EGF receptor signaling is involved in expression of osmoprotective TonEBP target gene aldose reductase under hypertonic conditions

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Submitted 4 July 2008; accepted in final form 14 February 2009

Küper C, Steinert D, Fraek M-L, Beck F-X, Neuhofer W. EGF receptor signaling is involved in expression of osmoprotective TonEBP target gene aldose reductase under hypertonic conditions. Am J Physiol Renal Physiol 296: F1100–F1108, 2009. First published February 18, 2009; doi:10.1152/ajprenal.90402.2008.—Renal medullary cells adapt to their hyperosmotic environment by enhanced expression of various osmoprotective genes. Although it is clearly established that TonEBP contributes to the expression of these genes, neither the precise signaling mechanism by which hypertonicity activates TonEBP is completely understood, nor is it known whether a membrane-bound osmosensor, corresponding to yeast and bacteria, is present in mammalian cells. We found evidence that metalloproteinase (MMP)-dependent activation of the epidermal growth factor receptor (EGFR) signals to TonEBP and stimulates the expression of the TonEBP target gene aldose reductase (AR) under hypertonic conditions. Phosphorylation of EGFR and the downstream MAP kinases ERK1/2 and p38 was significantly enhanced by high NaCl in Madin-Darby canine kidney (MDCK) cells. Conversely, the broad-spectrum MMP inhibitor GM6001 or the EGFR inhibitor AG1478 diminished phosphorylation of EGFR, p38, and ERK1/2, the induction of AR mRNA and protein, and AR promoter reporter activity in response to hypertonicity. Accordingly, neutralizing antibodies against the putative EGFR ligand transforming growth factor-α (TGF-α) abolished AR induction during osmotic stress. Furthermore, toxicity-induced phosphorylation of p38 and ERK1/2 and expression of AR were reduced significantly in MDCK cells transfected with a dominant-negative Ras construct. These effects were not caused by reduced nuclear abundance of TonEBP during osmotic stress; however, inhibition of EGFR or p38 diminished TonEBP transactivation activity under hypertonic conditions. The contribution of MMP/EGFR signaling in vivo was confirmed in C57BL/6 mice, in which treatment with GM6001 was associated with reduced AR induction following dehydration. Taken together, these results indicate that osmotic stress induces MMP-dependent activation of EGFR, likely via shedding of TGF-α, and downstream activation of Ras and the MAP kinases p38 and ERK1/2, which stimulate TonEBP transactivation activity. This EGFR-Ras-MAPK pathway contributes to TonEBP transcriptional activation and targets gene expression during osmotic stress, thus establishing a membrane-associated signal input that contributes to the regulation of TonEBP activity.

osmotic stress; Ras signaling; MAP kinases; osmoadaptation

THE URINARY CONCENTRATING mechanism is of central importance for systemic water conservation, but necessarily entails extreme interstitial osmolalities in the renal papilla. In humans, papillary interstitial osmolality may reach 1,200 mosmol/kgH2O, whereas it may exceed 3,000 mosmol/kgH2O in water-deprived rodents (2). NaCl and urea account for the greatest part of renal medullary osmolality in the concentrating kidney, each contributing to roughly equally to interstitial osmolality in this kidney region. While urea equilibrates rapidly across most cellular membranes, and thus is not considered a major osmotic stressor, NaCl is functionally excluded from the intracellular space. Accordingly, a rise in extracellular NaCl concentration causes cell shrinkage and a rise in cellular ion strength due to a water shift from the intracellular to the interstitial compartment. In the longer term, i.e., hours to days, renal medullary cells balance intracellular and extracellular osmolality by accumulating small, nonperturbing organic compounds, i.e., compatible organic osmolytes, thereby reducing cellular ion strength. These substances are either taken up from the extracellular space, or synthesized intracellularly, the latter being the case for sorbitol, which is produced via reduction of glucose by the rate-limiting enzyme aldose reductase (AR). Sorbitol contributes substantially to the intracellular organic osmolytes in renal papillary cells (1). The enhanced expression of AR and other osmoprotective genes during osmotic stress is regulated by a common transcriptional activator, tonicity responsive enhancer binding protein (TonEBP), also known as nuclear factor of activated T cells (NFAT5), or osmotic response element binding protein (OREBP) (15, 43); in the following referred to as TonEBP.

In yeast, the adaptive response to hyperosmotic stress requires the activation of Hog1 MAPK, the homolog of mammalian p38, which mediates enhanced expression of enzymes required for synthesis of glycerol, which serves as a major osmolyte in these cells (34). Two distinct membrane-associated osmosensors signaling downstream to Hog1 have been defined in yeast (i.e., Sln1 and Sho1) (23), and a related two-component osmosensing machinery has been found in bacteria (9). A corresponding mechanism upstream from mammalian MAPK has not yet been described. We and others observed activation of the epidermal growth factor receptor (EGFR), p38, and ERK1/2 in cells exposed to osmotic stress (20, 50). In addition, previous studies suggested that both p38 and ERK1/2 are involved in induction of TonEBP target genes under hypertonic conditions (17, 38). It thus appears possible that EGFR and associated proteins represent a membrane-associated osmosensing complex. Activation of this machinery would entail downstream signaling to p38 and ERK1/2, which in turn contribute to enhanced transcriptional activity of TonEBP and induction of osmoprotective genes. The present study addressed this hypothesis using Madin-Darby canine kidney (MDCK) cells as a cell culture model.

METHODS

Cell culture and experimental protocol. MDCK (CCL 34 cells) obtained from the American Type Culture Collection (ATCC; Man-
assays, VA) were maintained in DMEM containing 10% FBS, 50 U/ml penicillin, and 50 µg/ml streptomycin at 37°C in 95% air-5% CO2. The cells were plated in 85-mm, 60-mm (Greiner, Frickenhausen, Germany), or 24-well plates (Nunc, Roskilde, Denmark) and used for experiments 2 days after reaching optical confluence. In experiments with pharmacological inhibitors AG1478 (Biozol, Eching, Germany), GM6001 (Biomol, Hamburg, Germany), SB202190 (Sigma, Deisenhofen, Germany), or U0126 (Sigma) or neutralizing TGF-α antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), the compounds or the respective vehicle were added 1 h before toxicity increase. For elevating the medium osmolality to 500 mosmol/kgH2O, the required volume of a 4 M NaCl stock solution was added drop by drop to the dishes and the cells were further incubated for the indicated periods and processed as indicated.

Transfection of cells. For disruption of Ras signaling, stably transfected MDCK cells expressing dominant-negative Ras (pCMV-Ras-N17; Clontech, La Jolla, CA) and control cells expressing wild-type Ras (pCMV-Ras; Clontech) were generated. pCMV-Ras-N17 expresses a dominant-negative form of the Ras protein that contains a serine-to-asparagine mutation at position 17, which blocks endogenous Ras signaling in mammalian cells. Cells were transfected using the calcium phosphate method as previously described (29). Following selection with 800 µg/ml geneticine, 24 clones obtained for each vector construct were expanded and further characterized. To identify stable MDCK cell clones with significant disruption of Ras signaling, each clone was transiently transfected with a serum-responsive element (SRE)-driven reporter construct based on secreted alkaline phosphatase (pSEAP-SRE; Clontech) followed by stimulation with 100 ng/ml transforming growth factor (TGF)-α. From the 24 clones initially obtained in cells transfected with pCMV-Ras-N17, 3–4 showed substantially repressed SRE reporter activity following exposure to TGF-α. These clones were pooled and used in further experiments. Pooled clones transfected with wild-type Ras were used as controls.

Northern blot and qRT-PCR analysis. Following the experiments, total RNA was recovered using TrisTrat Reagent (Péqlab, Erlangen, Germany) according to the manufacturer’s recommendations. For Northern blot analysis, 20 µg of total RNA were separated on 1% agarose/formaldehyde gels, blotted onto nitrocellulose membranes (Hybond N+; Amersham, Freiburg, Germany), and immobilized by ultraviolet crosslinking. The mRNA abundance of the respective genes was monitored using the following biotinylated cDNA probes: human AR, 1.0 kb EcoRI-HindIII fragment in pBluescript SK+ (32) and human GAPDH (X01677) (31). Generation of biotinylated probes with biotin-11-dUTP, hybridization conditions, and stringency washings were performed as described in detail elsewhere (20, 31). Hybridized probes were detected using a chemiluminescent nucleic acid detection kit (Pierce, Rockford, IL) according to the manufacturer’s protocol. Band intensity on X-ray films was quantified by Image J software (National Institutes of Health, Bethesda, MD) and the integrated band intensity on X-ray films was quantified by Image J software.

For qRT-PCR analysis of AR and actin expression in the renal inner medulla of mice, total RNA from the papilla was recovered using TrisTrat Reagent (Péqlab). Reactions were set up using the 2xSensiMix One Step Kit (Péqlab) with SYBR Green I to which gene-specific forward and reverse PCR primers were added (AR: forward, 5'-ATC GGA GCC AAG CAC AAT AA-3'; reverse, 5'-AGC AAT GGC TCT TGG TGA C-3'; actin: forward, 5'-CTA AGG CGG ACC GTG AAA AG-3'; reverse, 5'-ACC AGA GGC ATA CAG GGA CA-3'). PCR reactions were performed according to the manufacturer’s instructions using MX 3000 pPCR cycler (Stratagene). Specificity of PCR product formation was confirmed by monitoring melting point analysis and by nondenaturing polyacrylamide gel electrophoresis.

Preparation of nuclear and cytosolic extracts. Nuclear and cytosolic proteins were isolated using nuclear and cytoplasmic extraction reagent (NE-PER; Pierce) according to the recommendations of the supplier with broad specificity protease inhibitor cocktail (Sigma) added at 1:100 (vol/vol). Briefly, following the respective treatments, the nuclei in 60-mm dishes were washed with chilled PBS of equal osmolality as the experimental medium, and the cells were directly harvested by the addition of 200 µl cellular extraction reagent. After centrifugation at 13,000 g for 10 min at 4°C, the supernatant containing cytosolic proteins was saved and the pellet containing nuclear proteins was lysed by addition of 50 µl nuclear extraction reagent. Subsequently, nuclear and cytosolic protein fractions were stored at −80°C until use.

Western blot analysis. For determination of the phosphorylation status of EGFR, ERK1/2, and p38, the cells were washed with ice-cold PBS and lysed directly by addition of 1× SDS sample buffer. Nuclear and cytosolic protein extracts were prepared as described above. Aliquots of 30 µg protein were separated on 10% polyacrylamide gels and subsequently blotted onto nitrocellulose. Non-specific binding sites were blocked with 5% nonfat dry milk in PBS containing 0.1% Tween-20 (PBS-T) for 1 h at room temperature. Immunodetection of the respective proteins was performed by incubation with the following primary antibodies in PBS-T containing 5% nonfat milk overnight at 4°C: 1:10,000 for polyclonal anti-phospho-ERK1/2 (Thr202/Tyr204; Santa Cruz Biotechnology); 1:2,000 for polyclonal anti-phospho-p38 (Thr180/Tyr182; New England Biolabs, Beverly, MA); 1:2,000 for polyclonal TonEBP antibody (Calbiochem, Darmstadt, Germany); 1:1,000 for polyclonal anti-phospho-EGFR (Santa Cruz Biotechnology); 1:5,000 for polyclonal anti-actin (Sigma); 1:5,000 for monoclonal anti-histone H1 (Millipore, Billerica, MA); 1:5,000 for anti-rat lens AR (Sigma); and 1:5,000 for anti-GRB2 (Santa Cruz Biotechnology). Following incubation with primary antibodies, the blots were washed three times with PBS-T for 5 min each and subsequently incubated with peroxidase-conjugated goat anti-rabbit secondary antibody (1:5,000) for 1 h at room temperature. After being washed with PBS-T three times for 5 min each, the immunocomplexes were visualized by enhanced chemiluminescence (Pierce). Band intensity was analyzed by Image J software.

Reporter gene assays. Transcriptional activity of TonEBP was assessed using the SEAP system (Clontech). pSEAP-AR contains a 1.5-kb fragment (nucleotides −1505 to +22) derived from the 5′-flanking region of the human AR open reading frame which contains three TonE in the native context (18), followed by SEAP. The AR promoter fragment was amplified by PCR from genomic DNA from HEK 293 cells as described previously (30). MDCK cells were transfected using Effectene (Qiagen, Hilden, Germany). Briefly, exponentially growing MDCK cells were cotransfected with 20 µg plasmid DNA [pSEAP-AR and pcDNA3.1 (Intronig, Karlsruhe, Germany) at a ratio of 20:1], followed by selection with 600 µg/ml genetecin (Sigma), and clones with highest toxicity-inducible SEAP activity were pooled and used in experiments. SEAP activity was determined as described in detail elsewhere (30).

TonEBP transactivation assay. TonEBP transactivation activity was determined using the GAL4 binary assay as initially described by Ferraris et al. (7). pGAL4-TonEBP-TAD contains the yeast GAL4 DNA binding domain fused in-frame to the transactivation domain (TAD) of TonEBP (amino acids 548–1531; kindly provided by Dr. J. Ferraris, National Institutes of Health, Bethesda, MD). pFR-SEAP contains five tandem repeats of the GAL4 binding site upstream of a minimal promoter and the SEAP gene (Stratagene, Amsterdam, The Netherlands). Briefly, 4 × 104 cells were electroporated with 20 µg pGAL4-TonEBP-TAD and 20 µg pFR-SEAP at 350 V and 950 µF using a BioRad Gene pulser Xcell apparatus (Bio-Rad, Hercules, CA), and subsequently plated into 4–6 wells of a 24-well plate. After 24–48 h, the cells were treated as indicated and SEAP activity in the medium was determined as described above.

Animal studies. All experiments were conducted in accordance with German federal laws relating to animal experimentation. The review committee Regierung von Oberbayern approved the animal.
experiments in Munich. Male C57BL/6 mice (20–25 g) were injected intraperitoneally with 10 mg·kg\(^{-1}\)·day\(^{-1}\) GM6001 in 100 μl PBS or only vehicle PBS for 3 consecutive days. During the last 48 h of the experiment, the animals were water deprived or had free access to tap water. At the end of the experiment, the mice were anesthetized with pentobarbital sodium and the inner medullas from both kidneys were immediately isolated, snap-frozen in liquid nitrogen, and stored at −80°C.

Presentation of data and statistical analysis. Data are presented as means ± SE. Differences between the means were tested with Student’s t-test for statistical significance. \(P < 0.05\) was regarded as significant.

RESULTS

Osmotic stress activates a metalloproteinase-sensitive EGFR-dependent MAPK pathway. To determine the role of auto-/paracrine EGFR activation in osmoadaptation, the activation of the latter and downstream events was investigated in MDCK cells. High osmolality increased phosphorylation of EGFR, which was significantly reduced by preincubation with the metalloproteinase (MMP) inhibitor GM6001 (Fig. 1A), suggesting that the inhibitory effect of GM6001 on EGFR activation is due to reduced shedding of a putative pro-EGFR ligand under these conditions. We also investigated the activation of the downstream regulated MAPK p38 and ERK1/2. Phosphorylation of both kinases was significantly enhanced during hyperosmotic conditions which were abolished by GM6001 or by the EGFR inhibitor AG1478 (Fig. 1A), suggesting that activation of p38 and ERK1/2 depends on EGFR.

We next investigated involvement of the GTP-binding protein Ras, which plays a central role in receptor tyrosine kinase signal transduction pathways. In the “classical” MAP kinase pathway, EGFR-induced Ras activation mediates the phosphorylation of ERK1/2. Ras, however, may also participate in the activation of p38 (4, 14, 40). To determine whether Ras participates in the activation of ERK1/2 and/or p38, MDCK cells were transfected stably with dominant-negative Ras (MDCK-Ras-N17) or wild-type Ras (MDCK-Ras). In MDCK-Ras, osmotic stress activated both ERK1/2 and p38 (Fig. 1B). In contrast, phosphorylation of both p38 and ERK1/2 was diminished in MDCK-Ras-N17, indicating involvement of Ras in EGFR-mediated activation of p38 and ERK1/2.

EGFR signaling via MAP kinases is involved in toxicity-induced TonEBP activation and target gene expression. To address the question of whether EGFR activation in hypertonicity signals to TonEBP, the effects of the broad-spectrum MMP inhibitor GM6001 and the EGFR inhibitor AG1478 on hypertonicity-induced expression of AR, an established TonEBP target gene, were assessed in MDCK cells. Both compounds significantly attenuated the induction of AR at the mRNA (Fig. 2, A–B; protein (Fig. 2, C–D) level under hypertonic conditions, suggesting that shedding of a putative EGFR ligand with subsequent auto-/paracrine EGFR activation is involved. As already previously shown (35), inhibition of p38 also repressed toxicity-induced TonEBP target gene expression. We were able to extend these previous results, demonstrating that inhibition of p38 reduces both AR mRNA and protein abundance under hypertonic stress. Surprisingly, ERK1/2 inhibition with U0126 also diminished AR mRNA expression, although there was no significant effect at the protein level (Fig. 2, C–D). The reason for this unexpected observation is currently not clear.

We previously observed shedding of the EGFR ligand TGF-α with subsequent EGFR activation in MDCK cells under hypertonic conditions (20). To assess whether TGF-α may also be the ligand responsible for EGFR activation involved in toxicity-induced AR expression, MDCK cells were preincu-
bated either with a goat polyclonal TGF-α antibody, raised against the COOH terminus of TGF-α, or a mouse monoclonal TGF-α antibody, raised against an epitope corresponding to a domain encompassing cysteine 16, and measured tonicity-induced AR expression. As shown in Fig. 3, AR expression in cells preincubated with both TGF-α antibodies was reduced compared with cells incubated with preimmune sera of both species, probably due to neutralization of TGF-α. These results suggest that TGF-α may be the ligand responsible for tonicity-induced EGFR activation (Fig. 3).

To establish whether the effects on AR expression are paralleled by changes in TonEBP activity, MDCK cells were transfected stably with a reporter construct driven by a 1.5-kb fragment of the human AR promoter, which contains three TonE sites in the native context. Consistent with the results described above, AR promoter-driven reporter activity was strongly induced by hypertonicity, and this induction was diminished significantly by inhibition of EGFR and p38, as was the case for the MMP inhibitor GM6001, while the ERK1/2 inhibitor U0126 had no significant effect (Fig. 4).

Expression of AR was also tested in MDCK-Ras cells and in MDCK-Ras-N17 cells, in which tonicity-induced signaling from EGFR to p38 and ERK1/2 is impaired due to the dominant-negative Ras. As expected, induction of AR, both at the
transcriptional and translational level, was evident in MDCK-Ras cells in response to osmotic stress (Fig. 5, A–C). In contrast, the degree of induction was significantly reduced in MDCK-Ras-N17 cells, clearly demonstrating the importance of EGFR-Ras-MAPK signaling for TonEBP target gene expression.

**TonEBP expression and nuclear accumulation during high osmolality are not affected by inhibition of EGFR or MAPKs.**

The overall activity of TonEBP is regulated at different stages, e.g., by directed trafficking between the cytoplasm and the nucleus (6) and by enhanced expression during hypertonicity (5). Thus, we determined TonEBP protein levels and cytosolic and nuclear abundance of TonEBP under various conditions after 6 and after 24 h. Under isotonic conditions, TonEBP was detected predominantly in the cytoplasmic protein fraction, with only minor amounts in the nuclear fraction (Fig. 6). Following osmotic stress, nuclear TonEBP abundance increased substantially. Inhibition of EGFR, or ERK1/2 and p38, however, had no significant effect on nuclear accumulation of TonEBP neither after 6 (Fig. 6) nor 24 h (data not shown), suggesting that EGFR signaling is not involved in nuclear transition of TonEBP during osmotic stress.

**Inhibition of EGFR or p38 affects transactivation activity of TonEBP during high osmolality.** TonEBP contains a COOH-terminal tonicity-inducible transactivation domain (TonEBP-TAD; aa 872–1271). To investigate whether EGFR and MAPKs modulate transactivation activity of TonEBP, a GAL4 binary system was used. An expression vector encoding the broad-spectrum metalloproteinase inhibitor GM6001 (30 μM), the EGFR inhibitor AG1478 (10 μM), the p38 inhibitor SB202190 (10 μM), or vehicle DMSO under isotonic conditions was cotransfected into MDCK cells together with the reporter vector pFR-SEAP, in which expression of the SEAP gene is under the control of a synthetic promoter with five tandem repeats of the yeast GAL4 DNA binding sites. In the resulting reporter system, the amount of SEAP in the medium correlates with the activity of the TonEBP-TAD (7). As expected, high osmolality increased TonEBP-TAD activity severalfold (Fig. 7), which was blunted by inhibition of EGFR or p38. In contrast, inhibition of ERK1/2 had no significant effect (Fig. 7), suggesting that the EGFR-Ras-p38 pathway modulates the activity of TonEBP-TAD during hypertonicity.

**Effect of GM6001 on AR expression in the renal papilla in vivo.** To test whether the results obtained in vitro in MDCK cells may also be relevant in vivo, 6-wk-old male C57/BL6 mice were treated with GM6001. One group of mice was deprived of water for 48 h to stimulate the urinary concentrating mechanism, while the other group had free access to drinking water. The expression of AR in the renal inner medulla was determined at the transcriptional level by qRT-PCR and at the translational level by Western blotting. These results showed that AR expression was enhanced in water-deprived mice, suggesting that antidiuresis stimulates AR expression, as expected (Fig. 8, A–C). Administration of GM6001 reduced AR expression in both groups compared with controls (Fig. 8, A–C), although these changes did not reach statistical significance of P < 0.05. Nevertheless, the in vivo data support a role of the TGF-α-EGFR-MAPK-TonEBP signaling pathway in AR expression in vivo.
DISCUSSION

The precise signal transduction pathway(s) leading to enhanced expression of osmoprotective genes in mammalian cells exposed to osmotic stress is still under debate. Osmosensing appears to be conserved through evolution from yeast to mammals, including signaling from the cell membrane to the nucleus. While this mechanism is well described for yeast and bacteria, a potential membrane-bound osmosensor has not yet been identified in mammalian cells (33, 39). It is well-established that the expression of genes involved in osmolyte accumulation and that of specific heat shock proteins like HSP70 and OSP94 is regulated by TonEBP (43). In response to osmotic stress, the transcriptional activity of TonEBP increases rapidly and drives the expression of TonEBP target genes by binding to tonicity-responsive elements (TonE) in their 5' region (43). Several kinase pathways have been implicated in TonEBP activation, including MAPKs, Fyn, Syk, ATM kinase, and DNA-PK (3, 11, 13, 26, 35, 49). All three major members of the MAPK family have been demonstrated to be activated following osmotic stress (37, 48). Among these, signaling through p38 to TonEBP has been demonstrated in kidney cells (33), and it was reported that osmotic induction of the TonEBP target gene OSP94 is sensitive to inhibition of both p38 and ERK1/2 (19), although the upstream events are poorly charac-
characterized, in particular a membrane-associated input remains to be identified.

In the present study, we provide evidence that the EGFR is activated via a MMP-sensitive mechanism in response to osmotic stress and that EGFR inhibition with AG1478 blunts the expression of TonEBP transcriptional activity and the expression of AR, an established TonEBP target gene. Furthermore, MMP inhibition diminished EGFR phosphorylation and inhibition of EGFR resulted in loss of phosphorylation of p38 and ERK1/2 under hypertonic conditions, suggesting that both MAPK receive MMP-dependent EGFR input during osmotic stress. EGFR activation entails Ras-dependent ERK1/2 phosphorylation by localizing MAPK kinase kinase (MKKK) to the plasma membrane where it is activated (24, 42). Our observations that tonicity-induced phosphorylation of p38 decreases in MDCK cells during MMP and EGFR inhibition and in MDCK cells transfected with dominant-negative Ras suggest that not only ERK1/2 activation but also EGFR-dependent p38 activation are mediated by Ras. Previous findings showed an involvement of MEKK3 in hypertonicity-induced p38 activation (33), hence it is conceivable that EGFR activates a Ras→MEKK3→p38 axis.

Several mechanisms are conceivable by which MAPKs could modulate expression of TonEBP-regulated genes during hypertonic stress. 1) Relocalization of TonEBP. It is well-established that TonEBP is localized primarily in the cytoplasm in isotonicity, whereas it translocates to the nucleus.
during osmotic stress (27, 44). Phosphorylation of serines 155 and 158 is involved in this process (6, 45). However, the data from the present study do not support the notion of a role of EGFR, ERK1/2, or p38 in TonEBP trafficking between the cytoplasm and the nucleus, as inhibition of EGFR or the MAP kinases did not influence TonEBP localization. 2) Activation of TonEBP-associated factors. Like other members of the NFAT family, TonEBP interacts with other transcriptional factors, e.g., activator protein (AP)-1, resulting in stabilization of the ternary complex at the DNA level, thus enhancing transcriptional activity (22). p38 is known to activate the AP-1 factors c-Jun (25) and c-Fos (36), hence it is conceivable that toxicity-induced activation of the EGFR-Ras-MAPK pathway influences TonEBP activity via enhanced AP-1 recruitment. Interestingly, Irrazabal et al. (12) showed recently that AP-1 is needed for full activity of the so-called TonEBP enhanceosome in kidney cells and that p38 is required for the action of AP-1 in this process. However, contribution of this mechanism in toxicity-induced AR expression remains to be elucidated.

3) Regulation of the TonEBP TAD. TonEBP stimulates transcriptional activity by a COOH-terminal TAD. (21). In the present study, we used a GAL4 binary reporter system to clarify the question whether EGFR signals to the TonEBP-TAD during osmotic stress. Our results indicate that an EGFR-Ras-p38 pathway stimulates the activity of the TAD of TonEBP, while ERK1/2 seems not to contribute to this process. Both p38 and ERK1/2 have been reported to be involved in modulation of the TonEBP-TAD activity (17, 38). Details of the modulatory mechanism of p38 and ERK1/2 on TonEBP-TAD activity are currently not known. However, although the TAD is subject to phosphorylation, the latter event seems not correlate with transactivation activity. While the present results clearly demonstrate involvement of EGFR and p38 in toxicity-induced TonEBP activation in MDCK cells, our results regarding the role of ERK1/2 remain ambiguous; inhibition of ERK1/2 clearly reduced transcription of the AR gene, but AR protein levels, AR reporter activity, and TonEBP-TAD activity were unaffected. The reason for these unexpected results is not clear.

Activation of the EGFR under hypertonic conditions is probably mediated, at least partially, by one or more MMPs. Administration of the broad-band MMP inhibitor GM6001 in MDCK cells significantly reduced hypertonicity-induced EGFR phosphorylation and attenuated AR expression and AR promoter reporter activity. Furthermore, GM6001 also decreased AR expression in the renal inner medulla of C57/BL6 mice, indicating that this signal transduction pathway also contributes to AR expression in vivo. Several putative mechanisms may cooperate during the activation of EGFR under hypertonic conditions. As shown in the present study, activation of MMPs is necessary for maximum activity of EGFR during osmotic stress. This is probably mediated by MMP-dependent shedding of a pro-EGFR ligand (like EGF, HB-EGF, or TGF-α) (8, 10, 20). In the present study, we found strong evidence that TGF-α plays an important role in MDCK cells, since preincubation with different TGF-α neutralizing antibodies blunted the toxicity-induced AR expression. This is in accordance with a previous study from our laboratory, demonstrating that pro-TGF-α is highly abundant in the renal inner medulla and that pro-TGF-α shedding with subsequent EGFR and MAPK activation is involved in induction of cyclooxygenase (COX)-2 expression during osmotic stress (20). Additionally, ligand-independent mechanisms of EGFR activation by impairing the balance between intrinsic autophosphorylation activity of EGFR and dephosphorylation, mediated by various phosphatases (41, 47), may also occur. For both ligand-dependent and -independent mechanisms of EGFR activation, generation of reactive oxygen species (ROS) may play a crucial role. It is known that MMPs can be activated by ROS (28), and phosphatases involved in EGFR dephosphorylation are inhibited by ROS (16, 46). This explanation would also be consistent with the observations of Zhou et al. (51), who reported that during hypertonic stress, an increase in ROS generation contributes to an enhanced TonEBP activity.

In conclusion, the present study provides evidence that EGFR has a central function in a membrane-associated osmosensing complex that contributes to the regulation of osmoadaptive responses, reminiscent to the findings obtained in yeast and bacteria. The signal transduction pathway comprises ligand-dependent EGFR activation with subsequent Ras-mediated MAPK activation and contributes to enhanced expression of the TonEBP target gene AR, but also to non-TonEBP-regulated osmosensitive genes like COX-2 (20) during osmotic stress.

ACKNOWLEDGMENTS

Critical reading of the manuscript by Dr. J. Davis is gratefully acknowledged.

GRANTS

The present work was supported by grants from the Deutsche Forschungsgemeinschaft, the Friedrich-Baur-Stiftung Munich, and the Münchener Medizinische Wochenschrift.

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