

Modulation of gastric mucosal inflammatory responses to *Helicobacter pylori* by ghrelin: Role of cNOS-dependent IKK- β S-nitrosylation in the regulation of COX-2 activation

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ABSTRACT

Disturbances in nitric oxide synthase (NOS) and cyclooxygenase (COX) isozyme systems, manifested by the excessive NO and prostaglandin (PGE₂) generation, are well-recognized features of gastric mucosal inflammatory responses to *H. pylori* infection. In this study, we report that *H. pylori* LPS-induced enhancement in gastric mucosal inducible (i) iNOS expression and COX-2 activation was accompanied by the impairment in constitutive (c) cNOS phosphorylation, up-regulation in the inhibitory κ B kinase- β (IKK β) activation and the increase in the transcriptional factor, NF- κ B, nuclear translocation. Further, we show that abrogation of cNOS control over NF- κ B activation has led to induction of iNOS expression and COX-2 activation through S-nitrosylation. Moreover, we demonstrate that the modulatory effect of peptide hormone, ghrelin, on the LPS-induced changes was reflected in the increase in Src/Akt-dependent cNOS activation through phosphorylation and the suppression of IKK- β activity through cNOS-mediated IKK- β protein S-nitrosylation. As a result, ghrelin exerted the inhibitory effect on NF- κ B nuclear translocation, thus causing the repression of iNOS gene induction and the inhibition in COX-2 activation through iNOS-dependent S-nitrosylation. Our findings point to cNOS activation as a pivotal element in the signaling cascade by which ghrelin exerts modulatory control over proinflammatory events triggered in gastric mucosa by *H. pylori* infection.

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

1. INTRODUCTION

Infection with *Helicobacter pylori* is a primary factor in

the etiology of gastric disease, and the excessive nitric oxide (NO) and prostaglandin (PGE₂) generation along with up-regulation in proinflammatory cytokine production, are well-documented features of *H. pylori*-induced gastritis as well as characterize gastric mucosal inflammatory responses to *H. pylori* lipopolysaccharide (LPS) [1-4]. While NO and PGE₂ generated by the constitutive (c) nitric oxide synthase (cNOS) and cyclooxygenase-1 (COX-1) enzymes are responsible for maintaining normal physiological functions, and are recognized as essential elements of gastric mucosal defense mechanism, the overexpression of inducible (i) iNOS and cyclooxygenase-2 (COX-2) has been intimately implicated as the promoting event in *H. pylori*-associated chronic gastritis and gastric cancer [2,4-6].

A growing body of evidence, moreover, supports the existence of a functional and signaling relationship between NO generated by NOS isozyme system, and the formation of PGE₂ synthesized from arachidonic acid by the action of COX systems [7-10]. The cross-talk between the products of NOS and COX pathways is supported by the studies indicating that stimulation of NO production through iNOS induction leads to COX enzymes activation and the increase in PGE₂ generation, whereas NOS gene deletion or inhibition of NOS enzymes with pharmacological agents results in a decrease in PGE₂ formation [9-12]. The role of cNOS in the iNOS-dependent COX-2 activation has also been suggested [10,13], and we have reported that the disturbances in NO and PGE₂ generation elicited by *H. pylori* LPS are reflected in the massive up-regulation of iNOS and COX-2 activity, and the suppression in Src/Akt-dependent cNOS activation [14-16].

Investigations into LPS-induced signaling events underlying the expression of proinflammatory mediators indicate that *H. pylori* LPS, like LPS of other Gram-negative bacteria, is known to trigger the activation of toll-like receptor-4 (TLR-4), which through downstream ef-

factors leads to activation of transcriptional factors involved in iNOS and COX-2 gene induction [10,12,17,18]. While the induction of iNOS gene expression in response to LPS involves activation of transcriptional factor NF- κ B [8,10,19], the role of NF- κ B in the transcriptional control of COX-2 expression is less apparent and remains controversial [17,18,20]. Depending on the cell type, the regulation of COX-2 expression has been attributed to transcriptional factors, NF- κ B, activator protein-1 (AP-1), cAMP response element binding protein (CREB), and CCATT/enhancer binding protein (C/EBP), as well as kinases of MAPK and PKC family [10,12,18,20,21]. Moreover, up-regulation in COX-2 activation has been linked to posttranslational modification of the enzyme protein through S-nitrosylation via LPS-elicited induction in iNOS expression [9,10]. Indeed, as demonstrated recently, the induction in iNOS expression by *H. pylori* LPS leads to COX-2 S-nitrosylation that results in an excessive PGE₂ generation [16].

In this study, we investigated further the role of cNOS in the signaling cascade of *H. pylori* LPS-induced expression of iNOS and COX-2 in gastric mucosal cells. Our results demonstrate that the LPS-induced abrogation of cNOS control over NF- κ B activation results in the induction of iNOS expression and leads to COX-2 activation through S-nitrosylation. Moreover, our results show that peptide hormone, ghrelin, recognized for its modulatory control over NOS and COX enzyme systems [16,22-25], suppresses these untoward consequences of the LPS through up-regulation in cNOS activation that interferes with NF- κ B nuclear translocation, thus causing the repression of iNOS gene induction and the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation.

2. MATERIALS AND METHODS

2.1. Gastric Mucosal Cell Incubation

The cells were collected from the mucosa of freshly dissected rat stomachs with a blunt spatula, and suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 μ g/ml), penicillin (50 U/ml), streptomycin (50 μ g/ml), and 10% fetal calf serum. The cells were then gently dispersed by trituration with a syringe, settled by centrifugation, and following rinsing resuspended in the medium to a concentration of 2×10^7 cell/ml [14]. Cell aliquots (1 ml) were then transferred to DMEM in culture dishes and incubated under 95% O₂ - 5% CO₂ atmosphere at 37°C for up to 16h in the presence of 0 - 100 ng/ml of *H. pylori* LPS [15]. *H. pylori* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 4350 [3]. In the experiments evaluating the effect of ghrelin (rat, Sigma), cNOS inhibitor, L-NAME, iNOS inhibitor, 1400W, Akt inhibitor, SH-5,

Src inhibitor, PP2, NF- κ B inhibitors, Bay11-7082 and PPM-18 (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 [15], was greater than 97%.

2.2. 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-[³H] arginine to L-[³H] 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 Ca²⁺-independent iNOS) or 6 mM CaCl₂ (for Ca²⁺-dependent cNOS), and centrifuged. The aliquots of the resulting supernatant were incubated for 30 min at 25°C in the presence of 50 μ Ci/ml of L-[³H] arginine, 10 mM NAPDH, 5 μ M tetrahydrobiopterin, and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250 μ l. Following addition of stop buffer and Dowex-50 W (Na⁺) resin, the mixtures were transferred to spin cups, centrifuged and the formed L-[³H] citrulline contained in the flow through was quantified by scintillation counting [15].

2.3. COX-1 and COX-2 Activity Assay

The cyclooxygenase activity of COX-1 and COX-2 isoforms in gastric mucosal cells was measured with the COX Activity Assay Kit (Cayman) by monitoring the appearance of oxidized TMPD at 590 nm [16]. The cells from the control and various experimental treatments were settled by centrifugation, rinsed with phosphate-buffered saline, and homogenized in 0.3 ml of cold sample buffer containing 0.1 M TRIS-HCl, pH 7.8, and 1 mM EDTA, centrifuged at $12,000 \times g$ for 10 min at 4°C, and the supernatant collected. The COX-1 activity in 40 μ l sample aliquots was measured in the absence and the presence of COX-2 inhibition (DuP-697), while the COX-2 activity assays were conducted in the absence and the presence of COX-1 inhibition (SC-560), according to manufacturer's instruction.

2.4. Nuclear Protein Extraction

The aliquots of gastric mucosal cell suspension from the control and various experimental conditions were settled by centrifugation at $1500 \times g$ for 5 min, rinsed with phosphate-buffered saline, and lysed by incubation for 10 min on ice in the lysis buffer, containing 10 mM HEPES, pH 7.9, 10 mM KCl, 0.1 mM EDTA, 0.5% Nonidet P-40, 1 mM dithiothreitol, and 0.5 mM PMSF [26]. Following centrifugation at $12,000 \times g$ for 10 min at 4°C, the supernatant was subjected to centrifugation at

100,000 × g for 1 h and the obtained soluble fraction was used as source of cytosolic extract [27]. The pellets, from 12,000 × g centrifugation, containing crude nuclei were suspended for 20 min at 4°C in the extraction buffer, containing 20 mM HEPES, pH 7.9, 25% glycerol, 400 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF. The samples were centrifuged at 15,000 × g for 10 min at 4°C, and the supernatants containing nuclear extracts were collected and stored at -70°C until use.

2.5. IκB Kinase Activity Assay

To measure the IKK-β activity we utilized the ELISA-based detection kit, K-LISA™ (Calbiochem). The GST-IκB-α 50-amino acid peptide that includes the Ser³² and Ser³⁶ of IκB-α phosphorylation sites was used as a substrate [27]. The gastric mucosal cell cytosolic extracts were incubated a glutathione-coated 96-well plate with GST-tagged IκB-α at room temperature for 30 min, and the phosphorylated GST-IκB-α substrate was detected using anti-phospho IκB-α (Ser³²/Ser³⁶) as first antibody, followed by horseradish peroxidase-conjugated secondary antibody. Following washing the retained complex was probed TMB reagent for spectrophotometric quantification at 450 nm.

2.6. IKK-β and COX-2 Protein S-Nitrosylation Assay

A biotin switch procedure was employed to assess IKK-β and COX-2 protein S-nitrosylation [28,29]. The gastric mucosal cells were treated with ghrelin (0.5 μg/ml), or Akt inhibitor, SH-5 (20 μM) + ghrelin (0.5 μg/ml), and incubated 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 [29]. 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 [29]. The obtained proteins were then analyzed by Western blotting.

2.7. Immunoblot 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 [16]. Following brief sonication, the lysates were centrifuged at 12,000 × g for 10 min, and the supernatants were collected and analyzed for protein concentration using BCA protein assay kit (Pierce). The samples, including those subjected to biotin switch procedure, were then normalized with respect to protein content to ensure equal protein loading on SDS-PAGE. The proteins were suspended 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 IκB-α, COX-1, COX-2, iNOS, and cNOS (Calbiochem), NF-κB p65 and IKK-β (EMD Millipore). The phosphorylated cNOS (pcNOS) was analyzed using specific antibody (Calbiochem) directed against phospho-cNOS (mouse anti-eNOS, pSer¹¹⁷⁹).

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

3. RESULTS

Infection of gastric mucosa by *H. pylori* in humans or stimulation of gastric mucosal cells with *H. pylori* LPS elicits a rapid proinflammatory reaction characterized by the excessive NO and PGE₂ generation caused by the disturbances in NOS and COX isozyme systems [1-4,16]. Hence, to further ascertain the nature of the disturbances, we first assessed the time course of protein expression and the activity of NOS and COX isozyme systems in rat gastric mucosal cells exposed to *H. pylori* LPS. The results revealed that the LPS (100 ng/ml) elicited a significant induction in COX-2 (**Figure 1(a)**) and iNOS (**Figure 2(a)**) protein levels within 4 h of incubation, with further sustained increase for at least 16 h. This effect of the LPS, furthermore, was accompanied by a significant increase in the mucosal cell COX-2 (**Figure 1(b)**) and iNOS (**Figure 2(b)**) enzymatic activities. We have also observed that within the time frame of incubation, the LPS exerted no apparent effect on the mucosal cell ex-

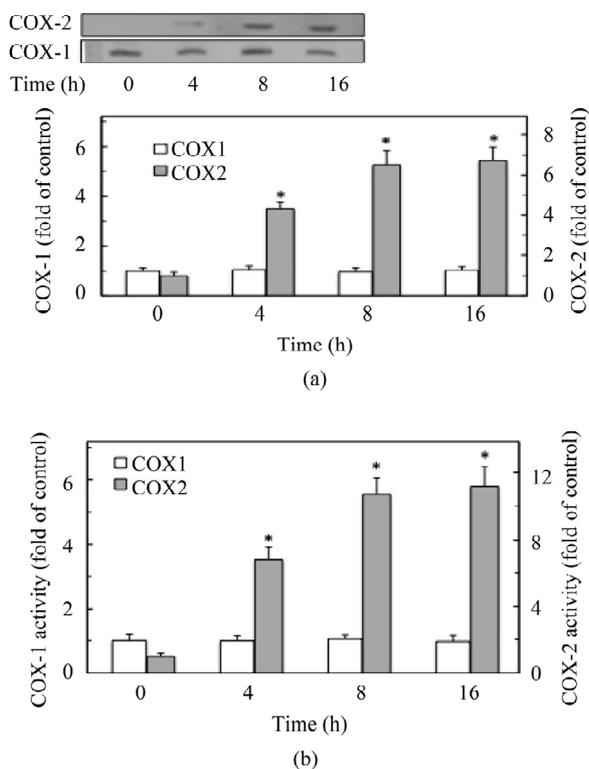


Figure 1. Time course of COX-1 and COX-2 protein expression (a), and the activity of COX-1 and COX-2 (b) in rat gastric mucosal cells subjected to *H. pylori* LPS. The cells were treated with the LPS at 100 ng/ml and incubated for up to 16 h. At the indicated time points, the cell lysates were analyzed by Western blotting with anti-COX-1 and anti-COX-2 antibody for protein expression, and the relative densities are expressed as fold of control. The activities of COX-1 and COX-2 were measured as described under “Materials and Methods”. The data represent the means \pm SD of four separate experiments. * P < 0.05 compared with that of control (time-0).

pression of COX-1 (**Figure 1(a)**) and cNOS (**Figure 2(a)**) proteins. However, while the COX-1 activity was not discernibly affected by the LPS (**Figure 1(b)**), the activity of cNOS in the presence of the LPS decreased markedly with the incubation time (**Figure 2(b)**).

Moreover, we established that preincubation of gastric mucosal cells with peptide hormone, ghrelin, lead to a concentration-dependent suppression of the LPS-induced COX-2 (**Figure 3(a)**) and iNOS (**Figure 3(b)**) activities, and the reversal in the LPS inhibitory effect on the cNOS activity. Ghrelin, however, showed no discernible effect on the mucosal cell activity of COX-1 enzyme. Furthermore, examination of the influence of the LPS and ghrelin on the gastric mucosal cell expression of cNOS, iNOS, COX-1, and COX-2 proteins revealed that the LPS-induced suppression in cNOS activity was associated with the inhibition in the enzyme phosphorylation, while up-regulation in cNOS activity by ghrelin was reflected in a marked increase in the enzyme phosphoryla-

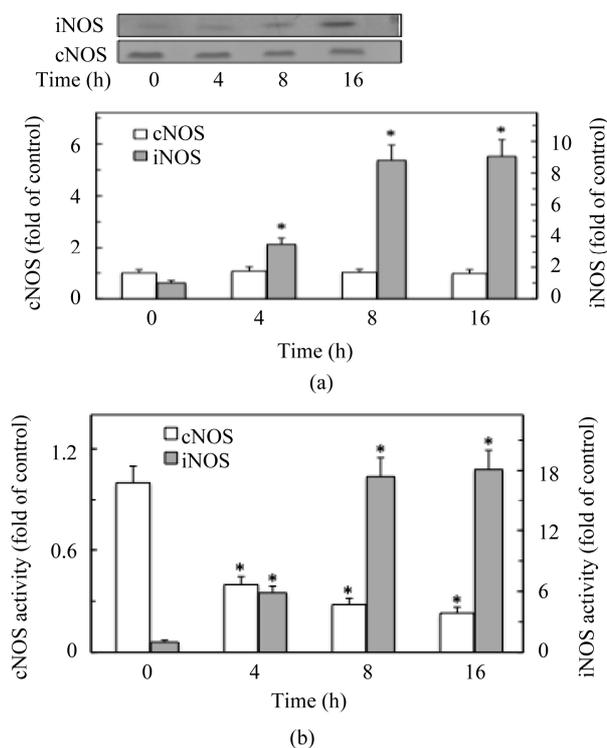


Figure 2. Time course of cNOS and iNOS protein expression (a), and the activity of cNOS and iNOS (b) in rat gastric mucosal cells subjected to *H. pylori* LPS treatment. The cells were treated with the LPS at 100 ng/ml and incubated for up to 16 h. At the indicated time points, the cell lysates were analyzed by Western blotting with anti-cNOS and anti-iNOS antibody for protein expression, and the relative densities are expressed as fold of control. The activities of cNOS and iNOS were measured as described under “Materials and Methods”. The data represent the means \pm SD of four separate experiments. * P < 0.05 compared with that of control (time-0).

tion at Ser¹¹⁷⁹ (**Figure 4**). We also found that the countering effect of ghrelin on the LPS elicited induction in iNOS and COX-2 activity was manifested in a marked inhibition of the iNOS protein expression, but no apparent change in COX-2 protein expression. Moreover, neither the LPS nor ghrelin affected the expression of COX-1 protein. These results point to a role of iNOS in the LPS-induced up-regulation in COX-2 activation.

Hence, to provide further leads as to the requirements for iNOS-dependent up-regulation in COX-2 activation by *H. pylori* LPS, the mucosal cells prior to incubation with ghrelin were pretreated with the inhibitors of cNOS phosphorylation, an Akt inhibitor, SH-5 and Src inhibitor, PP2, and assayed for COX-2 and iNOS activity. As shown in **Figure 5**, the ghrelin-induced suppression in COX-2 and iNOS activation was susceptible to reversal by both inhibitors. Moreover, the LPS-induced up-regulation in COX-2 and iNOS activity displayed susceptibility to the inhibitors of NF- κ B activation, PPM-18 and Bay11-7082. Further, we found that both these inhibitors

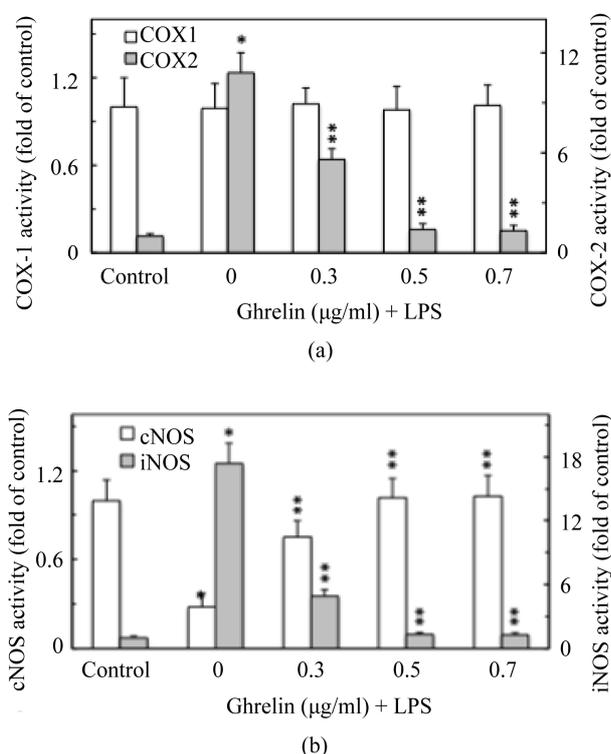


Figure 3. Effect of ghrelin on *H. pylori* LPS-induced expression of COX-1 and COX-2 (a), and cNOS and iNOS (b) activities in gastric mucosal cells. The cells, preincubated for 30 min with the indicated concentrations of ghrelin, were treated with the LPS at 100 ng/ml and incubated for 8 h. Values are presented as fold of control, and represent the means \pm SD of five experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of LPS alone.

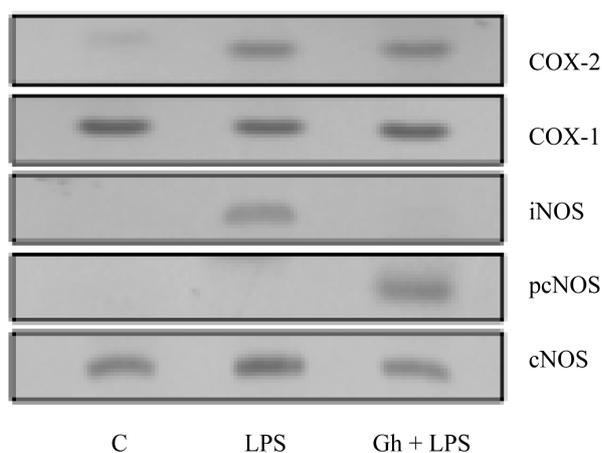


Figure 4. Effect of ghrelin (Gh) on *H. pylori* LPS-induced changes in expression of COX-1, COX-2, iNOS and cNOS proteins, and cNOS phosphorylation in gastric mucosal cells. The cells were treated with the LPS at 100 ng/ml or Gh at 0.5 μg/ml, and incubated for 8 h. Cell lysates were analyzed by Western blotting with anti-COX-1, anti-COX2, anti-iNOS, anti-cNOS, and phosphorylation specific cNOS (pcNOS) antibody. The immunoblots shown are representative of three experiments.

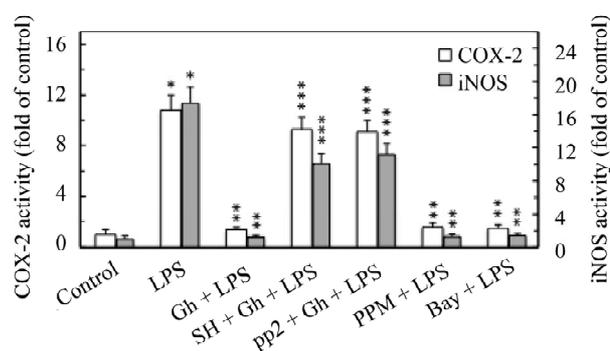


Figure 5. Effect of Akt inhibitor, SH-5, Src inhibitor, PP2, and NF- κ B inhibitors, PPM-18 and Bay 11-7082, on the ghrelin (Gh)-induced changes in COX-2 and iNOS activities in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 20 μM SH-5 (SH), 30 μM PP2, 15 μM PPM-18 (PPM), or 20 μM Bay 11-7082 (Bay), were treated with Gh at 0.5 μg/ml and incubated for 8 h in the presence of 100 ng/ml of LPS. Values represent the means \pm 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.

of NF- κ B activation, while showing no inhibitory effect on the LPS-induced expression of COX-2 protein, caused a marked inhibition in the LPS-induced expression of iNOS protein (**Figure 6**). Thus the countering effect of ghrelin on the LPS-induced COX-2 activation, like that of NF- κ B inhibitors, is the consequence of iNOS suppression at the transcriptional level, and depends on cNOS activation through phosphorylation.

Therefore, to understand the mechanism underlying up-regulation in COX-2 activation, we examined the effect of ghrelin on the LPS-induced NF- κ B activation. As NF- κ B activation requires degradation of the inhibitory protein I κ B- α that leads to translocation of p65 component of NF- κ B from the cytoplasm to nucleus [30-33], we exposed the gastric mucosal cells to *H. pylori* LPS in the absence or presence of ghrelin, and the cell lysates were analyzed for I κ B- α level, while the nuclear extracts were assessed for p65 NF- κ B. The results of immunoblots analysis revealed that, the LPS effect was manifested by a distinct reduction in I κ B- α protein level and a marked increase of p65 in the nuclear extract (**Figure 7**). We also found that in the presence of ghrelin, the LPS-induced I κ B- α degradation was significantly inhibited, while the extent of the LPS-induced nuclear translocation of p65 NF- κ B showed a significant decrease.

Next, we analyzed the influence of ghrelin on *H. pylori* LPS-induced changes in the activity of IKK- β , a key enzyme of NF- κ B activation pathway that controls the extent of I κ B- α phosphorylation and its proteasomal degradation. The results revealed that ghrelin exerted a profound countering effect on the LPS-induced up-regulation in gastric mucosal cell IKK- β activity (**Figure 8**).

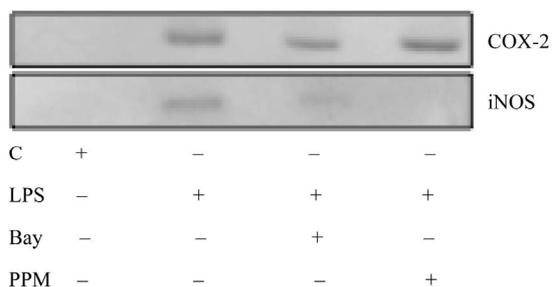


Figure 6. Effect of NF- κ B inhibitors, Bay11-7082 (Bay) and PPM-18 (PPM), on *H. pylori* LPS-induced expression of COX-2 and iNOS proteins in gastric mucosal cells. The cells were treated with the LPS at 100 ng/ml, or Bay at 20 μ M + LPS, or PPM at 15 μ M + LPS, and incubated for 8 h. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose, and probed with anti-COX-2 and anti-iNOS antibody. The immunoblots shown are representative of three experiments.

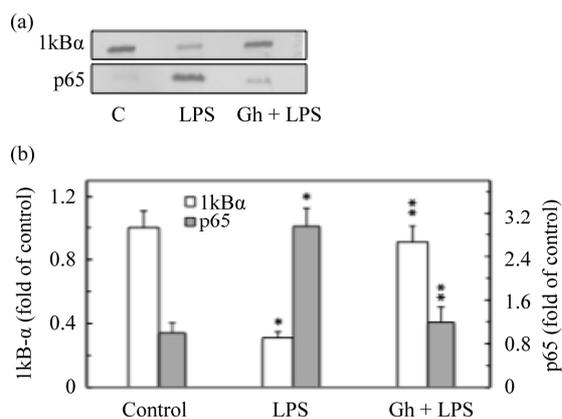


Figure 7. Effect of ghrelin on *H. pylori* LPS-induced I κ B- α protein degradation and p65 NF- κ B nuclear translocation in rat gastric mucosal cells. The cells were preincubated for 30 min with 0 or 0.5 μ g/ml of ghrelin and incubated for 30 min with the LPS at 100 ng/ml. Cell lysates were analyzed for I κ B- α by Western blotting with anti-I κ B- α antibody, while the level of p65 NF- κ B protein was assessed in the nuclear fraction with anti-p65 antibody (a). Relative densities are expressed as fold of control (b). The data represent the means \pm SD of four experiments. * P < 0.05 compared with that of control. ** P < 0.05 compared with that of LPS.

Moreover, the effect of ghrelin on LPS-induced up-regulation in IKK- β activity was subject to suppression by the inhibitors of Src/Akt pathway, PP2 and SH-5. A significant decrease in the countering effect of ghrelin on the LPS-induced up-regulation in IKK- β activity was also attained in the presence of cNOS inhibitor, L-NAME, while the inhibitor of NF- κ B activation, PPM-18, and iNOS inhibitor, 1400 W, had no effect. This indicates that the countering effect of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal IKK- β activation, and the suppression of COX-2 and iNOS enzymes,

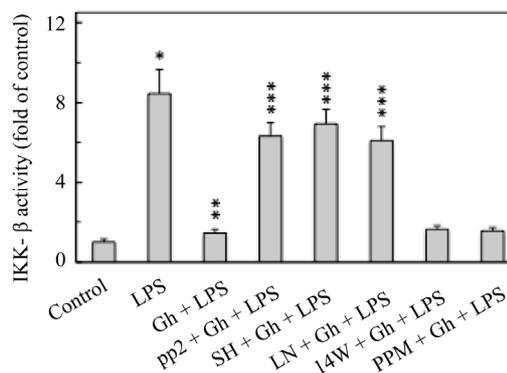


Figure 8. Effect of Src inhibitor, PP2, Akt inhibitor, SH-5, cNOS inhibitor, L-NAME, iNOS inhibitor, 1400W, and NF- κ B inhibitor, PPM-18, on the ghrelin (Gh)-induced changes in IKK- β activity in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 30 μ M PP2, 20 μ M SH-5 (SH), 200 μ M L-NAME (LN), 40 μ M 1400W (14W), or 15 μ M PPM-18 (PPM), were treated with Gh at 0.5 μ g/ml and incubated for 30 min in the presence of 100 ng/ml of LPS. Values represent the means \pm 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.

occurs with the involvement of Src/Akt-mediated cNOS activation, and shows dependence on NO generated by the cNOS system.

Consequently, to reveal further the role of NO generated by cNOS isozyme system in the regulation of the LPS-induced IKK- β and COX-2 activation, we assessed the effect of nitrosothiols reducing agent, ascorbate. While preincubation with ascorbate produced no discernible effect on the extent of the LPS-induced IKK- β activation, a marked decrease was observed in the LPS-induced COX-2 activation (Figure 9). In addition, ascorbate elicited amplification in the effect of ghrelin on COX-2 activity, and produced a significant relieve in the inhibitory effect of ghrelin on the LPS-induced IKK- β activity. Moreover, examination of the effect of the LPS and ghrelin on the IKK- β and COX-2 S-nitrosylation patterns by the biotin switch method [28,29], revealed that gastric mucosal cells exposed to *H. pylori* LPS showed a marked increase in COX-2 S-nitrosylation, while the countering effect of ghrelin on the LPS-induced up-regulation in IKK- β activity was manifested by an increase in IKK- β S-nitrosylation and the substantial loss in COX-2 S-nitrosylation (Figure 10). Furthermore, the up-regulation in IKK- β S-nitrosylation by ghrelin, like that of IKK- β enzymatic activity, was susceptible to suppression by Akt inhibitor, SH-5. We have also observed that Akt inhibition with SH-5 caused the reversal in the inhibitory effect of ghrelin on the LPS-induced COX-2 protein S-nitrosylation. Thus, ghrelin-induced IKK- β S-nitrosylation causes the repression of iNOS

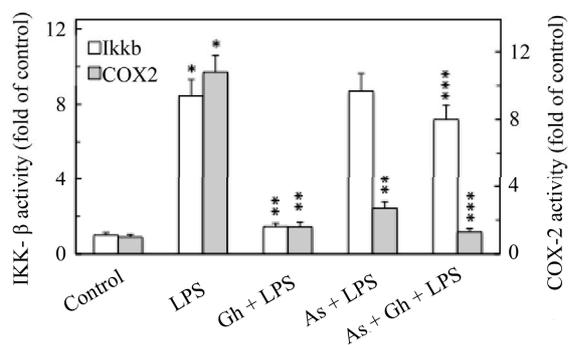


Figure 9. Effect of ascorbate on the ghrelin (Gh)-induced changes in the expression of IKK- β and COX-2 activities in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 300 μ M ascorbate (As), were treated with Gh at 0.5 μ g/ml and incubated for 30 min (in the case of IKK β) and 8 h (in the case of COX-2) in the presence of 100 ng/ml LPS. Values represent the means \pm 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.

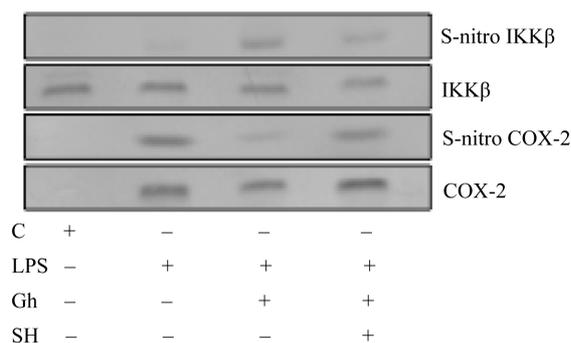


Figure 10. Effect of *H. pylori* LPS and ghrelin (Gh) on IKK- β and COX-2 protein S-nitrosylation in gastric mucosal cells exposed to Akt inhibitor, SH-5 (SH). The cells, were preincubated for 30 min with Gh at 0.5 μ g/ml, or SH at (20 μ M) + Gh, and incubated for 1 h (IKK β) and 8 h (COX-2) in the presence 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 remainder of the lysates, resolved on SDS-PAGE, transferred to nitrocellulose probed with anti-IKK β and anti-COX-2 antibody. The immunoblots shown are representative of three experiments.

induction and hence leads to the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation.

4. DISCUSSION

Colonization of gastric mucosa by *H. pylori* in humans or stimulation of gastric mucosal cells with *H. pylori* LPS is known to elicit a cascade of inflammatory responses resulting in the release of proinflammatory mediators that increase the risk of gastric disease [2,4-6,14]. Primary among these mediators, is up-regulation in pro-

inflammatory cytokine production and the excessive generation of NO and PGE₂ [2,4,14,16]. While the NO and PGE₂ generated by the constitutive cNOS and COX-1 enzyme systems are deemed essential for the maintenance of normal housekeeping functions, the overexpression of inducible iNOS and COX-2 isozymes, and consequent increase in NO and PGE₂ production, are considered of major importance in defining the extent of gastric mucosal inflammatory involvement [4,5,16]. The signaling events underlying the induction of iNOS and COX-2 enzymes by bacterial LPS involves the stimulation of TLR-4, which then through a series of downstream effectors triggers the activation of transcriptional factors that exert control over iNOS and COX-2 gene expression [10,17,18,34,35]. Principal among the factors implicated in the regulation of inflammatory responses to *H. pylori*, is the nuclear transcriptional factor, NF- κ B [34-36]. While the induction of iNOS gene by LPS has been convincingly linked to NF- κ B activation [10,19,26,30], the role of NF- κ B in COX-2 gene expression remains less apparent [17,18,20]. Hence, in the present study we assessed the relationship between *H. pylori* LPS-induced NF- κ B activation, iNOS gene induction, and COX-2 activation.

Our data, obtained with rat gastric mucosal cells, revealed that the LPS-elicited enhancement in the expression of iNOS COX-2 is accompanied by the impairment in cNOS phosphorylation, up-regulation in IKK- β activation, and the increase in NF- κ B nuclear translocation. Further, we demonstrated that abrogation of cNOS control over NF- κ B activation and its nuclear translocation leads to induction in iNOS expression, and COX-2 activation through S-nitrosylation. Moreover, we showed that ghrelin modulates the LPS-induced changes by exerting the inhibitory effect on NF- κ B nuclear translocation, thus causing repression of iNOS gene and the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation. Indeed, examination of gastric mucosal cell expression of COX and NOS proteins and the activity of the individual isozymes demonstrated that *H. pylori* LPS-induced enhancement in iNOS and COX-2 enzymatic activities was associated with the induction in iNOS and COX-2 protein levels. The LPS, however, had no apparent effect on the expression of COX-1 and cNOS proteins, and COX-1 activity, while the activity of cNOS showed a marked decrease. Furthermore, assessment of the effect of peptide hormone, ghrelin, revealed that the induced increase in cNOS phosphorylation at Ser¹¹⁷⁹ was associated with a significant up-regulation in cNOS activity, inhibition of iNOS expression, and the suppression in COX-2 activity without affecting its protein expression. These findings are thus in concordance with the rapidly accumulating literature data attesting to a central role of ghrelin in modulation of gastric mucosal

inflammatory responses to *H. pylori* colonization [14-16, 22-25], as well as point to cNOS activation through phosphorylation as a pivotal element of ghrelin signaling cascade.

The mechanism that underlies the regulation of NOS system by ghrelin involves the receptor (GHSR1a)-mediated activation of heterotrimeric G protein-dependent pathway that results in signal propagation through a multiple network of protein kinases, including that of Src/Akt cascade that controls the process of cNOS activation [14,15,24,37,38]. Indeed, we found that in keeping with the documented involvement of Src/Akt in posttranslational cNOS activation through phosphorylation at Ser¹¹⁷⁹ [37,38], the countering effect of ghrelin on the LPS-induced up-regulation in COX-2 and iNOS activation was susceptible to suppression by Akt inhibitor, SH-5, as well as Src inhibitor, PP2. Moreover, the LPS-induced increase in COX-2 and iNOS activity displayed susceptibility to PPM-18 and Bay 11-7082, the inhibitors of NF- κ B activation with different mechanism of action [39,40]. However, while this inhibitory effect on iNOS activity was also reflected in a marked suppression in the expression of iNOS protein, neither PM-18 nor Bay 11-7082 affected the LPS-induced expression of COX-2 protein. Hence, considering the fact that PPM-18 is a potent blocker of NF- κ B binding to its nuclear promoter response elements [39], while Bay 11-7082 inhibits the I κ B kinase- β (IKK- β) enzymatic activity [18,40], we concluded that NF- κ B does not appear to play a role in the regulation of *H. pylori* LPS-induced COX-2 protein expression and that the countering effect of ghrelin on the LPS-induced COX-2 activation, like that of NF- κ B inhibitors, is the consequence of iNOS suppression at the transcriptional level, and depends on cNOS activation through phosphorylation.

Indeed, our studies confirmed an important role for ghrelin in the inhibition of NF- κ B activation in response to *H. pylori* LPS-induced up-regulation in iNOS expression. NF- κ B is a rapid transcriptional activator that is held in the cytoplasm of resting cells bound to a family of inhibitory I κ B proteins. Upon stimulation by LPS or TNF- α , I κ B undergoes phosphorylation at two critical serine residues by the an I κ B kinase (IKK) complex, which targets I κ B for degradation through the ubiquitin-proteasomal pathway and leads to nuclear translocation of NF- κ B, its binding to promoter response elements, and activation of the target gene transcription [30-34]. In concordance with this classical pathway of NF- κ B activation [35,41], we have shown that the effect of *H. pylori* LPS was manifested by a distinct reduction in the inhibitory protein, I κ B- α and a marked increase in p65 NF- κ B nuclear translocation, whereas in the presence of ghrelin, the extent of the LPS-induced I κ B- α degradation and the nuclear translocation of NF- κ B decreased sig-

nificantly. Moreover, we found that countering effect of ghrelin on the LPS-induced up-regulation in gastric mucosal cell IKK- β activity was subject to suppression by the inhibitors of Src/Akt pathway, PP2 and SH-5. A significant decrease in the effectiveness of ghrelin to counter the LPS-induced IKK- β activation was also observed in the presence of cNOS inhibitor, L-NAME, while the inhibitor of NF- κ B activation, PPM-18, and iNOS inhibitor, 1400W, had no effect. From this, we inferred that the countering effect of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal IKK- β activation, as well as COX-2 and iNOS enzyme suppression, occurs with the involvement of Src/Akt-mediated cNOS activation, and shows dependence on NO generated by the cNOS. In this connection, it is pertinent to reiterate that signaling through Src/Akt pathway is known to occupy a central stage in the receptor (GHSR1a)-mediated responses to ghrelin stimulation [14,15, 24,38]. Deserving equally insightful consideration in furthering our understanding the signaling pathways of ghrelin are the reports indicating that the activity of IKK- β complex as well as that of COX-2 protein may also be regulated through S-nitrosylation [9,10,33,42-44].

Indeed, as demonstrated recently, the induction in iNOS expression by LPS leads to COX-2 S-nitrosylation that result in an excessive PGE₂ generation [9,10,16], and S-nitrosylation of a specific cysteine residue within the activation loop of IKK- β by endogenous NO donors everts the inhibitory effect on the extent of I κ B- α degradation, and hence affects the nuclear translocation of NF- κ B [33,42,44]. Hence, to examine further the role of cNOS in the regulation of *H. pylori* LPS-induced IKK- β and COX-2 activation by ghrelin, we assessed the effect of nitrosothiol reducing agent, ascorbate. While preincubation with ascorbate produced no discernible effect on the extent of the LPS-induced IKK- β activation, a marked decrease was observed in the LPS-induced COX-2 activity. Moreover, ascorbate elicited an amplification in the inhibitory effect of ghrelin on COX-2 activity and produced a significant relieve in the inhibitory effect of ghrelin on the LPS-induced IKK- β activity. Therefore, consistent with our findings, the regulation of IKK- β activity as well as the activation of COX-2 appears to be intimately linked to the events of S-nitrosylation by NO generated by the cNOS system. This contention is supported further by the results of biotin switch assay [28, 29]. Western blot analysis of COX-2 and IKK- β protein S-nitrosylation patterns revealed that gastric mucosal cells exposed to the LPS alone showed a marked increase in COX-2 S-nitrosylation, while the countering effect of ghrelin on the LPS-induced up-regulation in IKK- β activity was manifested by an increase in the kinase S-nitrosylation as well as the substantial loss in COX-2

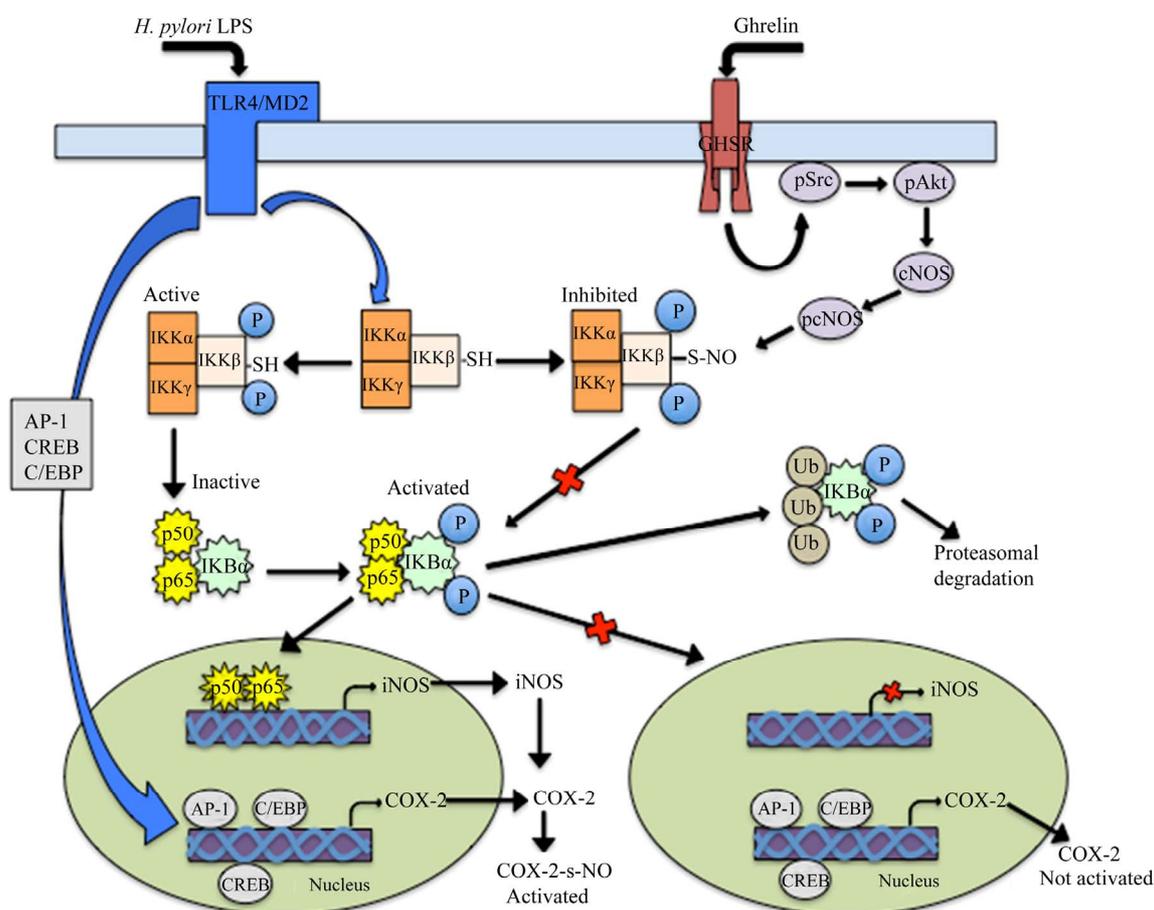


Figure 11. Proposed mechanism of ghrelin action in countering the gastric mucosal proinflammatory events triggered by *H. pylori* LPS. Binding of the LPS to Toll-like receptor 4 (TLR4)/MD2 triggers the activation and nuclear translocation of transcriptional factors involved in the induction of COX-2 (AP-1, CREB and C/EBP), and iNOS (NF- κ B) genes transcription. The up-regulation in NO generated by iNOS leads to COX-2 protein S-nitrosylation that results in the induction of COX-2 enzymatic activity and the excessive PGE₂ production. Binding of ghrelin to growth hormone secretagogue receptor (GHSR) triggers up-regulation in Src/Akt-dependent cNOS activation through phosphorylation that leads to the inhibition of the LPS-induced IKK- β ctivation by cNOS-mediated IKK- β S-nitrosylation, which interferes with I κ B- α proteasomal degradation and NF- κ B nuclear translocation, thus causing repression of iNOS gene induction and the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation. AP-1, activator protein-1; CREB, cAMP response element binding protein; C/EBP, CCAAT/enhancer binding protein.

S-nitrosylation. Moreover, the induced by ghrelin increase in IKK- β S-nitrosylation was susceptible to suppression by Akt inhibitor, SH-5, which also caused the reversal of the countering effect of ghrelin on the LPS-induced COX-2 S-nitrosylation. Thus, our findings lend further support to increasingly apparent assertion that cNOS activation through Src/Akt-mediated phosphorylation is a pivotal element in the signaling cascade by which ghrelin exerts the modulatory control over proinflammatory events triggered in gastric mucosa by *H. pylori* infection [14-16,24,26,41].

Together, the data provided in our study demonstrate that *H. pylori* LPS-induced abrogation of cNOS control over NF- κ B activation leads to the induction of iNOS expression that triggers up-regulation COX-2 activation

through S-nitrosylation that results in an excessive PGE₂ generation (**Figure 11**). We also show that peptide hormone, ghrelin, counters these untoward consequences of the LPS via up-regulation in Src/Akt-dependent cNOS activation that results in up-regulation in cNOS-mediated IKK- β S-nitrosylation which interferes with I κ B- α proteasomal degradation and NF- κ B nuclear translocation, thus causing repression of iNOS gene induction and hence the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation

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