Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells

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Michea, L., D. R. Ferguson, E. M. Peters, P. M. Andrews, M. R. Kirby, and M. B. Burg. Cell cycle delay and apoptosis are induced by high salt and urea in renal medullary cells. Am. J. Physiol. Renal Physiol. 278: F209–F218, 2000.—We investigated the effects of hyperosmolality on survival and proliferation of subconfluent cultures of mIMCD3 mouse renal collecting duct cells. High NaCl and/or urea (but not glycerol) reduces the number of viable cells, as measured with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT). Raising osmolality from a normal level (300 mosmol/kg) to 550–1,000 mosmol/kg by adding NaCl and/or urea greatly increases the proportion of cells in the G2M phase of the cell cycle within 8 h, as measured by flow cytometry. Up to 600 mosmol/kg the effect is only transient, and by 12 h at 550 mosmol/kg the effect reverses and most cells are in G1. Flow cytometry with 5-bromodeoxyuridine (BrdU) pulse-chase demonstrates that movement through the S phase of the cell cycle slows, depending on the concentrations of NaCl and/or urea, and that the duration of G2M increases greatly (from 2.5 h at 300 mosmol/kg to more than 16 h at the higher osmolalities). Addition of NaCl and/or urea to total osmolality of 550 mosmol/kg or more also induces apoptosis, as demonstrated by characteristic electron microscopic morphological changes, appearance of a subdiploid peak in flow cytometry, and caspase-3 activation. The number of cells with subdiploid DNA and activated caspase-3 peaks at 8–12 h. Caspase-3 activation occurs in all phases of the cell cycle, but to a disproportionate degree in G2/G1 and S phases. We conclude that elevated NaCl and/or urea reduces the number of proliferating mIMCD3 cells by slowing the transit through the S phase, by cell cycle delay in the G2M and G1, and by inducing apoptotic cell death.

excessive concentrations kill the cells in culture (24, 32). The mechanism of increased cell death related to high NaCl and/or urea concentration remains controversial. One previous report concluded that high NaCl kills murine inner medullary collecting duct cells (mIMCD3) by necrosis (41), whereas another report attributed the death of these cells to apoptosis (32). Interestingly, although adding a given osmotic concentration of urea or NaCl kills approximately the same number of cells, when the NaCl and urea are combined to reach the same total osmolality the effect is much smaller (32).

High NaCl (20, 32) and/or urea (32) also retards mIMCD3 cells in the G2/M phase of their cell cycle. Associated with this cell cycle delay, high salt greatly increases the expression of some of the growth arrest and DNA damage-inducible proteins (GADDs) (20), which were originally identified by their association with genotoxic stresses such as ionizing radiation (17). The increased expression of GADDs raised the still speculative possibility that extreme hypertonicity might damage cellular DNA.

There is a compelling, but incompletely understood, relation between regulation of the cell cycle and apoptosis. DNA damage, such as that caused by ionizing radiation, activates checkpoints that delay the cell cycle at different points, including G1 and the G2/M boundary (27, 28). During the checkpoint delay, DNA repair systems are induced that can fix the damage and permit normal cell cycle progression. Alternatively, DNA damage can also lead to apoptosis (6). Cyclins and cyclin-dependent kinases (CDKs) were identified from their role in controlling the cell cycle. However, their expression also correlates with the onset of apoptosis, and overexpression of CDKs increases the incidence of apoptosis (22, 43).

The purpose of the present study was further to examine the cell cycle arrest and the cell death mechanism induced in proliferating mIMCD3 cells by high NaCl and/or urea and how these processes might be related. We find that high NaCl and/or urea induces a specific concentration-dependent reduction of cell number, associated early with delay in the G2/M phase of the cell cycle, slower movement of cells through the S phase, and later a delay in G1. We also find that high salt and/or urea concentrations kills cells by apoptosis.

DURING THE PRODUCTION of concentrated urine, cells of the renal medulla are subjected to high concentrations of solutes, particularly of NaCl and urea. The cells, both in vivo and in cell culture, adapt to these adverse conditions by a number of mechanisms, including accumulation of a variety of organic osmolytes (4, 15) and induction of heat shock proteins (10, 24, 33). However, there are limits to the concentration of salt and urea that even kidney medullary cells can withstand, and

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The implication of these results and possible mechanisms are discussed.

METHODS

Cell culture. Murine inner renal medullary collecting duct cells, cell line mIMCD3, generously provided by S. Gullans (30) between passages 10 and 17, were cultured on Falcon plates (10-cm diameter) in 1:1 Irvine Dulbecco’s modified Eagle’s medium/Ham’s F-12 containing 10% fetal calf serum and 2 mM L-glutamate at 37°C in 5% CO2. The osmolality of the basal medium was 300 mosmol/kg (µ-Osmette, Precision Systems).

MTT assay. 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay was performed essentially as previously described (12). Briefly, mIMCD3 cells were seeded on 96-well plates (Costar 3596) and were allowed 6 h to attach. Then, control and experimental media were substituted and remained for the time required by the experiment. At the termination of the experiment, all media were replaced with serum-free control medium, containing MTT, 1 mg/ml, for 2 h at 37°C. After aspirating the MTT solution, the blue formazan reaction product was extracted from the cells with 1-propanol for 10 min with shaking, then the optical density was read on a Labsystems Multiskan MCC/340 microplate scanning spectrophotometer at 540 nm with background subtraction at 690 nm. The result reported for each condition is the mean of eight determinations on separate wells.

Flow cytometry. In the first set of experiments, DNA cell cycle analysis and apoptosis were measured on 70% ethanol-fixed, propidium iodide (PI)-stained cells. A combination of previously described methods (3, 14, 34) was used. In brief, mIMCD3 cells were seeded (1 × 10^6 cells/dish) in 10-cm dishes in 1:1 Irvine Dulbecco’s modified Eagle medium, and the plates were incubated for variable periods of time up to 24 h. The floating cells and particles suspended in the media were harvested by centrifugation at 500 g for 5 min, and the pellet was suspended in PBS. The attached cells from the subconfluent cultures were harvested by trypsinization, combined with the floating cells, centrifuged, and resuspended in 500 µl PBS. Then, the cells were fixed by adding the cell suspension into ice-cold 70% ethanol and stored at −20°C. After PI staining, the fixed cells were washed once with PBS and incubated in the presence of PI (80 µg/ml) plus 1 mg/ml RNase A in PBS for 30 min at room temperature in the dark. Analysis was performed with a Coulter flow cytometer (model EPICS-XL-MCL). Cell cycle compartments were deconvoluted from a single-parameter DNA histogram of 30,000 events using WinMDI (Phoenix Flow Systems, San Diego, CA), after exclusion of cell doublets (Win Mdi 2.7, Joseph Trotter), and results were expressed as the G1/G2M ratio for each sample.

5-Bromodeoxyuridine (BrdU) incorporation was measured in exponentially growing cells 48 h after seeding (1 × 10^6 cells/dish). mIMCD3 cells were fed with isotonio or experimental media and 4 h later were pulsed with 10 µM BrdU (Sigma) for 30 min. Cells were harvested and fixed in 70% ethanol, as described above, up to 16 h after the BrdU pulse. BrdU content was analyzed after DNA denaturation with 2N HCl and 0.1 M Na2B4O7 buffer, pH 8.5. The content was analyzed after DNA denaturation with 2N HCl and 0.1 M Na2B4O7 buffer, pH 8.5. The osmolality of the basal medium was 300 mosmol/kg (µ-Osmette, Precision Systems).

Electron microscopy. The cells were rinsed with PBS and then fixed on their coverslips by immersion in phosphate-buffered 2.5% glutaraldehyde. For scanning electron microscopy (SEM), the coverslips with the cells attached were dehydrated through graded acetones, dried by the critical point method, mounted on stubs, coated with a thin layer of palladium-gold in a sputter coater, and examined using a Hitachi 570 SEM operating at 20 kV. For light and transmission electron microscopic (TEM) examination, the glutaraldehyde-fixed samples were scraped off their coverslips and centrifuged down in conical tubes to form a pellet. The pellets of cells were then rinsed in PBS and postfixed for 1 h in phosphate-buffered 1% osmium tetroxide. The pellets were then dehydrated through graded acetones and embedded in Spurr embedding medium. Semi-thick sections (1–2 µm) were cut, stained with toluidine blue, and examined with an Olympus OM2 light microscope. Thin sections (50–80 mm) of
this material were poststained with uranyl acetate and lead citrate and examined using a JEOL 1200EX TEM operating at 60 kV.

Statistical methods. The results are presented as means ± SE. Significance was analyzed with the GraphPad Instat program, using ANOVA (Student-Newman-Keuls multiple comparison test). P < 0.05 is considered significant.

RESULTS

High concentrations of urea and/or NaCl reduce the number of growing cells. Cell number was estimated with the MTT assay, which measures the reduction of the tetrazolium salt, MTT, to form a blue formazan product by the mitochondria of living cells (12). Figure 1 shows the results of an experiment in which 1,000 mMCD3 cells were seeded per well on 96-well culture plates and followed for 4 days during the log phase of their proliferation. Addition of urea (Fig. 1A), NaCl (Fig. 1B), or a mixture of equiosmolar amounts of both (Fig. 1C) reduces the number of cells and the rate at which they increase. In this respect the effects of NaCl and urea are equal (Fig. 1D). Furthermore, when NaCl and urea are combined and added together, the effect is the same as with either alone (Fig. 1D). In other words, the effects of NaCl and urea on cell number are not additive.

The actions of NaCl and urea are not due simply to the elevation of osmolality. Equally high concentrations of glycerol affect cell growth much less (Fig. 1D).

The results with MTT do not distinguish whether NaCl and urea reduce cell number by slowing the cell cycle, killing cells, or both and, if the cells are dying, do not identify the mechanism. We evaluated these possibilities by flow cytometry, electron microscopy, and analysis of DNA fragmentation.

High urea and/or NaCl induces G1/M and G1/G0 delay and prolongs the S phase of the cell cycle. The effect of high urea and/or NaCl on the cell cycle of exponentially growing mIMCD3 cells was analyzed by measuring cell DNA content by flow cytometry. The proportion of cells in G1/M increases initially under all the hyperosmolar conditions tested, as reflected by the decreased ratio of G1/G2M (Fig. 2A). Urea initially reduces the G1/G2M ratio to a similar minimum value regardless of its concentration. The reduction occurs faster at lower urea concentrations (Fig. 2B). Furthermore, when urea is added to a total osmolality of 550 or 600 mosmol/kg, the effect reverses after 8 or 12 h. What is more, at 550 mosmol/kg, G1/G2M is actually elevated by 12 h. High NaCl causes similar results, but G1/G2M does not fall as far as with urea. Finally, when equiosmolar amounts of NaCl and urea are added together to a total osmolality of 1,000 mosmol/kg, the effect is the same as with NaCl alone added to 650 mosmol/kg. Again, the effects of NaCl and urea are not additive.

The preceding results are consistent with G1/M delay induced by high urea and/or salt. However, the inverse relationship between the increase in urea concentration and the rate of decrease of the G1/G2M ratio is intriguing. It could be the consequence of more complex effects over the cell cycle, like differences in the S phase transit time and/or the time needed for the installation of the G1/M arrest. We analyzed these possibilities by using BrdU pulse-chase to quantify the G1/M delay and the G1/G2M delay. Thus we pulsed the cells for 30 min with BrdU, a thymidine analog that is incorporated in the DNA of cells in S phase, and harvested them at different times for bivariate analysis of DNA content (PI staining) vs. BrdU incorporation (FITC-conjugated antibody). The percentage of BrdU+ cells was the same in the controls and experimental groups immediately following the pulse, indicating that a similar proportion of cells were synthesizing DNA at that time in all the tested conditions (data not shown). By 4 h of chase, the movement of BrdU+ cells through the S phase is evidently slowed when urea and/or NaCl is high (Fig. 3A). Calculation of the RM of cells though the S phase shows that the BrdU+ cells in 300 mosmol/kg medium start with an average value of 0.53 ± 0.01 (Fig. 3B), meaning that the average DNA content of cells in S phase is half way between that of G1/G2 and G1/M. These control cells progress at a relatively constant speed through S phase and reach G2M (RM = 1) 6.3 h after the pulse. In contrast, cells in high urea start with a significantly higher RM and their RM increases...
from the time for initial appearance of BrdU.

The duration of G2/M in each condition was calculated (big arrow). The final stages of apoptosis are demonstrated by catalytic cleavage of the proenzyme generating caspase-3, which is considered a marker of apoptosis, by 10.220.33.1 on June 25, 2017. High NaCl has a similar effect. At the highest concentrations of NaCl and/or urea complete G2/M arrest is sustained throughout the experimental period of 16 h.

In summary, high NaCl and/or urea slow the S, G1, and G2/M phases of the cell cycle in a concentration- and time-dependent fashion.

High salt and/or urea causes apoptosis. Apoptosis is characterized by rounding up and detachment of cells, disappearance of microvilli, appearance of surface blebs, and cytoplasmic and nuclear condensation. When viewed by SEM, control cells in 300 mosmol/kg medium (Fig. 4A) form a flat pavement epithelial layer with well-marked borders, surface microvilli, and small rudimentary cilia. Incubation for 24 h in medium to which urea is added to 650 mosmol/kg (Fig. 4B) causes the cells to separate from each other and reduces numbers of microvilli. Some cells (marked by arrows) are rounding up, and others (marked by arrow heads) have already become rounded. Blebs are evident on many cells (marked by asterisks). When NaCl is added to 600 mosmol/kg (data not shown) or an equiosmolal mixture of NaCl and urea is added to 1,000 mosmol/kg (Fig. 4C), the same features are apparent.

A TEM image of control cells is shown in Fig. 5A. Treatment for 24 h with urea to 650 mosmol/kg (Fig. 5B), causes the cells to separate and round up, increases cytoplasmic density, and reduces numbers of mitochondria. Some cells show surface blebbing and condensation of chromatin at the margins of the nuclei. Cells treated with NaCl (Fig. 5C) for the same period demonstrate less marked rounding and separation, but the chromatin changes are evident (small arrows), as is an apoptotic cell remnant containing a nuclear fragment (big arrow). The final stages of apoptosis are shown in the example of cells subjected to the equiosmol mixture of NaCl and urea at 1,000 mosmol/kg for 24 h (Fig. 5D). Cells are small and rounded, some surface blebs are visible, and the nuclei (arrows) are small and pyknotic.

Thus mIMCD3 cells subjected to high NaCl and/or urea display morphological changes characteristic of apoptosis.

Quantification of the apoptosis by flow cytometry.

When apoptotic cells are fixed and stained with PI they appear as a subdiploid peak in flow cytometry (3, 34). This occurs when the osmolality is raised by adding NaCl and/or urea (Fig. 6A). The number of apoptotic cells is concentration dependent and peaks at 8 and 12 h in the presence of high urea or high salt, respectively, followed by a significant reduction at 24 h (Fig. 6B). Interestingly, equiosmol mixture of NaCl and urea added up to 1,000 mosmol/kg results in a rapid increase in apoptotic particles which persists over 24 h.

Apoptosis may be signaled by different pathways, but those converge on the activation of the so-called effector caspases, such as caspase-3 (16, 22). The activation of caspase-3, which is considered a marker of apoptosis, involves catalytic cleavage of the proenzyme generat-

at a progressively slower rate (Fig. 3B). High NaCl slows passage of cells through S even more than urea does (Fig. 3B). In fact, there is practically no progression when NaCl is added to an osmolality of 650 mosmol/kg. Interestingly, the presence of urea apparently ameliorates this effect of NaCl to some extent. There is some progression through S phase with a combination of NaCl and urea added to a total osmolality of 1,000 mosmol/kg, whereas no progression is evident with NaCl alone added to a total osmolality of 650 mosmol/kg (Fig. 3B).

The duration of G2/M in each condition was calculated from the time for initial appearance of BrdU· in cells in G1, G2/M is 2.5 h for cells in isotonic media (Fig. 3C). High urea prolongs G2/M in a concentration-dependent fashion, reaching more than 16 h with urea added to a total osmolality of 650 mosmol/kg. High NaCl has a similar effect. At the highest concentrations of NaCl and/or urea complete G2/M arrest is sustained throughout the experimental period of 16 h.

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Apoptosis may be signaled by different pathways, but those converge on the activation of the so-called effector caspases, such as caspase-3 (16, 22). The activation of caspase-3, which is considered a marker of apoptosis, involves catalytic cleavage of the proenzyme generat-
ing the mature caspase-3 and exposing a conformational epitope that can be recognized by specific antibodies. Therefore, to study the relationship between the cell cycle and apoptosis, we analyzed caspase-3 activation and DNA content of cells with high salt and/or urea by using dual parameter cytograms, in which the cell cycle status of apoptotic cells is determined. The cells were harvested, fixed in methanol, labeled with a

Fig. 3. Cell cycle analysis by 5-bromodeoxyuridine (BrdU) pulse chase. A: bivariate DNA/BrdU cytograms from pulse-chase experiments. Culture medium bathing subconfluent mIMCD3 cells was replaced with medium that was isotonic (300 mosmol/kg) or made hyperosmolar by adding urea, NaCl, or an equiosmolar combination of both. Four hours after medium change, cells were incubated for 30 min in presence of BrdU (10 µM), washed, fed with control or experimental medium, and harvested 4 h later. Cells were fixed in 70% ethanol and stained for bivariate analysis of DNA content (PI) and BrdU incorporation (monoclonal Ab-FITC). Final total osmolality and harvesting time (hours after media change) are indicated in each contour histogram from a representative experiment. B: relative movement (RM) through S phase. Cells were treated as described in A and harvested at different intervals up to 16 h after the BrdU pulse. RM of BrdU cells was calculated as described in METHODS and plotted as a function of time. Each point represents the mean ± SE of 3–4 independent experiments, isotonic (open circles), hyperosmolar NaCl or urea (solid symbols), and equiosmolar combination of both (1,000 mosmol/kg, open squares). C: appearance of BrdU cells in G1. Percentage of BrdU cells in G1 was calculated as described in METHODS and plotted as in B. Each point represents the mean ± SE of 3–4 independent experiments.

Fig. 4. Scanning electron microscope images of IMCD cells. A: control cells are flat and exhibit microvillous projections. Rudimentary cilia are present (arrows). B: urea added to 650 mosmol/kg for 24 h. Some cells are rounded (arrowheads), and others appear to be in the process of rounding up (arrows). Many surface blebs are present (asterisks). C: equiosmolar mixture of NaCl and urea added to 1,000 mosmol/kg for 24 h. Cells are rounded and exhibit significant blebbing. Apoptotic bodies are present (arrowheads). Bars = 10 µm.
phycoerythrin conjugated to antibody against active caspase-3, and stained with 7-AAD for DNA content measurement. The bivariate analysis shows very few active caspase-3-positive cells under isotonic conditions (Fig. 7). High NaCl significantly increases the population of active caspase-3-positive cells (Fig. 7). High urea produces a similar but less pronounced effect, as does the combined addition of both salt and urea. The time course of active caspase-3 cell abundance is similar to that obtained when measuring the subdiploid peak in PI stained cells. The peak values (reached at 12 h, n = 3) are 18.9 ± 2.6% for added NaCl and 12.2 ± 3.1% for added urea, compared with 1.5 ± 0.3% for cells in isotonic media. With both salt and urea added to 1,000 mosmol/kg total osmolality, the peak of apoptosis at 12 h is 22 ± 2.8%. The high level with added NaCl and urea persists at 24 h (18.5 ± 0.6%). Finally, comparison of linear DNA histograms of the whole cell population vs. the DNA histograms of active caspase-3-positive cells demonstrates the presence of apoptotic cells at all phases of cell cycle, with a relative predominance of active caspase-3 cells in G1 and S phase cells (Fig. 7).

DISCUSSION

High salt and/or urea slow movement through the S phase of the cell cycle and cause G2/M delay, followed by G1 delay. The earliest detectable changes in the cell cycle are G2/M delay and slower transit through the S phase. These results extend the previous observations that high NaCl causes growth arrest and G2 delay (20) and that both high NaCl and high urea increase the proportion of cells in G2/M in confluent cultures (32). Thus we know that the G2/M delay is transient at relatively low NaCl and urea concentrations. In fact, with urea or NaCl added to total osmolality of 550 mosmol/kg, the effect on G2/M reverses by 24 h, at which point most cells are in G1/M. Similarly, when the cells are chronically adapted through many passages to high NaCl or urea (total osmolality 650 mosmol/kg) or to both NaCl and urea (total osmolality 1,000 mosmol/kg) the G2/G1 phase of the cell cycle predominates during the slow growth that remains (data not shown). We speculate that the acute G2/M cell cycle delay that follows elevation of NaCl and/or urea provides time for appropriate adaptative responses prior to mitosis. However, in the longer term, the hyperosmolar stress induces a G1 delay and/or reduction in the number of cells going through the cell cycle. This follows from previous theories that suggest that following genotoxic stress there may be a transient delay in cell cycle progression to allow time for repair, so dangerous mutations are not passed into daughter cells (17).

It is surprising that the effects of high NaCl and urea on the cell cycle are so similar, considering that NaCl and urea regulate different sets of genes in renal epithelial cells (7, 9, 19). Osmolality, per se, is not the
common factor, because glycerol has much less effect than NaCl or urea (Fig. 1). Although the different pathways by which high NaCl and urea modulate the cell cycle are not yet known in detail, it may be that the pathways converge on some common molecular mechanisms such as the CDK cdc2-cyclin B-cdc25 machinery that regulates the G2M progression (26, 39).

Although the analysis of the RM through S phase shows concentration dependence for both NaCl and urea, there is a quantitative difference. A given osmotic concentration of NaCl retards RM through S phase considerably more than does the same concentration of urea (Fig. 3B). This is in agreement with the reduction in DNA synthesis rate previously observed with high NaCl but not urea (8). On the other hand, high urea not only reduces progression through S phase less than high NaCl does, but the presence of urea apparently reduces the effect of high NaCl (Fig. 3B). Along the same line, high urea increases [3H]thymidine incorporation two- to threefold without induction of polyploidy or aneuploidy in kidney epithelial cells, consistent with enhanced DNA synthesis (8). Thus urea might specifically activate DNA repair and/or synthesis mechanisms and thus ameliorate an inhibition of DNA synthesis caused by high NaCl.

High NaCl and/or urea causes apoptosis. Very high concentrations of NaCl or urea kill cells in culture (24, 32). The mechanism of cell death is somewhat controversial. One previous report concludes that high NaCl kills mIMCD3 cells by necrosis (41), whereas another report attributes the death of these cells to apoptosis (32). In the present studies, we find clear evidence of apoptosis. Thus high concentrations of NaCl, urea, or both cause changes in cell size, morphology, PI staining in flow cytometry (subdiploid peak), and caspase-3 activation that together are diagnostic of apoptosis. The apoptosis is time and concentration dependent.

Comparison of the effects of high NaCl and urea individually vs. when they are combined is interesting. Our results on cell proliferation are essentially the same as those in the previous study (32), but we have a more limited interpretation. Thus, in both studies, a given osmolar concentration of NaCl reduces cell number to approximately the same extent as that concentration of urea does. However, the number of cells at a given concentration of NaCl or urea remains the same when an equal concentration of the other solute is added.

The broader interpretation (32) is that the combined actions of NaCl and urea enhance survival because of differences in Hsp70 expression. This interpretation implies that total osmolality is the appropriate independent variable, so the effects should vary with total osmolality. However, when glycerol is added to the same high osmolality as that of either NaCl or urea alone or to the higher osmolality of the combination of NaCl and urea, then the glycerol has only a minor effect on cell number compared with the large effects of NaCl and urea (Fig. 1). Moreover, contrasting with the previous study, we found that a combination of NaCl and urea induces an additive increase in apoptosis (Fig. 6).

The study of the G2/M ratio time course and duration of G2M delay shows that high salt or urea produce very similar dose-response curves, and the effects of a combination of NaCl and urea are nonadditive. Interestingly, studies on mIMCD3 or MDCK cells show that Hsp70 expression is induced by NaCl added to the medium up to 600 mosmol/kg (total osmolality) but not by urea or higher concentrations of salt (24, 32). Thus NaCl or urea specifically trigger a similar dose-dependent response affecting the cell cycle unrelated to Hsp70 expression. We demonstrate that apoptosis significantly increases when total osmolality rises over 600 mosmol/kg by adding high salt or urea. However,
Neuhofer et al. (25) had recently shown that blocking the Hsp72 expression with a specific antisense construct does not affect MDCK cell survival under high NaCl but reduces cell survival upon subsequent addition of urea in the medium. Therefore, we prefer to limit our interpretation to the observation that final effects of NaCl and urea are not additive.

Activation of caspase-3 by high NaCl and/or urea. We find time-dependent activation of caspase-3 following elevation of NaCl and/or urea levels, which is consistent with the general role of caspase-3 as an executioner of programmed cell death (13). Caspase-3 activity is modulated by some of the other systems whose expression increases during osmotic stress and vice versa. For example, part of the effectiveness of Hsp70 in enhancing viability of stressed cells may involve its prevention of the activation of caspase-3 (23) and its inhibition of proteolytic activity of caspase-3 (18). On the other hand, caspase-3 can modulate key molecules of signaling cascades regulated by hyperosmolality such as mitogen-activated kinase-kinase 1 (MEKK1), which is known to be activated by high osmolality in kidney epithelial cells (21). Active membrane-associated MEKK1 is a proteolytic substrate for caspase-3 (11, 40). In human embryonic kidney cells, the MEKK1 cleavage by caspase-3 generates a pro-apoptotic kinase which translocates to the cytosol, transforming MEKK1 in an apoptotic signal (5, 11, 40). Thus it is conceivable that the caspase-3-cleaved MEKK1 functions as a part of the apoptotic cascade, mediating the nuclear changes involved in high osmolality-induced programmed cell death in our model.

Relation between cell cycle delay in G1 and apoptosis caused by NaCl and/or urea. Although high NaCl and urea are known to differ in their effects on cell metabolism and DNA expression (4, 9, 31, 32, 35, 36), it is not clear how each causes apoptosis and whether the mechanisms differ between NaCl and urea. A variety of in vitro experiments have demonstrated strong effects of osmotic strength on DNA structure and transcriptional activity (29, 38). A wide variety of DNA-damaging agents trigger growth arrest, G1 and/or G2M delay, and apoptosis (27). Moreover, in mIMCD3 cells, high NaCl and/or urea induces expression of GADD-45 and GADD-153, proteins believed to be involved in DNA damage repair (20). Similarly, high urea also increases GADD-153 expression in mIMCD3 cells (42). Thus we speculate that one important consequence of hyperosmotic stress is the DNA damage. The cellular adaptive response could involve the tumor suppressor protein p53 (19, 20), which is known to be a central

Fig. 7. Relation between cell cycle and caspase-3 activation. Cells were exposed to isotonic (A) or hyperosmolar media prepared by adding NaCl (B) or urea (650 mosmol/kg total osmolality; C) or an equiosmolar combination of both (1,000 mosmol/kg total osmolality; D). Cells were harvested after 12 h, fixed in methanol 100% and stained for DNA content (7-amino actinomycin D (7AAD)) and active caspase-3 (polyclonal Ab-PE). Representative results are shown as dual-parameter histograms in each experimental condition. Left: cytograms for all the recorded events (DNA log scale vs. active caspase-3 and DNA linear scale vs. active caspase-3). Activation of caspase-3 under hyperosmotic stress is detected by the appearance of cells with high PE fluorescence (upper quadrants of each cytogram). Right: cytograms for DNA content (linear scale) of active caspase-3-positive and of all cells.

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element in DNA damage recognition and repair and a transcriptional factor for GADD proteins.

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