierce, Rockford, IL).

**RESULTS**

The role of PAF in mediation of detrimental influence of *P. gingivalis* on the synthesis of salivary mucin was investigated using mucous acinar cells of rat sublingual salivary glands exposed to a specific PAF antagonist, BN52020. Under the employed incubation conditions, the incorporation of [3H]glucosamine used as a marker of mucin synthesis increased steadily with time for at least 16 h, and the cell viability monitored by Trypan blue uptake remained over 96% for up to 20h, with only marginal (0.9%) cellular damage as judged by lactate dehydrogenase release. The effect of *P. gingivalis* LPS on the synthesis of mucus glycoprotein in the absence and the presence of BN52020 is presented in Fig. 1. The incorporation of [3H]glucosamine into mucus glycoprotein showed a dose-dependent decrease with the LPS concentration up to 500 ng/ml, at which point a 58.4% reduction in mucin synthesis was observed (Fig. 1A). A specific PAF antagonist, BN52020, introduced to the incubation medium 30 min prior to the LPS, countered the severity of the LPS-induced decrease in mucin synthesis. The
The effect of BN52020 was dose-dependent, and at 20 µM resulted in an 83.7% impedance of the LPS inhibitory effect on the mucin incorporation of glucosamine (Fig. 1B). The data on the effect of BN52020 on *P. gingivalis* LPS-induced acinar cell apoptosis and the induction of TNF-α are summarized in Figs. 2 and 3, respectively. The results of assays revealed that comparing to the controls the LPS at 500 ng/ml evoked a 6.9-fold increase in apoptosis (Fig. 2), and elicited an 11.2-fold induction in the expression of TNF-α (Fig. 3). Further enhancement in apoptosis and the induction in TNF-α occurred in the presence of wortmannin, an inhibitor of PI3K, which at its optimal concentration increased the LPS-induced apoptosis by 26% and that of TNF-α by 20.5%. The effect of the LPS was countered in a dose-dependent fashion by BN52020, which at 20 µM produced a 74.8% decrease in apoptosis (Fig. 2), and caused a 76.1% impedance in the LPS-induced expression of TNF-α (Fig. 3). However, the preventive effects of PAF antagonist BN52020, on the LPS-induced increase in apoptosis and TNF-α, were subject to suppression by the pretreatment with wortmannin which evoked a 3.1-fold increase in apoptosis (Fig. 2) and a 3.8-fold increase in TNF-α (Fig. 3).
The countering effect of BN52020 on the LPS-induced enhancement in apoptosis and TNF-α was also reflected in the agent's capacity to impede (82.6%) the LPS-induced acinar cells NO generation (Fig. 4). On the other hand, wortmannin produced a 28.3% increase in the LPS-induced NO production, an inhibitor of NOS-2, 1400W, exerted profound inhibitory (89.5%) effect, and only marginal changes in the LPS-induced NO production was observed with cNOS inhibitor, L-NNA. Pretreatment with BN52020 substantially prevented the effect of wortmannin (39.1%) on the LPS-induced NO production and caused a 74.6% decrease in NO production in the presence of L-NNA, but countered (by 30.9%) the inhibitory effect on the LPS-induced NO production by 1400W (Fig. 4).

Figure 5 illustrates the effect of NOS-2 inhibitor, 1400W, on P. gingivalis LPS-induced changes in the synthesis of mucin by sublingual salivary gland acinar cells incubated in the presence of BN52020, whereas the effect of cNOS inhibitor, L-NNA, is presented in Fig. 6. In the absence of BN52020 the pretreatment with NOS-2 inhibitor, 1400W, led to a dose-dependent impedance (up to 73%) in the severity of the LPS-induced reduction in mucin synthesis, while the cNOS inhibitor, L-NNA, caused a dose-dependent potentiation (up to 30.5%) in the severity of the LPS-induced inhibitory effect. Moreover, NOS-2 inhibitor exerted a potentiating effect on the impedance by BN52020 of the detrimental
effect of *P. gingivalis* LPS on mucin synthesis (*Fig. 5*), while cNOS inhibitor caused a reduction in the impedance effect of BN52020 on the LPS-induced decrease in mucin synthesis (*Fig. 6*).

The results on the effect of PI3K inhibitor, wortmannin, on *P. gingivalis* LPS-induced changes in mucin synthesis by sublingual salivary gland acinar cells are summarized in *Fig. 7*. The data revealed that pretreatment of the acinar cells with wortmannin led to a profound exacerbation (38.9%) of the LPS-induced inhibition in mucin synthesis, and the effect was further amplified (by 19.8%) in the presence of cNOS inhibitor, L-NNA. A substantial reduction in the potentiating effect of wortmannin on the LPS-induced decrease in mucin synthesis was, however, achieved in the presence of PAF antagonist BN52020 and NOS-2 inhibitor 1400W.

**DISCUSSION**

Lipopolysaccharide, a component of outer membrane of *P. gingivalis* bacterium colonizing the oral cavity, is recognized as a key factor in the development of chronic mucosal inflammations and periodontal lesions that lead

---

*Fig. 3.* Effect of PAF antagonist, BN52020, and PI3K inhibitor, wortmannin, on *P. gingivalis* LPS-induced TNF-α expression in sublingual salivary gland cells. The cells, preincubated with 0 - 40µM BN52020 (BN) or 100nM wortmannin (Wt), were incubated for 16 h in the absence or presence of the LPS at 500 ng/ml. Values represent the means ± SD of five experiments. *P < compared with that of LPS alone (Normal). **P < compared with that of BN (20µM)+LPS.
to periodontal disease (4, 5). The pathways of inflammatory LPS stimulation converge upon the transcriptional factor NF-κB, recognized for its central role in initiating and sustaining inflammatory reactions through the induction of a wide variety of proinflammatory mediators. The molecular mechanisms that attenuate the extent of NF-κB activation and the induction of inflammatory genes, however, are not well discerned. Moreover, recent studies into the regulatory pathways of NF-κB activation in response to inflammatory stimulus suggest that the most proximal mediator released in reaction to LPS is platelet-activating factor (PAF) (9, 10). This potent messenger molecule, rapidly generated from membrane alkylacyl-glycerophosphorylcholine through the concerted action of phospholipase A₂ and acetyl-transferase enzymes by a variety of cells, including those of mucosal tissue, plays a major role in a number of diverse pathological conditions associated with bacterial infection and inflammatory diseases (9, 10, 23, 24).

Indeed, the available literature data show that the release of PAF in response to LPS stimulus triggers an early phase of NF-κB activation that leads to up-regulation in TNF-α expression and the induction of genes encoding proinflammatory cytokines, adhesion molecules, growth factors, and inducible
enzymes such as NOS-2 (10, 11). Moreover, we have shown recently that stimulation of NO production through exogenous NO donor or NOS-2 induction by LPS stimulus leads to the enhancement in apoptosis and TNF-α, and results in the inhibition of salivary mucus glycoprotein synthesis (7, 13). Also, the inhibition of PAF activation has been found to suppress the LPS-induced up-regulation in mucosal expression of TNF-α and NOS-2 activity during oral mucosal ulcer healing (16).

In the study presented herein, using mucin-producing acinar cells of sublingual salivary gland, we assessed the effect of a specific PAF antagonist, BN52020, on the interplay of factors involved in the disturbances in salivary mucin synthesis evoked by LPS of periodontopathic bacterium, P. gingivalis. The results revealed that exposure of acinar cells to the LPS led to a dose-dependent decrease in mucin synthesis, accompanied by a marked increase in apoptosis, induction in TNF-α, and the enhancement in NO generation. Inhibition of PAF receptor activation with a specific antagonist, BN52020, countered the severity of the LPS-induced decrease in mucin synthesis and elicited a significant reduction in apoptosis, NO generation, and the expression of TNF-α. Further, we showed that the impedance by BN52020 of the LPS inhibitory effect on mucin synthesis was subject to suppression by wortmannin, a specific inhibitor of PI3K, which

**Fig. 5.** Effect of NOS-2 inhibitor, 1400W, and PAF antagonist, BN52020, on P. gingivalis LPS-induced changes in the synthesis of mucin by sublingual salivary gland cells. The cells, preincubated with 0 - 15 µM 1400W (14W) or 20 µM BN52020 (BN), were incubated for 16 h in the absence or presence of the LPS at 500 ng/ml. Values represent the means ± SD of five experiments. *P < compared with that of LPS alone (None). **P < compared with that of BN + LPS.
also obviated the inhibitory effect of BN52020 on the LPS-induced upregulation in acinar cell apoptosis, expression of TNF-α, and the capacity for NO production. The findings thus single out PAF as a key mediator of the early phase of inflammatory responses to P. gingivalis LPS that affect elaboration of salivary mucins, and suggest that the modulatory effect of PAF antagonist on mucin synthesis is directly linked to the events associated with PI3K-Akt pathway activation, namely inhibition of apoptosis, suppression of TNF-α production, and the control of NO release. This is keeping with the literature data demonstrating that a constitutively active PI3K inhibited induction of NOS-2 gene expression in human astrocytes, and that inhibitors of PI3K-Akt pathway cause the enhancement in LPS-induced NOS-2 induction and NO production, as well as potentiate the expression of TNF-α (14, 15, 25, 26).

While the role of PI3K-Akt pathway activation in the suppression of NO generation by Ca²⁺-independent inducible NOS-2 is relatively well recognized, the emerging new evidence suggests that PI3K also plays a crucial role in the activation of Ca²⁺-dependent constitutive cNOS which provide precise pulses of NO for a fine tuning of the processes pertinent to cellular survival (27, 28). These studies revealed that pharmacological inhibitors of PI3K inhibit NO release by...
constitutively expressed NOS, and that PI3K-Akt activation leads to the enhancement in cNOS activity even in the absence of a transient increase in cytosolic Ca\(^{2+}\) (28, 29). As shown recently, this enhancement in cNOS activity is a consequence of PI3K-depending phosphorylation of the enzyme protein on Ser\(^{1177}\), a process that increases electron flux through the cNOS reductase domain with a reduced rate of calmodulin dissociation at resting Ca\(^{2+}\) levels (28, 29). Interestingly, in our study PI3K inhibitor, wortmannin, and cNOS inhibitor, L-NNA, exerted inhibitory effect on the impedance by BN52020 in the LPS-induced NO production, while NOS-2 inhibitor, 1400W, caused potentiation of the inhibitory effect of BN52020 on NO production. Thus, the activation of PAF release in salivary gland acinar cells in response to \textit{P. gingivalis} LPS stimulus serves as a negative regulator of PI3K/Akt pathway controlling the processes associated with NO production.

To gain further insight into the nature of disturbances in salivary mucin caused by \textit{P. gingivalis}, we assessed the role of NOS isozymes in the impedance by PAF antagonist of the LPS-induced changes in mucin synthesis. The results revealed that while NOS-2 inhibitor, 1400W, exerted a potentiating effect on the impedance by BN52020 of the LPS detrimental effect on mucin synthesis, a

**Fig. 7.** Effect of PAF antagonist, BN52020, NOS-2 inhibitor, 1400W, and cNOS inhibitor, L-NNA, on \textit{P. gingivalis} LPS-induced changes in the synthesis of mucin by sublingual salivary gland cells treated with wortmannin. The cells, preincubated with 20 µM BN52020 (BN), 10 µM 1400W (14W) or 100µM L-NNA (LN), were treated with 100 nM wortmannin (Wt) and incubated for 16 h in the absence or presence of the LPS at 500 ng/ml. *P < 0.05 compared with that of LPS alone (Normal). **P < 0.05 compared with that of Wt+LPS.
reduction in the impedance effect of BN52020 occurred in the presence of cNOS inhibitor, L-NNA. However, the L-NNA caused further exacerbation of the potentiating effect of wortmannin on the LPS-induced reduction in mucin synthesis, whereas NOS-2 inhibitor, 1400W, and PAF antagonist, BN52020, countered the potentiating effect of wortmannin on the LPS-induced decrease in mucin synthesis. The fact that cNOS inhibition, while exacerbating the potentiating effect of wortmannin on the LPS-induced decrease in mucin synthesis, caused a reduction in the impedance effect of PAF antagonist provides a strong indication as to the importance of cNOS activity in the regulation of salivary mucin synthesis. The findings thus implicate PAF as a pivotal factor affecting the nature and extent of pathological consequences of \textit{P. gingivalis} infection on salivary glands capacity for mucin elaboration.

As salivary mucins are recognized as an essential component of oral mucosal mucus coat that acts as a barrier protecting soft oral tissue against microbial insults (6, 30), the results our study supply an important new clue into the mechanism by which \textit{P. gingivalis} is capable of compromising the pre-epithelial element of oral mucosal defense and affect the progression of periodontal disease.

REFERENCES


Received: August 4, 2003
Accepted: January 22, 2004

Author’s address: Dr. B.L. Slomiany, Research Center, C875, UMDJN-NJ Dental School, 110 Bergen Street, P.O. Box 1709, Newark, NJ 07103 - 2400, USA, Phone 973 -972-7052
E-mail: slomiabr@umdnj.edu