A combination of NaCl and urea enhances survival of IMCD cells to hyperosmolality

BENTO C. SANTOS, ALEJANDRO CHEVAILE, MARIE-JOSÉ HÉBERT, JANE ZAGAJESKI, AND STEVEN R. GULLANS
Renal Division, Department of Medicine, Brigham and Women’s Hospital
and Harvard Institutes of Medicine, Boston, Massachusetts 02115

Abstract

The process of creating a concentrated urine requires that renal medullary cells survive and function in high concentrations of NaCl and urea. Interestingly, renal epithelial cells exhibit distinctly different responses to NaCl and urea. Hyperosmolar NaCl is known to activate multiple families of mitogen-activated protein kinases (14, 20, 31, 39, 40), stimulate gene transcription via a tonicity/osmotic response element (11, 38), and enhance expression of molecular chaperones (8, 30, 33) and genes responsible for accumulating organic osmolytes (2, 15). In contrast, hyperosmolar urea activates protein kinase C, stimulates extracellular signal-regulated kinase (ERK) and, via the actions of serum response factor (SRF) and ERK-responsive transcription factor (EGR-1), enhances expression of two transcription factors, Egr-1 and c-fos (4, 5). These actions of hyperosmolar urea are urea specific and renal epithelial cell specific (6), suggesting that urea, acting through a urea sensor/receptor, activates a specific program of gene expression (7). Hyperosmolar urea does not increase accumulation of the same set of genes as NaCl, and, to date, the downstream physiological responses to urea remain largely unknown.

In the present study, we evaluated the effects of hyperosmolar NaCl and urea on the survival and function of murine inner medullary collecting duct (mIMCD3) cells. Exposure to hyperosmolar NaCl or urea caused comparable dose- and time-dependent decreases in cell viability, such that 700 mosmol/kgH₂O killed >90% of the cells within 24 h. In both cases, cell death was an apoptotic event. For NaCl, loss of viability at 24 h paralleled decreases in RNA and protein synthesis at 4 h, whereas lethal doses of urea had little or no effect on these biosynthetic processes. Cell cycle analysis showed both solutes caused a slowing of the G₂/M phase. Surprisingly, cells exposed to a combination of NaCl + urea were significantly more osmotolerant such that 40% survived 900 mosmol/kgH₂O. Madin-Darby canine kidney cells but not human umbilical vein endothelial cells also exhibited a similar osmotolerance response. Enhanced survival was not associated with a restoration of normal biosynthetic rates or cell cycle progression. However, the combination of NaCl + urea resulted in a shift in Hsp70 expression that appeared to correlate with survival. In conclusion, hyperosmolar NaCl and urea activate independent and complementary cellular programs that confer enhanced osmotolerance to renal medullary epithelial cells.

Santos, Bento C., Alejandro Chevaile, Marie-José Hébert, Jane Zagajeski, and Steven R. Gullans. A combination of NaCl and urea enhances survival of IMCD cells to hyperosmolality. Am. J. Physiol. 274 (Renal Physiol. 43): F1167–F1173, 1998.—Physiological adaptation to the hyperosmolar milieu of the renal medulla involves a complex series of signaling and gene expression events in which NaCl and urea activate different cellular processes. In the present study, we evaluated the effects of NaCl and urea, individually and in combination, on the viability of murine inner medullary collecting duct (mIMCD3) cells. Exposure to hyperosmolar NaCl or urea caused comparable dose- and time-dependent decreases in cell viability, such that 700 mosmol/kgH₂O killed >90% of the cells within 24 h. In both cases, cell death was an apoptotic event. For NaCl, loss of viability at 24 h paralleled decreases in RNA and protein synthesis at 4 h, whereas lethal doses of urea had little or no effect on these biosynthetic processes. Cell cycle analysis showed both solutes caused a slowing of the G₂/M phase. Surprisingly, cells exposed to a combination of NaCl + urea were significantly more osmotolerant such that 40% survived 900 mosmol/kgH₂O. Madin-Darby canine kidney cells but not human umbilical vein endothelial cells also exhibited a similar osmotolerance response. Enhanced survival was not associated with a restoration of normal biosynthetic rates or cell cycle progression. However, the combination of NaCl + urea resulted in a shift in Hsp70 expression that appeared to correlate with survival. In conclusion, hyperosmolar NaCl and urea activate independent and complementary cellular programs that confer enhanced osmotolerance to renal medullary epithelial cells.

Hyperosmotic stress; renal medulla; protein synthesis; apoptosis; heat shock protein 70; ribonucleic acid synthesis; cell cycle

METHODS

Cell culture and viability assays. mIMCD3 cells were grown to confluence in plastic dishes in Dulbecco’s modified Eagle’s medium/ Ham’s F12 (1:1) supplemented with 10% fetal bovine serum (JRH Biosciences) and 2% penicillin/streptomycin (Life Technologies). For hyperosmolality experiments, cells were exposed to either isosmolar or hyperosmolar medium supplemented with NaCl, urea, or both. The crystal violet assay was used to assess viability, as has been done in many previous studies (17, 21, 23–25, 32, 36, 37). Cells were seeded at 10⁴ cells/well in 96-well flat-bottom plates, incubated in 5% CO₂ atmosphere at 37°C until they reached confluence, and treated for 4–24 h, under the appropriate osmotic conditions. After treatment, DNA of remained adherent cells was stained with 20 µl/well of 0.75% crystal violet in 30% acetic acid for 15 min, rinsed, and dried. Methanol was added to solubilize the stained cells, and the absorbance of each well was read at 630 nm with a Vmax-Kinetic Microplate Reader (MoTo Molecular Devices) (12). Percent viability of treated cells was defined as the absorbance relative to control cells. Independent analysis of viability using trypan blue exclusion confirmed the results of the crystal violet assay. Assessment of mIMCD3 cells by light microscopy. mIMCD3 cells were seeded in 12-well plates until they reached confluence and treated with hyperosmolar medium (NaCl or urea). After 24 h, the supernatants and trypsinized cells were collected. Both collections were cytocentrifuged onto a slide,
at 750 rpm, for 6 min, fixed, stained with Wright-Giemsa, and examined by light microscopy in a blinded-label fashion, and, whenever possible, at least 100 cells (adherent and nonadherent) were evaluated (19).

[^3H]Juridine and [^3H]leucine incorporation. mIMCD3 cells were seeded in 96-well plates and grown to confluence in the presence of complete medium. The medium was replaced by isosmotic or hyperosmotic medium (NaCl, urea, or both). Concomitantly, cells received a pulse of labeled substrate (NEN) as follows: 1 µCi/ml [^3H]Juridine and 0.5 µCi/ml [^3H]leucine. After 4 h, cells were trypsinized for 30 min and harvested, using a 1205 Betaplate system (Wallack, Finland). The results were obtained by scintillation counting in the presence of Betaplate Scint.

Northern analyses. As described previously (22), at appropriate time points, cells were washed twice with phosphate-buffered saline (PBS), and total RNA was isolated using the RNAzol B method (Tel-Test). Total RNA (10 µg) was fractionated in a 1% agarose/0.7% formaldehyde denaturing gel and transferred overnight to a nylon membrane. cDNA probes for the cells. To examine this issue, RNA and protein processes, including RNA and protein synthesis (5, 6), which could potentially explain the loss of viability by the cells. To examine this issue, RNA and protein synthesis rates were measured in cells during the first 4 h of exposure to hyperosmolality (Fig. 2). Hyperosmolar NaCl greatly inhibited synthesis of RNA and protein, and the dose dependence was similar to that observed for viability at 24 h. In comparison, urea had no significant adverse effects on biosynthesis even at osmolalities higher than 900 mosmol/kgH2O. Of particular note, 750 mosmol/kgH2O failed to alter biosynthesis at 4 h but resulted in nearly complete cell death at 24 h. The combination of NaCl and urea gave an intermediate response that appeared to correlate with the enhanced survival observed at 24 h (Fig. 1C).

Previous work found that hyperosmolar NaCl can increase Hsp70 expression (8, 30, 33). Moreover, enhanced Hsp70 expression can prevent apoptosis in cells exposed to heat shock, ethanol, osmotic shock, H2O2, and ultraviolet irradiation (13, 26). To investigate whether expression of molecular chaperones correlated...
with cell survival, we measured expression of Hsp70 4 h after exposure to hyperosmolality (Fig. 3). As shown previously in MDCK cells (8, 33), hyperosmolar NaCl but not urea increased inducible Hsp70 mRNA expression. Notable in the response to NaCl was the fact that Hsp70 expression was robust at lower osmolalities (500–600 mosmol/kgH2O) when survival was greatest and was absent at the highest osmolalities when cell viability was lower. The combination of NaCl and urea shifted the peak of Hsp70 response to higher osmolalities (750 mosmol/kgH2O), which correlated with the enhanced survival. The constitutive Hsc70 was unchanged with hyperosmolality.

Previous work in MDCK cells demonstrated that hyperosmolar NaCl slows cell cycle progression at the G2/M phase (6), a stage known for its sensitivity to external stresses (34). To evaluate whether a combination of NaCl and urea could restore normal cell cycle progression, we performed flow cytometric analysis of cellular DNA content on mIMCD3 cells after 4 h of exposure to hyperosmolality, a time point that preceded significant cell death. As shown in Fig. 4, mIMCD3 cells exposed to hyperosmolar NaCl or urea (500 mosmol/kgH2O) exhibited a decrease in the proportion of cells in the G0/G1 stage and a corresponding increase in the proportion of cells in G2/M. Thus NaCl and urea have similar effects on cell cycle progression and appear to slow progression at the G2/M stage. Exposure to a combination of NaCl and urea (600 mosmol/kgH2O) produced a similar change in the cell cycle profile, with
cells shifting from G₀/G₁ to G₂/M. Identical results were also observed with 450 mosmol/kgH₂O NaCl and urea. Thus exposure to a combination of NaCl and urea slowed cell cycle progression. Cell cycle analysis also revealed that, under diverse hyperosmolar conditions (500–750 mosmol/kgH₂O), there was no increase in the sub-G₀/G₁ population of cells, confirming the absence of significant apoptosis at this early time point.

**DISCUSSION**

Under normal physiological circumstances, cells of the renal medulla survive and function, despite being exposed to extremely high concentrations of NaCl and urea. In the present study, we found that mIMCD3 cells possess an inherent mechanism that confers enhanced survival during exposure to a combination of NaCl and urea. In particular, mIMCD3 cells exposed to hyperosmolar NaCl + urea could survive osmolalities 200–300 mosmol/kgH₂O greater than cells exposed to either solute alone. This enhanced osmotolerance was also observed in MDCK cells but not in HUVECs, suggesting it is a characteristic of renal cells. The survival response required the presence of serum, suggesting a role for other unknown prosurvival cofactors. Morphological analysis and DNA laddering revealed that cell death was an apoptotic event. In this regard, cell death was time dependent, with reduced viability apparent within 4 h and reaching maximal levels after 8–24 h of hyperosmolar stress.

Individually NaCl and urea had quantitatively similar effects on cell survival. In both cases, ~50% of the cells were killed after 24 h of exposure to 600 mosmol/kgH₂O. However, these treatments had entirely different effects on the synthesis of RNA and protein. Hyperosmolar NaCl suppressed biosynthetic rates starting at 500 mosmol/kgH₂O, whereas hyperosmolar urea had no adverse effects until osmolality exceeded 900 mosmol/kgH₂O. Thus, at 700–750 mosmol/kgH₂O, an osmolality that killed 90% of the cells under both conditions, only NaCl suppressed cellular biosynthesis. These observations confirm and extend those made previously.

**Fig. 2.** A comparison of effects of hyperosmolar NaCl and/or urea on rates of RNA (A) and protein (B) synthesis. mIMCD3 cells were exposed to increasing concentrations of hyperosmolar NaCl and/or urea for 4 h. To measure RNA and protein synthesis, cells were pulsed with [³H]uridine and [³H]leucine throughout the 4-h time period. When used in combination, NaCl and urea were added in equimolar amounts. Each point is the mean ± SE of 7–18 observations corrected by the number of cells. Majority of points that comprise the NaCl + urea curve are statistically different from NaCl or urea alone (P < 0.05).
Moreover, in vivo Hsp70 expression is highest in the renal medulla where osmolality is highest (27).

The role of Hsp70 in hyperosmolality has not been explicitly delineated. Of interest, Hsp70 is known to modulate apoptosis (13, 26). In U-937 and PEER human lymphoid cells, overexpression of Hsp70 prevented apoptosis induced by heat shock, osmotic shock, ethanol, or ultraviolet irradiation (13). Thus it is reasonable to speculate that Hsp70 may serve a similar role in renal medullary cell adaptation to hyperosmolality, such that moderate stress, which a cell can tolerate, will generate an Hsp70 response, thereby preventing cell suicide. With more severe stress, the Hsp70 response is either not activated or is unable to be activated, resulting in programmed cell death.

Flow cytometric analysis of DNA content revealed that hyperosmolar stress caused a shift in the cell cycle profile of mIMCD3 cells. Four hours of hyperosmolar NaCl or urea failed to induce Hsp70 mRNA expression in mIMCD3 cells. This is consistent with a slowing of the G2/M phase, which has been observed previously with a variety of stress conditions (9, 34). A combination of NaCl and urea, while enhancing survival, did not alter this cell cycle profile, suggesting that slowing of the cell cycle is a normal physiological response and is not necessarily a sign of cell death.

The synergistic actions of NaCl and urea in promoting cell survival complement previous observations that NaCl and urea activate independent signal transduction systems. In renal epithelial cells and a variety of other cell types, hyperosmolar NaCl activates three families of signaling mitogen-activated protein kinases, including jun kinase (JNK) (14, 31), p38 (40), and ERK (20). This appears to be a response to tonicity and can lead to enhanced transcription via a specific enhancer element within a variety of genes (11, 38). In contrast, urea activates ERK family in a MEK-dependent fashion (3) and subsequently activates gene transcription via an SRF/EElk-1-dependent mechanism. The urea response is specific for renal epithelial cells (6). The present study indicates that the combined actions of NaCl and urea, presumably via these independent signaling pathways, enhance cell survival. This dual program of activation and survival suggests that epithelial cells of the renal medulla are endowed with a complex proactive program that confers enhanced survival and likely other physiological functions that characterize IMCD cells. Finally, it is reasonable to speculate that many of the signs of stress that are observed with moderate hyperosmolality, such as altered biosynthesis, slowing of the cell cycling, and stress protein induction, are not a response to toxic injury but, rather, represent normal adaptive responses of the cells. Moreover, exceeding the limits of adaptation activates a program of cell death, whereas moderate hyperosmolality promotes survival and other phenotypic changes that are characteristic of the IMCD.

This work was supported by National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-51606 and DK-36031. S. R. Gullans was supported as an Established Investigator of the
American Heart Association. B. C. Santos was supported by a Fellowship from the Conselho Nacional de Desenvolvimento Científico e Tecnológico, Brazil. 20026-94-2(NV). A. Chevalle was supported by a fellowship from the International Society of Nephrology. M.-J. Hébert was supported by a fellowship from the Medical Research Council of Canada.

Address for reprint requests: S. R. Gullans, Harvard Institutes of Medicine, Rm. 554, 77 Ave. Louis Pasteur, Boston, MA 02115.

Received 3 November 1997; accepted in final form 4 March 1998.

REFERENCES


34. Smith, C. N., C. D. Lindsay, and D. G. Upshall. Presence of methenamineglutathione mixtures reduces the cytotoxic effect