Toll-like receptor 4 activates NF-κB and MAP kinase pathways to regulate expression of proinflammatory COX-2 in renal medullary collecting duct cells

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Küper C, Beck FX, Neuhofer W. Toll-like receptor 4 activates NF-κB and MAP kinase pathways to regulate expression of proinflammatory COX-2 in renal medullary collecting duct cells. Am J Physiol Renal Physiol 302: F38–F46, 2012. First published September 21, 2011; doi:10.1152/ajprenal.00590.2010.—Binding of bacterial LPS to the Toll-like receptor 4 (TLR4) complex of inner medullary collecting duct (IMCD) cells plays a central role in recognition of ascending bacterial infections and activation of proinflammatory responses. Since proinflammatory cyclooxygenase (COX)-2 is induced in IMCD cells upon LPS exposure, the present study addressed the question of whether TLR4 mediates COX-2 induction in IMCD cells and characterized the underlying signaling mechanisms. Enhanced COX-2 expression and activity in the presence of LPS was diminished by TLR4 inhibition. LPS induced a TLR4-dependent stimulation of NF-κB and the MAPKs p38, ERK1/2, and JNK. Activation of NF-κB was under negative control of JNK, as inhibition of JNK increased NF-κB activity and COX-2 expression. Phosphorylation of p38 and ERK1/2 required TLR4-dependent release of TGF-α with subsequent activation of the epidermal growth factor receptor (EGFR), whereas JNK activation was EGFR independent. Inhibition of p38 or ERK1/2 had no significant effect on LPS-induced NF-κB activation, nor on activator protein 1-, cAMP response element-, or serum response element-driven reporter constructs. However, the transcriptional regulator SP-1 appears to contribute to COX-2 expression after LPS exposure. In conclusion, these results propose that LPS mediates enhanced COX-2 expression in IMCD cells by 1) TLR4-mediated activation of the NF-κB signaling pathway, 2) TLR4-dependent release of TGF-α with subsequent activation of the EGFR and downstream MAPKs p38 and ERK1/2, and 3) TLR4-mediated, EGFR-independent activation of JNK that negatively regulates NF-κB activation.

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PYELONEPHRITIS IS MOST OFTEN CAUSED BY GRAM-NEGATIVE BACTERIA LIKE ESCHERICHIA COLI, ASCENDING FROM URINARY TRACT INFECTIONS. EPITHELIAL SURFACES, OFTEN CONSIDERED AS BARRIERS PREVENTING PATHOGEN INVASION, HARBOR TOLL-LIKE RECEPTORS (TLR) THAT ARE KEY COMPONENTS IN THE DETECTION OF PATHOGENS OR PATHOGEN-ASSOCIATED MOLECULAR PATTERNS AND INITIATE COMPLEX SIGNAL TRANSDUCTION PATHWAYS TO ACTIVATE EARLY INFLAMMATORY RESPONSES. THE TLR4 RECEPTOR COMPLEX IS THE ESSENTIAL RECEPTOR FOR DETECTION OF LPS, COMPONENTS OF THE OUTER MEMBRANE OF GRAM-NEGATIVE BACTERIA (34). BINDING OF LPS TO THE TLR4 RECEPTOR COMPLEX INITIATES THE RECRUITMENT OF VARIOUS ADAPTOR PROTEINS AND TRIGGERS THE ACTIVATION OF SEVERAL SIGNALING PATHWAYS, FINALLY RESULTING IN THE ACTIVATION OF MAP KINASES AND NF-κB TO INDUCE EXPRESSION OF PROINFLAMMATORY GENES (19). THE IMPORTANCE OF TLR4 SIGNALING DURING ASCENDING BACTERIAL INFECTIONS IS DEMONSTRATED BY THE FACT THAT TLR4-NEGATIVE MICE ARE PARTICULARLY PRONE TO URINARY TRACT INFECTIONS (11, 34, 40).

An important downstream-target of TLR4 signaling in innate immune cells is the inducible cyclooxygenase (COX)-2 (37), a key enzyme in the synthesis of prostaglandins, prostacyclins, and thromboxanes. Stimulation of COX-2 expression by LPS not only in immune cells, but also in inner medullary collecting duct (IMCD) cells was first shown by Yang et al. (48). In rats, expression of TLR4 and COX-2 in cells of the cortical and the medullary thick ascending limb and in IMCD cells has been observed, and COX-2 was further increased in these nephron segments in experimental sepsis models (7, 8). The exact role of renal COX-2-mediated prostanoid synthesis during urinary tract infections has not been characterized as yet; however, several mechanisms are conceivable. PGE2 exerts antiapoptotic effects in renal medullary cells (13, 26) and thus may favor cell survival of IMCD cells to preserve the integrity of the renal epithelium barrier during bacterial infections. COX-2 increases expression of Tamm-Horsfall protein, which in turn attenuates bacterial colonization of the urinary bladder (6). Finally, prostanooids may help to regulate renal expression of chemokines during bacterial infections (49). Although controlled renal COX-2 expression may have beneficial effects for cell survival and host defense during bacterial infections, dysregulation of COX-2 expression can promote the well-known adverse effects of renal inflammation (25).

Although there is experimental evidence for the existence of LPS-induced COX-2 expression in renal medullary epithelial cells, the underlying signal transduction pathways are barely characterized. Chassin et al. (3) has examined various TLR4-dependent or -independent signaling pathways in renal collecting duct cells that were induced by uropathogenic E. coli. These pathways included activation of NF-κB and MAPKs. As similar pathways are known to be involved in LPS-induced COX-2 expression in macrophages (18), it is conceivable that they also contribute to COX-2 expression in renal epithelial cells.

We have recently identified a signal transduction scheme in renal collecting duct cells in which osmotic stress activates a TNF-α-converting enzyme (TACE)-dependent release of transforming growth factor-α (TGF-α) with subsequent activation of the epidermal growth factor (EGFR) and MAPKs, finally resulting in induction of COX-2 expression (27). The aim of the present study was to investigate whether similar signaling events are also involved in TLR4-mediated activation of MAPKs and NF-κB to gain better insight into the signal transduction pathways underlying LPS-induced COX-2 expression in IMCD cells.
MATERIALS AND METHODS

Materials. LPS from *E. coli* O111:B4, NF-κB inhibitor Bay 11–7082, SP-1 inhibitor mithramycin A, EGFR inhibitor AG1478, p38 inhibitor SB202190, ERK1/2 inhibitor U0126, and JNK inhibitor SP600125 were obtained from Sigma (Deisenhofen, Germany), and TLR4 inhibitor TAK242 (also known as CLI-095) was from Invivogen (San Diego, CA). Anti-COX-2 antibody was from Cayman Chemical (Ann Arbor, MI); anti-actin antibody was from Sigma; anti-TACE, anti EGFR, anti-phospho-EGFR (Tyr1173), anti SP-1, and anti-TGF-α were from Santa Cruz Biotechnology (Santa Cruz, CA); anti-p65, anti-IκBα, anti-phospho-p65 (Ser536), anti-phospho-IκBα (Ser32), anti-phospho-ERK1/2 (Thr202/Tyr204), anti-phospho-JNK (Thr183/Tyr185), and anti-phospho-p38 (Thr180/Tyr182) antibodies were purchased from Cell Signaling (Beverly, MA); horseradish peroxidase-conjugated anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). Unless otherwise indicated, other reagents were purchased from Biomed (Hamburg, Germany), Biozol (Eching, Germany), Carl Roth (Karlsruhe, Germany), or Sigma.

Animal studies. Animal studies were approved by the institutional animal care board and by local authorities. All experiments were conducted in accordance with German federal laws relating to animal experimentation. Male Wistar rats (~200 g) were injected intraperitoneally with LPS (5 mg/kg body wt, dissolved in a final volume of 250 μl PBS) or only vehicle (PBS). After 24 h, the rats were anesthetized with pentobarbital sodium, and outer and inner medullas from both kidneys were immediately isolated, snap-frozen in liquid nitrogen, and stored at −80°C. Urine samples for estimation of PGE2 excretion were taken directly from the bladder of anesthetized animals and stored at −80°C.

Cell culture. IMCD-3 cells were obtained from the American Type Culture Collection (CRL-2123; ATCC, Manassas, VA) and maintained in F12 medium supplemented with 10% fetal bovine serum (Biochrom, Berlin, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen, Karlsruhe, Germany) at 37°C in a humidified atmosphere (5% CO2-95% air). In experiments with pharmacological inhibitors, confluent monolayers of IMCD-3 cells were preincubated with the appropriate compound as indicated or with vehicle alone for 30 min before addition of LPS (5 μg/ml) to the medium. Thereafter, the cells were incubated for the indicated periods and processed as indicated.

**qRT-PCR analysis.** For determination of TLR4, COX-2, and β-actin mRNA expression levels, total RNA from LPS-treated IMCD-3 cells was prepared by adding TRIReagent (Peqlab, Erlangen, Germany) according to the manufacturer’s recommendations. The primers (Metabion, Martinsried, Germany) used in this experiment were TLR4 forward (5′-CTG TCA TTA CAT ACA CAC GAG TGA TTG-3′), TLR4 reverse (5′-CTG ATC CAT GCA TGT GGA GTT C-3′), Cox-2 forward (5′-GGA TGC TCT ATC ACT TTT TCT A-3′), Cox-2 reverse (5′-AGA GAG CCA GCT TGT GGA A-3′), β-actin forward (5′-CGA GAA ACA GGA CTT AAG CAC C-3′), and β-actin reverse (5′-ACC AGA GAG ACA TCA GAG CAA C-3′). Experiments were carried out on a Roche LightCycler 2.0, using the SensiMix SYBR One-Step Kit (Peqlab) according to the manufacturer’s recommendations. Specificity of PCR product formation was confirmed by monitoring melting point analysis and by nondenaturing PAGE.

**Western blot analysis.** Following the experiments in 24-well plates, the cells were washed with chilled PBS and lysed by the addition of 50 μl 1× SDS sample buffer/well. For detection of COX-2 in rat kidneys, frozen kidney specimens dissected into outer and inner medullary portions were homogenized in a solution containing 8 M urea/PBS (100 μl/10 mg tissue) using a Potter-Elvehjem homogenizer. Aliquots (30 μg protein) were subjected to 10% SDS-PAGE and blotted onto nitrocellulose membranes (Amersham Pharmacia Biotech, Buckinghamshire, UK). Nonspecific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween 20 (PBS-T) at room temperature for 1 h. Samples were incubated with primary antibodies in PBS-T containing 5% nonfat dry milk overnight at 4°C. Subsequently, the blots were washed three times with PBS-T for 5 min each. Subsequently, the membranes were incubated with appropriate secondary antibody at room temperature for 1 h in PBS-T containing 5% nonfat dry milk. After washing with PBS-T three times for 5 min each, immunocomplexes were visualized by enhanced chemiluminescence (Pierce, Rockford, IL).

**PGE2 measurement.** PGE2 concentrations in 100-μl medium aliquots or in urine were determined using a commercially available assay (Correlate EIA PGE2-Kit; Assay Designs, Ann Arbor, MI) according to the manufacturer’s protocol. Plates were read at 405 nm in a plate reader, and PGE2 concentrations were determined from the standard curve. Urinary PGE2 levels were normalized by reference to urinary creatinine, which was determined by the method of Jaffé (2).

**Reporter gene assays.** Activation of transcription factors in response to LPS was assessed using the secreted alkaline phosphatase system (SEAP, Clontech, Heidelberg, Germany) with reporter constructs, in which the SEAP open reading frame is under control of the following trans-acting elements: 1) pNF-κB-SEAP, containing four copies of the NF-κB response element; 2) pAP-1-SEAP, containing four copies of the activator protein 1 (AP-1) enhancer; 3) pCRE-SEAP, containing three copies of the cAMP response element (CRE); and 4) pSRE-SEAP, containing three copies of the serum response element (SRE). For transfection, IMCD-3 cells were grown to ~80% confluence, trypsinized, washed in PBS, and 106 cells were finally resuspended in 200 μl modified HBS electroporation buffer (0.5% HEPES, 1% glucose, 0.5% Ficoll, 5 mM NaCl, 135 mM KCl, 2 mM MgCl2, pH 7.4) together with 10 μg of the appropriate reporter vector. Electroporation was performed with a Gene Pulser Xcell Electroporation System (Bio-Rad) at 150 V and 950 μF (exponential decay pulse) in a 2-mm cuvette, and cells were subsequently seeded immediately in 24-well plates. After growing to confluence, the cells were treated as indicated and SEAP activity in the medium was determined as described in detail elsewhere (32).

**Small interfering RNA knockdown.** Accell SMARTpool small interfering RNA (siRNA) constructs for knockdown of TACE, EGFR, or SP-1 or Accell nontargeting siRNA (no. 2) were obtained from Thermo Fisher Scientific (Epsom, UK). Knockdown in IMCD-3 cells was performed according to the manufacturer’s instructions. The concentration of siRNA constructs was 500 nM in Accell delivery medium, containing 2% FCS. Cells were incubated for 5 days, and knockdown efficiency was determined by Western blot analysis.

**Presentation of data and statistical analysis.** Data are presented as means ± SE. The significance of differences between the means was established using Student’s t-test. *P* < 0.05 was regarded as significant.

**RESULTS**

**LPS-induced renal COX-2 expression and PGE2 synthesis in vivo.** In male Wistar rats, COX-2 expression in the inner medulla was generally considerably higher than in the outer medulla. In LPS-treated animals, COX-2 expression, particularly in the inner medulla, was significantly higher than in the control group (Fig. 1A). Furthermore, urinary PGE2, an indicator of renal PGE2 synthesis, was elevated in LPS-treated animals (Fig. 1B), indicating that LPS activates renal COX-2 expression, and hence PGE2 synthesis, in vivo.

**LPS induces COX-2 expression and PGE2 synthesis in IMCD-3 cells.** To further characterize the signal transduction pathways involved in LPS-induced COX-2 expression, we employed cells of the IMCD line IMCD-3, originating from the terminal segment of the murine collecting duct (36). Addition of LPS to the growth medium induced a significant increase in COX-2 mRNA and protein abundance (Figs. 2, A and B).
**TLR4 REGULATES RENAL PROINFLAMMATORY COX-2 EXPRESSION**

LPS induces activation of EGFR and MAPKs. We have shown previously that shedding of the EGFR ligand TGF-α mediates EGFR and downstream MAPK activation in MDCK cells in response to osmotic stress (27, 28). The effect of LPS on activation of EGFR and MAPKs was tested by immunoblotting, using phospho-specific antibodies. As shown in Fig. 4A, LPS caused a rapid phosphorylation of the EGFR and the MAPKs JNK, p38, and ERK1/2 within 15 min; activation peaked after ~60 min (Fig. 4A), except for JNK, which was analyzed. As expected, LPS induced TLR4-dependent phosphorylation and subsequent degradation of IkBα, even in the absence of LPS induced-COX-2 expression (Fig. 2, A and B). Two principal signaling pathways have been described for LPS-induced COX-2 expression in other cell types: activation of a NF-κB pathway and activation of MAP kinases p38, ERK1/2, and JNK. We therefore tested the effects of pharmacological inhibitors of these pathways on LPS-induced COX-2 expression. Inhibition of NF-κB attenuated COX-2 expression and PGE2 synthesis significantly, as did inhibition of EGFR, p38 kinase, and ERK1/2 kinase. Unexpectedly, the JNK inhibitor SP600125 enhanced LPS-induced COX-2 expression (Fig. 2, A and B); additional experiments demonstrated that treatment with SP600125 even in the absence of LPS induced-COX-2 expression (data not shown) and PGE2 synthesis.

**LPS induces activation of NF-κB.** The activation state of NF-κB in the presence of LPS was monitored by means of a reporter construct driven by four NF-κB sites. As expected, LPS stimulated reporter gene activity by about fourfold. This could be blocked by pharmacological inhibition of TLR4 or NF-κB. Inhibition of EGFR, p38, or ERK1/2 had no significant effect on reporter gene activity. In contrast, addition of the JNK inhibitor Bay11–7082 (20 μM), the Toll-like receptor 4 (TLR4) inhibitor TAK242 (2.5 μM), the EGFR inhibitor AG1478 (10 μM), the p38 inhibitor SB202190 (10 μM), the ERK1/2 inhibitor U0126 (10 μM), the JNK inhibitor SP600125 (10 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. A; for mRNA analysis, the cells were further incubated for 2 h and subsequently processed for COX-2 mRNA expression by qRT-PCR analysis as described in MATERIALS AND METHODS. Relative COX-2 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Values are means ± SE; n = 3. #P < 0.05 vs. control. *P < 0.05 vs. LPS. B: for protein analysis, the cells were further incubated for 24 h, and subsequently medium samples were saved and PGE2 concentration was determined as described in MATERIALS AND METHODS. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown. C: for analysis of PGE2 synthesis, the cells were further incubated for 24 h. Subsequently, medium samples were saved and PGE2 concentration was determined as described in MATERIALS AND METHODS. Values are means ± SE; n = 3. #P < 0.05 vs. control. *P < 0.05 vs. LPS.

**Fig. 1.** LPS induces renal medullary cyclooxygenase-2 (COX-2) expression and PGE2 synthesis in vivo. Rats were injected with LPS (5 mg/kg ip) or only vehicle PBS. A: for analysis of medullary COX-2 expression, tissue samples from the outer (OM) and the inner medulla (IM) were harvested 24 h after LPS injection and processed for immunoblotting as described in MATERIALS AND METHODS. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown. B: for analysis of PGE2 synthesis, urinary PGE2 concentration was determined and normalized to urinary creatinine as described in MATERIALS AND METHODS. Values are means ± SE; n = 3. #P < 0.05 vs. control. Time-dependent analysis of COX-2 mRNA abundance revealed that transcription occurred within 1 h after LPS administration, peaked after about 2 h, and subsequently declined and remained slightly elevated (data not shown).

**Fig. 2.** COX-2 expression and PGE2 synthesis in IMCD-3 cells. Confluent IMCD-3 cells were preincubated as indicated with the NF-κB inhibitor Bay11–7082 (20 μM), the Toll-like receptor 4 (TLR4) inhibitor TAK242 (2.5 μM), the EGFR inhibitor AG1478 (10 μM), the p38 inhibitor SB202190 (10 μM), the ERK1/2 inhibitor U0126 (10 μM), the JNK inhibitor SP600125 (10 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. A; for mRNA analysis, the cells were further incubated for 2 h and subsequently processed for COX-2 mRNA expression by qRT-PCR analysis as described in MATERIALS AND METHODS. Relative COX-2 mRNA abundance was normalized to that of β-actin to correct for differences in RNA input. Values are means ± SE; n = 3. #P < 0.05 vs. control. *P < 0.05 vs. LPS. B: for protein analysis, the cells were further incubated for 24 h, and subsequently medium samples were saved and PGE2 concentration was determined as described in MATERIALS AND METHODS. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown. C: for analysis of PGE2 synthesis, the cells were further incubated for 24 h. Subsequently, medium samples were saved and PGE2 concentration was determined as described in MATERIALS AND METHODS. Values are means ± SE; n = 3. #P < 0.05 vs. control. *P < 0.05 vs. LPS.
which exhibited maximal activation already after 15–30 min. Accordingly, inhibition of TLR4 attenuated EGFR and MAPK phosphorylation. Activation of p38 and ERK1/2 required activation of EGFR, as pharmacological inhibition of EGFR by

![Graph showing inhibition of NF-κB activity by various treatments](image)

Fig. 3. Activation of the NF-κB pathway. A: IMCD-3 cells were transfected with the reporter vector NF-κB-SEAP. Confluent cells were preincubated as indicated with the NF-κB inhibitor Bay11–7082 (20 μM), the TLR4 inhibitor TAK242 (2.5 μM), the EGFR inhibitor AG1478 (10 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml, and the cells were further incubated for 24 h. Thereafter, medium samples were taken and NF-κB driven reporter activity was assessed as described in MATERIALS AND METHODS. Basal activity in unstimulated cells was defined as 1. Values are means ± SE; n = 3. *P < 0.05 vs. control. #P < 0.05 vs. LPS. B: IMCD-3 cells were preincubated as indicated with the TLR4 inhibitor TAK242 (2.5 μM), the JNK inhibitor SP600125 (10 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. After 0.5 or 2 h, the cells were lysed and abundance of phosphorylated or total IκBα and p65 was determined by immunoblotting. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown.

![Graph showing time course of EGFR and MAPK activation](image)

Fig. 4. Activation of EGFR and MAPKs ERK1/2, p38, and JNK. A: time course of EGFR and MAPK activation. LPS was added to a final concentration of 5 μg/ml to IMCD-3 cells. At the indicated times, the cells were lysed and phosphorylation of EGFR, JNK, ERK1/2, and p38 was determined by immunoblotting. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown. B: IMCD-3 cells were preincubated as indicated with the TLR4 inhibitor TAK242 (2.5 μM), the EGFR inhibitor AG1478 (10 μM), the p38 inhibitor SB202190 (10 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. After 45 min, the cells were lysed and phosphorylation of EGFR, JNK, ERK1/2, and p38 was determined by immunoblotting using phospho-specific antibodies. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown.
AG1478 not only abolished EGFR phosphorylation but also phosphorylation of p38 and ERK1/2. In contrast, phosphorylation of JNK was not affected by EGFR inhibition (Fig. 4B).

LPS induces ectodomain shedding of pro-TGF-α. Next, we determined whether shedding of TGF-α is also involved in EGFR-MAPK activation and COX-2 expression in IMCD-3 cells exposed to LPS. Pro-TGF-α is abundantly expressed in the medullary collecting duct (27). As shown in Fig. 5A, LPS treatment increased the concentration of soluble TGF-α, demonstrating that LPS induces ectodomain shedding of pro-TGF-α. Release of mature TGF-α in the medium was largely prevented by the TLR4 inhibitor TAK242 or by the matrix metalloproteinase (MMP) inhibitor GM6001 (Fig. 5A), indicating that TLR4 activation causes MMP-dependent processing of pro-TGF-α by LPS treatment.

We further investigated the role of TGF-α on EGFR and MAPK activation. TLR4 inhibition blunted phosphorylation of EGFR and all three MAPKs. Simultaneous addition of recombinant TGF-α restored phosphorylation of EGFR, p38, and ERK1/2 (Fig. 5B), but not that of JNK. Similar results were obtained with the MMP inhibitor GM6001, as phosphorylation of EGFR, p38, and ERK1/2 was diminished but could be restored by TGF-α. In contrast, GM6001 and TGF-α had no effect on LPS-induced JNK phosphorylation (Fig. 5B).

Finally, the role of TGF-α during LPS-induced COX-2 expression was addressed. As shown in Fig. 5C, GM6001 significantly diminished COX-2 expression, which was counteracted by TGF-α. Furthermore, preincubation of IMCD-3 cells with a TGF-α-neutralizing antibody significantly attenuated LPS-induced COX-2 expression (Fig. 5C). In the absence of LPS, TGF-α alone was not sufficient to induce COX-2 expression (Fig. 5C). In conclusion, these results suggest that binding of LPS to the TLR4 receptor complex triggers the release of TGF-α to activate EGFR and downstream MAPKs p38 and ERK1/2, while activation of JNK appears to be independent from TGF-α-induced EGFR activation.

Knockdown of TACE and EGFR. TACE, also known as ADAM17, is usually described as the MMP responsible for ectodomain shedding of pro-TGF-α (42), while EGFR is described as the receptor tyrosine kinase which is activated by mature TGF-α (15). To prove specific roles for TACE and EGFR in LPS-induced COX-2 expression, we transfected IMCD-3 cells with siRNA for TACE or EGFR and observed the effect on LPS-induced COX-2 expression by immunoblot analysis. Transfection with the respective siRNA resulted in a knockdown efficiency of 60–80%, depending on the target.

Fig. 5. Role of pro-TGF-α ectodomain shedding on EGFR-MAPK activation and COX-2 expression. A: TLR4 and metalloproteinase dependence of pro-TGF-α ectodomain shedding. IMCD-3 cells were preincubated as indicated with the TLR4 inhibitor TAK242 (2.5 μM), the matrix metalloproteinase (MMP) inhibitor GM6001 (50 μM), or vehicle DMSO for 1 h. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. After 2 h, medium samples were taken and concentration of mature TGF-α was determined as described in MATERIALS AND METHODS. Values are means ± SE; n = 3. *P < 0.05 vs. non-LPS control. #P < 0.05 vs. LPS. B: role of TGF-α in EGFR and MAPK activation. Confluent IMCD-3 cells were preincubated as indicated with the MMP inhibitor GM6001 (50 μM) or vehicle DMSO for 1 h. Recombinant TGF-α (50 ng/ml) was added where indicated. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. After 60 min, the cells were lysed and phosphorylation of EGFR, JNK, ERK1/2, and p38 was determined by immunoblotting. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown. C: role of TGF-α in COX-2 expression. Confluent IMCD-3 cells were preincubated as indicated with the TLR4 inhibitor TAK242 (2.5 μM), the MMP inhibitor GM6001 (50 μM), a TGF-α-neutralizing antibody (10 μg/ml), or vehicle DMSO for 1 h. In experiments with GM6001, recombinant TGF-α (50 ng/ml) was added where indicated. Subsequently, LPS was added to the medium to a final concentration of 5 μg/ml. The cells were further incubated for 24 h and subsequently processed for immunoblotting. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown.
gene (Fig. 6). Each siRNA construct significantly decreased COX-2 induction by LPS, compared with nonspecific control siRNA (Fig. 6). These results further indicate that TACE and EGFR are critical signaling molecules for LPS-induced COX-2 expression.

**SP-1 contributes to LPS-induced COX-2 expression.** To identify the transcriptional regulator(s) activated by p38 and ERK1/2, IMCD-3 cells were transfected transiently with various reporter gene constructs containing binding sites for CRE binding protein (CREB), serum response factor (SRF), or AP-1, respectively. However, none of the tested constructs exhibited elevated reporter gene activity in the presence of LPS or a declined basal activity in response to pharmacological inhibitors of p38 or ERK1/2 (data not shown), indicating that neither CREB nor SRF nor AP-1 contributes to LPS-induced COX-2 expression.

Since also the transcription factor SP-1 has been reported to be involved in MAPK-mediated induction of COX-2 expression (46), we tested the effect of the SP-1 inhibitor mithramycin A on COX-2 expression. At concentrations of 1 μM, mithramycin A had no effect on COX-2 expression, while at 10 μM COX-2 expression was significantly decreased (Fig. 7). Additionally, siRNA-mediated knockdown of SP-1 attenuated LPS-induced COX-2 expression (Fig. 6). Taken together, these results indicate that activation of SP-1 contributes to COX-2 expression.

In conclusion, the results presented here indicate a complex regulation pattern, by which LPS induces COX-2 expression in IMCD-3 cells, and which is summarized in Fig. 8.

**DISCUSSION**

The aim of the present study was to gain further insight into the mechanisms involved in COX-2 induction and PGE2 syn-
thesis in IMCD cells exposed to LPS as present in the medullary collecting duct during ascending bacterial infections. The underlying signaling mechanisms are of interest because COX-2-derived PGE2 is regarded as an important proinflammatory mediator (49). Various pathways were activated by binding of LPS to the TLR4 receptor complex and contribute to COX-2 expression: 1) an NF-κB pathway, 2) ectodomain shedding of pro-TGF-α with subsequent auto-/paracrine activation of EGFR, 3) an EGFR-dependent activation of the MAPKs p38 and ERK1/2; and 4) an EGFR-independent activation of JNK, the MAPK which negatively regulates NF-κB activation and hence COX-2 expression (Fig. 8).

Activation of an NF-κB pathway by LPS is essential for induction of COX-2 expression, which was not surprising as this signaling pathway has been previously reported in various cell types and tissues (4, 24, 33). However, our findings show that NF-κB activity is negatively regulated by JNK, which probably accounts for the observation that pharmacological inhibition of JNK increases LPS induced COX-2 expression. This phenomenon was unexpected, as, to our knowledge, JNK has only been described as a positive regulator of renal COX-2 expression (10, 48, 50). Negative regulation of NF-κB activity is probably mediated via the transcription factor c-jun, as knockdown of c-jun increases NF-κB activity in mouse fibroblasts (39) and in murine renal collecting duct cells (38). The underlying mechanism has been investigated in more detail in Drosophila: LPS-induced activation of JNK activates AP-1, consisting of c-jun and c-fos, which binds to NF-κB-activated promoters and recruits histone-deacetylase to these promoters. Modifications of the local histone acetylation pattern can subsequently downregulate the activity of the respective promoters (20). JNK may also regulate NF-κB activity by additional mechanisms. We found that inhibition of JNK slightly enhanced phosphorylation and subsequent degradation of IkBα and significantly enhanced phosphorylation of p65 and thereby stimulates NF-κB activity. These results are in accordance with a study on multiple myeloma cell lines (16), in which the authors demonstrated that JNK inhibition stimulates IKK phosphorylation and hence NF-κB p65 subunit phosphorylation. The negative regulation found in the present study may be necessary for fine tuning of COX-2 expression in IMCD-3 cells and/or to prevent an inadequate proinflammatory response through excessive NF-κB activation.

Expression of COX-2 in renal medullary cells has been investigated particularly with respect to the response to osmotic stress or dehydration. Regulation of COX-2 under these conditions showed some similarities but also some differences compared with LPS-induced COX-2 expression. In renal epithelial cell lines, MAPK activation seems to be a common signaling pathway during toxicity-induced COX-2 expression (27, 47, 50). In contrast, NF-κB signaling has not been described in toxicity-induced COX-2 expression in these studies. We examined NF-κB-driven reporter activity in the renal epithelial cell lines IMCD-3 and Madin-Darby canine kidney (MDCK) cells in response to osmotic stress and found no activation in both cell lines (data not shown), indicating that activation of NF-κB seems not to occur during toxicity-induced COX-2 expression in these cells. In contrast, in medullary interstitial cells toxicity-induced COX-2 expression appears to depend on NF-κB rather than on MAPK signaling (13).

Although it has been known for almost two decades that LPS can stimulate MAPK signaling (5, 12), data regarding the molecular details of the mechanism by which the TLR4 receptor complex or its adaptor proteins trigger MAPK activation are limited. In the present study, we found evidence that EGFR signaling is involved in activation of p38 and ERK1/2. Activation of EGFR may occur by ligand-dependent and -independent mechanisms (17). A common principle in ligand-dependent EGFR activation is the shedding of a membrane-resident pro-EGFR ligand with subsequent auto-/paracrine receptor activation (30). In the present study, we found evidence that binding of LPS to the TLR4 receptor triggers ectodomain shedding of pro-TGF-α and release of mature TGF-α with subsequent activation of EGFR and downstream MAPKs p38 and ERK1/2. Ectodomain shedding of pro-TGF-α is mediated by the MMP TACE (42), which is abundantly expressed in the IMCD (data not shown). Accordingly, siRNA-mediated knockdown of TACE or EGFR significantly decreased LPS-induced COX-2 expression in IMCD-3 cells (Fig. 6), indicating that the activation of a TACE→TGF-α→EGFR→MAPK axis is involved in LPS-induced COX-2 expression in renal epithelial cells. This signal transduction scheme seems to be of general importance for the regulation of renal medullary COX-2, as we have previously described activation of this signaling pathway also during toxicity-induced COX-2 expression in MDCK cells (27).

Activation of the EGFR in epithelial cells by pathogen-associated molecular patterns has been reported previously in other tissues (22, 23, 41, 45). Particularly, Finzi et al. (9) observed TGF-α release in human biliary carcinoma cells in response to LPS with downstream activation of EGFR and ERK1/2 to activate COX-2 expression. These findings and the observations of the present study suggest that TLR4-mediated activation of EGFR may play an important role in triggering the innate immune response in epithelial cells in response to bacterial invasion.

ERK1/2 or p38 inhibition had no substantial effect on NF-κB activity (Fig. 3A), suggesting that other transcriptional factors are stimulated by the EGFR-MAPK axis to enhance COX-2 expression. The COX-2 promoter contains various cis elements, which are probably differentially activated under changing physiological or pathophysiological conditions in the kidney (14). Activation of AP-1 (21) or CREB (31) has been observed during LPS-induced COX-2 expression in macrophages; however, reporter gene assays showed that neither AP-1 nor CREB was activated by LPS in IMCD-3 cells. We identified the transcriptional activator SP-1 as the most promising candidate to be activated by p38 and ERK1/2, as the SP-1 inhibitor mithramycin A (Fig. 7) or siRNA-mediated knockdown of SP-1 (Fig. 6) decreased LPS-induced COX-2 expression in IMCD-3 cells. This is in accordance with previous studies, in which SP-1 has been shown to be activated by an EGFR-p38 cascade to increase COX-2 expression in human glioma cells (46); SP-1 activation is also reportedly involved in neuronal COX-2 expression in response to oxidative stress (29).

In summary, we have shown that TLR4 regulates the expression of COX-2 in IMCD cells in response to LPS by multiple mechanisms. In particular, we have identified TGF-α-mediated activation of EGFR-MAPK signaling and activa-
tion of NF-kB, negatively regulated by JNK, as critical steps for transcriptional activation of COX-2.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

Author contributions: C.K. performed experiments; F.-X.B. edited and revised manuscript; F.-X.B. approved final version of manuscript; W.N. conception and design of research.

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