Urea sensitizes mIMCD3 cells to heat shock-induced apoptosis: protection by NaCl

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Urea sensitizes mIMCD3 cells to heat shock-induced apoptosis: protection by NaCl. Am J Physiol Cell Physiol 280: C614–C620, 2001.—Urea, with NaCl, constitutes the osmotic gradient that allows water reabsorption in mammalian kidneys. Because NaCl induces heat shock proteins, we tested the responses to heat shock of mIMCD3 cells adapted to permissive urea and/or NaCl concentrations. We found that heat-induced cell death was stronger after adaptation to 250 mM urea. This effect was reversible, dose dependent, and, interestingly, blunted by 125 mM NaCl. Moreover, we have shown that urea-adapted cells engaged in an apoptotic pathway upon heat shock, as shown by DNA laddering. This sensitization is not linked to a defect in the heat shock response, because the induction of HSP70 was similar in isotonic and urea-adapted cells. Moreover, it is not linked to the presence of urea inside cells, because washing urea away did not restore heat resistance and because applying urea and heat shock at the same time did not lead to heat sensitivity. Together, these results suggest that urea modifies the heat shock response, leading to facilitated apoptosis.

IN MAMMALS, the urinary concentrating mechanism relies on water reabsorption driven by the renal corticopapillary osmotic gradient. Cells from the renal medulla are thus uniquely exposed to fluctuating hyperosmolarity; sodium chloride; adaptation

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(11). We have recently shown that the cell death pathways triggered by acute or progressive urea increases are different, with typical apoptosis resulting from progressive urea increases (10). To our knowledge, other cellular effects of permissive urea concentrations (i.e., concentrations that allow mIMCD3 cells to grow in vitro) have not been addressed. As mentioned above, urea imposes a stress on target cells. We (10) and others (12) have shown that the long-term effects of this stress are not sufficient to protect cells from subsequent hyperosmotic injuries. However, it could not be excluded that urea-induced stress was able to modify cell responses to another stress, a point that would give important indications on the long-term cellular effects of urea at the molecular level. Phenotypic interactions between cell responses to different kinds of stress have already been reported, essentially because of the potential beneficial protective consequences of nonlethal stresses, a phenomenon called hormesis (1). In addition, exploring the interactions between hyperosmotic NaCl and/or urea and another kind of stress could help in individualizing the respective effects of global hyperosmolarity and of the different solutes.

Therefore, we have analyzed the responses of urea-adapted cells to heat shock.

METHODS

Cell culture. Mouse inner medullary collecting duct (mIMCD3) cells (14) were obtained from the American Type Culture Collection. The cells were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium and F-12 medium (Life Technologies) supplemented with 10% fetal bovine serum (Sigma) and 2 mM l-glutamine (Sigma; referred to as iso medium) at 37°C with 5% CO2. Cells were adapted to hyperosmotic media with different solutes (NaCl, urea, glycerol, or a combination of NaCl and urea). Cells grown in iso medium were subcultured at a ratio of 1 to 10 and grown overnight, and the medium was exchanged for a freshly prepared hyperosmotic medium. Osmolarity increases were never >200 mosmol/lH2O, whatever the solute used. Cells were then grown to confluence in this hyperosmotic condition, subcultured at a ratio of 1 to 5, and grown overnight, and another osmolarity increase could be performed. When the desired osmolarity was reached, cells were grown and subcultured in these hyperosmotic conditions for 10–15 passages (1.5–2 mo). Experiments were performed with cells adapted to the desired osmolarity for at least 1 wk. Hyperosmotic media were prepared extemporaneously by adding the desired volumes of solute stock solutions (2.5 M NaCl, 5 M glycerol, or 5 M urea; all stock solutions were prepared in iso medium and sterile filtered). It should be noted that urea stock solutions were kept no longer than 3 days to avoid spontaneous degradation. Viable cells were assayed by manual counting of at least 100 trypsinized adherent cells in a trypan blue solution. Heat shocks were performed by placing culture flasks in a prewarmed 42°C incubator in the presence of 5% CO2.

Analysis of genomic DNA by agarose electrophoresis and flow cytometry. Genomic DNA was extracted from detached cells by lysis in 20 mM Tris, 10 mM EDTA, and 0.5% Triton X-100, pH 7.5. After repeated pipetting, cell lysates were centrifuged for 5 min at 15,000 g, and the supernatants were treated with 0.2 mg/ml proteinase K and 0.2 mg/ml RNase A for 1 h at 42°C. Samples were analyzed on an agarose electrophoresis gel in the presence of ethidium bromide and visualized by ultraviolet fluorescence. For the analysis of cell DNA content, floating and adherent cells were pooled, washed with phosphate-buffered saline (PBS; Sigma), fixed in 1× PBS with 70% ethanol at −20°C for 10 min, washed once in PBS, and treated with 100 μg/ml RNase A for 30 min at 37°C. Samples were then incubated for 10 min in the dark with 20 μg/ml propidium iodide and analyzed with a FACScan calibrating flow cytometer (Becton Dickinson). Cells were first selected on a forward scatter/side scatter dot plot, and propidium iodide fluorescence was analyzed on the FL2 channel. Single cells were selected on a FL2-A/FL2-W dot plot, and the DNA content was determined as FL2-H.

Western blotting. Proteins were extracted by lysis for 60 min at 4°C in cell lysis buffer [1× PBS, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS)] in the presence of protease inhibitors (1 μg/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml pepstatin, and 1 μg/ml aprotinin), followed by centrifugation at 15,000 g for 20 min at 4°C. Protein content was assayed with the Bio-Rad protein assay. Western blots were saturated for 2 h at room temperature in PBS supplemented with 0.3% (vol/vol) Tween 20 and 5% (wt/vol) nonfat dry milk (PBS-TM). The anti-HSP70 monoclonal antibody (clone C92F3A-5; Stressgen) was diluted in the same buffer and incubated on the membrane for 1.5 h. After five washes, the secondary antibody (peroxidase-coupled goat anti-mouse; Promega) was added after dilution in PBS-TM and incubated for 45 min at room temperature. The membrane was washed three times in PBS-TM, twice in PBS-T, and then once in PBS before bound antibodies were revealed with the ECL+ enhanced chemiluminescence kit (Amersham) according to the manufacturer’s instructions.

Statistical analysis. Simple comparisons were performed by unpaired Student’s t-test. Multiple comparisons were performed by ANOVA, followed by Fisher’s protected least significant difference test. Statistical significance was set at 5% (P < 0.05).

RESULTS

Urea sensitizes mIMCD3 cells to heat shock-induced apoptosis. mIMCD3 cells were first adapted (as described in METHODS) to media made hypertonic with 250 mosmol/lH2O NaCl [125 mM NaCl added, final osmolality 540 ± 7 mosmol/kgH2O (mean ± SD, n = 17); referred to as Na medium], or 250 mosmol/lH2O urea (250 mM urea added, final osmolality 558 ± 12 mosmol/kgH2O, n = 14; U medium), or both (final osmolality 804 ± 20 mosmol/kgH2O, n = 63; NaU medium). The osmolality of iso medium was found to be 317 ± 7 mosmol/kgH2O (n = 27). The proliferation rates of these cells were similar (doubling time ~15 h), except for cells adapted in NaU medium, which exhibited a doubling time of ~21 h (see Fig. 2). Adapted cells were then seeded at a density of 6 × 104 in 25-cm2 flasks, grown for 16–20 h at 37°C, and submitted to a 42°C heat shock for various periods of time. Results are presented as the percentage of viable adherent cells relative to the initial number of cells (Fig. 1). In the absence of heat shock, we observed a similar prolifer-
is able to inhibit the greater heat sensitivity of cells adapted to hyperosmotic urea. The inhibition was not complete because the difference between the number of cells in Na and NaU medium was found to be significant ($P = 0.03$). It should be noted that, in this experimental setting, Na on its own had no effect on the heat sensitivity of mIMCD3 cells ($P$ values of 0.08, 0.05, and 0.82 after a 2-, 5-, or 8-h heat shock, respectively).

To determine whether these results could be detected after short heat shocks, we treated cells for 2 h at 42°C and then incubated them for different periods of time at 37°C. The number of viable cells as a function of time is presented in Fig. 2, A–D. Again, urea-adapted cells showed a greater sensitivity to this reduced heat shock (Fig. 2C). At 48 h, only 5 ± 3% ($n = 5$) of the urea-adapted cells were still viable, compared with 123 ± 20% for cells grown in iso medium ($n = 6$; $P < 0.05$). This effect of urea adaptation was blocked by NaCl, because cells grown in NaU medium (Fig. 2D) behaved essentially like cells grown in isotonic conditions (Fig. 2A) except for their basal proliferation rate. The calculated doubling time for cells grown in NaU medium in the absence of heat shock was 21 h, compared with 14 h for cells grown in iso medium. It should be noted that, in this experimental setting, there was no obvious difference between cells adapted to hypertonic NaCl (Fig. 2B) and cells maintained in iso medium (Fig. 2A). Genomic DNA was extracted from nonadherent cells at 24 and 48 h and analyzed on an agarose gel (Fig. 2E). The observation of an apoptotic DNA laddering pattern indicated that urea-adapted cells were engaged in an apoptotic pathway after the 2-h heat shock. A quantitative analysis of the heat-shock induced apoptosis could thus be performed by quantifying via flow cytometry the number of events in the sub-G$_1$ peak of propidium iodide labeled cells after heat shock (Fig. 2F). The number of heat shock-dependent apoptotic events could then be calculated by subtracting the number of apoptotic events in the absence of heat shock. Urea adaptation raised this value from 9.7% to 50.7%.

Cells were then adapted for at least 1 wk to various urea concentrations, and the response of adapted cells to an 8-h heat shock was analyzed (Fig. 3A). No effect of urea could be detected at urea concentrations under 50 mM. Cells revealed a greater sensitivity to heat shock at urea concentrations of 100 mM ($P < 0.003$ between heat shocked and non-heat shocked cells) and higher. It should be noted that this concentration of urea can be reached in the plasma of uremic patients.

Long-term phenotypic changes of cell lines maintained in vitro can reflect either adaptation of the whole cell population or selection of a subpopulation of cells. Because the second hypothesis would require another interpretation of our data, in particular that of the protective effect of NaCl, we analyzed the reversibility of urea adaptation with the idea that selection of a subpopulation cannot be reversed by shifting the cells back to the initial culture conditions. Therefore, cells were adapted to U medium for at least 1 wk and then either shifted or not shifted back to iso medium.
for 24 h before being tested for their resistance to an 8-h heat shock (Fig. 3B). Cells grown in iso medium were analyzed in parallel. The heat-sensitive phenotype was essentially reversible. After an 8-h heat shock, remaining viable cells were 37 ± 4% for urea-adapted cells and 278 ± 15% after reversion (P < 0.0001). It should be noted that the small difference between cells grown in iso medium and cells reversed from U medium is actually statistically significant (322 ± 6% vs. 278 ± 15%; P < 0.02).

Fig. 3. Dose response and reversibility of the urea effect on the heat shock response. A: cells were adapted for at least 1 wk to media made hyperosmotic with the indicated concentrations of urea. The response to heat shock was analyzed as described in Fig. 1, with a heat shock length of 8 h. Results are presented as means ± SE of at least 5 independent experiments. B: cells adapted to U medium were shifted (U/iso) or not shifted back (U) to iso medium for 24 h, and the heat shock response was analyzed as in A. Cells grown in iso medium were used as a control. Results are presented as means ± SE of 3 independent experiments.

Fig. 2. Urea-adapted cells engage in an apoptotic pathway upon heat shock. A–D: cells adapted to isotonic medium (A) or to Na (B), U (C), or NaU medium (D) were seeded at 10^5 cells per 25-cm² flask and incubated 16–20 hrs at 37°C. They were then heat shocked (+ HS) for 0 (○) or 2 h (●) and incubated at 37°C for the indicated periods of time. Values presented are percentages of viable adherent cells relative to the number of seeded cells. Results are presented as means ± SE of at least 5 independent experiments. E: at 24 and 48 h after a 2-h heat shock, genomic DNA was extracted and analyzed on an agarose gel electrophoresis. F: the number of heat shock-induced apoptotic events was estimated by analyzing the DNA content of cells grown in iso medium or adapted to urea 48 h after a 5-h heat shock (iso + HS or U + HS, respectively). As a control, the same experiment was performed on heat-shocked cells (iso and U). Identification of the different peaks (sub-G₁, G₁, and G₂/M) is indicated. PI, propidium iodide.
Urea adaptation does not block heat shock protein expression. Because NaCl has been claimed to protect cells from subsequent stresses by inducing a heat shock response, we reasoned that urea might sensitize cells by inhibiting this heat shock response. Thus we analyzed the expression of the HSP70 protein, as a marker of this response, in cells adapted to different media, before and after heat shock (Fig. 4). We first confirmed that HSP70 was overexpressed in cells grown in media containing NaCl (Na medium and NaU medium) but not in cells adapted to urea (Fig. 4, U, lane 0). The expression of the HSP70 protein was induced by heat shock in cells maintained in iso medium, as expected, and was overinduced in cells grown in Na and NaU medium. More interestingly, cells adapted to hyperosmotic urea were found to be able to overexpress HSP70 in response to heat shock at levels similar to those observed in cells maintained in isotonic conditions (cf. Fig. 4, U, lanes 5 and 8 vs. iso, lanes 5 and 8).

Effect of acute urea changes. One possible way to explain our data would be that urea and heat shock could have qualitative and/or quantitative additive effects on protein denaturation. Because urea is a freely permeant solute, such additive effects should be detectable when urea and heat shock are applied at the same time. We thus treated mIMCD3 cells with NaCl, urea, or glycerol (as a control of the effect of an hyperosmotic shock with a permeant solute) and incubated the cells immediately at 42°C for 8 h. As shown in Fig. 5A, this treatment had no statistically significant effect on cell survival. Reciprocally, washing urea away (with two 15-min washes in isotonic medium) should have blocked the hypersensitivity of urea-adapted cells. However, this was not the case (Fig. 5B, Urea, closed vs. hatched bars). Again, glycerol-adapted cells were not hypersensitive to heat shock either before or after the two isotonic washes. Altogether, these experiments show that the cellular effects of urea adaptation are not immediately mediated by urea.

DISCUSSION

Cells from the kidney medulla are generally exposed for long periods of time to high osmolarities resulting from the reabsorption of NaCl and urea. The long-term effects of NaCl on cell physiology have been studied by different laboratories (2). They consist essentially of the triggering of both a general stress response (as revealed by the induction of heat shock proteins) and a specific osmotic response leading to the cytoplasmic accumulation of compatible organic osmolytes. Together, these responses facilitate cell survival in this stressful environment. In contrast, little is known of the long-term effects of hyperosmotic urea on cell physiology.

Our data demonstrate that cells adapted to hyperosmotic urea show a greater sensitivity to heat shock than cells grown in isotonic conditions. This sensitivity is linked to the engagement of a larger proportion of urea-adapted cells into an apoptotic pathway upon
heat shock treatment. Of note is the fact that this effect was found to be dose dependent and could be detected at urea concentrations of 100 mM. Such a concentration can be reached in uremic patients (G Deschênes, personal communication), suggesting that nonrenal cells are submitted to these hyperosmotic urea conditions under pathophysiological situations. The clinical relevance of our findings are not clear at this point, but our results suggest that they should be explored not only in renal systems but also in nonrenal cells. In any case, the findings stress the differences between acute and chronic hyperosmolarity treatments. Only cells adapted to hyperosmolar urea were hypersensitive to heat shock, whereas acute urea treatment did not lead to similar results (Fig. 5A). We have previously shown that progressive increases in NaCl or urea concentrations lead to cell survival or typical apoptosis, respectively (10). Again, this is in contrast with the results obtained by us and others on acute osmolarity increases (10, 11, 15). Because in vivo osmolarity changes are not acute, our data suggest that results obtained from in vitro models of acute hyperosmolarity changes might miss some aspects of the cellular responses to osmotic stress.

The functional bases of this long-term effect of urea require further investigation to be fully understood at the molecular level. However, three points can be stressed from our data. 1) Although urea is known to denature proteins at a high concentration, the greater sensitivity of urea-adapted cells to heat shock is not linked to an additive effect of heat and urea on protein denaturation. Such an additive effect should be detectable upon simultaneous treatment with urea and heat, which is not the case. Furthermore, it should be blocked by washing out urea, which also is not the case. Thus it is tempting to suppose that the specific signaling pathways triggered by urea are able to induce cellular phenotypic changes that might include this greater heat sensitivity. 2) The long-term effect of urea is specific in the sense that glycerol, another freely permeant solute, does not sensitize cells to heat shock. In other words, hyperosmolarity per se is not sufficient to induce the long-term effects induced by hyperosmotic urea. This point again suggests that the specific signaling events triggered by urea could play a major role in the induction of a heat-sensitive phenotype. 3) The effect of urea is not due to an inhibition of the heat shock response, because the HSP70 induction is conserved in heat-sensitized cells. Thus the mechanism of heat-sensitization by urea is different from the mechanism of stress protection induced by NaCl, which relies most probably on the induction of a heat shock response.

Interestingly, the urea-induced sensitization is blunted by NaCl. From a physiological point of view, urea is both a waste product and a major functional molecule involved in resistance to water limitation in several metazoans. For instance, amphibians are able to increase their urea plasma levels to retain water in their “milieu intérieur.” In mammals, this increase in osmolarity is limited to the kidney medulla, where urea concentrations can reach very high levels. Our results are in line with the emerging notion of a transprotection mechanism between NaCl and urea because they demonstrate that NaCl protects cells not only from the acute toxic effects of urea but also from the long-term consequences of the accumulation of this solute. Thus, beyond its role as a driving force for water reabsorption, it appears that NaCl plays a role as a cellular protectant against the deleterious effects of urea, most probably because of its capacity to induce both an osmotic and a general stress response. In particular, because the induction of HSP70 has been shown to be a critical event in NaCl-mediated protection against urea toxicity (13) and because this protein is known to induce thermotolerance in several cellular models (8), it might be speculated that the same protein is involved in NaCl-mediated protection against urea sensitization to heat shock. Whether other NaCl-induced mechanisms also play a role is an open question.

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REFERENCES


