Mre11-Rad50-Nbs1 complex is activated by hypertonicity

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Sheen, Mee Rie, Seung Whan Kim, Ju-Young Jung, Joon Young Ahn, Juong G. Rhee, H. Moo Kwon, and Seung Kyoong Woo. Mre11-Rad50-Nbs1 complex is activated by hypertonicity. Am J Physiol Renal Physiol 291: F1014–F1020, 2006; doi:10.1152/ajprenal.00153.2006.—When exposed to hypertonic conditions, cells accumulate double-strand DNA breaks (DSBs) like they are exposed to ionizing radiation. It has been proposed that inactivation of the Mre11-Rad50-Nbs1 (MRN) complex due to nuclear exit is responsible for the accumulation of DSBs as cells fail to repair DSBs produced during normal cellular activity. In this study, we examined the MRN complex in cells switched to hypertonicity. Surprisingly, we found that the MRN complex stayed in the nucleus and remained intact in response to hypertonicity. In fact, the MRN complex was dramatically activated after 4 h of switch to hypertonicity in a dose-dependent manner as shown by formation of foci. Activation of ATM and the MRN complex by hypertonicity and bleomycin was additive as was activation of their downstream targets including γH2AX and Chk2 indicating that the cellular response to DSB was intact in hypertonic conditions. Activation of Chk2 in response to hypertonicity was not observed in mutant cells with functionally impaired MRN complex confirming that they are in the same pathway. After 20 h of a switch to hypertonicity, MRN foci and γH2AX returned to a control level, suggesting that cells adapted to hypertonicity by repairing DNA. We conclude that cells respond normally to DSB and repair the DNA damages induced by hypertonicity.

ATM; double-strand DNA breaks; renal medulla; cell cycle checkpoint; DNA repair; histone H2AX; Chk2

A UNIQUE FEATURE of the mammalian kidney is that the osmolality of the medulla is very high. Osmolality of the renal medulla reaches over 1,000 mosmol/kgH2O in humans and over 3,000 mosmol/kgH2O in the mouse depending on the status of hydration (3). The hyperosmolality is essential for the urinary concentration as it provides driving force for water reabsorption in the medullary collecting duct (15). Unfortunately, hyperosmolality imposes a great deal of stress on cells. Salt and urea, the two principal solutes in the interstitium of the renal medulla, differ in their cellular effects. Hyperosmotic salt is hypertonic and causes double-strand DNA breaks (DSB) like ionizing radiation (18). Urea does not cause DSB but induces single-strand DNA breaks and oxidative lesions (30).

The cellular response to DSB caused by ionizing radiation has been extensively studied. Upon DSB, eukaryotic cells stop progression in the cell cycle until the damage is repaired or else activate an apoptotic pathway to avoid propagation of the mutations (2, 22). The Mre11-Rad50-Nbs1 (MRN) complex and ATM are crucial for the initiation of signaling in response to DSB (2). Genetic mutations in ATM, Mre11, and Nbs1 cause ataxia telangiectasia (AT), AT-like disorder (ATLD), and Nijmegen breakage syndrome (NBS), respectively. Patients with these diseases display similar symptoms, indicating that the functions of the proteins are related (27). The MRN complex physically associates with DSB and is involved in DNA repair by homologous recombination and nonhomologous end joining (2, 27). The MRN complex also contributes to the signaling of ATM. In response to DSB, ATM is activated via autophosphorylation on Ser1981 (1). Although activated ATM phosphorylates p53 at Ser15 and ATM itself at Ser1981 via autophosphorylation on Ser1981 (1). Although activated ATM phosphorylates p53 at Ser15 and ATM itself at Ser1981, p53 is phosphorylated at Ser15. ATM in response to ionizing radiation (2, 16).

Recently, several studies examined ATM and the MRN complex in response to hypertonicity (9–12, 14). Although ATM and p53 are activated by hypertonicity (11, 12, 14), Mre11 is inhibited as it is shifted from the nucleus to the cytoplasm (9). The cytoplasmic shift of Mre11 is associated with disruption in some of the DNA damage signaling pathways in that phosphorylation of histone H2AX (γH2AX) in response to ionizing radiation and Chk1 in response to UV are inhibited in hypertonic conditions (9). It has been proposed that inhibition of Mre11 accounts for DSB in response to hypertonicity (10).

This study was undertaken to investigate the molecular mechanism underlying the cytoplasmic shift of Mre11 by hypertonicity. Unexpectedly, we did not observe the cytoplasmic shift of Mre11 in response to hypertonicity. Our data demonstrate that Mre11 is activated in response to hypertonicity leading to stimulation of the downstream events including phosphorylation of H2AX and Chk2.

MATERIALS AND METHODS

Cell culture and treatments. HeLa, immortalized mouse embryonic fibroblast (MEF), and COS7 cells were maintained in DMEM supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Immortalized mouse inner medullary collecting duct (mIMCD3) cells were maintained in DMEM/F12 (1:1) supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin (29). LX1N and NBS1 cell lines were maintained

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in DMEM supplemented with 15% fetal bovine serum, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 μg/ml streptomycin, and 500 μg/ml G418 (5). LX1N and NBS1 cells were derived from an immortalized human fibroblast cell line originated from a patient with a hypomorphic mutation in Nbs1 (NBS-ILB1) by transfecting an expression vector alone and with the human Nbs1 gene, respectively (5). Introduction of the Nbs1 gene corrected the phenotypes observed in the NBS cells including radiation sensitivity and inability to form MRN foci (5). To make hypertonic medium, additional 100 mM or 150 mM NaCl was added to the medium as indicated. To study effects of DNA-damaging agents, cells were treated with 10 mU/ml bleomycin, 1% Triton X-100, and protease inhibitor cocktail (Roche) for 30 min at 4°C. Protein concentration was determined by Bradford method (Bio-Rad).

Immunoprecipitation analysis. To prepare total lysate, cells were lysed in a lysis buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% Triton X-100, and protease inhibitor cocktail (Roche) for 30 min at 4°C. Protein concentration was determined by BCA (Pierce) or the Bradford method (Bio-Rad).

To immunoprecipitate Mre11 from the lysate, 150 μg of total lysate were incubated with 2 μl of anti-Mre11 antiserum (Novus Biologicals) for 1 h at 4°C and further incubated for 1 h after addition of 40 μl of 50% slurry of protein A-conjugated agarose beads (Sigma). The beads were then separated from the supernatant by centrifugation.

Immunocytochemistry and detection of MRN foci. Cells grown on glass coverslips were fixed for 15 min in 4% paraformaldehyde in PBS and permeabilized with 0.5% Triton X-100 in PBS-buffered saline (TBS) for 15 min. Antibodies for Nbs1 (Novus Biologicals) and Mre11 (Novus Biologicals) were diluted to 1:200 in PBS and further incubated for 1 h after addition of 40 μl of 50% slurry of protein A-conjugated agarose beads (Sigma). The beads were then separated from the supernatant by centrifugation. After being washed with 1 ml of the lysis buffer twice, the immune-complex containing Mre11 was eluted from the beads with SDS sample buffer. To facilitate comparison of abundance of the MRN complex between supernatant and precipitated fraction, the volume of the eluted sample was increased to the identical volume of the supernatant.

Immunoblot analysis. Total lysates were prepared as described above for immunoblot analysis except for immunoblot analysis of phospho-H2AX. Nuclear and cytoplasmic extracts were prepared using NE-PER kit from Pierce. For immunoblot analysis of phospho-H2AX, lysates were prepared in an acid extraction buffer containing 10 mM HEPES (pH 7.9), 1.5 mM MgCl2, 10 mM KCl, and 0.2 N HCl. The resulting lysates were dialyzed against 0.1 N acetic acid and water for 1 h each as described (17). An equal amount of the total lysate was separated on a SDS-polyacrylamide gel and blotted onto a polyvinylidene difluoride (PVDF) membrane. To detect a specific protein, the blots were incubated with individual antiserum/antibody in PBS containing 0.1% Tween 20 and 5% nonfat milk. Antibodies for phospho-H2AX (Ser139), Chk1, phospho-Chk1 (Ser345), Chk2, and phospho-Chk2 (Thr68) were purchased from Cell Signaling Technology. Antibodies for Nbs1, Nbs1, and Rad50 were purchased from Novus Biologicals. Anti-phospho-ATM (Ser1981) antiserum was obtained from Rockland. Antibodies for Ku80 (Abcam), H2A (Upstate), H2AX (Stressgen), and H2B (Stressgen) were purchased. Antibodies for Ku80, another protein with affinity to DBD, were purchased from Stressgen. The MRN complex is not disrupted by hypertonicity. Nuclear localization of Mre11 is dependent on the integrity of the MRN complex because Mre11 is cytoplasmic in the Nbs1-mutant cell lines (4, 5). We hypothesized that the cytoplasmic shift of Mre11 in response to hypertonicity (9) was due to disruption of the MRN complex. To test the hypothesis, we examined the integrity of the MRN complex employing coimmunoprecipitation analysis (Fig. 1). HeLa cells were cultured in isotonic or hypertonic medium containing additional 100 or 150 mM NaCl, and the MRN complex was immunoprecipitated using an Mre11 antibody. Practically all Mre11 was immunoprecipitated along with all of Nbs1. On the other hand, about half of Rad50 was brought down with Mre11. Abundance of β-actin was measured to validate equal loading. The immunoprecipitation was specific because Ku80, another protein with affinity to DBD, was not precipitated. More importantly, the pattern more than five foci per nucleus was counted. The experiment was repeated four times, and results were represented as means ± SE.

RESULTS

The MRN complex is not disrupted by hypertonicity. Nuclear localization of Mre11 is dependent on the integrity of the MRN complex because Mre11 is cytoplasmic in the Nbs1-mutant cell lines (4, 5). We hypothesized that the cytoplasmic shift of Mre11 in response to hypertonicity (9) was due to disruption of the MRN complex. To test the hypothesis, we examined the integrity of the MRN complex employing coimmunoprecipitation analysis (Fig. 1). HeLa cells were cultured in isotonic or hypertonic medium containing additional 100 or 150 mM NaCl, and the MRN complex was immunoprecipitated using an Mre11 antibody. Practically all Mre11 was immunoprecipitated along with all of Nbs1. On the other hand, about half of Rad50 was brought down with Mre11. Abundance of β-actin was measured to validate equal loading. The immunoprecipitation was specific because Ku80, another protein with affinity to DBD, was not precipitated. More importantly, the pattern more than five foci per nucleus was counted. The experiment was repeated four times, and results were represented as means ± SE.

Immunohistochemistry of rat kidney. All animal studies were approved by the Institutional Animal Care and Use Committee at the University of Maryland. Kidneys from male Sprague-Dawley rats producing average urine osmolality of 1,760 mosmol/kg H2O (n = 3) were used. Periodate-lysine-paraformaldehyde (PLP) was used to fix the kidneys for optimal results with the immunoperoxidase staining (28). The fixed kidneys were embedded in wax and cut serially at a thickness of 5 μm on a rotary microtome (Leica). The sections were washed with PBS, blocked with normal donkey serum (Jackson ImmunoResearch Laboratories), and incubated with diluted (1:500) Mre11 antibody (Novus Biologicals) overnight at 4°C. After being washed with PBS, the sections were incubated with biotinylated secondary antibody for 2 h and further incubated with Vectastain ABC reagent (Vector Laboratories) containing avidin and biotinylated horseradish peroxidase. To detect expression of the proteins, the sections were incubated with a mixture of 0.05% 3,3′-diaminobenzidine and 0.01% H2O2 for 2 min at room temperature. The images were observed under a light microscope.

The MRN complex is not disrupted by hypertonicity.
and efficiency of coimmunoprecipitation were not affected by medium hypertonicity. These results do not support the hypothesis that the integrity of the MRN complex is disrupted by hypertonicity.

The MRN complex stays in the nucleus in hypertonicity. To directly measure nucleocytoplasmic shift of the MRN complex, HeLa cells were fractionated into nuclear and cytoplasmic fractions and analyzed by immunoblotting (Fig. 2A). The fractionation was validated by immunoblots analyses of the cytoplasmic marker lactate dehydrogenase (LDH) and a nuclear protein toxicity-responsive enhancer binding protein (TonEBP). Mobility of the TonEBP band was slower in hypertonic condition due to phosphorylation (6). The constitutive form of heat shock protein 70 (HSC70) was measured as a loading control for both the cytoplasmic and nuclear fractions as described earlier (25). We noted that the cytoplasmic abundance of Mre11, Nbs1, and Mre11 increased moderately in response to hypertonicity. However, these proteins were predominantly detected in the nuclear fraction of HeLa cells cultured in isotonic or hypertonic medium. Because nuclear localization of the MRN complex is critical for its function (9, 10), these data suggest that the MRN complex is unaffected by hypertonicity.

Next, immunocytochemistry was performed on HeLa cells cultured in isotonic or hypertonic medium (Fig. 2B). Mre11 (green) colocalized with Nbs1 (red) as indicated by yellow in merged images. In hypertonic conditions, there were changes in the staining pattern of the MRN complex within the nuclei, more signal on the nuclear periphery and aggregations within the nucleoplasm. This pattern was unique to HeLa cells and not seen in other cells. At any rate, the MRN complex was predominantly nuclear in both isotonic and hypertonic conditions. These data confirm the results of Fig. 2A that in HeLa cells the MRN complex does not exit the nucleus in hypertonic conditions.

Because mIMCD3 cells were used in the report of the cytoplasmic shift of Mre11 in response to hypertonicity (9, 10), we examined the MRN complex in these cells. Although all the available antibodies of Mre11, Nbs1, and Rad50 did not work in immunocytochemistry, we were able to detect Mre11 in mIMCD3 by immunoblotting as in the previous studies (9, 10). Mre11 was detected predominantly in the nuclear fraction, and hypertonicity did not affect the nuclear localization (data not shown). In sum, we did not observe a cytoplasmic shift of Mre11 in response to hypertonicity in HeLa or mIMCD3 cells. Mre11 was nuclear in both isotonic and hypertonic conditions.

Nuclear localization of Mre11 in kidney in vivo. We examined localization of Mre11 in the kidney for two reasons. First, nonphysiological artifacts associated with cultured cells would be eliminated. Second, cells in isotonic conditions (cortex) as well as those in hypertonic conditions (medulla) would be examined. Figure 3 summarizes immunohistochemical analysis of Mre11 in normal rat kidneys. In the isotonic cortex, immunoreactivity was not detected in the glomerulus, Bowman’s capsule, and proximal tubule, whereas moderate immunoreactivity was seen in the nuclei of distal tubule and cortical collecting duct (Fig. 3, A to C). In the outer medulla, the immunoreactivity was prominent in the nuclei of the collecting duct, whereas it was weak in the descending thin limb and thick ascending limb (Fig. 3D). In the inner medulla, intense immunoreactivity was present in the nuclei of the inner medullary collecting duct and medullary interstitial cells (Fig. 3, E and F). Overall, Mre11 expression was higher in the renal medulla than the cortex. As in HeLa or mIMCD3 cells, Mre11 was nuclear in the isotonic cortex and hypertonic medulla demonstrating that hypertonicity did not affect the nuclear localization of the MRN complex in the kidney.

Hypertonicity induces MRN foci. Because the MRN complex remained nuclear in hypertonic conditions, we asked whether the MRN complex was activated in response to hypertonicity. To answer this question, we measured formation of MRN foci that were known to represent the activated form of the MRN complex (4, 23). In HeLa cells, MRN foci were not observed in response to hypertonicity. Instead, aggregation and concentration in the nuclear boundary were observed. It is not clear whether these changes are related to formation of MRN foci. On the other hand, Nbs1 foci were clearly observed in MEF and COS7 cells in response to hypertonicity in a dose-dependent manner (Fig. 4). Thirty-five percent and 58% of cells contained more than five foci per nucleus of MEF and COS7 cells, respectively, after 2 h in hypertonic medium containing additional 150 mM NaCl compared with 2–7% of

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Fig. 2. Localization of the MRN complex in isotonic and hypertonic conditions. HeLa cells were cultured for 2 h in isotonic medium or hypertonic medium containing additional 150 mM NaCl. A: cells were fractionated into a cytoplasmic (Cyt) and nuclear fractions (Nuc) and immunoblotted using antibodies indicated left. B: Mre11 (Green) and Nbs1 (Red) were detected by immunocytochemistry on HeLa cells grown on coverslips. Merged images are shown at right. Magnification was ×400.
cells in isotonic medium (Fig. 4B). In these cells, the MRN complex was not disrupted by hypertonicity (data not shown) as in HeLa cells, indicating that the Nbs1 foci represented foci of the MRN complex. These data demonstrate that hypertonicity activates the MRN complex presumably because of DSBs generated in response to hypertonicity. The MRN foci disappeared after 20 h of hypertonicity, suggesting that the DSBs were repaired over time (Fig. 4A).

Activation of MRN complex-dependent signaling by hypertonicity. Because we had evidence that the MRN complex was activated by hypertonicity, we examined signaling events downstream of the MRN complex. First, we examined phosphorylation of histone H2AX on Ser139 (γH2AX) which required activated ATM and the MRN complex (2, 17). As shown in Fig. 5A, γH2AX was dramatically induced by hypertonicity in a dose-dependent manner in both HeLa and mIMCD3 cells. The level of γH2AX peaked at 4 h and then returned to basal level in 8 h in mIMCD3 cells (Fig. 5B) and 16 h in HeLa cells (not shown). The time course is consistent with the activation of MRN complex discussed above.

Because previous reports (9, 10) indicated that hypertonicity inhibited DNA repair and signaling in response to ionizing radiation and UV, we examined effects of bleomycin and hydroxyurea in combination with hypertonicity in HeLa cells. Bleomycin induces DSB and activates preferentially ATM, leading to phosphorylation of Chk2 (13, 26). On the other hand, hydroxyurea induces stalled replication forks and activates preferentially ATR, leading to phosphorylation of Chk1 (13, 20). Hypertonicity moderately stimulated phosphorylation of ATM on Ser1981 in a dose-dependent manner (Fig. 5C, 1st panel) as reported earlier (14). Bleomycin strongly stimulated the phosphorylation of ATM as expected. It should be noted that the effects of hypertonicity and bleomycin were additive. While hydroxyurea stimulated moderately the phosphorylation of ATM in isotonic conditions, this was inhibited by hypertonicity. These data demonstrate that activation of ATM in response to DSB is not inhibited by hypertonicity, whereas activation of ATM in response to hydroxyurea is.

Phosphorylation of Chk2 on Thr68 was stimulated by hypertonicity and bleomycin (Fig. 5C, 2nd panel) as expected from the activation of ATM. The effects of hypertonicity and bleomycin on phosphorylation of Chk2 were additive, indicating that cellular signaling by ATM and the MRN complex was...
not inhibited in hypertonic conditions. On the other hand, phosphorylation of Chk1 on Ser345 was not affected by hypertonicity (Fig. 5C, 4th panel). As expected, hydroxyurea stimulated this phosphorylation which was inhibited by hypertonicity. We conclude that while signaling in response to DNA damage by hydroxyurea is inhibited by hypertonicity at the level of ATR and Chk1, signaling in response to bleomycin is not.

The MRN complex is required for phosphorylation of Chk2 in response to hypertonicity. To confirm that the phosphorylation of Chk2 induced by hypertonicity was due to an activated MRN complex, LX1N and NBS1 cells were analyzed. As expected, Nbs1 was not detectable in LX1N cells while a significant amount of Nbs1 was expressed in NBS1 cells as shown in Fig. 6A. Expression of Mre11 was comparable between LX1N and NBS1 cells (Fig. 6A), although nuclear localization of the Mre11 was impaired in the LX1N cells (data not shown) as reported (5). Phosphorylation of Chk2 changed little in LX1N cells in response to hypertonicity (Fig. 6B). On the other hand, in Nbs1 cells the phosphorylation of Chk2 increased by 50 and 120% over control in response to hypertonic media containing additional 100 and 150 mM NaCl, respectively. Phosphorylation of Chk1 was not increased by hypertonicity in either cell line. These results demonstrate that the induction of Chk2 phosphorylation by hypertonicity is dependent on MRN complex as in ionizing radiation (2, 17).

**DISCUSSION**

Using comet assay, we had observed significant DNA damages in mIMCD3 cells cultured for 4 h in hypertonic medium (data not shown) confirming the accumulation of DSBs in response to hypertonicity reported previously (18). The original goal of this study was to investigate the idea (9) that inactivation of Mre11 due to cytoplasmic shift accounts for the hypertonicity-induced accumulation of DSBs as cells fail to repair DSBs generated from normal cellular activity. Our data presented here do not support this idea. We observe that the MRN complex remains intact and stays in the nucleus in hypertonic conditions. Immunohistochemical analysis of kidney confirms the nuclear localization of Mre11 in isotonic and hypertonic conditions in vivo. In fact, we have unequivocal evidence that the MRN complex is activated in response to hypertonicity. When switched to hypertonic conditions, cells...
form MRN foci in a dose-dependent manner. This is associated with activation of downstream signaling pathways including induction of γH2AX and activation of Chk2. The Chk2 activation does not occur in mutant cells with an inactive MRN complex. In aggregation, our data demonstrate that the cellular response to hypertonicity is remarkably similar to that of ionizing radiation in terms of activation of the MRN complex, ATM, and their downstream pathways.

There two areas in which our data do not agree with the previous reports (9, 10). One is that we do not observe the cytoplasmic shift of Mre11 in response to hypertonicity. We find that the MRN complex stays in the nucleus under hypertonic conditions. The other is that we observe clear induction of γH2AX in response to hypertonicity. We do not understand the basis for the differences in observation except to recognize that antibodies were obtained from different sources.

Recent studies have reported that cells in the hypertonic renal medulla or cultured cells adapted to hypertonicity contain numerous DNA breaks (9, 10). These observations are seemingly puzzling because these cells survive, proliferate, and function normally prompting the investigators to propose “alternative DNA damage response pathways” operating under hypertonic conditions (7, 8). Our data provide new insight into this conundrum. First, it should be noted that these studies (9, 10) employed an alkali comet assay and incorporation of BrdUTP by terminal deoxynucleotidyltransferase, both of which detect single-strand DNA breaks as well as DSBs. Our data show that the cellular response to DSB leading to activation of Chk2 is normal. Although the cellular response to hydroxyurea, which induces stalled DNA replication forks, leading to activation of Chk1, is inhibited moderately, it is still quite active. Thus a normal or nearly normal level of DNA damage response pathways are maintained in hypertonic conditions. There is no need for alternative pathways to sense and repair DNA damages.

While we observe MRN foci and induction of γH2AX after 4 h of acute exposure to hypertonicity, they disappear by 20 h. These data suggest that DSBs are repaired in time as cells adapt to hypertonicity. This is consistent with a previous report in which cell cycle arrest is found to be transient, lasting about 18 h, when proliferating mIMCD3 cells are switched to hypertonicity (19). After the initial arrest, cells in hypertonicity proliferate at the same rate as cells in isotonicity, strongly suggesting that DNA damages are minimal, i.e., repaired. There is only one study that used a neutral comet assay to measure exclusively DSBs in cultured cells treated with hypertonicity (18). This study shows that the number of DSBs decreases when cells are kept in hypertonicity longer than 24 h. In addition, treatment with LY 294002 increases the number of DSBs dramatically in hypertonic conditions, suggesting that DSBs are vigorously repaired in hypertonicity. All these data are consistent with the idea that cells in hypertonicity do adapt to hypertonicity and repair DSBs given sufficient time, 18–20 h. In fact, cells in the hypertonic renal medulla do not display MRN foci (Fig. 3), indicating that the cells adapted to hypertonic conditions in vivo do not contain DSBs.

In sum, our data demonstrate that the cellular response to DSB is intact in hypertonicity in terms of activation of the MRN complex, ATM, and their downstream pathways. While the underlying mechanism for generation of DSB in response to hypertonicity is not understood, there is no evidence for derangement of DNA repair due to inactivation of the MRN complex.

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