High NaCl increases TonEBP/OREBP mRNA and protein by stabilizing its mRNA

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Cai, Qi, Joan D. Ferraris, and Maurice B. Burg. High NaCl increases TonEBP/OREBP mRNA and protein by stabilizing its mRNA. Am J Physiol Renal Physiol 289: F803–F807, 2005. First published May 17, 2005; doi:10.1152/ajprenal.00448.2004.—Hyperosmolarity increases mRNA and protein abundance of the transcription factor toxicity-responsive enhancer/osmotic response element binding protein (TonEBP/OREBP), contributing to increased transcription of downstream osmoprotective genes. Previously, this was attributed to increased transcription of TonEBP/OREBP because no change was found in its mRNA stability. However, there is no direct evidence for increased transcription, and the 3'-untranslated region (UTR) of TonEBP/OREBP contains numerous adenylate/uridylate-rich elements, which can modulate RNA stability. Therefore, we have reinvestigated the effect of hyperosmolarity on TonEBP/OREBP mRNA stability. We find that, in mouse inner medullary collecting duct cells, raising osmolality from 300 to 500 mosmol/kg H2O by adding NaCl increases TonEBP/OREBP mRNA to a peak of 2.3-fold after 4 h, followed by a decline. TonEBP/OREBP protein increases to a sustained peak of 3.0-fold at 8 h. To determine the stability of TonEBP/OREBP mRNA, we measured the rate of its decrease after inhibiting transcription with actinomycin D, finding that it is stabilized for 6 h after addition of NaCl. This stabilization is sufficient to explain the increase in mRNA without any change in transcription. To investigate how hyperosmolarity stabilizes TonEBP/OREBP mRNA, we tested luciferase reporters containing parts of the TonEBP/OREBP mRNA UTR. Inclusion of both the 5'- and 3'- UTR increases reporter activity, consistent with mRNA stabilization. Surprisingly, however, it is the 5'-UTR that stabilizes; the 3'-UTR, by itself, decreases reporter activity. We concluded that 1) hyperosmolarity stabilizes TonEBP/OREBP mRNA, contributing to its increase, and 2) stabilization depends on the presence of the 5'-UTR.

mRNA stability; hyperosmolarity

WHEN THE TRANSCRIPTION factor, toxicity-responsive enhancer/osmotic response element binding protein (TonEBP/OREBP, also called NFAT5) is activated by hyperosmolarity, it increases transcription of osmoprotective genes, including those involved in increased expression of organic osmolytes (11) and heat shock proteins (3). Several mechanisms contribute to hyperosmoticity-induced activation of TonEBP/OREBP, including translocation from cytoplasm to nucleus (15, 18), transactivation (9), and increased TonEBP/OREBP protein abundance (18).

In Madin-Darby canine kidney (MDCK) cells (26), hyperosmolarity (200 mM raffinose added) increases TonEBP/OREBP mRNA within 6 h, reaching a maximum increase of almost threefold by 12 h and falling to twofold at 18 h. TonEBP/OREBP mRNA stability was measured beginning 12 h after raffinose was added by following the rate of decrease of its mRNA after transcription was stopped by actinomycin D. Stability of TonEBP/OREBP mRNA, measured in this fashion, is not affected by hyperosmolarity, which led to the conclusion that the increase in TonEBP/OREBP mRNA resulted from increased transcription. However, the attempt to demonstrate this directly was unsuccessful because nuclear run-on experiments proved not to be feasible. The increase in TonEBP/OREBP mRNA is accompanied by an approximately equal increase in TonEBP/OREBP protein abundance and synthesis rate. The rate of TonEBP/OREBP protein degradation is unaffected. Hypertonicity produced by adding 100 mM NaCl is as effective as adding 200 mM raffinose. In HeLa cells (15), adding 100 mM NaCl increases TonEBP/OREBP mRNA abundance within 2 h, which reaches a maximum at 6 h and decreases to the basal level at 12 h.

TonEBP/OREBP mRNA contains 29 adenylate/uridylate-rich elements (AREs) in its 3' untranslated region (3'-UTR). AREs destabilize mRNAs, modulated by ARE binding proteins, including HuR, which stabilizes mRNAs (4), and AUF1, which destabilizes them (13, 23). The level of ARE binding proteins and their binding to mRNA are regulated by extracellular conditions, particularly stresses (2, 10, 23, 25). In addition to the 3'-UTR, cis-acting elements that regulate mRNA stability can also be located in the coding regions of mRNAs (17, 19), and in 5'-UTRs (6, 14).

Given the lack of direct evidence that the hypertonicity-induced increase in TonEBP/OREBP mRNA and protein results from increased transcription and the presence of AREs in the 3'-UTR of its mRNA, we have reinvestigated the possibility that mRNA stability accounts for the increased abundance. We find that high NaCl stabilizes TonEBP/OREBP mRNA, mediated by elements within its 5'-UTR.

MATERIALS AND METHODS

Materials. Low-glucose DMEM was from Irvine Scientific (Irvine, CA), and Coon's improved medium mF-12 was from BioSource (Camarillo, CA). Restriction enzymes were purchased from New England Biolabs (Beverly, MA). Pfu polymerase was from Stratagene (La Jolla, CA). QiaShredder column, RNeasy mini kit, RNase-free DNase, and QIAquick gel extraction kit were from Qiagen (Valencia, CA). Lipofectamine 2000 was from Invitrogen (Carlsbad, CA). ABI Prism 7900 sequence detection system, TaqMan reverse transcription reagents kit, TaqMan PCR Master Mix, mixture of 18S rRNA primers and 18S probe, and primers and probes for real-time PCR were from Applied Biosystems (Foster City, CA). Passive lysis buffer and luciferase assay system were from Promega (Madison, WI). TonEBP/OREBP mRNA stability was measured beginning 12 h after raffinose was added by following the rate of decrease of its mRNA after transcription was stopped by actinomycin D. Stability of TonEBP/OREBP mRNA, measured in this fashion, is not affected by hyperosmolarity, which led to the conclusion that the increase in TonEBP/OREBP mRNA resulted from increased transcription. However, the attempt to demonstrate this directly was unsuccessful because nuclear run-on experiments proved not to be feasible. The increase in TonEBP/OREBP mRNA is accompanied by an approximately equal increase in TonEBP/OREBP protein abundance and synthesis rate. The rate of TonEBP/OREBP protein degradation is unaffected. Hypertonicity produced by adding 100 mM NaCl is as effective as adding 200 mM raffinose. In HeLa cells (15), adding 100 mM NaCl increases TonEBP/OREBP mRNA abundance within 2 h, which reaches a maximum at 6 h and decreases to the basal level at 12 h.

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OREBP (NFAT5) antibody was from Affinity Bioreagents (Neshanic Station, NJ), goat anti-rabbit horseradish peroxidase-conjugated secondary antibody was from Cell Signaling (Beverly, MA), and the enhanced chemiluminescence-plus Western blotting detection system was from Amersham Biosciences (Piscataway, NJ). Mammalian protein extraction reagent was from Pierce (Rockford, IL), and complete mini-protease inhibitor tablets were from Roche (Indianapolis, IL).

**Cell culture.** Subconfluent cultures of mouse inner medulary collecting duct (mIMCD3) cells (21) (a gift from Dr. S. Gullans, Harvard University, Boston, MA) were cultured at 37°C in 5% CO2 atmosphere in 45% low-glucose DMEM plus 45% Coo’s improved medium mF-12 plus 10% FBS with 100 U/ml penicillin added. Osmolality of the control medium was 300 mosmol/kgH2O.

**Immunoblotting.** Cells were lysed with mammalian protein extraction reagent (Pierce), according to the manufacturer’s instructions, with added protease inhibitors. An equal amount of protein was loaded onto each lane of 4–12% gradient acrylamide-Tris-glycine gels with transfer electrophoretically to polyvinylidene difluoride membranes. Membranes were blocked for 1 h at room temperature with 5% nonfat dry milk and then incubated overnight at 4°C with the primary TonEBP/OREBP antibody (diluted 1:2,000), followed by goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (diluted 1:2,000) for 1 h at room temperature. Horseradish peroxidase was visualized with the enhanced chemiluminescence-plus Western blotting detection system. The band densities were quantitated by laser densitometry.

**RNA isolation.** Total RNA from mIMCD3 cells was isolated using QiaShredder columns, followed by Qiagen RNeasy columns, according to the manufacturer’s directions. RNA was treated with DNase while bound to the RNeasy column. Total RNA concentration was measured by spectrophotometry, and the RNA was run on agarose gels to assess its quality.

**Reverse transcription and real-time PCR.** Real-time RT-PCR was performed as previously described (9). Briefly, 2 μg of total RNA were reverse transcribed with random hexamers, using a TaqMan reverse transcription reagents kit, following the manufacturer’s recommendations. Specific primers and oligonucleotide probes containing a 5′ fluorescent dye (6-FAM) and a 3′ quencher (TAMRA; TaqMan probes) were designed for mouse TonEBP/OREBP mRNA (GenBank accession no. AF453571) and luciferase mRNA, using Primer Express software. For TonEBP/OREBP mRNA, the forward primer was 5′-GGTACACACTCTTCTCTCTACAGCAA-3′, the reverse primer was 5′-TCTTATGCTTCGCTGTTCTCCATCA-3′, and the probe was 5′-6FAM-CCAGAGACTTCCCCACTGGCCTCCT-TAMRA-3′. For Photinus luciferase mRNA, the forward primer was 5′-GGTCCCTATGATTATGCTGGTGTT-3′, the reverse primer was 5′-TGTAGGACCCTCCTCTGCATGTCA-3′, and the probe was 5′-6FAM-TCCGGAAGCGACCAACGCCTT-TAMRA-3′. Multiplex PCR was performed with an ABI Prism 7900 sequence detection system, using TaqMan PCR Master Mix to which were added specific primers and probes for TonEBP/OREBP or Photinus luciferase and 18S rRNA primers and 18S probe. The 18S probe was labeled with the fluorescent dye VIC. The amplified 18S cDNA served as an internal control for reverse transcription and cDNA loading. Triplicates of each sample were analyzed in each PCR run. We analyzed the results using ABI Prism 7900 system software.

### RESULTS

**Raising osmolality to 500 mosmol/kgH2O by adding NaCl increases TonEBP/OREBP mRNA in mIMCD3 cells (Fig. 2A).** The level peaks at 2.4-fold at 4 h and then falls. TonEBP/OREBP protein starts rising after 4 h. It reaches a peak 2.4-fold at 4 h and then falls. TonEBP/OREBP including the poly(A) signal. The sequence of all constructs was confirmed.

**Luciferase assay.** mIMCD3 cells grown at 300 mosmol/kgH2O were transfected with luciferase reporter plasmid containing the 5′-UTR and/or 3′-UTR of TonEBP/OREBP mRNA, using Lipofectamine 2000 according to the manufacturer’s instructions. Sixteen hours later, osmolality was increased from 300 to 500 mosmol/kgH2O by addition of NaCl for 8 h or was kept constant; cells were then harvested in 200 μl of passive lysis buffer. Total protein was measured, and luciferase activity was determined on duplicate 10-μl aliquots with the luciferase assay system, using a Monolight 2010 Luminometer (Analytical Luminescence Laboratory, San Diego, CA).

**Statistical analysis.** Statistical analysis was performed using InStat 3 software. Data are presented as means ± SE, with n = number of independent experiments. P ≤ 0.05 (paired t-test) is regarded as significant.

**Results**

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To test whether increased mRNA stability contributes to the elevation of TonEBP/OREBP mRNA, we added actinomycin D to stop transcription 2 h after increasing NaCl and then measured the rate at which TonEBP/OREBP mRNA decreased (Fig. 3). At 300 mosmol/kgH2O the half life of TonEBP/OREBP mRNA is ~6 h. However, after osmolality is increased to 500 mosmol/kgH2O by adding NaCl, there is no
degradation of mRNA for 4–6 h. Then, it begins falling at about the same rate as at 300 mosmol/kgH2O. We conclude that high NaCl stabilizes TonEBP/OREBP mRNA for 6 h.

Given that the half life of TonEBP/OREBP mRNA is 6 h at 300 mosmol/kgH2O, if we assume that transcription of TonEBP/OREBP mRNA is not affected by high NaCl, a 6-h delay in its degradation is sufficient to double its level, as observed. We conclude that stabilization of TonEBP/OREBP mRNA accounts for its high-NaCl-induced increase.

mRNA stability generally is regulated by cis-acting elements in the UTR and trans-acting factors (14). Therefore, we next tested whether the 5′-UTR or 3′-UTR of TonEBP/OREBP mRNA is involved in its high NaCl-induced stabilization, using a chimeric luciferase reporter construct (12, 16, 24). The reporter consisted of the Photinus luciferase gene preceded by the TonEBP/OREBP mRNA 5′-UTR and/or followed by its 3′-UTR in pGL3-null plasmid. Raising osmolality from 300 to 500 mosmol/kgH2O for 8 h by adding NaCl increases luciferase activity ~1.8-fold when the full-length 5′-UTR is present (Fig. 4A). As a control, luciferase activity is not significantly affected using a reporter that contains only the tonicity-responsive enhancer/osmotic response element binding protein (TonEBP/OREBP) adenylate/uridylate-rich element (ARE) signal (Fig. 4A). In contrast, high NaCl decreases luciferase activity by ~35% when the reporter contains the full-length 3′-UTR of TonEBP/OREBP mRNA (Fig. 4A). When both the 3′-UTR and 5′-UTR are present, high NaCl increases luciferase activity 1.3-fold (Fig. 4A). We conclude that cis-acting elements in the TonEBP/OREBP mRNA 5′-UTR are involved in high NaCl-induced stabilization of the mRNA. cis-Acting elements in the 3′-UTR have the opposite effect of destabilizing it but to a lesser extent; thus the net effect is stabilization.

The reporter that we used does not directly measure mRNA stability. The assumption is that its strong T7 promoter is unaffected by tonicity, so that luciferase expression depends entirely on mRNA stability and not on transcription. To test directly for stability of the luciferase mRNA, we added actinomycin D to stop transcription 2 h after increasing NaCl and then measured the rate at which luciferase mRNA decreased. High NaCl reduces the rate of degradation of the chimeric luciferase mRNA containing 5′-UTR of TonEBP/OREBP mRNA (Fig. 4B), confirming the validity of the assays of luciferase activity in Fig. 4A.

DISCUSSION

High NaCl causes a transient increase in TonEBP/OREBP mRNA abundance. The peak is reached in 12 h in MDCK cells (26), 6 h in HeLa cells (15), and 4 h in mIMCD3 cells (Fig. 2A) and is followed by a decline toward the baseline level.

In the present study of mIMCD3 cells, we find that high NaCl increases TonEBP/OREBP mRNA by stabilizing it. This was not observed in the previous study of MDCK cells (26).
We attribute the difference to timing. The stabilization that we observed is transient, lasting only \( \frac{1}{10} \) h after NaCl is increased (Fig. 3). In the study of MDCK cells, the measurement of stability did not begin until 12 h after raising NaCl (26). Therefore, such a transient stabilization of TonEBP/OREBP mRNA would have been missed.

The rate of translation of proteins is affected by mRNA abundance. mRNA level, in turn, is determined by the relative rates of mRNA synthesis and degradation. Stabilizing an mRNA that is rapidly turning over is a quick and energy-saving way to increase its abundance. mRNA stability is regulated by numerous cis- and trans-acting factors (14).

The 3′-UTR of TonEBP/OREBP mRNA contains 29 AREs. AREs are cis-acting elements that destabilize mRNA. Proteins that bind to AREs can mask endonuclease sites and thus regulate degradation of the ARE-containing mRNAs (27). Binding of proteins to AREs is regulated. For example, cytokines and UV radiation increase the binding (1, 25). We had anticipated that AREs in the 3′-UTR of TonEBP/OREBP would be involved in its high-NaCl-induced stabilization. That is clearly not the case because inclusion of this 3′-UTR destabilizes mRNA, rather than stabilizes it (Fig. 4A). However, binding of proteins to AREs can also destabilize mRNAs (23). We speculate that the stress of high NaCl affects binding of some unidentified protein to AREs in the 3′-UTR of TonEBP/OREBP mRNA, accounting for more rapid degradation of mRNA containing this 3′-UTR (Fig. 4A). A previous example of stress-induced reduction of binding to mRNA of a protein known to regulate mRNA stability is that heat shock dissociates HuR from AREs in the 3′-UTR of cytoplasmic mRNAs (10).

Despite high-NaCl-induced destabilization of mRNAs containing the 3′-UTR of TonEBP/OREBP, high NaCl actually
stabilizes TonEBP/OREBP mRNA (Fig. 3). That stabilization is mediated by the 5'-UTR (Fig. 3). 5'-UTRs can be important regulators of mRNA stability. For example, a JNK-response element in the 5'-UTR of IL-2 mRNA is involved in its stabilization during T cell activation (6, 10). This is mediated by two RNA binding proteins, YB-1 and nucleolin (7). The role of 5'-UTRs may depend on the ongoing rate of translation (14). Thus inhibition of translation initiation by kasugamycin promotes mRNA stability (22). This is pertinent because hypertonicity inhibits protein synthesis, as previously demonstrated in endothelial (20) and MDCK cells. In a cell-free system, high NaCl inhibits both translation initiation and elongation (5). We speculate that 5'-UTR-directed stabilization of TonEBP/OREBP mRNA is mediated by high-NaCl-induced decrease in translation. However, further study is required to define the mechanism by which high NaCl increases stability of TonEBP/OREBP mRNA.

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