

# Cells adapted to high NaCl have many DNA breaks and impaired DNA repair both in cell culture and *in vivo*

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**Acute exposure of cells in culture to high NaCl damages DNA and impairs its repair. However, after several hours of cell cycle arrest, cells multiply in the hypertonic medium. Here, we show that, although adapted cells proliferate rapidly and do not become apoptotic, they nevertheless contain numerous DNA breaks, which do not elicit a DNA damage response. Thus, in adapted cells, Mre11 exonuclease is mainly present in the cytoplasm, rather than nucleus, and histone H2AX and chk1 are not phosphorylated, as they normally would be in response to DNA damage. Also, the adapted cells are deficient in repair of luciferase reporter plasmids damaged by UV irradiation. On the other hand, the DNA damage response activates rapidly when the level of NaCl is reduced. Then, Mre11 moves into the nucleus, and H2AX and chk1 become phosphorylated. Renal inner medullary cells *in vivo* are normally exposed to a variable, but always high, level of NaCl. As with adapted cells in culture, inner medullary cells in normal mice exhibit numerous DNA breaks. These DNA breaks are rapidly repaired when the NaCl level is decreased by injection of the diuretic furosemide. Moreover, repair of DNA breaks induced by ionizing radiation is inhibited in the inner medulla. Histone H2AX does not become phosphorylated, and repair synthesis is not detectable in response to total body irradiation unless NaCl is lowered by furosemide. Thus, both in cell culture and *in vivo*, although cells adapt to high NaCl, their DNA is damaged and its repair is inhibited.**

A fast, coordinated response to DNA damage, including activation of cell cycle checkpoints and initiation of accurate DNA repair is believed to be necessary for maintenance of genomic integrity and prevention of oncogenic transformations. Transient DNA breaks occur normally during DNA replication, transcription, and recombination but are rapidly repaired by DNA damage response networks (1). If repair is inhibited, as by mutation or elimination of members of a damage response network, cells may die (2–5) or the resulting genomic instability may be tumorigenic (6, 7).

High NaCl can be genotoxic, as evidenced by the appearance of DNA breaks when NaCl concentration is acutely increased in cell culture medium (8). In examining this phenomenon, we found that acute elevation of NaCl inhibits the DNA damage response, preventing DNA repair (9). The Mre11 exonuclease complex is a central component of DNA damage response. This complex assembles at sites of DNA damage where it processes DNA ends for subsequent activation of repair, and it initiates cell cycle checkpoints (10). High NaCl causes the Mre11 exonuclease to translocate from nucleus to the cytoplasm, rather than localizing to sites of DNA damage, and also prevents the phosphorylation of histone H2AX that normally occurs at DNA breaks and leads to DNA repair. We suggested that acute elevation of NaCl prevents repair of normally occurring DNA breaks, resulting in accumulation of DNA damage.

Although acute elevation of NaCl is genotoxic, many types of cells normally exist in a high NaCl environment. Our particular interest is cells in the renal medulla, where the level of NaCl varies with the concentration of urine, but normally remains much higher than in the rest of the body (11). Cells, including

those derived from the renal inner medulla, are conventionally grown at  $\approx 300$  milliosmol (mosmol)/kg, which is normal for most bodily tissues. However, cells can be adapted to high NaCl in culture by gradually increasing NaCl over several days. Such adapted cells proliferate indefinitely in high NaCl medium (12, 13), which provides a convenient model. The initial response to acute elevation of NaCl is cell cycle arrest that lasts for several hours (14). Based on responses to other genotoxic stresses (15), it seemed likely that the transient arrest of proliferation provided time for DNA repair (14, 16), but unlikely that DNA damage would persist in adapted cells and even less likely that it would exist *in vivo*.

In the present studies, we examined DNA damage, DNA damage-activated signaling, and DNA repair both in adapted cells in culture and in renal inner medullary cells *in vivo*. The unanticipated result is that, in both situations, although NaCl concentration remains high, DNA damage is extensive and DNA repair is suppressed, but, if NaCl concentration is reduced, repair ensues rapidly and the DNA damage is corrected.

## Methods

**Cell Cultures.** Subconfluent cultures of mouse inner medullary collecting duct cells (mIMCD3) (17) were used in passages 12–20. Mouse embryonic fibroblasts were a gift from A. Nussenzweig (National Cancer Institute, Bethesda). The medium contained 45% DME Low Glucose, 45% Coon's Improved Medium mF-12 (Irvine Scientific), and 10% FBS (HyClone). Cells were incubated at 37°C and gassed with 5% CO<sub>2</sub>, 95% air during growth and during all experiments. Osmolality of control (isotonic) medium was 320 mosmol/kg. Hypertonic medium was prepared by adding NaCl to total osmolality indicated on figures and in the text. To adapt cells to high NaCl, osmolality of the medium was increased every 2 days in increments of 50 mosmol/kg. Cells were passaged when  $\approx 90\%$  confluent to maintain logarithmic rate of growth.

**Animal Experiments.** Two- to 3-month-old male 129/SVE mice (Taconic Farms) were used. Furosemide (1.5 mg per mouse, Abbott) and BrdUrd labeling reagent (0.3 ml per mouse, no. 00-0103, Zymed) were injected i.p. Mice were exposed to 2.5 Gy of ionizing radiation from a <sup>137</sup>Cs source in a Shepherd Mark I irradiator. Mice were killed by cervical dislocation, and kidneys were fixed in 4% paraformaldehyde.

**Measurements of Mitotic Index.** Mitosis was identified by immunostaining with antiphospho-histone H3 antibody (mitotic marker) (no. 06-570, Upstate Biotechnology, Lake Placid, NY). The percentage of mitotic cells was determined by laser scanning cytometry, as described (18).

Abbreviations: mosmol, milliosmol; IR, ionizing radiation.

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**Analysis of DNA Damage by Alkali Comet Assay.** Comet Assay kit (no. 4250-050-K, Trevigen, Gaithersburg, MD) was used according to the manufacturer's instructions. mIMCD3 cells were rinsed with PBS, scraped off the dish, and resuspended in PBS. Renal inner medulla or a piece of renal cortex in a drop of PBS was disrupted by using fine forceps to release cells, which were suspended first in PBS, then in low melting point agarose, and spread on microscopic slides. Slides were incubated for 1 h in lysis solution, then for 1 h in alkaline solution (pH 12). Electrophoresis was performed at 4°C for 30–45 min in a horizontal apparatus at 1 V/cm and 300 mA in the alkaline solution. DNA was stained with SYBR Green. Distribution of DNA between the tail and the head of the comet was analyzed with SCION IMAGE software (Scion, Frederick, MD) to calculate comet moment, which is the ratio of DNA in the tail to total DNA (head plus tail). Seventy-five randomly selected cells per sample were analyzed.

**Repair of Renilla Luciferase Reporter Vector.** pRL-CMV Vector (no. E2261, Promega) was damaged by UV in a Stratilinker UV Crosslinker (Stratagene). Normal or UV-damaged pRL-CMV Vectors were transfected into control and adapted cells by using TransFast Transfection Reagent (no. E2431, Promega). Luciferase was quantified after 16 h with a Renilla luciferase assay system (no. E2820, Promega). To estimate repair efficiency, the results were normalized to values obtained from undamaged plasmids.

**Proteins Preparation, Western Blotting, and Immunodetection.** To prepare total protein extracts from mIMCD3 cell, cells were rinsed with PBS, adjusted with NaCl to the same osmolality as the medium, then lysed with RIPA lysis buffer (50 mM Tris-HCl/1% Nonidet P-40/150 mM NaCl/1 mM EDTA/1 mM NaF/1 mM Na<sub>3</sub>VO<sub>4</sub>) and protease inhibitors (no. 1836170, Roche Molecular Biochemicals). For total protein extracts from renal inner medullas and renal cortices, they were homogenized in the RIPA lysis buffer and centrifuged for 20 min at 15,000 × g at 4°C; then, the protein content of supernatants was measured by using the BCA Protein Assay (Pierce). To extract histones, the pellets containing histones bound to DNA were resuspended in Laemmli sample buffer and boiled for 5 min. Nuclear and cytoplasmic proteins were extracted separately, by using a Pierce N-Per kit (no. 78833). Western analysis was performed by immunoblot of proteins separated by SDS/PAGE. Equality of protein loading was confirmed by staining gels with Coomassie blue. Immunoblots used specific antibodies against Mre11 (PC388, Oncogene), Chk1 (sc-7898, Santa Cruz Biotechnology), phospho-Chk1 (Ser-345, no. 2341, Cell Signaling Technology, Beverly, MA) and phospho-H2AX (Ser-139, Upstate Biotechnology, 07-164).

**Analysis of Histone H2AX Phosphorylation in mIMCD3 Cells by Immunostaining.** Cells were grown on eight-chamber slides and immunostained with anti-phospho-H2AX (Ser-139) antibody (Upstate Biotechnology, 07-164). Primary antibodies were detected with Alexa 488 goat anti-rabbit IgG (green fluorescence) (no. A-11034, Molecular Probes). DNA was stained with propidium iodide (red fluorescence). The percentage of phospho-H2AX-positive cells was determined by Laser Scanning Cytometer (LSC, CompuCyte, Cambridge, MA), as described (18).

**Immunohistological Detection of Phosphorylated Histone H2AX.** Mouse kidneys were fixed in 4% paraformaldehyde, paraffin-embedded, and cut, and sections were mounted on silanized slides (American Histolabs, Gaithersburg, MD). Sections were deparaffinized with xylene and rehydrated in a graded series of ethanol. Endogenous peroxidase was quenched by placing the slides in 3% hydrogen peroxide in methanol for 10 min. Heat-

induced epitope retrieval was performed by boiling the slides for 6 min in citrate buffer solution, pH 6.0 (no. 00-5000, Zymed). Slides were washed with PBS and stained with anti-phospho-H2AX antibody (no. 07-164, Upstate Biotechnology) by using Histostain-Plus Kit (Zymed) according to the manufacturer's instructions. Briefly, sections were blocked with serum-blocking solution, then incubated successively with primary antibody, biotinylated secondary antibody, and streptavidin-FITC conjugate (green fluorescence). DNA was stained with propidium iodide (red fluorescence).

**Immunohistological Detection of BrdUrd in Kidney Sections.** Mouse kidneys were fixed in 4% paraformaldehyde, paraffin-embedded, and cut, and the sections were mounted on silanized slides (American Histolabs). Slides were stained by using a BrdUrd detection kit (no. 93-3943, Zymed), according to the manufacturer's instructions, but with modifications made in protocol to increase sensitivity, as follows. An additional blocking step with a Blocking Endogenous Antibody Technology (BEAT) Blocker Kit (no. 50-300, Zymed) was added, which eliminated nonspecific staining that occurred in the absence of primary antibody. Slides were incubated with biotinylated anti-BrdUrd antibody and streptavidin-peroxidase conjugate from the BrdUrd detection kit. Peroxidase was detected by using Tyramide Signal Amplification Kit (no. T-20915, Molecular Probes) in which Thyramid-Alexa 594 deposit (red) is produced by reaction with peroxidase (19). Adjacent sections were stained with hematoxylin for histology (no. 00-8001Zymed).

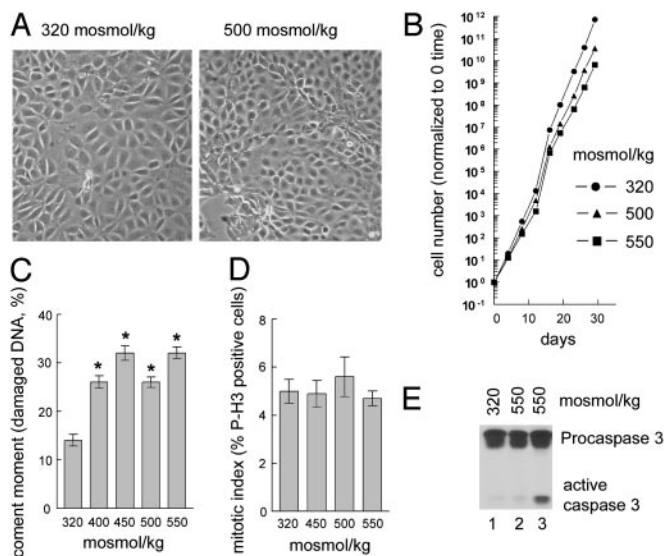
**Detection of DNA Breaks in Kidney Tissue Sections.** DNA fragmentation Detection Kit (no. QIA80, Oncogene) was used according to the manufacturer's instructions. Briefly, DNA breaks were detected by *in vitro* labeling of their 3' OH ends with BrdUTP in a reaction catalyzed by terminal deoxynucleotidyltransferase (TdT). TdT catalyzes the addition of BrdUTP and unlabeled deoxynucleotides to the 3' OH ends. BrdUTP was detected with biotinylated anti-BrdUrd antibody and streptavidin-peroxidase conjugate. Peroxidase activity was analyzed by addition of 3,3'-diaminobenzidine tetrahydrochloride (DAB) substrate, which produces a brown colored deposit on reaction with peroxidase.

## Results

We previously found that acute elevation of NaCl inhibits the response to DNA damage, leading to inhibition of DNA repair and accumulation of DNA breaks (9). However, cells adapt to elevated NaCl, proliferating in culture (12, 13) and functioning without apoptosis in the renal inner medulla *in vivo* (20, 21) where NaCl normally is always high. The question addressed in the following studies is whether the adaptation to high NaCl involves restoration of the DNA damage response and repair of the DNA damage.

We first examined the DNA damage response in mIMCD3 cells that have been adapted to high NaCl by adding NaCl in increments that elevated osmolality by 50 mosmol/kg every 2 days to a final osmolality of up to 550 mosmol/kg. At 500 mosmol/kg, the adapted mIMCD3 cells appear the same as cells at 320 mosmol/kg (Fig. 1A), and, up to 550 mosmol/kg, they proliferate at nearly the same rate (Fig. 1B and D). However, despite this apparent normality, the adapted cells have many more DNA breaks, measured by comet assay, than cells that remained at 320 mosmol/kg (Fig. 1C). DNA breaks in proliferating cells lead to accumulation of mutations and apoptosis (15, 22), but we find no evidence of apoptosis. The cells appear normal (Fig. 1A) and, despite continuous proliferation, caspase 3 is not activated (Fig. 1E) unless the cells are allowed to overgrow beyond confluence for several days (Fig. 1E, lane 3).

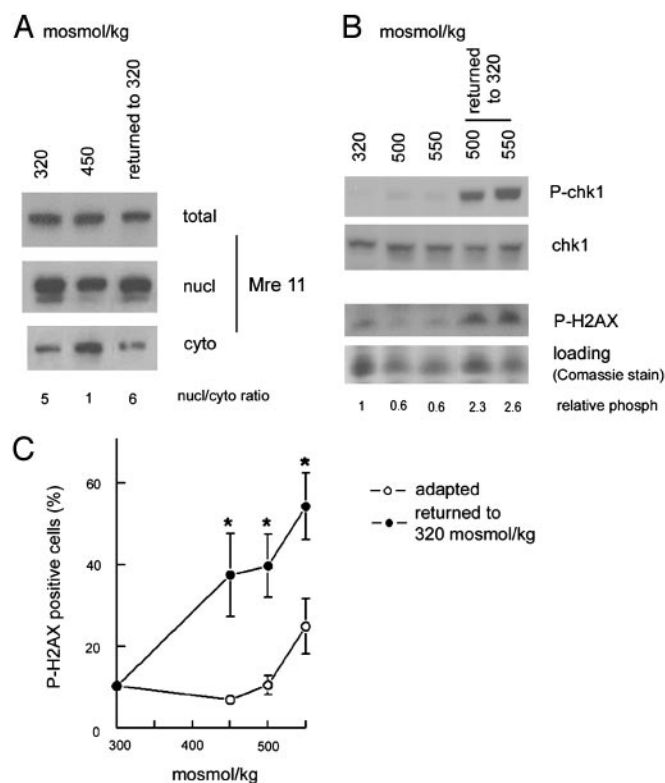
The initial response to DNA damage in proliferating cells normally is cell cycle arrest, during which the DNA is repaired



**Fig. 1.** Cells adapted to high NaCl have many DNA breaks, yet they survive and grow well. Cells were adapted to high NaCl, as described in *Methods*, then studied from passage 13 to 20. (A) Cells adapted to high NaCl (osmolality raised gradually to 500 mosmol/kg) appear similar to cells kept at 320 mosmol/kg. (B) Cells adapted by gradually raising NaCl to 500–550 mosmol/kg proliferate nearly as rapidly as cells kept at 320 mosmol/kg. (C) DNA breaks measured by single cell electrophoresis. Adapted cells have many DNA breaks. (D) Mitotic index, measured by immunostaining with anti-phospho-histone H3 antibody. Mitotic index is not altered in adapted cells, indicating absence of G<sub>2</sub> arrest. (E) Caspase 3 activation, as a measure of apoptosis. Western blot detects cleavage to active form. There is no evidence of apoptosis (caspase 3 activation) whereas the adapted cells are proliferating, but caspase 3 is activated if they become overcrowded because they are allowed to grow beyond confluence (lane 3).

(15). However, despite the presence of DNA breaks (Fig. 1C), the adapted mIMCD3 cells continue to proliferate rapidly (Fig. 1B), their mitotic index is not reduced (Fig. 1D), and checkpoint kinase 1 is not phosphorylated (Fig. 2B), as it would be if it were signaling G<sub>2</sub>/M delay in response to DNA damage (3). Therefore, we find no evidence of cell cycle arrest in the adapted cells, despite their DNA being damaged.

DNA breaks normally cause a DNA damage response that includes repair of the damage. However, similar to our previous observations with acute elevation of NaCl (9), DNA repair is deficient in mIMCD3 cells chronically adapted to high NaCl. Mre11, which normally accumulates at DNA breaks, is elevated in the cytoplasm, compared with the nucleus (Fig. 2A). Histone H2AX, which is normally phosphorylated during repair of double-stranded breaks, is not phosphorylated in response to the DNA breaks in adapted cells (Fig. 2B and C). As a possible explanation, we considered that the DNA breaks induced by high NaCl might differ qualitatively from breaks induced by other stresses, resulting in their not triggering the DNA damage response. However, lowering NaCl to reduce osmolality to 320 mosmol/kg immediately activates a robust response to the DNA breaks. Mre11 moves into the nucleus (Fig. 2A), histone H2AX becomes phosphorylated on Ser-139 (Fig. 2B and C), and chk1 becomes phosphorylated on Ser 345 (Fig. 2B). Thus, despite adaptation in other respects, cells chronically exposed to high NaCl apparently remain deficient in repair of DNA damage as long as the NaCl remains high. More direct evidence for this conclusion comes from comparison between repair of reporter plasmids damaged by UV irradiation in cells kept at 320 mosmol/kg vs. repair in cells adapted to high NaCl (Fig. 3). The efficiency of the repair is much lower in cells adapted to high NaCl. Taken together, these findings indicate that cells adapted

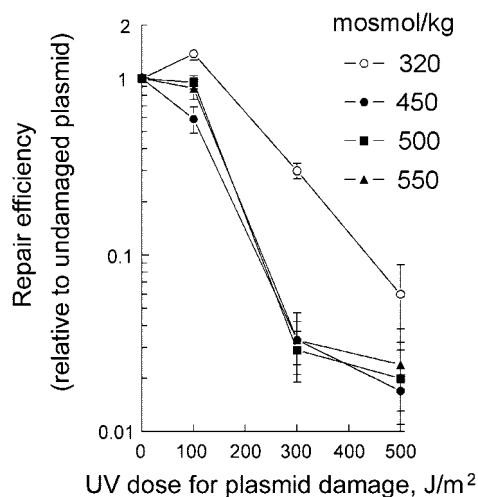


**Fig. 2.** The classical DNA damage response is not activated in cells adapted to high NaCl and is activated when the level of NaCl is reduced. Adapted cells were returned to 320 mosmol/kg medium for 30 min. (A) Western blot analysis of Mre11 intracellular localization. In adapted cells, the proportion Mre11 in the cytoplasm increases, but the Mre11 translocates into nucleus after return to 320 mosmol/kg medium. (B) Western blot analysis of phosphorylation of chk1 and histone H2AX. Chk1 and H2AX are not phosphorylated in adapted cells, despite presence of DNA breaks, but become phosphorylated after return to 320 mosmol/kg. (C) Analysis of histone H2AX phosphorylation by immunocytochemistry. The percentage of P-H2AX-positive cells increases when adapted cells are returned to 320 mosmol/kg medium, indicating activation of DNA repair.

to high NaCl in culture maintain high levels of DNA damage without accumulating lethal mutations and without activating classical DNA damage response. Nevertheless, the DNA damage response is available and switches on rapidly when the level of NaCl is reduced.

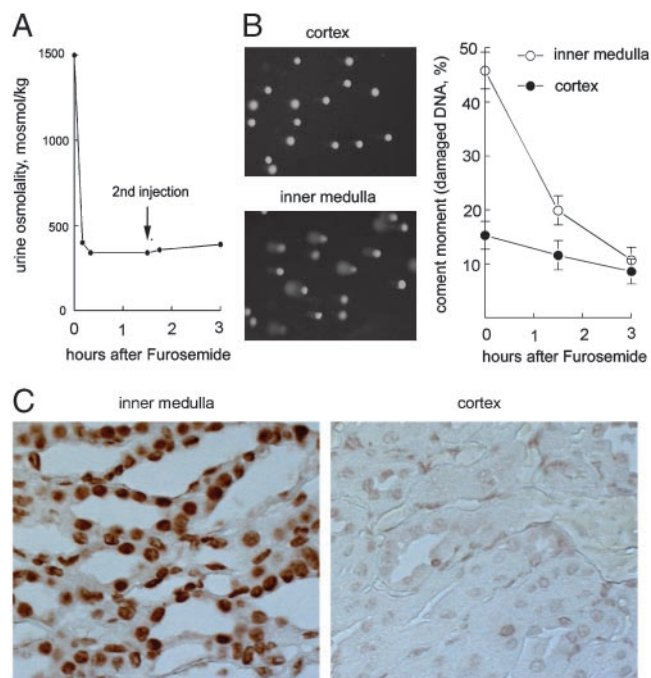
Concentrations of both urea and NaCl are high in renal inner medullary interstitial fluid. There is considerable evidence that cells in culture adapt better to elevation of a combination of NaCl and urea than to elevation of either one alone (23–25). Therefore, it seemed possible that increasing both urea and NaCl might result in less damage to DNA in adapted cells than adding NaCl alone. However, although mIMCD3 cells adapt well to gradual increase in osmolality to 770 mosmol/kg, adding 250 mosmol/kg NaCl and 200 mosmol/kg urea, DNA breaks and inhibition of repair are no less than when the NaCl is added alone (Fig. 7, which is published as supporting information on the PNAS web site). Also, gradual increase in osmolality to 500 mosmol/kg by adding urea, alone, does not increase DNA breaks significantly (Fig. 7).

We next asked whether chronic exposure to high NaCl also causes DNA breaks and suppresses DNA damage response *in vivo*. Although NaCl concentration normally varies in inner medullary interstitial fluid, depending on the concentration of the urine, it is always much higher than in the rest of the body (11). We first tested for DNA breaks in the inner medullas of

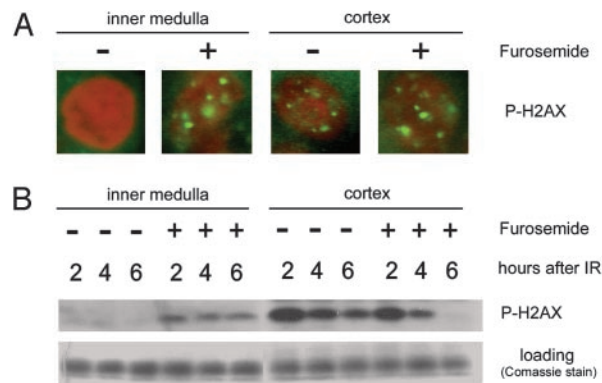


**Fig. 3.** Repair of reporter plasmids damaged by UV is impaired in cells adapted to high NaCl. Cells were transfected with pRL-CMV-luciferase vector damaged by the doses of UV radiation that are shown. Luciferase expression, which depends on DNA repair within the cells, was measured 16 h after transfection.

normal mice by using single cell electrophoresis (“comet assay”). The unexpected result is that cells in mouse inner medulla have many more DNA breaks than do cells in the renal cortex (Fig. 4B), in which extracellular NaCl is not elevated. Because the



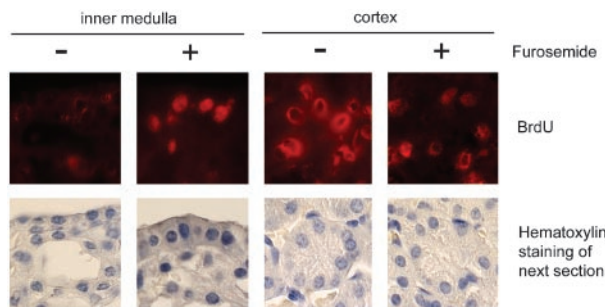
**Fig. 4.** DNA damage exists in mouse inner medullas *in vivo* under the normal condition of high NaCl and urea and is repaired rapidly when medullary osmolality is decreased by furosemide. (A) Time course of urine osmolality after furosemide injection (see *Methods* for details). (B) Single cell gel electrophoresis (comet) assay of DNA damage in cells from inner medulla and cortex. Damaged DNA appears in the “tails” of the “comets.” (Left) Representative nuclei stained with SYBR Green before furosemide treatment. (Right) The percentage of DNA in comet tails. (C) Terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling assay of DNA breaks performed on mouse kidney sections. DNA damage is widespread in the inner medulla but not in cortex.



**Fig. 5.** Histone H2AX is not phosphorylated in response to ionizing radiation at the normally elevated inner medullary osmolality *in vivo* but becomes phosphorylated when medullary osmolality is decreased by furosemide. Mice were given total body irradiation of 2.5 Gy, and some were injected with furosemide. (A) Immunocytochemical staining of phospho-H2AX on kidney sections. Red, DNA stained with propidium iodide; green, phospho-H2AX foci at locations of DNA damage. (B) Western blot analysis of H2AX phosphorylation in inner medulla and cortex.

presence of DNA breaks in a normal, healthy tissue seemed rather surprising, we confirmed this finding by an independent method. We treated sections of inner medulla with terminal deoxynucleotidyltransferase in the presence of BrdUrd, which becomes incorporated in extensions of any disconnected 3' OH ends of DNA. BrdUrd incorporated in the DNA was detected by immunocytochemistry (26). This assay confirms the widespread DNA breaks in the inner medulla (Fig. 4C) detected by the comet assay. The diuretic furosemide rapidly reduces urine osmolality to 300 mosmol/kg. Under these conditions, urine osmolality represents the osmolality in the medullary interstitium (27). If the injection of furosemide is repeated, the osmolality remains low for several hours (Fig. 4A). Within 3 hours after inner medullary interstitial NaCl is reduced by furosemide, the DNA breaks in the inner medulla disappear (Fig. 4B).

Because in cell culture the high NaCl-induced accumulation of DNA breaks is accompanied by impairment of DNA repair (ref. 9 and Figs. 1–3), we next examined whether the high NaCl normally present in inner medullary interstitial fluid inhibits DNA repair in inner medullary cells. Histone H2AX becomes phosphorylated immediately after DNA is damaged by ionizing radiation (IR), marking the activation of DNA repair (28, 29). Acute elevation of NaCl inhibits histone H2AX phosphorylation in response to IR in mIMCD3 cells (9). To test for this *in vivo*, we exposed mice to total body irradiation, then compared the response to the resultant DNA damage that occurs in the renal inner medulla, where NaCl is high, to that in renal cortex, where it is not. IR (2.5 Gy) increases histone H2AX phosphorylation in cortex, but not in inner medulla (Fig. 5). However, lowering inner medullary NaCl with furosemide rapidly induces H2AX phosphorylation (Fig. 5), consistent with activation of DNA repair. To confirm this finding, we measured DNA synthesis, which generally is involved in DNA repair. To label newly synthesized DNA, we injected mice with BrdUrd at the time of irradiation and visualized BrdUrd incorporation in the newly synthesized DNA by immunostaining of kidney sections. After IR, BrdUrd is incorporated in nuclei in the cortex, but not in the inner medulla (Fig. 6), consistent with impaired DNA repair in the inner medulla. However, when, under otherwise identical conditions, inner medullary NaCl is lowered by furosemide, DNA synthesis occurs (Fig. 6). Taken together, these results indicate that the high osmolality normally present in renal inner medulla impairs repair of DNA breaks induced by IR *in vivo*.



**Fig. 6.** DNA repair synthesis does not occur after induction of DNA damage by ionizing radiation at the normally high inner medullary osmolality *in vivo* but does when medullary osmolality is decreased by furosemide. Mice were given total body irradiation of 2.5 Gy, and some were injected with furosemide. All mice were injected with BrdUrd at the time of irradiation to label newly synthesized DNA. (Upper) Immunocytochemical staining of BrdUrd on kidney sections. (Lower) Staining of adjacent section with hematoxylin to show tissue structure.

Although renal medullary cells are normally exposed to very high NaCl, cells elsewhere in the body are not. To test whether they might respond like renal cells, we adapted mouse embryonic fibroblasts (MEFs) to high NaCl, gradually raising osmolality to 500 mosmol/kg. The MEFs adapt to high NaCl and appear normal (Fig. 8A, which is published as supporting information on the PNAS web site). Their rate of proliferation is rapid but is reduced at a lower osmolality than mIMCD3 cells (compare Fig. 1B with Fig. 8B). Similar to mIMCD3 cells, their DNA damage response apparently is inhibited whereas NaCl concentration remains high. At 500 mosmol/kg, Mre11 is elevated in the cytoplasm relative to the nucleus (Fig. 8C), and Chk-1 phosphorylation is reduced (Fig. 8D). Further, as with the renal cells, when NaCl is lowered, Mre11 translocates to the nucleus (Fig. 8C), and Chk1 becomes phosphorylated (Fig. 8D). Thus, the adaptation of nonrenal cells to high NaCl resembles that of renal cells, even though the nonrenal cells are not normally exposed to high NaCl.

## Discussion

There have been many studies of how cells adapt to hypertonicity. The known protective responses include accumulation of organic osmolytes (30), up-regulation of heat shock proteins (12, 13), and activation of Na/K ATPase (12). In cell culture, high NaCl causes DNA damage (8, 9), but also induces transient cell cycle arrest (14, 16, 18), which was believed to provide time for repair of the DNA. Premature cessation of the cell cycle arrest causes apoptosis (31). After the cell cycle delay, cells in culture can adapt to hypertonicity and resume apparently normal growth (12, 13) (Fig. 1). Cellular proliferation is rare in the renal inner medulla *in vivo*, and there is little apoptosis despite chronically elevated NaCl (20, 21). Thus, despite the fact that hypertonicity causes DNA damage, there has been no reason to suspect that the DNA damage is sustained once cells adapt, either in culture or *in vivo*. Therefore, we were surprised to observe in the present studies that DNA damage persists in cells adapted to high NaCl both in cell culture and *in vivo*. Further, these DNA breaks do not prevent proliferation in culture, nor do they activate apo-

ptosis either in cell culture or *in vivo*, and in both situations DNA repair is not activated unless the NaCl concentration is reduced.

These findings raise a number of questions. (i) What is the cause of the DNA breaks? We have proposed (9) that, because transient DNA breaks are continuously created during transcription and replication (1), inhibition of DNA repair will, of itself, increase the number of existing DNA breaks. However, there are other possibilities, including effects on chromatin (32). (ii) Are such DNA breaks pathological, or are they simply attributes of normal cellular physiology under stressful conditions? One might suppose that extending the duration of even the normal transient DNA breaks would increase the risk of aberrant transcription or replication. Perhaps the cells can stabilize these DNA breaks in a way that prevents harmful consequences to transcription, replication, and other processes involving DNA. (iii) Are there novel forms of DNA damage response and repair that occur in the presence of high NaCl that, although limited, nevertheless protect essential functions? Even though known components of DNA damage response, such as Mre11, H2AX and Chk1, are not activated, there might be alternative pathways that provide some essential repair. The fact that DNA damage does not continue to increase indefinitely in adapted cells indicates that some equilibrium is reached where rate of repair matches the rate of damage. If so, what are those alternative damage response and repair pathways? (iv) What are the consequences in renal inner medullas of deficient repair of DNA breaks induced by external genotoxic agents? Our results indicate that repair of DNA breaks induced by IR (Figs. 5 and 6) or UV irradiation (Fig. 3) is impaired while NaCl is high. Does this result also apply to nephrotoxicity of drugs and xenobiotics that have DNA damaging potential? (v) Normally, there is little cellular proliferation in renal medullas (20, 21). Is activation of proliferation dangerous for the cells? Excessive ingestion of nonsteroidal analgesic drugs (NSAIDs) damages inner medullas (33). Early passage rat inner medullary cells exposed to a high level of NaCl and urea, similar to that *in vivo*, grow slowly. However, the NSAID acetaminophen activates their proliferation and causes apoptosis (34). (vi) The similarity of the response of renal and nonrenal (mouse embryonic fibroblasts) cells to high NaCl suggests that high NaCl might damage DNA and impair its repair in cells exposed to high NaCl in other organisms, like marine invertebrates, that retain an extracellular ionic composition similar to seawater ( $\approx 1,000$  mosmol/kg) (35) or desert tortoise *Gopherus agassizii*, which can concentrate its body fluids to 600 mosmol/kg under extreme conditions (36).

Thus, our results add a perspective to consideration of mechanisms of adaptation to hypertonicity. The classical view is that hypertonicity can be compensated for by maintaining normal cell volume and intracellular ion composition by replacement of inorganic ions with compatible osmolytes (30), thus ensuring biochemical and metabolic stability. Now, we find that those compensations do not prevent DNA damage or preserve the DNA damage response in adapted cells. Thus, there must be additional mechanisms that protect the cells from consequences of the DNA damage.

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- Vilenchik, M. M. & Knudson, A. G. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 12871–12876.
- Luo, G., Yao, M. S., Bender, C. F., Mills, M., Bladl, A. R., Bradley, A. & Petrini, J. H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 7376–7381.
- Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., et al. (2000) *Genes Dev.* **14**, 1448–1459.
- Xiao, Y. & Weaver, D. T. (1997) *Nucleic Acids Res.* **25**, 2985–2991.

- Zhu, J., Petersen, S., Tessarollo, L. & Nussenzweig, A. (2001) *Curr. Biol.* **11**, 105–109.
- Charames, G. S. & Bapat, B. (2003) *Curr. Mol. Med.* **3**, 589–596.
- Celeste, A., Petersen, S., Romanienko, P. J., Fernandez-Capetillo, O., Chen, H. T., Sedelnikova, O. A., Reina-San-Martin, B., Coppola, V., Meffre, E., Difilippantonio, M. J., et al. (2002) *Science* **296**, 922–927.
- Kultz, D. & Chakravarty, D. (2001) *Proc. Natl. Acad. Sci. USA* **98**, 1999–2004.

9. Dmitrieva, N. I., Bulavin, D. V. & Burg, M. B. (2003) *Am. J. Physiol. Renal Physiol.* **285**, F266–F274.
10. D'Amours, D. & Jackson, S. P. (2002) *Nat. Rev. Mol. Cell. Biol.* **3**, 317–327.
11. Bankir, L. (1996) in *The Kidney*, eds. Brenner, B. M. & Rector, F. C. (Saunders, Philadelphia), 5th Ed., pp. 571–606.
12. Capasso, J. M., Rivard, C. J. & Berl, T. (2001) *Am. J. Physiol. Renal Physiol.* **280**, F768–F776.
13. Santos, B. C., Pullman, J. M., Chevaile, A., Welch, W. J. & Gullans, S. R. (2003) *Am. J. Physiol. Renal Physiol.* **284**, F564–F574.
14. Michea, L., Ferguson, D. R., Peters, E. M., Andrews, P. M., Kirby, M. R. & Burg, M. B. (2000) *Am. J. Physiol. Renal Physiol.* **278**, F209–F218.
15. Zhou, B. B. & Elledge, S. J. (2000) *Nature* **408**, 433–439.
16. Kultz, D., Madhany, S. & Burg, M. B. (1998) *J. Biol. Chem.* **273**, 13645–13651.
17. Rauchman, M. I., Nigam, S. K., Delpire, E. & Gullans, S. R. (1993) *Am. J. Physiol.* **265**, F416–F424.
18. Dmitrieva, N. I., Bulavin, D. V., Fornace, A. J., Jr., & Burg, M. B. (2002) *Proc. Natl. Acad. Sci. USA* **99**, 184–189.
19. Van Heusden, J., de Jong, P., Ramaekers, F., Bruwieri, H., Borgers, M. & Smets, G. (1997) *J. Histochem. Cytochem.* **45**, 315–319.
20. Terada, Y., Inoshita, S., Hanada, S., Shimamura, H., Kuwahara, M., Ogawa, W., Kasuga, M., Sasaki, S. & Marumo, F. (2001) *Kidney Int.* **60**, 553–567.
21. Zhang, Z., Cai, Q., Michea, L., Dmitrieva, N. I., Andrews, P. & Burg, M. B. (2002) *Am. J. Physiol. Renal Physiol.* **283**, F302–F308.
22. Bielas, J. H. & Heddle, J. A. (2000) *Proc. Natl. Acad. Sci. USA* **97**, 11391–11396.
23. Neuhofer, W., Muller, E., Burger-Kentscher, A., Fraek, M. L., Thurau, K. & Beck, F. (1998) *Pflügers Arch.* **435**, 407–414.
24. Santos, B. C., Chevaile, A., Hebert, M. J., Zagajeski, J. & Gullans, S. R. (1998) *Am. J. Physiol.* **274**, F1167–F1173.
25. Zhang, Z., Tian, W. & Cohen, D. M. (2000) *Am. J. Physiol. Renal Physiol.* **279**, F345–F352.
26. Li, X. & Darzynkiewicz, Z. (1995) *Cell Prolif.* **28**, 571–579.
27. Beck, F., Dorge, A., Rick, R. & Thurau, K. (1985) *Pflügers Arch.* **405**, Suppl. 1, S28–S32.
28. Rogakou, E. P., Pilch, D. R., Orr, A. H., Ivanova, V. S. & Bonner, W. M. (1998) *J. Biol. Chem.* **273**, 5858–5868.
29. Paull, T. T., Rogakou, E. P., Yamazaki, V., Kirchgessner, C. U., Gellert, M. & Bonner, W. M. (2000) *Curr. Biol.* **10**, 886–895.
30. Garcia-Perez, A. & Burg, M. B. (1991) *Physiol. Rev.* **71**, 1081–1115.
31. Iliakis, G., Wang, Y., Guan, J. & Wang, H. (2003) *Oncogene* **22**, 5834–5847.
32. Kultz, D. (2000) in *Cell and Molecular Response to Stress: Environmental Stressors and Gene Responses*, eds. Storey, K. B. & Storey, J. M. (Elsevier Science, London), pp. 157–179.
33. Bach, P. H. & Nguyen, T. K. (1998) *Toxicol. Pathol.* **26**, 73–91.
34. Cai, Q., Dmitrieva, N. I., Michea, L. F., Rocha, G., Ferguson, D. & Burg, M. B. (2003) *J. Pharmacol. Exp. Ther.* **306**, 35–42.
35. Withers, P. C. (1992) *Comparative Animal Physiology* (Saunders, Philadelphia), pp. 777–830.
36. Martin, K. L. M. & Nagy, K. A. (1997) in *Amniote Origins: Completing Transition to Land*, eds. Sumida, S. S. & Martin, K. L. M. (Academic, New York), pp. 399–423.