Role of epidermal growth factor receptor transactivation in the amplification of Helicobacter pylori-elicited induction in gastric mucosal expression of cyclooxygenase-2 and inducible nitric oxide synthase

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Abstract

Introduction
In this study, we report on the role of epidermal growth factor receptor (EGFR) transactivation in H. pylori lipopolysaccharide (LPS)-elicited induction in gastric mucosal expression of COX-2 and iNOS.

Materials and methods
We aimed to demonstrate that the LPS-induced p38 activation along with MMPs are of critical significance to EGFR transactivation that leads to up-regulation in the activation of ERK signalling cascade, amplification in iNOS and COX-2 induction, and consequently to the excessive increase in gastric mucosal PGE2 and NO generation.

Results
EGFR transactivation results in the amplification of the LPS-induced ERK phosphorylation and up-regulation in ERK-mediated IKK-β activation for the enhanced induction of NFκB-dependent expression of iNOS. The rise in iNOS-dependent NO generation, in turn, leads to an up-regulation in COX-2 activation through S-nitrosylation and excessive PGE2 production.

Conclusion
Taken together, our data provide strong indications of the involvement of EGFR transactivation in the amplification of ERK signalling cascade associated with up-regulation in gastric mucosal induction of iNOS and COX-2, and excessive NO and PGE2 generation in response to H. pylori.

Introduction
Infection with Helicobacter pylori is recognized as a primary factor in the aetiology of gastric disease, and results in the excessive generation of prostaglandin (PGE2) and nitric oxide (NO) triggered by disturbances in cyclooxygenase (COX). Nitric oxide synthase (NOS) isoyme systems are considered to be of major importance in defining the extent of gastric mucosal inflammatory involvement1–6. Studies on the signalling events underlying the up-regulation of PGE2 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 NOS and COX-2 gene expression7–9. The engagement of TLR-4 by LPS, moreover, is known to elicit the activation 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 factor activation through phosphorylation10–15. Indeed, we have recently shown that stimulation of gastric mucosal cells with H. pylori LPS elicits the activation of JNK, p38 and ERK and have linked the involvement of JNK/p38 in the transcription factor AP-1 activation, as well as provided evidence for the role of ERK activation in the regulation of factors linked to the induction of COX-2 and inducible iNOS (iNOS) expression for the increase in PGE2 and NO generation9,14.

Moreover, literature indicates that LPS-induced activation of MAPK cascade is also associated with epidermal growth factor receptor (EGFR) transactivation15–17. EGFR is a transmembrane glycosylated protein with intrinsic tyrosine kinase activity that controls a wide variety of cell functions that are of significance to mucosal defence and repair. These include processes such as cellular proliferation, differentiation, migration and modulation of apoptosis18,19. Studies indicate that in addition to direct EGFR activation by its cognate EGF peptide ligand, transactivation of EGFR requires an extracellular matrix metalloproteinase (MMP)-mediated shedding of heparin-binding EGF-like growth factor (HB-EGF) and its subsequent binding to the receptor19,20. Furthermore, the crosstalk between TLR4 and EGFR signalling systems has been linked to rapid changes in the extent of mucosal inflammation with bacterial invasion21,22. In particular, there are strong indications that p38 MAPK activation plays an important role in MMP-2 and MMP-9 activation and that EGFR transactivation results in up-regulation in ERK MAPK pathway stimulation23–26.

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As gastric mucosal responses to *H. pylori* are reflected in MAPK activation\(^9,14\), in this study we investigated the relationship between *H. pylori* LPS-induced MAPK activation and the processes associated with EGFR transactivation. Our results demonstrate that the LPS-induced p38 activation along with MMPs are of critical significance to EGFR transactivation that leads to up-regulation in the activation of ERK signalling cascade, amplification in iNOS and COX-2 induction and consequently to the excessive increase in gastric mucosal PGE\(_2\) and NO generation.

### Materials and methods

The protocol of this study has been approved by the relevant ethical committee related to our institution in which it was performed. Animal care was in accordance with the institution guidelines.

### Cell incubation

The gastric mucosal cells, collected from dissected rats 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% foetal calf serum. After gentle trituration with a syringe, the dispersed cells were settled by centrifugation and resuspended in the medium at a concentration of 2 × 10\(^7\) cells/mL. 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 to 8 h in the presence of 0–100 ng/mL of *H. pylori*\(^5\). *H. Pylori* used for LPS preparation was cultured from clinical isolates obtained from ATCC No. 4350\(^5\). In the experiments evaluating the effect of EGF, EGFR kinase inhibitor, AG1478, and ascorbate (Sigma), p38 MAPK inhibitor, SB202190, ERK1/2 inhibitor, PD98059, Src inhibitor, PP2, Raf-1 kinase inhibitor, iNOS inhibitor, 1400W, and a broad-spectrum MMPInhibitor, GM6001 (Calbiochem), 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 experiment, assessed by Trypan blue dye exclusion assay\(^6\), was greater than 97%.

### iNOS activity assay

The activity of iNOS enzyme in gastric mucosal cells 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 NADPH, 5 µM tetrahydrobiop- terin and 50 mM Tris-HCl buffer, pH 7.4, in a final volume of 250 µL. Following the 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 flowthrough was quantified by scintillation counting\(^7\).

### COX-2 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\(^7\). 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 presence of COX-1 inhibition (SC-560), by monitoring the appearance of oxidized TMPD at 590 nm\(^14\).

### EGFR transactivation assay

Measurement of EGFR transactivation was conducted with PhosphoDetect Elisa kit (Calbiochem). The gastric mucosal cells from the control and various experimental conditions were washed twice with phosphate-buffered saline, treated with the receptor extraction buffer and centrifuged at 1500× g for 10 min at 4°C. The supernatant was then incubated at room temperature for 2 h with anti-EGFR antibody, followed by 30-minute incubation with protein A agarose and centrifugation\(^8\). After washing, the pellet was suspended in kinase reaction buffer, and 100 µL aliquots were used for EGFR phosphotyrosine assay following the manufacturer’s instructions.

### ikB kinase activity assay

To measure the inhibitory xBKinase-β (IKK-β) activity, we utilized the ELISA-based detection kit, K-LISA (Calbiochem). The GST-\(\beta\)-x50-amino acid peptide that includes the Ser\(^32\) and Ser\(^36\) of ikB-α phosphorylation sites was used as a substrate\(^27,29\). The gastric mucosal cell cytosolic extracts were incubated with a glutathione-coated 96-well plate with GST-tagged \(\beta\)-x and \(\alpha\)-x at room temperature for 30 min, and the phosphorylated GST-\(\beta\)-x substrate was detected using anti-phospho-\(\beta\)-x (Ser\(^32\)/Ser\(^36\)) as first antibody, followed by horseradish peroxidase-conjugated secondary antibody. Following washing, the retained complex was probed with 3,3',5,5'-tetramethylbenzidine reagent for spectrophotometric quantification.

### 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. 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 ERK1/2, phosphoryso-ERK1/2, p38, phosphoryso-p38, COX-2 and iNOS (Calbiochem), EGFR, phospho-EGFR (Tyr1068), IKK-β (EMD Millipore) and phospho-IKK-β (Cell Signalling). The anti-β-actin was from Sigma.

**Data analysis**

All experiments were carried out using duplicate sampling, and the results are expressed as means ± SD. Analysis of variance 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 \( P < 0.05 \).

**Results**

Recently, we reported that gastric mucosal inflammatory responses to *H. pylori* infection, characterized by the excessive NO and PGE2 generation, are primarily linked to the LPS-induced activation of MAPK. Indeed, as illustrated in Figure 1, incubation of rat gastric mucosal cells with *H. pylori* LPS leads to a significant induction in the level of iNOS and COX-2 proteins. Moreover, upon further examination, we found that the LPS-induced expression of iNOS protein exhibited susceptibility to inhibition by the ERK1/2 inhibitor, PD98059, while the expression of COX-2 protein was subject to suppression by the inhibitor of p38, SB202190. Further, following on recent literature evidence on the role of LPS in EGFR transactivation, we assessed the MAPK requirement for *H. pylori* LPS-induced EGFR transactivation. For this, we examined the effect of EGFR kinase inhibitor, AG1478, on the LPS-induced phosphorylation of ERK1/2 and p38. The results of assays revealed that pretreatment of gastric mucosal cells with AG1478 leads to a substantial suppression of the LPS-induced ERK1/2 phosphorylation, while the extent of p38 phosphorylation remained unaffected (Figure 2). This attests to the involvement of EGFR transactivation in the mediation of the LPS-induced ERK1/2 activation.

To gain further understanding of the mechanism of *H. pylori* LPS-induced EGFR transactivation, we next focused our attention on the pathways of EGFR activation. As EGFR activation in response to LPS...
has been reported to occur with the involvement of nonreceptor tyrosine kinase, Src as well as MMP-mediated release of EGFR ligand, we employed Src kinase-specific inhibitor, PP2, and a broad-spectrum MMP inhibitor, GM6001, in the assessment of the LPS-induced gastric mucosal EGFR phosphorylation. The results of Western blot analysis revealed that while Src inhibitor, PP2, had no effect on the extent of H. pylori LPS-induced EGFR phosphorylation, a significant decrease in the EGFR phosphorylation was attained in the presence of MMP inhibitor, GM6001 (Figure 3). Moreover, the LPS-induced EGFR phosphorylation was susceptible to inhibition by p38 MAPK inhibitor, SB202190. However, neither the MMP nor p38 inhibitor affected the EGFR phosphorylation induced by EGF. Furthermore, similar requirements for H. pylori LPS-induced gastric mucosal EGFR activation were also observed using EGF protein tyrosine kinase activity assay (Figure 4). As shown in Figure 4, the LPS-induced EGFR activity was not affected by the inhibitors of Src and ERK1/2 kinases, but showed susceptibility to the inhibitors of p38 and MMP, the effect of which was additive. Collectively, these results indicate that EGFR transactivation by H. pylori LPS occurs with the involvement of p38 MAPK and MMP and do not appear to require Src participation.

As gastric mucosal responses to H. pylori LPS associated with the activation of MAPK ERK through phosphorylation have been linked to the processes of IKK-β activation for the induction in NO and PGE2 production, we next examined the role of EGFR transactivation in the LPS-induced ERK and IKK-β activation. Western blot analysis of the LPS-induced ERK1/2 phosphorylation in the presence of EGFR inhibitor, AG1478, revealed a partial decrease in ERK1/2 phosphorylation, while the specific inhibitors of ERK1/2, PD98059 and RAF kinase, Raf-1 inhibitor, evoked nearly complete suppression in the ER1/2 phosphorylation (Figure 5). Further, we found that the inhibitors of ERK1/2 and RAF kinase, PD9809 and Raf-1, exerted profound inhibitory effect on the LPS-induced activity of IKK-β, a key enzyme of NF-κB activation pathway (Figure 6). A significant decrease in the LPS-induced up-regulation in gastric mucosal cell IKK-β activity was also attained in the presence of EGFR inhibitor, AG1478, p38 MAPK inhibitor, SB202190, and MMP inhibitor, GM6001, while the inhibitor of Src, PP2, neither affected the LPS-induced ERK1/2 phosphorylation (Figure 5) nor the activity of IKK-β (Figure 6). These findings thus clearly point to the role of H. pylori LPS-induced EGFR transactivation in the amplification of ERK-mediated up-regulation in gastric mucosal IKK-β activity and subsequent induction of proinflammatory iNOS and COX-2 enzymes.

Consequently, to provide additional support as to the role of EGFR transactivation in H. pylori LPS-induced up-regulation in NO and PGE2 generation, we assessed the activity of iNOS and COX-2 enzymes in the presence of inhibitors of EGFR kinase, ERK and p38 MAPK. The results revealed that the LPS-elicited induction in the activity of iNOS and COX-2 enzymes was susceptible to suppression by EGFR inhibitor, AG1478, as well as by the inhibitor of ERK1/2, PD98059 (Figure 7). The effect of p38 MAPK inhibitor, SB202190, was reflected mainly in the suppression COX-2 activity, whereas the inhibitor of Src, PP2, had no effect on either of the two enzymes. Moreover, preincubation with nitrilotriol reducing nitric oxide synthase. OA Inflammation 2013 Apr 01;1(1):1.

**Figure 3:** Effect of Src, p38 and MMP inhibitors on H. pylori LPS-induced EGFR transactivation in gastric mucosal cells. The cells, preincubated for 30 min with 30 µM PP2, 30 µM SB202190 (SB) or 100 µM GM6001 (GM), were treated with 100 ng/mL of LPS or 10 ng/mL EGF and incubated for 10 min. Cell lysates were analysed by Western blotting for total and phosphorylated EGFR (a) and the relative density of pEGFR protein (b) is expressed as a fold of control. Total EGFR was used as a loading control. The data represent the means ± SD of four experiments. *P < 0.05 compared with that of control. **P < 0.05 compared with that of LPS.

**Figure 4:** Effect of MAPK, Src and MMP inhibitors on H. pylori LPS-induced gastric mucosal EGFR protein tyrosine kinase activity. The gastric mucosal cells, preincubated for 30 min with 30 µM PP2, 30 µM PD98059 (PD), 20 µM SB202190 (SB) or 100 µM GM6001 (GM), were incubated for 15 min with LPS at 100 ng/mL. Values represent the means ± SD of four experiments. *P < 0.05 compared with that of control. **P < 0.05 compared with that of LPS.

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**Discussion**

Invasion of gastric mucosa by *H. pylori* or stimulation of gastric mucosal cells with the bacterium LPS elicits a pattern of inflammatory responses characterized by the activation of MAPK cascade, induction in iNOS and COX-2 gene expression and the excessive generation of NO and PGE$_2$.

The data on the signalling events underlying the up-regulation in NO and PGE$_2$ generation by LPS, moreover, point to the role of MAPK activation in the control of cognate transcription factors that exert control over iNOS and COX-2 gene expression. Along these lines, we have recently provided evidence for the involvement of *H. pylori* LPS-elicited activation of p38 and ERK MAPK in the regulation of factors linked to the induction of gastric mucosal NO and PGE$_2$ production. As recent literature evidence indicates that LPS-induced activation of MAPK cascade is also associated with EGFR transactivation, in the present study we investigated the role of EGFR transactivation in *H. pylori* LPS-stimulated induction in gastric mucosal iNOS and COX-2 expression.

Our data demonstrate that EGFR transactivation in gastric mucosal cells exposed to stimulation with *H. pylori* LPS is dependent on p38 MAPK and MMP activation and does not involve nonreceptor tyrosine kinase, Src, participation. We further show that the LPS-induced EGFR transactivation results in the amplification of ERK activity, up-regulation in ERK-mediated IKK-β activation and subsequent induction in the expression of iNOS and COX-2 enzymes. Indeed, we found that the LPS-induced EGFR phosphorylation as well as tyrosine kinase activity showed susceptibility to the inhibitors of p38 MAPK and MMP, SB202190 and GM6001, but not that of Src inhibitor, PP2. Furthermore, neither MMP nor p38 inhibitor affected the phosphorylation of EGFR induced by EGF, whereas blocking EGFR kinase activity with...
AG1478 leads to a substantial suppression in the phosphorylation of ERK1/2, but had no effect on the extent of the LPS-induced phosphorylation of p38 MAPK. The fact that broad-spectrum MMP inhibitor, GM6001, affected the LPS-induced EGFR phosphorylation suggests the involvement of MMPs in the cleavage and release of EGFR ligand. While the nature of these MMPs was not investigated, the demonstrated dependence of EGFR transactivation on the LPS-induced p38 MAPK activation points to MMP-2 or MMP-9, as the literature data indicate that p38 MAPK plays an important role in the activation of both these MMPs.

Considering the demonstrated role of LPS-induced activation of Ras/Raf/MEK/MAPK pathway in the regulation of factors coupled to the induction of iNOS and COX-2 expression, and the evidence as to the involvement of EGFR transactivation in ERK MAPK activation, we further addressed the consequences of H. pylori LPS-induced EGFR transactivation on the gastric mucosal ERK1/2 phosphorylation. A partial, but significant, reduction in the LPS-induced ERK1/2 phosphorylation was attained with EGFR kinase inhibitor, AG1478, while RAF and ERK inhibitors, Raf-1 and PD98059, evoked nearly complete inhibition of the LPS-induced ERK1/2 phosphorylation. These data thus suggest that EGFR transactivation associated with H. pylori infection could play a substantial role in the amplification of the LPS-induced gastric mucosal consequences of MAPK signalling cascade activation, including that of excessive NO and PGE generation. Indeed, EGFR transactivation induced by LPS has been linked to COX-2 activation through S-nitrosylation and the increase in PGE production, and we have recently shown that suppression of H. pylori LPS-induced COX-2 S-nitrosylation by iNOS inhibition results in the inhibition of PGE2 generation. These data and the finding that the LPS-induced up-regulation in the activity of IKK-β was also susceptible to suppression by the inhibitors of ERK as well as EGFR transactivation, provide strong indication as to the role of EGFR transactivation in the amplification of ERK signalling cascade associated with up-regulation in gastric mucosal PGE and NO generation in response to H. pylori.

**Conclusion**

The data present in this report demonstrate the critical role of MAPK signalling cascade in the mediation of gastric mucosal inflammatory responses to H. pylori LPS that lead to an up-regulation in gastric mucosal NO and PGE generation. The LPS-induced p38 MAPK activation along with MMPs plays a major role in EGFR transactivation that leads to amplification in ERK phosphorylation, up-regulation in ERK-mediated IKK-β activation and increase in NF-xB nuclear translocation for the induction of iNOS expression and NO generation. The rise in iNOS-dependant NO, in turn, leads to COX-2 activation through S-nitrosylation and excessive PGE production (Figure 8). Thus, the extent of EGFR transactivation elicited by H. pylori LPS may be of primary importance in defining the degree of gastric mucosal inflammatory involvement that could also be of significance to the development of gastric carcinoma.

**Abbreviations list**

- COX: cyclooxygenase;
- DMEM: Dulbecco’s modified Eagle’s minimal essential medium;
- EGFR: epidermal growth factor receptor;
- ERK: extracellular signal-regulated kinase;
- HB-EGF: heparin-binding EGF-like growth factor receptor; ERK, extracellular signal-regulated kinase; HB-EGF, heparin-binding EGF-like growth factor receptor; NF-xB, nuclear factor-xB; PG, prostaglandin; TNF, tumor necrosis factor; LPS, lipopolysaccharide; iNOS, inducible nitric oxide synthase; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase.

**Competing interests** None declared.

**Conflict of interests** None declared.

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growth factor; IKK-β, inhibitory κB kinase-β; iNOS, inducible nitric oxide synthase; JNK, Jun N-terminal kinase; LPS, lipopolysaccharide; MAPK, mitogen-activated protein kinase; MMP, matrix metalloproteinase; NO, nitric oxide; NOS, nitric oxide synthase; PGE₂, prostaglandin; TLR, Toll-like receptor 4.

References


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