

# Ghrelin Suppression of *Helicobacter pylori*-Induced Gastric Mucosal Expression of iNOS is Mediated through the Inhibition of IKK- $\beta$ Activation by cNOS-Dependent S-Nitrosylation

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Received November 7, 2011; revised December 3, 2011; accepted December 12, 2011

## Abstract

Excessive nitric oxide generation, caused by the disturbances in nitric oxide synthase (NOS) isozyme system, plays a key role in defining the extent of gastric mucosal inflammatory response to *H. pylori* infection. Here, we report that *H. pylori* LPS-induced enhancement in gastric mucosal inducible (i) iNOS expression and the impairment in constitutive (c) cNOS activity was associated with up-regulation in the inhibitory  $\kappa$ B kinase- $\beta$  (IKK $\beta$ ) activation through phosphorylation, rise in I $\kappa$ B- $\alpha$  degradation, and the increase in the transcriptional factor, NF- $\kappa$ B, nuclear translocation. Further, we show that the countering effect of peptide hormone, ghrelin, on the LPS-induced disturbances in NOS isozyme system was reflected in the increase in Src/Akt-dependent cNOS activation through phosphorylation and the suppression of IKK- $\beta$  activity through cNOS-mediated IKK- $\beta$  protein S-nitrosylation. As a consequence, ghrelin exerted the inhibitory effect on the LPS-induced rise in I $\kappa$ B- $\alpha$  degradation and NF- $\kappa$ B nuclear translocation, thus leading to iNOS gene suppression and the repression of iNOS induction. These results point to a central role of cNOS activation in controlling the signaling pathways of the LPS-triggered iNOS gene induction. Moreover, our findings suggest a molecular mechanism by which ghrelin suppresses the gastric mucosal proinflammatory consequences of *H. pylori* infection.

**Keywords:** *H. Pylori*, Gastric Mucosa, Ghrelin, cNOS Activation, IKK- $\beta$  S-Nitrosylation

## 1. Introduction

Cell-wall lipopolysaccharide (LPS) of *H. pylori*, a Gram-negative bacterium colonizing the gastric mucosa of over 50% of the human population, is recognized as a potent endotoxin capable of eliciting mucosal inflammatory changes that characterize gastritis and duodenal ulcer [1-3]. The stimulation of gastric mucosa with *H. pylori* LPS is known to provoke a marked up-regulation in pro-inflammatory cytokine production, massive rise in epithelial cell apoptosis, and the excessive nitric oxide (NO) generation caused by the disturbances in nitric oxide synthase (NOS) system [4-6]. A growing body of evidence, moreover, points to the disturbances in NO production associated with *H. pylori* colonization, and reflected in continual activation of iNOS and the suppression of cNOS isozyme system, as the event defining the

extent of gastric mucosal inflammatory involvement. [4, 7-9]. Moreover, studies indicate that the expression of proinflammatory mediators induced by *H. pylori* infection is regulated by the activity of the nuclear transcription factor, NF- $\kappa$ B [6,10-12].

Indeed, NF- $\kappa$ B is ubiquitously present in all types of mammalian cells, where it controls a variety of genes involved in immune, inflammatory, and proliferative responses, including those of the NOS isozyme system that are responsible for NO production [12-14]. While the factor is known to consist of five homo- and heterodimers of the Rel protein family, the most characterized NF- $\kappa$ B complex is a p50/p65 heterodimer, which is sequestered in the cytoplasm of resting cells bound to a family of inhibitory I $\kappa$ B proteins [14-16]. Activation of NF- $\kappa$ B in response to such stimuli as TNF- $\alpha$  or LPS occurs through a rapid I $\kappa$ B- $\alpha$  phosphorylation by an I $\kappa$ B

kinase (IKK) complex, that targets I $\kappa$ B- $\alpha$  for degradation through the ubiquitin-proteasomal pathway, thus allowing nuclear translocation of NF- $\kappa$ B, its binding to promoter response elements, and activation of the transcription of genes implicated in immunoregulation, inflammation and cellular proliferation [14-17].

The IKK complex consists of two catalytic subunits, IKK- $\alpha$  and IKK- $\beta$ , and one regulatory subunit, IKK- $\gamma$ , also known as NEMO (NF- $\kappa$ B essential modulator) [12, 14,15,17]. While the activation of IKK complex requires phosphorylation of IKK- $\alpha$  and IKK- $\beta$  on the specific conserved serine residues in the activation loops, the IKK- $\gamma$  regulatory subunit appears to be responsible for IKK complex stabilization and the interaction with upstream regulatory proteins [15,17]. Furthermore, although IKK- $\alpha$  and IKK- $\beta$  show a high degree of homology in their structural domains, the available literature indicates that IKK- $\beta$  displays up to 50 fold higher level of kinase activity towards I $\kappa$ B proteins, and the gene deletion studies demonstrated that it is IKK- $\beta$  and not IKK- $\alpha$  which is responsible for NF- $\kappa$ B activation in response to stimulation by TNF- $\alpha$  or bacterial LPS [18- 20]. In addition, recent reports indicate that the activity of IKK complex may also be regulated through S-nitrosylation of a specific cysteine residue within the activation loop of IKK- $\beta$ , by endogenous NO donors, leading to suppression of I $\kappa$ B- $\alpha$  degradation and the inhibition of NF- $\kappa$ B nuclear translocation, thus blocking the transcription of NF- $\kappa$ B-regulated gene products [17,21,22].

As peptide hormone, ghrelin, is recognized as an important modulator of gastric mucosal inflammatory responses to *H. pylori* through the regulation of NOS system [4,9,23-26], in this study we investigated the effect of *H. pylori* LPS on the activation of NF- $\kappa$ B signaling pathway in gastric mucosal cells, and the mechanism of ghrelin modulatory action. Our results demonstrate that the LPS elicit induction in iNOS expression via up-regulation in IKK- $\beta$  activation through phosphorylation. We further show that ghrelin inhibits the LPS-induced activation of IKK- $\beta$  through cNOS-mediated IKK- $\beta$  protein S-nitrosylation, thus leading to suppression of I $\kappa$ B- $\alpha$  phosphorylation and degradation, inhibition of NF- $\kappa$ B nuclear translocation, and the repression of iNOS gene induction.

## 2. Materials and Methods

### 2.1 Gastric Mucosal Cell Incubation

The cells were collected by scraping 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  $\mu$ g/ml), penicillin (50 U/ml), streptomycin (50  $\mu$ 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  $2 \times 10^7$  cell/ml [9]. Cell aliquots (1 ml) were then transferred to DMEM in culture dishes and incubated under 95% O<sub>2</sub> - 5% CO<sub>2</sub> atmosphere at 37°C for 16 h in the presence of 0 - 100 ng/ml of *H. pylori* LPS [25]. *H. pylori* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 4350 [5]. In the experiments evaluating the effect of ghrelin (rat, Sigma), cNOS inhibitor, L-NAME, iNOS inhibitor, 1400W, Akt inhibitor, SH-5, 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, assessed by Trypan blue dye exclusion assay [9], 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-[<sup>3</sup>H] arginine to L-[<sup>3</sup>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<sup>2+</sup>-independent iNOS) or 6 mM CaCl<sub>2</sub> (for Ca<sup>2+</sup>-dependent cNOS), and centrifuged. The aliquots of the resulting supernatant were incubated for 30 min at 25°C in the presence of 50  $\mu$ Ci/ml of L-[<sup>3</sup>H] arginine, 10 mM NADPH, 5  $\mu$ M tetrahydrobiopterin, and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250  $\mu$ l. Following addition of stop buffer and Dowex-50 W (Na<sup>+</sup>) resin, the mixtures were transferred to spin cups, centrifuged and the formed L-[<sup>3</sup>H] citrulline contained in the flow through was quantified by scintillation counting.

### 2.3. Nuclear Protein Extraction

The aliquots of gastric mucosal cell suspension from the control and various experimental conditions were settled by centrifugation at 1500 xg 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 [13]. Following centrifugation at 12,000 xg for 10 min at 4°C, the supernatant was subjected to centrifugation at 100,000 xg for 1h and the obtained soluble fraction was used as source of cytosolic extract [27]. The pellets, from 12,000 xg

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 MgCl<sub>2</sub>, 1 mM EDTA, 1 mM dithiothreitol, and 1 mM PMSF. The samples were centrifuged at 15,000 xg for 10 min at 4°C, and the supernatants containing nuclear extracts were collected and stored at -70°C until use.

## 2.4. 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 Ser<sup>32</sup> and Ser<sup>36</sup> of IκB-α phosphorylation sites was used as a substrate [28]. 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 (Ser<sup>32</sup>/Ser<sup>36</sup>) 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.

## 2.5. IKK-β Protein S-Nitrosylation Assay

A biotin switch procedure was employed to assess IKK-β protein S-nitrosylation [29,30]. 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 for 1h in the presence of 100ng/ml of *H. pylori* LPS. Following centrifugation at 500 xg 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 [30]. 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 [30]. The obtained proteins were then analyzed by Western blotting.

## 2.6. 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 [25]. Following brief sonication, the lysates were centrifuged at 12,000 g for 10 min, and the supernatants were collected and 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 IκB-α and cNOS (Calbiochem), phospho-IκB-α and phospho-IKK-β (Cell Signaling), 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, pSer<sup>1179</sup>).

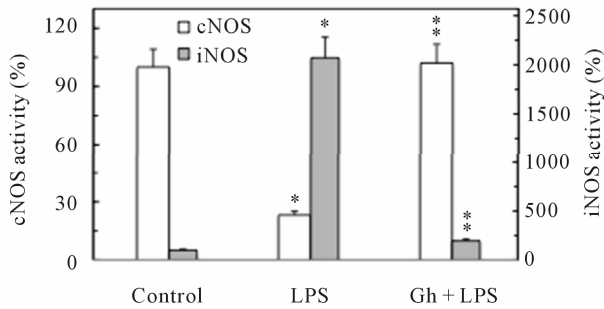
## 2.7. Data Analysis

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

We have reported that the modulatory effect of ghrelin on gastric mucosal inflammatory responses to *H. pylori* LPS, characterized by the disturbances in NO production, was reflected in the cNOS activation through phosphorylation and the suppression of iNOS protein expression [9,25]. Indeed, as illustrated in **Figure 1**, the LPS at 100 ng/ml elicited a 19.8-fold increase in the mucosal cell iNOS activity, whereas the cNOS activity showed a 4.3-fold decrease. Moreover, preincubation with ghrelin at 0.5 μg/ml lead to a 90.2% reduction in the LPS-induced iNOS activity, while the activity of cNOS increased by a 77%. Since iNOS expression is controlled primarily by the transcription factor, NF-κB [10-12], we further investigated the effect on ghrelin on the LPS-induced NF-κB activation.

As NF-κB activation requires phosphorylation and degradation of the inhibitory protein IκB-α, we exposed

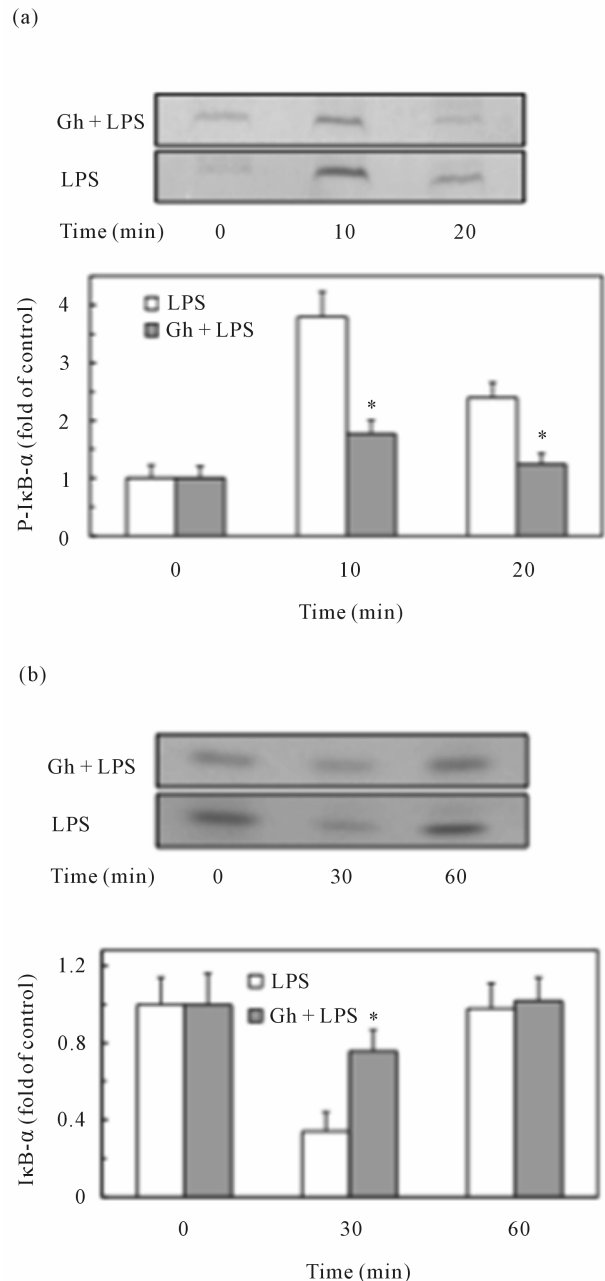


**Figure 1.** Effect of ghrelin on *H. pylori* LPS-induced expression of cNOS and iNOS activities in gastric mucosal cells. The cells, in the absence and presence of preincubation with ghrelin (0.5  $\mu\text{g/ml}$ ), were treated with *H. pylori* LPS (100 ng/ml) and incubated for 16 h. Values are presented as percent 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 the LPS.

the gastric mucosal cells for different time periods to *H. pylori* LPS in the absence or presence of ghrelin, and the cell lysates were analyzed for I $\kappa$ B- $\alpha$  phosphorylation and degradation. As shown in **Figure 2(a)**, the LPS induced a marked phosphorylation of I $\kappa$ B- $\alpha$  at 10 min, while a distinct reduction in I $\kappa$ B- $\alpha$  protein level occurred at 30 min (**Figure 2(b)**). Moreover, we found that in the presence of ghrelin, the LPS-induced phosphorylation of I $\kappa$ B- $\alpha$  was significantly inhibited (**Figure 2(a)**), and the extent of the induced I $\kappa$ B- $\alpha$  protein degradation showed a significant decrease (**Figure 2(b)**).

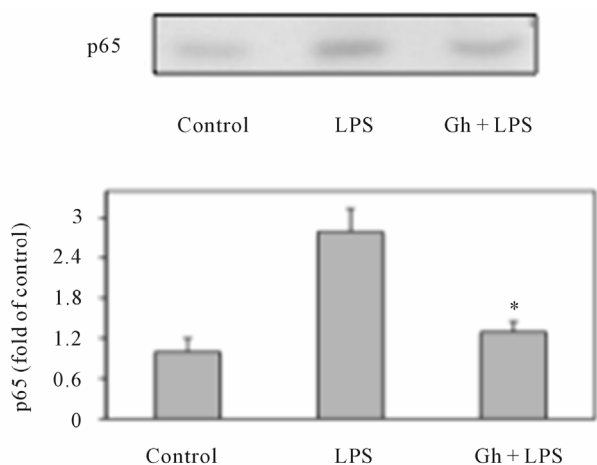
Further, as I $\kappa$ B- $\alpha$  degradation leads to translocation of p65 NF- $\kappa$ B from the cytoplasm to nucleus [14-17], we assessed the nuclear content of p65 NF- $\kappa$ B in response to *H. pylori* LPS alone and in the presence of preincubation with ghrelin. The results of immunoblots analysis revealed that incubation of the mucosal cells with the LPS lead to an increase of p65 NF- $\kappa$ B in the nuclear extracts (**Figure 3**), while in the presence of preincubation with ghrelin, the LPS-induced nuclear translocation of p65 NF- $\kappa$ B was significantly diminished (**Figure 3**).

As the extent of I $\kappa$ B- $\alpha$  phosphorylation and its proteasomal degradation is mediated through IKK- $\beta$  of the IKK complex [12,14,17], we next analyzed the influence of ghrelin on changes in IKK- $\beta$  activity in gastric mucosal cells exposed to *H. pylori* LPS. The results revealed that the LPS (at 100 ng/ml) induced a 8.4-fold increase in the mucosal cell IKK- $\beta$  activity, and that preincubation with ghrelin lead to a concentration-dependent suppression of the LPS effect (**Figure 4**). As a result the LPS-induced activity of IKK- $\beta$ , in the presence 0.5  $\mu\text{g/ml}$  of ghrelin, showed an 83% decrease. Moreover, as the suppression of the LPS-induced iNOS protein expression by ghrelin is associated with rapid cNOS activation through phosphorylation [24,25], we have also examined the

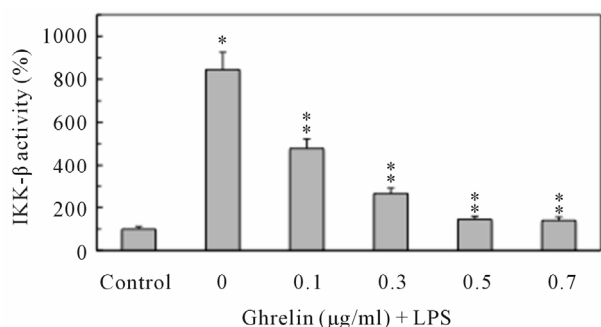


**Figure 2.** Effect of ghrelin on *H. pylori* LPS-induced I $\kappa$ B- $\alpha$  serine phosphorylation (a) and I $\kappa$ B- $\alpha$  protein degradation (b) in rat gastric mucosal cells. The cells were preincubated for 30 min with 0 or 0.5  $\mu\text{g/ml}$  of ghrelin and incubated for the indicated time periods with the LPS at 100 ng/ml. Cell lysates were analyzed by Western blotting with anti-phospho-I $\kappa$ B- $\alpha$  or anti-I $\kappa$ B- $\alpha$  antibody. Relative densities are expressed as fold of control (time, 0). Values represent the means  $\pm$  SD of four separate experiments. \* $P < 0.05$  compared with that of LPS.

mucosal cell IKK- $\beta$  activity in the presence of the inhibitors of cNOS phosphorylation. For this, the gastric mucosal cells prior to incubation with the LPS were pre-

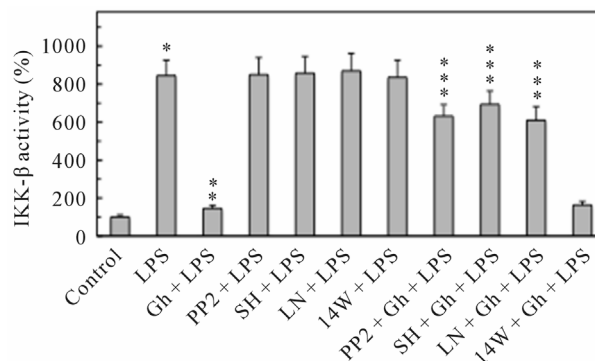


**Figure 3.** Effect of ghrelin on *H. pylori* LPS-induced nuclear translocation of p65 NF- $\kappa$ B in rat gastric mucosal cells. The cells were preincubated for 30 min with 0 or 0.5  $\mu$ g/ml of ghrelin and incubated for 30 min with the LPS at 100 ng/ml. Cells were lysed and the level of p65 NF- $\kappa$ B protein in the nuclear fraction was analyzed immunochemically. Relative densities are expressed as fold of control. The data represent the means  $\pm$  SD of four separate experiments. \* $P$  < 0.05 compared with that of LPS.



**Figure 4.** Effect of ghrelin on *H. pylori* LPS-induced changes in IKK- $\beta$  activity 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 30 min. 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.

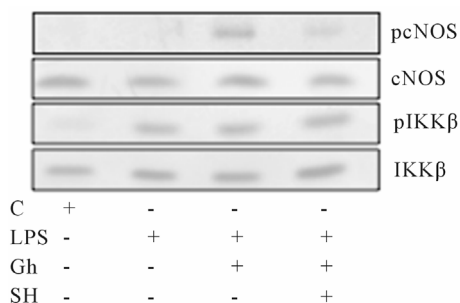
treated with the inhibitors of Src/Akt pathway in the absence or presence of ghrelin, and assayed for IKK- $\beta$  activity. We found that the countering effect of ghrelin on *H. pylori* LPS-induced up-regulation in the mucosal cell IKK- $\beta$  activity was subject to suppression by both, the inhibitor of Src, PP2, as well as Akt inhibitor, SH-5 (Figure 5). A significant decrease in the effectiveness of ghrelin on the LPS-induced IKK- $\beta$  activation, furthermore, was observed in the presence of cNOS inhibitor, L-NAME, whereas the inhibitor of iNOS, 1400 W, had no effect (Figure 5). Further, examination of the effect of *H. pylori* LPS and ghrelin on IKK- $\beta$  and cNOS phos-



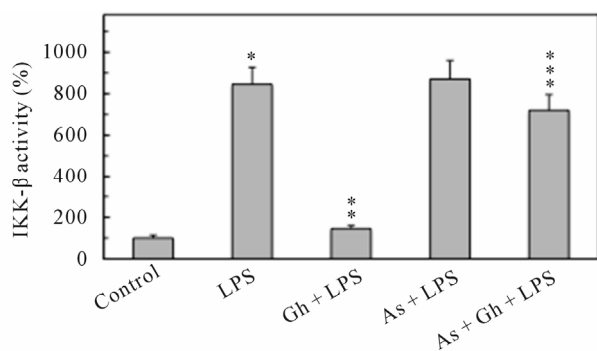
**Figure 5.** Effect of Src inhibitor, PP2, Akt inhibitor, SH-5, cNOS inhibitor, L-NAME, and iNOS inhibitor, 1400 W, on the ghrelin (Gh)-induced changes in IKK- $\beta$  activity in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 30  $\mu$ M PP2, 20  $\mu$ M SH-5 (SH), 200  $\mu$ M L-NAME (LN), or 40  $\mu$ M 1400W (14W), were treated with Gh at 0.5  $\mu$ 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.

phorylation revealed that the LPS-induced up-regulation in IKK- $\beta$  activity was associated with a marked increase in IKK- $\beta$  phosphorylation and the suppression in cNOS protein phosphorylation (Figure 6). Moreover, while preincubation with ghrelin lead to the increase in cNOS phosphorylation that was susceptible to suppression by Akt inhibitor, SH-5, ghrelin produced no apparent effect of the extent of the LPS-induced IKK- $\beta$  phosphorylation (Figure 6). Thus, the countering effect of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal IKK- $\beta$  activation shows an apparent dependence on NO generated by the cNOS system.

To ascertain further the role of NO generated by cNOS system in the modulatory influence of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal IKK- $\beta$  activation, we assessed the effect of nitrosothiols reducing agent, ascorbate. As show in Figure 7, preincubation with ascorbate lead to a significant relieve in the inhibitory effect of ghrelin on the LPS-induced IKK- $\beta$  activity. However, ascorbate produced no discernible effect on the extent of the LPS-induced IKK- $\beta$  activation. Moreover, we also examined the dependence of IKK- $\beta$  S-nitrosylation on the ghrelin-induced cNOS activation through Akt-mediated phosphorylation by the biotin switch method [29,30]. The gastric mucosal cells were incubated with *H. pylori* LPS or ghrelin + LPS in the absence and presence of Akt inhibitor, SH-5, and the lysates following the biotin switch procedure were examined for IKK- $\beta$  protein S-nitrosylation (Figure 8). Western blot analysis revealed that ghrelin countering effect on the LPS-induced up-regulation in IKK- $\beta$  active-



**Figure 6.** Effect of *H. pylori* LPS and ghrelin (Gh) on cNOS and IKK- $\beta$  phosphorylation in gastric mucosal cells exposed to Akt inhibitor, SH-5 (SH). The cells were preincubated for 30 min with Gh at 0.5  $\mu$ g/ml, or SH (20  $\mu$ M) + Gh, and incubated for 1 h in the presence of 100 ng/ml of LPS. Cell lysates were resolved on SDS-PAGE, transferred to nitrocellulose and probed with phosphorylation specific cNOS (pcNOS) and pIKK $\beta$  antibody, and after stripping reprobed with anti-cNOS and anti-IKK $\beta$  antibody. The immunoblots shown are representative of three experiments.

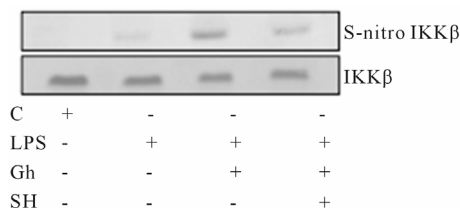


**Figure 7.** Effect of ascorbate on the ghrelin (Gh)-induced changes in the expression of IKK- $\beta$  activity in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 300  $\mu$ M ascorbate (As), were treated with Gh at 0.5  $\mu$ g/ml and incubated for 30 min in the presence 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.

ity was manifested in a marked increase in the enzyme protein S-nitrosylation, and that this effect of ghrelin on IKK- $\beta$  S-nitrosylation was subject to suppression by Akt inhibitor, SH-5. These data suggest that ghrelin counteracting effect of the detrimental consequences of up-regulation in IKK- $\beta$  activity by *H. pylori* LPS is mediated through cNOS-dependent IKK- $\beta$  protein S-nitrosylation.

#### 4. Discussion

Infection with gastric pathogen, *H. pylori*, initiates the course of mucosal inflammatory events that increases the risk of chronic gastritis, peptic ulcer disease, and gastric



**Figure 8.** Effect of *H. pylori* LPS and ghrelin (Gh) on IKK- $\beta$  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  $\mu$ g/ml, or SH at (20  $\mu$ M) + Gh, and incubated for 1 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 remainder of the lysates, resolved on SDS-PAGE, transferred to nitrocellulose probed with anti-IKK $\beta$  antibody. The immunoblots shown are representative of three experiments.

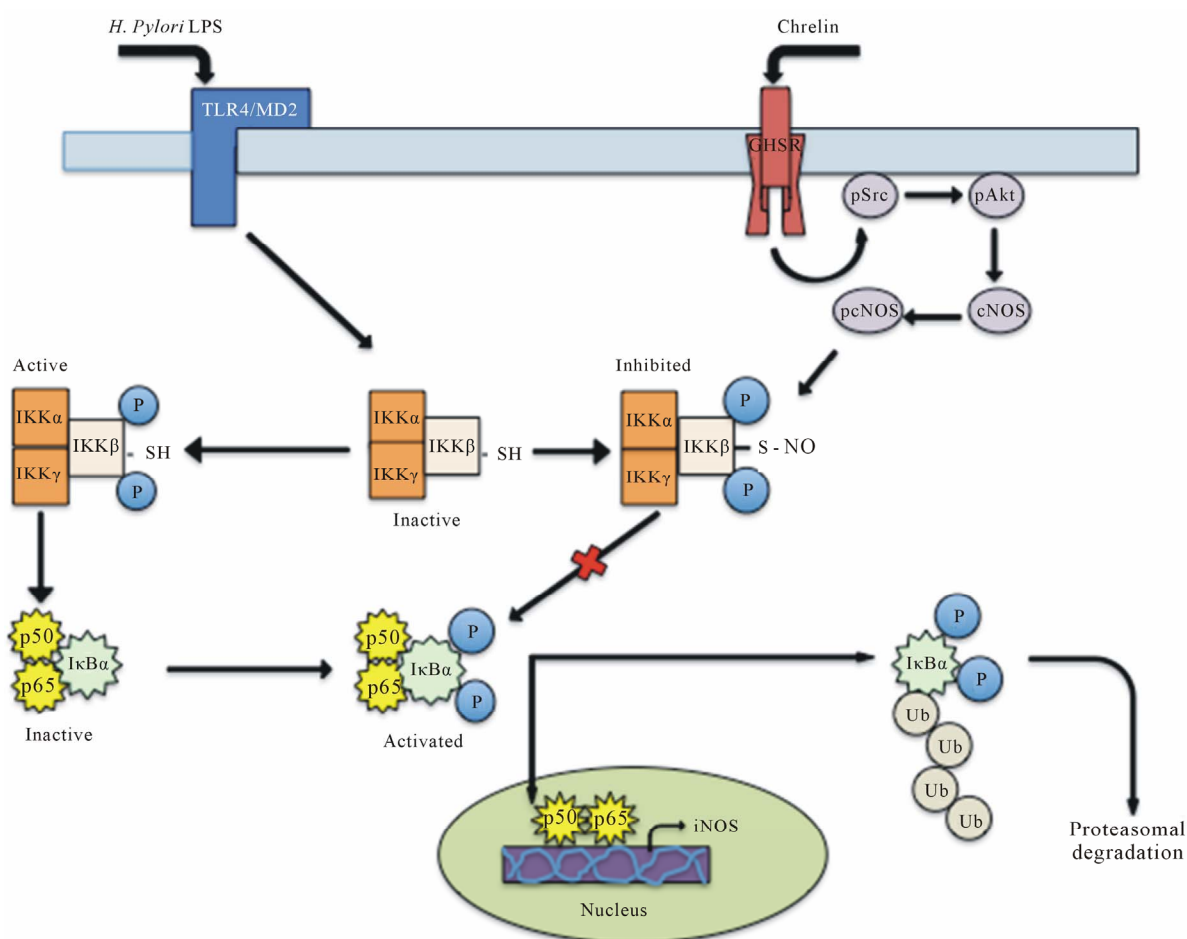
adenocarcinoma [1-3,12]. Indeed, colonization of gastric mucosa by *H. pylori* in humans or stimulation of gastric mucosal cells with *H. pylori* LPS is known to induce up-regulation in proinflammatory cytokine release, enhances the epithelial cell apoptosis, and causes the excessive NO generation due to disturbances in NOS isozyme system [4-7,9]. These events define the extent of gastric mucosal inflammatory involvement, and are carefully regulated at the early response stage by a family of transcriptional factors, known as NF- $\kappa$ B, which transduce the inflammatory stimulus into the activation of a variety genes involved in ant-microbial responses, including those accountable for NO generation [6, 10-12, 31,32]. As gastric mucosal inflammatory responses to *H. pylori* as well as its LPS are reflected in continual induction of iNOS and the suppression of cNOS activation, and a peptide hormone, ghrelin, is recognized as an important regulator of NOS system [4,5,7,9,23-26], the objective of this study was to ascertain the influence of ghrelin on the processes associated with NF- $\kappa$ B activation by *H. pylori* LPS.

The data obtained with rat gastric mucosal cells revealed that *H. pylori* LPS-induced enhancement in the expression of iNOS and the impairment in cNOS activity was associated with up-regulation in IKK- $\beta$  activation through phosphorylation, rise in I $\kappa$ B- $\alpha$  degradation, and the increase in NF- $\kappa$ B nuclear translocation. These findings are thus in keeping with the preponderant literature evidence as to the involvement of bacterial LPS in the classical signaling pathway of NF- $\kappa$ B activation [14-17]. In this pathway, NF- $\kappa$ B activity is regulated by cytosolic sequestration through interaction with inhibitory I $\kappa$ B proteins. Activation of NF- $\kappa$ B by proinflammatory stimuli, such as LPS or TNF- $\alpha$ , involves phosphorylation of the inhibitory I $\kappa$ B- $\alpha$  at two conserved N-terminal serine residues (Ser<sup>32</sup> and Ser<sup>36</sup>) by I $\kappa$ B-kinase (IKK) complex, a process that signals I $\kappa$ B- $\alpha$  for degradation by the ubiquitin-proteasomal pathway [15-18]. Then, the released

activated NF- $\kappa$ B, usually consisting of p50 and p65 subunits, translocates to the nucleus, and activates the transcription of various proinflammatory target genes, including those coding for cytokines, apoptotic factors, and iNOS [10-12,31,32]. Indeed, we have found earlier that the induction in gastric mucosal iNOS enzyme protein expression in response to *H. pylori* LPS was susceptible to suppression by the inhibitor of NF- $\kappa$ B activation, PM-18 as well as peptide hormone, ghrelin [9,33]. Furthermore, as shown herein, ghrelin exerted the inhibitory effect on *H. pylori* LPS-induced rise in gastric mucosal IkB- $\beta$  degradation and NF- $\kappa$ B nuclear translocation.

Hence, to reveal further insights into the mechanism by which ghrelin exerts modulatory control over gastric mucosal inflammatory disturbances caused by *H. pylori* infection, we examined the influence of ghrelin on *H.*

*pylori* LPS-induced changes in the activity of IKK- $\beta$  a key enzyme of NF- $\kappa$ B activation pathway that controls the extent of IkB- $\alpha$  phosphorylation and its proteasomal degradation [12,14,17]. We found that countering effect of ghrelin on the LPS-induced up-regulation in gastric mucosal cell IKK- $\beta$  activity was subject to suppression by Src inhibitor, PP2 as well as Akt inhibitor, SH-5. A significant decrease in the effectiveness of ghrelin to counter the LPS-induced IKK- $\beta$  activation was also observed in the presence of cNOS inhibitor, L-NAME, while the inhibitor of iNOS, 1400 W, had no effect. Furthermore, in consonance with the documented involvement of Akt in rapid cNOS activation through phosphorylation at Ser<sup>1179</sup> [9,24,33], ghrelin-induced up-regulation in cNOS activity was reflected in the increase of enzyme phosphorylation that was susceptible to sup-



**Figure 9.** Schematic diagram illustrating modulatory mechanism of ghrelin action in countering the gastric mucosal proinflammatory events triggered by *H. pylori* LPS-induced NF- $\kappa$ B activation. Binding of the LPS to Toll-like receptor 4 (TLR4)/MD2 leads to IKK complex activation by phosphorylation at its IKK- $\beta$  catalytic subunit, thus triggering up-regulation in IkB- $\alpha$  phosphorylation and its proteasomal degradation. This causes the increase in NF- $\kappa$ B translocation to the nucleus to promote iNOS gene transcription. Ghrelin, through binding to growth hormone secretagogue receptor (GHSR), inhibits the LPS-induced IKK activation via up-regulation in Src/Akt-dependent cNOS activation that results in cNOS-mediated IKK- $\beta$  S-nitrosylation, thus leading to suppression of IkB- $\alpha$  phosphorylation and degradation, inhibition of NF- $\kappa$ B nuclear translocation, and the repression of iNOS gene transcription.

pression by Akt inhibitor, SH-5. However, the hormone produced no apparent effect on the extent of the LPS-induced phosphorylation of IKK- $\beta$ . From this, we concluded that the countering effect of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal IKK- $\beta$  activation and iNOS induction occurs with the involvement of Akt-mediated cNOS activation. Indeed, signaling through Src/Akt pathway is known to occupy a central stage in the receptor (GHS-R)-mediated responses to ghrelin stimulation [26,34], and the recent reports suggest that the activity of IKK complex may also be subject to inhibition through S-nitrosylation of a specific cysteine residue within the activation loop of IKK- $\beta$  by the endogenous NO donors [21,22,31].

Therefore, to address further the role of cNOS in the regulation of IKK- $\beta$  activation by ghrelin, we assessed the effect of nitrosothiol reducing agent, ascorbate. We found that while ascorbate produced no discernible effect on the extent of the LPS-induced IKK- $\beta$  activation, the agent elicited a marked relieve in the inhibitory effect of ghrelin on the LPS-induced IKK- $\beta$  activity. Hence, taking into consideration known susceptibility of S-nitrosylated proteins to reduction by ascorbic acid [26,29,30, 33], we surmised that the countering effect of ghrelin on *H. pylori* LPS-induced IKK- $\beta$  activation are intimately linked to the events of IKK- $\beta$  protein S-nitrosylation by NO generated by the cNOS system. Indeed, examination of IKK- $\beta$  S-nitrosylation by the biotin switch method, revealed that ghrelin countering effect on the LPS-induced up-regulation in IKK- $\beta$  activity was manifested by a marked increase in the kinase protein S-nitrosylation, and subject to suppression by Akt inhibitor, SH-5. These findings, together with the results of IKK- $\beta$  activity assays, suggest that proinflammatory events elicited in gastric mucosa by *H. pylori* and manifested by the induction in iNOS expression are the result of the LPS-induced up-regulation in IKK- $\beta$  activation through phosphorylation. Further, it is also evident that ghrelin exerts the inhibitory effect on the LPS-induced activation IKK- $\beta$  via cNOS-mediated IKK- $\beta$  S-nitrosylation, thus leading to suppression in I $\kappa$ B- $\alpha$  phosphorylation and degradation, inhibition in of NF- $\kappa$ B nuclear translocation, and the repression of iNOS gene transcription. Consequently, the role of ghrelin in modulation of the extent of gastric mucosal inflammatory involvement appears to be intimately linked to signaling events associated with Src/Akt-mediated cNOS activation through phosphorylation.

Taken together, our results suggest that the initial phase gastric mucosal proinflammatory events triggered by *H. pylori* involves the disturbances in Src/Akt kinase-dependent cNOS phosphorylation that leads to abrogation of cNOS-mediated control over IKK $\beta$  activity through

S-nitrosylation and the increase in proteasomal degradation of I $\kappa$ B- $\alpha$ , thus allowing NF- $\kappa$ B translocation to the nucleus to promote iNOS gene transcription. We also report that ghrelin counters these untoward consequences of *H. pylori* LPS via up-regulation in Src/Akt-dependent cNOS activation through phosphorylation that results in up-regulation in cNOS-mediated IKK- $\beta$  S-nitrosylation which interferes with I $\kappa$ B- $\alpha$  phosphorylation and proteasomal degradation, thereby preventing the nuclear translocation of NF- $\kappa$ B and the activation of iNOS gene transcription. A schematic diagram illustrating the proposed mechanism of anti-*H. pylori* action of ghrelin is depicted in **Figure 9**.

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