Ectodomain shedding of pro-TGF-α is required for COX-2 induction and cell survival in renal medullary cells exposed to osmotic stress

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Küper C, Bartels H, Fraek ML, Beck FX, Neuhofer W. Ectodomain shedding of pro-TGF-α is required for COX-2 induction and cell survival in renal medullary cells exposed to osmotic stress. Am J Physiol Cell Physiol 293: C1971–C1982, 2007. First published October 17, 2007; doi:10.1152/ajpcell.00404.2007.—In the renal medulla, cyclooxygenase (COX)-2 is induced by osmotic stress as present in this kidney region during antidiuresis. Increasing evidence suggests that EGF receptor (EGFR) signaling is involved in this process. The aim of the present study was to examine the mechanisms responsible for COX-2 expression and PGE2 production during hypertonic conditions and to identify potential autocrine/paracrine EGFR ligands. Immunohistochemistry and Western blot analysis revealed abundant expression of the pro-EGFR ligand pro-transforming growth factor (TGF)-α in renal medullary cells in vivo and in cultured Madin-Darby canine kidney cells. In Madin-Darby canine kidney cells, hypertonicity rapidly increased TNF-α converting enzyme (TACE)-dependent ectodomain shedding of pro-TGF-α; phosphorylation of EGFR, p38, and ERK1/2; expression of COX-2; and production of PGE2. Conversely, TACE inhibition prevented TGF-α release; EGFR, p38, and ERK1/2 activation; and COX-2 expression. Furthermore, cell survival was reduced substantially, a response that could be reversed by the addition of PGE2. Simultaneous addition of recombinant TGF-α during TACE inhibition restored EGFR and MAPK phosphorylation, COX-2 expression, PGE2 production, and cell survival during osmotic stress. These results indicate that hypertonicity induces TACE-mediated ectodomain shedding of pro-TGF-α, which subsequently activates COX-2 expression in an autocrine/paracrine fashion, via EGFR and MAPKs. We conclude that tonicity-induced TGF-α release is required for COX-2 expression, PGE2 synthesis, and survival of renal medullary cells during osmotic stress.

During antidiuresis, renal medullary cells are exposed physiologically to extremely high interstitial solute concentrations, in particular NaCl and urea. Because most cellular membranes are readily permeable to urea, urea does not cause cell shrinkage and, therefore, does not represent a major osmotic challenge. In contrast, NaCl is functionally excluded from the intracellular space by virtue of the action of the Na-K-ATPase. Therefore, extracellular solute concentrations in the form of NaCl initially cause cell shrinkage and a rise in cellular ionic strength. Cell survival under this condition depends on the activation of various osmosensitive genes, including those mediating the intracellular accumulation of compatible organic osmolytes and those of specific heat shock proteins (23). The enhanced expression of transporters and synthesis enzymes for organic osmolytes and several osmosensitive heat shock proteins during osmotic stress requires the interaction of the tonicity inducible transcription factor tonicity-responsive enhancer binding protein (TonEBP)/nuclear factor of activated T cells (NFAT) 5, with tonicity-responsive elements in the promoter region of the respective target genes (31).

Several other tonicity-inducible proteins that are not regulated by TonEBP/NFAT5 have been identified in recent years, including cyclooxygenase (COX)-2. COX-2 is induced in epithelial and interstitial cells of the renal medulla in vivo during antidiuresis and in cultured medullary cells exposed to elevated ambient tonicity (7) and is believed to account for the majority of PGE2 formed in the renal papilla in the concentrating kidney (33). COX-2-derived PGE2 is vitally important for the integrity and function of renal medullary cells by modulating medullary blood flow and tubular solute reabsorption and by stimulating the expression of osmoprotective genes (14, 15, 21, 24). Because release of free arachidonic acid (AA) by the enzyme cytosolic phospholipase A2 (cPLA2) represents the rate-limiting step for prostaglandin synthesis, the coordinated action of both cPLA2 and COX-2 is a prerequisite for PGE2 formation during osmotic stress.

COX-2 is induced by various stimuli, such as osmotic stress, phorbol esters, mitogens, or UV irradiation (3, 8, 36). Induction of COX-2 by hypertonicity was first demonstrated in renal medullary cells by Cowley et al. (7). In recent years, interest in the mechanism of tonicity-induced COX-2 expression has increased in view of the latter’s pivotal role for the integrity and function of renal medullary cells during antidiuresis. However, COX-2 is not regulated through the TonEBP/NFAT5 pathway. Recent studies by Zhao et al. (39) and Yang et al. (32) have suggested that epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) signaling are implicated in COX-2 induction in response to hypertonicity. In addition, EGFR-dependent activation of cPLA2 and AA release in an EGFR-dependent manner has been demonstrated in keratinocytes exposed to osmotic shock (27). Therefore, the EGFR may play a central role for the coordinated action of both cPLA2 and COX-2 during a rise in ambient tonicity in renal medullary cells.

Activation of EGFR may occur by ligand-dependent and -independent mechanisms (5, 16, 30). A common principle in ligand-dependent EGFR activation is the shedding of a membrane-resident pro-EGFR ligand with subsequent autocrine/paracrine receptor activation (11). The family of autocrine/paracrine pro-EGFR ligands contains EGF, transforming growth factor (TGF)-α, heparin-binding EGF-like growth factor, amphiregulin, betacellulin, and epiregulin (19, 26). A
major enzyme responsible for the ectodomain shedding of pro-EGFR ligands is the metalloproteinase TNF-α converting enzyme (TACE) (29). TACE belongs to a family of zinc-dependent, transmembrane proteases, also referred to as a disintegrin and metalloproteinase-17 (2). TACE activation has been reported in response to various stimuli, including reactive oxygen species (ROS), nitric oxide, and phorbol 12-myristate 13-acetate, all of which also increase COX-2 expression (10, 13, 34, 35).

It thus appears possible that a putative autocrine/paracrine pro-EGFR ligand mediates osmotic stress-induced COX-2 expression in renal medullary cells, although such a ligand has not been identified so far. Nor is it known whether EGFR activation occurs in a ligand-dependent or -independent manner, or whether a potential pro-EGFR ligand is involved in tonicity-induced COX-2 expression. The present study thus addressed the mechanism of tonicity-induced COX-2 expression and ePLA2 activation in renal medullary cells using a cell culture model, with focus on the dependence on a potential pro-EGFR ligand-EGFR interaction and the subsequent intracellular events. An additional aim was to address the functional relevance of this series of events with regard to cell survival during osmotic stress.

MATERIALS AND METHODS

Materials. TNF-α processing inhibitor (TAPI)-1 was obtained from Peptides International (Louisville, KY). AG1478, SB-202190, U-0126, SP-600125, AA, PGE2, N-acetylcysteine (Nac), and NS-398 were from Sigma (Deisenhofen, Germany). Arachidonyl trifluoromethyl-ketone was obtained from Biozol (Eching, Germany). TGF-α was purchased from PeproTech (London, UK). Polyclonal anti-COX-2 antibody was from Cayman Chemicals (Ann Arbor, MI).

Fig. 1. Expression of pro-transforming growth factor (TGF)-α in renal medullary cells. A: intrarenal distribution of pro-TGF-α in different kidney zones of normal rat kidney, as determined by immunoblot analysis. To demonstrate that equal amounts of protein from the different kidney regions were loaded, the blots were additionally stained for actin. B: immunohistochemistry for pro-TGF-α in paraffin-embedded tissue sections of cortex and outer and inner medulla of normal rat kidney. The bar represents 100 μm. C: expression of pro-TGF-α in Madin-Darby canine kidney (MDCK) cells. Confluent MDCK cells were exposed to isotonic (300 mosmol/kgH2O) or hypertonic medium (600 mosmol/kgH2O by adding NaCl) for 20 h. To demonstrate equal protein loading, the blots were additionally stained for actin.
Polyclonal anti-actin antibody was from Sigma. Monoclonal anti-TGF-α antibody was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal goat anti-TGF-α antibody was from R&D Systems (Wiesbaden, Germany). Polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204) antibody and anti-phospho-EGFR (Tyr1173) were from Santa Cruz Biotechnology. Polyclonal anti-phospho-cPLA2 (Ser505) and anti-phospho-p38 (Thr180/Tyr182) antibodies were purchased from New England Biolaboratories (Beverly, MA). Rabbit polyclonal anti-mouse IgG and horseradish peroxidase-conjugated anti-rabbit IgG were from Jackson Immunoresearch (West Grove, PA). Biotin-conjugated rabbit anti-goat antiserum was obtained from Vector Laboratories (Burlingame, CA). 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) was from Roth (Karlsruhe, Germany). Arachidonoyl-phosphorylcholine was purchased from Alexis (Lörrach, Germany).

Cell culture and experimental protocol. Madin-Darby canine kidney (MDCK) cells obtained from the American Type Culture Collection (CCL-34; ATCC, Manassas, VA) were maintained in DMEM 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 MDCK cells were preincubated with the respective compound as indicated or with vehicle alone for 1 h before the medium was changed. Medium osmolality was raised gradually by addition of NaCl in 10 steps (30 mosmol/kgH2O each) to a final osmolality of 600 mosmol/kgH2O over a period of 4.5 h by dropwise addition of the required volume from a 4 M NaCl stock solution. Thereafter, cells were incubated for the indicated periods. In experiments measuring lactate dehydrogenase (LDH) release, medium osmolality was raised in one step to 700 mosmol/kgH2O by addition of NaCl in 10 steps (30 mosmol/kgH2O each) to a final osmolality of 700 mosmol/kgH2O over a period of 4.5 h. In experiments assessing the phosphorylation status of EGFR, ERK1/2, p38, and cPLA2, and cPLA2 activity, and for measurement of soluble TGF-α, the cells were preincubated as described above, but medium osmolality was raised in one step to 600 mosmol/kgH2O by dropwise addition of the required volume from a 4 M NaCl stock solution, and the cells were incubated further for the indicated times. Northern blot analysis. Following the experimental treatments, the cells were lysed by the addition of 0.5 ml Trisfast reagent (Peqlab, Erlangen, Germany), and total RNA was recovered according to the manufacturer’s recommendations. Aliquots (20 µg) were subjected to electrophoresis through 1% agarose/formaldehyde gels, blotted onto nylon membranes (Hybond-N+; Amersham, Freiburg, Germany), and immobilized by ultraviolet-cross-linking. For detection of COX-2 mRNA expression, a biotinylated COX-2 cDNA probe was prepared. Therefore, gel purified COX-2 cDNA was subjected to random-primed extension by the large fragment of DNA polymerase Klenow (MBI Fermentas, St. Leon-Rot, Germany) in the presence of dNTPs and biotin-11-dUTP (MBI Fermentas). For Northern blot analysis, the blots were prehybridized for 2 h at 55°C in a solution containing 50% formamide, 5% SSC, 0.1% SDS, and 10% blocking reagent (Roche, Mannheim, Germany) and were hybridized overnight in the same solution containing 20 ng/ml biotinylated probe. After hybridization, the membranes were washed twice for 15 min each with 2× SSC/0.1% SDS at room temperature and twice for 15 min with 0.1× SSC/0.1% SDS at 55°C. Nonradioactive detection procedures were as described elsewhere (22). To correct for differences in RNA loading, the membranes were stripped and rehybridized with biotinylated cDNA probe specific for glyceraldehyde phosphate dehydrogenase (22).

Western blot analysis. Following the experiments in 24-well plates, the cells were washed three times with chilled PBS and lysed by the addition of 50 µl 8 M urea/PBS per well. For determination of the phosphorylation status of EGFR, ERK1/2, p38, and cPLA2, the cells were washed and lysed directly by addition of 1× SDS sample buffer. For detection of pro-TGF-α in rat kidney, frozen kidney specimens dissected into cortex and outer and inner medulla 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 Biotechnology, 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 over night at 4°C with the following dilutions: 1:10,000 for COX-2 and phospho-ERK1/2; 1:5,000 for actin, TGF-α, and anti-phospho-cPLA2; 1:2,000 for anti-phospho-p38; and 1:1,000 for anti-phospho-EGFR and anti-TGF-α. Following incubation with primary antibodies, the blots were washed three times with PBS-T for 5 min each. Subsequently, the membranes were incubated with appropriate secondary and/or tertiary antibodies conjugated with horseradish peroxidase at room temperature for 1 h at a dilution of 1:5,000. After washing with PBS-T three times for 5 min each, immunocomplexes were visualized by enhanced chemiluminescence (SuperSignal West Pico Stable Peroxide Solution, Pierce, Rockford, IL).

Fig. 2. Ectodomain shedding of pro-TGF-α during osmotic stress. A: time course of pro-TGF-α shedding under hypertonic conditions. Confluent MDCK cells remained in isotonic medium (osmolality 300 mosmol/kgH2O) or were exposed to a increase in tonicity to a final osmolality of 600 mosmol/kgH2O by NaCl addition. At the indicated times, medium samples were collected, and the concentration of soluble TGF-α was determined as described in MATERIALS AND METHODS. Values are means ± SE for n = 3 per point. *P < 0.05 vs. isotonic control. B: effect of TNF-α protease inhibitor (TAPI-1) on pro-TGF-α shedding under hypertonic conditions. Confluent MDCK cells were incubated for 1 h in isotonic medium (osmolality 300 mosmol/kgH2O) or in hypertonic medium (osmolality 600 mosmol/kgH2O) by adding NaCl in the presence of the TNF-α converting enzyme (TACE) inhibitor TAPI-1 (10 µM) or vehicle (DMSO). Thereafter, medium samples were collected, and the concentration of soluble TGF-α was determined as described in MATERIALS AND METHODS. Values are means ± SE for n = 3. #P < 0.05 vs. isotonic control. *P < 0.05 vs. hypertonic + vehicle.
**Immunohistochemistry.** All experiments were conducted in accordance with German federal laws relating to animal experimentation. Normal rat kidneys were cut into 2-mm slices along the corticomedullary axis, fixed overnight in 4% paraformaldehyde in PBS, embedded in paraffin, and cut into 5-μm-thick sections. Endogenous peroxidase activity was blocked with 3% H2O2 for 10 min. Thereafter, the sections were rinsed with PBS, and nonspecific binding sites were blocked by incubation with 3% rabbit serum in PBS for 30 min at room temperature. Subsequently, sections were incubated with a goat polyclonal anti-TGF-α antiserum, diluted 1:10 in 3% rabbit serum in PBS overnight at room temperature, and subsequently with secondary biotin-conjugated rabbit anti-goat antiserum (1:200 in 3% rabbit serum in PBS). Immunocomplexes were visualized using an ABC detection kit, according to the manufacturer’s protocol (Vectastain; Vector Laboratories) with diaminobenzidine tetrahydrochloride/0.1% H2O2 in PBS as chromogen substrate. Finally, the sections were counterstained with hemalaun and mounted. In negative controls, the primary antibody was omitted.

**Measurement of soluble TGF-α.** For measurement of soluble TGF-α, confluent MDCK cells were preincubated in serum-free medium for 1 h with TAPI-1 (10 μM) (9) or vehicle alone, as indicated. Subsequently, medium tonicity was increased to 600 mosmol/kgH2O by adding NaCl, and the cells were incubated further for 10 min to 6 h. Thereafter, the supernatant was collected, and sodium deoxycholate was added to a final volume of 0.02%. Following incubation on ice for 30 min, trichloroacetic acid was added to a final volume of 15%, and the samples were further incubated on ice over night. Subsequently, the samples were centrifuged at 14,000 g for 15 min at 4°C, the supernatants discarded, and the precipitates were washed with acetone and finally resuspended in PBS containing 1% BSA. TGF-α content in the samples was determined using a commercially available ELISA system (R&D Systems), according to the manufacturer’s protocol. Briefly, 96-well plates were coated with 0.4 μg/ml TGF-α capture antibody (diluted in PBS) overnight at room temperature, washed with PBS-T three times, and then incubated with PBS containing 1% BSA for 1 h. After being washed once with

![Fig. 3. Role of the TACE-TGF-α-epidermal growth factor receptor (EGFR) axis on hypertonicity-induced cyclooxygenase (COX)-2 mRNA expression. Confluent MDCK cells were preincubated with TAPI-1 (10 μM), AG1478 (10 μM), or vehicle DMSO under isotonic conditions (300 mosmol/kgH2O) for 1 h. In some experiments, recombinant TGF-α (50 ng/ml) was added where indicated. Subsequently, the medium tonicity was increased gradually over a period of 4.5 h to 600 mosmol/kgH2O by NaCl addition (controls remained in isotonic medium). A: after reaching the final osmolality of 600 mosmol/kgH2O, the cells were further incubated for 2 h and subsequently processed for COX-2 mRNA expression by Northern blot analysis. To demonstrate equal RNA loading, the blots were stripped and reprobed for GAPDH. Representative blots from 3 independent experiments are shown. B: quantification of the results. Relative COX-2 mRNA abundance was normalized to that of GAPDH to correct for differences in RNA loading. Values are means ± SE for n = 3. #P < 0.05 vs. isotonic. *P < 0.05 vs. hypertonic + vehicle.](http://ajpcell.physiology.org/content/full/293/6/C1974/F3)

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PBS-T, each well was incubated with 100 μl of samples or TGF-α standard for 2 h at room temperature. Following washing, the wells were incubated with biotin-conjugated anti-TGF-α antibody (0.3 μg/ml) for 2 h at room temperature. Following color development, the reactions were stopped by the addition of 50 μl H2SO4 (0.5 N). Finally, the plates were read at 450 nm in a plate reader, and TGF-α concentrations were determined from the standard curve.

**PGE2 measurement.** PGE2 concentrations in 100-μl medium aliquots 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.

**LDH assay.** LDH release into the medium was employed as an index of cell death. MDCK cells were grown in 96-well plates and treated as indicated. Subsequently, 100-μl aliquots of supernatant were removed from each well and saved, and the remaining adherent cells were lysed in 100-μl 1% Triton X-100 for 30 min. Subsequently, aliquots of 100 μl of both fractions were incubated with 100-μl chromogenic LDH substrate (Promega, Madison, VA), and the reaction was incubated for 30 min at room temperature and read with a microplate reader at 492 nm. Relative LDH activity was expressed as the ratio of LDH activity in the supernatant to total LDH activity (LDH activity in supernatant plus LDH activity in lysate).

**Determination of cPLA2 activity.** The glycerophospholipid arachidonoyl-thio-phosphorylcholine was used as substrate in this assay. Following the hydrolysis of the arachidonoyl-thioester bond at the sn-2 position by cPLA2, the free thioester is detected by the addition of Ellman’s reagent DTNB (25). Briefly, MDCK cells were lysed by passing 10 times through a 20-gauge needle and centrifuged at 10,000 g for 10 min at 4°C, and cPLA2 activity was measured in the supernatant. To eliminate measurement of any calcium-independent PLA2 activity in the samples, bromoenol lactone, a specific inhibitor...
of calcium-independent PLA₂, was added at 5 μM, and the samples were incubated for 15 min at 25°C. For measurement of cPLA₂ activity, aliquots of 10 μl were mixed with 5-μl assay buffer containing (in mM) 80 HEPES, pH 7.4, 150 NaCl, 10 CaCl₂, 4 Triton X-100, 30% glycerol, and 1 mg/ml BSA. The reaction was initiated by the addition of 200 μl 1.5 mM arachidonoyl-thio-phosphorylcholine in 0.5× assay buffer, and the mixture was incubated for 60 min at room temperature. The reaction was stopped and developed by the addition of 10 μl of a solution containing 475 mM EGTA and 25 mM DTNB. The color was allowed to develop for 5 min at room temperature, and absorbance was read at 405 nm using a plate reader.

RESULTS

Pro-TGF-α is highly abundant in the renal medulla. The expression of pro-TGF-α was determined in the cortex and outer and inner medulla of the normal rat kidney. While only low amounts were detected in the cortex by immunoblotting, substantially higher amounts were found in the outer and inner medulla (Fig. 1A). The apparent molecular mass of pro-TGF-α, ~20 kDa, is in agreement with published data (17). The same intrarenal distribution was observed after immunohistochemical analysis. While in cortex, only distal tubules were positive, staining for pro-TGF-α increased robustly in tubular epithelial cells along the corticomedullary axis, with the most intense signals observed in outer medullary collecting duct cells (Fig. 1B). Only minimal immunoreactivity was found in vascular structures and renal medullary interstitial cells. The presence of pro-TGF-α was also confirmed in MDCK cells, which are frequently used as a model system for collecting duct cells. In immunoblots, a band with a molecular mass of ~50 kDa could be detected (Fig. 1C), which is in accordance with the reported molecular mass of canine pro-TGF-α of ~49 kDa (20a). Incubation in hypertonic medium had no significant effect on the amount of pro-TGF-α (Fig. 1C).

Ectodomain shedding of pro-TGF-α during osmotic stress. To establish whether pro-TGF-α is processed by ectodomain cleavage in response to hypertonic stress, the concentration of soluble TGF-α released by MDCK cells into the medium was determined. To this end, medium tonicity was raised in one step to 600 mosmol/kg H₂O by NaCl addition, and medium samples were assessed for soluble TGF-α at times between 10 and 360 min after onset of osmotic stress. As shown in Fig. 2A, processing of pro-TGF-α occurred very rapidly and time dependently with increased concentrations of soluble TGF-α within 10 min. Pro-TGF-α processing has been reported to be mediated by the metalloprotease TACE. To test whether TACE is also involved in hypertonicity-induced pro-TGF-α ectodomain shedding in MDCK cells, the cells were preincubated with TAPI-1, a specific pharmacological TACE inhibitor. As demonstrated in Fig. 2B, TGF-α release during osmotic stress was almost completely prevented by TAPI-1, suggesting that pro-TGF-α processing during osmotic stress is mediated by TACE. Qualitatively similar results were obtained when the medium tonicity was raised nearly linearly over several hours (data not shown).

TGF-α mediates COX-2 expression and PGE₂ synthesis during osmotic stress. To determine whether pro-TGF-α shedding during hypertonicity is involved in COX-2 expression, we assessed COX-2 mRNA (Fig. 3) and protein (Fig. 4) abundance under various experimental conditions. As evident from Figs. 3 and 4, COX-2 expression was almost absent in cells incubated in isotonic medium. Conversely, raising the medium tonicity strongly increased COX-2 expression. Preincubation of the cells with the TACE inhibitor TAPI-1 attenuated hypertonicity-induced COX-2 expression significantly, both at the mRNA (Fig. 3) and protein levels (Fig. 4). This effect could be reversed by additional supplementation with recombinant TGF-α. Furthermore, EGFR inhibition by AG1478 again decreased COX-2 expression, suggesting that TACE-mediated pro-TGF-α shedding and subsequent EGFR activation are required for COX-2 induction during osmotic stress. To establish whether addition of TGF-α is sufficient for COX-2 induction in the absence of osmotic stress, recombinant TGF-α was

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**Fig. 5.** Role of the TACE-TGF-α-EGFR axis on hypertonicity-induced and PGE₂ synthesis. Confluent MDCK cells were preincubated with TAPI-1 (10 μM), AG1478 (10 μM), or vehicle DMSO under isotonic conditions (300 mosmol/kg H₂O) for 1 h. In some experiments, recombinant TGF-α (50 ng/ml) was added where indicated. Subsequently, the medium tonicity was increased gradually over a period of 4.5 h to 500 mosmol/kg H₂O by NaCl addition, and the cells were incubated for a further 20 h (controls remained in isotonic medium). Subsequently, medium samples were saved, and PGE₂ concentration was determined as described in MATERIALS AND METHODS. Values are means ± SE for n = 3. #P < 0.05 vs. isotonic. *P < 0.05 vs. hypertonic + vehicle.
added to the medium under isotonic conditions. As shown in Figs. 3 and 4, TGF-α, however, failed to induce COX-2 expression when added to isotonic medium.

To address the question of whether TGF-α is the major EGFR ligand required for COX-2 expression, or whether other EGFR ligands are involved in this process, the cells were preincubated with TGF-α-neutralizing antibody before increasing the medium tonicity. As demonstrated in Fig. 4, this maneuver almost completely abolished COX-2 expression, further illustrating the major role of TGF-α in this process (Fig. 4).

As expected, PGE₂ concentrations in the medium, a measure of COX activity, correlated with expression of COX-2 under various experimental conditions (Fig. 5). Accordingly, PGE₂ concentration was increased in hypertonic medium, and this increase was prevented by pharmacological inhibition of TACE or EGFR. The effect of TACE inhibition was counteracted by simultaneous addition of recombinant TGF-α, while addition of recombinant TGF-α to isotonic medium had no effect on PGE₂ concentration.

TGF-α mediates EGFR and MAPK phosphorylation during osmotic stress. Previous reports have suggested that activation of EGFR and the MAP kinases ERK1/2 and p38 under hypertonic conditions are involved in COX-2 expression in different renal cell systems (32, 39). Consistent with these results, preincubation with the EGFR inhibitor AG1478, the p38-inhibitor SB-202190, or the ERK1/2 inhibitor U-0126 abolished COX-2 expression almost completely during osmotic

Fig. 6. Effect of MAP kinase inhibitors on tonicity-induced COX-2 expression. MDCK cells were preincubated with SB-202190 (10 μM), SP-600125 (20 μM), U-0126 (10 μM), or with vehicle DMSO for 1 h under isotonic conditions (300 mosmol/kgH₂O). Subsequently, the medium tonicity was increased gradually over a period of 4.5 h to 600 mosmol/kgH₂O by NaCl addition, and the cells were incubated for a further 20 h (controls remained in isotonic medium). Thereafter, COX-2 expression was determined by immunoblotting. To demonstrate comparable protein loading, the blots were also probed for actin. Representative blots from 3 independent experiments are shown.

Fig. 7. Activation of EGFR, ERK1/2, and p38 by TGF-α. Confluent MDCK cells were preincubated with TAPI-1 (10 μM), AG1478 (10 μM), or vehicle DMSO under isotonic conditions (300 mosmol/kgH₂O) for 1 h. In experiments with TAPI-1, recombinant TGF-α (50 ng/ml) was added where indicated. Subsequently, the medium tonicity was increased in one step to 600 mosmol/kgH₂O by NaCl addition, and the cells were incubated further for 1 h (controls remained in isotonic medium). Thereafter, phosphorylation of EGFR, ERK1/2, and p38 was determined by immunoblotting using phospho-specific antibodies, as described in MATERIALS AND METHODS. To demonstrate comparable protein loading, a representative immunoblot for actin is shown.

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stress, while preincubation with the JNK inhibitor SP-600125 had no significant effect (Fig. 6). Based on this observation, we determined whether TACE-mediated pro-TGF-α shedding is involved in activation of EGFR, p38, and ERK1/2 during osmotic stress. Exposure of MDCK cells to hypertonic medium (final medium osmolality of 600 mosmol/kgH$_2$O) for 60 min significantly increased phosphorylation of EGFR, p38, and ERK1/2 (Fig. 7). Phosphorylation of all three molecules was decreased substantially by preincubation with TAPI-1 and could be restored by additional supplementation with recombinant TGF-α (Fig. 7). Furthermore, preincubation of the cells with the EGFR inhibitor AG1478 prevented activation of EGFR, as expected, and also decreased activation of p38 and ERK1/2, indicating that activation of both MAP kinases depends on EGFR. Addition of recombinant TGF-α to the medium under isotonic conditions caused phosphorylation of EGFR and ERK1/2, but not of p38 (Fig. 7).

**Activation of cPLA$_2$ during hypertonicity.** Another important enzyme in prostaglandin synthesis is cPLA$_2$, which catalyzes the hydrolysis of glycerophospholipids at the sn-2 position to release free AA. To address the question of whether osmotic stress also increases cPLA$_2$ activity and consequently AA release as substrate for COX-2, activation of cPLA$_2$ was determined by assessing phosphorylation at Ser505 and by enzyme activity assay. MDCK cells were incubated for 1 h in isotonic medium (300 mosmol/kgH$_2$O) or in hypertonic (600 mosmol/kgH$_2$O by NaCl addition) medium under various experimental conditions. As shown in Fig. 8, basal cPLA$_2$ activity and phosphorylation were even detectable when the cells were incubated in isotonic medium. Switching the cells to hypertonic medium increased both cPLA$_2$ activity and phosphorylation two- to threefold (Fig. 8). This observation suggests that cPLA$_2$ phosphorylation correlates with enzyme activity and that hypertonicity increases cPLA$_2$ activity.

![Phosphorylation and activation of cPLA$_2$](image_url)
Inhibition of the EGFR by AG1478 diminished cPLA₂ phosphorylation and activity under hypertonic conditions, while neither the addition of TAPI-1 nor simultaneous addition of TAPI-1 and recombinant TGF-α had significant effects (Fig. 8). Both p38 and ERK1/2 reportedly regulate phosphorylation (and therefore activity) of cPLA₂ (18, 27). To determine which kinase is essential for cPLA₂ phosphorylation and activation during osmotic stress, the cells were preincubated with either TAPI-1, AG1478, or NS-398 under isotonic conditions (300 mosmol/kgH₂O) for 1 h. TGF-α (50 ng/ml), arachidonic acid (AA; 10 μM), or PGE₂ (20 μM) were also added where indicated. Subsequently, the cells remained in isotonic medium (controls), or were subjected to a gradual tonicity increase to 700 mosmol/kgH₂O over 4.5 h by NaCl addition (NaCl). A: 20 h after reaching the final osmolality of 700 mosmol/kgH₂O, cell survival was determined by measuring lactate dehydrogenase (LDH) release. LDH release is expressed as percentage of total LDH activity of the respective cultures. Values are means ± SE for n = 3. *P < 0.05 vs. NaCl + vehicle. #P < 0.05 vs. NaCl + respective pharmacological inhibitor. B: representative phase contrast micrographs of cells remaining attached to the culture dish after 20 h under the indicated experimental conditions.
SB-202190 or U-0126 before elevating the medium tonicity. While treatment with U-0126 had no significant effect, treatment with SB-202190 diminished phosphorylation and activity to a level similar to isotonic conditions, indicating that p38 regulates phosphorylation and activity of cPLA2 in MDCK cells during osmotic stress in an EGFR-dependent manner.

Functional role of the TACE-TGF-α-EGFR axis in cell survival during osmotic stress. The functional relevance of the findings described in the previous section was assessed. Medium osmolality was increased nearly linearly to 700 mosmol/kgH2O by NaCl addition under the conditions described in Fig. 9. Comparable tonicity increases have been observed in the papilla of the mammalian kidney in vivo 2-4 h following acute stimulation of the urinary concentrating mechanism (1). Thereafter, the cells were incubated for a further 20 h, and cell viability was assessed by LDH release, and detachment of cells was estimated by microscopy. As demonstrated in Fig. 9A, LDH activity was slightly increased in cells exposed to hypertonic medium compared with isotonic controls. Preincubation with the TACE-inhibitor TAPI-1 before tonicity increase resulted in a drastic increase in cell death, which was reversed to control values by simultaneous addition of recombinant TGF-α or PGE2, clearly illustrating the significance of TGF-α-mediated PGE2 synthesis for cell survival during osmotic stress. Consistently, pretreatment with the EGFR inhibitor AG1478 caused substantial LDH release, which was prevented by simultaneous addition of PGE2. Moreover, interference with PGE2 synthesis by inhibition of either cPLA2 with arachidonoyl trifluoromethyl-ketone, an inhibitor of cPLA2, or COX-2 inhibition with NS-398, a selective COX-2 inhibitor, drastically elevated LDH release. Again, these effects were reversible upon simultaneous supplementation with AA or PGE2, respectively.

Qualitatively similar results were evident when assessing the number of the cells remaining attached to the culture dish, as shown in Fig. 9B, thereby illustrating the functional significance of the TACE-TGF-α-EGFR-COX-2 axis for cell survival during osmotic stress in view of PGE2 synthesis.

Involvement of ROS in pro-TGF-α shedding. Finally, the role of ROS in TGF-α release in MDCK cells was investigated. Generation of ROS in renal medullary cells during osmotic stress has been extensively documented both in vitro and in vivo (20, 34) as well as ROS-mediated activation of TACE (38). When H2O2 was added to isotonic medium, the release of TGF-α was significantly increased compared with the controls (Fig. 10). Conversely, preincubation with the antioxidant Nac caused a significant increase in TGF-α release (Fig. 10). Taken together, these results suggest that generation of ROS during osmotic stress contributes to pro-TGF-α ectodomain shedding in MDCK cells.

**DISCUSSION**

The aim of the present study was to gain further insight into the mechanisms involved in COX-2 induction and PGE2 synthesis in medullary cells exposed to osmotic stress, as present in the renal medulla during antidiuresis. The underlying signaling mechanism is important because COX-2-derived PGE2 is assumed to constitute the majority of prostanooids formed in the renal medulla of the concentrating kidney, and inhibition of COX-2 activity is associated with damage to renal medullary cells in vivo and in vitro (28). Activation of the EGFR and downstream signaling by all three members of the MAPK family (i.e., ERK1/2, p38, JNK) have been reported to be involved in COX-2 expression in medullary collecting duct cells during osmotic stress (32, 39). Activation of EGFR can occur by ligand-dependent and -independent mechanisms (5, 16, 30). The latter has been described recently in human keratinocytes exposed to osmotic shock (6). Since a potential autocrine/paracrine pro-EGF ligand responsible for toxicity-induced COX-2 expression has not yet been identified so far, the precise mechanism regulating COX-2 expression in renal medullary cells remained uncertain.

In the present study, we demonstrate that osmotic stress causes rapid shedding of pro-TGF-α and release of soluble TGF-α, mediated by the metalloproteinase TACE.

Enhanced production of ROS has been reported repeatedly in vivo and in vitro in renal medullary cells in response to hypertonicity (20, 37). Furthermore, Yang et al. (34) showed that generation of ROS is a prerequisite for COX-2 expression during osmotic stress. The present study gives a possible explanation for this observation: TACE contains an inhibitory prodomain and a catalytic metalloproteinase domain. It is presumed that, in the inactive state, a cysteine residue from the prodomain interacts with a zinc atom within the catalytic domain. Disruption of this cysteine-zinc bond leads to activation of TACE. ROS reportedly activate TACE by oxidizing the cysteine residue in the prodomain (2, 38). This would be consistent with the finding of enhanced shedding of pro-TGF-α upon exposure to H2O2. Furthermore, preincubation of MDCK cells with the antioxidant Nac efficiently attenuated TGF-α release during osmotic stress. Together, these results indicate that ROS, generated during osmotic stress, mediate toxicity-induced COX-2 expression via activation of TACE and enhanced ectodomain-shedding of pro-TGF-α.

Pharmacological inhibition of TACE by TAPI-1 significantly reduced release of TGF-α; phosphorylation of EGFR,
p38, and ERK1/2; and, finally, expression of COX-2 and PGE 
2 synthesis. Conversely, simultaneous addition of recombinant TGF-α during TACE inhibition restored phosphorylation of EGFR, p38, and ERK1/2; expression of COX-2; PGE 
2 synthesis; and cell survival during osmotic stress. Accordingly, pre-
incubation of MDCK cells with a TGF-α-neutralizing antibody 
drastically decreased hypertonicity-induced COX-2 expression. 
Although we cannot completely rule out the possibility 
that other EGFR ligands (e.g., amphiregulin or heparin-bind-
ing-EGF) also play a role in hypertonicity-induced COX-2 
expression, the fact that the TGF-α-neutralizing antibody sup-
pressed >80% of COX-2 expression suggests that TGF-α 
plays the predominant role in this process. The notion that 
shedding of pro-TGF-α is involved in the regulation of COX-2 
in the renal medulla in vivo is further supported by the 
intrarenal distribution of pro-TGF-α. Pro-TGF-α was 
abundantly expressed in the outer and inner medulla of rat kidney 
and in MDCK cells, which are frequently employed to address 
questions relating to osmosadaptation of renal medullary cells. 

Addition of recombinant TGF-α was not sufficient for COX-2 
induction under isotonic conditions, although activation of 
EGFR and ERK1/2, but not p38, was evident. Thus the lack 
for p38 activation by TGF-α in isotonicity probably explains 
the failure to induce COX-2 expression under this condition. 
This observation suggests the existence of additional, yet unknown 
signaling pathways, necessary for p38 activation during os-
motic stress. In addition to the mechanism underlying toxicity-
induced COX-2 expression, we also explored activation of 
cPLA 
2. cPLA 
2 is activated by phosphorylation on serine-505 
by p38 and/or ERK1/2, depending on the cell type and the 
stimulus (12, 18). Interestingly, EGFR-dependent cPLA 
2 activation 
has been demonstrated in HaCaT keratinocytes following 
osmotic shock (27). In the present study, we demonstrate 
that cPLA 
2 shows basal activity under isotonic conditions in 
MDCK cells and that enzyme activity is increased two- to 
threefold within 1 h after onset of osmotic stress. Pharmacolo-
logical inhibition of EGFR and p38, but not of ERK1/2, 
diminished cPLA 
2 phosphorylation and activity, indicating that 
an EGFR-p38-dependent pathway is involved in hypertonicity-
induced activation of cPLA 
2. Addition of TAPI-1, however, 
had no significant effect, despite attenuation of EGFR and p38 
phosphorylation. This finding may be explained by the fact that 
preincubation with TAPI-1 substantially decreased hypertonicity-
induced phosphorylation of EGFR and p38, but did not 
completely abolish it. Therefore, it is conceivable that osmotic 
stress induces EGFR activation in a ligand-independent fash-
ion. As mentioned above, osmotic stress induces the generation 
of ROS, causing oxidative stress in the cell. The EGFR exhibits 
significant spontaneous protein kinase activity (16). The phos-
phoryl groups can be removed from the EGFR by tyrosine 
phosphatases, but oxidative stress in the cell inhibits these 
phosphatases by a reversible sulfhydryl group oxidation in the 
active center of the enzymes, resulting in an enhanced EGFR 
phosphorylation (16). This could explain a basal, ligand-inde-
pendent EGFR activation during osmotic stress, which may be 
sufficient for cPLA 
2 activation, but not for COX-2 expression, 
the latter requiring more robust ligand-dependent EGFR acti-
vation, which was the case in view of TGF-α, as demonstrated 
in the present study.

The functional relevance of the present findings was finally 
addressed by examining cell survival during osmotic stress. 
Inhibition of TACE and pro-TGF-α ectodomain shedding 
caus ed a dramatic increase in cell death, effects that were 
prevented by simultaneous addition of recombinant TGF-α 
(Fig. 9). Accordingly, inhibition of EGFR, cPLA 
2, or COX-2 
had deleterious effects on cell viability, which could be pre-
vented by the addition of AA or PGE 
2, further demonstrating 
the importance of the TGF-α-EGFR-cPLA 
2-COX-2-PGE 
2 axis 
for cell survival during hypertonicity.

Taken together, we conclude that hypertonicity induces 
TACE-mediated ectodomain-shedding of pro-TGF-α in renal 
medullary cells. Subsequently, soluable TGF-α activates an 
EGFR-MAPK signaling pathway by autocrine/paracrine mech-
anisms, leading finally to expression of COX-2 and production 
of PGE 
2. Hypertonicity also increases the activity of cPLA 
2 in an 
EGFR-dependent mechanism, thereby releasing AA as 
substrate for COX-2. Both processes provide the basis for 
synthesis of PGE 
2, which is a critical process for cell survival 
in the harsh environment of the renal medulla during anti-
diuresis.

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