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Lipopolysaccharide Initiates a Positive Feedback of Epidermal Growth Factor Receptor Signaling by Prostaglandin E\textsubscript{2} in Human Biliary Carcinoma Cells\textsuperscript{1}

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Bacterial products (e.g., LPS) are viewed as critical stimuli in inflammation-associated cancer. Cyclooxygenase 2 (COX-2), a major effector of LPS, and EGFR, are key to carcinogenesis, notably in the hepatobiliary tract. In this study, we tested the hypothesis that LPS can initiate an interaction between the epidermal growth factor receptor (EGFR) and COX-2 pathways. We examined the effect of LPS in biliary carcinoma cells that displayed constitutive COX-2 expression and PGE\textsubscript{2} production and in normal human biliary epithelial cells in which COX-2/PGE\textsubscript{2} expression was virtually absent. LPS induced early phosphorylation of EGFR and ERK1/2 in both types of cells, which reached maximum levels within 30 min (first phase). However, only the carcinoma cells showed a second significant rise in both EGFR and ERK phosphorylation 6 h after exposure to LPS (second phase). Inhibition of COX-2/PGE\textsubscript{2} production prevented the second, but not the first, phase of EGFR and ERK1/2 phosphorylation, implicating COX-2/PGE\textsubscript{2} in the second phase of phosphorylation. LPS induced COX-2-derived PGE\textsubscript{2} production at 4 h, which was before the rise in the second phosphorylation that occurred at 6 h. Exogenous PGE\textsubscript{2} also caused EGFR activation via a signaling pathway involving TACE-dependent TGF-\textalpha release. Inhibition of the second phase of EGFR phosphorylation with EGFR or COX-2 inhibitor prevented LPS-induced cell invasion in vitro, demonstrating the biological importance of this COX-2 feedback signaling in cancer cells. We conclude that LPS triggers a positive feedback loop involving COX-2/PGE\textsubscript{2} in biliary carcinoma cells and that this second phase of EGFR phosphorylation is implicated in cell invasion by LPS. The Journal of Immunology, 2009, 182: 2269 –2276.
Materials and Methods

Materials

DMEM, DMEM/Ham’s F12, and Moloney murine leukemia virus reverse transcriptase were purchased from Life Technologies. Ultrasor G was from Bioeropa and human type IV collagen from Tebu. Protease type XIV from Streptomyces griseus, LPS, 1,3-dimethyl-2-thiourea, and n-propyl gallate were provided by Sigma-Aldrich. Tyrphostin AG 1478, PD 98059, TFN-α protease inhibitor (1 TAPI-1), GM 6001, EGFR monoclonal-neutralizing Ab (Ab-3), TGF-α monoclonal-neutralizing Ab (Ab-3), and COX-2 mAb were purchased from Calbiochem. Anti-EGF Ab was obtained from R&D Systems. Phospho-EGFR (Tyr992) and EGFR polyclonal Abs were purchased from Cayman Chemical.

Cell culture

The human biliary carcinoma cell line Mz-ChA-1, which is derived from a gallbladder adenocarcinoma (18), was provided by Dr. A. Knuth (Zurich University Hospital, Zurich, Switzerland). The human cholangiocarcinoma cell line KMBC was a gift from Dr. G. Gores (Mayo Clinic College of Medicine, Rochester, MN). Mz-ChA-1 and KMBC cells were cultured in DMEM supplemented with 10% FBS and 1% HEPES. Experiments were performed in confluent cells that had been serum-deprived for 24 h earlier. Primary cultures of human biliary epithelial cells derived from the gallbladder were obtained from subjects who underwent cholecystectomy during liver surgery. Epithelial cells were isolated by incubation of the gallbladder samples in 0.075%/w/v protease type XIV for 12 h at 4°C. Isolated cells were suspended in DMEM/Ham’s F12 supplemented with 5.35 g/L d-glucose, 14 mmol/L NaHCO₃, 2% Ultroser G, and 200,000 IU/200 mg/L penicillin G-streptomycin (pH 7.4) and were plated in culture dishes coated with human type IV collagen. The cells were incubated with 95% air and 5% CO2 at 37°C.

Expression of COX-2 was examined in cell cultures of the human epithelial cells and in biliary carcinoma cells. A, NHBE cells (NHBEC) or a biliary carcinoma cell line, Mz-ChA-1 cells, were grown to confluence. Cell lysates were collected and TGF-β2 production in each sample was related to total protein in cell lysate using the TGF-β2 ELISA kit (Calbiochem).

**FIGURE 1.** Expression of COX-2 and production of PGE2 in normal biliary epithelial cells and in biliary carcinoma cells. A, NHBE cells (NHBEC) or a biliary carcinoma cell line, Mz-ChA-1 cells, were grown to confluence. Cell lysates were collected to detect COX-2 protein expression by Western blot using anti-COX-2 Ab. β-Actin was used as an internal control, showing equal loading of protein samples. Results are representative of three different preparations. B, NHBE cells and Mz-ChA-1 cells were grown to confluence and were then serum (or serum substitute) starved for 24 h. Cell supernatants were collected to measure concentrations of PGE2 protein using PGE2 ELISA as described Materials and Methods. PGE2 protein was normalized to total protein in cell lysates. Values represent means ± SEM of three different experiments performed in duplicate. *, p < 0.05, compared with NHBE cells.

Immunoblotting and EGFR phosphorylation assay

After various treatments, cells were lysed on ice in PBS lysis buffer containing 25 mmol/L Tris-HCl, 300 mmol/L NaCl, 1 mmol/L CaCl₂, 1% Triton X-100 (pH 7.4), and protease inhibitor (Complete Mini; Roche). Lysates were precleared by centrifugation at 14,000 rpm for 20 min at 4°C. Protein concentration was determined by the bicinchoninic acid-based BCA Protein Assay kit (Pierce/Perbio Science France). Fifty micrograms of proteins was subjected to 7.5% SDS-PAGE electrophoresis and transferred to a polyvinylidene difluoride membrane (Bio-Rad), which was blocked with 5% BSA, probed with primary Abs, washed with PBS, and then probed with secondary Abs conjugated to HRP. Immunoreactive bands were visualized by ECL using an ECL kit (Amersham Biosciences). The detected bands were quantified with the National Institutes of Health image software.

EGFR phosphorylation in some studies was also analyzed using the PhosphoDetect EGFR phosphorylation ELISA kit (Calbiochem) according to the manufacturer’s instructions.

PGE2 production assay

Cells were incubated in serum-free medium (SFM) and then exposed or not to LPS (10 μg/ml) for various times. In inhibitory studies, the cells were preincubated with inhibitors for 30 min to 2 h before LPS was added. At the end of the experiments, cell supernatants were collected and the PGE2 concentration was determined by ELISA kit (Cayman Enzyme Immunoassay Kit) according to the manufacturer’s instructions. The amount of PGE2 production in each sample was related to total protein in cell lysate and was expressed as ng of PGE2 per mg of total cellular protein.

TGF-α shedding assay

The shedding (cleavage and release) of TGF-α was analyzed in human epithelial cells incubated with PGE2 (10 μM) or LPS (10 μg/ml) for 2 h. To prevent soluble TGF-α from binding to EGFR, an EGFR-neutralizing Ab (4 μg/ml) was added 2 h before PGE2 or LPS. In inhibitory studies, the cells were preincubated with inhibitors for 30 min to 2 h before PGE2 or LPS was added. At the end of the experiments, cell supernatants were collected and TGF-α was measured using the TGF-α ELISA kit (R&D Systems) according to the manufacturer’s instructions.

All statistical analyses were performed with the StatView 5.01 software (SAS Institute). The paired Student t test was used to analyze statistical differences among groups. In the time course analysis, comparison among groups was also performed using Newman-Keuls multiple range test. Differences of p < 0.05 were considered statistically significant.

Results

**Expression of COX-2 and production of PGE2 in biliary epithelial cells**

The expression of COX-2 was examined in cell cultures of the human biliary carcinoma cell line Mz-ChA-1 and compared with normal human biliary epithelial (NHBE) cells. Although COX-2 was not detected by Western blot in NHBE cells, strong expression was demonstrated in Mz-ChA-1 carcinoma cells (Fig. 1A). Whereas COX-2-derived PGE2 protein production was barely detectable in the supernatants of NHBE cells, a high level of PGE2 was produced by Mz-ChA-1 carcinoma cells (Fig. 1B). Thus, biliary cancer cells overexpress COX-2 and up-regulate PGE2 production.

**LPS triggers different temporal patterns of EGFR and ERK1/2 activation in biliary carcinoma vs normal epithelial cells**

LPS is known to stimulate both the EGFR and COX-2/PGE2 signaling pathways (15, 20). Because NHBE and Mz-ChA-1 cells display different expression patterns of COX-2/PGE2, we examined the effect of LPS on the temporal activation of an EGFR cascade: incubation of NHBE cells with LPS increased EGFR phosphorylation, which occurred as early as 15 min, was maximal at ~30 min, and decreased by 2 h (Fig. 2A). Because ERK1/2 plays important roles in multiple cellular functions and is one of the most common effectors downstream of EGFR, we also
examined ERK1/2 activation by LPS. ERK1/2 displayed a pattern of activation similar to EGFR, although after 30 min, ERK1/2 phosphorylation showed more variability before returning to baseline (Fig. 2B). However, LPS stimulation of Mz-ChA-1 carcinoma cells induced a biphasic temporal pattern of EGFR (Fig. 2C) and ERK1/2 (Fig. 2D) activation, with a rapid increase in phosphorylation within 30 min, followed by another peak at ~6 h. Western blot analyses with Abs that recognize total EGFR and ERK proteins showed that EGFR and ERK1/2 protein levels remained unchanged in both normal epithelial cells and carcinoma cells throughout the experiments. Next, we examined whether LPS induced the first phase of EGFR activation via binding to its putative receptor, TLR4, in the plasma membrane. We preincubated cancer cells with neutralizing Abs that block the ligand binding site on the extracellular domain of TLR4 and compared with TLR5 (used as a control). Preincubation of the cells with TLR4 Ab but not TLR5 Ab inhibited LPS-induced EGFR phosphorylation (Fig. 2E), confirming the role of TLR4 in LPS-induced responses. To investigate whether this LPS-induced biphasic phosphorylation of EGFR is limited to a specific cancer cell line (i.e., Mz-ChA-1), we examined two other cancer cell lines, biliary carcinoma KMBC cells and lung adenocarcinoma A549 cells, which were previously reported to express COX-2 (21, 22). Both cancer cell lines showed a similar pattern of biphasic EGFR phosphorylation in response to LPS stimulation (Fig. 2F; data not shown for A549 cells). Thus, these studies show that LPS induces different temporal patterns of EGFR and ERK1/2 activation in these cancer vs normal biliary epithelial cells.

The second phase of LPS-induced EGFR and ERK1/2 activation is dependent on COX-2 signaling in biliary carcinoma cells

LPS can induce EGFR activation in lung cancer cells via TNF-α-converting enzyme (TACE)-dependent release of the EGFR ligand TGF-α (15). However, the mechanism(s) of the second phase of EGFR phosphorylation in response to LPS stimulation (Fig. 2F; data not shown for A549 cells). Thus, these studies show that LPS induces different temporal patterns of EGFR and ERK1/2 activation in these cancer vs normal biliary epithelial cells.
activation in the carcinoma cells is caused by COX-2-derived PGE2 production induced by LPS. According to this hypothesis, LPS stimulates EGFR phosphorylation (first phase, within 30 min), leading to COX-2 up-regulation and increased production of PGE2, which then induces EGFR activation (second phase, at and after 6 h). To test this hypothesis, we examined the temporal production of PGE2 by LPS. In Mz-ChA-1 cells, LPS induced a significant increase in PGE2 production at 4 h (Fig. 3A), which preceded the second phase of EGFR activation. Then, we examined the pathway of PGE2-induced EGFR activation, because NHBE cells contain receptors for PGE2 but do not produce PGE2 constitutively. We examined the effect of exogenously delivered PGE2 on EGFR signaling: stimulation of NHBE cells with PGE2 induced rapid EGFR phosphorylation (Fig. 3B). These results indicate that NHBE cells activate EGFR. They also suggest that the second phase of EGFR activation induced by LPS in cancer cells could be due to PGE2 generation via COX-2. To test this hypothesis, we pretreated cancer cells with a selective COX-2 inhibitor (NS-398) that prevented PGE2 production in the cancer cells (Fig. 3C). As expected, NS-398 prevented the second, but not the first, phase of LPS-induced activation of EGFR (Fig. 3D) and ERK1/2 (Fig. 3E). From these results, we conclude that LPS induces the second phase of EGFR activation via COX-2-dependent PGE2 production in biliary cancer cells.

**LPS induces PGE2 production via an EGFR cascade in biliary carcinoma cells**

Having determined that PGE2 production is responsible for the LPS-induced second phase of EGFR and ERK1/2 activation, we examined the mechanism involved in LPS-induced PGE2 production. Previously, we have shown that an EGFR cascade is involved in LPS-induced MUC5AC mucin and IL-8 production in human airway epithelial cells (15, 23). Whether an EGFR cascade also mediates LPS-induced PGE2 production in biliary cancer cells is presently unknown. Pretreatment of the cells with a selective EGFR tyrrosine kinase inhibitor (AG1478) prevented LPS-induced PGE2 production (Fig. 4), implicating EGFR activation in LPS-induced PGE2 production. Preincubation of the cells with an EGFR-blocking Ab, which binds and occupies the ligand binding sites on the extracellular domains of EGFR, also prevented LPS-induced PGE2 production, implicating ligand-dependent EGFR phosphorylation in the PGE2 response (Fig. 4). PGE2 production by LPS was also inhibited by the ERK1/2 inhibitor (PD98059), implicating ERK1/2 (Fig. 4). From these results, we conclude that LPS induces PGE2 production in biliary cancer cells via EGFR ligand-dependent EGFR and ERK1/2 activation.

**PGE2 induces EGFR activation via a TACE-TGF-α signaling pathway**

Next, we examined the epithelial pathway utilized by PGE2 in signaling EGFR phosphorylation. Mz-ChA-1 carcinoma cells express COX-2 and PGE2 constitutively, whereas the expressions are minimal or absent in normal biliary epithelial cells. To avoid constitutive COX-2 and PGE2 effects, we chose normal biliary epithelial cells for these experiments and we stimulated the cells exogenously with PGE2. To determine whether PGE2-induced EGFR activation involves ligand binding to EGFR, we pretreated the cells...
with an EGFR-blocking Ab. This pretreatment suppressed PGE2-induced EGFR phosphorylation (Fig. 5A), implicating ligand-dependent EGFR activation in the response to PGE2. Pretreatment of the cells with a neutralizing Ab to the EGFR ligand TGF-α also suppressed PGE2-induced EGFR phosphorylation (Fig. 5A). Preincubation with an EGF-neutralizing Ab also reduced PGE2-induced EGFR phosphorylation slightly, but the inhibition was not significant (Fig. 5A). These results suggest that TGF-α plays a major role in PGE2-induced EGFR phosphorylation in biliary epithelial cells.

Metalloprotease TACE mediates multiple stimuli-induced EGFR activation via cleavage of EGFR proligand (e.g., pro-TGF-α) (7, 15, 24). To examine whether TACE mediates PGE2-induced TGF-α release and EGFR activation, we pretreated cells with metalloprotease inhibitors GM 6001 and TAPI-1. These two inhibitors blocked both PGE2-induced TGF-α release (Fig. 5B) and EGFR phosphorylation (Fig. 5C). LPS-induced TGF-α release in Mz-ChA-1 cells was also suppressed by the inhibitors (Fig. 5B), suggesting the involvement of a metalloprotease, most likely TACE. To confirm this, we used TACE small interfering RNA

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**FIGURE 4.** Effect of inhibition of EGFR cascade on LPS-induced PGE2 production. Mz-ChA-1 cells were pretreated with or without a selective EGFR inhibitor, AG 1478 (AG; 10 μM), a selective ERK1/2 inhibitor PD 98059 (PD; 30 μM), and an EGFR-neutralizing Ab (EGFR-Ab; 4 μg/ml) for 1 h and then stimulated with LPS for 4 h. Supernatants were collected to measure the concentration of PGE2 using ELISA. Data are means ± SEM (n = 5). *, p < 0.05, compared with LPS alone.

**FIGURE 5.** Effect of inhibition of TACE-TGF-α cascade on PGE2-induced EGFR activation. A, NHBE cells were preincubated with or without an EGFR-neutralizing Ab (4 μg/ml) or neutralizing Abs against TGF-α or EGF (4 μg/ml) for 1 h and then stimulated with PGE2 (10 μM) for 5 min. EGFR phosphorylation was detected using Western blot. Quantification of EGFR phosphorylation is expressed as described above. Data are means ± SEM (n = 5). *, p < 0.05, compared with the basal level. ***, p < 0.05, compared with LPS alone. B, Mz-ChA-1 cells were incubated with an anti-EGFR-neutralizing Ab for 30 min to block EGFR ligand binding sites and with metalloprotease inhibitors GM 6001 (GM; 10 μM) or TAPI-1 (TAPI; 10 μM) for 30 min and then incubated with PGE2 (10 μM) or LPS (10 μg/ml) for 2 h. Supernatants were collected to measure TGF-α using ELISA. Data are means ± SEM (n = 3). *, p < 0.05, compared with the basal level (without PGE2 or LPS). C, NHBE cells were pretreated with or without metalloprotease inhibitors GM (10 μM) or TAPI (10 μM) for 30 min and then stimulated with PGE2 (10 μM) for 5 min. EGFR phosphorylation was detected and analyzed as described previously. D, Mz-ChA-1 and KMBC cells at 50–70% density were transfected with or without TACE siRNA (100 nM) or negative control (NC) siRNA (100 nM). Seventy-two hours later, these cells were treated with or without PGE2 (10 μM) for 30 min to analyze EGFR phosphorylation by Western blot.
(siRNA) to knockdown TACE expression in two biliary cancer lines (Mz-ChA-1 and KMBC) and then examined EGFR phosphorylation in response to PGE2. TACE siRNA knocked down TACE gene expression (data not shown) and inhibited PGE2-induced EGFR phosphorylation in these two cell lines (Fig. 5D, Mz-ChA-1; upper panels) and KMBC (lower panels), implicating TACE in the responses. From these results, we conclude that TACE mediates PGE2-induced EGFR activation.

**COX2/PGE2-mediated second phase of EGFR phosphorylation by LPS is involved in cancer cell invasion**

Having determined the two phases of LPS-induced EGFR phosphorylation, we proceeded to examine the biological significance of the biphasic EGFR phosphorylation. We chose to perform in vitro cell invasion studies for the following reasons: 1) LPS exposure is implicated in tumor progression in animal models (25, 26) and 2) cell invasion is a critical step in tumor progression. First, we examined whether LPS can increase cancer cell invasion in vitro: Exposure of Mz-ChA-1 cancer cells to LPS for 24 h induced cell invasion significantly (Fig. 6A). Then, we examined the role of EGFR phosphorylation in LPS-induced cell invasion. We treated cells with AG1478 at various time points before and after LPS stimulation. Pretreatment with AG1478 for 1 h prevented LPS-induced cell invasion; treatment starting at 4 h after LPS stimulation, which preserved the first phase but prevented the second phase of the EGFR phosphorylation, also inhibited LPS-induced cell invasion significantly. Treatment with AG1478 starting at 12 h after LPS stimulation (2–4 h after the second phase EGFR phosphorylation) did not have significant inhibition of LPS-induced cell invasion (Fig. 6A). These results suggest that the second (COX-2/PGE2) phase of EGFR phosphorylation is important in LPS-induced cell invasion.

![Figure 6](http://www.jimmunol.org/Downloaded_from.png)
To further examine the role of the second phase of EGFR phosphorylation on cell invasion, we pretreated the cells with the COX-2 inhibitor NS-398 to prevent the second (COX-2/PGE2-dependent) phase. This pretreatment also inhibited LPS-induced cell invasion significantly (Fig. 6A), implicating COX-2/PGE2 in LPS-induced cell invasion. Together, these results show that in addition to the effects of the first phase of EGFR phosphorylation, subsequent activation of the COX-2/PGE2-dependent second phase of EGFR phosphorylation is important in cell invasion in response to LPS.

Discussion

In the present study, we show that in biliary carcinoma cells, LPS, a pathophysiological stimulus, induces biphasic EGFR phosphorylation: the early phase occurs at ∼30 min after stimulation, involving TACE-dependent EGFR ligand release; the delayed phase occurs at ∼6 h after stimulation via a signaling pathway involving COX-2/PGE2-dependent TACE activation and EGFR ligand release. However, in normal biliary epithelial cells, LPS induces only the early phase of EGFR activation due to the lack of COX-2 expression and PGE2 production. We propose a novel model showing the pathways mediating this biphasic EGFR activation (Fig. 6B).

Our current studies extend the previous discovery that LPS can induce EGFR phosphorylation (30 min) via a TACE-TGF-α cascade in human airway epithelial cells (15) to biliary epithelial cells. In this study, we report that LPS can induce a second phase of EGFR phosphorylation, which occurs at ∼6 h after the stimulation. We found that COX-2-dependent PGE2 production is responsible for the second but not the first phase of LPS-induced EGFR phosphorylation.

LPS induces COX-2-derived PGE2 production in various epithelia, including enterocytes, hepatocytes, and biliary epithelial cells (9–12). Recently, Fukata et al. (12) showed that LPS induced EGFR phosphorylation (at 30 min) in a human intestinal epithelial cell line (SW-480) and suggested that this is caused by LPS-induced COX-2 up-regulation and PGE2 production that did not require EGFR activation. However, they also showed that LPS did not induce COX-2 up-regulation significantly until 4 h after stimulation, suggesting that COX-2/PGE2 is unlikely to be responsible for the EGFR phosphorylation that they found at 30 min. Our studies found that the COX-2 inhibitor NS-398 inhibited the second but not the first phase of EGFR phosphorylation, confirming our hypothesis. In this study, we also found that LPS enhanced PGE2 production in biliary carcinoma (Mz-ChA-1) cells, which express COX-2 constitutively, via an EGFR signaling cascade. In Mz-ChA-1 cells, LPS-induced PGE2 production was prevented completely by inhibitors of EGFR and ERK1/2. These findings indicate that COX-2-derived PGE2 synthesis caused by LPS is mediated by the activation of an EGFR signaling cascade. It was previously reported that EGFR activation induces COX-2 expression and PGE2 production (4, 8). In this study, we show that activation of EGFR and subsequent ERK1/2 phosphorylation occurs within 30 min after LPS stimulation, in agreement with the temporal pattern of LPS-induced EGFR phosphorylation observed in other epithelial cells (15). This activation in carcinoma cells results in PGE2 production, which is maximal 4 h after exposure to LPS, preceding the second phase of EGFR activation.

In relation to the second phase of EGFR activation, we investigated the response to PGE2 in normal biliary epithelial cells. These cells do not express COX-2 constitutively, which allowed us to ascertain specifically the effect of exogenous PGE2. It was previously established that PGE2 exerts its biological actions via specific G protein-coupled receptors (GPCRs) (EP-1, -2, -3, -4) (27).

It was initially proposed that EGFR transactivation was mediated via an intracellular signaling pathway (ligand-independent EGFR phosphorylation), based on the rapid kinetics of the transactivation signal and the absence of detectable levels of soluble EGFR ligands (28). However, recent studies have shown unequivocally that GPCR activation leads to the cleavage of EGFR proligand, allowing the secreted soluble ligand to bind EGFR (5, 29), although the metalloprotease responsible for the proteolytic cleavage event was not determined. In this study, we show that PGE2, a GPCR stimulus, activates the EGFR ligand by causing the activation of the metalloprotease TACE, which cleaves EGFR ligands, shown here to consist, at least in part, of TGF-α.

The EGFR ligand TGF-α is synthesized as a transmembrane precursor molecule that requires proteolytic cleavage by transmembrane metalloproteases known as ADAMs. TACE, an ADAM family member, causes the ectodomain shedding of TGF-α (30, 31). We found that PGE2-induced TGF-α release was blocked by a metalloprotease inhibitor, GM6001, and by a TACE inhibitor, TAPI-1 (24). Together, these data indicate that PGE2 initiates an epithelial cell surface signaling cascade involving TACE/TGF-α-EGFR phosphorylation.

The major contribution of the present work is the demonstration that the two phases of EGFR activation described above are integrated in a positive feedback loop initiated by the bacterial product LPS. We further document that this loop is present in carcinoma cells (probably a major mechanism making these cancer cells different from the normal cells). Our experiments were designed to analyze EGFR phosphorylation over a prolonged time period in two types of cells (i.e., normal cells vs carcinoma cells) incubated with LPS. They allowed us to show that in biliary carcinoma cells, LPS caused two phases of EGFR phosphorylation. Because the initial transient activation of an EGFR cascade occurred before (and was responsible for) the activation of the COX-2/PGE2 pathway, we suggested that the second phase of EGFR phosphorylation could be the consequence of COX-2/PGE2 activation. This assumption is based on the fact that this late phase response was prevented by a selective COX-2 inhibitor in the carcinoma cells. That PGE2 release was the cause of the second phase of EGFR phosphorylation was further supported by the observation that the normal cells, which do not produce PGE2 constitutively, lack a second phase of EGFR phosphorylation. The temporal correlation between LPS-induced PGE2 release and the occurrence of the second phase of EGFR activation is also fully consistent with this mechanism. Importantly, LPS induced an EGFR-mediated positive feedback loop involving COX-2/PGE2 production, causing rephosphorylation of EGFR, resulting in a significant stimulation of cell invasion in these cancer cells, a critical aspect of tumor progression. Future studies will address a variety of potential outcomes of this important feedback pathway, including carcinogenic effects and effects on cell differentiation. Thereby, this signaling cascade can provide further insights into the pathophysiology of epithelial inflammation and tumorogenesis.

The present results are in keeping with recent computational analyses showing that positive feedback loops allow cells to modulate the amplitude and the duration of signaling responses (32). They are of particular interest with respect to chronic inflammation and its relationship to cancer. Thus, LPS triggers a positive feedback loop involving COX-2/PGE2 and the EGFR signaling cascade, which could contribute to the promotion of carcinogenesis, notably in the biliary tract where carcinoma generally arises in a background of chronic inflammation.

Disclosures

The authors have no financial conflict of interest.
References

1. Clevers, H. 2004. At the crossroads of inflammation and cancer. Cell 118: 645–654.

2. Williams, C. S., M. Mann, and R. N. DuBois. 1999. The role of cyclooxygenases in inflammation, cancer, and development. Oncogene 18: 7908–7916.

3. Smith, W. L., D. L. DeWitt, and R. M. Garavito. 2000. Cyclooxygenases: structural, cellular, and molecular biology. Annu. Rev. Biochem. 69: 145–182.

4. Coffey, R. J., C. J. Hawkey, L. Damstrup, R. Graves-Deal, V. C. Daniel, P. J. Dempsey, R. Chinery, S. C. Kirkland, R. N. DuBois, T. L. Jetton, and J. D. Morrow. 1997. Epidermal growth factor receptor activation induces nuclear targeting of cyclooxygenase-2, basolateral release of prostaglandins, and mitogenesis in polarizing colon cancer cells. Proc. Natl. Acad. Sci. USA 94: 657–662.

5. Pai, R., B. Soreghan, I. L. Szabo, M. Pavelka, D. Baatar, and A. S. Tarnawski. 2002. Prostaglandin E2 transactivates EGF receptor: a novel mechanism for promoting colon cancer growth and gastrointestinal hypertrophy. Nat. Med. 8: 289–293.

6. Buchanan, F. G., D. Wang, F. Bargiacchi, and R. N. DuBois. 2003. Prostaglandin E2 regulates cell migration via the intracellular activation of the epidermal growth factor receptor. J. Biol. Chem. 278: 35451–35457.

7. Shao, J., B. M. Evers, and H. Sheng. 2004. Prostaglandin E2 synergistically enhances receptor tyrosine kinase-dependent signaling system in colon cancer cells. J. Biol. Chem. 279: 14287–14293.

8. Han, C., and T. Wu. 2005. Cyclooxygenase-2-derived prostaglandin E2 promotes human cholangiocarcinoma cell growth and invasion through EPI receptor-mediated activation of the epidermal growth factor receptor and Akt. J. Biol. Chem. 280: 24053–24063.

9. Collejas, N. A., M. Casado, M. J. Diaz-Guerra, L. Bosca, and P. Martin-Sanz. 2001. Expression of cyclooxygenase-2 promotes the release of matrix metalloproteinase-2 and -9 in fetal rat hepatocytes. Hepatology 33: 860–867.

10. Kim, H. J., S. K. Lee, M. H. Kim, D. W. Seo, and Y. I. Min. 2003. Cyclooxygenase-2 mediates mucin secretion from epithelial cells of lipopolysaccharide-treated canine gallbladder. Dig. Dis. Sci. 48: 726–732.

11. Grishin, A. V., J. Wang, D. A. Potoka, D. J. Hackam, J. S. Upperman, P. Boyle, R. Zamora, and H. R. Ford. 2006. Lipopolysaccharide induces cyclooxygenase-2 expression in intestinal epithelium via a noncanonical p38 MAPK pathway. J. Immunol. 176: 580–588.

12. Fukata, M., A. Chen, A. Klepper, S. Krishnareddy, A. S. Vannadevan, L. S. Thomas, R. Xu, H. Inoue, M. Arditi, A. D. Nannenga, and M. T. Abreu. 2006. Cox-2 is regulated by Toll-like receptor-4 (TLR4) signaling: role in proliferation and apoptosis in the intestine. Gastroenterology 131: 862–877.

13. Guilhot, L., S. Medjane, K. Le-Barillier, V. Balloy, C. Danel, M. Chignard, and M. Si-Tahar. 2004. Response of human pulmonary epithelial cells to lipopolysaccharide involves Toll-like receptor 4 (TLR4)-dependent signaling pathways: evidence for an intracellular compartmentalization of TLR4. J. Biol. Chem. 279: 2712–2718.

14. Harada, K., S. Ohira, K. Inose, S. Omote, Y. Zen, Y. Sato, and Y. Nakanuma. 2003. Lipopolysaccharide activates nuclear factor-kB through Toll-like receptors and related molecules in cultured biliary epithelial cells. Lab. Invest. 83: 1657–1667.

15. Shao, M. X., I. F. Ueki, and J. A. Nadel. 2003. Tumor necrosis factor alpha-convertase enzyme mediates MUC5AC mucin expression in cultured human airway epithelial cells. Proc. Natl. Acad. Sci. USA 100: 11618–11623.

16. Oses, T., O. Sandstad, V. Skar, and M. Oses. 1997. Lipopolysaccharides and beta-glucuronidase activity in choledochal bile in relation to choledochothiasis. Digestion 88: 437–443.

17. Sasatomi, K., K. Noguchi, S. Sakisaka, M. Sata, and K. Tanikawa. 1998. Abnormal accumulation of endotoxin in biliary epithelial cells in primary biliary cirrhosis and primary sclerosing cholangitis. J. Hepatol. 29: 409–416.

18. Knuth, A., H. Gabbart, W. Dippold, O. Klein, W. Sachsse, D. Bitter-Suermann, W. Prellwitz, and K. H. Meyer zum Buschenfelde. 1985. Biliary adenocarcinoma: characterisation of three new human tumor cell lines. J. Hepatol. 1: 579–596.

19. Housset, C., A. Carayon, B. Housset, C. Legendre, L. Hannoun, and R. Poupon. 1993. Endothelin-1 secretion by human gallbladder epithelial cells in primary culture. Lab. Invest. 69: 750–755.

20. Elopoulos, A. G., C. D. Dimitti, C. C. Wang, J. Cho, and P. N. Tsichlis. 2002. Induction of COX-2 by LPS in monocytes is regulated by Tp2-dependent CREB activation signals. EMBO J. 21: 4831–4840.

21. Yuan, J. H., A. E. Canbay, N. W. Werneburg, S. P. Lee, and G. J. Gores. 2004. Oxysterols induce cyclooxygenase-2 expression in cholangiocytes: implications for biliary tract carcinogenesis. Hepatology 39: 732–738.

22. Shin, Y. K., J. S. Park, H. S. Kim, H. J. Jun, G. E. Kim, C. O. Suh, Y. S. Yun, and H. Pyo. 2005. Radiosensitivity enhancement by celecoxib, a cyclooxygenase (COX)-2 selective inhibitor, via COX-2-dependent cell cycle regulation on human cancer cells expressing differential COX-2 levels. Cancer Res. 65: 9501–9509.

23. Nakamura, T., J. A. Nadel, I. F. Ueki, J. L. Koff, and M. X. Shao. 2007. Regulation of interleukin-8 via an airway epithelial signaling cascade. Am. J. Physiol. 292: L1289–L1296.

24. Shao, M. X., and J. A. Nadel. 2005. Neutrophil elastase induces MUC5AC mucin production in human airway epithelial cells via a cascade involving protein kinase C, reactive oxygen species, and TNF-alpha-convertase enzyme. J. Immunol. 175: 4009–4016.

25. Harmey, J. H., C. D. Bucana, W. Lu, A. M. Byrne, S. McDonnell, C. Lynch, D. Boucher-Hayes, and Z. Dong. 2002. Lipopolysaccharide-induced metastatic growth is associated with c-fos, c-jun, CREB activation signals. J. Immunol. 179: 42603–42612.

26. Pidgeon, G. P., J. H. Harmey, E. Kay, M. Da Costa, H. P. Redmond, and D. J. Boucher-Hayes. 1999. The role of endotoxin/lipopolysaccharide in sputum induced tumour growth in a murine model of metastatic disease. Br. J. Cancer 81: 1311–1317.

27. Naramiya, S., Y. Sugimoto, and F. Ushikubi. 1999. Prostanoid receptors: structures, properties, and functions. Physiol. Rev. 79: 1193–1226.

28. Tsuch, H. F., U. Weiss, C. Wallasch, and A. Ullrich. 1996. Role of transactivation of the EGF receptor in signalling by G-protein-coupled receptors. Nature 379: 557–560.

29. Collins, D. F., S. J. Keely, R. J. Coffey, and K. E. Barrett. 2002. Transactivation of the epidermal growth factor receptor in colonic epithelial cells by carbachol requires extracellular release of transforming growth factor-a. J. Biol. Chem. 277: 42603–42612.

30. Peschon, J. J., J. L. Slack, P. Reddy, K. L. Stocking, S. W. Summarbog, D. C. Lee, W. E. Russell, B. J. Castner, R. S. Johnson, J. N. Fitzner, et al. 1998. An essential role for ectodomain shedding in mammalian development. Science 282: 1281–1284.

31. Black, R. A., C. T. Rauch, C. J. Kozlosky, J. J. Peschon, J. L. Slack, M. F. Wolfson, B. J. Castner, K. L. Stocking, P. Reddy, S. Strimuvas, et al. 1997. A metalloproteinase disintegrin that releases tumour-necrosis factor-a from cells. Nature 385: 729–733.

32. Shvartsman, S. Y., M. P. Hagan, A. Yacoub, P. Dent, H. S. Wiley, and D. A. Lauffenburger. 2002. Autocrine loops with positive feedback enable context-dependent cell signaling. Am. J. Physiol. 282: C545–C559.