MEKK3-mediated signaling to p38 kinase and TonE in hypertonically stressed kidney cells

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Padda, Ranjit, Ann Wamsley-Davis, Michael C. Gustin, Rebekah Ross, Christina Yu, and David Sheikh-Hamad. MEKK3-mediated signaling to p38 kinase and TonE in hypertonically stressed kidney cells. Am J Physiol Renal Physiol 291: F874–F881, 2006. First published May 9, 2006; doi:10.1152/ajprenal.00377.2005.—Mitogen-activated protein kinase (MAPK) cascades contain a trio of kinases, MAPK kinase kinase (MKKK) → MAPK kinase (MKK) → MAPK, that mediate a variety of cellular responses to different signals including hypertonicity. The signaling response to hypertonicity is conserved across evolution from yeast to mammals in that it involves activation of p38/SAPK. However, very little is known about which upstream protein kinases mediate activation of p38 by hypertonicity in mammals. The MKKKs, MEKK3 and MEKK4, are upstream regulators of p38 in many cells. To investigate these signaling proteins as potential activators of p38 in the hypertonicity response, we generated stably transfected MDCK cells that express activated versions of MEKK3 or MEKK4, utilized RNA interference to deplete MEKK3, and employed pharmacological inhibition of p38 kinase. MEKK3-transfected cells demonstrated increased betaine transporter (BGT1) mRNA levels and upregulated tonicity enhancer (TonE)-driven luciferase activity under isotonic (basal) and hypertonic conditions compared with empty vector-transfected controls; small-interference RNA-mediated depletion of MEKK3 downregulated the activity of p38 kinase and decreased the expression of BGT1 mRNA. p38 Kinase inhibition abolished the effects of MEKK3 activation on BGT1 induction. In contrast, the response to hypertonicity in MEKK4-kA-transfected cells was similar to that observed in empty vector-transfected controls. Our data are consistent with the existence of an input from MEKK3 → p38 kinase → TonE.

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MAPK kinase kinases (MKKKs; see review in Ref. 52). MEK kinase 1 (MEKK1; 1 of the MKKKs) is linked to JNK activation, whereas MEKK2 is linked to JNK and ERK activation (reviewed in Ref. 27). On the other hand, MEKK3 may activate ERK, JNK, and p38, whereas MEKK4 may activate JNK and p38 kinase (27). Hence, we hypothesized that MEKK3 and/or MEKK4 are likely mediators of p38 kinase activation in kidney cells under hypertonic conditions. Our data are consistent with the existence of MEKK3 → → → p38 kinase input to drive TonE-mediated gene expression.

EXPERIMENTAL PROCEDURES

 Constructs. For stable transfections, hemaglutinin antigen (HA)-tagged kinase active MEKK3 [HA-MEKK3-kA; corresponding to nt 1257–2200 of mouse MEKK4 (accession no. U43187); aa 309–622] and HA-tagged kinase active MEKK4 [HA-MEKK4-kA; corresponding to nt 3936–5153 of mouse MEKK4 (accession no. BC058719; aa 1302–1597)] were cloned in pCDNA3 (a generous gift from Dr. Gary Johnson, Univ. of Colorado, Denver, CO) (6, 16). For TonE-driven luciferase reporter assays, we used a 132-bp human aldolase reductase promoter fragment (nt 2032–2163, accession no. AF032455) containing TonE (23), and its surrounding sequence (51) or an insert spanning 1.5 kb of the human AR gene (nt 1801–3300, accession no. AF032455), which includes the TonE and other identified elements of the AR promoter (23, 41, 45, 51) to drive the pGL3 basic vector (constructs were a generous gift from Dr. Kurt Bohren, Baylor College of Medicine).

 Tissue culture and transfections. MDCK cells (ATCC, Manassas, VA), within passages 63–68, were grown to confluence in isotonic medium [315 mosmol/kgH2O; made of equal volumes of DMEM, low glucose and Coon’s-F12 media, supplemented with 10% fetal calf serum, 1% penicillin-streptomycin, and 2 mM glutamine (GIBCO BRL)]. Hypertonic conditions were generated by the addition of NaCl to basic isotonic media, lacking serum, betaine, inositol, or choline (a precursor for the organic solute glycerophosphorylcholine), bringing the final osmolality to 565 mosmol/kgH2O. Cells were exposed to hypertonic media for 16 h. In some experiments, we utilized 50 μM SB203580 or vehicle (equal volume of DMSO) for inhibition of the p38 kinase pathway. While lower concentrations of the inhibitor (5–10 μM) are usually sufficient for p38 kinase inhibition in most cells, MDCK cells require higher concentrations of the inhibitor for optimal p38 kinase inhibition, as we have shown previously (46). All cultures were maintained in 5% CO2–95% air at 37°C.

For stable transfection of MEKK3 or MEKK4, cells (0.25 × 106) were seeded into 10-cm-diameter polystyrene plates and grown for 24 h. The cells were transfected with 8 μg DNA/dish using FuGENE 6 as per the manufacturer’s instructions (Roche Diagnostics, Indianapolis, IN). Cells were transferred to regular media 24 h later, and selection was initiated by the addition of G418 at a concentration of 1,000 μg/ml. Following colony selection, cells are maintained in media containing 500 μg/ml G418. Surviving colonies were propagated and tested for incorporation of the desired constructs by PCR amplification of the construct sequence from genomic DNA, using vector-specific primers (data not shown). Transfected cells appeared morphologically similar to empty vector-transfected controls and formed domes after reaching confluence, a typical feature of MDCK cells. To eliminate inherent bias related to the integration site of the plasmid, we used pooled stable transfectants for all experiments. These pooled colonies exhibited stable phenotype and demonstrated highly reproducible results, even after multiple passages.

For small-interference (si)RNA experiments, human embryonic kidney-derived cells (HEK-293) were transfected (using DharmaFECT1; Dharmacon, Chicago, IL) with scrambled siRNA or SMARTpool MEKK3 siRNAs (total of 3; Dharmacon) for 8 h before experimental manipulation with hypertonicity and analysis of MEKK3 mRNA and protein abundance. BGT1 mRNA, p38 kinase, and phospho-p38 kinase. These cells were chosen for this experiment due to the ready availability of effective siRNAs for human MEKK3 sequences and the difficulty we encountered in establishing effective siRNAs for canine MEKK3. According to data sheets provided by the manufacturer, the scrambled siRNA (proprietary sequence, Dharmacon catalog no. D-001210–01-20) does not affect gene expression, as determined by gene array experiments. SMARTpool MEKK3 siRNAs were as follows: 1) 5’-GAU AGA AGC UCA AGC AUG AUU-3’; 2) 5’-AAA CUC AGC UUU AUG ACA AUU-3’; and 3) 5’-CCA AGC AGG UCC AUU UUG AUU-3’.

Reporter gene assays. For reporter gene assays, cells were grown to 50% confluence in 10-cm-diameter polystyrene dishes and were transiently cotransfected with pRL-TK (Renilla luciferase) and either 132-bp TonE-pGL3 (firefly luciferase) or 1.5-kb AR promoter-driven pGL3 (41), using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), and subjected to isotonic or hypertonic medium for 16 h. Transfection efficiency was determined using green fluorescent protein-expressing plasmids and averaged 40–50%. After hypertonic stress, cells were harvested by scraping with a cell scraper and centrifugation, and lysed at 4°C in 1 ml Triton lysis buffer (TLB; see kinase assay). The lysate was cleared of debris by centrifugation for 10 min (microcentrifuge, 14,000 rpm, 4°C), and 20 μl of the supernatant were analyzed for dual firefly and Renilla luciferase activity (10), using a dual luciferase kit (Promega) and a TD-20/20 luminometer (Turner Design Instruments, Sunnyvale, CA). Firefly luciferase activity was normalized to Renilla luciferase activity in each sample, and each experiment was carried out in triplicate.

Northern blot analysis. MDCK cells, stably transfected with MEKK3-ka, MEKK4-ka, or empty vector were grown to confluence and exposed to isotonic or hypertonic medium for 16 h as described above. The cells were scraped and harvested by centrifugation (5,000 g for 5 min at 4°C). Total RNA was isolated from the cell pellet using RNAzol (Tel-test). Equal amounts of RNA per lane were loaded onto a 1% agarose-2.2 M formaldehyde gel. The gel was electrophoresed and transferred to GeneScreen membrane (New England Nuclear, Boston, MA). Canine BGT1 cDNA (a gift from Dr. Moo Kwon, Johns Hopkins School of Medicine) (54) and full-length human β-actin cDNA (Clontech) were labeled with [γ-32P]ATP (Random Primed DNA Labeling Kit, Boehringer Mannheim) for use as probes. Probes were hybridized to the blots overnight at 42°C in a solution containing 40% formamide, 5× SSC (0.75M NaCl, 75 mM trisodium citrate, pH 7), 5× Denhardt’s solution [0.5% (wt/vol) polyvinylpyrrolidone, 0.5% (wt/vol) Ficoll 400], 0.5% SDS, 250 μg/ml salmon sperm DNA, 10 mM Tris, pH 7.5, and 10% dextran sulfate. The blots were washed under high stringency at 65°C as follows: twice in 3× SSC, 0.5% SDS for 30 min, and twice in 0.3× SSC, 0.5% SDS for 30 min. The blots were autoradiographed, and band intensities were quantitated using Image Tool software (University of Texas Health Science Center, San Antonio, TX). Relative band intensities were normalized to β-actin.

Real-time RT-PCR. Total RNA was isolated from the HEK-293 cell pellet using RNAzol (Tel-test), and real-time RT-PCR was performed to determine the abundance of MEKK3 and BGT1 mRNAs relative to GAPDH mRNA, essentially as was previously described (31). One-step RT was carried out using Maloney murine leukemia virus reverse transcriptase and oligo-dT to generate cDNAs (Invitrogen), followed by PCR using specific primers, SYBR Green PCR Reagents (Bio-Rad, Hercules, CA), and the Opticon DNA Engine (MJ Research, Water- town, MA) according to the manufacturers’ instructions. Five micrograms of total RNA were reverse transcribed followed by PCR (denaturation at 94°C for 2 min followed by 40 cycles of 94°C for 15 s, 58°C for 30 s, 72°C for 30 s, and a final extension at 72°C for 10 min). Primers used in this study included MEKK3, forward 5’-GAT GCC AGA AGC ACA TTT-3’, reverse 5’-ACC CAT GTT CTC GCC ATT-3’; BGT1, forward 5’-AGC CAG TTT GTC GTG GAG TCT-3’, reverse 5’-ACA GCA ATG GCA AGG ATG A-3’; and GAPDH, forward 5’-CAA TGA CCC CTT CAT TGA-3’.
CC-3', reverse 5'-GTT CAC ACC CAT GAC GAA CAT G-3'.

Reaction specificity was confirmed by electrophoretic analysis of products before real-time PCR, and bands of expected size were detected. Ratios for MEKK3/GAPDH and BGT1/GAPDH mRNAs were calculated for each sample and expressed as means ± SD.

**Kinase assays.** MAPK activity was determined as previously described (18, 39, 43) with slight modifications. The cells were exposed to isotonic or hypertonic medium for 16 h, scraped into the experimental medium, and harvested by centrifugation. The cell pellet was lysed in TLB, consisting of 20 mM Tris (pH 7.4), 137 mM NaCl, 2 mM EDTA, 1% Triton X-100, 25 mM β-glycerophosphate, 1 mM Na orthovanadate, 2 mM Na pyrophosphate, 10% glycerol, 1 mM PMSF, and 1 μg/ml leupeptin. The supernatant was collected by centrifugation at 15,000 g for 10 min. Anti-ERK1/2 (Upstate Biotechnology; the antibody cross reacts with ERK1 and ERK2), anti-MEK3 (Santa Cruz Biotechnology, Santa Cruz, CA), anti-MEK4 (Santa Cruz Biotechnology), or anti-HA antibodies (Santa Cruz Biotechnology) were bound to protein A and G-agarose. Cell lysates (100 μg protein) were then added to the agarose beads, and the immobilized kinases were precipitated by the antibody-agarose complex. The beads were washed three times in TLB, followed by three additional washes in kinase buffer (25 mM HEPES, pH 7.4, 25 mM β-glycerophosphate, 25 mM MgCl2, 2 mM DTT, 0.1 mM Na vanadate). Myelin basic protein (Sigma, St. Louis, MO) in kinase buffer containing 25 μM [γ-32P]dATP was added to the beads and incubated for 20 min at 30°C. The kinase reaction was stopped by centrifugation at 12,000 g for 2 min. The supernatant was resolved on a 15% SDS-PAGE, and the gel was dried and autoradiographed. Kinase activity was determined by the extent of incorporation of 32P into the myelin basic protein substrate. For JNK1, JNK2, p38α, or p38β assays, the above procedure was used except for the following: anti-p-p38α, anti-p38β, anti-JNK1 (Santa Cruz Biotechnology), or anti-JNK2 (StressGen, Victoria, AB) antibodies were used for immunoprecipitation, and Pansorbin (Calbiochem) was used to immobilize the antibodies; ATF-2 (Santa Cruz Biotechnology) was used as a substrate. The reaction supernatant was resolved on 10% SDS-PAGE. Each experiment was carried out in triplicate. Band intensities were quantitated using Image Tool software (University of Texas Health Science Center, San Antonio, TX), and statistical analysis was carried out using ANOVA.

**SDS-PAGE.** This method is based on Laemmli (28) with slight modifications. Briefly, equal amounts of protein were run on 12% reducing SDS-PAGE. Proteins were transferred overnight (4°C, 40 V) onto Hybond-ECL membrane (Amersham, Arlington Heights, IL), in Laemmli buffer (25 mM Tris, 52 mM glycine, pH 8.3). Blots were then blocked for 1 h at room temperature (RT) in TBST (20 mM Tris, pH 7.6, 137 mM NaCl, 0.05% Tween 20) containing 5% dry milk. This was followed by overnight incubation at 4°C with anti-p38 kinase (at a dilution of 1:500; Santa Cruz Biotechnology), anti-phospho-p38 kinase (at a dilution of 1:200; Santa Cruz Biotechnology), or rabbit anti-p-ERK5 polyclonal antibody (anti-Thr218/Tyr220 at a dilution of 1:1,000; Cell Signaling Technology, Beverly, MA). Incubations were carried out in TBST containing 5% fraction V bovine serum albumin. After a 15-min wash in TBST, the blots were incubated for 1 h (RT) with peroxidase-conjugated secondary antibody and diluted (1:2,000) in TBST containing 5% milk. The blots were then washed for 15 min in TBST, and protein bands were visualized using the ECL-Plus detection system (Amersham Life Sciences, Little Chalfont, Buckinghamshire, UK) as per the manufacturer’s instructions.

For HA, MEK3, and GAPDH detection, proteins were transferred overnight (4°C, 40 V) onto Hybond-ECL membrane in Laemmli buffer containing 10% methanol. Blots were blocked for 1 h at RT in PBST (50 mM NaPO4, pH 7.5, 100 mM NaCl, 0.05% Tween 20) containing 5% dried milk. This was followed by 1-h incubation at RT with polyclonal anti-HA (at a dilution of 1:50; Santa Cruz Biotechnology), goat anti-MEK3 (C-20; at a dilution of 1:500; Santa Cruz Biotechnology), or anti-GAPDH (at a dilution of 1:1,000; Santa Cruz Biotechnology) suspended in PBST containing 5% milk. After a 15-min wash in PBST, blots were incubated for 1 h (RT) with peroxidase-conjugated secondary antibody and diluted (1:1,000) in PBST containing 5% milk. Blots were then washed for 15 min in PBST and protein bands were visualized as discussed above.

**Statistical analysis.** Statistical analysis was carried out using Student’s t-test and ANOVA. A P value of <0.05 was considered statistically significant.

**RESULTS**

MEKK3 (but not MEKK4) activation upregulates the expression of BGT1 mRNA under isotonic and hypertonic conditions, whereas siRNA-mediated MEKK3 depletion diminishes BGT1 mRNA expression. Expression of MEKK3 and MEKK4 constructs was verified by Western blotting using anti-HA antibodies (Fig. 1). To document upregulation of MEKK3- and MEKK4-specific kinase activities, we examined the in vitro phosphorylation of myelin basic protein by HA-MEK3-kA and HA-MEK4-kA, using anti-HA antibodies to pull down the respective kinases (Fig. 2). The activity of HA-MEK3-kA, but not HA-MEK4-kA, was slightly higher under hypertonic conditions compared with isotonic conditions, consistent with hypertonicity-mediated activation of MEKK3, but not MEKK4. Of interest, stimulation of native MEKK3 activity by hypertonicity was not detected. This observation may reflect negative autoregulation. The NH2-terminal portions of MEKK3 contain PB1 domains, which function in an inhibitory capacity (42). Thus native MEKK3 activity 16 h into hypertonic stress may be attenuated, reflecting negative autoregulation; this autoregulation is absent in HA-MEK3-kA as the NH2-terminal portion containing the PB1 is not included in the construct, and hence, sustained activation of MEKK3 signaling is expected in MEKK3-kA-transfected cells. Similarly, native MEKK4 activity was not altered in HA-MEK4-kA-transfected cells (data not shown; because the results in MEKK4-kA-transfected cells were negative, we focused our illustrations in Fig. 2 on the MEKK3-relevant data). To determine the effect of HA-MEK3-kA and HA-MEK4-kA on the expression of hypertonic stress-relevant genes, we examined mRNA abundance of betaine transporter BGT1 under isotonic and hypertonic conditions. MDCK cells, stably transfected with kinase-active MEKK3, but not kinase active MEKK4 or empty vector (pcDNA3.1), showed upregulation of BGT1 under isotonic (Fig. 3A) and hypertonic (Fig. 3B) conditions.
3B) conditions. While the activity of HA-MEKK4-kA was not as robust as that of HA-MEKK3-kA, we believe that MEKK4 activation was sufficient for detection of gene-target effects. We observed a small decrease in the BGT1 mRNA level in MEKK4-kA-transfected cells under hypertonic conditions compared with pcDNA3.1-transfected cells, suggesting negative input from MEKK4 to BGT1, or potential interference by MEKK4-kA in native MEKK3-mediated signaling; however, this observation requires further analysis before definite conclusions are drawn. Incubation of HA-MEKK3-kA-transfected cells with SB203580 (a highly-specific p38 kinase inhibitor) blocked the induction of BGT1 mRNA in MEKK3-kA-transfected cells (Fig. 3C). The data suggest that BGT1 is a downstream target for MEKK3 and that the effect of MEKK3 on BGT1 is p38 kinase mediated.

To confirm the involvement of MEKK3 in the expression of BGT1 under hypertonic conditions, we examined BGT1 expression in HEK-293 cells treated with MEKK3 siRNA. These cells were chosen for this experiment because of the ready availability of effective siRNAs for human MEKK3 (but not canine MEKK3) and the fact that HEK-293 cells (thought to be of distal nephron origin) are osmotically active and have been previously utilized to study hypertonicity responses (14, 29, 30). As shown in Fig. 4A, treatment of HEK-293 cells with pooled MEKK3 siRNAs provides a >95% reduction in MEKK3 mRNA and an ~70% reduction in MEKK3 protein level (Fig. 4B) compared with scrambled siRNA-treated cells. Scrambled siRNA had no effect on MEKK3 protein abundance compared with nontreated control (data not shown). MEKK3 siRNAs-treated cells demonstrated a 75% reduction in p38 kinase activity (phospho-p38α kinase; Fig. 4B) and diminished BGT1 mRNA expression under isotonic and hypertonic conditions compared with scrambled siRNA-treated cells (BGT1 mRNA was 15% of control under hypertonic conditions and 60% of control under isotonic conditions). These data confirm the involvement of MEKK3 in p38 kinase activation and BGT1 expression.

MEKK3-kA enhances the expression of TonE under isotonic and hypertonic conditions. The regulation of many genes by hypertonicity, including those that contribute to the accumulation of organic osmolytes in hypertonically stressed cells, is similar. Promoter analyses of BGT1 (betaine transporter), SMIT (inositol transporter), taurine transporter, and AR suggest that the transcription of these genes is dependent on the
that TonEBP is subject to MEKK3 regulation. Thus, we aimed to determine whether MEKK3-mediated input to TonE and thus suggest a role for MEKK3 in the adaptive response of kidney cells to hypertonic stress. To elucidate the mechanism of MEKK3-mediated signaling to TonE, we examined MDCK cells stably transfected with empty vector, MEKK3-kA, or MEKK4-kA for the expression of luciferase reporter constructs (transient transfection) driven by the 1.5-kb AR promoter or the AR promoter-derived 132-bp fragment containing TonE. TonE-driven luciferase reporter gene expression in MEKK3-kA-transfected cells was 1.5-fold higher under isotonic conditions and 7-fold higher under hypertonic conditions compared with empty vector-transfected control (Fig. 5A). Similarly, the expression of a luciferase reporter construct driven by the 1.5-kb AR promoter was 1.5-fold higher under isotonic conditions and 3-fold higher under hypertonic conditions in MEKK3-kA-transfected cells, compared with empty vector-transfected controls (Fig. 5B). However, the expression of both reporter constructs in MEKK4-kA-transfected cells was similar to that seen in empty vector-transfected controls. Our data are consistent with an input from MEKK3 to TonEBP and TonE in the respective promoters. To elucidate the mechanism of BGT1 induction by MEKK3, we examined MDCK cells stably transfected with empty vector, MEKK3-kA, or MEKK4-kA for the expression of luciferase reporter constructs (transient transfection) driven by the 1.5-kb AR promoter or the AR promoter-derived 132-bp fragment containing TonE. TonE-driven luciferase reporter gene expression in MEKK3-kA-transfected cells was 1.5-fold higher under isotonic and hypertonic conditions, measured using real-time RT-PCR in MEKK3 siRNA-treated cells, and expressed as percentage of values observed in scrambled siRNA-treated controls. Results were normalized to GAPDH and represent the means of 3 independent determinations. To elucidate the mechanism of p38 kinase activity in this experiment represents the steady-state condition after 16-h incubation in isotonic or hypertonic media, which is different from the response of these kinases to acute (minutes) hypertonic stress, where activation of all MAPKs normally occurs (20, 53). In summary, our data are consistent with an MEKK3-mediated input to drive ERK5 and p38 kinase.

**DISCUSSION**

These data on gene expression in MEKK3-transfected cells provide novel insight into the mechanism of p38 kinase activation in the context of hypertonicity. MDCK cells expressing MEKK3-kA show increased BGT1 mRNA and TonE-mediated reporter gene expression, a response correlated with up-regulation of p38α and ERK5 and downregulation of ERK1/2. However, our data do not suggest a role for MEKK4 in the adaptive response of kidney cells to hypertonic stress. Thus induction of TonE-mediated gene expression in MDCK cells is consistent with an input from MEKK3 to p38 kinase or interaction between the transcription factor TonEBP and TonE.
MEKK3 to ERK5. Alternatively, it may result from downregulation of an inhibitory input from ERK1/2. However, addition of SB203580 (a specific inhibitor of p38 kinase) to the experimental media abolished the stimulatory effects of MEKK3 on BGT1 induction, suggesting that the effects of MEKK3 on BGT1 are p38 kinase mediated. Early studies suggested that ERK activity was not essential for transcriptional regulation of BGT1 and SMIT (26) or the stimulation of inositol uptake (5), and thus the observed differences in ERK activity in MEKK3-kA-transfected cells compared with pcDNA3.1-transfected controls were statistically significant (P < 0.01, using Student’s t-test and ANOVA).

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In yeast, cell survival in a hypertonic environment (7) and the induction of glycerol-3-phosphate dehydrogenase 1, which is responsible for the synthesis of glycerol in response to osmotic stress, are dependent on the MAPK HOG1, a homolog of p38 kinase (2, 7). Consistent with that, p38 kinase is required for induction of BGT1 mRNA as well as TonE-mediated gene expression under hypertonic conditions in mammalian cells (11, 22, 41, 46).

p38 Kinase is activated by the upstream MAPK kinases MKK3 and MKK6 (27). Both enzymes are highly selective for p38 and do not normally activate JNK or ERK (9, 12). They differ, however, in their substrate specificity with regard to p38 isoforms. Whereas MKK3 activates p38α and p38β preferentially, MKK6 can activate all known p38 isoforms (α, β, γ, and δ). Thus both MKK3 and MKK6 may be relevant to TonE-mediated gene expression, and whereas previous data employ-
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