Does the intracellular ionic concentration or the cell water content (cell volume) determine the activity of TonEBP in NIH3T3 cells?

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Rodgaard T, Schou K, Friis MB, Hoffmann EK. Does the intracellular ionic concentration or the cell water content (cell volume) determine the activity of TonEBP in NIH3T3 cells? Am J Physiol Cell Physiol 295: C1528–C1534, 2008. First published October 8, 2008; doi:10.1152/ajpcell.00081.2008.—The transcription factor, tonicity-responsive enhancer binding protein (TonEBP), is involved in the adaptive response against hypertonicity. TonEBP regulates the expression of genes that catalyze the accumulation of osmolytes, and its transcriptional activity is increased by hypertonicity. The goal of the present investigation was to investigate whether cell shrinkage or high intracellular ionic concentration induced the activation of TonEBP. We designed a model system for isotonically shrinking cells over a prolonged period of time. Cells swelled in hypotonic medium and performed a regulatory volume decrease. Upon return to the original isotonic medium, cells shrank initially, followed by a regulatory volume increase. To maintain cell shrinkage, the RVI process was inhibited as follows: ethyl-isopropyl-amiloride inhibited the Na+/K+ antiport, bumetanide inhibited the Na+-K+-2Cl- cotransporter, and gadolinium inhibited shrinkage-activated Na+ channels. Cells remained shrunken for at least 4 h (isotonically shrunken cells). The activity of TonEBP was investigated with a Luciferase assay after isotonic shrinkage and after shrinkage in a high-NaCl hypertonic medium. We found that TonEBP was strongly activated after 4 and 16 h in cells in high-NaCl hypertonic medium, but not after 4 or 16 h in isotonically shrunken cells. Cells treated with high-NaCl hypertonic medium for 4 h had significantly higher intracellular concentrations of both K+ and Na+ than isotonically shrunken cells. This strongly suggested that an increase in intracellular ionic concentration and not cell shrinkage is involved in TonEBP activation.

cell volume; cell ionic strength; cell ionic concentration; NIH3T3 cells; isosmotic shrinkage; tonicity-responsive enhancer binding protein

LONG-TERM EXPOSURE TO HYPERTONIC media results in the transcription of a number of osmoregulatory genes that are primarily involved in the uptake and synthesis of nonionic (compatible) osmolytes. This mechanism protects the cell against the harmful effects of hypertonicity (1), including DNA damage (5, 17) and inhibition of DNA repair (5). Unless healthy osmotic conditions are restored, these effects can lead to cell death (6, 21). The tonicity-responsive enhancer binding protein ([TonEBP]; also known as osmotic response element (ORE) binding protein (OREBP) or nuclear factor of activated T cells (15)) is a transcription factor that is activated by hypertonicity and regulates expression of osmoregulatory proteins, including the sodium-chloride-betaine cotransporter (22), the sodium-myo-inositol cotransporter (28), and aldose reductase (AR) (8, 16). TonEBP is a highly conserved protein (19), and TonEBP mRNA has been found in all tissues (10, 23). TonEBP is predominantly described in the kidneys where cells are often exposed to hypertonicity. However, its function in most nonrenal tissues is currently not understood. It was speculated that, since it is active under isonictonic conditions (32), TonEBP may act as a housekeeping protein in nonrenal tissues (31).

TonEBP is activated with increases in external osmolarity via several pathways: 1) at the mRNA level, it is upregulated under hypertonic conditions and downregulated under hypotonic conditions, or after accumulation of organic osmolytes (2, 32); 2) the phosphorylation of TonEBP seems to be important for its nuclear localization during hypertonic incubation (4,18), which is associated with the DNA damage-induced activation of the phosphatidylinositol 3-kinase-related kinase, ataxia telangiectasia mutated (33); 3) TonEBP activation is associated with p38 MAPK (15, 24), which is potently activated by hypertonic shrinkage in NIH3T3 cells (9), and mitogen-activated protein kinase kinase/extracellular signal-regulated kinases have also been found to regulate TonEBP activity (30).

Although a lot is known about the function and regulation of TonEBP, its mechanism for sensing changes in extracellular tonicity is not entirely clear. Previous studies showed that the activity of TonEBP correlated with intracellular ionic strength (32). However, in various kidney cells (25, 26), it was shown that significant increases in intracellular ionic strength did not correlate with increased activity of TonEBP under conditions in which increases in cell volume were concurrent with increases in ionic strength. It was thus concluded that the water content or cell volume was as important as ionic strength in determining the activity of TonEBP. In a recent review, Ho (11) suggested that changes in water content or cell volume were the main regulators of TonEBP activity.

In the present study, we investigated the roles of ionic concentration vs. cell shrinkage in TonEBP activation. We measured the activation of TonEBP and the cellular ion concentrations in isotonically shrunken cells and in hypertonically shrunken cells in high-NaCl medium.

EXPERIMENTAL PROCEDURES

Reagents and Solutions

Unless otherwise stated, reagents were purchased from Sigma-Aldrich (St. Louis, MO), Merck (Darmstadt, Germany), or Baker (Deventer, The Netherlands) and were of the highest analytic grade. 5’-[(N-ethyl-N-isopropyl) amiloride (EIPA) (Invitrogen, Carlsbad, CA) was dissolved at 10 mM in DMSO. 3-(Aminosulfonyl)-5-[(butylamino)-4-phenoxybenzoic acid (bumetanide) (Sigma-Aldrich) was dissolved at 10 mM in DMSO and was added to the medium at a final concentration of 10 μM. Amiloride (99% pure, Sigma-Aldrich) was added to the medium at a final concentration of 10 μM. All other chemicals were obtained from Sigma-Aldrich.

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dissolved at 5 mM in ethanol. Both were stored at −20°C. GdCl₃ (gadolinium) (also from Sigma-Aldrich) was dissolved at 100 mM in H₂O (distilled and deionized) and stored at −80°C.

Cell Culture

NIH3T3 fibroblasts were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) fortified with 10% fetal calf serum and 1% penicillin/streptomycin (Invitrogen) and incubated at 37°C, 5% CO₂, and 95% humidity. Cells were passaged every 4th or 5th day and used from passages 5 to 30. Cells were grown to 80% confluence in isotonic medium before being treated with experimental mediums. Normal isotonic medium contained 110 mM NaCl. Hypertonic medium was obtained by increasing the concentration of NaCl to 192 mM. Hypotonic (161.5 mosM) medium was obtained by dilution with a buffer containing 4.6 mM MOPS, 3.3 mM TES, and 5 mM HEPES.

Regulatory volume increase after regulatory volume decrease protocol. Cells were transferred to a hypotonic medium for 30 min [regulatory volume decrease (RVD)] and then returned to an isotonic medium [regulatory volume increase (RVI)] for 4 or 16 h.

Isotonic shrinking protocol. Cells were treated with the RVI after RVD protocol, but, during the last 5 min of the RVD treatment, ion transporter inhibitors were added: EIPA, the Na⁺/H⁺ exchanger inhibitor; bumetanide, the Na⁺ channel inhibitor; gadolinium, the shrinkage-activated Na⁺/H⁺ exchanger inhibitor; and gadolinium, the shrinkage-activated Na⁺ channel inhibitor. Then cells were returned to isotonic conditions in the continued presence of inhibitors.

Inhibitors alone protocol. Cells were maintained in isotonic medium containing the ion transport inhibitors.

Measurements of TonEBP/OREBP Transcriptional Activity

The ORE reporter plasmid, −1233/−1105 IL2min-GL3, was a gift from Dr. Joan Ferraris from the National Institutes of Health. It was constructed by inserting nucleotides from the S' flanking region (−1233 to −1105) of the human AR gene into MluI/NheI sites upstream of the human IL-2 minimal promoter, as previously described (29, 34). The AR gene is a target for TonEBP, and transcription of this gene is taken as a measure of TonEBP activity. The ORE reporter was transfected into NIH3T3 cells grown in six-well plates, using 1 μg of DNA and lipofectamine 2000 (Invitrogen), following the manufacturer’s instructions. Experimental treatments were performed 24–48 h after transfection. After treatment, the cells were harvested and combined with Luciferase working substrate from either Sigma-Aldrich or Promega (Madison, WI) in 96-well black plates (Perkin Elmer, Wellesley, MA), and Luciferase activity was measured in a FluorStar Optima luminometer (BMG, Offenburg, Germany). Cell protein was determined with the bicinchoninic acid protein assay kit (Pierce, Rockford, IL).

Measurements of Cell Volume Using Large-angle Light Scatter

Cell volume measurements were carried out essentially as described by Pedersen et al. (27). Briefly, the cells were grown to confluence (80%) on rectangular (10 × 50 mm) HCl- and ethanol-cleaned coverslips in four-well plates. The cells were placed at an angle of 50° relative to the excitation light in thermo-stated cuvettes and seated in a PTI RatioMaster spectrophotometer with a 75-W xenon lamp. The cell volumes were measured by exciting the cells at 550 nm and measuring emission at 555 nm. The cells were continuously perfused at 0.7 ml/min with the experimental medium, and rapid solution changes were performed by briefly increasing flow to 3.5 ml/min. It was controlled that this in itself did not induce a cellular volume response. Volume was measured as the inverse of the 555-nm emission. To obtain relative cell volumes, the values were normalized to those obtained during isotonic conditions. Because there is scatter from ingredients in the medium, the diluted medium used under hypotonic conditions changed the scatter. Thus we could not accurately measure the volume in the initial RVD period. Traces from known RVD curves (27) are shown with dotted lines in the figures (see Fig. 2, B and C).

Measurements of Caspase Activity

Cells were grown to confluence in 10-cm petri dishes. The caspase activity was measured with the ApoTarget Caspase-3/CPP32 Colorimetric assay from BioSource International (Nivelles, Belgium). After the appropriate treatment, cells were lysed with ice-cold lysis buffer (Tris-buffered saline containing a nonspecified detergent), transferred to cooled Eppendorf tubes, and incubated for 10 min on ice. Cells were sonicated twice for 10 s to fully disrupt and disperse cell debris and then centrifuged at 10,000 g for 1 min at 4°C to precipitate nonsoluble material. The supernatant was then transferred to new Eppendorf tubes and stored on ice. Protein content of the supernatant was determined, and the volume was adjusted with lysis buffer to achieve similar protein concentrations for activity measurements (1–4 μg/μl). Caspase 3 activity in the cell lysate was estimated in 96-well plates by adding the peptide substrate acetyl-Asp-Glu-Val-Asp p-nitroanilide (Ac-DEVD-pNA) and estimating the p-nitroanilide (pNA) production. Absorbance was measured at 405 nm with a microplate reader (BMG LabTechnologies, Offenburg, Germany).

Measurements of Intracellular Potassium Content and Potassium and Sodium Concentrations

Cells were grown to 80% confluence in 10-cm petri dishes. After the various treatments, the cells were washed three times with 0.1 M Mg(NO₃)₂ and then dried with 96% EžOH. Intracellular ions were extracted with washing with 1 ml of water for 1 h (ion fraction), and the remaining cell lysate was denatured with 1 ml of 1 M NaOH containing 0.5% sodium deoxycholate for 10 min. After centrifugation of the lysate (20,000 g for 5 min at 4°C), the supernatant was used for protein determination using the bicinchoninic acid assay (Pierce). Potassium levels were measured in the ion fraction with an Atomic Absorption Spectrophotometer (Perkin Elmer). Potassium content was expressed as K⁺ per gram protein. Cells used for measurements of potassium and sodium concentrations were harvested as above with the following changes. The media was changed 1 h before harvesting with equivalent media containing 2.3 μl ¹⁴C 3-O-methyl-d-glucose per 10 ml media to measure water content of the cells, and the cells were washed four times with 0.1 M Mg(NO₃)₂ containing 1 mM phlorizin to block the glucose transporter. The water content was calculated per gram protein. The potassium and sodium contents were measured in the ion fraction with a FLM3 Flamephotometer (Radiometer, Copenhagen) and calculated per gram protein. Concentrations were calculated from ion content by dividing with water content and were expressed as millimoles.

Statistical Analysis

Data are expressed as means ± SE for separate independent experiments. Statistical analyses were performed by paired or unpaired t-tests or repeated ANOVA, as appropriate. Post hoc comparisons were made by Tukey’s test. P < 0.05 was considered significant; n denotes the number of independent experiments.

RESULTS

To study the effect of hypertonicity on the TonEBP/OREBP transcriptional activity in NIH3T3 cells, we measured the activity of the ORE reporter in cells maintained in isotonic medium or in hypertonic high-NaCl medium (500 mosM). The activity of TonEBP was significantly increased under hypertonic conditions compared with isotonic conditions by 5- and 12-fold after 4 and 16 h, respectively (Fig. 1).
followed by an uptake of water and concomitant Na caused an increase in intracellular ionic concentration that was of NaCl in other cell types (14), the cells initially shrank. This

Previously, when osmolarity was increased by the addition of NaCl in other cell types (14), the cells initially shrank. This caused an increase in intracellular ionic concentration that was followed by an uptake of water and concomitant Na\(^+\), K\(^+\), and Cl\(^-\) (RVI). This resulted in cells with normal cell volume and increased intracellular ionic concentrations. Similarly, we found that NIH3T3 cells initially shrank when exposed to hypertonic treatment (500 mosM) by addition of NaCl, and this was followed by an RVI response that resulted in a normal cell volume after less than 2 h (Fig. 2A). Thus cells exposed to hypertonicity exhibited a biphasic change in cell volume.

To clarify whether the increase in TonEBP activity in NIH3T3 cells under hypertonic conditions was dependent on an increased intracellular ionic strength or the biphasic change in cell volume, we compared the hypertonic condition to two different experimental conditions (Fig. 2). In the first, NIH3T3 cells were treated with the RVI after RVD protocol (Fig. 2B). We observed that, under these conditions, cells regulated cell volume back to the isotonic cell volume immediately after they were returned to isotonic media. We assumed this treatment resulted in normal intracellular ion concentrations, as was previously shown in other cell types (14). In the second condition, cells were isotonically shrunken (Fig. 2C). To achieve this, cells were treated with the RVI after RVD protocol, but, during the last 5 min of hypotonicity, we added the ion transporter inhibitors, 10 \(\mu\)M EIPA (Na\(^+\)/H\(^+\) exchanger), 50 \(\mu\)M bumetanide (Na\(^+\)-K\(^+\)-2Cl\(^-\) cotransporter), and 100 \(\mu\)M gadolinium (shrinkage-activated Na\(^+\) channels). Then cells were returned to isotonic conditions in the continued presence of the inhibitors. As expected, the inhibitors prevented the cells from performing a RVI response, and the cells remained significantly shrunken for at least 4 h under isotonic conditions (Fig. 2C). We assumed this treatment also resulted in normal intracellular ion concentrations, as was previously shown in other cell types (14). At the end of this treatment, cells began to fall off the coverslips, and the light scatter measurements were stopped. It should be mentioned that addition of EIPA or bumetanide alone did slow down, but did not prevent, a RVI response. Furthermore, the addition of 50 \(\mu\)M gadolinium to EIPA or bumetanide had no significant effect.

We then tested the TonEBP activity after 4 and 16 h under the three conditions described above (see Fig. 3). We observed no activation of TonEBP either during the RVI after RVD protocol or during the RVI after RVD protocol with inhibitors (isotonic shrinkage). In both conditions, the cells were expected to have normal ionic concentrations, but, in the former condition, they had only a very short shrinkage period, and, in the latter condition, the cells were isotonically shrunken for a long period. This was compared with the direct addition of high salt, where cells were biphasically shrunken and were expected to have a prolonged increase in intracellular ion concentration. In that case, the TonEBP activity had increased 5- and 12-fold after 4- and 16-h treatment, respectively (Fig. 1). Thus significantly high TonEBP activity only occurred

![Fig. 2. The changes in cell volume over time. NIH3T3 cells were kept in hypertonic medium (81.5 mM NaCl in a final 500 mosM) (A); incubated with hypotonic medium (165 mosM) for 30 min, followed by isotonic medium [regulatory volume increase (RVI) after regulatory volume decrease (RVD) protocol] (B); or incubated with hypotonic medium (165 mosM) for 30 min, with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium added for the last 5 min, followed by isotonic medium with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium for 4 h (isotonic shrinkage with inhibitors) (C). Cell changes were recorded in A and B until the level of cell volume was the same as before treatment. The dotted lines represent the changes in cell volume during hypotonic treatments. Values are means ± SE; \(n = 3\). *\(P < 0.05\) vs. isotonic control. All values during the isotonic shrinkage in C had \(P < 0.01\).]
when cells were treated with hypertonic high NaCl medium (as in Fig. 2A).

It should be noted that the inhibitors do have some negative effect on TonEBP activity under isotonic conditions (Fig. 3). We, therefore, also investigated the effect of the inhibitors on hypertonic activation of TonEBP. After 16 h, the activity of TonEBP is significantly lower in hypertonic conditions with inhibitors than under hypertonic conditions in the absence of inhibitors shown in Fig. 1 ($P = 0.023$). However, TonEBP activity is still significantly higher, $3.1 \pm 0.023$ times ($P = 0.0001$), under hypertonic conditions in the presence of inhibitors compared with the activity of TonEBP under isotonic shrinkage in the presence of inhibitors.

It was previously shown in NIH3T3 cells that 687 mosM gave a significant increase in caspase 3 activity after 4.5 h ($9$). To investigate whether apoptosis was induced by the different cell treatments used in the present study, we measured caspase 3 activity. Compared with the positive control (staurosporine), there was very little caspase 3 activity under the different situations (Fig. 4). This indicated that the lack of TonEBP activation observed during isotonic shrinkage (Fig. 3) was not due to apoptosis in the treated cells. Thus neither the 500 mosM nor the inhibitor concentrations were apoptotic.

After establishing that TonEBP was not activated by cell shrinkage as such, we further examined TonEBP activation by investigating changes in intracellular $K^+$ content (Fig. 5). The amount of $K^+$ in isotonically shrunken cells was significantly lower than in cells kept in isotonic media, both after 4 h (999 ± 50 vs. 1,215 ± 67 mmol/kg protein) ($P = 0.02$) and after 16 h (1,019 ± 52 vs. 1,303 ± 47 mmol/kg protein) ($P = 0.01$). The ionic loss had most likely occurred during the RVD process, and, when the cells were retransferred to isotonic medium, they could not replenish $K^+$ ions due to the inhibition of the ion uptake systems. The cell volume measurements performed by light scatter did not quantitatively measure the water content; thus we were unable to translate $K^+$ contents into concentrations. Significantly higher amounts of potassium were found after treatment with direct hypertonic media (500 mosM) than after any other treatment, both after 4 h (1,490 ± 28 mmol/kg protein) and after 16 h (1,467 ± 25 mmol/kg protein); however, the difference between direct hypertonicity and RVI after RVD was not quite significant ($P = 0.06$) at 16 h. Most importantly, $K^+$ content was significantly different between cells shrunken under isotonic and hypertonic conditions. The cells with high $K^+$ content (hypertonic high NaCl-treated cells) exhibited increased activation of TonEBP (Fig. 1), and those with low potassium content (isotonically shrunken cells) exhibited reduced activation of TonEBP (Fig. 3). This suggested that changes in intracellular potassium play a role in the activation of TonEBP.

To measure possible differences in concentrations of Na$^+$ and $K^+$ between the isotonically shrunken cells and the cells treated with hypertonic high NaCl medium, we measured the $K^+$, Na$^+$, and water content simultaneously at 4- and 16-h treatments (Fig. 6). It is seen that in cells treated with hypertonic medium both $K^+$ and Na$^+$ concentrations increase after 4 h, although the increase in $K^+$ is not quite significant ($P = 0.056$). In the period of 4–16 h, the $K^+$ concentration decreases to a value below the isotonic control (Fig. 6A), whereas the Na$^+$ concentrations stay elevated (Fig. 6B). In contrast, the isotonically shrunken cells essentially did not show any changes in either $K^+$ or Na$^+$ concentrations compared with isotonic control. Thus at 4 h, $K^+$ and Na$^+$ concentrations are significantly lower in isotonically shrunken cells than in cells treated with hypertonic high NaCl medium ($P < 0.01$).

Fig. 3. The activity of TonEBP measured with the ORE reporter in NIH3T3 cells. Cells were kept in 1) isotonic medium (isotonic control); 2) isotonic medium with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium (isotonic with inhibitors); 3) preincubated with hypotonic medium (165 mosM) for 25 min, followed by hypotonic medium (165 mosM) with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium for 5 min, followed by isotonic medium with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium (isotonic shrinkage with inhibitors); or 4) preincubated with hypotonic medium (165 mosM) for 30 min, followed by isotonic medium (RVI after RVD protocol). Cells were treated for 4 or 16 h. Values are means ± SE; $n = 11$ for 4-h samples and $n = 10–12$ for 16-h samples. *$P < 0.05$ vs. isotonic control.

Fig. 4. The activity of caspase 3 in NIH3T3 cells. Cells were kept in 1) isotonic medium (Iso); 2) isotonic medium with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium (Inhibitors); 3) preincubated with hypotonic medium (165 mosM) for 30 min, followed by isotonic medium (RVI after RVD); 4) preincubated with hypotonic medium (165 mosM) for 30 min with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium added during the last 5 min, followed by isotonic medium with $10 \mu M$ EIPA, $50 \mu M$ bumetanide, and $100 \mu M$ gadolinium (Iso shrinkage); 5) hypertonic medium (81.5 mM NaCl added to a final 500 mosM) (Hyper); or 6) isotonic medium with 1 mM staurosporin (Staurosporin) as a positive control. Cells were treated for 4 or 16 h, except with staurosporin, where cells were treated for 1 h. Values are means ± SE; $n = 3$ for 4-h and 16-h samples, and $n = 1$ for staurosporin-treated samples.
have previously been shown to activate TonEBP after hypertonic cell shrinkage. TonEBP might have other roles than that involved in long-term volume regulation (see Ref. 10).

When osmolarity was increased by addition of NaCl, NIH3T3 cells initially shrank and then exhibited a RVI response that resulted in normal cell volume after <2 h. This response was identical to that previously demonstrated in several other cell types. In Ehrlich Ascites tumor cells, both ion concentrations and water content were measured (14). They showed that the initial cell shrinking resulted in an increased intracellular ionic concentration, and the RVI process induced the uptake of Na\(^+\), K\(^-\), and Cl\(^-\), resulting in cells with normal cell volume and increased KCl concentrations where the uptake of Na\(^+\) was exchanged for K\(^+\) by the Na\(^+\)-K\(^+\) pump.

**DISCUSSION**

The present investigation demonstrated that, in NIH3T3 cells, during isotonic shrinkage, where the cell volume decreased (Fig. 2C), but the intracellular ionic concentration did not change (Fig. 6), TonEBP was not stimulated (Fig. 3). However, in cells treated with hypertonic high NaCl medium, where cell volume initially decreased and then normalized after ~2 h (Fig. 2A), and the intracellular K\(^+\) and Na\(^+\) concentrations increased (Fig. 6), TonEBP was strongly activated (Fig. 1). This suggested that the increase in ion concentration/strength, and not the cell shrinkage, was the important factor for activating TonEBP. It should be mentioned that it was already pointed out by Handler and Kwon (10) that it is unlikely that activation of TonEBP is mediated by cell size, since cells return to their normal size after hypertonic treatment within a relatively short period of time, whereas the activation of TonEBP is a much slower process. We found that the transcriptional activity of TonEBP increased steadily in NIH3T3 cells treated with hypertonic high NaCl medium (500 mosM). The increase after 16 h was much higher than the increase after 4 h, indicating that this is a relatively slow process compared with other known hypertonic stress responses. Our results are consistent with results in Madin-Darby canine kidney cells, where >3 h of hypertonicity were required for a detectable increase in TonEBP activity and >10 h for full activation (23).

The function of TonEBP has been elucidated in the renal medulla, in activated T cells (19), and to a certain extent in neurons (20). The roles of TonEBP in other cells are currently uncertain, but several cell types, including fibroblasts (13),

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**Fig. 5.** The cellular K\(^+\) content in NIH3T3 cells. Cells were kept in 1) isotonic medium (Isotonic control); 2) preincubated with hypertonic medium (165 mosM) for 25 min, followed by hypertonic medium (165 mosM) with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium for 5 min, followed by isotonic medium with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium (Isotonic shrinkage with inhibitors); 3) preincubated with hypertonic medium (165 mosM) for 30 min, followed by isotonic medium (RVI after RVD protocol); or 4) incubated in hypertonic medium (81.5 mM NaCl added to a final 500 mosM) (Hypertonic). Cells were treated for 4 or 16 h. Values are means ± SE; \(n = 3\). *\(P < 0.05\) vs. isotonic control. **\(P < 0.01\) vs. isotonic control.

**Fig. 6.** The cellular K\(^+\) (A) and Na\(^+\) (B) concentrations in NIH3T3 cells. Cells were kept in 1) isotonic medium (Isotonic control); 2) preincubated with hypertonic medium (165 mosM) for 25 min, followed by hypertonic medium (165 mosM) with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium for 5 min, followed by isotonic medium with 10 \(\mu\)M EIPA, 50 \(\mu\)M bumetanide, and 100 \(\mu\)M gadolinium (Isotonic shrinkage with inhibitors); or 3) incubated in hypertonic medium (81.5 mM NaCl added to a final 500 mosM) (Hypertonic). 1 h before harvesting, the media were changed to similar media containing \(^{14}\)C 3-O-methyl-D-glucose. Cells were treated for 4 or 16 h. Values are means ± SE; \(n = 5–6\). *\(P < 0.05\) and **\(P < 0.01\) vs. isotonic control. ###\(P < 0.001\) vs. hypertonic samples.
These results are consistent with the results presented in the current study, except that, after 4 h in hypertonic high NaCl medium, the NIH3T3 cells showed a significant increase in Na⁺ concentration as well, demonstrating that the Na⁺-K⁺ pump did not exchange all Na⁺ taken up for K⁺ in this cell type.

Previous studies have attempted to distinguish the importance of cell shrinkage vs. increases in ionic concentration, with no clear resolution. In one study, a correlation was found between mRNAs for TonEBP target genes and intracellular ionic strength, but, when intracellular ionic strength was raised with high extracellular potassium and/or with ouabain, no elevations of the mRNAs were observed. This suggested that cell swelling had counteracted the effect of increased ionic strength (26). The authors concluded that both the intracellular ionic strength and the water content were major factors in determining the activity of TonEBP. Moreover, it was shown that papillary collecting duct cells preconditioned in hypertonic medium and reexposed to isosmotic medium (the RVI after RVD protocol) showed significantly higher abundance of osmoregulatory mRNAs than isosmotic controls (25), although the intracellular ionic strengths did not differ. This supported the view that cell volume is an additional factor in stimulating TonEBP. In the present study, no TonEBP activation was observed after isosmotic cell shrinkage with the inhibited RVI after RVD protocol. However, comparisons should be made with caution because Neuhofer et al. (25) preincubated the cells in hypertonic medium for 12 h, and we pretreated in hypertonic medium for only 0.5 hour. Moreover, the kidney cells used by Neuhofer et al. might behave differently from the fibroblasts we used. On the other hand, our results are consistent with those of Cai et al. (3), who showed that the transcription of osmoregulatory genes was stimulated by a very slow rise in osmolality and isovolumetric regulation that increased the intracellular ionic strength but did not shrink the cells.

TonEBP mRNA is expressed in low levels in most cultured cells, and this low level is reduced further when cells are adapted to hypertonic medium (10). Based on this, we assumed, in the present investigation, that the small reduction in TonEBP expression observed after addition of inhibitors of the ion transporters and nonselective cation channels to the isosmotic medium was most likely due to a loss of intracellular ions. Handler and Kwon (10) speculated that the TonEBP expression levels observed in isosmotic media reflects an adaptation from an ancestral life in more hypotonic environments.

After our results showed the increase in ion concentration/strength was the stimulating factor for TonEBP activation, we were interested in determining whether a specific ion could be identified. Previous studies in Ehrlich Ascites tumor cells (12, 14) showed that, during the RVI process, there is an uptake of Na⁺, K⁺, and Cl⁻, but the Na⁺ is immediately exchanged for K⁺ by the Na⁺-K⁺-ATPase, resulting in a net uptake of KCl. To determine whether this was also the case in the present study, we measured K⁺ content in the various conditions. We found that cells that had undergone RVI after RVD had K⁺ contents identical to the isosmotic cells, the isotonically shrunken cells had a decreased K⁺ content, and the cells treated with high NaCl had a significantly increased K⁺ content. Thus the strong increase in K⁺ content apparently corresponded to the activation of TonEBP. Measuring the ion concentrations revealed, however, that, after 4 h in hypertonic high NaCl medium, the Na⁺ concentration was significantly increased, although slightly less than the K⁺ concentration, making it difficult to distinguish the importance of Na⁺ and/or K⁺ in the present investigation. Ferraris and Burg (7) have, however, provided good arguments against increased Na⁺ as a key factor in TonEBP activation, and, since cells with an effective Na⁺-K⁺-ATPase experience an increase in K⁺ rather than Na⁺ after treatment with hypertonic high NaCl and RVI, it is tempting to speculate that the increase in K⁺ concentration is the key factor for TonEBP activation in hypertonic high NaCl medium. If true, this might explain the finding that the addition of ouabain, an inhibitor that caused high intracellular Na⁺ but not high intracellular K⁺, did not activate TonEBP (26).

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