Rate of increase of osmolality determines osmotic tolerance of mouse inner medullary epithelial cells

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Cai, Qi, Luis Michea, Peter Andrews, Zheng Zhang, Gerson Rocha, Natalia Dmitrieva, and Maurice B. Burg. Rate of increase of osmolality determines osmotic tolerance of mouse inner medullary epithelial cells. Am J Physiol Renal Physiol 283: F792–F798, 2002. First published April 23, 2002; 10.1152/ajprenal.00046.2002.—Renal inner medullary cells survive and function despite interstitial osmolality of 600–1,700 mosmol/kgH2O or more. In contrast, much smaller changes kill cells in tissue culture. Using mouse inner medullary epithelial cells at passage 2, we defined factors that might account for the difference. Most of the factors that we tested, including addition of hormones (insulin-like growth factor I, epidermal growth factor, or deamino-8-D-arginine vasopressin), growth on porous supports, and presence of matrix proteins (collagen I, collagen IV, fibronectin, laminin, or fibrillar collagen I), have no significant effect. However, the time course of the change makes a major difference. When osmolality is increased from 640 to 1,640 mosmol/kgH2O by addition of NaCl and urea in a single step, only 30% of cells survive for 24 h. However, when the same increase is made linearly over 20 h, 89% of the cells remain viable 24 h later. We conclude that gradual changes in osmolality, e.g., in vivo, allow cells to survive much greater changes than do the step changes routinely used in cell culture experiments.

- cell survival; hyperosmolality; growth factor; extracellular matrix; porous support

ALONG WITH CHANGES IN URINE concentration, renal inner medullary cells are exposed to large alterations in interstitial salt and urea concentration (16). During water diuresis, NaCl transport by thick ascending limb cells maintains interstitial NaCl concentration at levels approximately twice that in peripheral plasma throughout the renal medulla (2). During antiuresis, NaCl and urea concentrations increase to high levels. At the tip of the inner medulla in rodents, interstitial osmolality can be 1,700 mosmol/kgH2O or more, depending on the species and conditions. Despite such osmotic stress, the cells evidently survive and function.

In contrast, much lower concentrations of salt and/or urea kill cells in renal inner medullary epithelial cell lines in tissue culture. For example, mouse inner medullary collecting duct (mIMCD3) cells that are immortalized by expression of simian virus 40 and cells from other continuous lines do not survive an acute increase of NaCl or urea concentration above a total osmolality of 600 mosmol/kgH2O (20, 28).

Second-passage mouse inner medullary epithelial (p2mIME) cells survive greater acute increases in osmolality than do mIMCD3 cells (32). The p2mIME cells were prepared and grown in a medium in which osmolality was elevated to 640 mosmol/kgH2O by addition of 260 mosmol/kgH2O NaCl and 80 mosmol/kgH2O urea to the 300 mosmol/kgH2O basal medium, thus mimicking the inner medullary osmotic composition during water diuresis. To provide direct comparison with the mIMCD3 cells that had been routinely grown at 300 mosmol/kgH2O, we decreased the p2mIME medium to 300 mosmol/kgH2O by eliminating urea and lowering NaCl for 48 h before acutely increasing NaCl or urea. Few mIMCD3 cells survived increase of NaCl or urea above 600 mosmol/kgH2O. In contrast, most p2mIME cells survived NaCl added to 1,100 mosmol/kgH2O and urea added to 700 mosmol/kgH2O. The difference was similar when NaCl and urea were added together. Therefore, less added NaCl and/or urea kills immortalized cells than is necessary to kill early-passage cells. Despite this difference, most p2mIME cells did not survive an acute increase in NaCl plus urea above 1,300 mosmol/kgH2O, which is less than the level normally reached in inner medullary interstitial fluid in vivo after 18 h of antidiuresis. The purpose of the present studies was to identify possible factors besides immortalization that account for the difference in osmotic tolerance between cell culture and in vivo conditions.

Epithelial and endothelial cells undergo apoptosis when detached from their extracellular matrix (ECM),

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so-called anoikis (8, 18). Anchorage-dependent survival is signaled by ECM components, such as fibronectin (1, 9), and by growth factors, such as insulin-like growth factor I (IGF-I) (4, 27). The effect of ECM proteins depends on the cell type. Laminin signals survival in mammary epithelium (7) and apoptosis in endothelium (31). Mesangial cells grown on wells treated with collagen or laminin are resistant to apoptosis induced by serum starvation (23). Therefore, we examined the effect of various ECM proteins (collagen I, collagen IV, fibronectin, laminin, and fibrillar collagen I) on osmotic tolerance of p2mIME cells. IGF-I rescues cardiac myocytes or human neuroblastoma cells from apoptosis induced by osmotic stress (17, 24); IGF-I and epidermal growth factor (EGF) reduce activation of caspase 3 by high NaCl (33). Therefore, we examined the effect on osmotic tolerance of p2mIME cells to IGF-I and EGF, as well as vasoressin, which is elevated during anti-diuretics.

Interstitial fluid bathes the basolateral surface of renal medullary epithelial cells in vivo, providing nutrients and signaling molecules. In contrast, cells are generally grown on solid supports in tissue culture, which limits access of the medium to their basolateral surface. The normal geometry can be mimicked by growing the cells on porous supports, so that the basolateral surface of the cells is exposed to the medium beneath the support. The appearance of cells grown on porous supports is different from that of cells grown on solid supports. For example, A6 cells develop active motile cilia (12), and mammary epithelial cells are more cuboidal-columnar (29). Also, A6 cells grown on porous supports developed a higher short-circuit current, equivalent to greater net sodium transport (12). Because it was conceivable that epithelial cells grown on porous supports might also have greater osmotic tolerance, we compared survival of p2mIME cells grown on plastic slides with that of p2mIME cells grown on porous supports.

The response to changes of osmolality can depend on the rate of change. Studies of osmotic effects in tissue culture generally have utilized step changes in osmolality that produce readily discernable responses. In contrast, increases in inner medullary interstitial osmolality in vivo are more gradual, occurring over a period of hours. When cells are exposed to a step increase or decrease, they immediately respond. Increases in inner medullary interstitial osmolality (25). Because it was conceivable that the rate of change of osmolality might affect osmotic tolerance, we compared the effect of a step increase with the effect of a linear increase of osmolality on survival of p2mIME cells.

**MATERIALS AND METHODS**

**Materials.** Mice were obtained from Taconic Farms (Germantown, NY). DMEM-Ham’s F-12 was purchased from GibCO BRL (Rockville, MD), collagenase B from Roche (Indianapolis, IN), hyaluronidase from Worthington Biochemical (Lakewood, NJ), DMEM and Coon’s modification of Ham’s F-12 from Mediatech Cellgro (Herndon, VA), selenium, insulin, transferrin, 3,3,5-triiodo-L-thyronine (T3), hydrocortisone, propidium iodide (PI), RNase, IGF-I, EGF, and deamino-8-d-arginine vasopressin (dDAVP) from Sigma (St. Louis, MO), cell culture inserts from Becton Dickinson Labware (Bedford, MA), eight-channel plastic slides from Nalge Nunc (Naperville, IL), Antibody from Molecular Probes (Eugene, OR), plastic culture dishes from Corning (Corning, NY), and an osmometer from Advanced Instruments (Norwood, MA).

**Mouse inner medullary cell culture.** Kidneys were harvested from mice killed by cervical dislocation. Renal inner medullas and papillas were dissected, minced (1–2 mm), and digested in 640 mosmol/kgH2O (80 mM urea and 130 mM NaCl added) enzyme solution (DMEM-Ham’s F-12 containing 50 mg of collagenase B and 18 mg of hyaluronidase per 25 ml). Tissues were incubated for 90 min at 37°C with continuous mixing (300 rpm) in a humidiﬁed incubator (5% CO2, 95% O2). The resulting cell suspension was centrifuged at 1,000 rpm/min for 1 min and washed three times with DMEM-Ham’s F-12 (640 mosmol/kgH2O) without enzyme. Cells were resuspended in 640 mosmol/kgH2O medium (10% fetal bovine serum, 45% DMEM, 45% Coon’s modiﬁcation of Ham’s F-12, 10 mM HEPES, pH 7.5, 2.5 mM T3, 5 mg/l transferrin, 10 nm selenium, 50 nm hydrocortisone, 2 mM l-glutamine, 100 U/ml penicillin G, and 100 µg/ml streptomycin sulfate) and maintained at 37°C in 5% CO2. Cells were fed each 24 h until confluent (48–72 h). The confluent cultures were split with 0.1% trypsin in Ca2+- and Mg2+-free 640 mosmol/kgH2O Dulbecco’s modiﬁed PBS into 6-cm plastic dishes (passage 1). When confluent, the cells were split onto cell culture inserts (40,000 cells/insert) or eight-chamber plastic slides (30,000 cells/chamber) for the experiments (passage 2). After 3 days, the 640 mosmol/kgH2O medium was replaced by an otherwise identical medium containing no serum, and that medium was replaced 48 h later by the experimental medium. The 1,640 mosmol/kgH2O medium was prepared from the 640 mosmol/kgH2O medium with increasing NaCl to 400 mM and urea to 800 mM. Media of intermediate osmolality were prepared by diluting the 1,640 mosmol/kgH2O medium with the 640 mosmol/kgH2O medium.

**Step vs. linear osmotic stress.** When the cells on cell culture inserts were confluent, the medium was changed to serum-free 640 mosmol/kgH2O medium (45% DMEM, 45% Coon’s modiﬁcation of Ham’s F-12, 10 mM HEPES, pH 7.5, 5 mM T3, 5 mg/l transferrin, 10 nm selenium, 50 nm hydrocortisone, 2 mM l-glutamine, and 5 mg/l insulin) for 48 h. To achieve a gradual increase in osmolality, the cell culture inserts were mounted in chambers with a constant ﬂow of medium bathing the lower surface of the inserts. The osmolality of the medium bathing the basolateral surface of the cells was increased linearly to 1,640 mosmol/kgH2O over 20 h using a gradient mixer and then maintained at 1,640 mosmol/kgH2O...
for an additional 24 h. The linear increase in osmolality was confirmed by repeated sampling of the fluid flowing through the chamber. Osmolality of the fluid contained in the insert (bathing the apical cell surface) equaled that bathing the lower surface of the inserts at the end of the linear increase.

To achieve a step increase to 1,640 mosmol/kgH$_2$O, the medium was changed inside and outside the insert to one otherwise identical to the 640 mosmol/kgH$_2$O medium, except NaCl was increased to 400 mM and urea to 800 mM for 24 h. So that all the cells could be harvested at the same time, the step increase was made 20 h after the linear increase was started.

**Cell fixation and PI staining.** Cells grown on cell culture inserts or plastic slides were fixed in ice-cold 100% methanol at −20°C for 15 min, washed with PBS, and incubated for 15 min with 1 mg/ml RNase and 20 μg/ml PI in PBS. The filters containing the cells were cut from the cell culture inserts and placed on glass slides. Antifade (200 μl) was added before the glass or plastic slides were sealed with glass coverslips.

**Light and electron microscopy.** After 48 h in serum-free medium, confluent cells were fixed in 2% paraformaldehyde for 1 h at room temperature and then in 2% phosphate-buffered gluteraldehyde overnight. Then they were postfixed for 45 min in 1% phosphate-buffered osmium tetroxide, dehydrated, and embedded in Spurr’s medium. For light microscopy, semithin sections (0.2–1 μm) were cut and mounted on glass slides, stained with toluidine blue, and examined and photographed using a light microscope (model BH-2, Olympus) equipped with a 35-mm camera. For transmission electron microscopy, ultrathin sections (20–50 nm) were cut and mounted on grids, stained with uranyl acetate and lead citrate, and examined with a Jeol transmission electron microscope operating at 60 kV.

**Laser scanning cytometry.** Cell survival was analyzed by laser scanning cytometry of the PI-stained cells (counting the number of cells [nuclei] and determining the maximal brightness [maximal pixel PI fluorescence] within each nucleus). Cells acutely exposed to hypertonicity undergo apoptosis (20), characterized by nuclear condensation, which results in increased brightness of PI staining. Cells with maximal brightness that did not exceed the maximal brightness of most cells at 640 mosmol/kgH$_2$O were considered to be viable.

**Statistical methods.** All values were normalized to those in 640 mosmol/kgH$_2$O medium (100%) and expressed as means ± SE (n = number of experiments). Statistical significance was determined using the paired t-test for single comparisons or ANOVA (with Student-Newman-Keuls multiple comparison test) for multiple comparisons (GraphPad Instat Software). P < 0.05 was considered significant.

**RESULTS**

**Effect of porous supports on p2mIME cell differentiation and tolerance of p2mIME cells to acute hyperosmolality.** The basal surface of epithelial cells in vivo is directly exposed to nutrients and signaling molecules in the interstitial fluid, whereas the basal surface of epithelial cells grown on solid tissue culture plastic is not directly exposed to the medium. Direct exposure of the basolateral surface to the medium does occur, however, when epithelial cells are grown on porous supports. Epithelial cells are more differentiated when grown on porous supports than when grown on a solid surface. This is manifested by appearance of cilia and basolamina in Madin-Darby canine kidney cells and higher short-circuit current and response to vasopressin in A6 cells (12). We hypothesized that p2mIME cells might be more differentiated and tolerate acute osmotic stress better if they were grown on porous supports. Therefore, we compared the appearance and osmotic tolerance of p2mIME cells grown on porous cell culture inserts with p2mIME cells grown on solid plastic.

When examined by light microscopy, p2mIME cells grown on solid plastic coverslips are flattened (Fig. 2A) compared with those grown on porous supports (Fig. 2B). To evaluate their fine structure, we compared by electron microscopy some of the tallest cells growing on coverslips (Fig. 2C) with cells growing on porous supports (Fig. 2D). The cells on the coverslips and their nuclei are relatively flat, and they contain fewer cytoplasmic organelles (i.e., mitochondria) and have fewer cell surface microprojections.

When osmolality is acutely increased above 640 mosmol/kgH$_2$O in a single step to 880, 1,100, 1,370 or viable cells decreased to the same extent whether or not the individual growth factors were added (Fig. 1).
1,640 mosmol/kgH$_2$O, the number of viable cells is not significantly affected up to 1,130 mosmol/kgH$_2$O, whether the cells are on solid plastic slides or on porous supports (Fig. 3). At higher osmolality, the number of viable cells decreases and the number of apoptotic cells (high maximal pixel PI fluorescence) increases to the same extent on both kinds of supports (Fig. 3). Therefore, although the cells on porous supports appear more differentiated, their osmotic tolerance is not different.

**Lack of effect of ECM proteins on tolerance of p2mIME cells to acute increase of osmolality.** In vivo, the inner medullary collecting duct cells are surrounded by basement membranes that are composed of a number of ECM proteins, including collagen IV, laminin, fibronectin, and heparin sulfate proteoglycan (26). Previous studies showed that ECM proteins influence survival of several types of cells and their susceptibility to apoptosis (7, 23). Therefore, we tested the effect on osmotic tolerance of coating porous plastic [poly(ethylene tetraphthalate)] filters with several different ECM proteins. Compared with the bare plastic alone, none of the ECM proteins, including collagen I, collagen IV, fibronectin, laminin, or fibrillar collagen I, significantly increase the number of cells that remain viable after osmolality is increased acutely from 640 to 1,640 mosmol/kgH$_2$O by addition of NaCl and urea to the apical and basolateral medium for 24 h (Fig. 4).

p2mIME cells survive a slow, linear increase of osmolality much better than a step increase. Inner medullary interstitial osmolality normally fluctuates, but the change is more gradual than the acute step increase in osmolality to which we exposed the p2mIME...
Fig. 4. Lack of effect of extracellular matrix proteins on tolerance of p2mIME cells to acute increase of osmolality. Cells were grown on porous plastic supports coated with collagen I, collagen IV, fibronectin, laminin, or fibrillar collagen I. When confluent, cells were switched to serum-free medium for 48 h, and NaCl and urea were added, elevating osmolality to 1,640 mosmol/kg H₂O in a single step for 24 h. Nuclei were stained with PI, and number of viable cells was counted by laser scanning cytometry. Values are means ± SE (n = 5).

cells in the preceding experiments. To test the effect of rapidity of osmotic change, we grew p2mIME cells on collagen I-coated porous supports and increased osmolality of the basolateral medium from 640 to 1,640 mosmol/kg H₂O by adding NaCl and urea in a single step or, more gradually, by using a gradient mixer. The gradient mixer provided a linear increase in osmolality over 20 h (Fig. 5A). In both cases, the number of viable cells was measured 24 h after the osmolality reached 1,640 mosmol/kg H₂O (Fig. 5B). Only 30% of the cells remained viable after the acute step increase of osmolality compared with 89% of the cells after a linear increase (Fig. 5D). The difference is similar using bare plastic porous supports or porous supports coated with the other ECM proteins listed above (results not shown).

Hyperosmolality-induced apoptosis is characterized by increased intensity of staining with PI (elevated maximal brightness of red fluorescence) in the hypercondensed nuclei of the apoptotic cells (3). A step increase in NaCl and urea greatly increases the number of p2mIME cells with elevated maximal brightness of red fluorescence 24 h later. In contrast, after a linear increase in osmolality, few p2mIME cells have elevated maximal brightness of red fluorescence (Fig. 5C). Thus many cells become apoptotic after the step increase but not after the linear increase.

DISCUSSION

Although renal inner medullary cells in vivo survive changes in salt and urea concentration between ~600 and ~1,700 mosmol/kg H₂O, much lower concentrations of salt and/or urea kill renal inner medullary epithelial cell lines in tissue culture. For example, mIMCD3 cells do not survive an acute increase of NaCl or urea concentration above a total osmolality of 600 mosmol/kg H₂O and increase of NaCl plus urea above 1,000 mosmol/kg H₂O (20, 28). At least part of the difference is due to immortalization of the cell lines and their routine culture in media with an osmolality appropriate for the rest of the body (300 mosmol/kg H₂O) but not the renal inner medulla (600 mosmol/kg H₂O). Thus p2mIME cells survive NaCl added to 1,100 mosmol/kg H₂O, urea to 700 mosmol/kg H₂O, or NaCl...
plus urea to 1,300 mosmol/kgH_2O but not higher (32). Thus the osmotic tolerance of p2mIME cells is greater than that of mIMCD3 cells but less than that of inner medullary cells in vivo. This discrepancy led us to examine additional factors that might account for the greater osmotic tolerance in vivo, namely, hormones that are present in vivo but not in routine tissue culture medium, growth on porous supports, connective tissue components, and the time course of the osmotic change.

IGF-I increases osmotic tolerance of cardiac myocytes and neuroblastoma cells (17, 24), reducing the apoptosis induced by hypertonicity. Also, IGF-I and EGF reduce activation of caspase 3 by high NaCl (33), and the level of vasopressin increases during antidiuresis, which might contribute to osmotic tolerance. Therefore, we examined the effect of IGF-I, EGF, and dDAVP on the osmotic tolerance of p2mIME cells. All the results were negative. None of these agents increased the tolerance of p2IME cells to acute increase of osmolality.

Growing cells on porous supports, so that the basolateral surface of the cells is exposed to the medium, more closely mimics their situation in vivo than does growing the cells on solid plastic. The appearance of cells grown on porous supports is different from that of cells grown on solid supports (12). Accordingly, the appearance of p2mIME cells grown on porous plastic inserts differs from their appearance when they are grown on solid plastic. p2mIME cells grown on porous supports are taller and have more organelles and microprojections than those grown on solid supports (Fig. 2). However, there is no corresponding difference in osmotic tolerance. At 24 h after an acute increase of osmolality, the number of viable cells does not differ between the two conditions (Fig. 3), so the difference in morphology is not associated with improved osmotic tolerance.

Anchorage-dependent survival of epithelial and endothelial cells is signaled by ECM components (1, 7, 9, 15). Therefore, we examined the effect of various ECM proteins (collagen I, collagen IV, fibronectin, laminin, and fibrillar collagen I) on osmotic tolerance of p2mIME cells. We found no difference in survival of p2mIME cells after osmotic stress, whether the cells are grown on bare porous supports or porous supports coated with the various ECM proteins (Fig. 4).

Increasing NaCl by 50 mosmol/kgH_2O from passage to passage conditioned mIMCD3 cells to proliferate at 900 mosmol/kgH_2O, which is higher than if the change is made acutely (5). However, the cells could not be conditioned to survive concentrations of urea that were similarly high but less than in vivo. Along the same lines, an initial small elevation of NaCl enhances survival of mIMCD3 after a later larger increase (13). These results suggested that the time course of increased osmolality might be important for cell survival. However, the stepwise time courses previously tested in cell culture models differ in an important way from the time course in vivo. With the onset of antidiuresis in vivo, inner medullary interstitial osmolality increases smoothly, not stepwise, and over a period of hours, not days or weeks. The difference could be important, because step changes of osmolality between passages could be selecting resistant cells, rather than inducing adaptation of all the cells. Furthermore, cells exposed to a step increase of osmolality immediately shrink, and such a rapid decrease in volume could be detrimental. On the other hand, when the rate of change of osmolality is slow enough, cell volume does not change measurably, despite a large cumulative change in osmolality (14, 25).

To produce a gradual change of osmolality in tissue culture, we grew the cells on top of porous supports and constantly perfused fluid through the chamber under the support. This protected the cell layer from disruption by the flowing fluid. By using a gradient mixer, we increased osmolality linearly over 20 h from 640 mosmol/kgH_2O (similar to that in inner medullary interstitial fluid during water diuresis) to 1,640 mosmol/kgH_2O (similar to that in antidiuresis). After osmolality increased to 1,640 mosmol/kgH_2O in this fashion, 89% of p2mIME cells survived for ≥24 h, whereas only 30% survived a step increase of this magnitude (Fig. 5).

What accounts for the cells surviving a linear increase in osmolality so much better than a step increase? We can only speculate on some possibilities. Previous studies show that the accumulation of compatible organic osmolytes plays an important role in the adaptation of renal inner medullary cells to hyperosmolality (10). The predominant renal medullary organic osmolytes are sorbitol, glycophosphorylcholine, glycinebetaine, and myo-inositol. Their accumulation permits maintenance of normal cell volume and intracellular electrolyte concentration, despite hypertonicity. Accumulation of the organic osmolytes is relatively slow. For example, when PAP-HT25 cells are switched to high-NaCl medium, sorbitol accumulation takes days (10). A more gradual increase of osmolality might provide more time for cells to accumulate the compatible organic osmolytes. Another possibility is suggested by the observation that a step increase in tonicity induces mitochondrial dysfunction in mIMCD3 cells (19). When the osmolality of the medium bathing mIMCD3 cells is acutely increased in a single step from 300 to 700 mosmol/kgH_2O by addition of NaCl, mitochondrial potential difference decreases rapidly, NADH leaves the mitochondria, and the cellular ADP-to-ATP ratio increases, indicating rapid cessation of mitochondrial function. Then most cells die by apoptosis. A possible explanation for the dramatic effect of hypertonicity on mitochondria is that matrix volume shrinks acutely, resulting in increased molecular crowding, which slows diffusion of metabolites. In support of this possibility, acute hypertonicity reduces diffusion of a metabolite-sized fluorescent probe in mitochondria in vitro (11). Furthermore, high sucrose (6) or NaCl (30) decreases mitochondrial matrix volume, mitochondrial respiration, and oxidative phosphorylation. IVR might avoid this by allowing the mitochondria to equilibrate osmotically without a marked decrease in matrix volume.

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In summary, we found that a slow rate of increase of osmolality promotes survival of renal medullary epithelial cells, but we did not find evidence that other factors, such as ECM proteins (collagen I, collagen IV, fibronectin, laminin, or fibrillar collagen I), hormones (IGF-I, EGF, or dDAVP), and growth on porous supports, promote osmotic tolerance.

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