Research Article

Ghrelin Protects against the Detrimental Consequences of *Porphyromonas gingivalis*-Induced Akt Inactivation through S-Nitrosylation on Salivary Mucin Synthesis

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Disturbances in nitric oxide synthase isozyme system and the impairment in salivary mucin synthesis are well-recognized features associated with oral mucosal inflammatory responses to periodontopathic bacterium, *P. gingivalis*. In this study, using rat sublingual gland acinar cells, we report that *P. gingivalis* LPS-induced impairment in mucin synthesis and associated suppression in Akt kinase activity were accompanied by a decrease in constitutive nitric oxide synthase (cNOS) activity and an induction in inducible nitric oxide synthase (iNOS) expression. The LPS effect on Akt inactivation was manifested in the kinase S-nitrosylation and a decrease in its phosphorylation at Ser[473]. Further, we demonstrate that a peptide hormone, ghrelin, countered the LPS-induced impairment in mucin synthesis. This effect of ghrelin was reflected in the suppression of iNOS and the increase in Akt activation, associated with the loss in S-nitrosylation and the increase in phosphorylation, as well as cNOS activation through phosphorylation. Our findings suggest that induction in iNOS expression by *P. gingivalis*-LPS leads to Akt kinase inactivation through S-nitrosylation that detrimentally impacts cNOS activation through phosphorylation as well as mucin synthesis. We also show that the countering effect of ghrelin on *P. gingivalis*-induced impairment in mucin synthesis is associated with Akt activation through phosphorylation.

1. Introduction

Porphyromonas gingivalis is a Gram-negative periodontopathic bacterium found in periodontal packets of patients with periodontitis, a chronic destructive inflammatory disease that affects 10–15% of the adult population and is the major cause of adult tooth loss [1, 2]. The oral mucosal responses to *P. gingivalis* and its key virulence factor, cell-wall lipopolysaccharide (LPS), are manifested by a marked increase in epithelial cell apoptosis and proinflammatory cytokine production, and the disturbances in nitric oxide synthase (NOS) isozyme system responsible for nitric oxide (NO) generation [3–5]. The inflammatory events associated with oral mucosal bacterial infection, furthermore, are reflected in the reduced secretion of saliva and the diminished protective performance of the saliva-derived mucosal mucus coat, caused by the impairment in salivary mucin synthesis and secretion [6, 7]. Indeed, using acinar cells of sublingual salivary gland, we have demonstrated that the impairment in salivary mucin synthesis caused by *P. gingivalis* LPS is directly linked to the disturbances in NO signaling pathways [4, 8].

Investigations into the nature of factors that influence the extent of mucosal inflammatory responses to bacterial infection along the alimentary tract, including that of oral cavity, have brought to focus the importance of a peptide hormone, ghrelin [9–11]. This 28-amino acid peptide, initially isolated from the stomach [9], and more recently identified in oral mucosa, saliva, and the acinar cells of salivary glands [11], has emerged as a principal modulator of the local inflammatory responses to bacterial infection through the regulation of nitric oxide synthase isozyme system [12, 13]. The signaling mechanism that underlies the regulation of NO by ghrelin involves the receptor- (GHS-R1a-) mediated

activation of heterotrimeric G protein-dependent pathway that results in signal propagation through a network of protein kinases, including that of kinase Akt, implicated in controlling the NOS isozyme system [12–14].

Activation of serine/threonine kinase, Akt, also known as protein kinase B (PKB) or Akt/PKB, in response to ghrelin occurs downstream of phosphoinositide 3-kinase (PI3K) and involves the generation of the lipid second messenger phosphatidylinositol-3,4,5-triphosphate, which accumulates in the plasma membrane and serves as a recognition site for the N-terminal PH (pleckstrin homology) domain of Akt [14, 15]. The induced conformational changes in Akt result in the exposure to phosphorylation within the activation (A) loop at Thr[308] and of Ser[473] located within the HM (hydrophobic motif) region of the C-terminal regulatory domain of Akt [14, 15]. Apparently, phosphorylation of the Thr[308] in the activation loop and the Ser[473] in the hydrophobic motif results in stabilization of the kinase domain in the active state and is required for full activation of Akt [16].

Moreover, a growing body of evidence indicates that the activity of Akt may be also regulated through S-nitrosylation at the kinase cysteine residues [16–18], and protein S-nitrosylation, with the involvement of NO generated by both constitutive and inducible forms of NOS system, is rapidly emerging as a posttranslational event of significance to a variety of biological processes [13, 17–20]. Indeed, S-nitrosylation of caspas-3 with the involvement of cNOS has been linked to the apoptogenic signal inhibition and the events of cytosolic phospholipase A_2 activation, whereas the NO generated by iNOS has been implicated in S-nitrosylation of proteins involved in insulin signal transduction and the reduced Akt activity in muscle cells of diabetic mouse [13, 19, 21, 22].

As the oral mucosal inflammatory responses to *P. gingivalis* are characterized by the disturbances in NOS system and the impairment in mucin production and since Akt kinase plays a major role in the regulation of NOS isozyme system [8, 15, 22, 23], in this study we investigated the influence of ghrelin on the Akt-mediated processes in *P. gingivalis* LPS-induced interference in salivary mucin synthesis. The results of our findings show that the countering effect of ghrelin on *P. gingivalis*-induced impairment in sublingual salivary gland acinar cell mucin synthesis is intimately associated with Akt activation, manifested by a decrease in the kinase S-nitrosylation and the increase in its phosphorylation.

2. Materials and Methods

2.1. Sublingual Gland Cell Preparation and Mucin Synthesis.
The acinar cells of sublingual salivary gland, collected from freshly dissected rat salivary glands, were suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone ($50 \mu g/mL$), penicillin ($50 U/mL$), streptomycin ($50 \mu g/mL$), and 10% fetal calf serum, and gently dispersed by trituration with a syringe, and settled by centrifugation [5]. After rinsing, the cells were resuspended in the medium to a concentration of 2×10^7 cell/mL, transferred in 1 mL aliquots to DMEM in culture dishes containing

[3H]glucosamine ($100 \mu Ci$), used as a marker of mucin synthesis, and incubated under 95% O_2—5% CO_2 atmosphere at $37°C$ for 16 h in the presence of 0–200 ng/mL of *P. gingivalis* LPS [5]. In the experiments evaluating the effect of ghrelin (rat, Sigma), cNOS inhibitor, L-NAME, iNOS inhibitor, 1400 W, Src inhibitor, Akt inhibitor, SH-5 (Calbiochem), and ascorbate (Sigma), the cells were first preincubated for 30 min with the indicated dose of the agent or vehicle before the addition of the LPS. The viability of cell preparations before and during the experimentation, assessed by Trypan blue dye exclusion assay [5], was greater than 98%. The concentration range of the LPS and the drugs used in the study was chosen based on the results of the dose-response experiments and the reported data as to their effectiveness [4, 5, 8, 13, 24]. The obtained values were not affected by exposure of the cells to ghrelin or tested agents without LPS challenge. At the end of the specified incubation period, the cells were centrifuged, washed with phosphate buffered saline, and the combined supernatants used for mucin analysis.

2.2. 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. The glycoprotein precipitates were dissolved in 6 M urea and chromatographed on Bio-Gel A-1.5 column, and the mucin fractions eluted in the excluded volume were subjected to analysis for total incorporation of radiolabel and protein content [8].

2.3. Porphyromonas gingivalis Lipopolysaccharide.
P. gingivalis used for LPS preparation was cultured from clinical isolates obtained from ATCC no. 33277 [8]. The bacterium was homogenized with liquid phenol-chloroform-petroleum ether and 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 $100,000 \times g$ for 4 h, and the resulting LPS sediment subjected to lyophilization. Analyses indicated that such obtained LPS preparation was essentially free of nucleic acids as determined by absorption at 260 nm, and its protein content, measured by BCA assay kit, was less than 0.2% [8].

2.4. NO Production and cNOS and iNOS Activity Assay.
NO production in the acinar cells was determined by measuring the stable NO metabolite, nitrite, accumulation in the culture medium using Griess reaction [23]. A $100 \mu L$ of spent culture medium was incubated for 10 min with 0.1 mL of Griess reagent (Sigma), and the absorbance was measured at 570 nm. The activity of cNOS and iNOS enzymes was measured by monitoring the conversion of L-[^{3}H]arginine to L-[^{3}H]citrulline using NOS-detect kit (Stratagene). The acinar cells from the control and experimental treatments were homogenized in a sample buffer containing either 10 mM EDTA (for Ca^{2+}-independent iNOS) or 6 mM $CaCl_2$ (for Ca^{2+}-dependent cNOS) and centrifuged [4, 13]. The aliquots of the resulting supernatant were incubated for 30 min

at 25°C in the presence of 50 μCi/mL of L-[^{3}H]arginine, 10 mM NAPDH, 5 μM tetrahydrobiopterin, and 50 mM Tis-HCl buffer, pH 7.4, in a final volume of 250 μL. Following addition of stop buffer and Dowex-50 W (Na$^+$) resin, the mixtures were transferred to spin cups and centrifuged, and the formed L-[^{3}H]citrulline contained in the flow through was quantified by scintillation counting.

2.5. Akt Activity Assay. The kinase activity of Akt in sublingual salivary gland acinar cells was measured with the Akt Activity Kit (Calbiochem), by quantifying phosphorylation of a biotinylated peptide substrate (GRPRTSSFAEG). The cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1% deoxycholate, 2 mM EDTA, 1 mM sodium orthovanadate, 1 mM PAF, and 1 mM NaF), containing protease inhibitor cocktail (Sigma), at 4°C for 30 min, centrifuged at 14,000 × g for 15 min, and immunoprecipitated with anti-Akt antibody for 1 h at 4°C. Protein A/G agarose beads were then added for an additional 1 h, and the immune complex was recovered by centrifugation and thoroughly washed with lysis buffer. The agarose beads were then suspended for 30 min at room temperature in the assay buffer and centrifuged, and the supernatants were used for the Akt activity assay by following the manufacturer's instruction.

2.6. Akt Phosphorylation Assay. Measurement of the phosphorylation status of Akt in sublingual gland acinar cells was performed using Akt (pThr^{308}) and Akt (pSer^{473}) ELISA kits (Calbiochem). The cells were lysed on ice for 30 min in lysis buffer (10 mM Tris-HCl, pH 7.4, 100 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1 mM NaF, 2 mM sodium orthovanadate, 1% Triton X-100, 10% glycerol, 0.1% SDS, 0.5% deoxycholate, and 1 mM PMSF), containing protease inhibitor cocktail, and centrifuged at 14,000 × g for 15 min. The supernatants diluted (1 : 10) in standard diluent buffer were pipetted in 100 μL aliquots into wells containing immobilized capture antibody, and after washing, the complex was reacted with antibody specific for Akt (pThr^{308}) or Akt (pSer^{473}). Following washing, the retained complex was labeled with horseradish peroxidase and probed with TMB reagent for spectrophotometric quantification at 450 nm.

2.7. Akt Protein S-Nitrosylation Assay. Assessment of Akt kinase S-nitrosylation in the acinar cells sublingual salivary gland was carried out using a biotin switch procedure for protein S-nitrosylation [25, 26]. The cells were treated with iNOS inhibitor, 1400 W (30 μM), or ghrelin (0.6 μg/mL), and incubated for 16 h in the presence of 100 ng/mL of *P. gingivalis* LPS. Following centrifugation at 500 × g for 5 min, the recovered cells were lysed in 0.2 mL of HEN lysis buffer (250 mM HEPES, 1 mM EDTA, 0.1 mM neocuproine, pH 7.7), and the unnitrosylated thiol groups were blocked with S-methyl methanethiosulfonate reagent at 50°C for 20 min [26]. The proteins were precipitated with acetone, resuspended in 0.2 mL of HEN buffer containing 1% SDS, and subjected to targeted nitrosothiol group reduction with sodium ascorbate (100 mM). The free thiols were then labeled with biotin, and the biotinylated proteins were recovered on streptavidin beads. The formed streptavidin bead-protein complex was washed with neutralization buffer, and the bound proteins were dissociated from streptavidin beads with 50 μL of elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.7) containing 1% 2-mercaptoethanol [26]. The obtained proteins were then analyzed by Western blotting.

2.8. Immunoblotting Analysis. The acinar cells from the control and experimental treatments were collected by centrifugation and resuspended for 30 min in ice-cold lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 10% glycerol, 1% Triton X-100, 2 mM EDTA, 1 mM sodium orthovanadate, 4 mM sodium pyrophosphate, 1 mM PMSF, and 1 mM NaF), containing 1 μg/mL leupeptin and 1 μg/mL pepstatin [13]. Following brief sonication, the lysates were centrifuged at 12,000 g for 10 min, and the supernatants were subjected to protein determination using BCA protein assay kit (Pierce). The samples, including those subjected to biotin switch procedure, were then resuspended in loading buffer, boiled for 5 min, and subjected to SDS-PAGE using 40 μg protein/lane. The separated proteins were transferred onto nitrocellulose membranes, blocked for 1 h with 5% skim milk in Tris-buffered Tween (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.1% Tween-20), and probed with the antibody against phosphorylated protein at 4°C for 16 h. After 1 h incubation with the horseradish peroxidase-conjugated secondary antibody, the phosphorylated proteins were revealed using an enhanced chemiluminescence. Membranes were stripped by incubation in 1 M Tris-HCl (pH 6.8), 10% SDS, and 10 mM dithiothreitol for 30 min at 55°C, and reprobed with antibody against total cNOS, iNOS or Akt. Immunoblotting was performed using specific antibodies directed against iNOS, cNOS (rabbit anti-eNOS) and phospho-cNOS (mouse anti-eNOS, pSer^{1179}), and Akt, phospho-Akt (Ser^{473}) (Calbiochem).

2.9. Data Analysis. All experiments were carried out using duplicate sampling, and the results are expressed as means ± SD. The data were analyzed by analysis of variance (ANOVA) for groups of not equal size and by nonparametric Kruskal-Wallis test for groups of equal size. Any difference detected was evaluated by means of post hoc Bonferroni test, and P value <.05 was considered significant.

3. Results

The role of serine/threonine kinase, Akt, in mediation of the signaling events associated with the detrimental influence of *P. gingivalis* on salivary mucin synthesis was investigated using primary culture of mucous cells of rat sublingual salivary gland exposed to the bacterium key virulence factor, LPS. Employing the cells incubated in the presence of [^{3}H]glucosamine as marker of mucin synthesis, in conjunction with the measurements of Akt activity and the cell capacity for NO generation, we demonstrated that *P. gingivalis* LPS-induced dose-dependent decrease in mucin synthesis was accompanied by a massive increase in NO
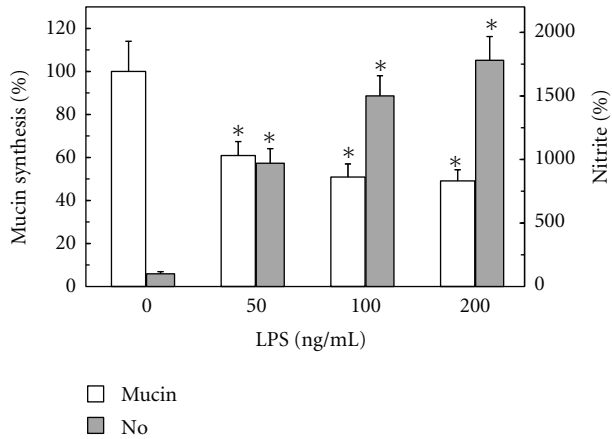
FIGURE 1: Effect of *P. gingivalis* LPS on mucin synthesis and nitrite production in rat sublingual salivary gland acinar cells. The cells were treated with the indicated concentrations of the LPS and incubated for 16 h in the presence of [³H]glucosamine used as a marker of mucin synthesis. Values represent the means ± SD of five experiments. *$P < .05$ compared with that of control (LPS − 0).
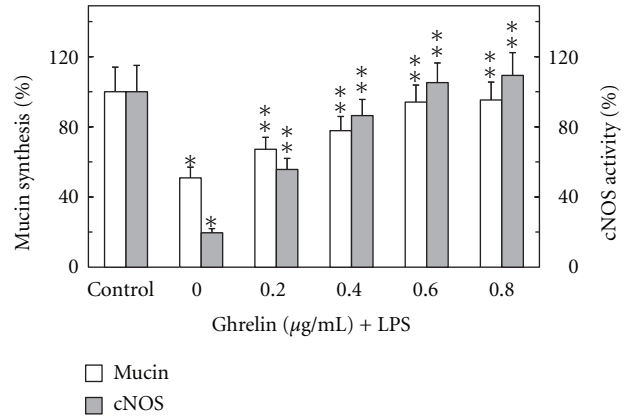

FIGURE 3: Effect of ghrelin on *P. gingivalis* LPS-induced changes in mucin synthesis and the expression of cNOS activity in the salivary gland acinar cells. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/mL and incubated for 16 h in the presence of [³H]glucosamine as a marker of mucin synthesis. Values represent the means ± SD of five experiments. *$P < .05$ compared with that of control. **$P < .05$ compared with that of LPS alone.
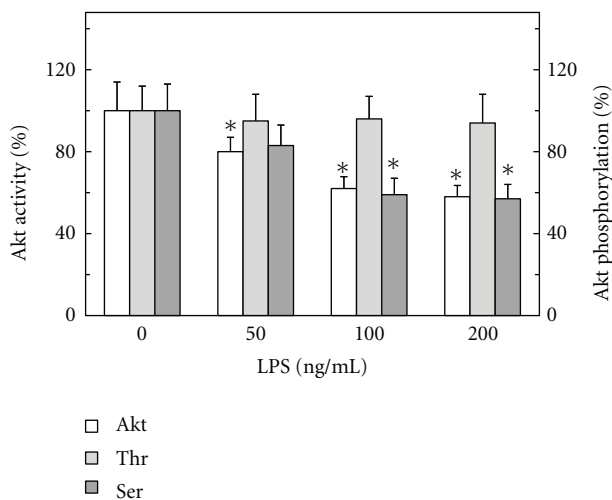
FIGURE 2: Effect of *P. gingivalis* LPS on Akt kinase activity and its phosphorylation on threonine (Thr³⁰⁸) and serine (Ser⁴⁷³) in rat sublingual salivary gland acinar cells. The cells were treated with the indicated concentrations of the LPS and incubated for 16 h. *$P < .05$ compared with that of control (LPS − 0).
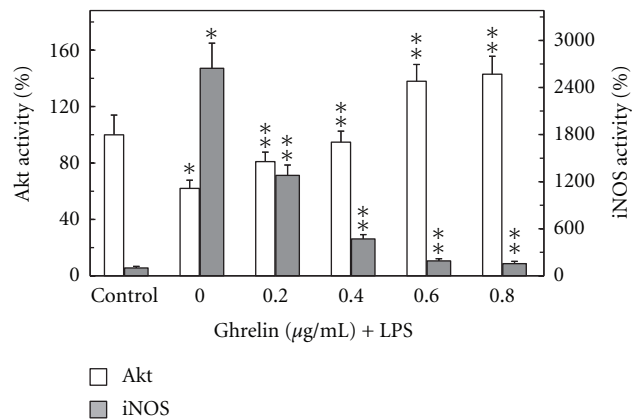

FIGURE 4: Effect of ghrelin on *P. gingivalis* LPS-induced changes in the expression of Akt kinase and iNOS activities in the salivary gland acinar cells. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/mL and incubated for 16 h. Values represent the means ± SD of five experiments. *$P < .5$ compared with that of control. **$P < .05$ compared with that of LPS alone.

generation and a drop in the acinar cell Akt activity. As shown in Figure 1, the LPS at 100 ng/mL concentration elicited a 49% drop in mucin synthesis, while the NO production showed a 15.1-fold increase. Moreover, the effect of the LPS (at 100 ng/mL) was manifested in a 38% decrease in the acinar cell Akt activity, which was reflected mainly in reduction of the extent in its phosphorylation at Ser⁴⁷³ (Figure 2).

We next examined the influence of peptide hormone, ghrelin, on the LPS-induced disturbances in NO generation system, Akt activation, and the interference with mucin synthesis. The results revealed that preincubation of the acinar cells with ghrelin led to a concentration-dependent reversal of the LPS-induced suppression of Akt, mucin synthesis, and cNOS activity, as well as countered the LPS-induced upregulation in iNOS. As a result, mucin synthesis, at 0.6 µg/mL of ghrelin, showed an 1.8-fold increase over that of the LPS, while the activity of cNOS increased 5.3-folds (Figure 3). Moreover, ghrelin at 0.6 µg/mL evoked a 2.2-fold increase in Akt activity and a 13.9-fold decrease in the LPS-induced activity of iNOS (Figure 4).

Further insights into the role of NOS isozyme system in ghrelin-induced up-regulation in Akt activity and mucin synthesis were ascertained with the use of cNOS and iNOS inhibitors. For this, the acinar cells prior to incubation with
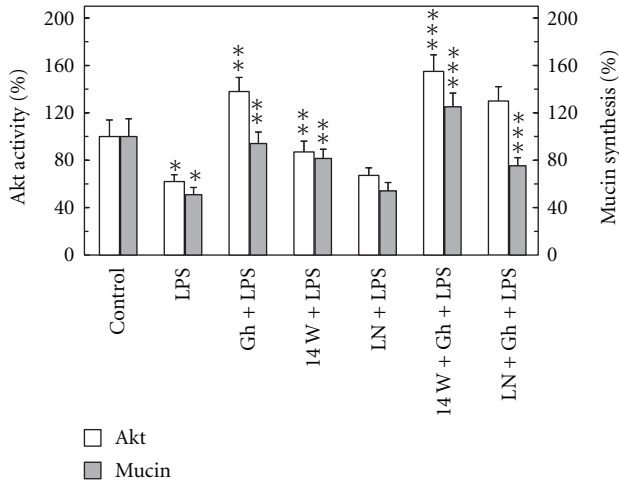
FIGURE 5: Effect of iNOS inhibitor, 1400 W, and cNOS inhibitor, L-NAME, on the ghrelin (Gh)-induced changes in Akt kinase activity and mucin synthesis in the acinar cells. The cells, preincubated with 30 μM 1400 W (14 W) or 200 μM L-NAME (LN), were treated with Gh at 0.6 μg/mL and incubated for 16 h with the LPS at 100 ng/mL, in the presence of [^{3}H]glucosamine as a marker of mucin synthesis. Values represent the means $\pm$ SD of five experiments. *$P < .05$ compared with that of control. **$P < .05$ compared with that of LPS alone. ***$P < .05$ compared with that of Gh + LPS.
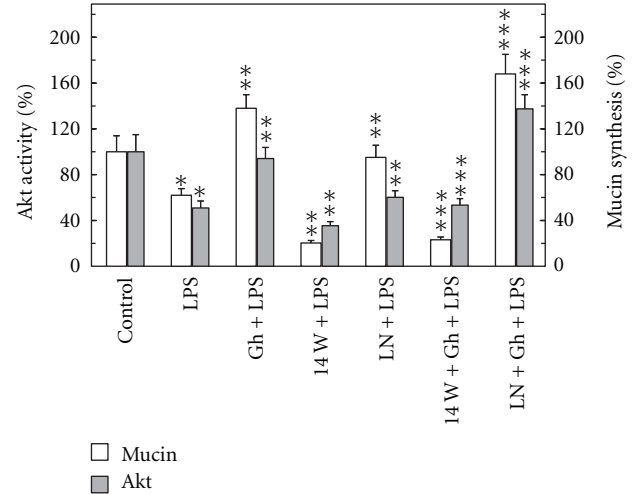

FIGURE 7: Effect of Akt inhibitor, SH-5, and ascorbate on the ghrelin- (Gh-) induced changes in mucin synthesis and Akt activity in the acinar cells exposed to *P. gingivalis* LPS. The cells, preincubated with 20 μM SH-5 (SH) or 300 μM ascorbate (As), were treated with Gh (0.6 μg/mL) and incubated for 16 h with the LPS at 100 ng/mL, in the presence of [^{3}H]glucosamine as a marker of mucin synthesis. *$P < .05$ compared with that of control. **$P < .05$ compared with that of LPS alone. ***$P < .05$ compared with that of Gh + LPS.
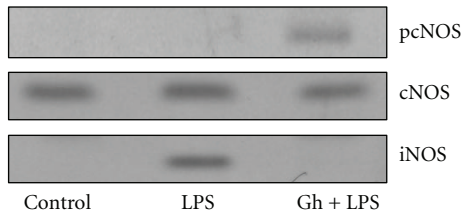

FIGURE 6: Effect of ghrelin on *P. gingivalis* LPS-induced changes in the expression of iNOS protein and cNOS phosphorylation in the acinar cells. The cells were treated with the LPS at 100 ng/mL or ghrelin (Gh) at 0.6 μg/mL + LPS and incubated for 16 h. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with anti-iNOS antibody or phosphorylation-specific cNOS (pcNOS) antibody, and after stripping reprobed with anti-cNOS antibody. The immunoblots shown are representative of three experiments.

ghrelin were pretreated with cNOS inhibitor, L-NAME, or iNOS inhibitor, 1400 W, and assayed for mucin synthesis and Akt kinase activity. We found that in addition to ghrelin, the LPS-induced inhibition in mucin synthesis and Akt activity was subject to partial suppression by iNOS inhibitor, 1400 W (Figure 5). Furthermore, iNOS inhibition with 1400 W produced amplification in the effect of ghrelin on Akt activity as well as mucin synthesis. On the other hand, the cNOS inhibition with L-NAME, while having no discernable effect on the extent of the LPS- and ghrelin-induced changes in Akt activity, was found to exert the inhibitory influence on ghrelin-induced increase in mucin synthesis (Figure 5).

The role of ghrelin in countering the LPS-induced disturbances in the processes of Akt activation and mucin synthesis was further assessed by examining the LPS influence on the expression iNOS protein and cNOS phosphorylation. For this, the acinar cells were incubated with the LPS or ghrelin plus LPS, and the lysates were probed with antibodies directed against iNOS, cNOS, and phosphorylated cNOS (Ser^{1179}). As shown in Figure 6, the LPS-induced up-regulation in iNOS activity, and accompanied reduction in mucin synthesis and Akt activity, was associated with the induction of in iNOS protein expression, while the suppression of the LPS effect by ghrelin was reflected in a marked reduction in the iNOS protein expression. Moreover, we observed that the countering effect of ghrelin on the LPS-induced suppression in cNOS activity was manifested by an increase in the enzyme protein phosphorylation at Ser^{1179} (Figure 6). Furthermore, the countering effect of ghrelin on the LPS-induced impairment in mucin synthesis was subject to suppression by Akt inhibitor, SH-5, while nitrosothiols reducing agent, ascorbate, produced amplification in the effect of ghrelin on Akt activity as well as mucin synthesis (Figure 7).

Hence, to ascertain the involvement of the LPS-induced Akt S-nitrosylation in the impairment in salivary mucin synthesis and to reveal further the countering role of ghrelin, the acinar cells were incubated with the LPS or ghrelin plus LPS, and the lysates following the biotin switch procedure were probed with antibodies directed against phospho-Akt (Ser^{473}) and total Akt (Figure 8). Western blot analysis revealed that the acinar cells exposed to the LPS alone showed a marked increase in Akt protein S-nitrosylation, while the effect of ghrelin was reflected in the loss in Akt S-nitrosylation and the increase in its protein phosphorylation.
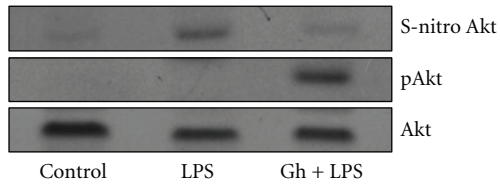
FIGURE 8: Effect of Ghrelin (Gh) on *P. gingivalis* LPS-induced Akt S-nitrosylation. The sublingual salivary gland cells were treated with the LPS at 100 ng/mL, or Gh at 0.6 μg/mL + LPS and incubated for 16 h. A portion of the cell lysates was processed by biotin switch procedure for protein S-nitrosylation and, along with the reminder of the lysates, subjected to SDS-PAGE, transferred to nitrocellulose, and probed with phospho-Akt (Ser^{473}) antibody, and after stripping reprobed with anti-Akt antibody. The immunoblots shown are representative of three experiments.

These finding suggest that ghrelin counters *P. gingivalis* LPS-induced impairment in salivary mucin synthesis through a decrease in Akt protein S-nitrosylation and the increase in its phosphorylation.

4. Discussion

Disturbances in salivary glands secretory function and the resulting diminished production of saliva, along with the ensuing weakening of the protective coating covering tooth enamel and oral mucosal surfaces, are the primary factors in determining susceptibility to bacterial infection and the development of chronic mucosal inflammation that leads to periodontal disease [2, 4, 6]. Studies indicate that the oral mucosal responses to periodontopathic bacterium, *P. gingivalis*, and its key virulence factor, cell wall LPS, are manifested by a massive rise in epithelial cell apoptosis and proinflammatory cytokine production and the disturbances in NO signaling pathways [3, 4, 13]. Moreover, *P. gingivalis* LPS exerts the detrimental effect on the synthesis of salivary mucin [5, 8], thus leading to weakening of the saliva-derived oral mucosal coating that constitutes pre-epithelial element of oral mucosal defense [6, 7]. A growing body of evidence, furthermore, links the impairment in mucin synthesis caused by *P. gingivalis* to the disturbances in NOS isozyme system responsible for NO generation [4, 8]. As serine/threonine kinase, Akt, plays a central role in controlling the activity of NOS isozyme system, and since a peptide hormone, ghrelin, has been implicated in the kinase regulation [14–16, 21], in this study we investigated the influence of ghrelin on the Akt-mediated signaling that leads to *P. gingivalis* LPS-induced interference in salivary mucin synthesis.

Our findings revealed that *P. gingivalis* LPS-induced changes in sublingual salivary gland mucin synthesis and accompanied suppression in Akt activity were associated with up-regulation in iNOS and a decrease in the activity of cNOS. Further, we found that the countering effect of ghrelin on the LPS-induced impairment in mucin synthesis was reflected in the increase in Akt activation through phosphorylation at Ser^{473}, reduction in iNOS activity, and up-regulation in cNOS activity. Moreover, an amplification in the countering effect of ghrelin on the LPS-induced

changes in mucin synthesis and Akt activity was attained with iNOS inhibitor, 1400 W, whereas the cNOS inhibition with L-NAME, while having no discernible effect on the changes in Akt activity, was found to exert the inhibitory influence on ghrelin-induced increase in mucin synthesis. Also, in agreement with well-documented involvement of Src/Akt pathway in GHS-R responses to ghrelin stimulation [14, 24], the countering effect of ghrelin on the LPS-induced changes in mucin synthesis was subject to suppression by Akt inhibitor, SH-5, which also abrogated the ghrelin-induced up-regulation in cNOS activity. The later finding suggests that Akt-mediated cNOS activation plays an essential role in the mechanism of ghrelin action on the LPS-induced impairment in mucin synthesis. This interpretation of our results is in concordance with the reports showing that rapid regulation of cNOS activity in response to external stimuli occurs through posttranslational process of enzyme protein phosphorylation at the critical Ser^{1179} with the involvement of Akt kinase [22, 27].

We next sought additional leads into the relationship between the LPS-induced up-regulation in iNOS activity and the changes in Akt activation and the acinar cell capacity for mucin synthesis. As up-regulation in iNOS activity in response to LPS involves transcriptional factor NF-κB transactivation of iNOS gene for the induction in the enzyme protein [17, 28], we analyzed the influence of ghrelin on the iNOS protein expression. We found that *P. gingivalis* LPS induction in iNOS activity was associated with the increase in the enzyme protein, while the countering effect of ghrelin was reflected in a marked inhibition in the iNOS protein expression. Interestingly, as reported recently with thiazolidinedione ligand to peroxisome proliferator-activated receptor γ(PPARγ), rosiglitazone [19], the repression of LPS-stimulated iNOS gene induction by the inhibition of NF-κB transactivation results in a reduced expression of iNOS activity as well as an increased Akt kinase activation. Moreover, we have shown earlier that the activation of PPARγ with thiazolidinedione ligand, ciglitazone, not only counters *P. gingivalis* LPS-induced up-regulation in the expression of iNOS activity, but also exerts the modulatory effect on salivary mucin synthesis [4]. These findings together with the demonstrated involvement of ghrelin in cNOS activation [13], attest to the importance of ghrelin in controlling the extent of inflammatory involvement in response to bacterial infection.

The accumulating evidence, furthermore, indicates that NO generated by iNOS in response to LPS challenge is involved in protein modification through S-nitrosylation that results in functional alterations [20, 21, 24]. Indeed, the NO produced by iNOS has been implicated in S-nitrosylation of the insulin receptor and proteins involved in early steps of the insulin signal transduction, and Akt S-nitrosylation was reported to be responsible for the reduced kinase activity in muscle cells of diabetic mice [19–21]. Hence, taking into consideration that enhancement in the effect of ghrelin on mucin synthesis and Akt activation was attained with iNOS inhibitor, 1400 W, we analyzed the effect of nitrosothiols reducing agent, ascorbate. We found that, in keeping with well-known susceptibility of S-nitrosylated

proteins to reduction by ascorbic acid [25, 26], preincubation of the acinar cells with ascorbic acid produced amplification in the countering effect of ghrelin on the LPS-induced changes in Akt activity as well as mucin synthesis. These data thus point to the involvement of iNOS in the LPS-induced sublingual salivary gland Akt kinase S-nitrosylation and suggests that S-nitrosylation exerts the detrimental effect on the kinase activity as well as mucin synthesis. Moreover, our results strongly imply that the countering effect of ghrelin on *P. gingivalis* LPS-induced impairment in salivary mucin synthesis is closely linked to the events of Akt activation.

The supporting evidence as to the role of ghrelin in countering *P. gingivalis* LPS interference with salivary mucin synthesis through the processes associated with Akt activation comes from the results of biotin switch assay. We found that the acinar cells exposed to incubation with the LPS alone showed a marked increase Akt protein S-nitrosylation, while the countering effect of ghrelin was reflected in the loss of in Akt S-nitrosylation and the increase in its phosphorylation at Ser^{473}. Hence, consistent with our results, the persistent induction in iNOS associated with oral mucosal response to *P. gingivalis* infection may be of major consequence defining the severity of oral mucosal inflammatory involvement and the extent of the impairment in salivary mucin production. As salivary mucins are recognized as a major component of oral mucosal mucus coat that acts as a barrier protecting soft oral tissue against microbial insults [6, 7], the findings provide further insight into the mechanism by which *P. gingivalis* is capable of compromising the pre-epithelial element of oral mucosal defense and affects the progression of periodontal disease.

In summary, our study demonstrates that the induction in iNOS expression, elicited in the acinar cells of salivary gland by *P. gingivalis* LPS, leads to Akt inactivation through S-nitrosylation that exerts the detrimental effect on salivary mucin synthesis. We also show that the countering effect of ghrelin on the LPS-induced impairment in mucin synthesis is associated with Akt kinase activation through phosphorylation at Ser^{473}. Furthermore, our data point to the importance of salivary ghrelin in countering the destructive consequences of chronic mucosal inflammation that characterizes periodontitis.

References

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