

# Alterations by Indomethacin in Proinflammatory Consequences of Salivary Gland Cytosolic Phospholipase A<sub>2</sub> Activation by *Porphyromonas gingivalis*: Role of Leptin

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## ABSTRACT

In this study, we report on the alterations by cyclooxygenase inhibitor, indomethacin, in the generation of proinflammatory lipid mediators in sublingual salivary gland acinar cells as a consequence of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>) activation by the lipopolysaccharide (LPS) of a periodontopathic *Porphyromonas gingivalis*, and assess the role of leptin in the process. We show that indomethacin while not affecting the LPS-induced enhancement in cPLA<sub>2</sub>-catalyzed arachidonic acid release, caused inhibition in prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production, up-regulation in apoptosis and platelet activating factor (PAF) generation, and potentiation of the impairment in mucin

synthesis. Preincubation with leptin countered the stimulatory effect of indomethacin on the LPS-induced PAF generation and apoptosis, and evoked reduction in the effect of indomethacin on the LPS-induced impairment in mucin. Moreover, the effect of indomethacin on the LPS-induced impairment in mucin synthesis was inhibited by PAF receptor antagonist, BN52020. Our findings demonstrate that indomethacin exacerbates the LPS-induced proinflammatory consequences of cPLA<sub>2</sub> activation by causing up-regulation in PAF generation that leads to enhancement in apoptosis and potentiation of the impairment in mucin synthesis. We also show that leptin counters the pathological implications of up-regulation in inflammatory lipid mediators production at the level of cPLA<sub>2</sub> activation.

## INTRODUCTION

Lipopolysaccharide (LPS), a component

of the outer membrane of *Porphyromonas gingivalis* bacterium colonizing the oral cavity, is recognized as a key virulence factor responsible for eliciting chronic mucosal inflammation and periodontal lesions that lead to periodontal disease.<sup>1-3</sup> The oral mucosal responses to *P. gingivalis* and its LPS are manifested by a marked increase in epithelial cell apoptosis and proinflammatory interleukin production, enhancement in nitric oxide and prostaglandin production, and the disturbances in NFκB (nuclear factor κB) and MAPK (mitogen-activated protein kinase) signaling cascades.<sup>2,4</sup> Moreover *P. gingivalis* LPS, through the induction in platelet activating factor (PAF) lipid messenger generation, has been shown to exert a detrimental effect on the synthesis of salivary mucin,<sup>4</sup> thus weakening the protective performance of the saliva-derived oral mucosal coating that constitutes the pre-epithelial element of oral mucosal defense.<sup>5-7</sup>

Although the molecular mechanisms that attenuate the extent of LPS-induced production of lipid mediators are not well discerned, a growing body of evidence suggests that the initial event in the generation of proinflammatory lipids is the liberation of arachidonic acid from membrane phospholipids by activation of cytosolic phospholipase A<sub>2</sub> (cPLA<sub>2</sub>).<sup>8-10</sup> The cleavage of arachidonic acid from the *sn*-2 (stereospecific numbering on carbon 2 of glycerol) position of membrane glycerophospholipids by the action of this highly selective Group IVA cPLA<sub>2</sub> is an initial and rate-limiting event in prostaglandin and leukotriene production, as well as a key step in the generation of 1-O-alkyl-glycerophosphorylcholine that is converted by the action of acetyl-transferase enzyme into a potent phospholipid messenger, PAF.<sup>11,12</sup> The central role of cPLA<sub>2</sub> in the production of proinflammatory lipid mediators is further supported by the experiments with cPLA<sub>2</sub>-deficient mice,

where the diminished ability for eicosanoid and PAF production was also reflected in the reduced inflammatory responses to LPS endotoxin.<sup>13,14</sup>

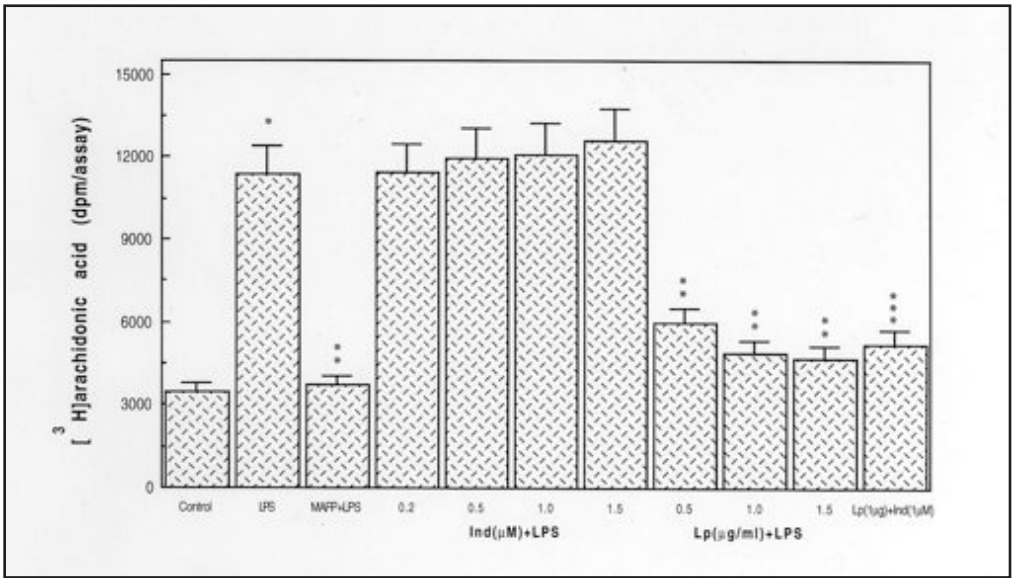
Interestingly, there are indications that the disturbances in arachidonic acid utilization for eicosanoids production and the ensuing elevation in arachidonic acid levels exert proapoptotic effects as well as affect the capacity for PAF generation.<sup>15,16</sup> This is consistent with the well-known proapoptotic effects of non-steroidal anti-inflammatory drugs and the findings that interference with PAF actions affects mucosal capacity for prostaglandin production.<sup>17,18</sup> Moreover, the cellular events associated with cPLA<sub>2</sub> activation appear to be influenced by leptin,<sup>19</sup> a pluripotent cytokine that emerged recently as an important regulator of mucosal inflammatory reaction to bacterial infection.<sup>20,21</sup> Furthermore, recent evidence supports the role of leptin in countering the detrimental consequences of *P. gingivalis* on the process of salivary mucin elaboration.<sup>22</sup>

In our previous report, we have shown that the detrimental effect of *P. gingivalis* LPS on salivary mucin synthesis involves extracellular signal-regulated kinase (ERK)-dependent cPLA<sub>2</sub> activation.<sup>23</sup> In this study, using mucin-producing acinar cells of sublingual salivary glands, we employed leptin in conjunction with the cyclooxygenase inhibitor, indomethacin, to investigate the consequences of alteration in the generation of proinflammatory lipid mediators in response to *P. gingivalis* LPS-induced cPLA<sub>2</sub> activation.

## **MATERIALS AND METHODS**

### **Sublingual gland cell isolation**

The study was conducted with Sprague-Dawley rats in compliance with the institutional Animal Care and Use Committee guidelines. Freshly dissected sublingual salivary glands were trimmed of fat and connective tissue, and minced



**Figure 1.** Effect of indomethacin (Ind) and leptin (Lp) on *Porphyromonas gingivalis* LPS-induced cPLA<sub>2</sub> activation in rat sublingual gland acinar cells. The [<sup>3</sup>H] arachidonic acid-labeled cells, preincubated with Ind, Lp, MAFP (20 μM) or Lp+Ind were treated with the LPS at 100 ng/mL and incubated for 2 hours. The medium was analyzed for the labeled arachidonic acid release. Values represent the means ± standard deviation (SD) of 5 experiments. \*P<0.05 compared with that of control; \*\*P<0.05 compared with that of 100 ng/mL LPS. \*\*\*P<0.05 compared with that of Ind (1 μM)+LPS.

by passage through a 50 mesh metal grid.<sup>4</sup> The minced tissue was suspended in 5 volumes of ice-cold Dulbecco's modified Eagle's minimal essential medium (DMEM) (Gibco, Grand Island, NY), 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. Following three consecutive rinses with DMEM, the cells were resuspended in the medium to a concentration of 2 x 10<sup>7</sup> cell/mL.

### Cell viability

Cell preparations before and during the experimentation were assessed for viability and cellular integrity using Trypan blue dye exclusion assay and the determination of lactate dehydrogenase released into the medium.<sup>4</sup> The results indicated that the cell viability during

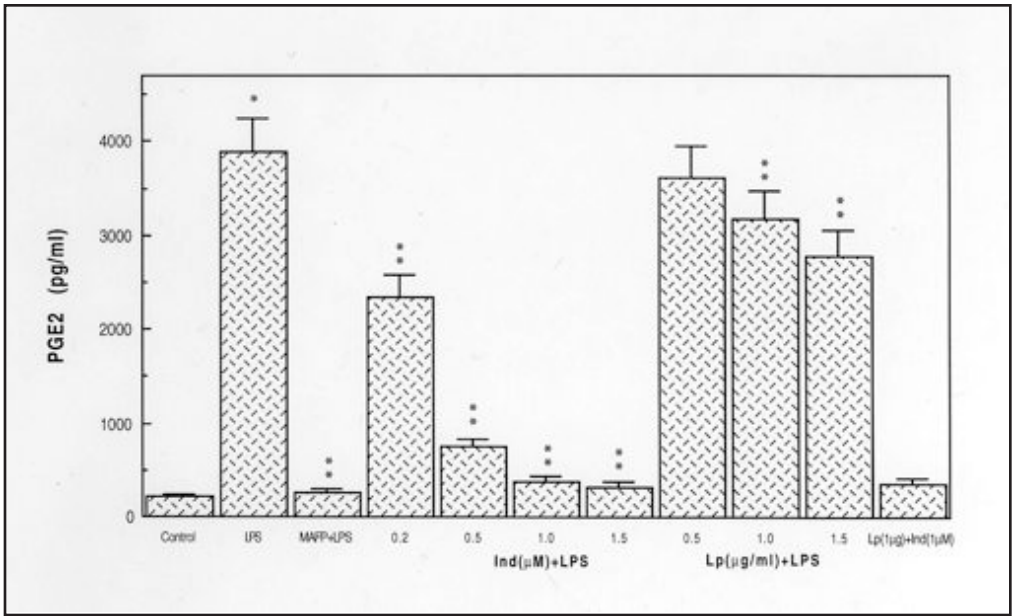
the experimentation with the agents used remained over 97%, with only marginal (0.8%) cellular damage.

### *P. gingivalis* LPS

*P. gingivalis* used for LPS preparation was cultured from clinical isolates obtained from ATCC (American Type Culture Collection, Rockville, MD) No. 33277.<sup>22</sup> The bacterium was homogenized with liquid phenol-chloroform-petroleum ether, centrifuged, and the LPS 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 x g for 4 hours, and the resulting LPS sediment subjected to lyophilization.<sup>4</sup>

### Phospholipid labeling and <sup>3</sup>H arachidonic acid release

Aliquots of cell suspension (1 mL) were



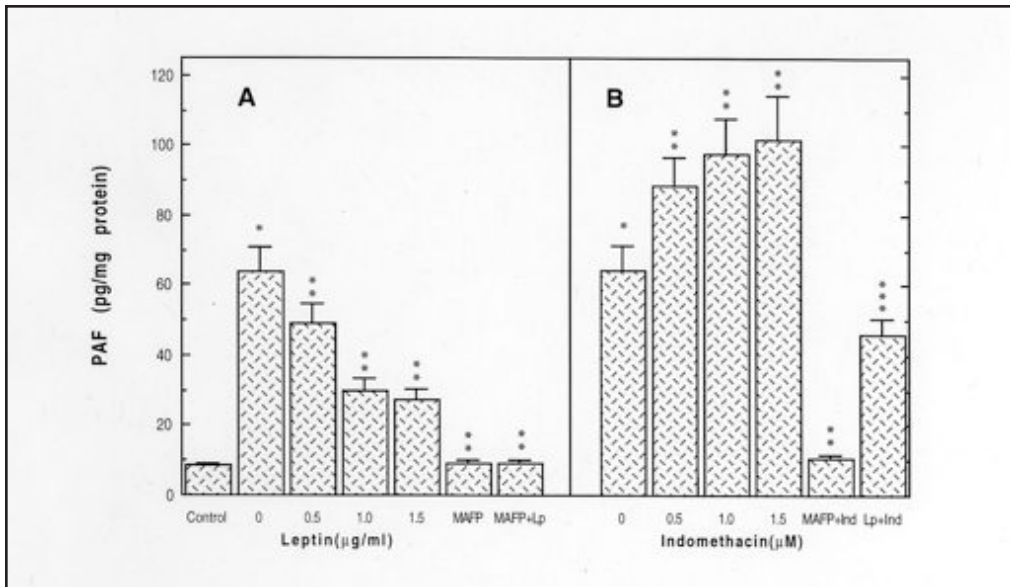
**Figure 2.** Effect of indomethacin (Ind) and leptin (Lp) on *P. gingivalis* LPS-induced generation of PGE<sub>2</sub>. The acinar cells, preincubated with Ind, Lp, MAFP (20 μM) or Lp+Ind, were incubated for 2 hours with the LPS at 100 ng/mL and the medium was analyzed for PGE<sub>2</sub> content. Values represent the means ± SD of 5 experiments. \*P<0.05 compared with that of control; \*\*P<0.05 compared with that of LPS.

transferred to DMEM in culture dishes containing 20 μCi of [5,6,8,9,11,12,14,15-<sup>3</sup>H]arachidonic acid (New England Nuclear, Boston, MA) and incubated for 4 hours under 95% O<sub>2</sub>/5% CO<sub>2</sub> atmosphere at 37°C.<sup>24</sup> The cells were then centrifuged at 300 x g for 5 minutes, washed 3 times with DMEM containing 5% albumin to remove free radiolabel, and resuspended in a fresh DMEM free of albumin. After a 5-minute equilibration period, the cells were transferred to a medium containing 0 or 100 ng/mL *P. gingivalis* LPS at 100 ng/mL and incubated for 2 hours.<sup>23</sup> In the experiments on the effect of indomethacin (Sigma Chemical Company, St. Louis, MO); leptin (mouse recombinant, Sigma Chemical Company); and cPLA<sub>2</sub> inhibitor, MAFP (methyl arachidonoyl fluorophosphonate; Calbiochem, La Jolla, CA); prior to the addition of the LPS, the cells were incubated for 30 minutes with the indicated dose of the

agent or the vehicle. At the end of the specified incubation period, the cells were centrifuged at 300 g for 5 minutes and the supernatant analyzed for the released <sup>3</sup>H arachidonic acid by scintillation spectrometry.

#### PAF and PGE<sub>2</sub> quantification

Prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) assays were carried out using a PGE<sub>2</sub> EIA kit (Cayman, Ann Arbor, MI) and 100 μL aliquots of spent culture medium, according to the manufacturer's instruction. The amount of PGE<sub>2</sub> released by the acinar cell incubates into the medium was expressed in pg/mL. For PAF analysis, the acinar cell incubates were homogenized with 10 volumes of chloroform-methanol-water (2:1:0.8, v/v/v) and the lipids contained in the organic phase were separated on silicic acid column into neutral lipid and phospholipid fractions.<sup>24</sup> The phospholipid fraction eluates were dried under nitrogen, the residue



**Figure 3.** Effect of indomethacin (Ind) and leptin (Lp) on *P. gingivalis* LPS-induced changes in PAF production by sublingual gland acinar cells. The cells, preincubated with Ind, Lp, MAFP (20  $\mu$ M) or Lp+Ind, were incubated for 2 hours with the LPS at 100 ng/mL and analyzed for the PAF content. Values represent the means  $\pm$  SD of 5 experiments. \* $P$ <0.05 compared with that of control. \*\* $P$ <0.05 compared with that of LPS alone (0). \*\*\* $P$ <0.05 compared with that of Ind (1  $\mu$ M)+LPS.

dissolved in saline, and subjected to PAF quantification by [ $^3$ H]PAF scintillation assay (Amersham, Piscataway, NJ). The protein content of samples was measured with BCA protein assay kit (Pierce, Rockford, IL).

### Apoptosis assay

For apoptotic measurements, the acinar cells were preincubated for 30 minutes with the indicated dose of leptin, MAFP, and PAF (Calbiochem, La Jolla, CA), alone or in combination with indomethacin before the addition of the LPS. After 16 hours of incubation, the cells were rinsed with 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 reacted with immobilized anti-histone antibody and the complex was reacted with anti-DNA peroxidase, and probed with ABTS reagent for spec-

trophotometric quantification.<sup>4</sup>

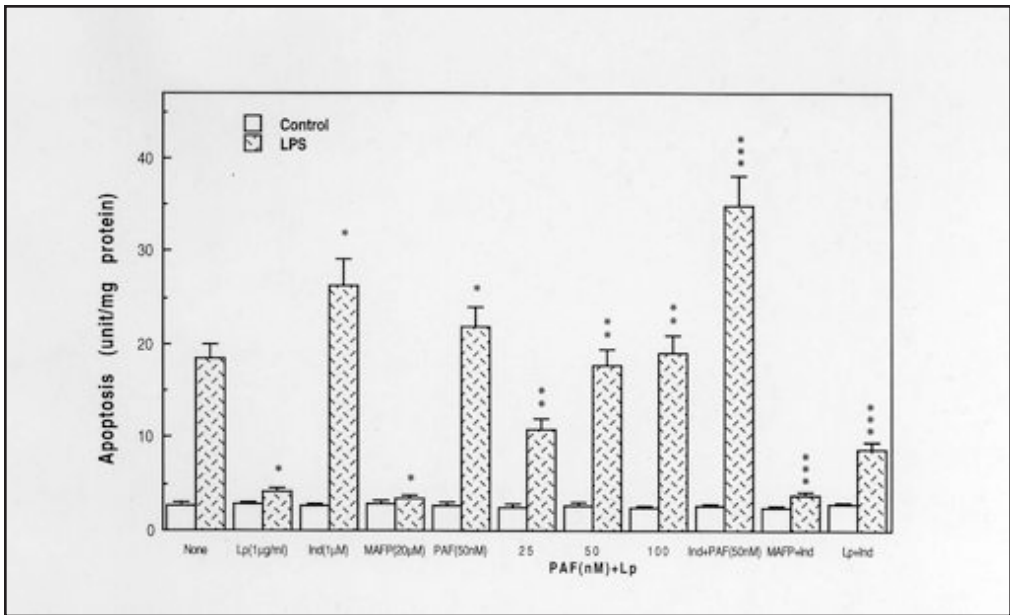
### Mucin synthesis

Aliquots of acinar cell suspension (1 mL) were transferred to DMEM in culture dishes containing 100 Ci of [ $^3$ H]glucosamine (NEN), and incubated according to previously described conditions in absence or the presence of *P. gingivalis* LPS at 100 ng/mL.<sup>23</sup> To study the effect of indomethacin; leptin; PAF; and PAF receptor antagonist, BN52020; the cells were preincubated for 30 minutes with the indicated dose of the agent or the vehicle before the addition of the LPS. At the end of the specified incubation period, the cells were centrifuged, washed with phosphate-buffered saline, and the combined supernatant used mucin analysis.<sup>4</sup>

### Data analysis

All experiments were carried out using duplicate sampling and the results are expressed as means  $\pm$  standard deviation (SD). Analysis of variance (ANOVA)





**Figure 4.** Effect of indomethacin (Ind), leptin (Lp), MAFP, and PAF on *P. gingivalis* LPS-induced salivary gland acinar cell apoptosis. The cells, preincubated with Ind, Lp, MAFP or PAF and PAF+Lp, Ind+PAF, or MAFP+Ind or Lp+Ind, were treated with the LPS at 100 ng/mL and incubated for 16 hours. Values represent the means  $\pm$  SD of 5 experiments. \* $P$ <0.05 compared with that of LPS alone (None). \*\* $P$ <0.05 compared with that of Lp+LPS. \*\*\* $P$ <0.05 compared with that of Ind+LPS.

was used to determine significance and the significance level was set at  $P$ <0.05.

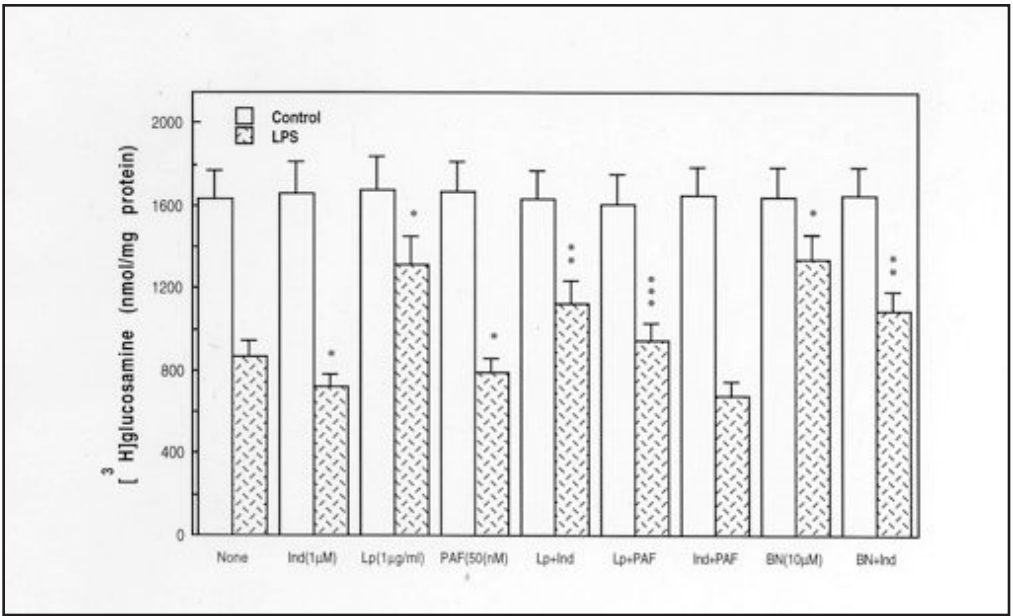
## RESULTS

The detrimental consequences of *P. gingivalis*-induced cPLA<sub>2</sub> activation on the signaling pathways associated with the generation of proinflammatory lipid mediators and the disturbances in salivary mucin synthesis was investigated in rat sublingual gland acinar cells exposed to *P. gingivalis* key virulence factor, LPS. Employing the acinar cells labeled with [<sup>3</sup>H]arachidonic acid, we demonstrated that the stimulatory effect of the LPS on the acinar cell arachidonic acid release was susceptible to inhibition by MAFP, a specific inhibitor of cPLA<sub>2</sub>, thus attesting to *P. gingivalis* LPS activation of cPLA<sub>2</sub> (Figure 1). Further, we found that the LPS-induced acinar cell cPLA<sub>2</sub> activation, reflected in up-regulation in arachidonic acid release was associated with the concomitant increase in PGE<sub>2</sub>

production (Figure 2), and the enhancement in PAF generation (Figure 3). Moreover, preincubation with cPLA<sub>2</sub> inhibitor, MAFP, led to the suppression of the LPS effect on the acinar cell capacity for PGE<sub>2</sub> production (Figure 2) as well as PAF generation (Figure 3A).

We next examined *P. gingivalis* LPS-induced acinar cell cPLA<sub>2</sub> activation in the presence of cyclooxygenase inhibitor, indomethacin. The results of assays revealed that while the pretreatment with indomethacin produced only negligible increase (9.5% at 1.5  $\mu$ M) in the LPS effect on cPLA<sub>2</sub> catalyzed arachidonic acid release (Figure 1), a profound concentration-dependent inhibition (up to 97.8%) of the LPS effect was attained in PGE<sub>2</sub> production (Figure 2). Moreover, the acinar cell capacity for PAF generation increased by 71.3% (Figure 3B).

As the recent data indicate that the events associated with cPLA<sub>2</sub> activation



**Figure 5.** Effect of indomethacin (Ind), leptin (Lp), PAF, and PAF antagonist BN52020 (BN) on *P. gingivalis* LPS-induced changes in the synthesis of mucin by sublingual salivary gland cells. The cells, preincubated with Ind, Lp, PAF or BN and Lp+Ind, Lp+PAF, Ind+PAF, or BN+Ind, were treated with the LPS at 100 ng/mL and incubated for 16 hours in the presence of (<sup>3</sup>H)glucosamine. Values represent the means ± SD of 5 experiments. \**P*<0.05 compared with that of LPS alone (None). \*\**P*<0.05 compared with that of Ind+LPS. \*\*\**P*<0.05 compared with that of Lp+LPS.

are impacted by leptin,<sup>19</sup> we employed this pluripotent cytokine in conjunction with indomethacin to sort further the processes influenced by *P. gingivalis* LPS-induced proinflammatory lipid generation. As depicted in Figure 1, preincubation of the acinar cells with leptin led to a dose-dependent suppression (up to 76.5%) of the LPS-induced arachidonic acid release, and was accompanied by a 28.4% reduction in PGE<sub>2</sub> production (Figure 2) and a 57.1% reduction in PAF generation (Figure 3A). Further, the inhibitory effect of leptin on the LPS-induced arachidonic acid release was not affected by the inclusion of indomethacin (Figure 1), but the acinar cell PGE<sub>2</sub> production in the presence of leptin was subject to suppression by indomethacin (Figure 2). Moreover, leptin produced a 52.5% reduction in the stimulatory effect of indomethacin on the LPS-induced acinar cell capacity for

PAF generation (Figure 3B).

Since the process of cPLA<sub>2</sub> activation and the ensuing eicosanoid and PAF production play a central role in controlling the balance between cell survival and death,<sup>15,16</sup> we investigated the influence of indomethacin and leptin on the apoptotic processes triggered by *P. gingivalis* LPS-induced cPLA<sub>2</sub> activation in the acinar cells. The results revealed that leptin at its optimal concentration (1 µg/mL) for the impedance of arachidonic acid release evoked a 77.2% decrease in the LPS-induced apoptosis, while a 43% enhancement in the LPS-induced apoptosis was attained in the presence of indomethacin (Figure 4). Moreover, the inhibitory effect of leptin on the LPS-induced apoptosis was subject to reversal by the addition of PAF, which also caused further amplification in the proapoptotic effect of indomethacin. On the other hand, prein-

cubation of the acinar cells with leptin or cPLA<sub>2</sub> inhibitor, MAFP, led to the impedance in the indomethacin effect on the LPS-induced apoptotic changes (Figure 4).

Further, we analyzed the impact of the LPS-induced cPLA<sub>2</sub> activation in the presence of indomethacin and leptin on the synthesis of mucin in the acinar cells. We found that the detrimental effect of the LPS on salivary mucin synthesis was subject to further potentiation by indomethacin as well as PAF, while preincubation with leptin or PAF receptor antagonist, BN52020, led to inhibition of the LPS effect (Figure 5). Furthermore, the potentiating effect of indomethacin was subject to reversal by preincubation with leptin as well as PAF receptor antagonist, BN52020, while the countering capacity of leptin of the LPS-induced reduction in mucin synthesis was inhibited by preincubation with PAF (Figure 5).

## DISCUSSION

Investigations into the nature of cellular responses to bacterial LPS increasingly assign the central role in mediation of LPS-initiated signaling events to the group of proinflammatory lipids that are generated by the action of the highly selective Group IVA cPLA<sub>2</sub>.<sup>8-10</sup> Indeed, the liberation of arachidonic acid from the *sn*-2 position of membrane alkylacylglycerophospholipids by the action of cPLA<sub>2</sub> is the initial and rate limiting event in prostaglandin and leukotriene production, as well as a key step in the generation of a potent phospholipid messenger, PAF.<sup>11,12</sup> Moreover, we have shown recently that the acinar cells of sublingual salivary gland respond to stimulation with *P. gingivalis* LPS by activation of cPLA<sub>2</sub>, as evidenced by the dose-dependent release of arachidonic acid.<sup>23</sup> The literature data, furthermore, indicate that the disturbances in eicosanoids production and the resulting

elevation in arachidonic acid levels affect the cellular capacity for PAF generation, and that the events of cPLA<sub>2</sub> activation are influenced by leptin.<sup>15,16,19</sup> More interestingly, the increased levels of leptin characterizes mucosal responses to bacterial infections, leptin deficiency enhances sensitivity to endotoxin-induced lethality, and the exogenous leptin has been demonstrated to protect gastric mucosa against injury caused by nonsteroidal anti-inflammatory drugs.<sup>20,21,25</sup>

In the study presented here, we employed leptin in conjunction with a well-known nonsteroidal anti-inflammatory agent and cyclooxygenase inhibitor, indomethacin, to examine the functional consequences of alteration in proinflammatory lipids generation by mucin-producing acinar cells of sublingual salivary gland in response to *P. gingivalis* LPS-induced cPLA<sub>2</sub> activation. We found that the LPS-induced acinar cell cPLA<sub>2</sub> activation, reflected in up-regulation in arachidonic acid release, was associated with the concomitant increase in apoptosis, enhancement in PGE<sub>2</sub> and PAF generation, and the impairment in mucin synthesis. Preincubation with indomethacin, while not affecting the LPS-induced rise in cPLA<sub>2</sub>-catalyzed arachidonic acid release, caused the inhibition in PGE<sub>2</sub> production, enhancement in apoptosis and PAF generation, as well as potentiated the LPS-induced impairment in mucin synthesis. Moreover, the effect of indomethacin on the LPS-induced acinar cell apoptosis was subject to further enhancement in the presence of exogenous PAF, whereas the PAF receptor antagonist, BN52020, countered the effect of indomethacin on the LPS-induced changes in mucin synthesis. These results, while consistent with the well-known proapoptotic effects of nonsteroidal anti-inflammatory drugs,<sup>17</sup> provide new leads as to the role of PAF in causing exacerbation of apoptotic events



triggered by these agents' inhibition of arachidonic acid utilization for eicosanoids production. Indeed, there are reports indicating that cells deficient in cPLA<sub>2</sub> enzyme have also diminished ability for PAF generation and exhibit impairment in apoptotic processes,<sup>10,13,16</sup> and our study demonstrated that inhibition of the acinar cell cPLA<sub>2</sub> activity with MAFP countered the proapoptotic as well as PAF stimulatory effect of *P gingivalis* LPS.

As the literature data suggests that mucosal injury caused by indomethacin can be prevented by leptin,<sup>25</sup> we further assessed the influence of leptin on the alterations in proinflammatory lipids generation induced by indomethacin. The results revealed that leptin suppression of the LPS-induced cPLA<sub>2</sub> activation, manifested by a drop in arachidonic acid release, was accompanied by a marked reduction in apoptosis and PAF generation, and a decline in PGE<sub>2</sub>. Moreover, while inhibitory effect of leptin on the LPS-induced arachidonic acid release was not affected by the inclusion of indomethacin, leptin elicited a profound reduction in the stimulatory effect of indomethacin on the LPS-induced acinar cell apoptosis and the capacity for PAF generation. Furthermore, we found that leptin countered the potentiating effect of indomethacin on the LPS-induced impairment in mucin synthesis. These findings thus provide an important insight into the mechanism of leptin involvement in controlling the detrimental consequences of alteration in proinflammatory lipid generation by indomethacin.

Apparently, by interfering with the LPS-induced cPLA<sub>2</sub> activation for the rapid cleavage of arachidonic acid from membrane phospholipids, leptin is capable of exerting a major modulatory effect on the acinar cell capacity for the generation of prostaglandins as well as

PAF. This demonstrated aspect of leptin function is of particular relevance with respect to understanding the complexity of actions of indomethacin. While majority of studies examining the anti-inflammatory actions of this agent have focused on its role as a cyclooxygenase inhibitor, our data suggest that the interference by indomethacin with cyclooxygenase function during inflammatory challenge by LPS leads to up-regulation in the generation of PAF. There are also indications that production of PAF is down regulated by concurrently produced PGE<sub>2</sub>.<sup>15</sup> Moreover, the involvement of PAF in a number of diverse pathological conditions associated with bacterial infection, allergic reactions, and inflammatory diseases is well documented.<sup>26,27</sup>

Taken together, our findings demonstrate that indomethacin exacerbates *P gingivalis* LPS-induced proinflammatory consequences of the acinar cell cPLA<sub>2</sub> activation by causing up-regulation in PAF generation that leads to enhancement in apoptosis and potentiation of the impairment in salivary mucin synthesis. We also show that leptin counters the pathological implications of up-regulation in inflammatory lipid mediators production at the level of cPLA<sub>2</sub> activation.

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