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Helicobacter pylori Induction in Gastric Mucosal Prostaglandin and Nitric Oxide Generation Is Dependent on MAPK/ERK-Mediated Activation of IKK-β and cPLA$_2$: Modulatory Effect of Ghrelin

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ABSTRACT

Among the key factors defining the extent of gastric mucosal inflammatory involvement in response to *H. pylori* is the excessive generation of prostaglandin (PGE_2) and nitric oxide (NO), caused by the overexpression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS), and triggered by the activation of MAPK/JNK, p38 and ERK, and nuclear translocation of the cognate transcription factors. In this study, we report on the role of MAPK/ERK in the regulation of *H. pylori* LPS-induced gastric mucosal expression of COX-2 and iNOS. We show that ERK activation by the LPS leads to phosphorylation of the inhibitory κB kinase-β (IKK-β) and cytosolic phospholipase A_2 ($cPLA_2$), and is reflected in the upsurge in NF-κB nuclear translocation, induction in COX-2 and iNOS expression, and up-regulation in $cPLA_2$ activity. The modulatory effect of peptide hormone, ghrelin, on the LPS-induced changes, although associated with further enhancement in ERK, IKK-β and $cPLA_2$ phosphorylation, was reflected in the suppression of IKK-β and $cPLA_2$ activity through S-nitrosylation. While the effect of ghrelin on S-nitrosylation was susceptible to suppression by the inhibitors of Src/Akt pathway, the inhibition of ERK activation caused the blockage in IKK-β and $cPLA_2$ phosphorylation as well as S-nitrosylation. Taken together, our data show that *H. pylori*-induced ERK activation plays a critical role in up-regulation of gastric mucosal PGE_2 and NO generation at the level of IKK-β and $cPLA_2$ activation, and that ghrelin counters these proinflammatory consequences of the LPS through Src/Akt-dependent S-nitrosylation.

Keywords: *H. pylori*; Gastric Mucosa; Ghrelin; PGE_2; NO; COX-2; iNOS; ERK; IKKβ; $cPLA_2$; S-Nitrosylation

1. Introduction

Infection with *Helicobacter pylori* is well-establish risk factor in etiology of gastric disease, and the excessive generation of prostaglandin (PGE_2) and nitric oxide (NO) caused by the overexpression of cyclooxygenase-2 (COX-2) and inducible nitric oxide synthase (iNOS) are considered of primary importance in defining the extent of gastric mucosal inflammatory involvement [1-6]. The signaling events underlying the up-regulation in PGE_2 and NO generation indicate that *H. pylori* cell wall lipopolysaccharide (LPS), like that of other Gram-negative bacteria, is capable of triggering the stimulation of Toll-like receptor-4 (TLR-4), which then through a series of downstream effectors causes the activation of transcriptional factors that exert control over iNOS and COX-2 gene expression [2,7-9].

While the induction of iNOS gene by LPS has been convincingly linked to transcriptional factor NF-κB activation [9-11], the nature of factors involved in transcriptional regulation of COX-2 expression is less apparent [7,12-14]. Depending on the cell type, at least four central response elements have been implicated in the regulation of COX-2 expression. These include, transcriptional factor, NF-κB, activator protein-1(AP-1), cAMP response element-biding protein (CREB), and CCATT/enhancer-binding proteins (C/EBP) β and δ [7,13-17]. Moreover, LPS is a potent activator of mitogen-activated protein kinase (MAPK) cascade, including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinase (JNK) and p38 kinase, which in turn exert their control over transcription factors activation through phosphorylation [12,14-16]. Indeed, we have shown recently that stimulation of gastric mucosal cells with *H. pylori* LPS elicits the activation of all three MAPK subtypes (JNK, p38, and ERK), and have linked the involvement of JNK/p38 in the transcription factor AP-1 activation [17].

Studies into the role of LPS-elicited ERK activation in the regulation of factors linked to the induction of COX-2 and iNOS expression indicate the involvement of MAPK/

ERK in the activation of transcriptional factors, C/EBP and CREB, implicated in the control of COX-2 expression, as well as the processes associated with the enhancement in NF-κB nuclear translocation and the induction of iNOS expression [14,15,18-20]. Moreover, *H. pylori* LPS-induced ERK activation plays an essential role in the phosphorylation $cPLA_2$, that facilitates the enzyme translocation form cytosol to membrane to gain access to phospholipid substrates for the increase in AA release [21]. The literature data, furthermore, point to similarities in the transcriptional regulation of COX-2 and $cPLA_2$ expression; the gene locus of $cPLA_2$ is located on chromosome I in the proximity of COX-2 gene, and the increased level of AA affects the COX-2 expression [22-24].

A substantial body of information also suggests that the systems involved in transcription factors activation are regulated through S-nitrosylation [9,25-27]. Indeed, S-nitrosylation has been linked to the processes of COX-2 and $cPLA_2$ activation, as well as the regulation of inhibitory κB kinase-β (IKK-β) activity responsible for IκB-α degradation and NF-κB nuclear translocation [26-29]. Moreover, we have recently shown that S-nitrosylation of IKK-β plays a role in the modulatory influence of peptide hormone, ghrelin, on the gastric mucosal inflammatory responses to *H. pylori* [11,17,30].

As gastric mucosal responses to *H. pylori* are associated with MAPK/JNK, p38 and ERK activation, while the modulatory influence of ghrelin is reflected in the inhibition of JNK and p38, but not the ERK, in this study we investigated the influence of *H. pylori* LPS and ghrelin on the processes affected by MAPK/ERK activation. Our results demonstrate that the LPS-induced ERK activation is of critical significance to up-regulation in gastric mucosal PGE_2 and NO generation at the level of IKK-β and $cPLA_2$ activation, and that ghrelin counters these proinflammatory consequences of the LPS through Src/Akt-dependent S-nitrosylation.

2. Materials and Methods

2.1. Gastric Mucosal Cell Incubation

The gastric mucosal cells, collected form freshly dissected rat stomachs by scraping the mucosa 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. After gentle trituration with a syringe, the dispersed cells were settled by centrifugation, and resuspended in the medium to a concentration of 2×10^7 cell/ml [31]. Cell aliquots (1 ml) were then transferred to DMEM in culture dishes and incubated under 95% O_2 - 5% CO_2 atmosphere at 37˚C for up to8 h in the presence of 0 - 100 ng/ml of *H. pylori* LPS [6]. *H. pylori* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 4350 [6]. In the experiments evaluating the effect of ghrelin (rat, Sigma), JNK inhibitor, SP600125, p38 MAPK inhibitor, SB202190, ERK1/2 inhibitor, PD98059, Akt inhibitor, SH-5, Src inhibitor, PP2, Raf-1 kinase inhibitor, NF-κB inhibitor, 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 [6], was greater than 97%.

2.2. COX-2 and iNOS Activity Assay

For measurements of COX-2 activity, the gastric mucosal 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 × g for 10 min at 4˚C, and the supernatant collected [5]. The COX-2 activity in 40 μl sample aliquots of the resulting supernatant was measured using COX activity assay kit (Cayman) in the absence and the presence of COX-1 inhibition (SC-560), by monitoring the appearance of oxidized TMPD at 590 nm. The activity of iNOS was measured by monitoring the conversion of L-[^{3}H] arginine to L-[^{3}H] citrulline using NOS-detect kit (Stratagene). The gastric mucosal cells from the control and experimental treatments were homogenized in a sample buffer containing 10 mM EDTA 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 Tris-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 [6].

2.3. cPLA2 Activity Assay

The $cPLA_2$ activity measurements were carried out using $cPLA_2$ assay kit (Cayman) with thioarachidonoylphosphatidylcholine as substrate. The gastric mucosal cells from the control and experimental treatments were settled by centrifugation, homogenized in 1 ml of 50 mM HEPES buffer, pH 7.4, containing 1 mM EDTA, and centrifuged at 10,000 × g for 15 min at 4˚C [29]. The supernatants were then filtered through an Amicon YM30 filter concentrators (m.w. cut-off 30 KDa) to remove any contamination with secretory PLA_2, followed by 15 min incubation with 5 μM calcium-independent PLA_2 inhibi-

tor, bromoenol lactone, and the aliquots (10 µl) of such prepared cell lysates were subjected to $cPLA_2$ assay according to manufacturer's instruction.

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^{32} and Ser^{36} of IκB-α phosphorylation sites was used as a substrate [32]. 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^{32}/Ser^{36}) as first antibody, followed by horseradish peroxidase-conjugated secondary antibody. Following washing the retained complex was probed TMB reagent for spectrophotometric quantification [11].

2.5. cPLA2 and IKK-β Protein S-Nitrosylation Assay

Detection of $cPLA_2$ and IKK-β protein S-nitrosylation was carried out utilizing a biotin switch method for protein S-nitrosylation [33,34]. The gastric mucosal cells were treated with ghrelin (0.5 µg/ml), or ERK inhibitor, PD 98059 (30 µM) + ghrelin, or Akt inhibitor, SH-5 (20 µM) + ghrelin, and incubated for 1h in the presence of 100ng/ml of *H. pylori* LPS. The cells were collected by centrifugation at 500 × g for 5 min, 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.

2.6. Nuclear Protein Extraction

The aliquots of gastric mucosal cell suspension from the control and various experimental conditions were settled by centrifugation at 500 × 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 [35]. Following centrifugation at 12,000 × g for 10 min at 4˚C, the supernatant was subjected to centrifugation at 100,000 × g for 1h and the obtained soluble fraction was used as source of cytosolic extract [11]. 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 $MgCl_2$, 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.

2.7. Immunoblot Analysis

The gastric 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 [5]. 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 antibodies directed against IκB-α, COX-2 and iNOS (Calbiochem), NF-κB p65 and IKK-β (EMD Millipore), and phospho-IKK-β, $cPLA_2$ and phospho-$cPLA_2$ (Cell Signaling) Antibodies directed against ERK1/2 and phospho-ERK1/2 MAPK were from Calbiochem, whereas ati-β-actin from Sigma.

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

In our previous study, we reported that ghrelin modulates *H. pylori* LPS-elicited induction in gastric mucosal COX-2 and iNOS expression by affecting p38 MAPK/ATF-2 and IKK-β/NF-κB activation pathways [17]. Indeed, as shown in **Figure 1**, exposure of rat gastric mucosal cells

to *H. pylori* LPS lead to a significant induction in the level of COX-2 and iNOS proteins (**Figure 1(b)**), accompanied by a marked increase in the mucosal cell COX-2 and iNOS enzymatic activities (**Figure 1(c)**). Further, preincubation with ghrelin lead to suppression of the LPS-induced iNOS and COX-2 activities. However, while the effect of ghrelin on iNOS activity was also manifested in a marked inhibition of the iNOS protein expression, less apparent change was observed in the expression of COX-2 protein (**Figure 1(b)**).

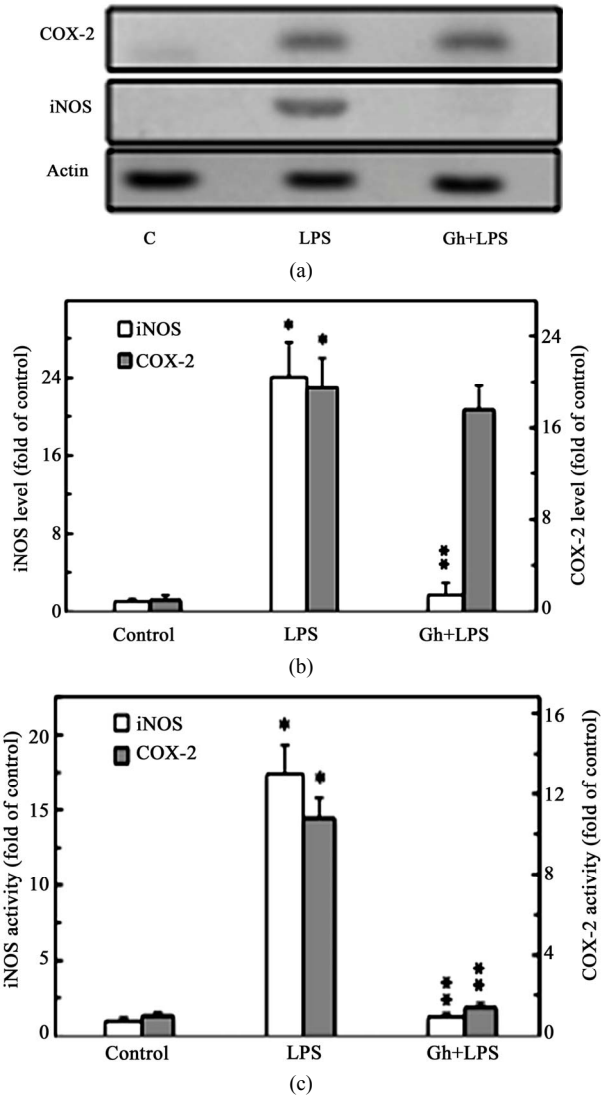

Figure 1. Effect of ghrelin (Gh) on *H. pylori* LPS-induced changes in the expression and activity of COX-2 and iNOS proteins in gastric mucosal cells. The cells were treated with the LPS at 100 ng/ml or Gh at 0.5 μg/ml + LPS, and incubated for 8 h. Cell lysates were analyzed by western blotting with specific antibodies (a) for COX2 and iNOS; their protein expression (b); and the enzymatic activity (c). Actin blot shows equal lane load. The data represent the mean ± SD of four experiments. ($^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS.)

Moreover, since induction of iNOS and COX-2 enzymes in response to LPS is controlled primarily by factors operating at the level of transcriptional activation [2,7-9,17], we assessed the expression of COX-2 and iNOS proteins in the presence of the inhibitors of MAPK and NF-κB activation. The results revealed that *H. pylori* LPS-induced expression of COX-2 protein showed susceptibility to inhibition by the p38 inhibitor, SB202190, whereas the LPS-induced expression of iNOS protein was subject to suppression by the inhibitor of NF-κB, PPM-18, as well as the inhibitor of ERK1/2, PD98059 (**Figure 2**). These results thus confirm the existence of an intimate relationship between the pathways of MAPK and NF-κB activation for the induction of proinflammatory iNOS and COX-2 expression in response to *H. pylori* colonization.

As gastric mucosal responses to *H. pylori* are associated with MAPK/JNK, p38 and ERK activation, while the modulatory influence of ghrelin is reflected in the inhibition of JNK and p38 phosphorylation but not that of ERK [17], we examined the influence of the LPS and

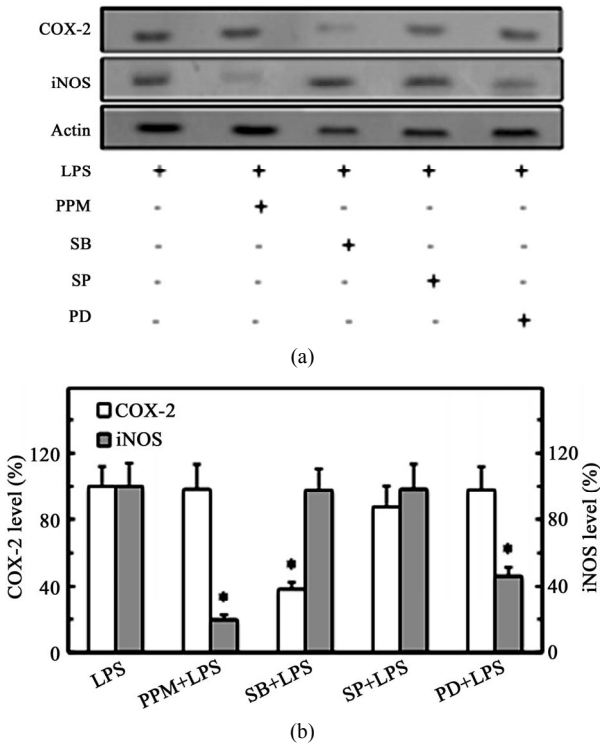

Figure 2. Effect of MAPK and NF-κB inhibitors on *H. pylori* LPS-induced changes in COX-2 and iNOS protein expression in gastric mucosal cells. The cells were treated with 30 μM PD98059 (PD), 10 μM SP600125 (SP), 20 μM SB202190 (SB), or 15 μM PPM-18 (PPM), and incubated for 8 h with the LPS at 100 ng/ml. Cell lysates were analyzed by western blotting for COX-2 and iNOS proteins (a) and their protein expression (b) are normalized to βactin. The data represent the mean ± SD of four experiments. ($^{*}P < 0.05$ compared with that of LPS.)

ghrelin on the processes affected by ERK activation. The results revealed that the LPS effect, manifested in ERK activation and further enhanced by ghrelin, was associated with IKK-β phosphorylation, and the inhibitory effect of PD98059 on ERK phosphorylation was also reflected in a reduced phosphorylation of IKK-β **Figure 3**). However, we found that in contrast to the LPS, the effect of ghrelin on IKK-β was associated with the inhibition of the LPS-induced IκB-α degradation in the cytosol and a marked decrease in the LPS-induced translocation of p65 NF-κB into nucleus (**Figure 4**). As a consequence, this effect of ghrelin is manifested in the suppression of iNOS gene induction at the level of NF-κB activation.

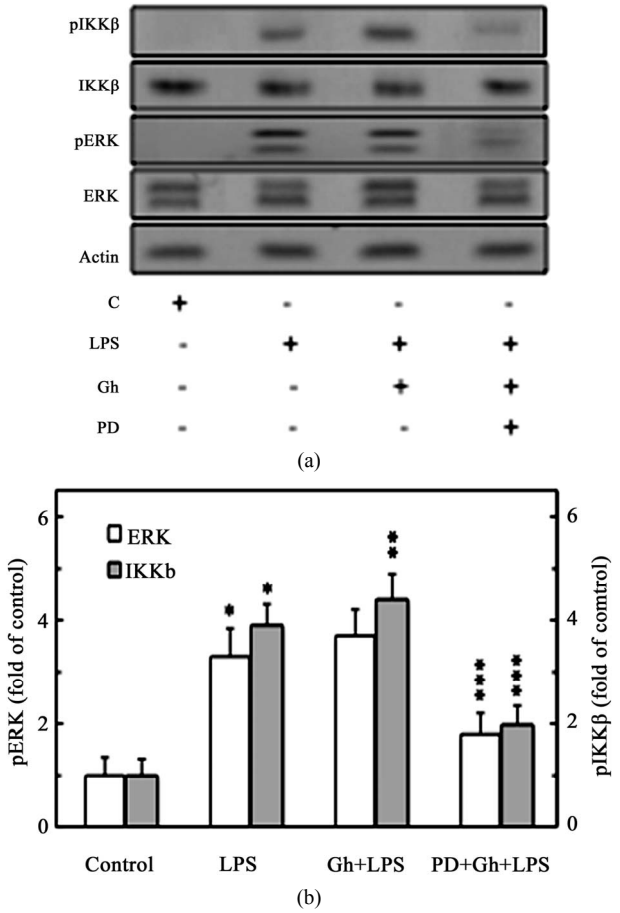

Figure 3. Effect of ghrelin (Gh) on *H. pylori* LPS-induced changes in gastric mucosal cell ERK and IKK-β phosphorylation. The cells, preincubated with 0 or 0.5 μg/ml ghrelin, or 30 μM PD98059 (PD) + Gh, were incubated for 30 min with the LPS at 100 ng/ml. Cell lysates were analyzed by western blotting for total and phosphorylated ERK and IKK-β with respective specific antibodies (a); and the relative densities of phosphorylated (pERK and pIKKβ proteins (b) are expressed as fold of control. Actin blot shows equal lane load. The data represent the mean ± SD of four experiments. ($^{*}P$ < 0.05 compared with that of control. $^{**}P$ < 0.05 compared with that of LPS. $^{***}P$ < 0.05 compared with that of Gh + LPS.)

Hence, in further assessment of the role of ERK activation in modulation of COX-2 and iNOS expression by ghrelin, we analyzed the changes in enzymatic activity of IKK-β in gastric mucosal cells exposed to *H. pylori* LPS. The results revealed that the LPS-induced up-regulation in IKK-β activity was subject to suppression by ghrelin as well as the inhibitors of ERK activation, PD98059 and Raf-1kinase inhibitor, and the effects were additive (**Figure 5**). Moreover, the effect of ghrelin on the LPS-induced up-regulation in IKK-β activity was susceptible to reversal by the inhibitors of Src/Akt pathway, PP2 and SH-5. The Src/Akt inhibitors, however, produced no discernible effect on the extent of the LPS-induced IKK-β

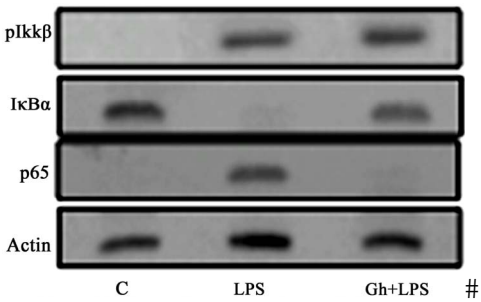

Figure 4. Effect of ghrelin (Gh) on *H. pylori* LPS-induced IKK-β phosphorylation, IκB-α degradation, and p65 NF-κB nuclear translocation in gastric mucosal cells. The cells, preincubated with 0 or 0.5 μg/ml Gh, were incubated for 30 min with the LPS at 100 ng/ml. Cell lysates were analyzed by western blotting for IκB-α and pIKKβ while the level of p65 NF-κB was assessed in the nuclear fraction. Actin blot shows equal line load. The immunoblots shown are representative of three experiments. #

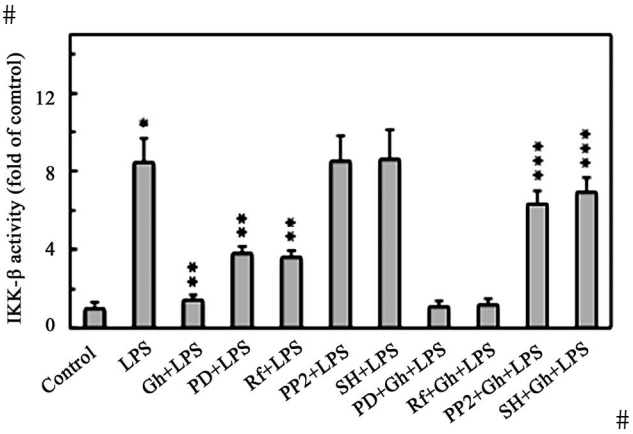

Figure 5. Effect of Src, Akt, Raf-1 kinase, and ERK inhibitors on the ghrelin (Gh)-induced changes in the expression of IKK-β activity in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 30 μM PP2, 20 μM SH-5 (SH), 30 μM PD98059 (PD), or 10 μM Raf-1 kinase inhibitor (Rf), were treated with Gh at 0.5 μg/ml and incubated for 30 min in the presence of 100 ng/ml of LPS. Values represent the mean ± SD of five experiments. ($^{*}P$ < 0.05 compared with that of control. $^{**}P$ < 0.05 compared with that of LPS. $^{***}P$ < 0.05 compared with that of Gh + LPS.)#

activity. This indicates that the countering effect of ghrelin on *H. pylori* LPS-elicited up-regulation in gastric mucosal IKK-β activation, and the suppression of proinflammatory COX-2 and iNOS enzymes induction, occurs with the involvement of pathways regulated by ERK and Src/Akt activation.

As the proinflammatory consequence of COX-2 induction by *H. pylori* is the massive up-regulation in gastric mucosal prostaglandin production, we have turned our attention to the processes associated with the release of AA from membrane phospholipids by the action of $cPLA_2$ [21]. Following on the documented involvement of ERK in the processes of $cPLA_2$ activation, we analyzed the effect of *H. pylori* LPS and ghrelin on gastric mucosal $cPLA_2$ phosphorylation. The results revealed that the LPS-induced ERK phosphorylation was associated with a marked increase in $cPLA_2$ phosphorylation, and that the phosphorylation of both enzymes, and $cPLA_2$ in particular, was further enhanced in the presence of ghrelin (**Figure 6**).

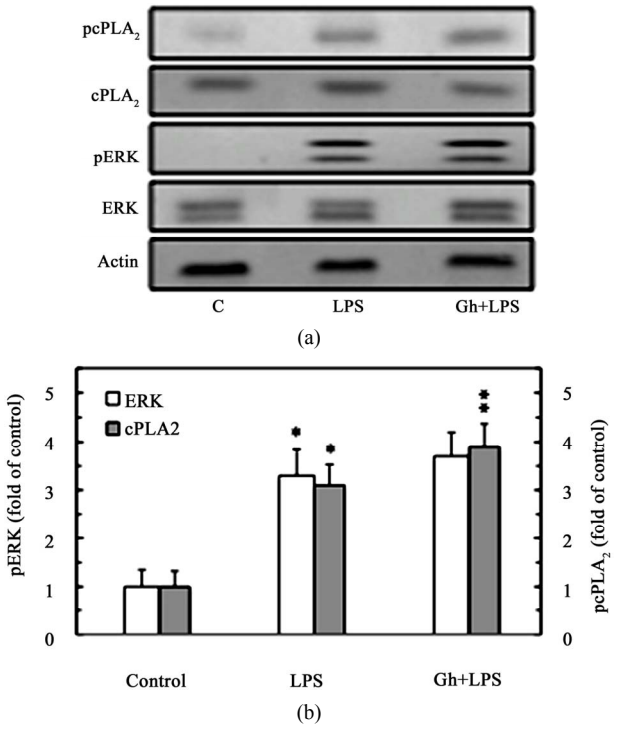

Figure 6. Effect of ghrelin (Gh) on *H. pylori* LPS-induced $cPLA_2$ and ERK phosphorylation in gastric mucosal cells. The cells, preincubated with 0 or 0.5 μg/ml ghrelin, were incubated for 30 min with the LPS at 100 ng/ml, and the lysates were analyzed by western blotting for total and phosphorylated $cPLA_2$ and ERK and with respective specific antibodies (a); and the relative densities of phosphorylated ($pcPLA_2$ and pERK proteins (b) are expressed as fold of control. Actin blot shows equal lane load. The data represent the mean ± SD of four experiments. ($^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS.)

Moreover, by measuring the mucosal cell $cPLA_2$ enzymatic activity, we found that the LPS-induced increase in the enzyme protein phosphorylation was reflected in up-regulation in $cPLA_2$ activity, and that this effect of the LPS was susceptible to suppression by ghrelin as well as the inhibitors of ERK, PD98059 and Raf-1 inhibitor (**Figure 7**). The countering effect of ghrelin on the LPS-induced up-regulation in $cPLA_2$ activity, furthermore, was subject to reversal by the inhibitors of Src/Akt pathway, PP2 and SH-5, but neither inhibitor produced any discernible alteration in the LPS-induced $cPLA_2$ activity. These results thus point to the role of ERK and Src/Akt pathways in the modulation of $cPLA_2$ activation by ghrelin in response to *H. pylori* LPS.

In further assessment of factors that influence the modulatory action of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal NO and PGE_2 production, we investigated the effect of nitrosothiols reducing agent, ascorbate, on the activity of IKK-β and $cPLA_2$ enzymes. The results revealed that, while preincubation with ascorbate produced no discernible result on the extent of the LPS-induced activation, the agent elicited a marked relieve in the inhibitory effect ghrelin on the LPS-induced IKK-β and $cPLA_2$ activity (**Figure 8**). However, ascorbate produced only negligible changes in the effect ERK inhibitor, PD98059, on the LPS-induced activity of both enzymes. Therefore, to ascertain further the relationship between the processes of IKK-β and $cPLA_2$ activation by *H. pylori* LPS, and the modulatory influence of ghrelin, we examined the patterns of IKK-β and $cPLA_2$ S-nitrosylation in conjunction with the phosphorylation requirements. We observed that gastric mucosal cells exposed to *H. pylori* LPS showed a substantial

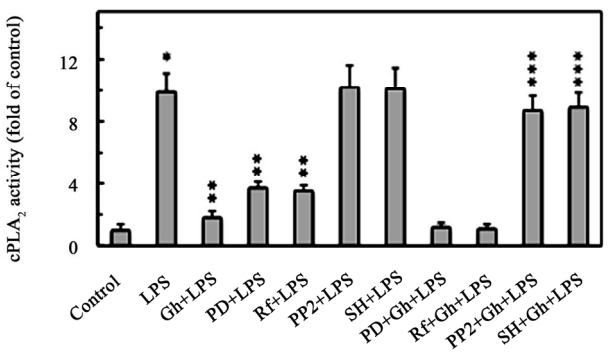

Figure 7. Effect of Src, Akt, Raf-1 kinase, and ERK inhibitors on the ghrelin (Gh)-induced changes in the expression of $cPLA_2$ activity in gastric mucosal cells exposed to *H. pylori* LPS. The cells, preincubated with 30 μM PP2, 20 μM SH-5 (SH), 30 μM PD98059 (PD), or 10 μM Raf-1 kinase inhibitor (Rf), were treated with Gh at 0.5 μg/ml and incubated for 30 min in the presence of 100 ng/ml of LPS. Values represent the mean ± SD of five experiments. ($^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS. $^{***}P < 0.05$ compared with that of Gh + LPS.)

increase in IKK-β and $cPLA_2$ phosphorylation, while the countering effect of ghrelin on the LPS-induced activation of the enzymes was manifested in a marked increase in S-nitrosylation of the both enzymes (**Figure 9**). Furthermore, preincubation with Akt inhibitor, SH-5, resulted in the blockage of the ghrelin-induced S-nitrosylation of IKK-β and $cPLA_2$, but it had no effect on phosphorylation of the proteins, whereas ERK inhibitor, PD98059, caused the blockage in IKK-β and $cPLA_2$ phosphorylation as well as S-nitrosylation. Thus, ghrelin affects the phosphorylation as well as S-nitrosylation of IKK-β and $cPLA_2$, and that in both cases the phosphorylation event is a prerequisite for S-nitrosylation. Further, the data suggest that *H. pylori* LPS-induced ERK activation plays a critical role in up-regulation of gastric mucosal nitric oxide and prostaglandin production at the level of IKK-β and $cPLA_2$ activation, and that ghrelin counters these untoward consequences of the LPS through Src/Akt-dependent S-nitrosylation.

4. Discussion

Colonization of gastric mucosa by *H. pylori* or stimulation of gastric mucosal cells with *H. pylori* LPS triggers the release of excessive amounts of NO and PGE_2, the overproduction of which is not only detrimental to bacterial survival but also cause tissue injury, and hence increase the risk of gastric disease [1,2,4-6]. The induction of iNOS and COX-2 genes responsible for rapid up-regulation in NO and PGE_2 generation is the result of stimulation by the LPS of gastric mucosal TLR-4 and the activation of cognate transcription factors as well as each of the three MAPK subtypes; JNK, p38 and ERK [2,17]. While the LPS-elicited induction in iNOS expression

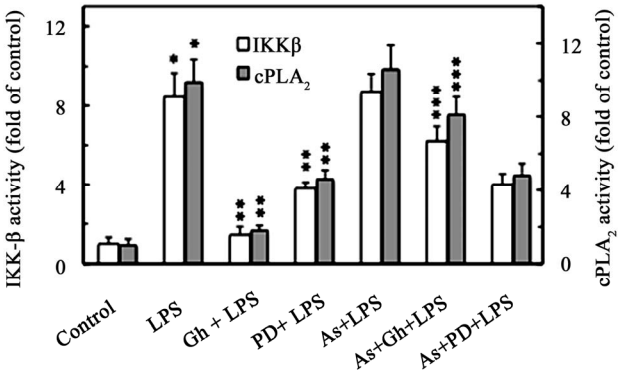

Figure 8. Effect of ascorbate on the LPS and ghrelin (Gh)-induced changes in the expression of IKK-β and $cPLA_2$ activities in gastric mucosal cells. The cells, preincubated with 300 μM ascorbate (As) or 30 μM PD98059 (PD), were treated with 0.5 μg/ml Gh and incubated for 30 min in the presence of 100 ng/ml LPS. Values represent the mean ± SD of five experiments. ($^{*}P < 0.05$ compared with that of control. $^{**}P < 0.05$ compared with that of LPS. $^{***}P < 0.05$ compared with that of Gh + LPS.)

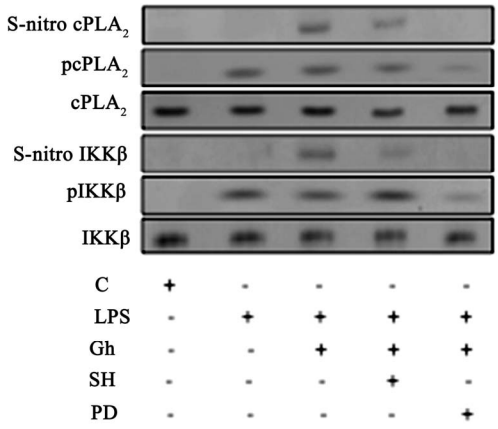

Figure 9. Effect of *H. pylori* LPS and ghrelin (Gh) on $cPLA_2$ and IKK-β protein S-nitrosylation in gastric mucosal cells. The cells, preincubated for 30 min with Gh at 0.5 μg/ml, or 30 PD98050 (PD) + Gh, or 20 μM SH-5 (SH) + Gh, and incubated for 1 h in the presence of 100 ng/ml LPS. A portion of the cell lysates was processed by biotin switch procedure for protein S-nitrosylation and, along with the reminder of the lysates, analyzed by western blotting for total and phosphorylated $cPLA_2$ and IKK-β proteins with respective specific antibodies. The immunoblots shown are representative of three experiments.

appears to be regulated by transcriptional factor, NF-κB, and the MAPK/JNK and p38 activation is associated with the induction in COX-2 expression through transcription factor, AP-1 activation, the data on the functional significance of LPS-induced MAPK/ERK activation remain at variance [9-12,16,17]. Depending on the cell type, the MAPK/ERK activation has been implicated in the control of COX-2 expression through transcription factors, C/EBP and CREB activation, as well as the enhancement in NF-κB nuclear translocation associated with the induction in iNOS expression [14,15,18-20].

Accordingly, in the present study, we explored the involvement of MAPK/ERK in the regulation of *H. pylori* LPS-induced gastric mucosal expression of COX-2 and iNOS. Our data, obtained with rat gastric mucosal cells, revealed that ERK activation by the LPS was associated with a marked increase in the phosphorylation of IKK-β and $cPLA_2$, and reflected in the upsurge in NF-κB nuclear translocation, induction in COX-2 and iNOS expression, and up-regulation in $cPLA_2$ activity. Moreover, the LPS-induced up-regulation in IKK-β and $cPLA_2$ activity was susceptible to suppression by the inhibitors of ERK phosphorylation, PD98059 and Raf-1 inhibitor, thus supporting the role of ERK in the processes of $cPLA_2$ activation for the increase in AA release for COX-2-mediated prostaglandin synthesis, as well as the NF-κB-dependent induction of iNOS expression for the increase in NO generation. Indeed, the literature data indicate that $cPLA_2$ activation requires MAPK/ERK-mediated enzyme phosphorylation on the critical Ser^{505} residue that

plays a crucial role in Ca^{2+}-dependent translocation of $cPLA_2$ form cytosol to membrane to gain access to phospholipid substrates [21,36]. Likewise, the ability of ERK to exert a stimulatory effect on IKK/NF-κB activation through Raf-1/MEK/ERK signaling cascade has been implicated in the regulation of adenosine A1 receptor mediated activation of NF-κB in human lymphocytic cells [19].

Further, we found that, while the LPS-induced phosphorylation of ERK, IKK-β and $cPLA_2$ was enhanced by preincubation with gastric hormone, ghrelin, the modulatory effect of the hormone was reflected in the suppression of IKK-β and $cPLA_2$ activity, as well as that of iNOS and COX-2. The effect of ghrelin on iNOS was manifested in a marked decline of the enzyme protein expression, and associated with the inhibition of the LPS-induced IκB-α degradation and a decrease in NF-κB nuclear translocation, while the effect on COX-2 was primarily manifested in the suppression of the LPS-induced up-regulation in COX-2 activity. Interestingly, NO stimulation through iNOS induction has been linked to COX-2 activation through S-nitrosylation and the increase in PGE_2 production [9,26], and we have recently shown that ghrelin suppression of *H. pylori* LPS-induced COX-2 S-nitrosylation results in the inhibition of PGE_2 generation [5]. Moreover, following our earlier leads as to the involvement of cNOS in the mechanism of ghrelin action [6,11], we revealed that the countering effect of ghrelin on the LPS-induced up-regulation in the activity of IKK-$\beta$and $cLPA_2$ enzymes was susceptible to suppression by the inhibitors of cNOS activation through phosphorylation, an Akt inhibitor, SH-5 and Src inhibitor, PP2. The Src/Akt inhibitors, however, produced no discernible alteration in the LPS-induced activity of IKK-β and $cPLA_2$ enzymes. These data and the finding that the LPS-induced up-regulation in the activities of IKK-β and $cPLA_2$ was also susceptible to suppression by the inhibitors of ERK activation, PD98059 and Raf-1, provide strong indication as to the role of ERK and Src/Akt pathways in the mediation of modulatory influence of ghrelin on the processes associated with up-regulation in gastric mucosal PGE_2 and NO generation in response to *H. pylori.*

While as an upstream kinase, cSrc phosphorylates a wide variety of substrates, and the signaling through Src/Akt pathway is known to occupy a central stage in the receptor (GHS-R)-mediated responses to ghrelin stimulation [6,11,37-39], it is becoming increasingly apparent that the hormone is also capable of exerting its modulatory influence through the process of protein S-nitrosylation. Indeed, the induction IKK-β S-nitrosylation by ghrelin exerts the inhibitory effect on the extent of IκB-α degradation, causing suppression in NF-κB nuclear translocation and resulting in the repression of iNOS gene induction [11,31]. Moreover, ghrelin has been implicated in the modulation of S-nitrosylation-dependent activation COX-2 and $cPLA_2$ enzymes, thus affecting the processes of PGE_2 generation [11,27,29,30]. Therefore, in further assessment of the modulatory action of ghrelin on *H. pylori* LPS-induced up-regulation in gastric mucosal generation of PGE_2 and NO, we evaluated the effect of nitrosothiol reducing agent, ascorbate on the activity of IKK-β and $cPLA_2$. We found that while ascorbate elicited a marked relieve in the inhibitory effect of ghrelin on the LPS-induced IKK-β and $cPLA_2$ activation, the agent produced only negligible changes in the effect of ERK inhibitor, PD98059, on the LPS-induced activity of both enzymes. Hence, consistent with the above findings and considering the fact that the changes in activity of both enzymes in the presence of ghrelin was susceptible to suppression by the inhibitors of cNOS activation, we concluded that the countering effect of ghrelin on *H. pylori* LPS-induced activation of IKK-β and $cPLA_2$ are intimately linked to the events of cNOS-dependent S-nitrosylation of these enzymes.

Consequently, to ascertain further the relationship between the processes of IKK-β and $cPLA_2$ activation by the LPS, and the modulatory action of ghrelin, we examined the patterns of IKK-β and $cPLA_2$ S-nitrosylation in conjunction with the phosphorylation requirements in the presence of the inhibitors of cNOS and ERK. We observed that the mucosal cells exposed to the LPS showed a marked increase in IKK-β and $cPLA_2$ phosphorylation, while the countering effect of ghrelin was associated with the increased phosphorylation as well as S-nitrosylation of the enzymes. Furthermore, preincubation with the inhibitor of cNOS activation, SH-5, lead to the blockage in ghrelin-induced S-nitrosylation of IKK-β and $cPLA_2$, but had no effect on phosphorylation of the enzymes, whereas ERK inhibitor, PD98059, caused the blockage in IKK-β and $cPLA_2$ phosphorylation as well as S-nitrosylation. This indicated that the phosphorylation event is a prerequisite for the IKK-β and $cPLA_2$ protein S-nitrosylation. Together, these findings suggest that the activation MAPK/ERK by *H. pylori* LPS plays a pivotal role in up-regulation in gastric mucosal PGE_2 and NO generation at the level of IKK-β and $cPLA_2$ activation through phosphorylation, and that ghrelin counters these proinflammatory consequences of the LPS through Src/Akt-mediated and cNOS-dependent S-nitrosylation of the IKK-β and $cPLA_2$ proteins.

In conclusion, the data present in this report add further support to our assertion as to the critical role of MAPK/ERK/JNK and p38 signaling cascades in mediation of gastric mucosal inflammatory responses to *H. pylori* LPS While the LPS-elicited induction in COX-2 expression relays primarily on JNK/p38-dependent activation of transcription factor AP-1 [17], the ERK

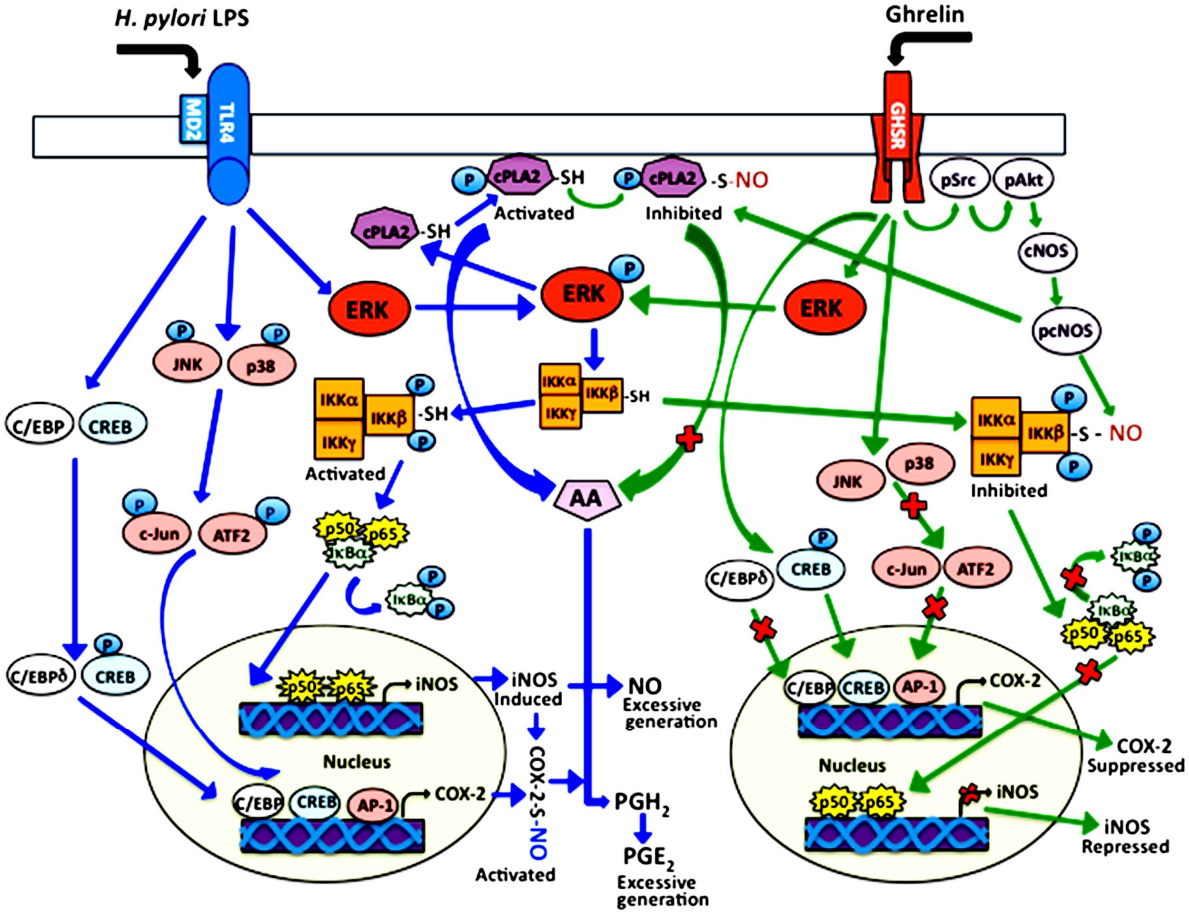

Figure 10. Schematic representation of the modulatory mechanism of ghrelin action in countering the excessive gastric mucosal NO and PGE_2 generation in response to *H. pylori* LPS. Binding of the LPS to Toll-like receptor 4 (TLR4)/MD2 triggers the activation of JNK, p38 and ERK1/2 MAPKs, and nuclear translocation of transcription factors involved in the induction of COX-2 (AP-1, CREB and C/EBP), and iNOS (NF-κB) genes transcription. While JNK and p38 MAPKs are involved in the regulation of AP-1 assembly [17], activation of ERK by the LPS leads to phosphorylation and activation of IKK-β and $cPLA_2$, which trigger up-regulation in arachidonic acid (AA) release and the upsurge in NF-κB nuclear translocation. The induction in iNOS and massive rise in NO leads to COX-2 activation through S-nitrosylation that results in the excessive PGE_2 generation. Engagement of the growth hormone secretagogue receptor (GHSR) by ghrelin leads to the inhibition of C/EBP and p38/JNK-mediated AP-1 activation, and hence results in the reduced COX-2 expression. Moreover, the effect of ghrelin is reflected in further enhancement in the LPS-induced ERK activation, and up-regulation in Src/Akt-dependent cNOS phosphorylation that leads to the inhibition of IKK-β and $cPLA_2$ activation by cNOS–mediated S-nitrosylation. This results in the repression of iNOS gene induction and the inhibition of COX-2 activation through iNOS-dependent S-nitrosylation, as well as the suppression of AA release. AP-1, activator protein-1; ATF-2, activating transcription factor-2; CREB, cAMP response element binding protein; C/EBP, CCAAT/enhancer binding protein; PGH_2, prostaglandin H_2.

activation is instrumental in promoting the signaling events leading to the activation of $cPLA_2$ and IKK-β thus triggering up-regulation in AA release for PGE_2 synthesis, and the increase in NF-κB nuclear translocation for the induction of iNOS gene. The induction in iNOS expression and concomitant rise in NO, in turn, leads to COX-2 activation through S-nitrosylation and the excessive PGE_2 generation (**Figure 10**). We also suggest that ghrelin exerts the modulatory influence over these proinflammatory events, precipitated in gastric mucosa by *H. pylori*-induced IKK-β and $cPLA_2$ activation, through Src/Akt-dependent S-nitrosylation.

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