Analysis of DNA breaks, DNA damage response, and apoptosis produced by high NaCl

Natalia I. Dmitrieva and Maurice B. Burg

Laboratory of Kidney and Electrolyte Metabolism, National Heart, Lung and Blood Institute, National Institutes of Health, Department of Health and Human Services, Bethesda, Maryland

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Dmitrieva NI, Burg MB. Analysis of DNA breaks, DNA damage response, and apoptosis produced by high NaCl Am J Physiol Renal Physiol 295: F1678–F1688, 2008.—We previously reported that, both in cell culture and in the renal inner medulla in vivo, elevating NaCl increased the number of DNA breaks, which persisted as long as NaCl remained high but were rapidly repaired when NaCl was lowered. Furthermore, those breaks did not induce the DNA repair protein γH2AX or cause activation of the MRN (Mre11, Rad50, Nbs1) complex. In contrast, others recently reported that high NaCl does induce γH2AX and MRN complex formation and concluded that those activities are associated with repair of the DNA (Sheen MR, Kim SW, Jung YJ, Ahn YJ, Rhee JG, Kwon HM, Woo SK. Am J Physiol Renal Physiol 291: F1014–F1020, 2006). The purpose of the present studies was to resolve the disparity. The important difference is that HeLa cells, which were the main subject of the later report, are much less tolerant of high NaCl than are the mIMCD3 cells, which were our main subject. mIMCD3 cells survive levels of NaCl that kill HeLa cells by apoptosis. Here we demonstrate that in both cell types raising NaCl to a level that the cells survive (higher for mIMCD3 than HeLa) increases DNA breaks without inducing γH2AX or activating the MRN complex and that the DNA breaks persist as long as NaCl remains elevated, but are rapidly repaired when it is lowered. Importantly, in both cell types, raising NaCl further to cause apoptosis activates these DNA damage response proteins and greatly fragments DNA, associated with cell death. We conclude that γH2AX induction and MRN activation in response to high NaCl are associated with apoptosis, not DNA repair.

DNA damage; hypertonicity; osmotic stress; HeLa cells; mIMCD3 cells

HIGH NaCl INCREASES THE NUMBER of DNA breaks in cells in tissue culture (12, 21) and in vivo in renal inner medullas (12), in Caenorhabditis elegans (13), and in marine invertebrates (14). The increase in DNA breaks has been detected by pulsed field gel electrophoresis (21), comet assay (8, 21), and bromodeoxyuridine (BrdU) terminal transferase-dUTP-nick-end labeling (TUNEL) (12). While NaCl remains high, the DNA breaks persist and yet the cells survive (12–14). If the high NaCl is lowered, however, the excess breaks disappear (8, 12, 14), and this occurs within 2 h in cell culture (8) and in renal inner medullas (12).

High NaCl also slows repair of damaged DNA. Thus in mIMCD3 cells and fibroblasts, DNA in plasmids that is damaged by UV radiation is repaired more slowly if NaCl is elevated (8, 12). Furthermore, following ionizing radiation (IR), no DNA repair synthesis is detected by BrdU incorporation in tissue culture (12, 21) and in vivo in renal inner medullas (12), in Caenorhabditis elegans (13), and in marine invertebrates (14). The increase in DNA breaks has been detected by pulsed field gel electrophoresis (21), comet assay (8, 21), and bromodeoxyuridine (BrdU) terminal transferase-dUTP-nick-end labeling (TUNEL) (12). While NaCl remains high, the DNA breaks persist and yet the cells survive (12–14). If the high NaCl is lowered, however, the excess breaks disappear (8, 12, 14), and this occurs within 2 h in cell culture (8) and in renal inner medullas (12).

Acute elevation of NaCl above a certain threshold causes apoptosis, which complicates analysis of high NaCl-induced DNA damage. DNA is greatly fragmented during apoptosis (34), despite induction of DNA damage response proteins, like γH2AX (33). Therefore, it is important to distinguish whether apoptosis accounts for any high NaCl-induced DNA breaks that are observed. mIMCD3 cells, which were derived from the renal inner medullary collecting duct of an SV40 transgenic mouse (32), are relatively tolerant of NaCl. They survive acute elevation of osmolality by adding NaCl up to 600 mosmol/kg H2O with little, if any, apoptosis (25, 35). Other types of DNA breaks continue to accumulate, however, and persist as long as NaCl remains high (8–10). As high NaCl is reduced, the excess breaks disappear (6, 8, 12, 21, 25, 35).

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cells, like the human macrophage cell line (U937 cells) (23), become apoptotic at lower levels of NaCl. DNA breaks increase in mIMCD3 cells at 400–550 mosmol/kgH2O, which the cells survive well without any evidence of apoptosis (12). Under those conditions, γH2AX is not induced (8, 12). Evidently, when testing is done of these responses in other cell types, it is important to know whether apoptosis is occurring.

Our present study of DNA breaks, DNA damage response, and apoptosis in response to high NaCl was motivated by a recent publication which reported that elevation of osmolality to 500–600 mosmol/kgH2O by adding NaCl results in activation of the MRN complex and induction of γH2AX. (37) The studies were mainly done in HeLa, a cell line derived from a human cervical adenocarcinoma. The authors concluded that DNA repair is activated in response to the DNA breaks induced by high NaCl, and they speculated that the breaks do not persist because they are repaired during adaptation to high NaCl. However, they did not directly measure DNA breaks or their repair and based their conclusions solely on the activation of the DNA damage response proteins. To resolve the disparity, in our present study we analyzed the response of HeLa cells to high NaCl in some detail, including osmotic tolerance of the cells, apoptosis, occurrence and repair of DNA breaks, and activation of γH2AX and the MRN complex. We find that the apparent DNA repair response in HeLa cells in fact accompanies apoptosis. The HeLa cells have a lower osmotic tolerance than mIMCD3, and when this lower osmotic tolerance is taken into account, the effects of high NaCl on DNA and its repair are the same in HeLa cells as in mIMCD3.

MATERIALS AND METHODS

Cell culture and exposure to high NaCl. HeLa cells purchased from ATCC (no. CCL-2) were grown in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT). mIMCD3 cells (32) were grown at ATCC (no. CCL-2) were grown in DMEM containing 10% fetal bovine serum (HyClone, Logan, UT). mIMCD3 cells (32) were grown in medium containing 45% DME Low Glucose (Invitrogen, Carlsbad, CA), 45% F12 Coon’s Modification (no. F6636, Sigma), and 10% fetal bovine serum (HyClone). mIMCD3 cells (32) were grown in medium containing 45% DME Low Glucose (Invitrogen, Carlsbad, CA), and 10% fetal bovine serum (HyClone). Osmolality of the control medium was measured with a Wallac 1420 multilabel counter (PerkinElmer Life Sciences, Turku, Finland). To calculate the nuclear-to-cytoplasmic ratio for Mre11, the amount of Mre11 in the nuclear fraction {IFnucl [Vnucl (total)/Vnucl (loaded)]} was divided by the amount of Mre11 in the cytoplasmic fraction {IFcyto [Vcyto (total)/Vcyto (loaded)]}, where V(loaded) is the volume of the sample loaded on the gel, V(total) is the volume of the entire sample, and IF is measured from the corresponding band on the immunoblot.

Identification of apoptotic cells with fluorochrome inhibitors of caspases. HeLa cells grown on eight chamber slides were labeled with carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase (FAM-VA-D-FMK; CaspaTag Pan-Caspase Assay Kit, no. APT420, Millipore). The inhibitor permeates cells and binds covalently to active caspases (15). The bound, labeled inhibitor is retained within the cell, while any unbound inhibitor diffuses out of the cell and is washed away. The green fluorescent signal is a measure of the amount of active caspase present in the cells at the time the reagent was added. The inhibitor working solution was prepared as specified in the kit and added to the cells for 1 h. The slides were fixed with 1.6% formaldehyde (no. 18814, Polysciences, Warrington, PA) in PBS for 10 min at room temperature. Following fixation, cells were permeabilized with 0.1% Triton X-100 in PBS and cellular DNA was stained with 4,6-diamidino-2-phenylindole (DAPI, Invitrogen). The slides were then washed with PBS, mounted with SlowFade Gold antifade reagent (no. S36936, Invitrogen), and subjected to microscopy (Leica SP1 laser-scanning confocal microscope) or analysis by a laser-scanning cytometer (LSC; CompuCyte, Cambridge, MA) (6, 39).

Immunostaining. Cells grown on eight chamber slides were fixed for 10 min in 1.6% formaldehyde (no. 18814, Polysciences) at room temperature, washed with PBS, permeabilized with 0.1% Triton X-100 in PBS, and blocked with 3% bovine serum albumin for 1 h at room temperature. Slides were incubated with primary antibodies for P-H2AX (no. 2577, Cell Signaling Technology), Mre11 (no. 4895, Cell Signaling Technology), or p95Nbs1 (no. PC269T, Oncogene, Gibbstown, NJ) at 4°C overnight, followed by secondary antibody labeled with Alexa Fluor 488 (Invitrogen). For simultaneous labeling of P-H2AX and cleaved caspase 3, cells were incubated with cleaved caspase-3 antibody (Alexa Fluor 488 Conjugate) and P-H2AX antibody (Alexa Fluor 488 Conjugate; no. 9669 and 9720, Cell Signaling Technology) simultaneously at 4°C overnight. After two washes with PBS, cells were stained with DAPI (DNA stain, Invitrogen), mounted with SlowFade Gold antifade reagent (no. S36936, Invitrogen), and subjected to microscopy (Leica SP1 laser-scanning confocal microscope) or analysis by a laser-scanning cytometer (LSC; CompuCyte, Cambridge, MA) (6, 39).

Fluorescence measurements. Cellular blue, green, and red fluorescence emission was measured using a LSC (CompuCyte), utilizing standard filter settings; fluorescence was excited at 405 (for DAPI), 488 [for fluorochrome inhibitors of caspases (FLICA) and Alexa 488], and 633 nm (for Alexa 647), respectively. The intensities of maximal pixel and integrated fluorescence were measured and recorded for each cell (6). At least 2,000 cells were measured per sample.

Analysis of DNA damage by alkaline comet assay. A Comet Assay kit (no. 4250-050-K, Trevigen) was used, according to the manufac-
HIGH NaCl-INDUCED DNA DAMAGE

RESULTS

High NaCl induces γH2AX in HeLa cells only when it is elevated to a level so high that the cells do not survive. We began by repeating the experiment presented in the recent publication (37) that reported induction of γH2AX in HeLa cells by elevating NaCl to a total osmolality of 500−600 mosmol/kg H2O. As shown on Fig. 1A, we confirm this observation. In our previous experiments with mIMCD3 cells, γH2AX was not induced by this level of NaCl (8). To explain the discrepancy, we proceeded to examine in detail the response of HeLa cells to high NaCl. First, we measured the osmotic tolerance of HeLa cells by determining how much acute elevation of NaCl they can withstand without apoptosis. The number of HeLa cells decreases within 24 h after an increase in osmolality above 400 mosmol/kg H2O by adding NaCl (Fig. 1B). Changes characteristic of apoptosis (cleavage of PARP and activation of caspase 3) occur within 4 h of elevating osmolality to 500 and 600 mosmol/kg H2O (Fig. 1A). It is striking that PARP is almost completely cleaved at 600 mosmol/kg H2O (Fig. 1A), suggesting that all of the cells rapidly become apoptotic at that osmolality and accounting for the disappearance of all the cells by 24 h (Fig. 1B). Less PARP is cleaved at 500 mosmol/kg H2O (Fig. 1A), indicating that only some of the cells become apoptotic at this level. At 400 mosmol/kg H2O, no cleaved PARP or activated caspase 3 is evident after 4 h and 7 days, indicating successful adaptation. mIMCD3 cells are known for their ability to adapt to much higher osmolalities than other cell lines (32). Unlike HeLa cells, only a small portion of the mIMCD3 cells become apoptotic at 600 mosmol/kg H2O, and it requires 700 mosmol/kg H2O to kill all mIMCD3 cells (25). Based on this difference and the fact that γH2AX is induced in apoptotic cells (33), we hypothesized that the induction of γH2AX by high NaCl in HeLa cells (37) (Fig. 1A) occurs in apoptotic cells and does not accompany DNA repair, as previously concluded (37). We tested this hypothesis in experiments that follow.

Single-cell analysis by LSC of effect of high NaCl on HeLa cells. The Western blots in Fig. 1A demonstrate that γH2AX is induced and apoptosis occurs in HeLa cells when NaCl is acutely elevated to a total osmolality of 500 mosmol/kg H2O or above. The following experiments were conducted to see whether γH2AX is induced specifically in apoptotic cells. We labeled proteins by immunocytochemistry and then measured γH2AX and markers of apoptosis in individual cells by LSC. We identified apoptotic cells with fluorochrome-labeled inhibitor of caspases (FLICA) (39) (Fig. 2). FLICA permeates living cells and binds covalently to active caspases (15). Binding of FLICA (green fluorescence) is evident within 4 h after osmolality is elevated to 500 mosmol/kg H2O by adding NaCl (Fig. 2A). We counted the number of cells labeled with FLICA by LSC at each osmolality. The number of FLICA-positive cells does not increase when osmolality is increased to 400 mosmol/kg H2O by adding NaCl (Fig. 2B). There is a small, but significant increase at 500 and a much larger increase at 600 mosmol/kg H2O (Fig. 2B). In addition to labeling of cells with FLICA, apoptotic cells can be identified by the chromatin condensation that occurs during apoptosis (6, 17). The intensity of DAPI staining increases when the chromatin in nuclei is condensed, raising maximal DAPI pixel intensity [DAPI (MP)]. Also, chromatin condensation reduces the size of the nucleus and, thus, the nuclear area. Cells with both increased DAPI (MP) and decreased nuclear area appear at 500 mosmol/kg H2O (Fig. 2C, left). FLICA binds to the cells with condensed chromatin (Fig. 2C, right), confirming the occurrence of apoptosis. These experiments demonstrate that individual HeLa cells survive acute addition of NaCl to 400 mosmol/kg H2O but are killed by apoptosis at 500 mosmol/kg H2O and above. In addition, they show that increased DAPI (MP) can be used to identify apoptotic HeLa cells.

We next tested whether the cells in which γH2AX is induced are apoptotic. When osmolality is increased by adding NaCl, the same cells whose γH2AX is increased have condensed chromatin, indicative of apoptosis (Fig. 3A). By the use of LSC, we extended this analysis to the entire cell population (Fig. 3B). Apoptotic cells [increased DAPI (MP) and decreased nuclear area, Fig. 3B, left] have a higher level of γH2AX than...
Apoptotic cells appear at 500–600 mosmol/kgH₂O but not at 400 mosmol/kgH₂O, confirming identification of apoptotic cells. Four hours after NaCl is added, FLICA binding and FLICA fluorescence intensity [FLICA (MP)] increase in cells containing condensed chromatin (red), which binds to active caspases in apoptotic cells. Cellular DNA was stained with 4,6-diamidino-2-phenylindole (DAPI; blue) after fixation. A: confocal images of cells exposed to 500 mosmol/kgH₂O (NaCl added) for 4 h. FLICA is present in cells containing condensed chromatin, characteristic of apoptosis. B: quantification of FLICA-positive cells. Blue (DAPI) and green (FLICA) fluorescence was measured in each cell by LSC. Left: FLICA maximal pixel fluorescence intensity [FLICA (MP)] vs. DAPI integral fluorescence. Right: percentage of cells with FLICA fluorescence greater than at 300 mosmol/kgH₂O. Values are means ± SE; n = 3, *P < 0.05.

C: correlation between chromatin condensation in apoptotic cells and FLICA binding. Left: nuclear area vs. DAPI (MP). Apoptotic cells (Apo; red) have decreased nuclear area and increased DAPI brightness due to chromatin condensation. Right: FLICA fluorescence intensity of cells with unchanged (black) or condensed (red) chromatin. Chromatin condensation correlates with FLICA binding, confirming identification of apoptotic cells. Four hours after NaCl is added, apoptotic cells appear at 500–600 but not at 400 mosmol/kgH₂O.

Figure 5 shows the time course of induction of DNA breaks and of H2AX phosphorylation after elevation of osmolality to 400 or 600 mosmol/kgH₂O by (NaCl added). We chose these particular osmolalities because they produce completely opposite results. HeLa cells adapt to 400 mosmol/kgH₂O without cell death, whereas elevation to 600 mosmol/kgH₂O kills all the cells by apoptosis (Figs. 1–3). The number of DNA breaks increases within 5–15 min to about the same extent at both 400 and 600 mosmol/kgH₂O (Fig. 5, A, B, and E). At 400 mosmol/kgH₂O, the number of DNA breaks remains elevated to the same extent through the duration of the experiment. In contrast, the number of DNA breaks increases sharply after 90 min in cells exposed to 600 mosmol/kgH₂O (Fig. 5E). Apoptosis and induction of γH2AX do not occur at 400 mosmol/kgH₂O (Fig. 5, C, F, and G). In contrast, although γH2AX is not induced during first 60 min at 600 mosmol/kgH₂O (Fig. 5, D, F, and G), it is markedly induced after 90 min, associated with a sharp increase in the number of DNA breaks (Fig. 5, D, F, and G).

We previously found that mIMCD3 cells adapted to high NaCl maintain an elevated number of DNA breaks for as long as NaCl remains elevated but that the breaks are rapidly repaired when osmolality is lowered to 300 mosmol/kgH₂O by removing the excess NaCl (8, 12). The situation is the same for HeLa cells. Elevating osmolality continuously to 400 mosmol/kgH₂O by adding NaCl increases DNA breaks in HeLa cells for at least 14 days, but the breaks are repaired within 2 h when NaCl is reduced (Fig. 6).

Figure 3. High NaCl increases histone H2AX phosphorylation in HeLa cells, but predominantly in those that are apoptotic, as indicated by condensation of chromatin. HeLa cells were exposed to high NaCl for 4 h and then immunostained for γH2AX. A: confocal images of cells exposed to 500 mosmol/kgH₂O (NaCl added). γH2AX-positive cells contain condensed chromatin, characteristic of apoptosis. B: correlation between chromatin condensation and γH2AX staining. Left: nuclear area vs. DAPI (MP). The cells indicated in red have decreased nuclear area and increased DAPI brightness due to the chromatin condensation characteristic of apoptosis. Right: γH2AX fluorescence intensity of cells with unchanged (black) or condensed (red) chromatin.

Time course of high NaCl-induced DNA breaks, apoptosis, and induction of γH2AX in HeLa cells. In mIMCD3 cells (8, 12, 21) and in mouse renal inner medullary cells in vivo (12), the number of DNA breaks is elevated when they are exposed to a level of NaCl that is high but one which they survive. The DNA breaks persist as long as NaCl remains high, but they are rapidly repaired when NaCl is reduced. In the present experiments, we used a comet assay (11) to estimate the number of DNA breaks in HeLa cells exposed to different levels of NaCl.
and Nbs1 to DNA increases (Fig. 7). In contrast, at 600 mosmol/kgH2O binding of Mre11 A time point, DNA breaks were measured by comet assay and sis decreases the amount of Mre11 bound to DNA (13). In the present studies, we tested for this in HeLa cells. Elevating osmolality to 400 mosmol/kgH2O by adding NaCl for 2 h or more does reversibly reduce the nuclear-to-cytoplasmic ratio of Mre11 in HeLa cells (Fig. 8), but the effect is smaller than previously observed in mIMCD3 cells (8).

Figure 9 shows the time course of cellular survival, of the appearance of DNA breaks, and of induction of γH2AX in mIMCD3 cells. In our previous studies of mIMCD3 cells, we increased NaCl only to a level that the cells survived. We found an increased number of DNA breaks that persisted without inducing γH2AX for as long as NaCl remained high. In the present studies, for comparison with HeLa cells, we repeated the previous studies both at 500 mosmol/kgH2O, which mIMCD3 cells survive, and also at 700 mosmol/kgH2O, which kills mIMCD3 cells by apoptosis (25, 35).

Effects of high NaCl on Mre11 and Nbs1 in HeLa cells. The nuclear MRN complex normally binds to DNA breaks and participates in DNA repair (5). We previously found, using mIMCD3 cells, that elevation of NaCl to a level which increases the number of DNA breaks without triggering apoptosis decreases the amount of Mre11 bound to DNA (13). In the present studies, we studied whether also occurs in HeLa cells. Elevating osmolality to 400 mosmol/kgH2O by adding NaCl for 4 h reduces the amounts of Mre11 and Nbs1 bound to DNA, as does longer adaption to 400 mosmol/kgH2O (Fig. 7A). In contrast, at 600 mosmol/kgH2O binding of Mre11 and Nbs1 to DNA increases (Fig. 7A). Proteins bound to breaks in DNA form foci that can be visualized by immunostaining (16). In HeLa cells, Mre11 and Nbs1 are evenly distributed within the nucleus at 300 and 400 mosmol/kgH2O but localize in foci at 600 mosmol/kgH2O. This suggests that activation of Mre11 and Nbs1 by high NaCl occurs only at levels of NaCl that kill the cells by apoptosis. It is interesting to note that formation of MRN foci at 600 mosmol/kgH2O occurs much earlier than induction of γH2AX and apoptotic DNA fragmentation. Thus Mre11 foci are already present in almost all cells 30 min after elevation of osmolality to 600 mosmol/kgH2O (Fig. 9, Supplemental Fig. 2) while apoptotic DNA fragmentation and H2AX phosphorylation do not start until 90 min after the increase in osmolality (Fig. 5). It is important to note that, although Mre11 foci are induced in this case, repair of DNA does not occur and DNA fragmentation continues through apoptotic cell death.

We also previously found in mIMCD3 cells that elevation of NaCl to a level that increases the number of DNA breaks without triggering apoptosis causes a substantial fraction of Mre11 to move from nucleus to cytoplasm (8, 12). In the present studies, we tested for this in HeLa cells. Elevating osmolality to 600 mosmol/kgH2O by adding NaCl for 2 h or more reversibly reduces the nuclear-to-cytoplasmic ratio of Mre11 in HeLa cells (Fig. 8), but the effect is smaller than previously observed in mIMCD3 cells (8).

Figure 5. Time course of appearance of DNA breaks and phosphorylation of H2AX in HeLa cells exposed to survivable (400 mosmol/kgH2O) or lethal (600 mosmol/kgH2O) levels of high NaCl. NaCl was elevated to final osmolality of 400 or 600 mosmol/kgH2O for the times indicated (from 5 to 180 min). At each time point, representative confocal images of the cells are shown (green, H2AX; blue, DAPI) (top). Fluorescence intensities were measured by LSC. A: quantification of viable cells (no chromatin condensation) with elevated H2AX vs. cleaved caspase 3, and counterstained with DAPI for DNA. Fluo-

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Figure 9 shows the time course of cellular survival, of the appearance of DNA breaks, and of induction of γH2AX when osmolality is elevated to either 500 or 700 mosmol/kgH2O by adding NaCl. Although an increase to 500 mosmol/kgH2O does not change the appearance or number of mIMCD3 cells (Fig. 9, A and F), 700 mosmol/kgH2O kills the cells progressively until almost all cells have detached by 30 h (Fig. 9, A and F). At 700 mosmol/kgH2O, apoptosis begins after 6–8 h, followed by the decrease in the number of attached cells (Fig. 9A). The number of DNA breaks increases within 1 h at both 500 and 700 mosmol/kgH2O (Fig. 9, B, C, and G). At 500 mosmol/kgH2O, the increased number of DNA breaks remains essentially constant through the duration of the experiment (Fig. 9, B and G). In contrast, the number of DNA breaks increases further after ~10 h in cells exposed to 700 mosmol/kgH2O (Fig. 9G), as the cells become apoptotic (Fig. 9C). Neither apoptosis nor induction of γH2AX occurs at 500 mosmol/kgH2O (Fig. 9, B, D, H, and I). At 700 mosmol/
kgH₂O, γH2AX increases after 1–2 h (Fig. 9E). This initial increase occurs in cells that do not yet display the increased DAPI (MP) associated with apoptotic chromatin condensation (Fig. 9E). A further sharp increase in γH2AX occurs later, as the cells become obviously apoptotic and DAPI (MP) increases (Fig. 9E).

Thus, when NaCl is elevated to levels that HeLa and mIMCD3 cells tolerate (lower for HeLa than for mIMCD3), both types of cells respond similarly. DNA breaks rapidly increase in both cell types, γH2AX is not induced, and the breaks persist as long as NaCl remains high. Lethally high levels of NaCl also produce essentially the same effect in both cell types, namely, apoptosis, greatly increased DNA fragmentation, and induction of γH2AX. Only the timing of apoptosis differs. Apoptosis occurs faster in HeLa cells, with virtually
simultaneous induction of γH2AX and chromatin condensation (Fig. 5). In mIMCD3 cells, on the other hand, a modest increase in γH2AX precedes apoptosis by several hours. In neither cell type does induction of γH2AX prevent or repair the progressive apoptotic DNA breaks.

**DISCUSSION**

Acute hypertonicity increases the number of DNA breaks (8, 21). While this conclusion apparently is generally accepted, disagreement has arisen about whether the breaks are promptly
repairs like DNA breaks from other causes (37) or whether hypertonicity inhibits repair of the breaks, so they persist as long as the osmolality remains elevated (9, 12, 14). The purpose of the present experiments was to resolve this controversy.

Nature of high NaCl-induced DNA damage. The initial studies of DNA damage caused by high NaCl (21) were motivated by previous findings that high NaCl causes genomic stress manifest by increased expression of p53 (7) and Gadd45 (22) and cell cycle delay (22, 25). Since neutral comet and pulsed-field gel electrophoresis assays showed increased breaks, the breaks were initially interpreted as double-strand breaks (DSBs), following the assumption that those assays detect DSBs (21). We later confirmed that breaks occur by using alkaline comet and BrdU TUNEL assays, and, although these methods measure single-strand breaks as well as DSBs (11, 27), we assumed that DSBs were present, as concluded from the initial observations (21). However, later we became aware that neutral comet and pulsed-field gel electrophoresis assays can detect single-strand breaks as well as DSBs (4, 11, 28). Therefore, we now remain uncertain of how many of the DNA breaks caused by high NaCl are DSBs, as opposed to other forms of DNA damage. We have continued to use alkaline comet and BrdU comet assays to measure DNA breaks but now understand that there may be single-strand breaks as well as DSBs. We are uncertain at this point of the exact nature of the increased DNA breaks, only that they occur.

Inhibition of repair of DNA breaks by high NaCl. The recent conclusion that cells promptly repair hypertonicity-induced DNA breaks in the same way that they do other forms of DNA damage was based mainly on studies of HeLa cells (37). It is important to note that those studies (37) did not directly address the issue since they did not include any direct measurements of DNA breaks or of their repair, but instead they relied solely on measurements of the activity of DNA damage response proteins. As demonstrated in the present experiments, HeLa cells have a relatively low tolerance for high NaCl, and the observed activation of these DNA damage proteins occurs in context of fragmentation of DNA, not during repair of DNA. In contrast, our numerous direct measurements of DNA breaks and repair show that in viable cells both in cell culture (Figs. 5, 6, and 9) (12) and in vivo (12, 14) the number of high NaCl-induced DNA breaks does not decrease as long as the hypertonicity persists but does decrease rapidly when the level of NaCl is reduced. Furthermore, our direct measurements of DNA repair both in cell culture and in vivo show that hypertonicity inhibits repair of DNA breaks (8, 12) whether the breaks are caused by the hypertonicity itself or by radiation (reviewed in Ref. 3). These findings are not limited to renal medullary cells or even to mammalia. High NaCl causes persistent DNA breaks in the soil nematode C. elegans (13) and in marine invertebrates from several different phyla (14). Strikingly, the DNA breaks in marine invertebrates are repaired when the osmolality of sea water is gradually reduced to 300 mosmol/kgH2O (14), which is well below the level at which they normally exist.

High NaCl-induced apoptosis. High NaCl kills cells by apoptosis (reviewed in Ref. 3). Since there is massive DNA fragmentation during apoptosis, it is important, when high NaCl-induced DNA damage is analyzed, to ascertain whether apoptosis is occurring. In the original experiments, demonstrating high NaCl-induced DNA breaks in mIMCD3 cells, apoptosis was excluded as a cause of the breaks (12, 21). However, osmotic tolerance differs widely among cell types. For example, acute elevation of osmolality to 500 mosmol/kgH2O by adding NaCl induces massive apoptosis in a macrophage cell line (U937 cells) (23), whereas primary cells from the mouse renal inner medulla survive up to 900 mosmol/kgH2O (43). In the present experiments, we find that acute elevation of NaCl to a total osmolality of 600 mosmol/kgH2O kills all HeLa cells by apoptosis within a few hours (Figs. 1, 2, and 5), whereas most mIMCD3 cells survive (25). Acute elevation to 700 mosmol/kgH2O eventually kills all mIMCD3 cells, but the time of death varies widely from cell to cell and is delayed compared with HeLa cells (Fig. 9).

High NaCl-induced inhibition of activation of DNA damage response proteins. To understand how high NaCl inhibits repair of DNA breaks, we examined the effect of high NaCl on several DNA damage response proteins. When DNA breaks occur in the absence of hypertonicity, an MRN complex assembles at sites of DNA damage, and H2AX and Chk1 become phosphorylated (5, 8, 24, 30). However, despite the fact that high NaCl increases DNA breaks in viable cells, MRN foci do not form (Fig. 7 and Supplemental Fig. 2), a portion of Mre11 exits from the nucleus, and H2AX and Chk1 do not become phosphorylated (Fig. 8) (8). Even when the mIMCD3 cells become chronically adapted to high NaCl and are proliferating, Mre11 exonuclease is mainly present in the cytoplasm, rather than the nucleus, and histone H2AX and Chk1 are not phosphorylated (12). Strikingly, when osmolality is reduced to 300 mosmol/kgH2O after acute or chronic hypertonicity, Mre11 moves back into the nucleus and H2AX and Chk1 become phosphorylated, associated with repair of the DNA breaks. Furthermore, in the renal inner medulla in vivo, total body radiation causes DNA breaks without phosphorylation of H2AX, but if the high interstitial NaCl is lowered by furosemide, H2AX rapidly becomes phosphorylated and DNA repair synthesis is induced (12). Based on these correlations, we proposed that these changes in the DNA damage response proteins could contribute to hypertonicity-induced...
slowing of the repair of DNA damage and increased number of DNA breaks (8, 9, 12). However, we recognize that we do not know how much any of these particular changes contributes to inhibition of DNA repair or what other factors might be involved.

Activation of DNA damage response proteins during apoptosis. The conclusion that high NaCl inhibits repair of DNA damage and prevents activation of DNA damage-induced proteins was called into question by the observation that in HeLa cells exposed to high NaCl (500–600 mosmol/kg H₂O total osmolality), the MRN complex stayed in the nucleus and has not yet occurred (Fig. 5). However, although not yet condensed and whose apoptotic DNA fragmentation Mre11 and Nbs1 (Fig. 7) are evident until 1 h later. Similarly, γH2AX appears, interpreted as indicating activation of DNA repair (37). However, DSBs generated during apoptotic DNA fragmentation are also known to trigger induction of γH2AX (33) as well as other DNA damage response proteins, including ATM, chk2, and the MRN complex (29, 31, 40, 42). In mIMCD3 cells, γH2AX appears in cells that will become apoptotic while fragmentation of DNA is still low and precedes chromatin condensation (Fig. 9). The same is true for formation of MRN foci in HeLa cells, as seen in Fig. 7B and Supplemental Fig. 2. Within 1 h after osmolality was raised to 600 mosmol/kg H₂O, foci of Mre11 and Nbs1 appear in cells that are destined to become apoptotic, but whose chromatin is not yet condensed and whose apoptotic DNA fragmentation has not yet occurred (Fig. 5). However, although γH2AX is induced and foci of MRN form, the existing DNA breaks are not repaired and apoptotic DNA fragmentation is not prevented.

High NaCl-induced DNA breaks in nonapoptotic cells. Increasing osmolality to a relatively low level that HeLa cells can survive (400 mosmol/kg H₂O, NaCl added), still causes DNA breaks, accompanied by significant movement of Mre11 into the cytoplasm (Fig. 8) and does not increase phosphorylation of H2AX (Fig. 5), similar to the result in mIMCD3 cells at higher osmolality. Also, the DNA breaks induced at 400 mosmol/kg H₂O in HeLa cells do not induce nuclear foci of Mre11 and Nbs1 (Fig. 7B). Thus elevation of NaCl to a level that HeLa cells survive induces DNA breaks without formation of MRN foci or induction of γH2AX. We conclude that apoptosis, not DNA repair, explains high NaCl-induced formation of the MRN complex and induction of γH2AX, and that, when their different osmotic tolerance is taken into account, the effects of high NaCl on DNA and its repair are essentially the same in HeLa cells as in mIMCD3.

Perspective. A model of high NaCl-induced DNA breaks, DNA damage response, and apoptosis is presented in Fig. 10. Each particular type of cell has a threshold of tolerance for acute elevation of NaCl. Below that threshold, cells continue to proliferate (after an initial cell cycle delay) (reviewed in Ref. 3) and function more or less normally. Acutely elevating NaCl above the threshold kills the cells by apoptosis within several hours (reviewed in Ref. 3). Regardless of whether the threshold is exceeded, the number of DNA breaks increases immediately. Below the threshold, the increased number of breaks stays unchanged as long as NaCl remains high. Above the threshold, after some hours, apoptosis occurs, accompanied by a large increase in the number of DNA breaks. The time lag to apoptosis differs among cell types. It is very rapid (~1.5 h) in HeLa cells and much slower (~10 h) in mIMCD3 cells. Although whether the cells eventually survive the initial increase in DNA breaks is essentially the same, the response of DNA damage response proteins differs. The MRN complex and γH2AX are not induced below the threshold of osmotic tolerance, despite the DNA breaks, but above the threshold, they are activated in cells that will become apoptotic. In the present study, Mre11 foci appear in HeLa cells as early as 30 min after NaCl increases to a lethal level, while apoptosis is not evident until 1 h later. Similarly, γH2AX appears in mIMCD3 cells within 2 h after NaCl increases to a lethal level, while apoptosis appears about 5 h later, at which time γH2AX increases much further. Although following other forms of DNA damage, γH2AX and MRN complexes initiate DNA repair, DNA is not successfully repaired when NaCl is lethally high.

Taken together, our results confirm that when cells are acutely exposed to levels of NaCl that are high, but which the cells survive, DNA breaks increase and persist until NaCl is lowered. Since failure to repair DNA breaks promptly usually is bad (1), it has been puzzling how the normally high renal medullary NaCl could raise the number of breaks without other apparent adverse effects. However, we recently identified at least one adverse effect, namely, that prolonged exposure to high NaCl induces cellular senescence (10), a known consequence of exposure to other stresses of various types, including increased DNA breaks (2, 36). Senescent cells accumulate faster with age in mouse renal inner medullas, where osmolality is high, than in the cortex, where osmolality is lower. The effect is slow, however, requiring months of constant exposure to high NaCl. We have proposed that cells adapted to high NaCl enter a new state with multiple complementary changes, including some that would be considered pathological under other circumstances. Interestingly, in the altered state, some of the seemingly pathological changes, including DNA lesions, elevated reactive oxygen species, and cytoskeletal rearrangement, may actually be protective in the sense that they apparently serve as sensors for activation of the osmoprotective transcription factor toxicity-responsive enhancer/osmotic response element-binding protein (reviewed in Ref. 3).

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