Urea protects from the proapoptotic effect of NaCl in renal medullary cells

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Zhang, Zheng, Wei Tian, and David M. Cohen. Urea protects from the proapoptotic effect of NaCl in renal medullary cells. Am J Physiol Renal Physiol 279: F345–F352, 2000.—Hypertonic NaCl upregulated two sensitive and specific biochemical indices of apoptosis, caspase-3 activation and annexin V binding, in a time- and dose-dependent fashion in renal medullary mIMCD3 cells. Pretreatment with urea (200 mM for 30 min) protected from the proapoptotic effect of hypertonic stress (200 mosmol/kgH2O) in this model. The protective effect of urea was dose dependent and was effective even when applied a short time (∼1 h) following NaCl exposure; this protective effect was not observed in the nonrenal 3T3 cell line. In both mIMCD3 and 3T3 cells, urea failed to protect from the proapoptotic stressor, ultraviolet (UV)-B irradiation. The ability of urea to protect from hypertonic stress was approximately comparable to the protective effect of peptide mitogens epidermal growth factor and insulin-like growth factor (IGF), but it potentiated the IGF effect. Interestingly, the tyrosine kinase inhibitor, genistein, potentiated the proapoptotic effect of urea yet abrogated the proapoptotic effect of hypertonic stress. In aggregate, these data indicate that urea protects from the proapoptotic effect of hypertonic stress in a potentially cell type-specific and stimulus-specific fashion.

Apoptosis; hypertonicity; caspase; annexin; stress; osmotic

ON AN OSMOLAR BASIS, urea and NaCl are the principal constituent solutes of the renal inner medullary interstitium. Each activates a unique subset of signaling intermediates and thereby regulates downstream expression of effector gene products in a relatively solute-specific fashion (1, 3). The interrelationship of these two solutes has come under increasing scrutiny in the context of medullary cell survival in this inhospitable milieu. Hypertonic NaCl appears to limit the proapoptotic effect of elevated concentrations of urea (19, 22). In the course of investigating signaling pathways conferring osmotic resistance, we uncovered the converse relationship: urea protects from the proapoptotic effect of hypertonic NaCl in the renal medullary mIMCD3 cell line, but not in nonrenal 3T3 cells.

Aberrant regulation of apoptosis has been implicated in developmental anomalies, neoplasia, and response to toxic or ischemic injury. The osmotic strength of the renal inner medulla is sufficient to cause apoptosis in diverse cell types in vitro. Resident cells of the inner medulla tolerate a wide range of ambient osmolarities, in part because of regulated accumulation of organic osmoles (reviewed in Ref. 12). Hyperosmotic urea and NaCl induce distinct programs of osmolyte accumulation (12). Urea signaling exhibits hallmarks of a peptide mitogen-like signaling pathway in cells of the renal medulla, including transcription (9) and expression (5) of immediate-early genes and activation of the signaling intermediates and receptor tyrosine kinase effectors ERK and Elk-1 (4); Shc, Grb2, SOS, and Ras (24, 27); phospholipase-Cγ (8); and PI3K, Akt, and p70 S6 kinase (27). Because it has recently been shown that the peptide mitogen insulin-like growth factor (IGF) is protective of hypertonicity-inducible apoptosis in cells of neural origin (17), the ability of urea to mediate an analogous phenomenon in renal medullary cells was investigated.

Earlier investigations relied upon relatively nonspecific endpoints of cell viability. The caspases, members of a family of aspartate-directed cysteine proteases, are responsible for much of the execution of the apoptotic program (reviewed in Ref. 23). The tetrapeptide recognition sequence differs among members of the caspase family. Caspase-3 specifically recognizes and cleaves the ASP-GLU-VAL-ASP (DEVD) motif (23); a modified peptide substrate incorporating this motif serves as the basis for a sensitive fluorometric assay of caspase-3 protease activity. An early consequence of initiation of the apoptotic program is disorganization of membrane lipid polarity. Phosphatidylserine, normally restricted to the inner leaflet of the cell membrane, translocates to the cell surface (16) where it can be labeled and detected by a fluorescent conjugate of the phosphatidylserine binding protein, annexin V (reviewed in Ref. 21). Herein, we used these sensitive and specific endpoints to examine the ability of urea to influence the proapoptotic effect of hypertonic and ultraviolet stress.

METHODS

Cell culture and treatment. Cells in culture (mIMCD3, Ref. 20; and 3T3) were maintained, propagated, and treated with...
in accordance with the manufacturer's directions for adherent cells (full details available at http://www.clontech.com/techinfo/manuals/PDF/PT3050–1.pdf). Cells were stained with propidium iodide (PI) or 7-aminactinomycin D (7AAD) and annexin V-FITC as directed and sorted (FL1 for annexin V-FITC, FL2 for PI, or FL3 for 7AAD; see below) on a Becton-Dickinson Calibur instrument, following gating upon a representative population of cells established by forward- and side-angle light scatter. Apoptosis (early) was ascribed to cells exhibiting annexin staining >40 fluorescence units (FL1) and PI staining <300 fluorescence units (FL2). For PI staining, cells labeled with only PI were used to compensate the FL2 signal out of FITC (FL1), and cells labeled with only FITC-annexin V were used to compensate the FITC signal out of FL2. Because the PI concentration was kept low, there was no excess signal overlapping into FL1. For the 7AAD protocol, FL3 was used as the DNA channel, and there was no need to compensate. Similar to the caspase-3 assay described above, there was a tendency for activation in response to solute stress to be greater in experiments exhibiting low basal annexin V binding activity. Subsequent to these studies, a preliminary observation was made that trypsinization, which is recommended by the manufacturer of the ApoAlert kit and was performed in these studies, may influence phosphatidylserine localization in the cell membrane (25). ApoAlert product literature indicated that the annexin binding assay may appear to overestimate apoptosis relative to other indices (Clontech). Whether this is a consequence of trypsinization or other manipulations is unexplored. We have been unable to reliably demonstrate annexin V-FITC binding to confluent (untrypsinized) monolayers by fluorescence microscopy, even when markedly proapoptotic stimuli (e.g., NaCl 400 mosmol/kgH2O) were applied for 1–24 h (data not shown). We have, however, qualitatively observed an increase in the abundance of PI-stained nuclei and of condensed PI-stained nuclei in confluent monolayers subjected to NaCl 200 mosmol/kgH2O relative to control treatment that was consistent with PI (or 7AAD) positivity in the trypsinized and sorted cells (see Fig. 2; two top quadrants).

RESULTS

NaCl induces apoptosis in mIMCD3 cells. To discriminate potentially subtle differences in proapoptotic events, a highly quantitative approach was adopted with respect to apoptosis detection. The renal medullary mIMCD3 cell line exhibits a dose-dependent increase in activity of the pivotal apoptotic protease, caspase-3, (as determined by cleavage of a fluorogenic substrate) in response to hypertonic NaCl at 100 mosmol/kgH2O (50 mM) and above (Fig. 1), consistent with the proapoptotic effect observed by others in this model (22).

To corroborate data obtained through the use of this assay, a second index of apoptosis was examined in parallel in critical experiments. Annexin V binding to disordered membrane phosphatidylserine orientation (a hallmark of apoptosis) was examined via fluorescence-activated cell sorting (FACS, Becton-Dickinson) analysis of cells labeled with fluorescein-conjugated annexin V. Under control conditions, comparatively few cells (4%) sorted to the “apoptotic quadrant” (Fig. 2A, bottom right; high annexin V/low 7AAD labeling). When cells were subjected to severe hypertonic stress as a positive control (NaCl 400 mosmol/kgH2O for 4 h),
there was a marked increase in the percentage of cells sorted to the bottom right quadrant (39%; Fig. 2B). These data were consistent with NaCl-inducible apoptosis. Lesser degrees of hypertonic stress exerted a lesser effect upon annexin V binding (see below).

NaCl (100 mM/200 mosmol/kgH2O) activated caspase-3 in mIMCD3 cells in a time-dependent fashion (Fig. 3), peaking at 4 h of treatment. Greater degrees of hypertonic stress resulted in a similar time course of caspase-3 activation (data not shown). Hypertonic NaCl (100 mM) also increased annexin V binding (Fig. 3); the kinetics of this upregulation closely paralleled that of NaCl-inducible caspase-3 activation.

Unlike impermeant solutes, urea fails to exert a pro-apoptotic effect in mIMCD3 cells. The effect of each of a panel of membrane-permeant and -impermeant solutes, applied in an equiosmolar fashion, was examined with respect to caspase-3 activity. The effect of the functionally impermeant solute mannitol was indistinguishable from that of NaCl (Fig. 4). Urea (200 mM) and the nonphysiological permeant solute glycerol (200 mM) failed to increase caspase-3 activity. The effect of glucose (200 mM) was similar to that of mannitol (n = 2 experiments; data not shown).

Urea pretreatment protects from NaCl-associated apoptosis in mIMCD3 cells. Others have suggested that NaCl may protect from the adverse effects of elevated urea concentration. In light of the ability of peptide mitogens to confer protection from hypertonic stress in cells of neural origin (17), the ability of urea to protect from hypertonicity was investigated in this cell line.

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Fig. 1. NaCl increases caspase-3 activity in a dose-dependent fashion. mIMCD3 monolayers were treated with the indicated concentrations of NaCl (expressed in mosmol/kgH2O) for 4 h. Caspase-3 (cpp32) activity (calculated as U/20 μg protein and calibrated with recombinant standard) is expressed relative to control. †P < 0.05 with respect to control (absence of supplemental NaCl).

Fig. 2. Severe NaCl-inducible hypertonic stress markedly increases annexin V binding. After receiving control treatment (A) or hypertonic NaCl [400 mosmol/kgH2O (N400) for 4 h; B], mIMCD3 monolayers were trypsinized, incubated with 7AAD and FITC-labeled annexin V, and subjected to fluorescence-activated cell sorting (FACS) analysis. 7AAD (y-axis in A and B) stains nuclei of dead (permeabilized) cells, and FITC-annexin V (x-axis in A and B) binds phosphatidylserine translocated to the outer leaflet of the cell membrane. Apoptotic cells (early phase) appear in the bottom right quadrant (4% of total for control vs. 39% of total for N400 for 4 h), corresponding to relatively high FITC-annexin V binding and relatively low 7AAD labeling (see METHODS). Representative data are depicted in C, where the y-axis indicates percent of cells staining with FITC-annexin V.

Fig. 3. Hypertonic NaCl increases caspase-3 activity and annexin V binding in a time-dependent fashion. mIMCD3 cells were treated with NaCl (200 mosmol/kgH2O) for the indicated interval (in hours), prior to determination of caspase-3 activity (expressed as units of cpp32 activity/20 μg protein) or annexin V binding [expressed as percentage of cells exhibiting both propidium iodide (PI) impermeance and FITC fluorescence by FACS analysis].
Treatment of mIMCD3 monolayers with NaCl (200 mosmol/kgH$_2$O for 4 h) resulted in a 2.5-fold increase in caspase-3 activity (Fig. 5A). Pretreatment of control cells treated with urea (200 mM for 30 min prior to sham treatment; 4.5 h total urea exposure) decreased caspase-3 activity by 23%, a degree that, although reproducible, did not achieve statistical significance. Pretreatment of mIMCD3 monolayers with urea (200 mM for 30 min) prior to NaCl treatment, however, inhibited caspase-3 activation by 37% (Fig. 5A). This represented a 61% inhibition of the increment in caspase-3 activity inducible by NaCl treatment. Urea also protected from the proapoptotic effect of nonionic solutes such as mannitol (data not shown). The protective effect of urea was evident out to 48 h of treatment (the last time point examined), suggesting that urea was not operating through a delay of apoptosis (data not shown).

In similar fashion, NaCl treatment (100 mM) increased annexin V binding by 2.1-fold relative to control (Fig. 5B). Urea treatment alone decreased annexin V binding by 18%; however, this did not achieve statistical significance. Urea pretreatment blocked NaCl-inducible annexin V binding by 34% (Fig. 5B). This represented a 63% inhibition of the increment in annexin V binding inducible by NaCl treatment. Therefore, urea pretreatment was protective of NaCl-inducible apoptosis with respect to two unrelated biochemical indices of apoptosis. Importantly, pretreatment with an impermeant solute (such as mannitol) in place of urea dramatically potentiated the proapoptotic effect of NaCl (data not shown).

The dose dependence of the protective effect of urea with respect to NaCl-inducible apoptosis is shown in Fig. 6. Statistical significance (protective effect) could not be demonstrated with urea concentrations less than 100 mM. Urea concentrations of 400 mM or greater exerted a modest proapoptotic effect (data not shown). Urea pretreatment (200 mM) also protected from NaCl 400 mosmol/kgH$_2$O; higher doses of urea (e.g., 400 mM) were ineffective in protecting from this greater degree of hypertonic stress (data not shown).

Of note, the protective effect of urea was not a universal phenomenon. In the fibroblastic 3T3 cell line, hypertonic NaCl (200 mosmol/kgH$_2$O) markedly increased caspase-3 activity (Fig. 8). Urea (100 mM) modestly increased caspase-3 activity, whereas 200 mM urea markedly increased caspase-3 activity. In contrast to the mIMCD3 data, pretreatment with either 100 or 200 mM urea failed to protect from the proapoptotic effect of NaCl in this fibroblastic model.
Others have shown that peptide growth factors may protect from the proapoptotic influence of other stressors (e.g., hypertonicity or UV stress) through an unknown mechanism (14, 17). Because of the similarity between urea signaling and mitogen signaling, the relationship among these phenomena was explored in preliminary fashion. Two peptide mitogens, IGF and epidermal growth factor (EGF), protected mIMCD3 cells from hypertonic NaCl (200 mosmol/kgH$_2$O) and to a degree approximately equivalent to urea (Fig. 9). When data were subjected to paired analyses, the effect of IGF exceeded that of urea in all experiments ($P < 0.05$), and the effect of urea exceeded that of EGF in all experiments ($P < 0.05$). Concentrations of urea and IGF affording maximal protection were used in these studies; interestingly, urea pretreatment substantially enhanced the protective effect of IGF (Fig. 9). It is difficult to establish whether the combined effects are less than additive, additive, or synergistic, because “percent protection” is maximal at 100. The ordinal relationship among these protectors (IGF > urea > EGF) was preserved in the setting of more extreme degrees of hypertonic stress (400 mosmol/kgH$_2$O NaCl). Under these conditions, IGF protected by 39%, urea protected by 13%, and EGF protected by a statistically insignificant 4% (data not shown).

UV-B stress is associated with apoptosis in diverse cell culture models. To determine the universality of the protective/antiapoptotic effect of urea in the mIMCD3 cell line with respect to adverse stimuli, the effect of urea upon UV-B-inducible apoptosis was ex-
A UV transilluminator (peak emission ~312 nm) was calibrated and found to deliver ~5 mW/cm² UV-B at 300 nm (see METHODS). When monitored through a plastic tissue-culture dish, this value was attenuated to ~2.3 mW/cm². On this basis, it was calculated that a 5-min exposure would provide ~6,000 J/m² UV-B. Possibility of UV-C contamination was excluded by measuring absorbance of the tissue culture plastic across the UV spectrum (Fig. 10A). UV-C transmission was blocked quantitatively by the tissue-culture plastic (Fig. 10A); therefore, virtually all of the cell exposure represented UV-B. As anticipated, increasing doses of UV-B produced increasing degrees of caspase-3 activation in the mIMCD3 cell model (Fig. 10B). Urea pretreatment, however, failed to protect mIMCD3 cells from the adverse effect of UV-B irradiation. Similar results were obtained in the nonrenal 3T3 cell line (data not shown).

With respect to the role of specific urea-inducible signaling events in the protective effect of urea, studies were performed with the tyrosine kinase inhibitor, genistein, which inhibits some urea-inducible signaling events (e.g., Ref. 8). Interestingly, genistein pretreatment (100 μM for 30 min) converted the modest ability of urea to diminish apoptosis under control conditions to a markedly proapoptotic effect (129 ± 45% increase in caspase-3 activity; n = 3; data not shown). The opposite phenomenon was observed with respect to NaCl-inducible apoptosis; specifically, genistein pretreatment substantially inhibited the proapoptotic effect of NaCl (28 ± 13%; n = 4; data not shown). Genistein appeared to have no effect upon the ability of urea to protect from NaCl-inducible apoptosis; however, this may have represented a combined effect of potentiation of urea-inducible apoptosis and inhibition of NaCl-inducible apoptosis. Therefore, urea and NaCl signaling exhibit markedly dissimilar responses to genistein pretreatment.

**DISCUSSION**

Using two independent biochemical indices of apoptosis, we show that pretreatment with urea protects renal medullary cells from the proapoptotic effect of hypertonic stress. The caspase-3 and annexin V assays were used rather than less quantitative approaches to maximize the likelihood of detecting a potentially subtle protective effect of urea. This protective effect is not complete; however, it does block greater than 60% of the increment in apoptosis inducible by modest degrees of hypertonic stress (e.g., 200–400 mosmol/kg H₂O) and is a function of the applied urea concentration. Whether the ability of 200 mM urea to protect most effectively from 200 mosmol/kg H₂O NaCl is a consequence of the equiosmolar solute ratio rather than the absolute concentration of urea remains unclear. A ratio-dependent protective effect has been described in the context of the counteracting osmolytes and urea stress (12). Detailed investigation of this possibility was precluded by the relative inability of very low doses of NaCl to exert a demonstrable apoptotic effect (Fig. 1) and by the proapoptotic effect associated with concentrations of urea markedly in excess of 200 mM.

**Urea, NaCl, and cell death.** Others have described a relationship among urea and NaCl concentration and cell viability that is converse to the present one (19, 22). Our studies, in contrast, utilized as outcome measures biochemical indices unique to apoptosis. Santos et al. (22) recently observed in the mIMCD3 model that high concentrations of either urea or NaCl decreased cell viability and that combining the two solutes enhanced cell survival of an osmotic shock. Neuhofer et
al. (19) showed that pretreatment of renal epithelial MDCK cells with hypertonic stress (a phenomenon known to induce heat shock protein expression; Ref. 10) was associated with subsequent enhanced survival of urea stress. This group later implicated NaCl-inducible hsp72 expression in this preconditioning effect (18). In our studies, medullary cells were much less sensitive to urea than they were to NaCl (on a mosmol/kgH2O basis) and subapoptotic doses of urea protected from proapoptotic doses of NaCl. This protective effect of the “activating” solute urea paralleled in many respects the ability of peptide growth factors to protect medullary cells from hypertonic stress (Fig. 9).

**Growth factor protection from hypertonic stress.** Matthews et al. (17) showed that IGF-I confers protection from hypertonic stress in a human neuroblastoma cell line. In Rat-1 fibroblasts, IGF-I protects from the proapoptotic effect of UV irradiation, and this phenomenon is Akt/PI3K dependent (14). We have previously implicated a PI3K-dependent signaling pathway in acquisition of resistance to both urea and hypertonic stress (27). Interestingly, in the Rat-1 model, overexpression of the relevant cell surface IGF receptor resulted in PI3K independence, implicating involvement of a second signaling pathway (14). We were unable to examine the role of these pathways in the present model, because pharmacological inhibition of PI3K-dependent signaling sharply sensitized medullary cells to osmotic stressors (27). Specifically, whereas PI3K inhibition exerted only a modest proapoptotic effect upon control-treated mIMCD3 cells in culture, it markedly potentiated the proapoptotic effect of high-dose urea (>400 mM) and both moderate (200 mosmol/kgH2O) and severe (400 mosmol/kgH2O) hypertonic stress (27). It was previously concluded on this basis that a PI3K-dependent pathway was potentially instrumental in protecting medullary cells from the adverse effects of hypertonic and urea stress. We sought to establish parallels between peptide mitogen-associated protection and that of urea in the renal medullary model. The tyrosine kinase inhibitor genistein inhibits elements of urea-mediated signaling in renal medullary cells (8); therefore, the effects of this compound were examined with respect to the protective effect of urea. Although the general tyrosine kinase inhibitor genistein failed to influence the protective effect of urea upon hypertonic stress, these data were confounded by the striking ability of genistein to prevent hypertonicity-inducible caspase-3 activation and by the ability of this inhibitor to potentiate the adverse effect of urea exposure upon this index of apoptosis. These data serve to further underscore the dissimilar physiological responses engendered by equiosmolar concentrations of urea and NaCl.

**Mechanism of urea protection.** We originally hypothesized that the effect of urea upon NaCl-inducible apoptosis would represent a signaling-dependent and not a physicochemical effect. Data presented here both support and refute this notion. The parallel abilities of urea, which exhibits selected biochemical hallmarks of a renal epithelial cell growth factor, and of the peptide mitogens, IGF and EGF, to protect from hypertonicity-inducible apoptosis support a signaling-dependent mechanism of protection. Efforts to ascertain the contribution of specific urea signaling effector pathways to the protective effect were confounded by the proapoptotic effect of the inhibitors themselves as described above (Ref. 27 and data not shown). Inhibition of the urea-activatable (and peptide mitogen-activatable) Ras effector arm through inducible high-level expression of a dominant negative-acting N17Ras mutant failed to influence mIMCD3 cell resistance to either urea or hypertonic stress (24). It could be argued, in contrast, that the ability of urea to add with a receptor-saturating dose of peptide mitogen does not support a common mechanism. By extension, however, the additivity of IGF- and EGF-inducible protection (n = 2; data not shown) from hypertonicity-inducible apoptosis would similarly implicate two distinct mechanisms, a less likely possibility. It would therefore appear plausible that a submaximal protective response is activated by either urea or peptide mitogen and that additivity need not equate to independence of the responsible signaling events.

**Limitations of the antiapoptotic effect of urea.** The ability of urea to protect from apoptosis is subject to at least three constraints. First, based upon the limited sampling presented here, it may exhibit cell-type specificity. Such specificity could represent a consequence of the unique ability of renal epithelial cells to respond to exogenous urea through the activation of receptor tyrosine kinase effector pathways. Activation of such effector pathways, in a fashion similar to the cell response to a peptide mitogen, could permit urea treatment to mimic the known protective effect of these mitogens in other contexts. We have been unable to confirm a promitotic effect of urea in the mIMCD3 cell line, although enhanced DNA synthesis and total DNA content have been observed in other renal epithelial cell lines in the absence of an increase in cell number (6, 7, 15). With respect to cell-type specificity, it is of interest that concentrations of NaCl and urea in the renal medulla are generally (but not universally) regulated in parallel. It is conceivable that urea protects from the proapoptotic influence of hypertonic NaCl in this milieu in vivo. The inability of urea to protect from NaCl stress in the nonrenal 3T3 cell culture model supports this teleological explanation. Second, the protective effect of urea is steeply dose dependent; urea concentrations in excess of 50 mM are required for demonstrable protection from hypertonicity. Interestingly, this protection is not confined to pretreatment; urea inhibited hypertonicity-inducible caspase-3 activation even when applied up to 60 min after initial exposure to hypertonic stress. Based upon this latter observation, it appears likely that a substantial interval must be traversed before biochemical commitment to irreversible caspase-3 activation (and ultimately apoptosis) occurs. Third, the protective effect of urea applies to only a subset of proapoptotic stimuli. Specifically, urea protected from hypertonicity (both NaCl and mannitol induced) yet failed to protect from the...
proapoptotic effect of UV-B irradiation. As both urea (26) and UV irradiation (2) are potential oxidative stressors, this failure of protection may represent a consequence of additive pro-oxidant stress.

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REFERENCES