Expression of osmotic stress-related genes in tissues of normal and hyposmotic rats

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TonEBP [also called OREBP (7), NFAT5 (10), NFATz (14), and NFATL1 (17)] is a transcription factor, originally identified from its role in adaptation of cells to increases in tonicity (13). This role is clear in the renal inner medulla, where tonicity is higher than in the rest of the body, and can vary greatly with urinary concentration (1). In inner medullary cells, TonEBP regulates transcription of genes that protect against hypertonicity, including genes such as those for aldose reductase (AR) and betaine (BGT1), inositol (SMIT), and taurine (TauT) transporters (2, 12, 13) that direct organic osmolyte accumulation and genes for heat shock proteins (21). TonEBP is also expressed in cultured cells derived from tissues not normally exposed to hypertonicity, for example, HEP G2 cells from liver (5), Chang liver cells (7), HeLa cells (7), embryonic stem (ES) cells (11), and HEK 293 cells (5). In these cells, as well as in kidney-derived cells such as Madin-Darby canine kidney (MDCK) (12, 20), COS 7 (7, 9), and mIMCD3 cells (21), TonEBP is expressed and is transcriptionally active in the ~300-mosmol/kgH2O media in which they are routinely grown. Furthermore, expression and transcrip- tional activity of TonEBP at ~300 mosmol/kgH2O are bidirectionally regulated by tonicity. These effects include that at ~300 mosmol/kgH2O 1) dominant negative TonEBP reduces expression of Ton-E-mediated luciferase constructs (13) and reduces the abundance of SMIT and BGT1 mRNA; and 2) reducing tonicity decreases abundance of TonEBP protein and mRNA and causes it to move out of the nucleus, opposite to the result of increasing tonicity (20). Because TonEBP mRNA falls more rapidly than can be accounted for by its half-life, hypotonicity apparently accelerates its turnover; 3) reducing tonicity decreases the transacti- vating activity of TonEBP, opposite to the effect of increasing tonicity (5, 13). In short, in cell cultures, TonEBP expression and transcriptional activity are present at ~300 mosmol/kgH2O and respond to decreases, as well as increases, in tonicity.

After its cloning, it became evident that TonEBP mRNA is constitutively expressed in virtually all tissues in vivo, including the majority that are never normally exposed to hypertonicity (10, 13, 17). Also, TonEBP protein was detected in adult murine thymus and testes (17). TonEBP is expressed in ES cells and throughout the stages of fetal development (11). Immunostaining shows expression of TonEBP in almost all developing tissues, including the brain, colon, heart, muscle, and eyes (11). These findings raise the question of what the role of TonEBP expression might be in nonrenal tissues. One possibility is that it serves an osmoregulatory role in these tissues, responding to decreases and increases in tonicity at ~300 mosmol/
kg H2O, as discussed above for tissue culture cells. However, other roles for TonEBP have also been identified. In lymphocytes, hypertonicity increases TonEBP transcriptional activity (16, 17), but so also do proinflammatory stimuli (6). However, the role of constitutive expression of TonEBP mRNA in most tissues is undefined.

In the present studies, we tested two hypotheses: 1) that previously undetected protein expression of TonEBP might occur in vivo in the tissues expressing its mRNA, and to test this we examined TonEBP protein expression in several tissues of rats; and 2) that TonEBP might serve an osmoregulatory role in nonrenal tissues in vivo, responding to decreases, as well as increases, in toxicity of ~300 mosmol/kg H2O. To test this, we looked for downregulation of TonEBP mRNA and protein expression in nonrenal rat tissues exposed to hypertonicity during hyposmolality and for decreases in RNA expression of genes that are transcriptional targets of TonEBP.

MATERIALS AND METHODS

Experimental animals. Osmolality of the blood in rats was reduced by using the vasopressin escape protocol previously described (4). Briefly, under isofluorane anesthesia, male Sprague-Dawley rats were subcutaneously implanted with osmotic minipumps (model 2002, Alzet, Palo Alto, CA), which delivered 20 ng dDAVP/h (Peninsula Laboratories, Belmont, CA) for 4 days. All rats received pellets of rat chow (Formula 53140000, Ziegler Brothers, Gardner, PA) and ad libitum water during this period. After 4 days, the rats were divided into control (3 rats for measurement of specific mRNAs) and experimental (hyposmolality, similarly 6 rats) groups. Vasopressin escape rats were given a daily water load via a gelled-agar diet (71% by volume) at room temperature with 5% nonfat dried milk and probed overnight at 4°C with rabbit polyclonal anti-NFAT5 COOH-terminal diluted antibodies (Affinity BioReagents, no. PA1–023; diluted to 1:1,000). Membranes were washed and exposed to secondary antibody (goat anti-rabbit IgG conjugated to horseradish peroxidase, Pierce no. 31463; diluted to 1:5,000) for 1 h at room temperature. After being washed, bands were visualized using a lumino-l-based enhanced chemiluminescence substrate (LumiGLO, Kirkegaard and Perry Laboratories, Gaithersburg, MD). Band densities were determined by laser densitometry (Personal Densitometer SI).

Sample preparation, RNA isolation, cDNA preparation, and real-time PCR for measurement of abundance of specific RNAs. Total RNA was isolated from brain, thymus, skeletal muscle, liver, and heart of three control and three hyposmotic rats, using an RNaseasy kit (Qiagen). This procedure includes treatment with DNase to minimize contamination by genomic DNA. cDNA was prepared with TaqMan reverse transcription reagents, using random hexamers, according to the manufacturer’s instructions (Applied Biosystems). Real-time PCR was performed in triplicate on both 8- and 80-ng aliquots of each cDNA sample using TaqMan universal PCR master mix in a total volume of 20 µl (ABI PRISM 7900HT Sequence Detection System, Applied Biosystems). In this system, the accumulation of the PCR product is monitored in real time by a fluorogenic 5′-nucleotide assay, using probes specific for each cDNA being tested. Primers and probes were designed from rat cDNA sequences. The PCR primers were designed to span an intron of genes that contain introns, namely TonEBP, AR, BGT1, TauT, and β-actin. This was not possible for SMIT and heat shock protein (HSP)70 genes, which contain no introns. The sequences of the primers and probes are shown in Table 1. We sequenced the PCR products produced by each primer set and found that all sequences

<table>
<thead>
<tr>
<th>Table 1. Primers and probes designed for TonEBP and downstream genes</th>
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<tr>
<td><strong>Forward Primer</strong></td>
</tr>
<tr>
<td>TonEBP</td>
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<tr>
<td>β-Actin</td>
</tr>
<tr>
<td>AR</td>
</tr>
<tr>
<td>BGT1</td>
</tr>
<tr>
<td>TauT</td>
</tr>
<tr>
<td>SMIT</td>
</tr>
<tr>
<td>Hsp70-1</td>
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<tr>
<td>Hsp70-2</td>
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AR, aldose reductase; Hsp, heat shock protein; TauT, taurine transporter; BGT1, betaine transporter; SMIT, inositol transporter.
matched those of the intended targets. Significant contamination by genomic DNA was excluded by failure to generate a product in PCR reactions on total RNA that was not reverse transcribed, using the SMIT- and HSP70-specific primers and probes.

Calculation of relative tissue mRNA abundance from the real-time PCR data. The detection system records the number of PCR cycles (Ct) required to produce an amount of product equal to a threshold value, which is a constant. From the Ct values, we calculated means ± SE of tissue mRNA abundance in each tissue from experimental animals relative to the mean of control animals (taken as 100%), using the following principles. 1) By definition, the number of specific cDNA molecules at the threshold (N_Ct) is constant for a given cDNA, independent of the number of cycles that it takes to reach it. 2) For a specific cDNA, the ratio N(exp)/N(cont), is independent of i, assuming only that the efficiency (E) of PCR for a specific template is constant and the same for samples from experimental and control animals, where i is the cycle number, and N(X), is the number of specific cDNA molecules in a sample (X = control or experimental) at cycle i. 3) The ratio of the number of specific cDNA molecules at a cycle, Ct, to the number at another cycle, i, is N_i/N_Ct = 1/E^{Ct–i}.

To normalize the comparison between control and experimental results, we compared all results to the number of specific molecules at an arbitrary cycle, I, chosen for convenience to be the largest whole number that is less than any of the experimental values of Ct. Then, we calculated N(exp)/N_i for each sample. From those results, i.e., avg[N(cont)/N_Ct], or the average of N(cont)/N_Ct, was obtained. Each result for a given tissue from an experimental animal was normalized to a mean control value of 100% by dividing each value of N(exp)/N_Ct (control and experimental) by avg[N(cont)/N_Ct] and multiplying by 100. Then, means ± SE were calculated for control and experimental samples.

Statistics. Statistical significance was calculated using the one-tail t-test to evaluate for each tissue the differences between control and experimental conditions (GraphPad InStat 3.0). Results are expressed as means ± SE. Differences were considered significant for P < 0.05.

RESULTS

Serum osmolality differs significantly (P < 0.0001) between rats in the control group (294 ± 1 mosmol/kg H_2O, n = 6) and the hyposmotic group (241 ± 2 mosmol/kg H_2O) after the latter are subjected to 3 days of water loading in the presence of dDAVP. The decrease is similar to that observed in earlier studies, using a similar protocol (4), in which differences in concentration of sodium and its associated anions accounted for most of the decrease in osmolality. Thus the lower plasma osmolality reflects hyponatremia and hypotonicity.

Effect of hypotonicity on TonEBP mRNA expression. Detectable levels of TonEBP mRNA are present in all tissues tested from control rats, but the abundance varies greatly from tissue to tissue. The lowest level is in skeletal muscle (mean Ct = 31.59 with 80 ng of cDNA reverse transcribed from total RNA). The levels in the other tissues are higher than in skeletal muscle: brain 209 times as high as skeletal muscle, thymus 251, heart 81, and liver 40.

Brains and livers from the hyposmotic rats contain less TonEBP mRNA than those from the control rats, but there is no significant difference in thymus, heart, or skeletal muscle (Fig. 1A). Representative amplification curves of TonEBP mRNA from brains of control and hyposmotic rats are shown in Fig. 2. Simultaneously measured 18s RNA does not differ significantly with serum osmolality in any tissue (Fig. 1B), confirming that reverse transcription, cDNA loading, and PCR are equivalent. Also, expression of β-actin does not differ (Fig. 1C), confirming that the changes in TonEBP in brain and liver do not reflect a general change in mRNA level in those organs.

![Fig. 1. Expression of specific RNAs in tissues from control and hyposmotic rats. Results are expressed relative to a mean control value of 100% (n = 3, *P < 0.05). A: TonEBP RNA expression. B: 18s RNA expression. C: β-actin RNA expression.](image)

![Fig. 2. Representative real-time PCR plot by the ABI Prism 7900HT Sequence Detection System. Points are mean of TonEBP result from brains of 3 control and 3 hyposmotic rats. Representative amplification curve showing cycle number of control and hyposmotic rats (broken line showing position of amplicon threshold).](image)
Effect of hypotonicity on TonEBP protein expression. Although TonEBP mRNA is detected in all tissues examined (Fig. 1), TonEBP protein is not detected in heart and skeletal muscle, even loading 80 µg of protein (Fig. 3). Hypotonicity significantly lowers TonEBP protein expression in thymus and liver but not in brain (Fig. 3).

Effect of hypotonicity on expression of other mRNAs. We also measured the mRNA abundance of several genes that are known transcriptional targets of TonEBP, namely AR, BGT1, SMIT, and TauT (Table 2) (2, 12, 13). AR mRNA does not differ significantly between osmotic conditions in any tissue. BGT1 decreases significantly with osmolality only in liver. SMIT decreases significantly with osmolality in liver and muscle. TauT decreases significantly with osmolality in brain and thymus.

HSP70 expression is known to be affected by hypertonicity. Hypertonicity increases HSP70 mRNA and protein expression in MDCK cells under isotonic and hypertonic conditions, consistent with regulation of transcription of HSP70 by TonEBP (21). Examination of HSP70 mRNA expression is complicated because two different HSP70 genes express virtually identical proteins (18, 21), and the names given in rat, human, and mouse for the homologous genes differ in a confusing fashion (Table 3) and are used inconsistently in the literature. In what follows a gene is designated by species and the name of the gene in that species, according to GenBank. The 5′-flanking region of the human HSP70-2 gene contains TonEs (21), as it does also in the homologous rat Hsp70-1 and mouse hsp70.1 genes. Hypertonicity increases mouse (mIMCD3 cells) hsp70.1 mRNA expression but not hsp70A1 expression (21). Also, transcription of a luciferase reporter construct containing ~4 kb of the 5′-flanking region of mouse hsp70.1 gene is increased by hypertonicity (21). Given this evidence that TonEBP regulates tonicity-dependent transcription of mouse hsp70.1, but not mouse hsp70A1, we measured the effect of hypotonicity on the homologous rat Hsp70-1 and rat Hsp70-2 genes. Surprisingly, hypotonicity causes a large increase of rat Hsp70-1 and Hsp70-2 mRNA in brain, thymus, heart, and skeletal muscle, without significant change in liver (Table 2).

DISCUSSION

The expression and transcriptional activity of TonEBP in immortalized cell lines at ~300 mosmol/kgH2O are

<table>
<thead>
<tr>
<th>mRNA</th>
<th>Brain</th>
<th>Thymus</th>
<th>Heart</th>
<th>Muscle</th>
<th>Liver</th>
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<tbody>
<tr>
<td>AR</td>
<td>97 ± 16</td>
<td>134 ± 16</td>
<td>106 ± 4</td>
<td>113 ± 7</td>
<td>88 ± 13</td>
</tr>
<tr>
<td>BGT1</td>
<td>153 ± 26</td>
<td>99 ± 4</td>
<td>91 ± 7</td>
<td>76 ± 4</td>
<td>41 ± 9*</td>
</tr>
<tr>
<td>TauT</td>
<td>66 ± 12*</td>
<td>81 ± 5*</td>
<td>81 ± 10</td>
<td>71 ± 17</td>
<td>38 ± 22</td>
</tr>
<tr>
<td>SMIT</td>
<td>76 ± 16</td>
<td>106 ± 8</td>
<td>97 ± 7</td>
<td>51 ± 5*</td>
<td>41 ± 16°</td>
</tr>
<tr>
<td>Hsp70-1</td>
<td>147 ± 16*</td>
<td>200 ± 22*</td>
<td>192 ± 29*</td>
<td>313 ± 37*</td>
<td>94 ± 28</td>
</tr>
<tr>
<td>Hsp70-2</td>
<td>284 ± 24*</td>
<td>210 ± 14*</td>
<td>163 ± 16*</td>
<td>235 ± 44*</td>
<td>118 ± 30</td>
</tr>
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</table>

Values are means ± SE. *P < 0.05 vs. control, n = 3.

Table 3. Comparison of nomenclature (from GenBank) of rat, mouse, and human HSP70 genes (18)

<table>
<thead>
<tr>
<th>HSP70 mRNA</th>
<th>Responds to Heat and Tonicity</th>
<th>Accession Number</th>
<th>Responds to Heat and Tonicity</th>
<th>Accession Number</th>
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<tbody>
<tr>
<td>Rat Hsp70-1</td>
<td>X77207</td>
<td>Hsp70-2</td>
<td>X77208</td>
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<tr>
<td>Human HSP70-2</td>
<td>M59830</td>
<td>HSP70-1</td>
<td>M59828</td>
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<tr>
<td>Mouse hsp70.1*</td>
<td>M35021</td>
<td>hsp70A1</td>
<td>M76613</td>
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Homologous genes are listed in the same column. *Hsp70.1 also called HSP70-2 (21), Hsp70-1 (18). Hsp70A1 also called HSP70-1 (21), Hsp70-3 (18).

Fig. 3. TonEBP protein expression in tissues from control and hypotonic rats. The 5 tissues shown were collected from each rat. Protein loading was 40 µg/lane from brain, liver, and thymus; 80 µg/lane from heart and skeletal muscle. In all blots, the left-most 3 lanes were from 3 individual control rats and the right-most 3 lanes were from 3 individual hypotonic rats.
bidirectionally regulated by tonicity, suggesting that they might also be bidirectionally regulated by tonicity at ~295 mosmol/kgH2O in the multiple nonrenal tissues in which they are expressed in vivo.

We used the rat “vasopressin escape” model (4) of hyposmolality to test this hypothesis. By infusing rats with dDAVP while maintaining a high water intake, we lowered their plasma osmolality from the normal 294 to 241 mosmol/kgH2O. Then, we measured in brain, liver, thymus, skeletal muscle, and heart TonEBP mRNA and protein and mRNA expression of AR, BGT1, SMIT, TauT, and HSP70 genes that are osmotically regulated by TonEBP. The results do not support the hypothesis that TonEBP generally responds to decreases, as well as increases, in tonicity around the normal plasma osmolality in vivo, which would be the case if there was a general osmoregulatory role for its ubiquitous expression in nonrenal tissues. In immortalized cells, hypotonicity decreases TonEBP mRNA and protein (20). In contrast, in vivo hypotonicity results in decreases in bothTonEBP mRNA and protein. In brain, TonEBP mRNA decreases, but TonEBP protein does not change. In thymus, TonEBP protein decreases, but mRNA does not change. In heart and skeletal muscle, TonEBP mRNA does not change and TonEBP protein is immeasurably low.

In immortalized cells in tissue culture, hypotonicity decreases AR, BGT1 mRNA abundance (5), and SMIT mRNA abundance (20). In contrast, in vivo hypotonicity (Table 2) does not change AR mRNA in any of the five tissues that were tested. It decreases BGT1, SMIT, and TauT mRNA in liver (although the latter reduction is not statistically significant), SMIT mRNA in muscle, and TauT mRNA in brain and thymus. Thus we find that hypotonicity decreases mRNA of some transcriptional targets of TonEBP in some nonrenal tissues in vivo, as might be expected if expression of these genes were under bidirectional constitutive osmotic regulation by TonEBP, but not all targets and not in all tissues. Liver is the only organ in which we found that hypotonicity consistently reduces not only TonEBP mRNA and protein but also BGT1, SMIT, and TauT mRNA. The latter results are reminiscent of a previous study of primary culture of rat liver sinusoidal endothelial cells (19) in which hypotonicity decreases BGT1, SMIT, and TauT mRNA and reduces uptake of betaine, inositol, and taurine. We infer that, although tonicity may bidirectionally control TonEBP activity in liver, other sources of regulation predominate in the other tissues in which it is constitutively expressed.

Thus TonEBP apparently plays a nonosmotic role in most nonrenal tissues in which it is constitutively expressed in vivo. Some nonosmotic roles of TonEBP are already known, namely in lymphocytes proinflammatory stimuli, as well as hypertonicity, increase TonEBP transcriptional activity, suggesting a role in signaling inflammation (17), and TonEBP is involved in promotion of carcinoma invasion downstream of integrin, suggesting a role in tumor metastasis (6). It seems likely that other nonosmotic roles of TonEBP remain to be discovered.

Liver is the only organ in which hypotonicity-induced decreases of TonEBP mRNA and protein expression correlate. In brain, TonEBP mRNA decreases but not protein; in thymus, TonEBP protein decreases but not mRNA. This lack of correlation is not surprising, however, because protein abundance is also regulated by translation and degradation, which may or may not follow mRNA abundance.

Hypertoncity increases HSP70 expression (3) because of TonEBP-mediated increase in transcription (21). Therefore, we expected that hypotonicity might decrease HSP70 in the nonrenal tissues that we studied in vivo. Surprisingly, we found that hypotonicity greatly increases Hsp70-1 and Hsp70-2 mRNA in brain, thymus, skeletal muscle, and heart. Greater induction of HSP70 mRNA and protein by heat in primary cultures of rat hepatocytes was observed at 205 than at 305 mosmol/kgH2O, but the result of hypotonicity alone was not reported (8). We are unaware of any other previous evidence that hypotonicity induces HSP70. A specific TonEBP-mediated response to hypotonicity per se seems unlikely, if only because Hsp70-2 is not mediated by TonEBP (21). Instead, we suppose that hypotonicity might trigger a general stress response akin to heat shock but unrelated to TonEBP.

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REFERENCES


17. **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.


