Increased reactive oxygen species contribute to high NaCl-induced activation of the osmoregulatory transcription factor TonEBP/OREBP

Xiaoming Zhou,1 Joan D. Ferraris,2 Qi Cai,2 Anupam Agarwal,3 and Maurice B. Burg2

1Division of Nephrology, Department of Medicine, Uniformed Services University of the Health Sciences, Bethesda, Maryland; 2Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland; and 3Division of Nephrology and Hypertension, University of Alabama at Birmingham, Alabama

Submitted 22 December 2004; accepted in final form 8 March 2005

Zhou, Xiaoming, Joan D. Ferraris, Qi Cai, Anupam Agarwal, and Maurice B. Burg. Increased reactive oxygen species contribute to high NaCl-induced activation of the osmoregulatory transcription factor TonEBP/OREBP. Am J Physiol Renal Physiol 289: F377–F385, 2005. First published March 15, 2005; doi:10.1152/ajprenal.00463.2004.—The signaling pathways leading to high NaCl-induced activation of the transcription factor TonEBP/OREBP from molecular proline/sorbitol transporters (TonEBP/OREBP) remain incompletely understood. High NaCl has been reported to produce oxidative stress. Reactive oxygen species (ROS), which are a component of oxidative stress, contribute to regulation of transcription factors. The present study was undertaken to test whether the high NaCl-induced increase in ROS contributes to toxicity-dependent activation of TonEBP/OREBP. Human embryonic kidney 293 cells were used as a model. We find that raising NaCl increases ROS, including superoxide. N-acetylcysteine (NAC), an antioxidant, and MnTBAP, an inhibitor of superoxide, reduce high NaCl-induced superoxide activity and suppress both high NaCl-induced increase in TonEBP/OREBP transcriptional activity and high NaCl-induced increase in expression of BGT1 mRNA, a transcriptional target of TonEBP/OREBP. Catalase, which decomposes hydrogen peroxide, does not have these effects, whether applied exogenously or overexpressed within the cells. Furthermore, NAC and MnTBAP, but not catalase, blunt high NaCl-induced increase in TonEBP/OREBP transcriptional activity. N4-hydroxy-arginine, a general inhibitor of nitric oxide synthase, has no significant effect on either high NaCl-induced increase in superoxide or high NaCl-induced increase in TonEBP/OREBP transcriptional activity. The effect of ROS on the transcription factor TonEBP/OREBP remains to be seen whether ROS also contribute to high NaCl-induced activation of the transcription factor, TonEBP/OREBP. We confirm that high NaCl increases ROS, including superoxide, and find that the ROS are necessary for full high NaCl-induced activation of TonEBP/OREBP.

MATERIALS AND METHODS

Cells, cell culture, and chemicals. Human embryonic kidney 293 (HEK293) cells, purchased from ATCC (Manassas, VA), were incubated in Eagle’s minimal essential medium plus 10% fetal bovine serum in 5% CO2-95% air at 37°C. The osmolality of this control “isotonic” medium was 300 mosmol/kgH2O. MnTBAP was purchased from Calbiochem (San Diego, CA), dihydroethidium (DHE) from Molecular Probes (Eugene, OR), and all other chemicals from Sigma (St. Louis, MO). All antioxidants and probes were freshly prepared. Cells overexpressing human catalase were from the same clone used in previous studies (8).

The costs of publication of this article were defrayed in part by the payment of page charges. The article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.
ROS CONTRIBUTE TO ACTIVATION OF TonEBP/OREBP

Measurement of ROS. 2′,7′-Dichlorofluorescin diacetate (DCFH-DA) permeates cell membranes. Within cells it is cleaved to produce DCFH, which is trapped because it is poorly permeating. DCFH oxidized by free radicals, producing fluorescence. HEK293 cells were detached by trypsinization, preloaded with 20 μM DCFH-DA in phenol red-free culture medium for 30 min, then transferred either to control culture medium or hypertonic medium (osmolality raised to 500 mosmol/kg H₂O by adding NaCl) for the periods indicated. The intensity of fluorescence was analyzed by flow cytometry (FACS Calibur, Becton Dickinson) with excitation at 488 nm and emission at 535 nm. A gate was set to exclude signals from debris and aggregates. Results are expressed as fold change compared with corresponding controls. Ten thousand cells were analyzed in each assay and assays were run in duplicate.

Measurement of superoxide anions. Dihydroethidium (DHE) enters cells freely. Superoxides oxidize DHE into ethidium or a structurally similar product, which intercalates into DNA, producing fluorescence (5). One to 1.2 × 10⁶ cells were grown overnight on the optical glass bottom of a dish (MatTek, Ashland, MA) and preincubated with 30 μM DHE for 30 min. Then, the medium was replaced with a dye-free one at 300 mosmol/kg H₂O or at 450 mosmol/kg H₂O (NaCl added) for 45 min. The fluorophore was excited at 488 nm. Fluorescent emission at 525 nm was recorded by confocal microscopy (LSM510 Meta, Zeiss, Thornwood, NY) and analyzed with MetaMorph software.

Measurement of TonEBP/OREBP transcriptional activity. The reporter, −1233/−1105 IL2min-GL3, was constructed by inserting nucleotides −1233 to −1105 of the 5′-flanking region of the human aldose reductase (AR) gene into MluI/NheI sites upstream of the human IL-2 minimal promoter (47). The parent vector, containing an IL-2 minimal promoter upstream of the Photinus pyralis luciferase gene, was a gift from Dr. S. N. Ho (University of California, San Diego, CA). This portion of the AR gene contains potentiation elements one (TGGAAAAATAT) and two (AAATTTTITCCA), an osmotic response element (ORE; TGGAAAAATAT) and an AP-1 binding site (TGAGTTCATAGAGTGATGGAGGAGAGGCTTTTCTCACTTTAG, (13). An otherwise identical reporter to which binding of TonEBP/OREBP is prevented by mutation of the potentiating and ORE elements and the adjacent AP-1 site was used to control for specificity to TonEBP/OREBP. In the mutated version, the nucleotide sequences were changed to TGGAGTAATAT, AAAAAATATCAGACA, TGTTCTATCATC, and TGCTTTCAC, respectively, Mutations were performed using a QuickChange site mutagenesis kit (Stratagene, Carlsbad, CA), blasticidin selection, and screening for luciferase activity to select clones with the highest expression; 3 × 10⁴ cells were seeded in 96-well white view plates (Packard, Wellesley, MA) and incubated for 24 h at 300 mosmol/kg H₂O, then the medium was changed for 16 h to one still at 300 mosmol/kg H₂O or elevated to 500 mosmol/kg H₂O by adding NaCl. Luciferase activity was measured with Bright-Glo substrate (Promega, Madison, WI) in a Victor3 luminometer (Perkin Elmer, Wellesley, MA). Cell protein was determined with the BCA reagent (Pierce, Rockford, IL).

Measurement of BGT1 mRNA expression. This was performed as previously described (16): 2.5 × 10⁵ cells/well were plated in a six-well dish, then incubated at the indicated osmolality for 16 h. Total RNA was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and reverse transcribed with Taqman reverse transcription reagents (Applied Biosystems, Foster City, CA). Amplicons were detected with an ABI Prism 7900HT detection system (Applied Biosystems). Primers for the human BGT1 mRNA were 5′-TGT-TACGCTCTTACCTTTCTGA-3′ and 5′-GCAATGCTCTGTTCTC-CAAAAG-3′. The 6-carboxyfluorescein (FAM)-labeled probe was 5′-CTGCCTTGGACGACCTGCAACAA-3′. As a control for reverse transcription efficiency and loading, 18S RNA was measured at the same time, using primers and a probe from Applied Biosystems. Fold differences in RNA abundance between conditions (F) was calculated, as F = 2^(ΔCt)/2. E (± 0.056, n = 18, means ± SE) is the efficiency of the reaction determined from the results of reactions containing 8 or 80 ng of cDNA template. Ct1 and Ct2 are the numbers of cycles required to reach the threshold of amplicon abundance in respective experimental conditions (16).

Measurements of TonEBP/OREBP transactivation. This was measured using a yeast binary GAL4 reporter assay system, as previously described (16). Briefly, the assay system comprises cotransfection of an expression vector containing the TonEBP/OREBP transactivation domain (TAD), GAL4dbd-TAD, and a reporter plasmid (GAL4US-GL3). GAL4dbd-TAD contains in-frame insertion of cDNA coding for amino acids 548–1531 of TonEBP/OREBP in a vector containing the neomycin resistance gene (pFA-CMV, Stratagene). An otherwise identical construct, in which the GAL4dbd construct did not contain a TAD, was used as a control for nonspecific effects. GAL4US-GL3 contains five tandem repeats of the yeast GAL4 binding site (upstream activating sequence) and a minimal promoter (TATAATA) derived from pFR-Luc (Stratagene) and inserted into the NheI/HindIII sites of pGL3 (Promega) upstream of the P. pyralis luciferase gene. GAL4US-GL3 was further modified for blasticidin resistance as described above. Stable reporter cell lines were established by blasticidin and neomycin selection and used as described above for assays of TonEBP/OREBP transcriptional activity.

Statistical analysis. Data are expressed as means ± SE. Statistical analyses were performed by paired t-test or repeated ANOVA, as appropriate. Post hoc comparison was made by Dunnett’s test. P < 0.05 is considered significant.

RESULTS

High NaCl increases ROS, including superoxides. Hypertonicity was previously found to activate TonEBP in HEK293 cells, which are renal epithelial cells of human origin (44). To examine whether hypertonicity increases ROS in HEK293 cells, we raised the osmolality from 300 to 500 mosmol/kg H₂O by adding NaCl and measured ROS by flow cytometry, using DCFH-DA as a probe. High NaCl raises the fluorescence intensity of DCFH within 30 min, indicating increased ROS (Fig. 1A). To examine whether high NaCl increases formation of superoxides, we measured superoxide activity by confocal microscopy, using DHE as a probe. High NaCl increases DHE fluorescence by 1.37-fold (Fig. 1B), indicating that high NaCl induces generation of superoxides. The effect of high NaCl on superoxide activity is reduced by N-acetylcysteine (NAC; 5 mM), a general antioxidant (Fig. 1B). Mn(III)tetrakis(4-benzoic acid)porphin chloride (MnTBAP; 40 μM), a SOD mimetic (3), and catalase (400 U/ml) do not significantly lower high NaCl-induced superoxide generation. MnTBAP increases superoxide activity in cells kept at 300 mosmol/kg H₂O (Fig. 1B).

Antioxidants attenuate high NaCl-induced TonEBP/OREBP transactivation activity. We measured transcriptional activity of TonEBP/OREBP in HEK293 cells stably expressing an ORE luciferase reporter. High NaCl increases ORE reporter activity 96- to 106-fold (Fig. 2A). NAC and MnTBAP, but not exogenously added catalase, significantly reduce high NaCl-induced ORE reporter activity (38 and 29%, respectively; Fig. 2A). To examine whether this effect is specifically mediated by
binding of TonEBP/OREBP to OREs, we used an otherwise identical reporter in which the ORE sites and the adjacent AP-1 site are mutated. This prevents both the binding of TonEBP/OREBP and high NaCl-induced increase of reporter activity (Fig. 2B). NAC and MnTBAP, but not catalase, inhibit high NaCl-induced BGT1 mRNA expression by 30 and 20%, respectively (Fig. 2A).

Exogenously added catalase does not affect ORE reporter activity (Fig. 2A) or BGT1 mRNA expression (Fig. 3A). Because it seemed possible that the exogenous catalase might not effectively reduce intracellular H$_2$O$_2$, we examined the effect of high NaCl on BGT1 mRNA expression in a clone of HEK293 cells that overexpresses human catalase (8). Overexpression of catalase does not reduce toxicity-dependent expression of BGT1 mRNA (Fig. 3B). The effectiveness of the

Fig. 1. A: high NaCl increases reactive oxygen species (ROS) activity in human embryonic kidney (HEK) 293 cells, as measured by flow cytometry with 2,7'-dichlorofluorescin diacetate (DCFH-DA) as a probe. Osmolality was increased from 300 to 500 mosmol/kgH$_2$O (NaCl added) for the period indicated. Results at 500 mosmol/kgH$_2$O are expressed relative to 300 mosmol/kgH$_2$O at each time (**) $P < 0.01$, $n = 4$). B: N-acetylcysteine (NAC; 5 mM) significantly inhibits high NaCl-induced superoxide activity. Superoxide activity was estimated by confocal microscopy, using dihydroethidium (DHE) as a probe. Osmolality of medium bathing HEK293 cells was increased from 300 to 450 mosmol/kgH$_2$O (NaCl added) for 45 min. Dyes and antioxidants were added 30 min before exposure to high NaCl. Results are expressed relative to control at 300 mosmol/kgH$_2$O (*$P < 0.05$, paired $t$-test, $n = 4$).

binding of TonEBP/OREBP to OREs, we used an otherwise identical reporter in which the ORE sites and the adjacent AP-1 site are mutated. This prevents both the binding of TonEBP/OREBP and high NaCl-induced increase of reporter activity (Fig. 2B). NAC and MnTBAP have no significant effect on activity of the mutated ORE reporter (Fig. 2B). We conclude that the full high NaCl-induced increase in TonEBP/OREBP transcriptional activity requires increased ROS, mainly superoxides.

The reporter that we used includes the AP-1 site adjacent to the OREs in the AR gene. Mutation of the AP-1 site reduces high NaCl-induced reporter activity (13). AP-1 is regulated by ROS (27, 30). To test whether antioxidants reduce ORE reporter activity by inhibiting AP-1 activity, we examined the effect of NAC on a different reporter, ORE-X, that does not include that AP-1 site. NAC reduces high NaCl-induced ORE-X activity by 41% (from 308 ± 124-fold increase, comparing 500 with 300 mosmol/kgH$_2$O, to 181 ± 68-fold increase, $P < 0.05$, $n = 5$), even in the absence of the AP-1 site, excluding the possibility that the effect of NAC is directly mediated by AP-1.

BGT1 is a transcriptional target of TonEBP/OREBP (21). Activation of TonEBP/OREBP by high NaCl elevates BGT1 mRNA abundance (21). To further examine the effects of antioxidants on TonEBP/OREBP activity, we measured the effect of NAC and MnTBAP on the high NaCl-induced increase in BGT1 mRNA expression, using real-time RT-PCR. NAC and MnTBAP, but not catalase, inhibit high NaCl-induced BGT1 mRNA expression by 30 and 20%, respectively (Fig. 3A).

Exogenously added catalase does not affect ORE reporter activity (Fig. 2A) or BGT1 mRNA expression (Fig. 3A). Because it seemed possible that the exogenous catalase might not effectively reduce intracellular H$_2$O$_2$, we examined the effect of high NaCl on BGT1 mRNA expression in a clone of HEK293 cells that overexpresses human catalase (8). Overexpression of catalase does not reduce toxicity-dependent expression of BGT1 mRNA (Fig. 3B). The effectiveness of the

Fig. 2. A: NAC (5 mM) and MnTBAP (40 μM), but not catalase, inhibit high NaCl-induced osmotic response element (ORE) reporter activity in HEK293 cells. B: NAC (5 mM) and MnTBAP (40 μM) have no significant effect on mutated ORE reporter activity. HEK293 cells were incubated with antioxidants for 30 min before increasing osmolality to 500 mosmol/kgH$_2$O by adding NaCl for 16 h. Results are expressed relative to control at 300 mosmol/kgH$_2$O (*$P < 0.05$, compared with the corresponding control, paired $t$-test, $n = 3$).
overexpressed catalase in these cells is attested by resistance of the cells to H$_2$O$_2$-mediated cell death induced by FK506 (53). Superoxides spontaneously react with nitric oxide to form peroxynitrite, which can induce gene expression in response to stress (24). For example, in human neuroblastoma cells, peroxynitrite induces DNA damage-inducible (Gadd) proteins 34, 45, 153 mRNA expression (37). $\text{N}^\text{G}$-monomethyl-l-arginine (l-NMMA) is a general inhibitor of nitric oxide synthases (4). To test whether nitric oxide is involved in high NaCl-induced activation of TonEBP/OREBP, we examined the effect of 100 $\mu$M l-NMMA. l-NMMA has no significant effect on high NaCl-dependent increase in superoxides (Fig. 5A), TonEBP/OREBP transcriptional activity (Fig. 5B), BGT1 mRNA (Fig. 5C), or TonEBP/OREBP transactivation (Fig. 5D). We conclude that high NaCl-induced activation of TonEBP/OREBP does not involve nitric oxide.
Increase of ROS in the absence of high NaCl is not sufficient to activate TonEBP/OREBP. We elevated ROS at 300 mosmol/kgH₂O by adding 1) xanthine oxidase (6 mU/ml) plus hypoxanthine (500 μM) to directly elevate ROS (23), 2) L-buthionine-[S,R]-sulfoximine (BSO; 100 and 400 μM) to deplete the reduced form of glutathione (19), or 3) diethyldithiocarbamate (100 μM and 1 mM) to elevate superoxide by inhibiting SOD (9). Although xanthine oxidase plus hypoxanthine increases superoxide (data not shown), ORE-X activity is unaffected at either 300 or 500 mosmol/kgH₂O (Fig. 6A). BSO (Fig. 6B) and diethyldithiocarbamate (data not shown) also do not affect ORE-X activity at either osmolality. We conclude that increase in ROS in the absence of other high NaCl-induced signals is not sufficient to activate TonEBP/OREBP.

Ouabain inhibits both high NaCl-induced superoxide activity and activation of TonEBP/OREBP. Ouabain is an inhibitor of Na-K-ATPase (18). In Madin-Darby canine kidney cells, ouabain reduces high NaCl-dependent increase of AR, SMIT and BGT1 mRNA abundance and of TonEBP/OREBP protein abundance (36). Those effects were attributed to cell swelling caused by ouabain and were proposed as evidence that TonEBP/OREBP activity is regulated by cell volume, as well as by intracellular ionic strength (36). However, ouabain also reduces the high NaCl-induced increase in superoxide in rat medullary thick ascending limbs (33). We reasoned that if ouabain should also reduce high NaCl-induced superoxide formation in renal epithelial cells, that could explain how ouabain reduces TonEBP/OREBP activity without invoking changes in cell volume. Therefore, we tested the effect of a low concentration of ouabain (12 nM) on high NaCl-induced superoxide level and TonEBP/OREBP activity in HEK293 cells.

Ouabain decreases superoxide (Fig. 7A), TonEBP/OREBP transcriptional activity (Fig. 7B), and TonEBP/OREBP transactivation (Fig. 7D). As controls for specificity, the effect on transcription does not occur in the absence of functional TonEBP/OREBP binding elements (Fig. 7C) nor on transactivation in the absence of the TonEBP/OREBP TAD (Fig. 7E). We conclude that ouabain-induced decrease in superoxide contributes to reduction by ouabain of the high NaCl-induced increase in TonEBP/OREBP activity.

DISCUSSION

Measurements of oxidized glutathione (41) and the effects of antioxidants (39, 52) originally indicated that osmotic stress causes oxidative stress. Subsequently, it was found that high NaCl increases ROS production and carbonylate proteins (indicative of oxidative damage to the proteins) in mIMCD3 cells and that protein carbonylation is abundant in normal renal medulla, associated with the normally high interstitial NaCl concentration in that tissue (51). In the present studies of HEK293 cells, we confirm by two independent methods, namely flow cytometry with DCFH as a probe (Fig. 1A) and confocal microscopy with DHE as an indicator (Fig. 1B), that high NaCl elevates ROS and superoxides. DCFH is oxidized to form DCF, which fluoresces. This was initially thought to require hydrogen peroxide (45). However, subsequent studies showed that DCFH can also be oxidized by enzymatic reactions and by other oxidants, including peroxynitrite and hypochlorous acid (45). DHE is oxidized by superoxides, but not by hydrogen peroxide, peroxynitrite, or hypochlo-
ROS CONTRIBUTE TO ACTIVATION OF TonEBP/OREBP

Fig. 6. Neither xanthine oxidase (A) nor l-buthionine-[S,R]-sulfoximine (BSO; B) has a significant effect on ORE-X activity either at 300 or 500 mosmol/kgH2O. HEK293 cells were incubated with xanthine oxidase (6 mU/ml) plus hypoxanthine (500 μM) for 30 min before increasing osmolality to 500 mosmol/kgH2O by adding NaCl for 8 or 16 h (A, representative experiment). HEK293 cells were also incubated with 100 or 400 μM BSO for 20 h to deplete reduced glutathione before increasing osmolality to 500 mosmol/kgH2O by adding NaCl for 6 h (B, representative experiment). Results are expressed relative to control at 300 mosmol/kgH2O.

ous acid (5), providing a specific probe for measuring superoxide activity (45).

ROS can serve as signaling molecules (17). In most cases, superoxides are the initial ROS produced. Examples include that, in vascular smooth muscle cells, IL-1β stimulates superoxide production, ERK activation, and matrix metalloproteinase-9 gene expression. Overexpression of SOD or addition of NAC inhibits all these effects (20). Similarly, ANG II activates NADPH oxidase, resulting in generation of ROS that, in turn, activate multiple transcription factors through the Raf-1-MAPK pathway, leading to increased vascular tone (54).

We used the antioxidants, NAC and MnTBAP, to study the role of ROS in high NaCl-induced activation of TonEBP/OREBP. These antioxidants reduce high NaCl-induced increase of ROS (Fig. 1B), TonEBP/OREBP transcriptional activity (Fig. 2, A and C), and the mRNA expression of TonEBP/OREBP’s transcriptional target, BGT1 (Fig. 3A). NAC, a membrane-permeating form of cysteine, is a precursor of glutathione. It serves as a general antioxidant by raising cellular reduced glutathione content, which alters redox balance, inhibiting formation of ROS (7). MnTBAP acts like SOD, dismutating superoxide to H2O2 (3).

Using these antioxidants, we found that ROS contribute to high NaCl-induced increase of TonEBP/OREBP transactivation (Fig. 4). At this point, we can only speculate on the mechanism. High NaCl increases phosphorylation of the TonEBP/OREBP TAD (14). Activation of a number of kinases, including PKAc, p38, and Fyn, is required for full high NaCl-induced increase in TonEBP/OREBP transactivation (14, 25). ROS can activate p38 (40). Thus ROS could transactivate TonEBP/OREBP through increased p38 kinase activity. An example of ROS affecting transactivation via phosphorylation by a MAP kinase is provided by the effect of PDGF. Activation of PDGF increases ROS, which contributes to activity of the transcription factor, AP-1 (32), mediated by ERK which increases phosphorylation of a TAD located at the COOH-terminal of c-Fos, a partner in AP-1 heterodimers (32). Phosphorylation of p38 by MEKK3 increases its activity (48) and dephosphorylation by protein tyrosine phosphatases (PTPs) can decrease it (34). Thiol groups of active site cysteines in PTPs are vulnerable to oxidation (17). Free radicals inhibit PTPs through either direct oxidation of the thiol groups or glutathionylation due to increased oxidized glutathione level (17). Hypertonicity inhibits the activity of PTPs, which can be prevented by NAC (40). Thus ROS may contribute to the activation of p38, and thereby of TonEBP/OREBP, by inhibiting PTPs.

We did not find any effect on TonEBP/OREBP transcriptional activity at 300 mosmol/kgH2O of exogenous agents that elevate ROS nor did the agents further increase the effect of high NaCl (Fig. 6). Thus, although increased ROS is necessary for full high NaCl-induced activation of TonEBP/OREBP, isolated elevation of ROS does not activate TonEBP/OREBP per se. This result is consistent with the previous conclusion that multiple signaling inputs are necessary for toxicity-induced activation of TonEBP/OREBP but that no one of the signals is sufficient by itself (22). A cogent previous example is provided by the DNA damage-inducible kinase, ATM (22). High NaCl damages DNA (11). Full high NaCl-dependent activation of TonEBP/OREBP requires the activation ATM (22). However, other agents such as high urea and radiation also activate ATM, but they do not increase TonEBP/OREBP transcriptional activity like high NaCl does (22) and high urea actually reduces toxicity-dependent activation of TonEBP/OREBP (46).

Hypertonicity shrinks cells by osmosis, resulting in increased concentration of all intracellular components, including intracellular ions (36) and macromolecules (6). Decrease in cell volume (12), increase in intracellular ionic strength (36), and increased macromolecular crowding (6) are all candidates to be sensors that trigger cellular responses to hypertonicity. Their relative importance remains controversial. Under conditions in which changes in cell volume are similar, TonEBP/OREBP activity correlates with the intracellular ionic strength.
Regardless of the external tonicity (36). On the other hand, inhibition of Na-K-ATPase by ouabain leads to decreased activity of TonEBP/OREBP despite a marked increase in the intracellular ionic strength. It was suggested that, because isotonic swelling is known to occur under these conditions, dilution of the cytoplasmic constituents inhibits the activity of TonEBP/OREBP (36). However, the present study affords an alternative explanation as the effect of ouabain on TonEBP/OREBP is associated with suppression of ROS formation (Fig. 7). Given that ROS contribute to the activation of TonEBP/OREBP, it is plausible that it is inhibition of ROS generation by ouabain that attenuates the activation of TonEBP/OREBP, rather than increased cellular water content.

It is interesting to speculate on how ouabain reduces ROS in cells stressed by high NaCl (Fig. 7). High NaCl increases Na-K-ATPase activity (38), presumably to energize increased Na and K transport. Production of ATP must increase to compensate for its greater consumption, which can entail greater mitochondrial oxidative metabolism. Ouabain reduces oxygen consumption by reducing Na and K transport and the associated consumption of ATP (2). Oxidative metabolism by mitochondria is associated with production of ROS (2). Putting all of this together, we propose that ouabain reduces high NaCl-induced increase in ROS by decreasing utilization of ATP by the Na-K-ATPase, thus reducing oxidative metabolism and the accompanying production of ROS. A similar process...
may be occurring in isolated rat medullary thick ascending limbs, where the Na transport inhibitors, dimethylaminozoide and ouabain, blunt the effect of high NaCl-induced superoxide activity (33).

ACKNOWLEDGMENTS
The authors thank Dr. J. Handler for thoughtful discussions during the course of the study. Dr. C. Combs for expert help with confocal microscopy, and A. Williams for assistance with flow cytometry.

GRANTS
This study was supported in part by a Grant-in-Aid from National Kidney Foundation/National Capital Area (to X. Zhou).

REFERENCES

Downloaded from http://ajprenal.physiology.org/ on July 6, 2017


47. Trama J, Lu Q, Hawley RG, and Ho SN. The NFAT-related protein NFATL1 (TonEBP/NFAT5) is induced upon T cell activation in a calcineurin-dependent manner. *J Immunol* 165: 4884–4894, 2000.


