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Role of constitutive nitric oxide synthase in regulation of *Helicobacter pylori*-induced gastric mucosal cyclooxygenase-2 activation through S-nitrosylation: mechanism of ghrelin action

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ABSTRACT

Gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide (LPS), are characterized by the excessive NO and prostaglandin (PGE_2) generation due to the disturbances in nitric oxide synthase (NOS) and cyclooxygenase (COX) systems. Here, we report that the LPS-induced enhancement in gastric mucosal inducible (i) iNOS) activity and up-regulation in PGE_2 production was associated with the suppression in Akt kinase activity and the impairment in constitutive (c) cNOS activation. The stimulatory effect of the LPS on PGE_2 production, furthermore, was susceptible to suppression by COX-2 inhibitor, NS-398, and iNOS inhibitor, 1400 W. Further, we show that the countering effect of peptide hormone, ghrelin, on the LPS-induced changes was reflected in up-regulation in Akt activity and the increase in cNOS activation through phosphorylation, and accompanied by the suppression in iNOS expression and the reduction in COX-2 activity associated with the loss in COX-2 protein S-nitrosylation. Moreover, the effect of ghrelin on the LPS-induced COX-2 S-nitrosylation was subject to repression by Akt inhibition. Our findings demonstrate that induction in iNOS with *H. pylori* infection leads to COX-2 activation through S-nitrosylation and up-regulation in PGE_2 generation, and that ghrelin counters these untoward consequences of the LPS through Akt-mediated up-regulation in cNOS activation required for the iNOS gene repression.

Keywords: *H. pylori*; Gastric Mucosa; iNOS Induction; COX-2 Activation; S-Nitrosylation; Ghrelin

1. INTRODUCTION

Helicobacter pylori, a Gram-negative bacterium colonizing the gastric mucosa, is recognized as a primary cause of gastric disease, and its cell-wall lipopolysaccharide (LPS) has been identified as a potent endotoxin capable of eliciting mucosal inflammatory changes that characterize gastritis and duodenal ulcer [1-3]. Indeed, the gastric mucosal inflammatory responses to *H. pylori* infection in humans as well as well as those characterizing mucosal inflammatory changes in the animal model of *H. pylori* LPS-induced gastritis are manifested by a marked increase in epithelial cell apoptosis and proinflammatory cytokine production, and the excessive nitric oxide (NO) and prostaglandin (PGE_2) generation [4-7]. A growing body of evidence, moreover, points to the disturbances in NO generated by nitric oxide synthase (NOS) isozyme system, and the formation of PGE_2 synthesized from arachidonic acid (AA) by the action of cyclooxygenase (COX) system, as the events defining the extent of gastric mucosal inflammatory involvement in response to *H. pylori* colonization [8-12].

The physiological and pathophysiological implications of NO and PGE_2 depend on the type of isozyme system involved in their generation, their subcellular targeting and the local concentrations [12-16]. Of the three NOS isozymes responsible for NO generation, the two expressed constitutively (cNOS) are calcium/calmodulindependent and provide precise pulses of NO for a fine modulation of the cellular processes [14,16]. The third, inducible isoform (iNOS) is calcium/calmodulin-independent, and its activation by proinflammatory cytokines and bacterial LPS provides the high output of NO that is importance to host defense against microbial invasion. However, its massive and sustained production is also associated with transcriptional disturbances and the induction of apoptosis [14-18]. The cyclooxygenase system consists of two COX isozymes, the constitutive isoform or COX-1, responsible for maintaining normal

physiological prostaglandin production, and the inducible form or COX-2, which accounts for up-regulation in PGE_2 production in response to inflammatory stimulus [8,9,12]. Moreover, a large volume of data supports the existence of functional and signaling cross-talk between the products of NOS and COX systems [13,14,19-22].

Indeed, the stimulation of NO production through iNOS induction or the exogenous NO donors leads to COX enzymes activation and the increase in PGE_2 generation, while a decrease in PGE_2 formation has been observed in the presence of pharmacological inhibitors of NOS system [20-23]. Furthermore, the COX-2 activation for the increase in PGE_2 synthesis has been linked to the enzyme protein S-nitrosylation via NO derived through LPS-elicited induction in iNOS [20]. There are also indications for the role of cNOS in the iNOS-dependent COX-2 activation [21,24]. Interestingly, the disturbances in NO and PGE_2 production associated with *H. pylori* colonization are reflected in the massive up-regulation in iNOS activity and the suppression of cNOS activation [4,5,25,26]. Moreover, we have recently shown that Akt-mediated cNOS activation through phosphorylation at Ser^{1179} plays an essential role in the modulatory influence of peptide hormone, ghrelin, on the extent of gastric mucosal inflammatory responses to *H. pylori* LPS [26].

As gastric ghrelin is recognized as an important regulator of NOS and COX enzyme systems, and implicated in the control of local inflammations, gastroprotection, and modulation of the mucosal inflammatory responses to bacterial infection [18,27-31], in this study we investigated the nature of inflammatory changes induced in gastric mucosal cells by *H. pylori* LPS and the mechanism of ghrelin modulatory influence on the cross-talk between the NOS and COX systems. Our data revealed that the LPS-elicited induction in iNOS leads to COX-2 activation through S-nitrosylation and up-regulation in PGE_2 generation, and that ghrelin counters these untoward consequences of the LPS through up-regulation in cNOS phosphorylation and the suppression of iNOS gene induction.

2. MATERIALS AND METHODS

2.1. Gastric Mucosal Cell Incubation

The mucosal cells, collected by scraping the mucosa of freshly dissected rat stomachs with a blunt spatula, 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 [5]. Following 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 and incubated under 95% O_2 - 5% CO_2 atmosphere at 37˚C for 16 h in the presence of 0 ng/ml - 200 ng/ml of *H. pylori* LPS [18]. In the experiments evaluating the effect of ghrelin (rat, Sigma), cNOS inhibitor, L-NAME, iNOS inhibitor, 1400 W, Akt inhibitor, SH-5 (Calbiochem), COX-1 inhibitor, SC-560, COX-2 inhibitor, NS-398 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 [11], was greater than 97%.

2.2. *Helicobacter pylori* Lipopolysaccharide

H. pylori used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 4350 [2,5]. 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 × g for 4 h, and the resulting LPS sediment subjected to lyophilization.

2.3. PGE_2 and NO Quantification

The aliquots of cell suspension from the control and various experimental conditions were centrifuged at 1500 × g for 5 min and the conditioned medium supernatant collected. PGE_2 assays were carried out using an enzyme-linked immunoassay (Cayman) and 100 μl aliquots of the spent medium supernatant, according to the manufacturer's instructions. The amount of PGE_2 released into culture medium was determined by measuring the absorbance at 405 nm [30]. To assess NO production in gastric mucosal cells, we measured the stable NO metabolite, nitrite, accumulation in the culture medium using Griess reaction [32]. A 100 μl of spent culture medium was incubated for 10 min at room temperature with 0.1 ml of Griess reagent and the absorbance was measured at 570 nm.

2.4. COX-2 Activity Assay

For measurements of COX-2 activity, the gastric mucosal cells from the control and various experimental conditions were settled by centrifugation, rinsed with phosphate-buffered saline, and homogenized in 0.3 ml cold sample buffer containing 0.1 M Tris-HCl, pH 7.8, and 1 mM EDTA, centrifuged at 12,000 × g for 10 min at 4˚C, and the supernatant collected. The COX-2 activity in 40 μl aliquots of the resulting supernatant was measured using COX activity assay kit in the absence and the presence of COX-1 inhibition (SC-560), according to the manufacturer's (Cayman) instruction. The absorbance was read at 590 nm.

2.5. cNOS and iNOS Activity Assay

Nitric oxide synthase activities of cNOS and iNOS enzymes in the gastric mucosal cells were measured by monitoring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline using NOS-detect kit (Stratagene). The 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+}-depenedent cNOS), and centrifuged. 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.

2.6. Akt Activity Assay

The kinase activity of Akt in gastric mucosal 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 [26]. The agarose beads were then suspended for 30 min at room temperature in the kinase assay buffer, centrifuged, and the supernatants used for the Akt activity assay by following the manufacturer's instruction.

2.7. COX-2 Protein S-Nitrosylation Assay

A biotin switch procedure was employed to assess COX-2 protein S-nitrosylation [33,34]. The gastric mucosal cells were treated with iNOS inhibitor, 1400 W (40 µM) or ghrelin (0.5 µg/ml), or Akt inhibitor, SH-5 (30 µM) + ghrelin (0.5 µg/ml), and incubated for 16 h in the presence of 100 ng/ml of *H. pylori* 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 neocuprin, pH 7.7), and the unnitrosylated thiol groups were blocked with S-methyl methanethiosulfonate reagent at 50˚C for 20 min [34]. 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-mercaptoethanol [34]. The obtained proteins were then analyzed by Western blotting.

2.8. Immunoblotting Analysis

The mucosal 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 [18]. Following brief sonication, the lysates were centrifuged at 12,000 g for 10 min, and the supernatants were normalized with respect to protein concentration 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 specific polyclonal rabbit antibodies directed against COX-1, COX-2, and iNOS (Calbiochem). The phosphorylated cNOS (pcNOS) was analyzed using specific antibody (Calbiochem) directed against phospho-cNOS (mouse anti-eNOS, $pSer^{1179}$), and following stripping, probed with antibody against total cNOS.

2.9. 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. 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 further ascertain the nature of gastric mucosal inflammatory responses to *H. pylori* and to reveal the modulatory role ghrelin, we investigated the interaction between the systems responsible for NO generation and prostaglandin production. Using rat gastric mucosal cells exposed to *H. pylori* key virulence factor, LPS, we

demonstrated that the LPS caused a dose-dependent increase in the mucosal cell PGE_2 and NO production, which at 100 ng/ml LPS reached the respective values of 12.7-fold and 14.5-fold over that of controls (**Figure 1**). Moreover, we also established that the LPS effect on NO production was reflected in a 20.5-fold up-regulation in the mucosal cell iNOS activity (0.42 cpm x 10^5/mg protein for the control vs. 8.67 cpm × 10^5/mg protein for 100 ng/ml LPS), and a 76.8% decrease (2.80 cpm × 10^5/mg protein for the control vs. 0.65 cpm × 10^5/mg protein for 100 ng/ml LPS) in the activity of cNOS (**Figure 2**).

To gain additional insights into the nature of the LPS-induced changes, we examined NO production and PGE_2 generation by gastric mucosal cell in the presence of NOS and COX systems inhibition. For this, the mucosal cells prior to incubation with the LPS were pretreated with cNOS inhibitor, L-NAME and iNOS inhibitor, 1400 W (**Figure 3**), or COX-1 inhibitor, SC-560 and COX-2 inhibitor, NS-398 (**Figure 4**), and assayed for PGE_2 generation and NO production. As shown in **Figure 3**, the effect of cNOS inhibition was reflected in a moderate up-regulation in the LPS-induced NO and PGE_2 production, while the inhibition of iNOS lead to a profound reduction in the LPS effect on the mucosal cell NO and PGE_2 production. Furthermore, the effect of the LPS on the mucosal cell capacity for NO and PGE_2 production was not discernibly affected by COX-1 inhibition (**Figure 4**), whereas preincubation with COX-2 inhibitor, NS-398, produced a marked reduction in PGE_2 generation but had no effect on the LPS-induced NO generation (**Figure 4**).

Further, we found that preincubation of gastric mucosal cells with ghrelin led to a concentration-dependent suppression of the LPS-induced effect on PGE_2 generation and the activity of iNOS (**Figure 5**), while the activity of cNOS showed an increase **(Figure 6**). Indeed, we

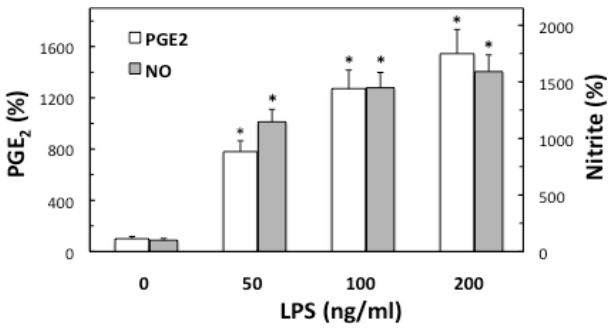

Figure 1. Effect of *H. pylori* LPS on PGE_2 and nitrite production in rat gastric mucosal cells. The cells were treated with the indicated concentrations of the LPS and incubated for 16 h. Values represent the means ± SD of five experiments. $^{*}P$ < 0.05 compared with that of control (LPS – 0).

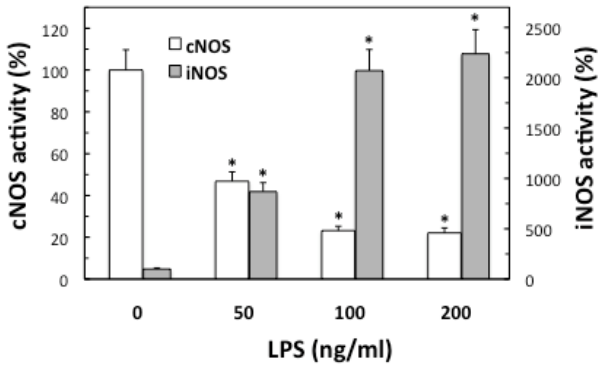

Figure 2. Effect of *H. pylori* LPS on the expression of cNOS and iNOS activities in rat gastric mucosal cells. The cells were treated with the indicated concentrations of the LPS and incubated for 16 h. Values represent the means ± SD of five experiments. $^{*}P$ < 0.05 compared with that of control (LPS - 0).

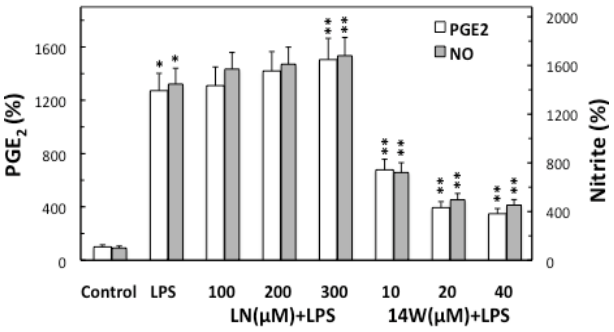

Figure 3. Effect of nitric oxide synthase inhibitors on *H. pylori* LPS-induced changes in the production of PGE_2 and nitrite by gastric mucosal cells. The cells, preincubated with the indicated concentrations of cNOS inhibitor, L-NAME (LN), or iNOS inhibitor, 1400 W, were treated with the LPS at 100 ng/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.

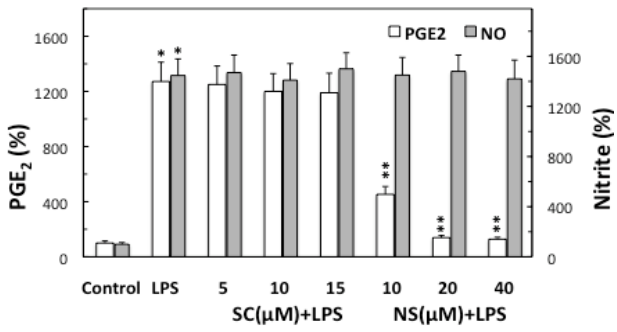

Figure 4. Effect of cyclooxygenase inhibitors on *H. pylori* LPS-induced changes in the production of PGE_2 and nitrite by gastric mucosal cells. The cells, preincubated with the indicated concentrations of COX-1 inhibitor, SC-560 (SC), or COX-2 inhibitor, NS-398 (NS), were treated with the LPS at 100 ng/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.

observed that ghrelin at 0.5 μg/ml, elicited an 84.1% drop in the LPS-induced mucosal cell PGE_2 generation and a 90.2% decrease in the activity of iNOS (**Figure 5**), whereas the activity of cNOS showed a 4.4-fold increase (**Figure 6**). Moreover, the increase in cNOS activation in the presence of ghrelin was associated with a concentration-dependent up-regulation in gastric mucosal cell Akt kinase activity, which at 0.5 μg/ml of ghrelin increased 2-folds over that of the LPS (**Figure 6**).

As cNOS is known to undergo a rapid posttranslational activation through phosphorylation at Ser^{1179} [18, 29,30], we have also examined the effect of the LPS and ghrelin on gastric mucosal cell cNOS phosphorylation. For this, the cells prior to incubation with ghrelin were pretreated with Akt kinase inhibitor, SH-5, and the lysates were probed with antibodies directed against cNOS and phosphorylated cNOS at Ser^{1179} (**Figure 7**). We observed that the LPS-induced suppression in cNOS activity was associated with the inhibition of the enzyme phosphorylation, while the up-regulation in cNOS activation by ghrelin was reflected in a marked increase in the enzyme protein phosphorylation at Ser^{1179}. Moreover, the suppression of ghrelin effect on cNOS phosphoryla- tion was attained in the presence of Akt inhibitor, SH-5 (**Figure 7**).

To gain further understanding of the events resulting in the suppression of *H. pylori* LPS-induced up-regulation in PGE_2 generation and iNOS activation by ghrelin, we examined the influence of the LPS and ghrelin on the gastric mucosal cell expression of iNOS, and COX-1 and COX-2 proteins (**Figure 8**). The results revealed that the LPS-induced up-regulation in iNOS activity and PGE_2 production was associated with the induction in iNOS and COX-2 proteins expression, while the suppression of the LPS effect by ghrelin was reflected in a marked inhibition of the iNOS protein expression, but no

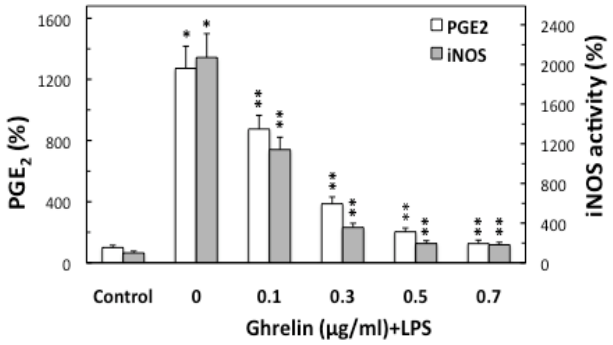

Figure 5. Effect of ghrelin on *H. pylori* LPS-induced changes in the production of PGE_2 and the expression iNOS activity in gastric mucosal cells. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/ml and incubated for 16 h. Values represent the means ± SD of five experiments. $^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS alone.

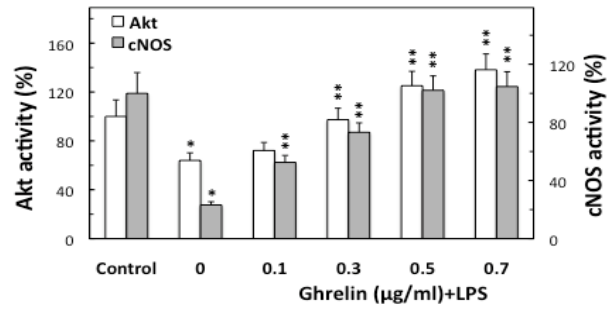

Figure 6. Effect of ghrelin on *H. pylori* LPS-induced changes in the expression of Akt kinase and cNOS activities in gastric mucosal cells. The cells, preincubated with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/ml and incubated for 16 h. Values represent the means ± SD of five experiments. $^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS alone.

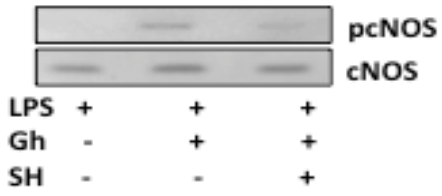

Figure 7. Effect of ghrelin (Gh) and Akt kinase inhibitor, SH-5 (SH), on *H. pylori* LPS-induced changes in cNOS phosphorylation in gastric mucosal cells. The cells were treated with ghrelin at 0.5 μg/ml, or Akt inhibitor, SH-5 (30 μM) + Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with phosphorylation-specific cNOS (pcNOS) antibody, and after stripping reprobed with anti-cNOS antibody. The immunoblots shown are representative of three experiments.

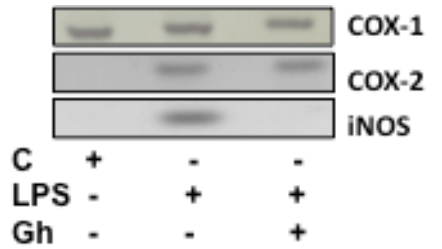

Figure 8. Effect of ghrelin on *H. pylori* LPS-induced expression of COX-1, COX-2, and iNOS proteins in gastric mucosal cells. The cells were treated with the LPS at 100 ng/ml or ghrelin (Gh) at 0.5 ng/ml + LPS and incubated for 16 h. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with anti-COX-1, anti-COX-2, or anti-iNOS antibody. The immunoblots shown are representative of three experiments.

COX-2 protein expression was discerned. We have also apparent diminution in observed that the COX-1 protein expression remained essentially unaffected by the inclusion of the LPS or ghrelin (**Figure 8**). Thus, the enzymatic activity of the LPS-induced COX-2 protein for up-regulation in PGE_2 production shows an apparent dependence on NO generated by the iNOS system.

Therefore, to ascertain the requirement of *H. pylori* LPS-induced up-regulation in COX-2 activation for NO, the mucosal cells prior to incubation with ghrelin were pretreated with iNOS inhibitor, 1400 W, or Akt inhibitor, SH-5, or nitrosothiol reducing agent, ascorbate, and assayed for COX-2 activity. We found that the LPS-induced up-regulation in COX-2 activity was subject to suppression not only by the pretreatment with ghrelin, but also showed susceptibility to iNOS inhibitor, 1400 W, whereas Akt inhibitor, SH-5, had no effect on the extent of the LPS-induced COX-2 activation (**Figure 9**). Moreover, while the iNOS inhibitor, 1400 W, produced amplification in the inhibitory effect of ghrelin on COX-2 activity, the Akt inhibitor, SH-5, caused the suppression in ghrelin effectiveness. Further, we found that the LPS-induced COX-2 activation displayed susceptibility to suppression by nitrosothiol reducing agent, ascorbate, which also produced enhancement in the effect of ghrelin on COX-2 activity (**Figure 9**).

Finally, we also examined the dependence of COX-2 S-nitrosylation on the LPS-induced iNOS activation by the biotin switch method [33,34]. The gastric mucosal cells were incubated with *H. pylori* LPS or ghrelin + LPS in the absence and presence of Akt inhibitor, SH-5, or iNOS inhibitor, 1400 W + LPS, and the lysates following the biotin switch procedure were examined for COX-2 protein S-nitrosylation (**Figure 10**). Western blot analysis revealed that COX-2 in the cells exposed to the LPS alone showed a marked increase in the protein S-nitrosylation, whereas the preincubation with iNOS inhibitor, 1400 W, resulted in the loss of the LPS-induced COX-2 S-nitrosylation. A pronounced decrease in the LPS-induced COX-2 S-nitrosylation was also attained in the presence of ghrelin. Moreover, this effect of ghrelin on COX-2 S-nitrosylation was subject to suppression by the inclusion of Akt inhibitor, SH-5. These data suggest that induction in iNOS elicited by *H. pylori* LPS leads to COX-2 activation through S-nitrosylation that results in an excessive PGE2 generation, and that ghrelin counters the detrimental consequences of iNOS induction by way of cNOS-dependent suppression of iNOS gene induction.

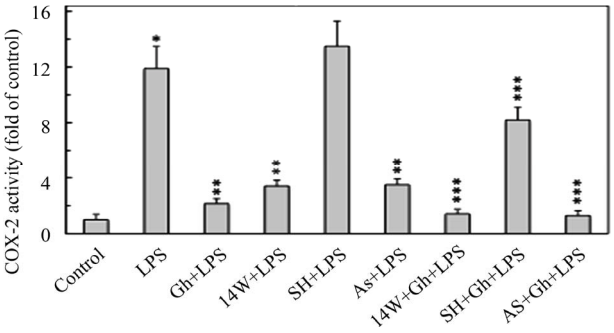

Figure 9. Effect of iNOS inhibitor, 1400 W, Akt inhibitor, SH-5, and ascorbate on the ghrelin (Gh)-induced changes in COX-2 activity in gastric mucosal cell exposed to *H. pylori* LPS. The cells, preincubated with 40 μM 1400 W (14 W), 30 μM SH-5 (SH), or 300 μM ascorbate (As), were treated with Gh at 0.5 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.

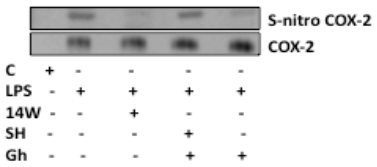

Figure 10. Effect of ghrelin (Gh) on *H. pylori* LPS-induced COX-2 S-nitrosylation. The gastric mucosal cells were treated with 40 μM of iNOS inhibitor, 1400 W (14 W), or Gh (0.5 μg/ml), or 30 μM Akt inhibitor, SH-5 (SH) + Gh, and incubated for 16 h in the presence of 100 ng/ml LPS. A portion of the 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 anti-COX-2 antibody. The immunoblots shown are representative of three experiments.

4. DISCUSSION

H. pylori lipopolysaccharide is recognized as a potent endotoxin capable of eliciting mucosal inflammatory responses akin to those that characterize gastritis and duodenal ulcer [1-3]. Indeed, *H. pylori* LPS, like LPS of other Gram-negative bacteria, is known to trigger a wide variety of transcriptional factors, including NF-κB and AP-1, that exert transcriptional control over such important mediators of inflammation as iNOS and COX-2 systems, which along with the constitutively expressed isozyme forms, cNOS and COX-1, are responsible for NO and PGE_2 production [4-6,20-22]. Moreover, a growing volume of data consistently points towards the existence of a functional and signaling relationship between the products of NOS and COX enzyme systems [13,14,19,20]. In particular, there are strong indications for the involvement of iNOS-derived NO in COX-2 activation through S-nitrosylation for the increase in PGE_2

generation [20-22]. Furthermore, the gastric mucosal inflammatory responses to *H. pylori* as well as its LPS are reflected in the suppression in cNOS activation [5,7,25,26]. Hence, in this study presented herein, we investigated the nature of interaction between the systems responsible for NO generation and PGE_2 production.

Using rat gastric mucosal cells exposed to *H. pylori* LPS, we demonstrated that the LPS-induced enhancement in iNOS activity and up-regulation in PGE_2 production was accompanied by the suppression in Akt kinase activity and the impairment in cNOS activation through phosphorylation. The stimulatory effect of the LPS on PGE_2 production was susceptible to suppression by COX-2 inhibitor, NS-398, as well as the inhibitor of iNOS, 1400 W. However, the LPS-induced up-regulation in the mucosal cell NO generation was not affected by the inhibitors of COX-1 and COX-2 systems. These latter findings are thus in keeping with the literature data as to the role of iNOS in the regulation of COX-2 activation for the increase in PGE_2 production [20-23].

Further, we found that preincubation of the mucosal cells with gastric hormone, ghrelin, recognized for its modulatory influence on the inflammatory responses to bacterial infection [18,27,29-31,35], exerted countering effect on the LPS-induced suppression in Akt activity and lead to the increase in cNOS activation through phosphorylation at Ser^{1179}. We also observed that these effects of ghrelin were accompanied by the suppression in iNOS protein expression and the reduction in COX-2 activity. Moreover, ghrelin-induced up-regulation in cNOS activation was susceptible to suppression by Akt inhibitor, SH-5, which also caused the abrogation in ghrelin-induced reduction in COX-2 activity. From this, we concluded that ghrelin countering effect on *H. pylori* LPS-induced inflammatory changes occurs with the involvement of Akt-mediated cNOS activation through phosphorylation, and that Akt kinase plays an essential role in the action of ghrelin on COX-2 activity. Indeed, Akt kinase occupies a central role in the receptor (GHS-R)-mediated responses to ghrelin stimulation [26,36], and the involvement of signaling through Akt for a rapid up-regulation in cNOS activity through the enzyme protein phosphorylation at Ser^{1179} is well documented [29, 30,37].

Our further examination of the influence of ghrelin on *H. pylori* LPS-induced up-regulation in iNOS activity and PGE_2 generation revealed that while the expression of COX-1 protein remained essentially unaffected by the LPS or ghrelin, the effect of the LPS was associated with the induction in iNOS and COX-2 proteins. The countering effect of ghrelin, moreover, was reflected in a marked inhibition of the iNOS protein expression, but no apparent diminution in COX-2 protein was discerned. Thus, in concordance with our earlier results, it is apparent that whilst the countering effect of ghrelin on the LPS-induced enhancement in iNOS activity is associated with the inhibition of iNOS gene expression at the transcriptional level [38], the suppression of the LPS-induced COX-2 activity by ghrelin does not involve the inhibition in the enzyme protein expression. However, the enzymatic activity of the LPS-induced COX-2 protein for up-regulation in PGE_2 production shows an apparent dependence on NO generated by the iNOS system. Indeed, the stimulation of NO production through iNOS induction has been reported to lead to COX-2 protein modification through S-nitrosylation that results in the enzyme activation and the increase in PGE_2 generation [20-22].

Hence, to reveal further insights into the mechanism of ghrelin suppression of the LPS-induced gastric mucosal inflammatory disturbances, we examined the COX-2 activity and its protein S-nitrosylation in the presence of the inhibitors of iNOS and Akt, as well as nitrosothiols reducing agent, ascorbate. We found that the countering effect of ghrelin on the LPS-induced up-regulation in COX-2 activity was further amplified in the presence of iNOS inhibitor, 1400 W, while the Akt inhibitor, SH-5, caused the suppression in ghrelin effectiveness. Moreover, the LPS-induced COX-2 activation displayed susceptibility to suppression by ascorbate, which also produced an amplification in ghrelin effect on the mucosal COX-2 activity. These data, together with the demonstrated susceptibility of the LPS-induced COX-2 activation to inhibition by iNOS inhibitor, 1400 W, as well as known vulnerability of S-nitrosylated proteins to reduction by ascorbate [20,21,26,33,34], suggest that the countering effect of ghrelin on *H. pylori* LPS-induced changes in COX-2 activation are intimately linked to the events of Akt activation and a rapid up-regulation in cNOS activity through Akt-mediated cNOS protein phosphorylation. Consequently, our results point to the involvement of cNOS in controlling the extent of iNOS-dependent COX-2 activation.

The supporting evidence as to the involvement of Akt-mediated cNOS activation in the modulatory action of ghrelin against *H. pylori* LPS-induced gastric mucosal consequences of iNOS induction and up-regulation in COX-2 activation comes from the results of biotin switch assay. We found that the mucosal cells exposed to incubation with *H. pylori* LPS showed a marked increase in COX-2 protein S-nitrosylation, while the suppression of the LPS-induced iNOS activity with a specific inhibitor, 1400 W, caused the loss in COX-2 S-nitrosylation. Further, we observed a pronounced drop in the LPS-induced COX-2 S-nitrosylation in the presence of ghrelin, the effect of which was subject to the repression by Akt inhibitor, SH-5. These findings, together with the results of COX-2 activity assays, suggest that induction

in gastric mucosal iNOS expression elicited by *H. pylori* LPS leads to COX-2 activation through S-nitrosylation that results in an excessive PGE_2 generation. It is also apparent that ghrelin counters these untoward consequences of *H. pylori* via Akt-mediated up-regulation in cNOS activation that is required for the suppression of iNOS gene induction.

While the intricate details of the role of cNOS in the regulation of iNOS gene induction remain obscure, there are indications that cNOS is capable of affecting transcriptional factor NF-κB activation and therefore to influence the extent of promoter activity and iNOS gene transcription [12,21,24]. More interestingly, the literature data suggest that S-nitrosylation of an inhibitor protein, IκB kinase complex (IKK) interferes with ubiquitinylation and proteasomal degradation of IκB, thus preventing the nuclear translocation of NF-κB, and resulting in its inability to promote target gene transcription [39-41]. Furthermore, we have shown that the LPS-induced suppression in cNOS activity was associated with the induction in iNOS protein expression, while the countering effect of ghrelin, like that NF-κB inhibitor, PPM-18, was reflected in a marked inhibition of the iNOS protein expression [38]. Moreover, the effect of ghrelin was intimately linked to Akt-mediated cNOS activation through phosphorylation [26].

Taken together, these observations lead us to postulate that the initial phase gastric mucosal inflammatory responses elicited by *H. pylori* involves the suppression in Akt kinase-dependent cNOS activation that leads to abrogation of cNOS-mediated IKK S-nitrosylation and the increase in proteasomal degradation of IκB, thus allowing NF-κB translocation to the nucleus to promote iNOS gene transcription. The induction in iNOS expression, in turn, leads to iNOS-mediated COX-2 activation through S-nitrosylation that results in an excessive PGE_2 generation. We also postulate that ghrelin counters these untoward consequences of *H. pylori* LPS via up-regulation in Akt-dependent cNOS activation through phosphorylation that results in up-regulation in cNOS-mediated IKK coplex S-nitrosylation which interferes with proteasomal degradation of IκB, thereby preventing the nuclear translocation of NF-κB and the induction in iNOS gene transcription. Hence, the repression of iNOS expression by ghrelin triggers the loss in COX-2 activation through iNOS-dependent S-nitrosylation and leads to a reduction in PGE_2 generation.

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