Acute hypertonicity causes cell cycle delay and apoptosis in mouse renal inner medullary collecting duct cells (mIMCD3) and increases GADD45 expression. Because the tumor suppressor protein p53 may be involved in these effects, we have investigated the role of p53 in mIMCD3 response to hyperosmotic stress. Acute elevation of osmolality with NaCl addition from the control level of 320 mosmol/kg to 500–600 mosmol/kg greatly increased the levels of total and Ser15-phosphorylated p53 within 15 min. However, similar elevation of osmolality with urea did not increase p53 levels. Our studies indicate that induced p53 is transcriptionally active because NaCl addition to 500–600 mosmol/kg stimulated transcription of a luciferase reporter containing a p53 consensus element and appropriately altered mRNA levels of known transcriptional targets of p53, i.e. increased MDM-2 and decreased BCL-2 levels. Elevating NaCl further to 700–800 mosmol/kg rapidly killed most of the cells by apoptosis. At these higher NaCl concentrations, p53 levels were further increased although Ser15 phosphorylation and transcriptional activity were significantly lower than at levels of 500–600 mosmol/kg. At NaCl-induced 500 mosmol/kg, apoptosis was rare in the presence of control, nonspecific oligonucleotide but highly prevalent upon addition of p53 antisense oligonucleotide that substantially reduced p53 levels. We conclude that induction of active p53 in mIMCD3 cells by hypertonic stress contributes to cell survival.

p53 is a tumor suppressor whose loss of function, observed in many types of cancer, contributes to genomic instability and malignancy (1–3). Numerous stresses cause increases in p53 activity, which results in either arrest of cell growth until damage is repaired or apoptosis, eliminating cells that are potentially dangerous to the organism. p53 is induced when DNA damage is caused by cytotoxic drugs, free radical formation, or ionizing radiation (4). p53 can also be induced in the absence of observed DNA damage by growth factor withdrawal, hypoxia, metabolic change, virus infection, cytokines, or deregulated expression of cell cycle genes (4).

High concentrations of solutes like NaCl or urea induce apoptosis in numerous types of cells including thymocytes (5), SH-SY5Y human neuroblastoma cells (6), DT40 chicken B cells (7), and murine renal inner medullary collecting duct cells (mIMCD3) (8, 9). Furthermore, raising medium levels of NaCl arrests growth of mIMCD3 (8–10) and increases levels of GADD45 (10), a growth arrest and DNA damage-inducible protein whose transcription is regulated by p53 (11). These results suggest that p53 might be induced by osmotic stress in these cells, an hypothesis supported by reports of nuclear accumulation of p53 in primary human skin fibroblasts in response to high NaCl levels (12).

The present studies were performed to determine what role p53 might have in the response of mIMCD3 cells to osmotic stress. We discovered that increasing medium NaCl to a total osmolality of 500 mosmol/kg increased the level of p53 and that this p53 is both phosphorylated on Ser15 and transcriptionally active. Our studies also indicate that the increase in p53 activity protects mIMCD3 cells, because when p53 activation was blocked by a specific p53 antisense oligonucleotide, a marked increase in apoptosis was observed.

**EXPERIMENTAL PROCEDURES**

**Cell Culture**—Subconfluent cultures of mIMCD3 cells (13) (generously provided by S. Gullans) were used in passages 13–17. The medium contained 5% Dulbecco’s modified Eagle’s medium, low glucose, 45% Coon’s improved medium (Irving Scientific), and 10% fetal bovine serum (Life Technologies, Inc.). Osmolality of control (designated “isosmotic” or “isotonic”) medium, was 320 mosmol/kg. Hyperosmotic medium, which was prepared by adding NaCl or urea, was substituted for the control medium as indicated. Cells were incubated at 37 °C and grown in 5% CO2, 95% air during all experiments.

**Protein Sample Preparation, Western Blotting, and Immunodetection**—Cells were rinsed with phosphate-buffered saline, adjusted with NaCl and/or urea to the same osmolality as the medium, and then lysed with 300 μl of lysis buffer (100 mM NaF, 50 mM Tris, 250 μM thimerosal, 1% Igepal (ν/ν), 16 mM Chaps, 5 mM activated NaVO4, 50 mg/liter Pefabloc, 100 mg/liter leupeptin, and 10 mg/liter aprotinin). Cell lysates were scraped and then homogenized with 10 strokes in Teflon/glass homogenizers (Wheaton) that were chilled in ice water. The combination of lysis buffer with physical homogenization was used to disrupt all membranes. After centrifugation for 20 min at 15,000 × g and 4 °C, the supernatant was aliquoted and stored at −80 °C. Protein content was measured using the BCA protein assay (Fierce). Proteins were separated by SDS-polyacrylamide gel electrophoresis. Equal amounts of protein (6 μg) were loaded onto each lane of 12% acrylamide/Tris/glycine gels, and electrophoresis was performed at 125 V constant voltage. Proteins were blotted onto Immobilon P membranes (Millipore Corp.) at 1 mA/cm2 constant current for 90 min. Immunodetection procedures were carried out using specific antibodies against p53 (Roche Molecular Biochemicals) or phospho-p53 (Ser15) (New England Biolabs). To measure p53 protein levels in immunoblots for p53 antisense experiments, cells grown in two-chamber plastic slides (Nalge Nune International) were lysed with 50 μl of lysis buffer, and 3 μg of protein were loaded onto each lane.

**Cell Fixation and Propidium Iodide (PI) Staining**—To detect apo...
ptosis in the antisense oligonucleotide experiments, cells grown on eight-chamber plastic slides (Nalge Nunc International) were fixed in 100% methanol at 220 °C for 15 min. After fixation, the cells were permeabilized with 0.1% Triton X-100, incubated with 1 mg/ml RNase (Sigma) for 15 min, stained with 10 mg/ml PI for 5 min, and then mounted with 200 µl of Antifade (Molecular Probes, Inc.).

Transfection and Luciferase Assays—p53 transcriptional activity was determined by transfecting with a reporter construct containing a p53 response element (basic TATA element and tandem repeats of the p53 enhancer element (TGCCTGGACTTGCCTGG) upstream of the Photinus pyralis (firefly) luciferase gene (p53-Luc, Stratagene). To control for transfection efficiency, cells were cotransfected with a constitutively active plasmid, pRL-B19 (14), containing a B19 parvovirus promoter subcloned upstream of the Renilla luciferase gene in the p53 Role in Response to Hypertonicity

Fig. 1. Effect of acute addition of NaCl to mIMCD3 cells. Cells grown in isosmotic medium were changed at time 0 to media made hypertonic by addition of NaCl to a total osmolality of 500, 600, or 700 mosmol/kg. The same area on a culture dish was photographed immediately before changing the medium (zero time) and at 6 and 24 h later.

Fig. 3. p53 (total or Ser15-phosphorylated) levels in mIMCD3 cells as a function of NaCl or urea concentration. Cells were exposed for 1 h to isotonic medium (320 mosmol/kg) or to medium to which NaCl or urea was added to different osmolalities (500, 600, 700, and 800 mosmol/kg). A, representative Western blot; B, densitometric analysis of total (○) or phosphorylated (●) p53 in NaCl-treated cells. Data are normalized to maximal densitometric values obtained for total or phosphorylated p53, respectively. Bars indicate ± S.E.; *, p < 0.05; n = 6.

Fig. 4. Effect of NaCl on p53 transcriptional activity. mIMCD3 cells were cotransfected with reporter constructs containing a p53 consensus DNA element upstream of firefly luciferase together with a constitutively active reporter expressing Renilla luciferase to normalize for transfection efficiency. Transfected cells were maintained in isosmotic medium (320 mosmol/kg) or exposed to medium made hypertonic by addition of NaCl (500, 600, or 700 mosmol/kg) for 6 h. Data are plotted as the ratio of firefly luciferase activity to Renilla luciferase activity, normalizing to that in isosmotic medium. Bars indicate mean ± S.E.; *, p < 0.01; n = 9.
promoterless pRL-null vector (Promega). mIMCD3 cells were grown in isosmotic medium in 10-cm dishes (Corning Inc.) and cotransfected with p53-Luc and pRL-B19 using a CellPhect transfection kit (Amersham Pharmacia Biotech). Transfected cells were seeded onto 35-mm dishes overnight. Then the medium was changed to the same isosmotic medium or to medium made hypertonic to 500, 600, or 700 mosmol/kg with NaCl. Cells were lysed with passive lysis buffer (dual luciferase reporter assay system, Promega). Lysates were analyzed for total protein using the BCA protein assay and for firefly and Renilla luciferase activities using the dual luciferase reporter assay system with a Monolight 2010C luminometer (Analytical Luminescence Laboratory). P. pyralis luciferase activity was normalized by Renilla luciferase activity to adjust for transcription efficiency, and all values were further normalized to the ratio in isosmotic medium. Statistical analyses were performed by the analysis of variance Friedman nonparametric repeated test followed by Dunn’s multiple comparison test.

RNA Isolation and Ribonuclease Protection Assay—mIMCD3 cells were grown to 40% confluence in isosmotic medium in 10-cm dishes (Corning Inc.) and cotransfected with p53 antisense oligonucleotide (for p53 antisense sequences see “Antisense Oligonucleotide Experiments—Antisense oligonucleotides and controls for regulating p53 expression have been designed and manufactured by Biognostik, Germany. Cells were grown on eight-chamber plastic slides. In experiments aimed at establishing the time course of oligonucleotide uptake by mIMCD3 cells, fluorescein-labeled randomized sequence phosphorothioate oligonucleotide (FITC-Control, Biognostik, Germany) was added for 0–48 h, followed by fixation in 100% methanol. Based on that result, cells were preincubated for 16 h with p53 antisense phosphorothioate oligonucleotide (GCTCAGTGCCTGAC) or the control, CG-matched randomized sequence phosphorothioate oligonucleotide (GACTGACGCTACGTG). Medium was changed to iso- or hypertonic containing the same oligonucleotides. After 6 h, cells were fixed with 100% methanol and stained with PI.

Laser Scanning Cytometry—Apoptosis was detected using a laser scanning cytometer (Compucyte Corp.) to measure total PI fluorescence and the peak intensity of PI fluorescence in cell nuclei. The data were displayed as bivariate cytograms, plotting peak PI fluorescence versus total PI fluorescence in each nucleus or particle. A gate representing the approximate limit of peak fluorescence in cells to which no oligonucleotide was added was set by eye. Peak fluorescence exceeding this limit identified chromatin condensation associated with apoptosis. To confirm apoptosis, representative cells in the different areas of the cytograms were located on slides using spatial coordinates recorded by the laser scanning cytometer, and morphology was recorded microscopically. High peak fluorescence corresponded to nuclei that were bright and shrunken, consistent with the hypercondensation of chromatin that occurs relatively early in apoptosis. Statistical analyses were performed by the analysis of variance Friedman nonparametric repeated test followed by Dunn’s multiple comparison test.

RESULTS

The increase of medium NaCl to a total osmolality of 600 mosmol/kg stopped proliferation of mIMCD3, but after approximately 18 h, cells resumed growth (10) (Fig. 1). At substantially higher levels of NaCl many of the cells died, and growth was suppressed (8, 9) (Fig. 1, 700, 24 h). Thus, we confirmed...
that mIMCD3 cells can survive acute increases in NaCl up to approximately 600 mosmol/kg but not to 700 mosmol/kg.

After acute elevation of NaCl to 600 mosmol/kg, there was a large increase in total and Ser15-phosphorylated p53 levels (Fig. 2, A and C). The initial increase in p53 levels following NaCl addition occurred within 15 min and persisted with some fluctuation for at least 24 h (Fig. 2, A and C). The NaCl-induced concentration-dependent increase in total p53 levels differed significantly from that of Ser15-phosphorylated p53 (Fig. 3). One hour after NaCl was added, p53 levels increased progressively between 400 and 700 mosmol/kg. In contrast, Ser15-phosphorylated p53 peaked at 600 mosmol/kg and decreased substantially at higher levels of NaCl (Fig. 3). Addition of urea to 600 mosmol/kg by adding NaCl at 500 or 600 mosmol/kg but not at 700 mosmol/kg. Moreover, addition of urea to 600 mosmol/kg reproducibly decreased p53 levels (Fig. 2B).

Because many of the actions of p53 depend on its activity as a transcription factor that binds to specific DNA elements on its target genes (17), we tested the effect of high NaCl on p53 transcriptional activity in mIMCD3 cells. We transiently transfected the mIMCD3 cells with a plasmid containing a p53 response element in a synthetic promoter driving a luciferase reporter gene. p53 transcriptional activity increased with added NaCl at 500 or 600 mosmol/kg but not at 700 mosmol/kg (Fig. 4).

We also tested the effect of high NaCl on mRNA levels of genes that are transcriptionally regulated by p53, namely MDM-2 (17), BCL-2 (18), and BAX-1 (17) (Fig. 5). Increasing NaCl to a total osmolality of 500–600 mosmol/kg substantially increases MDM-2 mRNA whereas it decreases BCL-2 mRNA, consistent with previous observations on the effect of p53 on transcription of these genes. In contrast, levels of BCL-x and BAX-1 mRNA were not significantly affected by NaCl addition.

Thus, the survival of mIMCD3 cells after an increase of NaCl to a total of 500–600 mosmol/kg correlated with increases in p53 phosphorylation on Ser15 and p53 transactivation. However, a large proportion of these cells did not survive when NaCl was added to a higher level (700 mosmol/kg), even though a further increase in total p53 was observed. The lower survival rate correlated with a lower degree of p53 phosphorylation on Ser15 and p53 transactivation as measured by luciferase assay. These results raised the possibility that the increased p53 activity at 500–600 mosmol/kg might contribute to the survival of the cells. To test this directly, we blocked the increase in p53 by adding p53 antisense oligonucleotides. To characterize oligonucleotide uptake by the mIMCD3 cells, fluorescein-labeled randomized sequence phosphorothioate oligonucleotide (FITC-oligonucleotide) was used. The fluorescein signal increased within 6 h and maintained a high level for at least 24 h (Fig. 6, A and B). On the basis of this experiment, the preincubation time for p53 antisense oligonucleotide was chosen, and its effect on p53 protein level was checked to test for persistence and function of the intact oligonucleotide in the cells. Incubation with p53 antisense oligonucleotide for 16 h greatly diminished p53 expression under both iso- and hyperosmotic conditions (Fig. 6C).

Laser scanning cytometry was used to determine the effect of p53 antisense oligonucleotide on cell survival. When osmolality was increased to 500 mosmol/kg with NaCl, p53 antisense oligonucleotide substantially reduced cell number (Fig. 7C) and correspondingly increased the proportion of the cells that displayed high peak intensity of PI staining within their nuclei (Fig. 7A). The increase in PI fluorescence integral (DNA content) was increased to 500 mosmol/kg with NaCl, p53 antisense oligonucleotide substantially reduced cell number (Fig. 7C) and correspondingly increased the proportion of the cells that displayed high peak intensity of PI staining within their nuclei (Fig. 7A, B, and D). Comparing with controls not exposed to oligonucleotides or exposed to the nonspecific oligonucleotide. Representative cells from the areas indicated by arrows in the cytograms in Fig. 7A were located on the original slides and examined microscopically. With NaCl added to 500 mosmol/kg, cells treated with control, nonspecific oligonucleotide had normal nuclear morphology (Fig. 7B). However, cells treated with p53 antisense oligonucleotide were clumped and nuclei were condensed, characteristic of apoptosis.
DISCUSSION

The level of p53 rises rapidly within minutes after acute hypertonic stress caused by increasing the concentration of NaCl in mIMCD3 cell medium. Rapid increases in p53 were previously noted to occur in response to a variety of other stresses such as DNA damage, arrest of DNA or RNA synthesis, and nucleotide depletion (reviewed in Refs. 2 and 3). The rapid rise in p53 levels generally occurs because of an increase in its half-life, i.e. from 5–20 min in untreated cells to about 150 min in UV-treated cells (19). MDM-2 is a major intracellular regulator of p53 stability. It binds to the transcriptional activation domain of p53 and targets p53 for rapid ubiquitin-mediated proteosomal degradation, accounting for the normally short half-life of p53. When MDM-2 binding to p53 is prevented, the intracellular levels of p53 increase.

The increase in total p53 level with hypertonicity was accompanied by phosphorylation on Ser 15. A number of kinases have been implicated in phosphorylation of Ser 15 in vitro, including DNA-PK (DNA-dependent protein kinase), ATM (ataxia telangietasia, mutated) and ATR (ataxia telangiectasia and rad3-related kinase) (1, 3, 20, 21). Presently, we do not know which kinase is involved in Ser 15 phosphorylation under hypertonic conditions. Nevertheless, the phosphorylation of Ser 15 has multiple effects including inhibiting MDM-2 binding, which increases p53 levels by reducing its rate of degradation (3). Ser 15 phosphorylation also increases transcriptional activity of p53 (3). As is shown in this study and others (22), increased p53 transactivation concerns the mdm-2 gene itself, which in turn results in increased levels of MDM-2 and a negative feedback loop for time-dependent regulation of p53 activity during stress. On the other hand, p53 transrepression of the bcl-2 gene has been reported for other stresses (23), a finding that is consistent with its transrepression during osmotic stress (Fig. 5). Interestingly, transcriptional activity and phosphorylation of p53 increase upon adding NaCl only up to approximately 600 mosmol/kg. At higher levels of NaCl, both phosphorylation and transcriptional activity fall dramatically even though there is a further increase in p53 levels.

p53 acts as a tumor suppressor either by signaling cell cycle arrest during repair of stress-induced damage or by causing apoptosis, which eliminates highly damaged cells (1–3). Thus, activation of p53 can either enhance or reduce survival of stressed cells. In the present studies, the phosphorylation of p53 and its transcriptional activation correlate with cell survival at 500–600 mosmol/kg, which suggests a protective role for p53. This hypothesis is supported by the observation that there is a large increase in apoptosis (Figs. 6 and 7) when, at 500 mosmol/kg, the rise in p53 is prevented with p53 antisense oligonucleotide. The mechanism by which increased p53 activity protects these cells after acute hypertonicity is uncertain. One possibility is induction of growth arrest. When NaCl is acutely increased to a total osmolality of 500–600 mosmol/kg, the mIMCD3 cell cycle is greatly slowed down (8, 9). This is also observed following p53 activation by other stresses and provides time for the repair of stress-induced damage (2, 24).

The rapid activation of p53 following acute but survivable hypertonicity may protect these cells during an initial period of vulnerability while longer acting protective systems are being activated. Renal medullary as well as other cells respond to hypertonicity over the long term by accumulating compatible organic osmoles (25, 26). This normalizes both cell volume, which is initially reduced with hypertonicity, and intracellular inorganic ion concentration, which is initially increased. However, it takes many hours to synthesize the transporters and enzymes that are responsible for accumulating the organic osmoles. The rapid activation of p53 could help protect the cells during this interval. The same role has been proposed for the increase in HSP 70 that also occurs in the early stages of acute hypertonic stress (27).

The mIMCD3 cells used in the present studies are immortalized by constitutive expression of SV40 (13). Immortalization by SV40 is known to involve its binding to p53 (28), which raises the question of possible influence of SV40 on the induction of p53 by hypertonicity. However, nuclear accumulation of p53 was also previously noted in human primary skin fibroblasts with addition of NaCl to 500–600 mosmol/kg (12), suggesting that SV40 expression is not required for hypertonic induction of p53.

An acute increase in the concentration of urea causes cell cycle delay and apoptosis to approximately the same extent as NaCl at the same osmolality (8, 9). Despite this, numerous differences exist in the cellular responses of high NaCl- and urea-treated cells (29). For example, high urea but not NaCl activates transcription of immediate-early genes via an extracellular signal-regulated kinase/Elk1-dependent pathway. Urea also activates multiple effectors characteristic of a tyrosine kinase-like signaling cascade. High NaCl, in contrast to urea, activates transcription of numerous toxicity-responsive and heat shock genes. Our finding that high NaCl, but not urea, activates p53 provides another distinction. Thus, despite similar effects of high NaCl and urea on the cell cycle and apoptosis, the two molecules may utilize different signaling pathways.

REFERENCES

1. Oren, M. (1999) J. Biol. Chem. 274, 36051–36054
2. Agarwal, M. L., Taylor, W. R., Chernov, M. V., Chernova, O. B., and Stark, G. R. (1998) J. Biol. Chem. 273, 1–4
3. Giaretta, A. J., and Kastan, M. B. (1998) Genes Dev. 12, 2973–2983
4. Meek, D. W. (1997) Pathol. Biol. 45, 804–814
5. Bortner, C. D., and Cidlowski, J. A. (1996) Am. J. Physiol. 271, C950–C961
6. Matthews, C. C., and Feldman, E. L. (1996) J. Cell. Physiol. 166, 323–331
7. Qin, S., Minami, Y., Kurosaki, T., and Yamamura, H. (1997) J. Biol. Chem. 272, 17994–17999
8. Santos, B. C., Chevaile, A., Hebert, M. J., Zagajeski, J., and Gallus, S. R. (1998) Am. J. Physiol. 274, F167–F173
9. Miech, L., Ferguson, D. R., Peters, E. M., Andrews, P. M., Kirby, M. R., and Burg, M. B. (2000) Am. J. Physiol. 278, F209–F218
10. Kultz, D., Madhany, S., and Burg, M. B. (1998) J. Biol. Chem. 273, 13645–13651
11. Zhan, Q., Chen, I. T., Antinore, M. J., and Fornace, A. J., Jr. (1998) Mol. Cell. Biol. 18, 2768–2778
12. Sugano, T., Nitta, M., Ohmori, H., and Yamazumi, M. (1995) Jpn. J. Cancer Res. 86, 415–418
13. Rauchman, M. I., Nigam, S. K., Delpierre, E., and Gallus, S. R. (1993) Am. J. Physiol. 265, F416–F424
14. Farber, J. D., Williams, C. K., Jung, K.-Y., Bedford, J. J., Burg, M. B., and Garcia-Perez, A. (1996) J. Biol. Chem. 271, 18318–18321
15. Furuya, T., Kamada, T., Murakami, T., Kurose, A., and Sasaki, K. (1997) Cytometry 29, 173–177
16. Bedner, E., Li, X., Gorecyza, W., Melamed, M. R., and Durrnynkiewicz, Z. (1999) Cytometry 35, 181–195
17. Levine, A. J. (1997) Cell 88, 323–331
18. Miyashita, T., Harigai, M., Hanada, M., and Reed, J. C. (1994) Cancer Res. 54, 3131–3135
19. Maltzman, W., and Czyzyk, L. (1984) Mol. Cell. Biol. 4, 1689–1694
20. Banin, S., Moyal, L., Shieh, S., Taya, Y., Anderson, C. W., Chessa, L., Smorodinsky, N. I., Priores, C., Reiss, Y., Shilois, Y., and Ziv, Y. (1998) Science 281, 1674–1677
21. Canman, C. E., Lim, D. S., Cimprich, K. A., Taya, Y., Tanai, K., Sakaguchi, K., Appella, E., Kastan, M. B., and Siliciano, J. D. (1998) Science 281, 1677–1679
22. Barak, Y., Lupo, A., Zauberman, A., Juen, T., Aloni-Grinstein, R., Gottlieb, E., Rotter, V., and Oren, M. (1994) Cold Spring Harbor Symp. Quant. Biol. 59, 225–235
23. Budhamahadeo, V., Morris, P. J., Smith, M. D., Midgley, C. A., Boxer, L. M., and Latchman, D. S. (1999) J. Biol. Chem. 274, 15237–15244
24. Taylor, W. R., Agarwal, M. L., Agarwal, A., Stacey, D. W., and Stark, G. R. (1999) Oncogene 18, 283–285
25. Yancey, P. H., Clark, M. E., Hand, S. C., Bowles, R. D., and Somero, G. N. (1982) Science 217, 1214–1222
26. Garcia-Perez, A., and Burg, M. B. (1991) Physiol. Rev. 71, 1081–1115
27. Cohen, D. M., Wasserman, J. C., and Gallus, S. R. (1991) Am. J. Physiol. 261, C594–C601
28. Kientz, T. D., and Tsvetich, M. J. (1993) J. Virol. 67, 1817–1829
29. Cohen, D. M. (1999) Clin. Exp. Pharmacol. Physiol. 26, 69–73

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