Urea inhibits hypertonicity-inducible TonEBP expression and action

WEI TIAN AND DAVID M. COHEN
Divisions of Nephrology and Molecular Medicine and Department of Cell and Developmental Biology, Oregon Health Sciences University and the Portland Veterans Affairs Medical Center, Portland, Oregon 97201

Received 24 October 2000; accepted in final form 29 January 2001

Tian, Wei, and David M. Cohen. Urea inhibits hypertonicity-inducible TonEBP expression and action. Am J Physiol Renal Physiol 280: F904–F912, 2001.—Tonicity-responsive genes are regulated by the TonE enhancer element and the tonicity-responsive enhancer binding protein (TonEBP) transcription factor with which it interacts. Urea, a permeant solute coexistent with hypertonic NaCl in the mammalian renal medulla, activates a characteristic set of signaling events that may serve to counteract the effects of NaCl in some contexts. Urea inhibited the ability of hypertonic stressors to increase expression of TonEBP mRNA and also inhibited tonicity-inducible TonEBP-dependent reporter gene activity. The permeant solute glycerol failed to reproduce these effects, as did cell activators including peptide mitogens and phorbol ester. The inhibitory effect of urea was evident as late as 2 h after the application of hypertonicity. Pharmacological inhibitors of known urea-inducible signaling pathways failed to abolish the inhibitory effect of urea. TonEBP action is incompletely understood, but evidence supports a role for proteasome function and p38 in action regulation; urea failed to inhibit proteasome function or p38 signaling in response to hypertonicity. Consistent with its effect on TonEBP expression and action, urea pretreatment inhibited the effect of hypertonicity on expression of the physiological effector gene, aldose reductase. Taken together, these data define a molecular mechanism of urea-mediated inhibition of TonEBP abundance in regulating TonE-mediated gene transcription.

tonicity-responsive enhancer binding protein; stress; renal; kidney; cell culture; proteasome; mitogen-activated protein kinase

ALL ORGANISMS ADAPT TO HYPERTONICITY at the cellular level through intracellular accumulation of osmotically active compounds. It is hypothesized that these compounds function primarily to offset a detrimental decrement in cell volume that accompanies profound hypertonic stress, however, other protective roles have been proposed as well. Cells of the mammalian kidney medulla are routinely subjected to a substantially elevated and fluctuating ambient osmolarity by virtue of the renal concentrating mechanism. Genes encoding proteins essential for osmolyte transport or synthesis are regulated by hypertonicity at the transcriptional level by a member of the nuclear factor of activated transcription (NFAT) family of transcription factors (12), tonicity-responsive enhancer binding protein (TonEBP) (15). TonEBP binds in its cognate DNA consensus sequence, which has been identified in the promoters governing osmotically responsive expression of these transporters and metabolic enzymes (reviewed in Ref. 4). Comparatively little is known about the mechanism of TonEBP regulation or action.

Urea coexists with hypertonic NaCl in the renal medulla. Some data have suggested a mutually protective role for these two solutes. Santos et al. (24) and Neuhofet al. (20) have shown that NaCl may protect renal epithelial cells from the potentially adverse effects of urea; we have shown the converse: urea protects medullary cells from the proapoptotic effect of NaCl (33). Interestingly, earlier data suggested that urea may abrogate the ability of (or necessity for) hypertonically stressed cells to maximally accumulate organic osmolytes (16–18).

For these reasons, we examined the effect of urea on tonicity-inducible TonEBP expression and action. Urea (but not the permeant solute, glycerol) suppressed hypertonicity-inducible TonEBP mRNA expression, TonE-mediated reporter gene transcription, and tonicity enhancer element (TonE)-dependent transcription of aldose reductase. These effects were not attributable to any of the previously described actions of urea and were not a consequence of urea inducing any of the known signaling events leading to enhanced TonEBP expression.

EXPERIMENTAL PROCEDURES

General methods. Cell culture and solute treatment were performed as previously described (23, 26). The following inhibitors and stimuli (purchased from Calbiochem unless otherwise indicated) were used: 200 mM urea (Sigma); 100 mM NaCl (200 mosmol/kg H2O; Sigma); 200 mM mannitol, (Sigma); 200 mM glycerol (Sigma); 100 mM epidermal growth factor (EGF; Sigma); 100 mM 12-O-tetradecanoyl

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.

http://www.ajprenal.org
phorbol-13-acetate (TPA); 50 μM PD98059; 10 μM LY294002; 30 mM N-acetylcysteine (Sigma); 50 μM SB203580; 30–100 μg/ml cycloheximide (Sigma); and 10 μM MG-132. Cells receiving pretreatment with an inhibitor or solute (i.e., urea) were generally pretreated for 30 min unless otherwise indicated and remained exposed to the pretreatment compound for the duration of the experiment until determination of the experimental end point. Depicted data represent means ± SE unless indicated; statistical significance is assigned to P < 0.05 (Excel; Microsoft).

**TonEBP RT-PCR and RNase protection assay.** Total cellular RNA was prepared using the Trizol reagent (Life Technologies) in accordance with the manufacturer’s directions. Murine TonEBP partial cDNA was generated by RT-PCR from mouse kidney poly-A+ RNA (Clontech) by using the Superscript preamplification system (Life Technologies) for first-strand cDNA synthesis in accordance with the manufacturer’s directions. The RT reaction was subjected to PCR amplification (30 cycles total at an annealing temperature of 60°C and an extension time of 90 s) using the OptiPrime system (buffer 1; Stratagene). 5’ Primer (Portland VA Molecular Biology Core Laboratory, Portland, OR) included sequences from nucleotides 3570–3590 of the mRNA encoding human TonEBP (GenBank accession AF089824 (15)); cttcgccatgcgaac; 3’ primer included nucleotides 4045–4065 from this sequence (read 5’ → 3’): tggctatggcatattgc. A ~500-bp amplified fragment was identified, subcloned into pcDNA3.1/V5/His/TOPO vector [using Eukaryotic TOPO TA Cloning Kit (Invitrogen) according to the manufacturer’s directions], and its identity was confirmed by sequencing (Core Facility, Vollum Institute for Advanced Biomedical Research, Portland, OR). Although not identical with human TonEBP, most differences at the amino acid level were conservative (Fig. 1); it was identical at the amino acid level with unpublished murine TonEBP expression sequences from nucleotides 3570–3590. cDNA was PCR generated for use in RPA with the Superscript preamplification system (Life Technologies) in accordance with the manufacturer’s directions, and its identity was confirmed by sequencing (Core Facility, Vollum Institute for Advanced Biomedical Research, Portland, OR).

**RESULTS**

Approximately 500-bp partial murine TonEBP cDNA was PCR generated for use in RPA with the murine inner medullary collecting duct (mIMCD-3) cell line. The sequence was virtually identical with subsequently deposited but unpublished GenBank sequences encoding murine NFAT5/CAG-8. Comparison of the conceptual translation of the partial murine clone to human TonEBP revealed an extremely high degree of similarity (Fig. 1), apart from a three-amino acid insertion contributing an additional negative charge.

By RPA, TonEBP expression in the murine renal medullary mIMCD-3 cell line appeared constitutive; upregulation of expression was observed with as little as 2 h of treatment with 100 mM (200 mosmol/kgH2O) NaCl (Fig. 2). Expression was much more robust at 4 and 6 h of treatment. Pretreatment with urea (200 mM × 30 min; see General methods) blunted the ability of hypertonic NaCl to increase TonEBP expression.

Previous data have indicated that some properties of the permeant solute urea are mimicked by other permeant solutes whereas others are not (e.g., Ref. 33). Therefore, the ability of urea to prevent hypertonicity-inducible TonEBP expression was compared with another permeant solute, glycerol. Urea pretreatment, but not glycerol pretreatment, inhibited NaCl-inducible TonEBP expression (Fig. 3A). In all repeats of this experiment, murine TonEBP expression was much more robust at 6 h of treatment with urea (200 mM × 30 min; see General methods) blunted the ability of hypertonic NaCl to increase TonEBP expression.

**DISCUSSION**

The data presented here further support the hypothesis that urea blocks the ability of hypertonic NaCl to increase TonEBP expression. The ability of TonEBP to respond to hypertonic NaCl was blunted by pretreatment with urea. This observation is consistent with previous data indicating that urea blunts the ability of hypertonic NaCl to increase TonEBP expression (33). The ability of urea to block the ability of hypertonic NaCl to increase TonEBP expression is consistent with the hypothesis that urea blocks the ability of hypertonic NaCl to increase TonEBP expression (33). The ability of urea to block the ability of hypertonic NaCl to increase TonEBP expression is consistent with the hypothesis that urea blocks the ability of hypertonic NaCl to increase TonEBP expression (33).
experiment, urea inhibited NaCl-inducible TonEBP expression by at least 25% (and generally much more) whereas glycerol, when applied at equiosmolar amounts, failed to reproduce this effect. Prolonged treatment with either 200 mM urea or glycerol failed to independently increase TonEBP expression. The effect of urea (and lack of effect of glycerol) was also apparent in the nonrenal 3T3 cell line (Fig. 3B). Results of densitometric reduction of autoradiograph data from three independent experiments using mIMCD-3 cells are depicted in Fig. 3C.

Because of the ability of urea to block TonEBP expression, the effect of urea on TonEBP-dependent transcription, a downstream event, was explored next. Cells were transfected with a luciferase reporter gene (Fig. 4A) under the control of two tandem repeats of the BGT tonicity-responsive enhancer element (14), as well

---

**Fig. 2.** Urea inhibits tonicity-inducible TonEBP expression. RNase protection assay (RPA) of TonEBP (filled arrowhead) and actin (Control; open arrowhead) mRNA expression from murine inner medullary collecting duct (mIMCD-3) cells treated with 100 mM NaCl (200 mosmol/kgH2O) for the indicated interval, in the absence (NaCl) or presence (Urea + NaCl) of 30-min pretreatment with 200 mM urea, is shown. P in this and subsequent figures indicates undigested probe alone (~10% of the activity added to RNA samples in other lanes). One of two replicate experiments is depicted.

**Fig. 3.** Glycerol fails to block TonEBP expression. RPA of TonEBP (filled arrowhead) and actin (Control; open arrowhead) mRNA from mIMCD-3 cells (A) and 3T3 cells (B) treated with 100 mM NaCl × 6 h, in the absence (−) or presence (+) of 30-min pretreatment with the indicated permeant solute (applied to 200 mM) is shown. C: densitometric reduction of autoradiograph data from 3 separate experiments using mIMCD-3 cells.

**Fig. 4.** Urea inhibits TonEBP-mediated transcription. Luciferase (Luc) activity (normalized to β-galactosidase activity from a lacZ-expressing constitutively active cotransfected plasmid) representing transcription of a thymidine kinase promoter under the control of 2 tandem repeats of the betaine-GABA transporter (BGT) tonicity-responsive consensus element (A) in mIMCD-3 (B) and 3T3 (C) cells receiving no pretreatment (Control) or 30-min pretreatment with either urea (+Urea; 200 mM) or glycerol (+Glycerol; 200 mM) before treatment with NaCl (100 mM × 6 h) is shown. Data in each panel are normalized to control pretreatment in the absence of NaCl treatment (first bar in graph) and represent the means ± SE of at least 4 separate experiments, each with determinations performed in duplicate or triplicate.
in the presence of urea pretreatment (200 mM Urea) or presence (+Urea) of urea pretreatment (200 mM × 30 min) is shown. Data are normalized to control treatment in the absence of urea pretreatment (first open bar in graph) and represent the means ± SE of at least 3 separate experiments, each with determinations performed in triplicate.

as a control plasmid harboring the lacZ gene with expression directed by the constitutively active CMV long-terminal repeat promoter. As anticipated, and consistent with the data of others, NaCl treatment (100 mM × 6 h) increased normalized reporter gene activity ~10-fold in mIMCD-3 cells (Fig. 4B). Urea pretreatment (200 mM × 30 min) substantially suppressed the effect of hypertonic NaCl, whereas glycerol pretreatment was again ineffective. By way of comparison, urea pretreatment resulted in a modest increase (~50%) in transcription from the control CMV LTR promoter, arguing against a global inhibitory effect of urea in this context. Similarly, urea pretreatment followed by NaCl treatment resulted in essentially no net effect on transcription from the CMV LTR (data not shown). As an additional control, the effect of urea on a nonosmotically responsive but specifically regulated promoter was examined. Cells (mIMCD-3) were transfected with a plasmid harboring a luciferase gene under the control of the glucocorticoid-responsive MMTV promoter (MMTV-Luc; Clontech). Dexamethasone treatment (300 nM × 24 h) increased luciferase activity from 170 ± 170 to 9,600 ± 2,400 relative light units; in the presence of urea pretreatment (200 mM × 30 min), dexamethasone increased luciferase activity from 590 ± 70 to 8,100 ± 300 relative light units (data not shown). Neither control nor dexamethasone-inducible values in the presence of urea pretreatment were statistically distinct from those obtained in the absence of urea pretreatment. Because some but not all effects of urea appear to be specific to cells of renal epithelial origin (e.g., MDCK and mIMCD-3 cells), the modulatory effect of urea was examined in nonrenal fibroblastic 3T3 cell line. Similar to the mIMCD-3 model, hypertonic NaCl (100 mM × 6 h) increased reporter gene activity by >10-fold (Fig. 4C). Also consistent with the mIMCD-3 model, urea pretreatment but not glycerol pretreatment substantially blocked the effect of hypertonic NaCl.

Some data have suggested that there is a unique relationship between urea and NaCl that does not extend to all hypertonic stressors. The effect of urea in the context of mannitol-associated hypertonicity was therefore examined in mIMCD-3 cells. The effect of mannitol (200 mM) was indistinguishable from that of equiosmolar NaCl (100 mM) at activating reporter gene activity (Fig. 5). Urea significantly blocked the effect of hypertonic mannitol on BGT-2X-Luc activity; despite the tendency toward a greater effect on NaCl-inducible transcription (84% inhibition) than mannitol-induced transcription (60% inhibition), there was no statistically significant difference between the two solutes with respect to protection afforded by urea.

The ability of urea to protect from hypertonicity-inducible apoptosis resembled that of peptide growth factors such as EGF and insulin-like growth factors (33). Therefore, the effect of other cell activators on hypertonicity-inducible transcription was explored. Neither the mitogen, EGF, nor the classic protein kinase C (cPKC)-activating TPA, affected toxicity inducibility of the luciferase reporter gene (Fig. 6). Specifically, induction by NaCl with no pretreatment was 9.9-fold, with EGF pretreatment, 9.2-fold, and with TPA pretreatment, 9.5-fold. In contrast, induction by hypertonicity was only 4.6-fold in the presence of urea pretreatment.

We next sought to determine whether the ability of urea to block TonEBP-mediated transcription required pretreatment with urea or whether concurrent administration would be suitable. Urea was added at varying concentrations performed in triplicate.
time points relative to the institution of hypertonic stress (from 60 min before to 120 min after), and the effect on tone-mediated transcription was determined (Fig. 7). NaCl treatment (200 mosmol/kgH$_2$O) dramatically increased reporter gene activity ninefold. Urea was equally inhibitory when added 60 or 30 min before, or simultaneously with NaCl. Even 2 h after NaCl treatment, urea still partially abrogated the effect of hypertonicity.

Multiple signaling pathways have been shown to be activated by urea treatment in the mMCD-3 model. Extracellular signal-regulated kinase signaling mediates, in part, the ability of urea to upregulate immediately early gene expression (5). Phosphoinositide 3-kinase signaling is instrumental in renal epithelial cell survival of urea and hypertonic stress (35). Oxidative stress mediates the ability of urea to activate transcription and expression of the stress-responsive transcription factor, Gadd153 (34). We therefore examined the effect of pharmacological inhibitors of each of these signaling events to assess whether the respective pathways mediated the ability of urea to block signaling to TonEBP expression and action. Although some of these compounds exhibited modest effects on basal and hypertonicity-inducible TonEBP action (i.e., reporter gene activity), none significantly influenced the ability of urea to block signaling by hypertonicity (Fig. 8).

Although urea has been shown by some to modestly activate p38, a pharmacological inhibitor of this pathway was not examined in the transient transfection assay (see Figs. 10 and 11) because it would be expected to independently inhibit potentially p38-mediated tonicity-inducible transcription (25), rendering the data uninterpretable.

TonEBP-mediated transcription is blocked by pharmacological inhibitors of proteasome function (29). It was therefore hypothesized that urea might act via inhibiting proteasome function. Consistent with the findings of Woo et al. (29), proteasome inhibition with MG-132 pretreatment substantially inhibited TonEBP-dependent transcription of the BGT-2X-Luc reporter gene (Fig. 9A); the degree of inhibition was comparable to that seen with urea pretreatment (e.g., Fig. 4). MG-132 treatment also resulted in significant accumulation of high- and low-molecular-weight ubiquitinated proteins (as determined via anti-ubiquitin immunoblotting; Fig. 9B) as would be anticipated with inhibition of proteasome-dependent catabolism; however, urea failed to mimic this effect. Therefore, urea does not appear to influence TonEBP signaling via inhibition of proteasomal function.

Activation of the p38 mitogen-activated protein kinase (MAPK) has also been variably implicated in tonicity-dependent gene regulation (25). Therefore, a possible role for urea in inhibiting tonicity-inducible p38 activation was explored. In mMCD-3 cells, hypertonic NaCl (200 mosmol/kgH$_2$O) dramatically increased p38 phosphorylation as determined by anti-P-p38 immunoblotting (Fig. 10), consistent with prior observations (3, 32). Not only did urea pretreatment fail to inhibit this effect, it exerted a modest potentiation of influence. As a correlate of these data, the ability of p38 to mediate the effect of NaCl on TonEBP expression was examined using the RPA assay. Pretreatment of mMCD-3 cells with the p38 inhibitor, SB203580, failed to influence NaCl-inducible TonEBP mRNA in-

![Fig. 7. Influence of pretreatment interval on ability of urea to protect from tonicity-inducible TonEBP-mediated transcription. Luc activity (normalized to β-galactosidase activity, and expressed relative to Control) in mMCD-3 cells cotransfected with BGT-2X-Luc and CMV-Gal and treated with 200 mM urea at the indicated time relative to treatment with 100 mM NaCl (e.g., "−30" indicates urea treatment precedes NaCl treatment by 30 min is shown). The dashed line labeled "+ NaCl Alone" indicates reporter gene activity in response to treatment with NaCl without urea, where the shaded region corresponds to ±SE; the dashed line labeled "No Treatment" indicates reporter gene activity in the absence of urea or NaCl treatment (there is no shaded region because SE = 0 following normalization to Control). Data are normalized to control treatment in the absence of pretreatment and represent the means ± SE of 3 separate experiments, each with determinations performed in triplicate.

![Fig. 8. Effect of inhibitors of urea signaling on urea-associated inhibition of TonEBP expression. Luc activity (normalized to β-galactosidase activity, and expressed relative to "Control" with "No addition") in mMCD-3 cells cotransfected with BGT-2X-Luc and CMV-Gal and pretreated for 30 min with the indicated inhibitors of known urea-inducible signaling events (50 µM PD98059; 10 µM LY294002; or 30 mM NAC) before treatment with NaCl alone (100 mM × 6 h), or urea (200 mM × 30 min) followed by NaCl. Data are normalized to control treatment in the absence of pretreatment (first open bar in graph) and represent the means ± SE of 3 separate experiments, each with determinations performed in triplicate.](http://ajprenal.physiology.org/)
duction (Fig. 11). Specifically, SB203580 inhibited basal TonEBP mRNA expression by 16% (n = 3; P < 0.05). NaCl treatment increased TonEBP expression by 150% (relative to control – SB203580) in the absence of SB203580 and by 80% (relative to control + SB203580) in its presence, but there was no statistically significant difference between NaCl treatment in the presence or absence of the p38 inhibitor (n = 3; data not shown).

Because urea and hypertonicity may influence protein synthesis, we also examined the dependence of NaCl-inducible TonEBP expression on protein synthesis. The nonspecific inhibitor of protein synthesis, cycloheximide, failed to influence the ability of hypertonic NaCl to increase TonEBP expression (Fig. 11). To confirm that an effective dose of cycloheximide had been used, the effect of cycloheximide on tonicity-inducible expression of the aldose reductase gene was examined (Fig. 11B). Consistent with a requirement for a prior round of protein synthesis (e.g., expression of the TonEBP protein), cycloheximide pretreatment dramatically inhibited the ability of hypertonic NaCl to increase aldose reductase mRNA expression without influencing the level of expression of the control actin gene.

Last, we sought to determine the functional consequence of the ability of urea to downregulate TonEBP signaling. Because osmotically responsive genes such as aldose reductase and the betaine cotransporter are regulated at the transcriptional level by TonEBP and the tonicity enhancer element, the effect of urea on tonicity-inducible expression of such genes was investigated. As anticipated, aldose reductase expression was dramatically upregulated by hypertonic mannitol or NaCl, whereas urea failed to increase expression (Fig. 12A). These changes occurred in the absence of any effect on actin gene expression (Fig. 12A). Consistent with data concerning the effect of urea on TonEBP expression and TonEBP-dependent reporter gene expression, urea pretreatment substantially inhibited the effect of either NaCl or mannitol on aldose reductase expression (Fig. 12B).

**DISCUSSION**

Urea inhibits the ability of hypertonicity to increase expression of TonEBP, and thereby blocks downstream TonEBP-mediated transcription. Because in the renal medulla, the only mammalian tissue routinely subjected to marked hypertonicity, this stress coexists with an elevated urea concentration, the inhibitory effect of urea may have significant implications in vivo. Much emphasis in hypertonicity-inducible gene regulation has focused on the role of posttranslational modification of the TonEBP transcription factor itself (15, 29); acute regulation of TonEBP mRNA or protein abundance has received less attention (e.g., Ref. 28).
Unexpectedly, expression of TonEBP itself appears to be regulated fairly early (2–6 h) following application of hypertonicity. In corresponding fashion, TonEBP-dependent transcription is also markedly upregulated by 6 h of hypertonic treatment. Miyakawa et al. (15) and Woo et al. (29) had earlier observed upregulated TonEBP expression at 18 h of hypertonic stress in the MDCK model. A single recent observation suggested upregulation in TonEBP mRNA abundance as early as 6 h; however, this increase was very modest (~100% increase) (28). Our finding that TonEBP expression and action are upregulated relatively early in this model might suggest a greater role for TonEBP abundance (in contrast to activation) in implementation of the downstream effector response to hypertonicity. In further support of this hypothesis is the observation that inhibition of TonEBP protein synthesis with cycloheximide blocks the tonicity-inducible upregulation in expression of the hypertonicity effector gene, aldose reductase (Ref. 1 and data herein). Such data, obtained through the use of a global inhibitor of protein synthesis such as cycloheximide, do not definitively establish the inhibition of TonEBP protein expression as the proximate cause of this effect. In contrast, the ability of urea to specifically block expression of both TonEBP and aldose reductase substantially strengthens this argument (i.e., urea is not a global inhibitor of protein synthesis (6) and is therefore a more specific inhibitor of TonEBP expression than is cycloheximide). In aggregate, however, these data do not establish that upregulated TonEBP expression is sufficient for the hypertonicity-inducible transcriptional response.

It is puzzling that urea, which is known to counteract some of the adverse effects of NaCl exposure (33), should block the ability of hypertonicity to increase TonEBP expression or action. Such data raise the provocative question of whether TonEBP expression is an adaptive or maladaptive event in the hypertonic stress response. Because genes deemed essential for adaptation to hypertonic stress (e.g., aldose reductase, betaine cotransporter, Na+–dependent myo-inositol cotransporter) represent downstream targets of TonEBP, it should stand to reason that suppression of TonEBP upregulation and action would sensitize cells to hypertonicity-inducible cell death. In fact, however, a number of studies have suggested a beneficial effect of combining urea and NaCl (20, 24, 33). Because inter-

Fig. 11. Effect of protein synthesis inhibition on expression of TonEBP and aldose reductase. Effect of pretreatment with the indicated concentration of cycloheximide (CHX; × 30 min) on control treatment (C) and hypertonicity-inducible (H; 100 mM NaCl × 6 h) expression of TonEBP (A) and the tonicity-responsive effector gene, aldose reductase (B), as determined by RPA is shown. One of two replicate experiments is depicted. The last 2 lanes in A (excluding probe-only lane (P)) indicate the effect on TonEBP expression of pretreatment with the p38 inhibitor, SB203580 (50 μM × 30 min). These latter data are representative of 3 such independent experiments.

Fig. 12. Urea inhibits tonicity-inducible aldose reductase gene expression. A: effect of urea (200 mM), mannitol (200 mM), and NaCl (100 mM) on aldose reductase and actin mRNA expression by RPA following 6 or 24 h of treatment in mIMCD-3 cells. B: effect of urea pretreatment (200 mM × 30 min) on NaCl- (100 mM × 6 h) and mannitol-inducible (200 mM × 6 h) TonEBP and actin expression by RPA. P indicates probe alone. For each figure, 1 of 2 replicate experiments is depicted.
ruprtion of signaling and gene expression events required for osmolyte accumulation are detrimental to cell and organismic functioning, it is highly likely that an ancillary non-TonEBP-dependent protective pathway is upregulated in the presence of urea pretreatment, limiting the requirement for maximal TonEBP expression in response to hypertonicity. The heat shock proteins may directly influence MAPK (e.g., p38) signaling (2), which has been implicated in TonEBP-mediated transcriptional regulation and acquisition of the hypertonically-stressed phenotype (11, 25, 27). Most (e.g., Refs. 8 and 21), but not all (13), studies have failed to demonstrate upregulated heat shock protein (e.g., hsp27 or hsp70) expression in response to urea stress; involvement of a subset of the heat shock proteins has, however, been inferred by some (19, 22). It remains possible that urea-inducible heat shock protein upregulation, or another as yet undescribed mechanism, simultaneously protects from hypertonic stress and abrogates TonEBP signaling.

The urea-initiated signaling events through which TonEBP expression is inhibited are unclear. Several groups have examined the role of p38 in toxicity-inducible transcriptional regulation and have arrived at contradictory conclusions (10, 25). Proteasome processing has also been recently implicated (29). Urea fails to influence either p38 signaling or proteasome function. In addition, none of several previously identified urea-inducible signaling cascades (31, 34, 35) appears to be involved.

The ability of urea to influence toxicity-inducible gene regulation may explain previously recognized phenomena in other contexts. In renal epithelial cells, urea treatment induces the accumulation of only glycophorosphorylcholine (GPC), and this is a consequence of decreased GPC degradation (reviewed in Ref. 9). Hypertonic NaCl treatment in these models induces accumulation of betaine and myo-inositol via increased transcription of the genes encoding their principal Na+-dependent cotransporters (reviewed in Ref. 4). Interestingly, treatment of renal epithelial cells with urea resulted in a decrement in intracellular betaine content in several models (16–18); this could represent the consequence of an inhibitory effect of urea on (basal) TonE-mediated transcription as is observed in the present data. Similarly, there has been a trend for treatment of cells with urea plus NaCl to effect a more modest accumulation of betaine than is observed in the presence of NaCl treatment alone (16–18). Again, the ability of urea to abrogate hypertonicity-inducible TonE-mediated transcription could account for this finding. Because of the purportedly protective effect of betaine with respect to urea stress (reviewed in Ref. 30), the inability of urea-treated cells to appreciably accumulate betaine has proved puzzling. A direct inhibitory effect of urea on the signaling events leading to upregulated betaine transport offers a mechanismistic (although not a teleological) explanation. Again, it would be anticipated that such a potentially maladaptive event would exacerbate NaCl-inducible apoptosis, rather than inhibit it (33); however, additional urea-responsive protective events are likely operative, obviating the need for maximally upregulated TonEBP-dependent signaling.

This work was supported by the National Institutes of Diabetes and Digestive and Kidney Diseases Grant DK-52494, the American Heart Association, and by the Department of Veterans Affairs.

REFERENCES


