

Cytosolic phospholipase A₂ S-nitrosylation in ghrelin protection against detrimental effect of ethanol cytotoxicity on gastric mucin synthesis

—Ghrelin in gastric mucosal protection

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ABSTRACT

Ghrelin, a peptide hormone produced mainly in the stomach, has emerged recently as an important regulator of nitric oxide synthase (NOS) and cyclooxygenase (COX) enzyme systems, the products of which play direct cytoprotective function in the maintenance of gastric mucosal integrity. In this study, using gastric mucosal cells, we report on the role of ghrelin in countering the cytotoxic effect of ethanol on mucin synthesis. We show that the countering effect of ghrelin on mucin synthesis was associated with the increase in NO and PGE₂ production, and characterized by a marked up-regulation in cytosolic phospholipase A₂ (cPLA₂) activity. The ghrelin-induced up-regulation in mucin synthesis, like that of cPLA₂ activity, was subject to suppression by Src inhibitor, PP2 and ERK inhibitor, PD98059, as well as ascorbate. Moreover, the loss in countering effect of ghrelin on the ethanol cytotoxicity and mucin synthesis was attained with cNOS inhibitor, L-NAME as well as COX-1 inhibitor SC-560. The effect of L-NAME was reflected in the inhibition of ghrelin-induced mucosal cell capacity for NO production, cPLA₂ S-nitrosylation and PGE₂ generation, whereas COX-1 inhibitor caused only the inhibition in PGE₂ generation. Our findings suggest that the activation of gastric mucosal cPLA₂ through cNOS-induced S-nitrosylation plays an essential role in the countering effect of ghrelin on the disturbances in gastric mucin synthesis caused by ethanol cytotoxicity.

Keywords: Ghrelin; Ethanol Cytotoxicity; Gastric Mucin; CNOS; cPLA₂; S-Nitrosylation

1. INTRODUCTION

Acute gastric mucosal lesions, mucosal inflammatory changes, and gastro-duodenal ulceration are well-recognized consequences of alcohol abuse on the health of gastrointestinal tract [1-3]. The gastric mucosal responses to ethanol cytotoxicity are manifested by microcirculatory changes, elevation in proinflammatory cytokine production, enhancement in epithelial cell apoptosis, and the impairment in prostaglandin and nitric oxide signaling pathways [2,4,5]. Furthermore, the disturbances in mucus producing gastric mucosal cells by ethanol affect the synthesis of mucins, the glycoproteins that maintain the integrity and strength of the protective mucus layer which constitutes the pre-epithelial element of gastric mucosal defense [6,7]. Hence, the alterations in the synthesis and processing of gastric mucins induced by ethanol are directly reflected in the impairment of mucus coat function that weakens the inherent resistance of the mucosa to injury, and facilitates the onset of gastric disease [3,6,7].

Recent advances in understanding the nature of factors involved in the maintenance gastric mucosal integrity revealed that the extent of mucosal protection against ethanol cytotoxicity is influenced by ghrelin, a 28-amino acid peptide hormone produced mainly in the stomach [4,8,9]. This endogenous ligand for the growth hormone secretagogue receptor, has been implicated in the control of local inflammations, healing experimentally induced gastric ulcers, and the protection of gastric mucosa against acute injury by ethanol [4,9,10]. Moreover, ghrelin emerged as an important regulator of the cross-talk between NOS and COX enzyme systems [9], the products of which, NO and PGE₂, play direct cytoprotective role in maintaining the gastric mucosal integrity under normal physiological conditions [11,12].

Indeed, the literature data support the existence of a functional relationship between the products of NOS and

COX systems, and there are strong indications that the enzyme compartmentalization and substrate availability determines the segregated utilization of the respective products in physiological and pathophysiological processes [13-15]. The stimulation of NO production through NOS induction or the exogenous NO donors leads to up-regulation in COX enzymes activation and the increase in prostaglandin generation [14-17], while the inhibition of NOS decreases prostaglandin formation. Moreover, it has been reported that the NO-induced enzyme protein S-nitrosylation impacts the events of cytosolic phospholipase (cPLA₂) activation, and hence influence the processes associated with arachidonic acid release for prostaglandin synthesis [18].

In our previous report, using rat gastric mucosal cells, we have shown that ghrelin protection against ethanol cytotoxicity involves cNOS-mediated cPLA₂ activation for the increase in PGE₂ production [19]. Here, we examined the influence of ghrelin on the disturbances in gastric mucin synthesis caused by ethanol.

2. MATERIALS AND METHODS

2.1. Cell Preparation and Mucin Synthesis

The gastric mucosal cells, collected from freshly dissected rat stomachs, were suspended in five volumes of ice-cold Dulbecco's modified (Gibco) Eagle's minimal essential medium (DMEM), supplemented with fungizone (50 µg/ml), penicillin (50 U/ml), streptomycin (50 µg/ml), and 10% fetal calf serum, and gently dispersed by trituration with a syringe, and settled by centrifugation [5]. Following rinsing, the cells were resuspended in the medium to a concentration of 2×10^7 cell/ml, transferred in 1 ml aliquots to DMEM in culture dishes containing [³H]glucosamine (110 µCi), used as a marker of mucin synthesis, and incubated under 95% O₂-5% CO₂ atmosphere at 37°C for 16 h [20]. After washing with DMEM containing 5% albumin to remove free radiolabel, the cells were resuspended in fresh DMEM and incubated for 2 h in the presence of 3% ethanol [5]. In the experiments evaluating the effect of ghrelin (rat, Sigma), cNOS inhibitor, L-NAME, iNOS inhibitor, 1400 W, Src inhibitor, PP2, ERK1/2 inhibitor, PD98059 (Calbiochem), COX-1 inhibitor, SC-560, COX-2 inhibitor, NS398, and ascorbate (Sigma), the cells were first preincubated for 30 min with the indicated dose of the agent or vehicle followed by incubation with ethanol. The viability of cell preparations before and during the experimentation, assessed by Trypan blue dye exclusion assay, was greater than 97%. At the end of the specified incubation period, the cells were centrifuged, washed with phosphate-buffered saline, and the combined supernatants used

for mucin assay.

2.2. Mucin Analysis and Ethanol Cytotoxicity Assay

The combined cell wash and incubation medium containing ³H-labeled mucin were treated at 4°C with 10 volumes of 2% phosphotungstic acid in 20% trichloroacetic acid for 4 h and the formed precipitates were collected by centrifugation. The glycoprotein precipitates were dissolved in 6 M urea and chromatographed on a Bio-Gel A-1.5 column, and the mucin fractions eluted in the excluded volume were subjected to analysis for incorporation of radiolabel and protein content [20]. For the measurement of ethanol-induced cytotoxicity, the aliquots of cell suspension from the control and various experimental conditions were centrifuged at $300 \times g$ for 5 min and the supernatants used for the assay of cytotoxicity using TOX-7 lactate dehydrogenase assay kit (Sigma).

2.3. PGE₂ and NO Quantification

The aliquots of cell suspension from the control and various experimental conditions were centrifuged at $1500 \times g$ for 5 min and the conditioned medium supernatant collected. PGE₂ assays were carried out using a PGE₂ EIA kit (Cayman) and 100 µl aliquots of the spent medium supernatant [5]. To assess nitric oxide production in rat gastric mucosal cells, we measured the stable NO metabolite, nitrite, accumulation in the culture medium using Griess reaction [21].

2.4. cPLA₂ Activity Assay

The measurement of cPLA₂ activity in the gastric mucosal cells following various experimental conditions was carried out using cPLA₂ assay kit (Cayman). The cells were homogenized in 1 ml of 50 mM Hepes buffer, pH 7.4, containing 1 mM EDTA, and centrifuged at $10,000 \times g$ for 15 min at 4°C. The supernatants were filtered through an Amicon YM30 filter concentrators, followed by 15 min incubation with 5 µM of calcium-independent PLA₂ inhibitor, bromoenol lactone, and the aliquots (10 µl) of such prepared cell lysates were subjected to cPLA₂ assay [19].

2.5. cPLA₂ S-Nitrosylation

A biotin switch procedure was employed to assess the cPLA₂ protein S-nitrosylation [22,23]. The gastric mucosal cells, treated with ghrelin (0.8 µg/ml) or L-NAME (300 µM)+ghrelin and incubated for 2 h in the presence of 3% ethanol, were lysed in 0.2 ml of HEN lysis buffer, pH 7.7, and the unnitrosylated thiol groups were blocked with S-methyl methanethiosulfonate reagent [23]. 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 μ l of elution buffer (20 mM HEPES, 100 mM NaCl, 1 mM EDTA, pH 7.7) containing 1% 2-mercaptoethanol [22]. The obtained proteins were then analyzed by Western blotting.

2.6. Western Blot Analysis

The mucosal cells, collected by centrifugation, were resuspended for 30 min in ice-cold lysis buffer [19], and following brief sonication the lysates were centrifuged at 12,000 g for 10 min and the supernatants subjected to protein determination using BCA protein assay kit (Pierce). The samples, including those subjected to biotin switch procedure, were then resuspended in loading buffer, boiled for 5 min, and subjected to SDS-PAGE using 50 μ g protein/lane. The separated proteins were transferred onto nitrocellulose membranes and probed with the antibody (Cell Signaling) against the cPLA₂, and after incubation with the horseradish peroxidase-conjugated secondary antibody, the protein bands were revealed using an enhanced chemiluminescence detection.

2.7. Data Analysis

All experiments were carried out using duplicate sampling, and the results are expressed as means \pm SD. Analysis of variance (ANOVA) was used to determine significance and the significance level was set at $P < 0.05$.

3. RESULTS

To assess the influence of ghrelin on the disturbances in gastric mucin synthesis caused by alcohol cytotoxicity, we employed rat gastric mucosal cells exposed to ethanol at the dose range (3%) that impairs the mucosal capacity for mucin synthesis and prostaglandin generation [1-3]. We determined that preincubation of the mucosal cells with ghrelin led to a concentration-dependent prevention of ethanol cytotoxicity, and afforded nearly complete protection at 0.8 μ g/ml of ghrelin (**Figure 1**). Further, our results revealed that cytotoxicity induced in gastric mucosal cells by 3% ethanol was reflected in a 32.6% decrease in mucin synthesis (**Figure 1**), as well as a 29.8% reduction in PGE₂ generation and a 51.7% drop in NO production (**Figure 2**). Ghrelin at its optimal concentration (0.8 μ g/ml) for the protection against ethanol

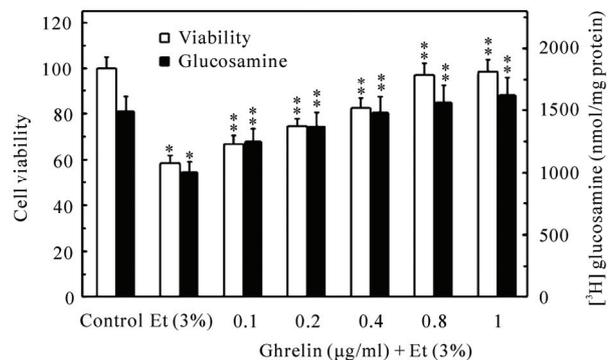


Figure 1. Effect of ghrelin on ethanol-induced cytotoxicity and the synthesis of mucin in rat gastric mucosal cells. The cells, labeled with [³H]glucosamine as a marker of mucin synthesis, were treated with the indicated concentrations of ghrelin and incubated for 2 h in the presence of 3% ethanol (Et). Values represent the means \pm SD of five experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of Et alone.

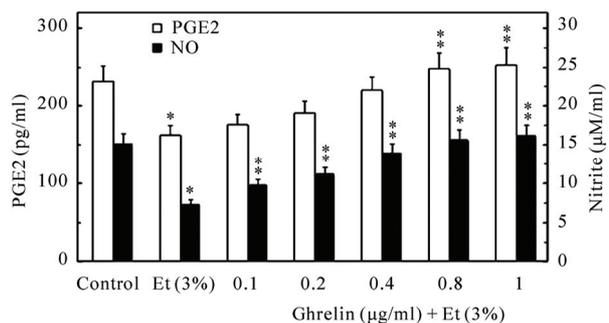


Figure 2. Effect of ghrelin on ethanol-induced changes in the production of PGE₂ and nitrite by gastric mucosal cells. The cells were treated with the indicated concentrations of ghrelin and incubated for 2 h in the presence of 3% ethanol (Et). Values represent the means \pm SD of five experiments. * $P < 0.05$ compared with that of control. ** $P < 0.05$ compared with that of Et alone.

cytotoxicity evoked a 55.4% increase in the mucosal cell capacity for mucin synthesis (**Figure 1**), while NO production increased 2.1-folds and PGE₂ generation by 53.1% (**Figure 2**).

Moreover, we found that significant loss in the preventive effect of ghrelin on the ethanol-induced toxicity and the cell capacity for mucin synthesis was attained with cNOS inhibitor, L-NAME as well as specific COX-1 inhibitor, SC-560, while selective iNOS inhibitor, 1400W and a specific COX-2 inhibitor, NS-398 had no effect (**Figure 3**). The effect of L-NAME, furthermore, was reflected in the inhibition of ghrelin-induced mucosal cell capacity for NO production as well as PGE₂ generation, whereas the pretreatment with COX-1 inhibitor,

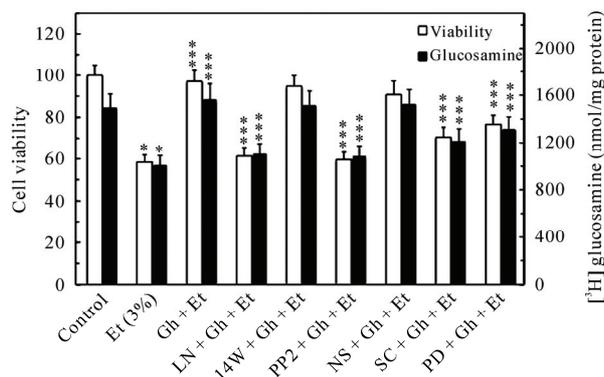


Figure 3. Effect of nitric oxide synthase and cyclooxygenase inhibitors on the ghrelin (Gh)-induced protection against ethanol (Et) cytotoxicity, and the synthesis of mucin in gastric mucosal cells. The cells, labeled with [³H] glucosamine, and preincubated with 300 μM L-NAME (LN), 20 μM 1400 W (14 W), 30 μM PP2, 30 μM NS-398 (NS), 20 μM SC-560 (SC) or 30 μM PD98059 (PD), were treated with Gh at 0.8 μg/ml and incubated for 2 h in the presence of 3% Et. Values represent the means ± SD of five experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of Et alone. ****P* < 0.05 compared with that of Gh + Et.

SC560, led only to the inhibition in ghrelin-induced PGE₂ generation (Figure 4). The stimulatory effect of ghrelin on the mucosal cell capacity for NO and PGE₂ production, however, was not affected by the inclusion of iNOS inhibitor 1400W and COX-2 inhibitor, NS-398 (Figure 4). Further, we found that the countering effect of ghrelin on the ethanol-induced changes in gastric mucosal cell capacity for mucin synthesis, and NO and PGE₂ production, were subject to suppression by Src kinase inhibitor, PP2, while the inhibitor of MAPK/ERK1/2, PD98059, caused the suppression in mucin synthesis and PGE₂ generation, but had no effect on NO production (Figures 3 and 4).

As the initial and rate limiting step in prostaglandin production is the liberation of arachidonic acid from membrane phospholipids by highly selective cPLA₂ [5,24], we next analyzed the effect of ghrelin on the mucosal cell cPLA₂ enzymatic activity. We found that ghrelin countering effect on the ethanol-induced cytotoxicity and the mucosal cell capacity for mucin synthesis was reflected in a 56.5% increase in cPLA₂ activity (Figure 5). Furthermore, the ghrelin-induced up-regulation in cPLA₂ activity, like that of mucin synthesis, was subject to suppression by Src inhibitor, PP2 and ERK1/2 inhibitor, PD98059, as well as the inhibitor of cNOS, L-NAME (Figure 5). The pretreatment with COX-1 inhibitor, SC-560, however, while not causing any discernible alteration in the cPLA₂ activity, exerted the inhibitory effect on mucin synthesis (Figure 5).

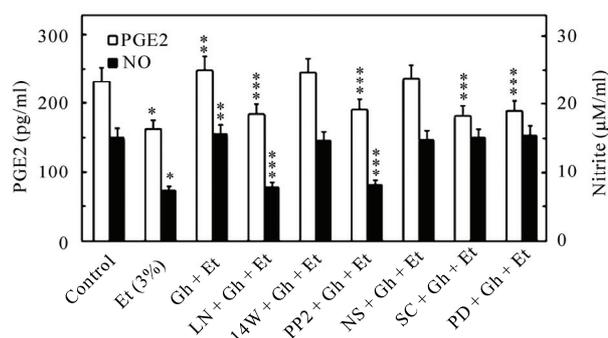


Figure 4. Effect of nitric oxide synthase and cyclooxygenase inhibitors on the ghrelin (Gh)-induced changes in the production of PGE₂ and nitrite by gastric mucosal cells in the presence of ethanol (Et). The cells, preincubated with 300 μM L-NAME (LN), 20 μM 1400 W (14 W), 30 μM PP2, 30 μM NS-398 (NS), 20 μM SC-560 (SC) or 30 μM PD98059 (PD), were treated with 0.8 μg/ml Gh and incubated for 2 h in the presence of 3% Et. Values represent the means ± SD of five experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of Et alone. ****P* < 0.05 compared with that of Gh + Et.

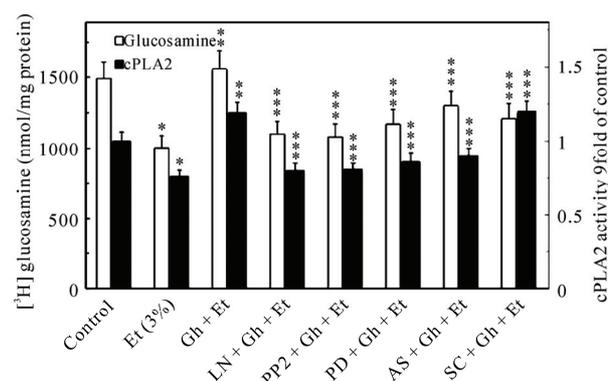


Figure 5. Effect of nitric oxide synthase and cyclooxygenase inhibitors on the ghrelin (Gh)-induced changes in mucin synthesis and cPLA₂ activity in gastric mucosal cells in the presence of ethanol (Et). The cells, labeled with [³H] glucosamine, and preincubated with 300 μM L-NAME (LN), 30 μM PP2, 30 μM PD98059 (PD), 300 μM ascorbate (As) or 20 μM SC-560 (SC), were treated with Gh at 0.8 μg/ml and incubated for 2 h in the presence of 3% Et. Values represent the means ± SD of five experiments. **P* < 0.05 compared with that of control. ***P* < 0.05 compared with that of Et alone. ****P* < 0.05 compared with that of Gh + Et.

Since in addition to phosphorylation, the activation of cPLA₂ involves the NO-dependent enzyme protein S-nitrosylation [18,19], we further analyzed the influence of S-nitrosylation on gastric mucosal cPLA₂ activity. The results revealed that ghrelin-induced up-regulation in cPLA₂ activity and the cell capacity for mucin synthesis was susceptible to suppression not only by the inhi-

bitor of cNOS, L-NAME, but also by ascorbate (**Figure 5**), which is in keeping with known susceptibility of S-nitrosylated proteins to this reducing agent [14,23]. Moreover, Western blot analysis of the mucosal cell lysates subjected to biotin switch procedure, with antibody against cPLA₂, revealed that ghrelin prevention of the ethanol-induced cytotoxicity was manifested in the increase in cPLA₂ protein S-nitrosylation, whereas preincubation with L-NAME blocked the ghrelin-induced cPLA₂ nitrosylation (**Figure 6**).

4. DISCUSSION

Acute gastric mucosal injury and gastro-duodenal ulcerations are the most common consequences of alcohol abuse on the health of gastrointestinal tract [1-3]. Moreover, the disturbances in gastric mucosal cells caused by ethanol cytotoxicity affect the synthesis of mucins, the glycoproteins that maintain the integrity and strength of the protective mucus layer, and hence weaken the inherent resistance of the mucosa to injury and facilitate the onset of gastric disease [6,7]. Therefore, considering the recent evidence for the involvement of ghrelin in gastric mucosal protection against injury by ethanol [4,9], in the study presented herein we examined the influence of this 28-amino acid peptide hormone on the synthesis of gastric mucin.

Using rat gastric mucosal cells exposed to ethanol at the concentration range that impairs mucosal cell capacity for mucin synthesis and prostaglandin generation [1-3], we demonstrated that the protective effect of ghrelin against ethanol cytotoxicity was associated with up-regulation in mucin synthesis, and accompanied by the increase in NO and PGE2 production, and the enhance-

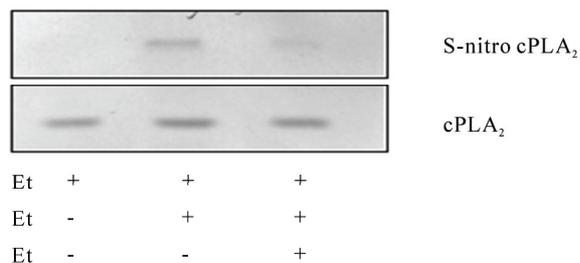


Figure 6. Effect of cNOS inhibitor, L-NAME (LN) on ghrelin (Gh)-induced cPLA₂ S-nitrosylation in gastric mucosal cells exposed to ethanol (Et). The cells were treated with Gh or L-NAME + Gh and incubated for 2 h in the presence of Et. A portion of the cell lysate was processed by biotin switch procedure for protein S-nitrosylation and, along with the remainder of the lysates, subjected to SDS-PAGE, transferred to nitrocellulose and probed with anti-cPLA₂ antibody. The immunoblots shown are representative of three experiments.

ment in cPLA₂ activity. A significant loss in the countering effect of ghrelin on the ethanol-induced cytotoxicity and the cell capacity for mucin synthesis was attained with cNOS inhibitor, L-NAME as well as specific COX-1 inhibitor, SC-560, whereas COX-2 inhibitor, NS-398, and iNOS inhibitor, 1400W, had no effect. These findings are thus in keeping with the results of earlier studies suggesting that the detrimental effect of ethanol on gastric mucin synthesis are associated with the impairment in NO and PGE2 generation [1-3,6,25], and lend further credence as to the role of ghrelin in regulation of the cross-talk between NOS and COX enzyme systems [9].

Further, we found that the countering effect of ghrelin on the ethanol-induced changes in mucin synthesis, and NO and PGE2 production, were subject to suppression by Src kinase inhibitor, PP2, whereas the inhibitor of MAPK/ERK, PD98059, elicited suppression in mucin synthesis and PGE2 generation, but had no effect on NO production. Thus the activation of Src appears to be a triggering event whereby ghrelin is capable of affecting the mucosal cell capacity for mucin synthesis as well as NO and PGE2 generation. Moreover, our data on the inhibition of ghrelin-induced mucosal cell capacity for mucin synthesis, and NO and PGE2 generation by L-NAME, and only that of PGE2 and mucin synthesis by COX-1 inhibitor, SC-560, suggest that ghrelin-induced up-regulation in mucin synthesis and PGE2 generation occurs with the involvement of cNOS-derived NO, and requires COX-1 participation. Indeed, the role of NO in modulation of COX enzymes activity in a variety of different systems is well documented [15-17].

As the initial and rate limiting event in prostaglandin production is the release of AA from membrane phospholipids by highly selective cPLA₂ [5,24], we further assessed the influence of ghrelin on the processes of cPLA₂ activation. We found that ghrelin elicited up-regulation in the mucosal cell cPLA₂ activation, which like that of mucin synthesis was subject to suppression by Src inhibitor, PP2, and ERK1/2 inhibitor, PD98059, as well as the inhibitor of cNOS, L-NAME. However, COX-1 inhibitor, SC-560, while not causing discernible alteration in the cPLA₂ activity, exerted the inhibitory effect on mucin synthesis. Hence, we concluded that the activation of gastric mucosal cPLA₂ by ghrelin for the increase in mucin synthesis to counter ethanol cytotoxicity occurs with the involvement of cNOS and requires Src kinase-dependent MAPK/ERK participation. Indeed, the literature data indicate that MAPK/ERK-dependent cPLA₂ phosphorylation on the critical Ser⁵⁰⁵ residue plays a crucial role in Ca²⁺-dependent translocation of cPLA₂ from cytosol to membrane to gain access to AA-rich phospholipid substrates [5,26].

Whilst regulation of the key enzymes for prostaglandin

production through phosphorylation is well recognized, recent evidence indicates that the increase in prostaglandin formation may also result from NO-induced enzyme protein S-nitrosylation [14,17-19]. Indeed, the post-translational modification through S-nitrosylation at the critical Cys⁵²⁶ residue has been linked to the NO-induced enhancement in catalytic activity of COX-2 [14], and cPLA₂ activation through S-nitrosylation at Cys¹⁵² was reported to be responsible for up-regulation in arachidonic acid release in human epithelial cells [18]. Therefore, to assess the role of ghrelin in cPLA₂ activation, we further analyzed the influence of S-nitrosylation on gastric mucosal cPLA₂ activity and the capacity for mucin synthesis. We found that ghrelin-induced up-regulation in cPLA₂ activity and the cell capacity for mucin synthesis was susceptible to suppression not only by the inhibitor of cNOS, L-NAME, but also by ascorbic acid, which is keeping with well known susceptibility of S-nitrosylated proteins to this reducing agent [14,23]. Moreover, Western blot analysis of the mucosal cell lysates subjected to biotin switch procedure with antibody against cPLA₂ revealed that ghrelin prevention of the ethanol-induced cytotoxicity was manifested in the increased cPLA₂ protein S-nitrosylation, whereas preincubation with cNOS inhibitor, L-NAME resulted in the blockage of the ghrelin-induced cPLA₂ protein S-nitrosylation. Therefore, consistent with our results, the cPLA₂ activation by ghrelin through S-nitrosylation is of direct relevance to the mucosal capacity for mucin synthesis and PGE₂ generation, and hence the protection of gastric mucosal cells against ethanol cytotoxicity.

In summary, our study demonstrates that the activation of gastric mucosal cPLA₂ through S-nitrosylation plays an essential role in the countering effect of ghrelin on the ethanol-induced disturbances in gastric mucin synthesis.

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