Urea Induces the Heat Shock Response in Human Neuroblastoma Cells

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ABSTRACT

Uremic encephalopathy is a complication of renal failure that reflects stresses exerted by as yet poorly defined uremic toxins. All cells respond to stresses by undergoing the “heat shock” response. Although urea kinetics and creatinine concentration are routinely used to assess dialysis adequacy, the roles of urea and creatinine as uremic toxins remain controversial. To investigate their potential roles in uremic encephalopathy, cultured human neuroblastoma cells (SK-N-SH) were exposed to 0.5 to 14 mg/dL creatinine, or to 20 to 200 mg/dL urea, or to mannitol, NaCl, or glycerol at equivalent osmolalities for 30 min to 48 h, and the induction of Hsp72 (heat shock) protein was used as a marker of cell stress. Although creatinine failed to elicit a heat shock response, urea, NaCl, or glycerol failed to exhibit this response. Protein extracts from cells exposed to urea showed significant carbamylation that increased as a function of time. These results demonstrate: (1) that urea is neurotoxic in vitro and that creatinine is not; (2) that the insult urea causes is not simply the result of hypertonicity; but rather (3) that urea, via breakdown to cyanate and ammonium ions, may cause cell stress because of its ability to cause carbamylation of cellular proteins. The cells' attenuation of the heat shock response after 10 h of exposure to urea suggests that they can adapt to the presence of urea or carbamylation. This may explain, in part, why the same degree of azotemia causes fewer neurological symptoms in patients with chronic as opposed to acute renal failure.

Key Words: Uremic encephalopathy, carbamylation, stress response, acute renal failure, hyperosmolality

Uremic encephalopathy is a common complication of severe acute and chronic renal failure, and is clinically characterized by difficulty with both motor and cognitive skills (1), muscle fatigue and cramping, involuntary movement of extremities (2), sleep disturbances (3–5), lethargy, irritability, and other emotional difficulties (2). As the uremic state worsens, the patient becomes disoriented, seizures and coma develop, and if the uremia is not treated, death results (2). The neurologic symptoms of acute uremia do not differ qualitatively from those of chronic uremia, however, patients who develop acute renal failure exhibit neurologic symptoms at lower azotemic levels, i.e., levels that cause few or no symptoms in patients with chronic renal failure (2,6). The reasons for this discrepancy are poorly understood.

Metabolic disturbances in the brain, secondary to uremia, have been noted. These include impaired ATP utilization, decreased glucose uptake and oxygen consumption, decreased Na-K-ATPase and Ca-ATPase activities, increased calcium content, altered brain cell permeability, abnormal amino acid content (such as increased levels of tryptophan) (7,8), and increased levels of the excitatory amino acids glutamate and aspartate (9). In addition, uremic patients exhibit major alterations of brain-wave activity (10). These manifestations suggest that the uremic environment is stressful to the brain, and in turn, should result in the induction of the heat shock response in affected brain cells.

The heat shock response is a means by which cells that have undergone some sort of damage are able to repair that damage, survive, and protect themselves from further damage (11,12). This response is generally characterized by a transient decrease in normal gene expression, accompanied by the vigorous production of a small set of evolutionarily conserved proteins known as heat shock proteins (11). The function of these proteins is to aid the cell in recovery from stressful events, and to protect the cell from future stresses (11). Once the cell has adapted to the presence of the stressor, or the stressful agent is removed, the heat shock response attenuates, as part of its normal regulation, and normal protein synthesis resumes (11). Previous results from ischemia models indicate that cells that survive and that are damaged because of ischemia have greatly increased synthesis of Hsp72, an inducible member of the 70-kd family of heat shock proteins (13–15). Likewise, brain cells that are damaged as a result of heat shock or physical injury exhibit markedly increased levels of heat shock proteins.
proteins (14). Thus, induction of the heat shock response is a suitable marker for a wide variety of cellular insults (14,16).

The causes of uremic encephalopathy are poorly understood, but are likely a result of the collective effects of various uremic toxins (6). Although urea kinetics and blood creatinine concentration are currently used as markers in the assessment of dialysis adequacy, the roles that these two substances themselves play as uremic toxins have been disputed (6). Early work by Grollman and Grollman found that bilaterally nephrectomized dogs that were treated by peritoneal dialysis for several days with a dialysate supplemented with urea rapidly became very ill and died. The BUN value of these dogs was maintained between 173 and 224 mg/dL (17). By contrast, earlier work by Bollman and Mann determined that a BUN concentration as high as 808 mg/dL could be maintained in dogs for weeks without any apparent ill effects (18). Similarly conflicting results have been obtained in experiments with humans (6).

Creatinine is a guanidino compound that, because of its accumulation in renal failure, has been suggested as a uremic toxin. Although it is the least potent of the guanidino compounds tested, it has been found to be a weak inhibitor of y-aminobutyric acid and glycine responses in mouse spinal cord neuronal cell cultures (19), and therefore, could potentially contribute to uremic encephalopathy.

Urea is a lipophilic endproduct of protein metabolism that serves to eliminate ammonia and carbonate. In molar concentrations, it is known to alter the structure of proteins. Although plasma urea levels in renal failure patients never reach such high concentrations, urea does exhibit other properties that might cause cellular stress.

Urea is known to break down in solution over time, and as a function of temperature, to ammonium and cyanate ions (20). This is a slow and reversible reaction whose equilibration takes place over a period of hours to days, depending on the concentration of urea and the temperature (20). Cyanate is a highly reactive species that readily, nonspecifically, and irreversibly carbamylates free amino groups. To a lesser extent, sulphydryl groups are reversibly carbamylated (21). It is well established that in patients with renal failure, the breakdown of urea to cyanate leads to the carbamylation of hemoglobin (22–26). More recent studies suggest that carbamylation of other proteins and peptides, such as insulin (27), LDL (28,29), and actin (30), as well as free amino acids (31,32), interferes with their activities or clearances, and may contribute to some of the pathology seen in renal failure patients. It is currently difficult to assess the extent to which proteins are carbamylated in vivo in renal failure patients (25,27), and most studies have indirectly assessed the effect either by carbamylating purified proteins of interest in vitro and assessing their activities (27,28, 30), or by purifying proteins of interest from renal failure patients and determining whether they exhibit the same properties as proteins that have been carbamylated in vitro (29). Because of such difficulties, the role, if any, that the breakdown of urea and subsequent carbamylation of proteins may play in uremia or uremic encephalopathy is unknown.

Because patients generally recover fully from uremic encephalopathy upon successful transplantation, it is apparent that uremic toxins cause neuronal dysfunction rather than extensive neuronal cell death. In other words, uremic toxins involved in uremic encephalopathy should be stressful, rather than lethal, to cells of the central nervous system. This property makes them difficult to identify or define by using classic methods. However, use of induction of the heat shock response as a molecular marker for cells that are adversely effected by a putative uremic toxin is ideally suited to the identification of such toxins because any cell or organism will undergo this response as a result of the presence of a stressful, rather than a lethal, agent.

This study was designed to further investigate the potential roles of urea and creatinine in the pathogenesis of uremic encephalopathy. Accordingly, we have performed dose-response and time-course studies in cultured neuronal cells exposed to varying concentrations of urea and creatinine, and assessed the heat shock response of treated cells by Western blot analysis of Hsp72 protein. The obtained response to urea was compared with that of equimolar concentrations of NaCl, mannitol, or glycerol. In addition, we determined the degree of protein carbamylation in cells that were exposed to urea.

METHODS

Cell Culture

SK-N-SH, human neuroblastoma cells (purchased from the American Type Culture Collection, Rockville, MD), were grown and passed in 25 cm2 flasks in 6 mL of modified Eagle's medium, pH 7.4 (MEM; Sigma Chemical, St. Louis, MO) supplemented with 10% newborn calf serum (MEM + NCS; Hyclone, Logan, UT) at 37°C (P<0.05; P<0.05). Cell Viability

Cell viability was determined by trypan blue exclusion as previously described (33). After 48 h of study, following 72 h of quiescence, control and test cells (those treated with urea, NaCl, mannitol, or glycerol) exhibited at least 90% cell viability.

Urea, NaCl, Mannitol, Glycerol, and Creatinine Experiments

Subconfluent cells (approximately 80% confluent) were made quiescent by replacing MEM + NCS with MEM and incubating the cells at 37°C for 72 h. A small volume (no greater than 240 µL) of a concentrated stock solution of sterile test substance (urea, mannitol, NaCl, glycerol, or creatinine), sufficient to raise the concentration to that described in this article's text and figure legends, was then added to each test flask. An equal volume of sterile water was added to the negative control flask for each experiment. The cells were then incubated in the presence of the test sub-
stance (or water) for the period of time indicated in the text and figure legends. Thereafter, cells were harvested by rinsing them twice with ice-cold phosphate-buffered saline (PBS) and scraping them into a small volume of PBS. The cells were then pelleted by centrifugation at 4°C and resuspended in 100 μL of ice-cold PBS. The number of cells in the samples was determined by hemocytometer and adjusted appropriately to ensure that extracts from similar numbers of cells (approximately 10^6) were loaded for Western blot analysis. An equal volume of 2X sample buffer (20% glycerol [vol/vol], 10% β-mercaptoethanol [vol/vol], 6% sodium dodecyl sulfate [wt/vol], 0.125 M Tris pH 6.8, 0.2% bromophenol blue [wt/vol]) was added to the cells, and the extracts were stored frozen at −20°C until ready for use. The test substance dose-response and time-course experiments were repeated three times.

The experiments outlined above were repeated in nonquiescent cells with no apparent differences found. The results shown in the figures are from experiments done on quiescent cells only.

Heat Shock Experiments

Cells were grown to subconfluence and made quiescent as described above. The medium was then removed from all flasks and replaced with medium that had been prewarmed either to 42°C (for test flasks) or to 37°C (for control flasks). The test flasks were then placed in a 42°C incubator for 1 h, while the control flasks were returned to the 37°C incubator. After 1 h, the 42°C flasks were transferred to the 37°C incubator and allowed to recover for the periods of time indicated in the text and figure legends. After this recovery period, the cells were harvested, counted, and stored at −20°C as described above. The control cells were harvested at the last time point for each experiment. This experiment was repeated three times.

The experiments outlined above were repeated in nonquiescent cells with no apparent differences found. The results shown in the figures are from experiments done on quiescent cells only.

Carbamylation of Cellular Proteins

Cells were grown to subconfluence and made quiescent as described above. (14C)-urea (specific activity of 4.4 mCi/mmol) was then added to the medium of the cells in sufficient quantity to raise the concentration of urea to 150 mg/dL. The cells were maintained at 37°C for 10, 24, and 48 h, at which times they were harvested. The extracts were examined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography by using Enzathen® (NEN DuPont, Boston, MA). As a control, no urea was added to one of the flasks, which was then maintained at 37°C for 48 h, harvested, and examined with the other samples as described above.

SDS-PAGE

Cells were lysed by boiling, and proteins were resolved by SDS-PAGE as previously described (34).

Western Blot Analysis

Western blot analysis was performed by using the procedures and reagents provided with the ECL Western blotting analysis system (Amersham, Arlington Heights, IL) and monoclonal antiserum against Hsp72 protein (Amersham) diluted 1:500. Each gel prepared for Western blot analysis was run in duplicate and Coomassie stained to compare protein content and ensure equal loading.

Osmolality Analysis

A 100-μl aliquot of medium was taken before addition of urea, mannitol, NaCl, or glycerol, immediately after addition of urea, mannitol, NaCl, or glycerol, and just before harvesting cells that had been exposed to urea, mannitol, NaCl, or glycerol. The osmolality of each sample was tested by using the procedures and reagents provided with the Wescor 5100C Vapor Pressure Osmometer (Logan, UT). A 200 mg/dL amount of urea was found to increase the osmolality of the solution by approximately 33 mosmol. This value was used for the concentrations of NaCl, mannitol, or glycerol to be added to the media. The osmolality of the media to which NaCl, mannitol, and glycerol were added also increased by approximately 33 mosmol, as expected.

Urea Concentration

Urea concentration was determined in all experiments involving urea. The level of urea was assessed by using the procedures and reagents provided with the BUN (endpoint) 20 kit (Sigma Chemical, St. Louis, MO). Urea concentrations increased as expected, and did not significantly decrease over time.

RESULTS

Heat Shock Response of SK-N-SH Cells

It was first determined whether the SK-N-SH cell line was able to undergo a heat shock response and produce Hsp72. To do so, the cells were exposed to a 1-h 42°C heat shock, allowed to recover for 1, 2, 3, and 4 h, and assessed by Western blot analysis for their ability to express Hsp72 protein as described in the Methods section. As shown in Figure 1, SK-N-SH cells do express Hsp72 in response to heat shock. This response is visible at a recovery time of 1 h, persists, and increases up to a recovery time of 4 h.

SK-N-SH Cells Response to Creatinine

To determine whether pathophysiologic concentrations of creatinine induce the heat shock response in SK-N-SH cells, sufficient quantities of creatinine were added to the media of quiescent cells to create final concentrations of 0.5, 1, 2, 3, 5, 10, and 14 mg/dL. The flasks that contained the cells were maintained in the 37°C incubator for 1, 5, 10, 24, 36, and 48 h, at which time the cells were harvested as described in the Methods section. Addition of 0.5 to 14 mg/dL creatinine resulted in no induction of the heat shock response up to 72 h after addition as assessed by Hsp72 expression (results not shown).

SK-N-SH Cell Response to Urea

To determine whether urea induces the heat shock response in SK-N-SH cells, final concentrations of 20, 40, 60, 100, 150, and 200 mg/dL were created in the media of quiescent cells. These concentrations correspond to urea nitrogen concentrations of 9.5, 19.1, 28.6, 47.6, 71.4, and 95.2 mg/dL, respectively. The
flasks that contained the cells were maintained in the 37°C incubator for 5 h, at which time the cells were harvested as described in the Methods section. As can be seen in Figure 2A, 20 mg/dL urea was insufficient to cause induction of the heat shock response. However, concentrations of 40 to 200 mg/dL resulted in production of Hsp72 protein. Scanning densitometry was performed on this blots, and the relative concentrations of Hsp72 protein (expressed as a multiple of the amount found in the 40 mg/dL lane) are shown in Figure 2B. As indicated in the figure, the intensity of expression of Hsp72 increases by approximately 1.7-fold up to 150 mg/dL of urea and then levels off.

**Time Course of Heat Shock Response to Urea**

To determine the length of duration and pattern of the heat shock response to urea, 200 mg/dL urea were added to quiescent test cells. These cells were then maintained in the 37°C incubator for 30 min, 1, 5, 10, 24, 33, and 48 h, after which times the cells were harvested and assessed for expression of Hsp72. An equivalent volume of sterile water was added to the control cells, and they were harvested at the 1-h time point. Control cells were also harvested at the 48-h time point and found not to have induction of Hsp72 protein (results not shown). From Figure 3A, it can be seen that Hsp72 is present 30 min after addition of urea, increases and peaks in intensity up to 10 h, and gradually declines and is gone by 48 h after addition of urea. Scanning densitometry (Figure 3B) shows the

fold increase in Hsp72 expression above the 30-min value. By 1 h, it has increased by approximately 1.2-fold over the 30-min value; by 5 h, approximately 4.9-fold; by 10 h, approximately 9.8-fold. By 24 h, the response has diminished to approximately 5.7-fold; by 33 h, it is only 1.8-fold increased; and by 48 h, no response is detectable.

**Heat Shock Response to Mannitol, NaCl, or Glycerol**

A 200 mg/dL amount of urea raised the osmolality of the medium by approximately 33 mosmol. To determine whether this increase in osmolality contributed to urea's induction of the heat shock response, a concentration of mannitol, NaCl, or glycerol sufficient to raise the osmolality by an equivalent amount was
Figure 3. Time course of the heat shock response to urea in SK-N-SH cells. Cells were treated as described in the Methods section and in the text with 150 mg/dL urea for 30 min to 48 h (Lanes 2 through 8). (A) Western blot of the time course. Lane 1: no urea; Lane 2: 30 min; Lane 3: 1 h; Lane 4: 5 h; Lane 5: 10 h; Lane 6: 24 h; Lane 7: 33 h; Lane 8: 48 h. (B) Results from scanning densitometry of the Western blot.

Figure 4. Time courses for induction of Hsp72 by mannitol and NaCl. Cells were treated as described in the Methods section and in the text with 33 mosmol mannitol (top) or NaCl (bottom) for 1 to 48 h. (Top) Lane 1: 1 h; Lane 2: 5 h; Lane 3: 10 h; Lane 4: 24 h; Lane 5: 33 h; Lane 6: 48 h; Lane 7: no mannitol; Lane 8: 1 h heat shock followed by a 2-h recovery. (Bottom) Lane 1: 1 h; Lane 2: 5 h; Lane 3: 10 h; Lane 4: 24 h; Lane 5: 33 h; Lane 6: 48 h; Lane 7: no NaCl; Lane 8: 1 h heat shock followed by a 3-h recovery.

As with mannitol, addition of 33 mosmol of glycerol resulted in no induction of the heat shock response as evaluated by Hsp72 expression (results not shown).

Carbamylation of Cellular Proteins

In each of the experiments involving urea, the concentration of urea in the medium was determined as described in the Methods section. Although there was no detectable decrease in urea concentration found over the time period studied, the possibility still existed that very small quantities of urea were breaking down to cyanate and ammonium ions. This in turn could lead to carbamylation of cellular proteins, and thus might contribute to induction of the heat shock response by urea. To test this possibility, 150 mg/dL [14C]-urea was added to the medium of quiescent cells. The cells were then maintained at 37°C for 10, 24, and 48 h, at which times they were harvested and their protein contents examined by SDS-PAGE and fluorography as described in the Methods section. If the added urea were breaking down to cyanate, and if the quantity of cyanate created were able to carbamylate cellular proteins, this would be manifest as generalized radiolabeling of cellular proteins. If urea were not breaking down significantly under the conditions described, then there would be no such labeling, as urea is a nonreactive species.

The results of this experiment clearly demonstrate that radiolabeling of cellular proteins occurs in a generalized fashion, is present 10 h after addition of [14C]-urea, and increases as a function of time (Figure 5).
Urea is Stressful to Neuronal Cells

Figure 5. Time course for carbamylation of cellular proteins through breakdown of urea to cyanate. Cells were treated as described in the Methods section and in the text with 150 mg/dL (14C)-urea. (A) Coomassie-stained SDS-PAGE of cellular extracts, Cells were exposed for 10 h (Lane 3), 24 h (Lane 2), or 48 h (Lane 1) to urea, or left untreated for 48 h (Lane 4). (B) Fluorography of the gel in (A).

DISCUSSION

The observation that urea can elicit the heat shock response at 40 to 200 mg/dL in SK-N-SH cells without decreasing cell viability (see “Methods”) is important because it indicates that an increase in urea concentration to clinically relevant levels is stressful to neuronal cells in vitro. Although it is possible that such results may be unique to the neuroblastoma cell line studied here and may prove not to be seen in vivo, the results do correlate to some extent with the development of uremic symptoms seen in renal failure patients, suggesting that they can be generalized to the in vivo situation. A BUN level of 40 mg/dL in patients constitutes mild azotemia. A BUN level of 100 mg/dL or above generally correlates with the appearance of uremic symptoms, often warranting initiation of dialysis therapy. Our in vitro finding that the addition of 40 mg/dL of urea (equivalent to a BUN of 19.1 mg/dL) begins to induce the heat shock response, and that this response gradually increases as urea concentrations are raised to 100 mg/dL (equivalent to a BUN of 47.6 mg/dL), remaining relatively constant from 100 to 200 mg/dL (equivalent to a BUN of 47.6 to 95.2 mg/dL), parallel to some extent the clinical development of the uremic symptoms. Although clinically, a BUN of 19.1 mg/dL is normal, a patient who suddenly develops renal failure would experience an increase in blood urea concentration above the baseline to which he is normally accustomed, e.g., an increase in BUN from approximately 20 mg/dL. In the tissue culture system, this baseline is 0 rather than 20 mg/dL. Therefore, a rise in urea concentration from 0 to 40 mg/dL (equivalent to a BUN of 19.1 mg/dL) in this system may constitute significant “azotemia.”

The attenuation of the heat shock response after a 10-h exposure to urea indicates that neuronal cells may be able to adapt to the presence of urea. This adaptation further supports the idea that it is an increase in BUN level over that to which the patient (or cells) has become accustomed that produces stress, rather than a particular concentration of urea. Such an adaptation could be one means by which chronic renal failure patients acquire a heightened tolerance to severe degrees of azotemia. Together with the results from the dose-response experiment, these results support the notion that urea is a uremic toxin and that it contributes to the pathophysiology of acute uremic encephalopathy. The fact that a creatinine concentration up to 14 mg/dL fails to elicit a heat shock response indicates that, unlike urea, this uremic retention product is not stressful to neuronal cells in pathophysiologic concentrations, and is therefore not likely a uremic neurotoxin.

The lack of heat shock response elicited by mannitol or glycerol, and the very weak and different pattern of response seen with NaCl, indicates that urea does not induce the heat shock response simply by increasing cellular osmolality. Although osmolality may partially contribute to some of the stress, much of the stress must be the result of some unique property of urea. Our results concerning such different patterns of heat shock protein expression from exposure to NaCl versus mannitol and glycerol are consistent with results from other laboratories. Work by Cohen et al. has shown that Hsp70 expression is increased by exposure to high levels of NaCl (515 mosmol) in MDCK cells (35). By contrast, 300 mosmol of mannitol and 200 mosmol of glycerol were found not to induce Hsp70 expression in these cells (35,36).

As osmotically active agents, urea, mannitol, glycerol, and NaCl will produce, in quantitative terms, comparable increases in cell osmolality. Thus, the differences seen in the degree and pattern of heat shock response in neuronal cells, elicited by the four substances, must reflect differences in the cells’ responses to them and differences in their respective properties. Whereas NaCl and mannitol differ from urea in their abilities to enter the cell, glycerol is similar in that it freely penetrates the cell membrane. Thus it is not likely that urea’s ability to enter the cell causes the cell stress.

One reason for the neuronal cells’ prolonged heat shock response to urea compared with NaCl could be a consequence of the breakdown of urea to cyanate and subsequent carbamylation of cellular proteins. The results presented here demonstrate that clinically relevant concentrations of urea do break down over a short period of time, and that this Indeed leads to carbamylation of proteins. Such carbamylation should have profound consequences on protein folding and/or function by creating alterations in charge and bulkier side chains. Stressors that lead to improper protein folding are classic inducers of the heat shock response (12). Thus the prolonged heat shock response seen for urea could be accounted for in the following manner. One could envision a situation...
where urea itself, in part through hyperosmolality, initiates a heat shock response, and where later, when the cells may have recovered or adapted to the presence of urea, urea breaks down to cyanate. Cyanate itself, or carbamylation of cellular proteins, in turn, could cause an additional stress by interfering with protein folding or function, thus prolonging the induction of the heat shock response. Because the heat shock response has subsided by 48 h, there must be further cellular response mechanisms that reduce the sensitivity to urea and protein carbamylation. For example, changes in the production of organic osmolytes, which is known to occur in the brains of uremic animals (Westenfelder C, Maddock AL, Baranowski RL: The effect of acute and chronic uremia on brain organic osmolytes. Kidney Int, manuscript in preparation) (37), may enable cells to adapt to the presence of urea.

In vivo, the liver converts cyanate to thio cyanate, a less toxic substance that is excreted by the kidneys. Because tissue-cultured neuronal cells are incapable of carrying out this reaction, it is possible that carbamylation of cellular proteins may not occur as readily in renal failure patients as seen in vitro. However, it is clear that carbamylation of hemoglobin (22–26) and free amino acids does occur extensively in renal failure patients (32), indicating that the hepatic conversion of cyanate to thio cyanate is insufficient to prevent carbamylation in renal failure. Kraus and Kraus have found that essential free amino acids are extensively carbamylated in chronic renal failure patients with BUN levels of approximately 60 mg/dL (32).

In summary, these studies have determined that the presence of clinically relevant concentrations of urea induce an intense prolonged heat shock response in cultured neuroblastoma cells, indicating that urea is stressful to neuronal cells and suggesting that, unlike creatinine, it is an acute uremic toxin involved in the pathogenesis of uremic encephalopathy. This stress cannot be simply the result of hypertonicity or its ability to enter the cell because equivalent osmolalities of NaCl, mannitol, or glycerol elicit a weak and transient or no heat shock response, respectively. Instead, it may well be because of urea’s ability to generate cyanate, which in turn, leads to carbamylation of cellular proteins. The fact that the heat shock response does eventually disappear indicates that neuronal cells are able to adapt to the presence of urea (and/or cyanate and carbamylated proteins) independently of the heat shock response. Such adaptation (mediated either through the heat shock response and/or some other as yet underdetermined system) may be one means by which chronic renal failure patients acquire a heightened tolerance to severe degrees of azotemia.

ACKNOWLEDGMENTS

This work was supported by grants from the Dialysis Research Foundation, Ogden, Utah, to A. Maddock and C. Westenfelder, as well as by Department of Veterans Affairs Medical Research Funds to C. Westenfelder. The technical assistance of Dr. R.L. Baranowski is gratefully acknowledged.

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