Ghrelin-induced cSrc activation through constitutive nitric oxide synthase-dependent S-nitrosylation in modulation of salivary gland acinar cell inflammatory responses to *Porphyromonas gingivalis*

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ABSTRACT

A peptide hormone, ghrelin, recognized for its role in the regulation of nitric oxide production has emerged as an important modulator of oral mucosal inflammatory responses to periodontopathic bacterium, *P. gingivalis*. As cSrc kinase plays a major role in controlling the activity of nitric oxide synthase (NOS) system, in this study we investigated the influence of *P. gingivalis* LPS on the processes of Src activation in rat sublingual gland acinar cells. The LPS-induced enhancement in the activity of inducible (i) iNOS and the impairment in constitutive (c) cNOS were reflected in the suppression in cSrc activity and the extent of its phosphorylation at Tyr^{416}. Further, we show that the countering effect of ghrelin on the LPS-induced changes in cSrc activity and the extent of its phosphorylation was accompanied by a marked reduction in iNOS and the increase in cNOS activation through phosphorylation at Ser^{1179}. Moreover, the effect of ghrelin on cSrc activation was associated with the kinase S-nitrosylation that was susceptible to the blockage by cNOS inhibition. Our findings suggest that *P. gingivalis*-induced up-regulation in iNOS leads to disturbances in cNOS phosphorylation that exerts the detrimental effect on the processes of cSrc activation through cNOS mediated S-nitrosylation. We also show that the effect of ghrelin on *P. gingivalis*-induced inflammatory changes are manifested in the enhancement in cSrc activation through S-nitro- sylation and the increase in its phosphorylation at Tyr^{416}.

Keywords: Ghrelin; *P. Gingivalis*; Salivary Acinar Cells; cNOS; cSrc Activation; S-Nitrosylation

1. INTRODUCTION

Ghrelin, a 28-amino acid peptide hormone, initially isolated from the stomach [1,2], and more recently identified in oral mucosa, saliva and the acinar cells of salivary glands [3], is recognized as an important modulator of oral mucosal inflammatory responses to periodontopathic bacterium, *P. gingivalis* through the regulation of nitric oxide synthase (NOS) isozyme system [4-6]. The signaling mechanism that underlies the regulation of NO by ghrelin involves the stimulation of growth-hormone secretagogue receptor type 1a (GHSR1a), a seven-transmembrane G-protein coupled receptor, that leads to activation of heterotrimeric G protein-dependent network of protein kinases, including that of cellular Src (cSrc), a member of the non-receptor protein tyrosine kinase Src family [7-10]. All currently known eleven members of the Src family kinases share similar structural organization, consisting of N-terminal myristoylation motif that facilitates membrane attachment, followed by the protein binding SH3 and SH2 homology domains, a protein - tyrosine kinase domain, and a C-terminal regulatory tail [8,10].

The 60 kDa phosphoprotein (pp60$^{c\text{-}src}$) Src is encoded by a physiological *c-src* gene, which is cellular homologue of the highly transforming *v-src* gene of Rouse sarcoma virus [8,11,12]. The activity of cSrc is tightly regulated by reversible phosphorylation on Tyr^{527} and Tyr^{416} amino acid residues, which either inactivates or activates the kinase. The inhibitory phosphorylation at Tyr^{527} of C-terminal tail locks the kinase in an inactive dormant conformation through the interaction with its SH2 domain [9]. The dormant form of the enzyme is destabilized by dephosphorylation or displacement of the inhibitory Tyr^{527} from the SH2 binding pocket, thus ex-

posing the activation loop to autophosphorylation at Tyr[416] which stabilizes the enzyme in its active state [9, 11]. Furthermore, there are indications that in addition to phosphorylation/dephosphorylation-based circuitry of Src activation, the activity of cSrc may be also regulated through S-nitrosylation at the kinase cysteine residues located within the C-terminal region of its catalytic domain [11,13,14].

Indeed, protein modification through targeted S-nitrosylation at the critical cysteine residues, with the involvement of both constitutive and inducible forms of NOS system, is gaining recognition as an important post-translational event of significance to a variety of biological processes affected by NO [4-6,13,14]. Moreover, the NO-induced Src protein S-nitrosylation has been shown to promote the kinase activation through autophosphorylation at Tyr[416] that appears to be independent of the phosphorylation status of C-terminal Tyr[527] autoinhibitory site [14-16].

As oral mucosal inflammatory responses to periodontopathic bacterium, *P. gingivalis,* are characterized by the disturbances in NO production, and since cSrc kinase plays a central role in transduction of signals that regulate the activity of NOS isozyme system [4,5,13,14], in this study we investigated the impact of *P. gingivalis* key virulence factor, LPS, on the cSrc kinase activity in sublingual salivary gland acinar cells. Moreover, considering the demonstrated role of ghrelin in the regulation of NOS system [4,5,17], we examined the influence of this peptide hormone on processes of cSrc activation through S-nitrosylation.

2. MATERIALS AND METHODS

2.1. Sublingual Salivary Gland Cell Incubation

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 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum, and gently dispersed by trituration with a syringe, and settled by centrifugation [18]. After rinsing, the cells were resuspended in the medium to a concentration of 2 $\times$ 10^7 cell/ml, transferred in 1 ml aliquots to DMEM in culture dishes and incubated under 95% O_2 - 5% CO_2 atmosphere at 37°C for 16h in the presence 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, PP2, (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, as-

sessed by Trypan blue dye exclusion assay [18], was greater than 98%.

2.2. *Porphyromonas Gingivalis* Lipopolysaccharide

P. gingivalis used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 33277 [19]. 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 $\times$ 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%.

2.3. NO Production, and cNOS and iNOS Activity Assay

NO production in the acinar cells of sublingual salivary gland was determined by measuring the stable NO metabolite, nitrite, accumulation in the culture medium using Griess reaction [20]. A 100 µl 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 [5,18]. 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, centrifuged and the formed L-[^{3}H] citrulline contained in the flow through was quantified by scintillation counting.

2.4. Src Kinase Activity Assay

Tyrosine kinase activity of cSrc in sublingual salivary gland acinar cells was measured by using polyE$_4$Y (Sigma) and [γ-^{32}P] ATP (Amersham) as the substrates [21]. The cells were lysed in lysis buffer (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1% Triton X-100, 0.5% sodium 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 $12,000 \times g$ for 10 min, and the supernatants were subjected to protein determination using BCA protein assay kit (Pierce). The supernatant samples containing equal total protein content were then immunoprecipitated with anti-Src antibody (Sigma) for 2 h at 4°C. Protein A/G agarose beads were 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 kinase assay buffer (10 mM Tris-HCl, pH 7.4, 150 mM NaCl, 5 mM MgCl$_2$, 5 mM MnCl$_2$, 2 mM EDTA, and 2 mM dithithreitol), centrifuged, and the supernatants used for the Src activity assay. For this, the samples containing 25 µg of the cell homogenate protein in 50 µl of Src kinase assay buffer were incubated with 1 mg/ml of polyE$_4$Y and 100 mM ATP containing 10 µCi of [γ-^{32}P] ATP, at 30°C for 20 min. The reaction was terminated by adding 25 µl of 40% trichloroacetic, the samples were spotted onto phosphorcellulose paper, and following thorough washing with 1% phosphoric acid and then acetone [22], the radioactivity incorporated into polyE$_4$Y was determined by liquid scintillation counting.

2.5. Src Kinase Phosphorylation Assay

Measurements of the phosphorylation status of cSrc at Tyr^{416} in sublingual salivary gland acinar cells were conducted using Phospho-Src (Tyr416) ELISA kits (Cell Signaling). The acinar cells were lysed on ice for 30 min in lysis buffer (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM sodium pyrophosphate,1 mM sodium orthovanadate, 1 mM β-glycerophosphate, 1 mM PMSF, 1 µg/ml leupeptin), and centrifuged at $12,000 \times g$ for 10 min. The supernatants diluted (1:1) in standard diluent buffer were pipetted in 100 µl aliquots into wells containing immobilized capture antibody specific for Src (pTyr^{416}), and following washing the complex was reacted with Src detection antibody. After washing, the retained complex was labeled with horseradish peroxidase and probed with TMB reagent for spectrophotometric quantification at 450 nm.

2.6. Src Protein S-Nitrosylation Assay

Assessment of cSrc kinase S-nitrosylation in the acinar cells of sublingual salivary gland was conducted using a biotin switch procedure for protein S-nitrosylation [23, 24]. The cells were treated with iNOS inhibitor, 1400W (30 µM) or cNOS inhibitor, L-NAME (200 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 $500 \times g$ 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 neocuprin, pH 7.7), and the unnitrosylated thiol groups were blocked with S-methyl methane-thiosulfonate reagent at 50°C for 20 min [24]. The proteins were precipitated with acetone, resuspended in 0.2 ml of HEN buffer containing 1% SDS, and subjected to targeted nitrothiol 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-mercap-toethanol [24]. The obtained proteins were then analyzed by Western blotting.

2.7. Immunoblotting Analysis

The acinar cells of sublingual salivary gland 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 [5]. Following brief sonication, the lysates were centrifuged at $10,000 \times g$ 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 1M Tris-HCl (pH 6.8), 10% SDS, and 10 mM dithiotreitol for 30 min at 55°C, and probed with antibody against total cNOS, iNOS or Src. Immunoblotting was performed using specific antibodies directed against iNOS, cNOS and phospho-cNOS (Ser^{1179}), (Calbiochem), and cSrc (monoclonal 327, Sigma) and phospho-Src (Tyr^{416}), (Cell Signaling).

2.8. Data Analysis

All experiments were carried out using duplicate sampling, and the results are expressed as means ± SD. Analysis of variance (ANOVA) and nonparametric Kruskal-Wallis tests were used to determine significance.
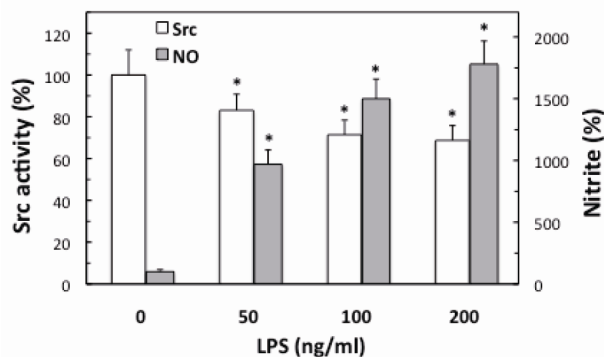
Figure 1. Effect of *P. gingivalis* LPS on the expression of Src kinase activity and nitrite production in rat sublingual salivary gland acinar cells. The acinar cells were treated with the indicated concentrations of the LPS and incubated for 16 h. Values represent the means ±SD of five experiments. *$p < 0.05$ compared with that of control (LPS - 0).
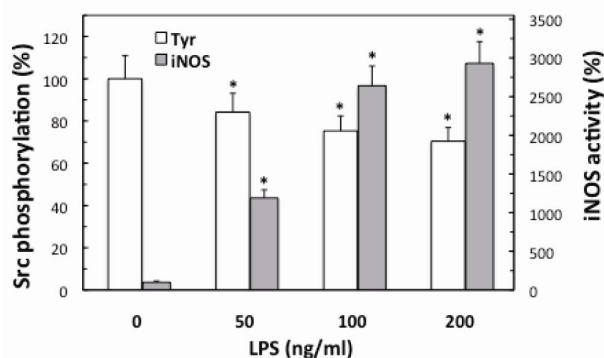

Figure 2. Effect of *P. gingivalis* LPS on Src kinase tyrosine (Tyr[416]) phosphorylation and the expression of inducible nitric oxide synthase (iNOS) activity in rat sublingual salivary gland acinar cells. The cells were treated with the indicated concentrations of the LPS and incubated for 16 h. *$p < 0.05$ compared with that of control (LPS - 0).

Any difference detected was evaluated by means of post hoc Bonferroni test, and the significance level was set at $p < 0.05$.

3. RESULTS

To examine the influence of periodontopathic bacterium, *P. gingivalis*, on the relationship between cSrc kinase activation and the disturbances in NO production, we employed rat sublingual salivary gland acinar cells exposed to *P. gingivalis* key virulence factor, LPS. We found that the LPS-induced massive increase in NO production was associated with a dose-dependent decrease in the acinar cell Src activity, which at 100 ng/ml LPS showed a 28.6% decrease (**Figure 1**). Moreover, the inhibitory effect of the LPS, at 100 ng/ml, on cSrc activity was reflected in a 25% drop in the enzyme Tyr[416] phosphorylation, and a 30% drop in the enzyme Tyr[416]
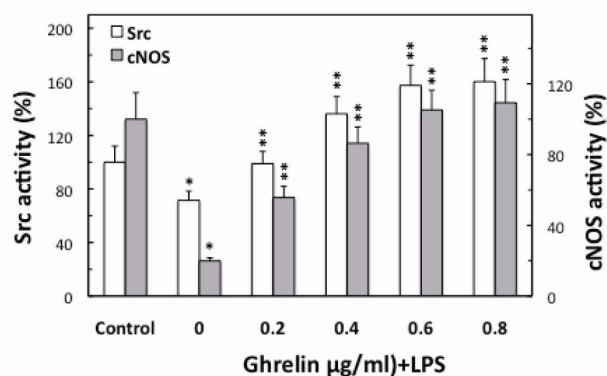

Figure 3. Effect of ghrelin on *P. gingivalis* LPS-induced changes in sublingual salivary gland acinar cell expression of Src kinase and cNOS activities. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/ml and incubated for 16 h. *$p < 0.05$ compared with that of control. **$p < 0.05$ compared with that of LPS alone.

phosphorylation occurred at 200 ng/ml LPS (**Figure 2**). We also established that the disturbances in NO production elicited in the acinar by *P. gingivalis* LPS at 100 ng/ml were manifested by a 26.4-fold up-regulation in iNOS activity (**Figure 2**), while the activity of cNOS showed a 5.2-fold decrease (**Figure 3**).

Our further results revealed that preincubation of the acinar cells with ghrelin led to a concentration-dependent reversal in the LPS-induced suppression of cSrc activity and the extent of its protein phosphorylation on Tyr[416]. As a result the activity of cSrc in the presence of 0.6 µg/ml ghrelin increased 2.2-fold over that of the LPS (**Figure 3**), while the Src protein phosphorylation at Tyr[416] showed a 2.3-fold increase (**Figure 4**). Further-
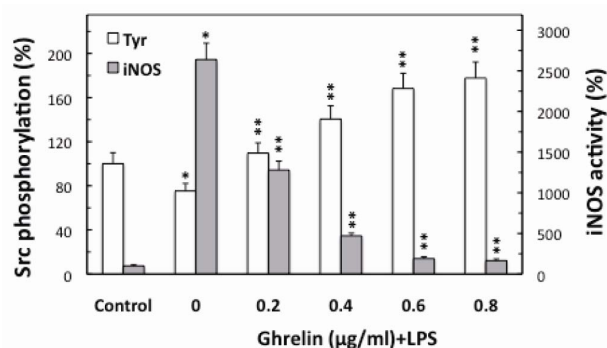

Figure 4. Effect of ghrelin on *P. gingivalis* LPS-induced changes in Src kinase tyrosine (Tyr[416]) phosphorylation and the expression of iNOS activity in sublingual salivary gland acinar cells. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100ng/ml and incubated for 16 h. Values represent the means ±SD of five experiments. *$p < 0.05$ compared with that of control. **$p < 0.05$ compared with that of LPS alone.
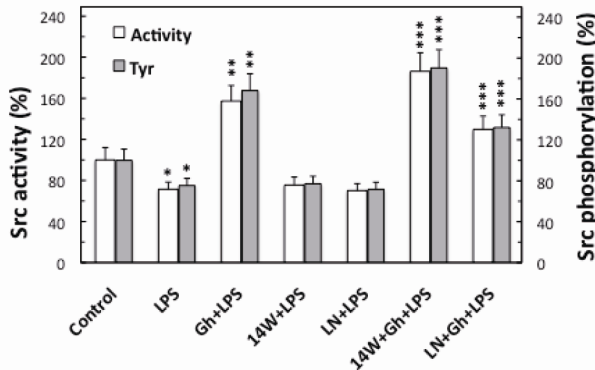
Figure 5. Effect of iNOS inhibitor, 1400W, and cNOS inhibitor, L-NAME, on the ghrelin (Gh)-induced changes in Src kinase activity and its phosphorylation at tyrosine (Tyr^{416}) in the salivary gland acinar cell exposed to *P. gingivalis* LPS. The cells, preincubated with 30 µM 1400 W (14 W), or 200 µM L-NAME (LN), were treated with Gh at 0.6 µg/ml and incubated for 16 h in the presence of 100 ng/ml LPS. Values represent the means ± SD of five experiments. *$p < 0.05$ compared with that of control. **$p < 0.05$ compared with that of LPS alone. ***$p < 0.05$ compared with that of Gh + LPS.
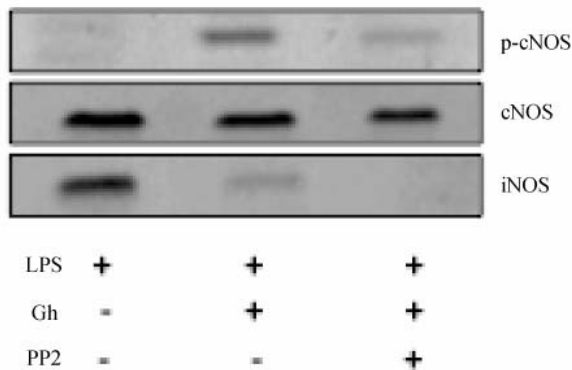

Figure 6. Effect of ghrelin (Gh) on *P. gingivalis* LPS-induced changes in the expression of iNOS protein and cNOS phosphorylation in the salivary gland acinar cells. The cells were treated with Gh at 0.6 µg/ml or Src inhibitor, PP2 at 20 µM + Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose and probed with phosphorylation specific cNOS (pcNOS) antibody, and after stripping the membranes were probed with anti-cNOS and anti-iNOS antibody. The immunoblots shown are representative of three experiments.

more, we observed that ghrelin at 0.6 µg/ml evoked a 5.3-fold increase in the acinar cell cNOS activity (**Figure 3**), and produced a 13.9-fold reduction in the LPS-induced iNOS activity (**Figure 4**).

To reveal further insight into the relationship between ghrelin-induced changes in the activity cNOS and iNOS enzymes and the acinar cell cSrc activation, we examined the role of NO generated by the NOS isozyme system. For this, the cells prior to incubation with ghrelin

were pretreated with cNOS inhibitor, L-NAME, or iNOS inhibitor, 1400 W, and assayed for cSrc activity as wells as the extent of its protein phosphorylation at Tyr^{416}. The results revealed that ghrelin-induced up-regulation in cSrc activity and Tyr^{416} phosphorylation was subject to inhibition by cNOS inhibitor, L-NAME, whereas preincubation with iNOS inhibitor, 1400 W, produced an amplification in the effect of ghrelin on Src activity and the extent of its protein phosphorylation at Tyr^{416} (**Figure 5**). These data, thus, indicate that ghrelin-induced Src activation through its Tyr^{416} phosphorylation occurs with the involvement of cNOS.

As NO generated by iNOS plays a role in *P. gingivalis* LPS-induced cNOS S-nitrosylation that interferes with the enzyme activation through phosphorylation at Ser^{1179} [5], we next examined the influence of ghrelin on the expression of iNOS protein and cNOS phosphorylation. The acinar cells prior to incubation with the LPS were pretreated with ghrelin or ghrelin plus Src inhibitor, PP2, and the lysates were probed with antibodies directed against iNOS, cNOS and phosphorylated cNOS (Ser^{1179}). As shown in **Figure 6**, the effect of the LPS was manifested in the induction of iNOS protein expression and the inhibition in cNOS phosphorylation, while the countering effect of ghrelin was reflected in a marked reduction in the iNOS protein expression and the increase in cNOS phosphorylation at Ser^{1179}. Moreover, the suppression of ghrelin effect on cNOS phosphorylation as well as further reduction in iNOS protein was observed in the presence of Src kinase inhibitor, PP2 (**Figure 6**), thus suggesting the involvement of cSrc in the regulation of NOS isozyme system at both translational and post-translational levels.
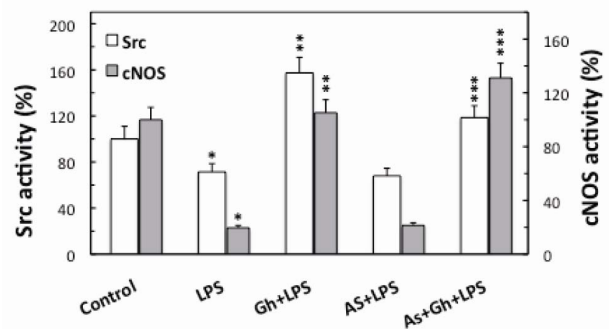

Figure 7. Effect of ascorbate on the ghrelin (Gh)-induced changes in the expression of Src kinase and cNOS activities in sublingual salivary gland acinar cells exposed to *P. gingivalis* LPS. The cells, preincubated with 300 µM ascorbate (As), were treated with Gh at 0.6 µg/ml and incubated for 16h in the presence of 100 ng/ml LPS. Values represent the means ± SD of five experiments. *$p < 0.05$ compared with that of control. **$p < 0.05$ compared with that of LPS alone. ***$p < 0.05$ compared with that of Gh + LPS.
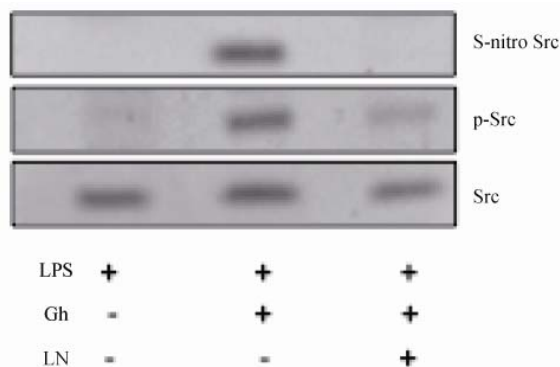
Figure 8. Effect of ghrelin (Gh) on *P. gingivalis* LPS-induced Src kinase S-nitrosylation. The salivary gland acinar cells were treated with Gh (0.6 µg/ml), or cNOS inhibitor, L-NAME (200 µM) + Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. A portion of the cell lysates was processed by biotin switch procedure for protein S-nitrosylation and, along with the reminder of the lysates, resolved on SDS-PAGE, transferred to nitrocellulose and probed with phospho-Src (Tyr[416]) antibody, and after stripping reprobed with anti-Src antibody. The immunoblots shown are representative of three experiments.

Further, we found that ghrelin countering effect on the LPS-induced Src activation in the acinar cells of salivary gland displayed susceptibility to suppression by nitrosothiols reducing agent, ascorbate, which also produced enhancement in the effect of ghrelin on cNOS activity (**Figure 7**). Hence, to ascertain the relationship between Src kinase S-nitrosylation and its activation through phosphorylation, we examined the dependence of cSrc S-nitrosylation on the ghrelin-induced cNOS activation by the biotin switch method [23,24]. The acinar cells were incubated with *P. gingivalis* LPS or ghrelin + LPS or cNOS inhibitor, L-NAME, + ghrelin + LPS, and the lysates following the biotin switch procedure were probed with antibodies directed against phospho-cSrc (Tyr[416)] and total Src. Western blot analysis revealed that ghrelin countering effect on the LPS-induced suppression in cSrc activity was manifested by a marked increase in both the Src kinase S-nitrosylation as well as its protein phosphorylation. Moreover, the blockage of cNOS activity with L-NAME, not only caused the loss in cSrc S-nitrosylation but was also accompanied by a substantial decrease in Src protein phosphorylation at Tyr[416] (**Figure 8**). Thus, ghrelin countering effects on *P. gingivalis*-induced inflammatory changes are manifested in the enhancement in Src activation through cNOS-dependent S-nitrosylation and the increase in its phosphorylation.

4. DISCUSSION

P. 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 and is a major cause of adult tooth loss [25, 26]. The oral mucosal responses to *P. gingivalis* and its key virulence factor, cell wall LPS, are characterized by a massive rise in epithelial cell apoptosis and proinflammatory cytokine production, and the disturbances in NOS isozyme system responsible for NO generation [4-6,19,27]. Investigations into the endogenous factors capable of influencing the extent of mucosal inflammatory responses along the alimentary tract, including that of oral cavity, have brought to focus the importance of a peptide hormone, ghrelin [1-4,28]. A growing body of evidence, furthermore, points to ghrelin as a principal modulator of the mucosal NOS [4-6,17]. The mechanism that underlies the regulation of NO signaling by ghrelin relies on the receptor (GHS-R1a)-mediated activation of G protein-dependent network of protein kinases, including that of membrane-associated non-receptor tyrosine kinase, cSrc [7-10].

As Src kinase plays a pivotal role in the transduction of signals that regulate the activity of NOS isozyme system [8,13,14], in this study we investigated the influence of *P. gingivalis* LPS on the processes associated with Src activation. Our findings revealed that the LPS-induced drop in sublingual salivary gland acinar cells activity of cNOS and up-regulation in iNOS was associated with the suppression in the activity of cSrc. Moreover, the suppression in cSrc activity was reflected in a decrease in the kinase phosphorylation at Tyr[416]. Furthermore, preincubation of the acinar cells with ghrelin exerted countering effect on the LPS-induced impairment in cSrc activity and the extent of its phosphorylation on Tyr[416], and was accompanied by an increase in the cNOS activity and a marked reduction in the activity of iNOS. These findings are thus in keeping with the literature data suggesting the involvement of cSrc kinase in the regulation of NOS isozyme system at both transcriptional and post-transcriptional levels [13,14].

Indeed, in concordance with the documented involvement of cSrc in post-translational cNOS activation through phosphorylation [5,18,29], we found that the induced up-regulation in cNOS activity by ghrelin was reflected in the increase of enzyme protein phosphorylation at Ser[1179]. Also, 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 [30-32], we analyzed the influence of ghrelin on the acinar cell iNOS protein expression. We found that *P. gingivalis* LPS induction in iNOS activity was associated with the increase in the enzyme protein expression, while the countering effect of ghrelin, was reflected in a marked inhibition of the iNOS protein expression that was further suppressed in the presence of

Src kinase inhibitor, PP2.

Next we addressed the relationship between the ghrelin-induced changes in activity of cNOS and iNOS enzymes, and the Src kinase activation. As up-regulation in cSrc activation through autophosphorylation on Tyr[416] has been reported in the presence of the exogenous NO donors as well as the NO produced by endothelial NOS [13-15], we examined the effect of NOS inhibitors on cSrc activity and the extent of its protein phosphorylation at Tyr[416]. We found that ghrelin-induced up-regulation in the acinar cell cSrc activity and Tyr[416] phosphorylation displayed susceptibility to cNOS inhibitor, L-NAME, while an amplification in the ghrelin effect on cSrc activation was attained with the inhibitor of iNOS, 1400 W. From this, we concluded that cNOS plays an essential role in ghrelin-induced activation of cSrc in the acinar cells. Furthermore, the countering effect of ghrelin on the LPS-induced changes in cSrc activation was susceptible to the suppression by nitrosothiols reducing agent, ascorbate, which also produced an enhancement in the effect of ghrelin on cNOS activity. These data, together with the known susceptibility of S-nitrosylated proteins to reduction by ascorbic acid [4,5,14,15,23,24], demonstrate that *P. gingivalis* LPS-induced disturbances in the acinar cell cNOS and cSrc protein S-nitrosylation patterns interfere with the activation of both cNOS and cSrc. Moreover, our results suggest that ghrelin-induced up-regulation in cSrc activity through phosphorylation at Tyr[416] is intimately linked to the events of the kinase protein S-nitrosylation by NO generated by the cNOS.

Our assertion is further supported by the literature evidence indicating that ascorbate treatment both increases cNOS activity and reduces the enzyme protein S-nitrosylation, and that the countering effect of ghrelin on the LPS-induced impairment in cNOS activity is associated with the loss of the enzyme protein S-nitrosylation and the increase in its phosphorylation at Ser[1179] [5,13,32]. Indeed, the accumulating evidence demonstrates that protein modification through targeted S-nitrosylation at the critical cysteine, with the participation of both constitutive and inducible forms of NOS system, is a post-translational event of significance to the regulation of signal transduction pathways by NO [4-6,13-15,17,32,33]. Moreover, NO-induced Src S-nitrosylation at the critical cysteine residues located within the C-terminal region of its catalytic domain has been reported to promote cSrc activation through autophosphorylation at Tyr[416] [13-15].

Hence, to assess the role of ghrelin in countering *P. gingivalis* LPS-induced interference with cSrc activation in the acinar cells, we examined the dependence of Src S-nitrosylation on the ghrelin-induced cNOS activation by the biotin switch assay. Western blot analysis of the

acinar cell lysates revealed that ghrelin countering effect on the LPS-induced suppression in cSrc activity was manifested by a marked increase in the kinase protein S-nitrosylation as well as phosphorylation at Tyr[416]. Furthermore, the suppression of ghrelin effect on NO production with cNOS inhibitor, L-NAME, caused the loss in cSrc S-nitrosylation and a decrease in the kinase phosphorylation. These findings thus imply that the changes evoked by *P. gingivalis* in the activity of NOS isozyme system involved in NO generation are of direct relevance to cSrc kinase activation through autophosphorylation at Tyr[416], and point to the role of cSrc S-nitrosylation in the protective mechanism of ghrelin action.

In conclusion, our findings demonstrate that *P. gingivalis*-induced up-regulation in the acinar cell iNOS leads to disturbances in cNOS phosphorylation that exerts the detrimental effect on the processes of cSrc kinase activation through cNOS mediated S-nitrosylation. We also show that the effects of ghrelin on *P. gingivalis*-induced inflammatory disturbances are manifested in the enhancement in Src activation through S-nitrosylation and the increase in its phosphorylation at Tyr[416].

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