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Dynamic Monitoring of Oxidative DNA Double Strand Break and Repair in Cardiomyocytes

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Abstract

DNA double strand breaks (DSBs) are most dangerous lesions. To determine whether oxidative stress can induce DSBs and how they are repaired in cardiomyocytes (CMs), cultured neonatal rat CMs were treated with different doses of H₂O₂ and followed for up to 72 hours for monitoring the spatiotemporal dynamics of DNA repair protein assembly/disassembly at DSB foci. The protein levels and foci numbers of histone H2AX phosphorylated at serine 139 (γ -H2AX) increased proportionally to 50, 100 and 200 μ mol/L H₂O₂ after 30 min treatment. When H₂O₂ was at or above 400 μ mol/L, γ -H2AX became predominantly pan-nuclear. After 30 min, 200 μ mol/L of H₂O₂ treatment, γ -H2AX levels were highest within the first hour and then gradually declined during the recovery and returned to basal levels at 48 hours. Among DNA damage transducer kinases, ataxia telangiectasia mutated (ATM) was significantly activated by H₂O₂ in contrast to mild activation of ataxia telangiectasia mutated and Rad3-related (ATR). DSB binding protein, p53 binding protein 1 formed distinct nuclear foci that colocalized with γ -H2AX foci and phosphorylated ATM. Our findings indicate that DNA double strand breaks can be induced by H₂O₂ and ATM is the main kinase to mediate DSB repair in cardiomyocytes. Therefore, monitoring DSB repair can assess oxidative injury and response in cardiomyocytes.

Keywords

DNA double strand breaks; H₂O₂; Cultured neonatal rat cardiomyocytes; DNA damage; Oxidative stress

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Disclosures

None

Introduction

The genome of all living organisms is constantly attacked by various insults during normal metabolism and under stress. It is estimated that every cell may suffer up to 10^5 DNA lesions per day [1]. To maintain genomic stability, a quick and accurate DNA damage response (DDR) has evolved to effectively and sufficiently repair single and double strand DNA breaks (SSBs and DSBs) and thus to insure genomic integrity and organism survival. Certain cellular processes such as cell cycle and gene transcription are modified or halted after DNA lesions are induced. Nuclear structure and chromatin organization are also remodeled to facilitate the recruitment of DNA repair proteins into DNA breakage sites. If damaged DNA is inappropriately repaired, genomic alterations such as translocation, deletion, or insertion can lead to genomic instability. On the other hand, ineffective DDR can cause persistent DDR and cellular dysfunction. Furthermore, apoptosis or senescence occurs when DNA damage is too severe to overload cellular DDR system.

DSBs are the most serious genomic lesions as they disrupt genomic integrity. Most DSBs are effectively repaired within 24 hours of insults [2]. However, DSBs may persist in certain genomic regions, especially when DDR is defective. DSBs are rejoined by two major DNA repair mechanisms: homologous recombination (HR) and nonhomologous end joining (NHEJ). HR is a preferred repair system as it repairs error free. However, HR is limited to the S and G2 phases of the cell cycle as a homologous template, usually a sister chromatid, is only available during these periods of the cell cycle [1]. The majority of adult mammalian cardiomyocytes (CMs) cannot enter the cell cycle [3] and so their DSBs cannot be repaired by HR. NHEJ mediates direct ligation of broken DNA ends and occurs throughout the cell cycle, but it is a less accurate repair system.

Ataxia telangiectasia mutated (ATM), a key DDR transducer kinase, is quickly recruited to DSBs and activated by autophosphorylation at serine 1981 (pATM) [4]. Another DDR transducer kinase, ataxia telangiectasia and Rad3-related (ATR) is mainly activated by single strand DNA (SSD) preceding HR or during DNA replication through phosphorylation at serine 428 (pATR). These DDR kinases phosphorylate histone at serine 139 (γ -H2AX) forming distinct nuclear foci that mark DSBs with one focus corresponding to one DSB [5, 6]. Subsequently, many other DNA repair factors are recruited to DSBs. These proteins and their modified forms also generate visible microscopic foci detectable by immunofluorescent labeling with specific antibodies or expressing GFP-tagged proteins [1]. DSB detection and repair dynamics has been mainly investigated in proliferating transformed or immortalized cell lines. It is not clear whether the findings from these pioneer investigations can be applied to differentiated CMs with limited cell cycle activity.

In CMs, β -adrenergic receptor (β -AR) stimulation increases ATM expression, but it is not known if ATM is also activated by β -AR agonist [7]. Doxorubicin, an effective and widely used chemotherapeutic drug causes DNA damage and activates ATM leading to life-threatening cardiotoxicity [8, 9]. In human failing hearts, γ -H2AX levels are increased [10]. It is not clear whether CMs with oxidative stress forms distinct nuclear γ -H2AX or ATM foci although they do evoke DDR after radiation injury [11]. Nevertheless, these findings

indicate DDR system is operational in CMs and its dysregulation may be involved in cardiac remodeling and dysfunction. However, DSB detection and repair dynamics has not been systemically investigated in CMs.

The most common damage to DNA from normal cellular metabolism comes from reactive oxygen species (ROS) that oxidizes DNA bases and induces DSBs [1]. ROS are significantly increased in many cardiac diseases [12–15]. Oxidized DNA bases are repaired by DNA glycosylases and base excision [16]. However, it remains to be determined whether H₂O₂ can induce DSB in CMs. Moreover, it is unknown how CMs respond and repair oxidative DSBs. Understanding the spatiotemporal dynamics of DNA repair protein assembly/disassembly at DNA damage sites can provide novel strategy to accelerate DNA damage repair and prevent cellular dysfunction. In this study, we investigated DDR in cultured neonatal rat CMs (NRCMs) after 30 min treatments with different concentrations of H₂O₂ as well as monitored dynamic changes of γ -H2AX foci, DNA damage sensing and repair factors for up to 3 days after 30 min, 200 μ mol/L H₂O₂ treatment. Our data revealed that γ -H2AX levels and foci increased proportionally to the severity of oxidative stress. pATM, but not pATR colocalized with γ -H2AX to form DNA damage foci. Another DNA repair protein, the tumor suppressor p53 binding protein1 (53BP1) also relocated to DSBs marked by γ -H2AX. Although γ -H2AX and pATM returned to basal levels, rare unrepaired DSBs marked by 53BP1, γ -H2AX, and pATM still remained 3 days after 30 min, 200 μ mol/L H₂O₂ treatment. These findings indicate that H₂O₂ can induce DSB and ATM is a critical kinase to mediate DSB repair in NRCMs.

Materials and Methods

Neonatal rat cardiomyocyte (NRCM) isolation and culture

The approval for using neonatal rats was granted by the University Committee on Animal Resources (UCAR) of University of Rochester (protocol number: 101341/2008-106). All procedures were performed in accordance with *the Guide for the Care and Use of Laboratory Animals* (US Department of Health, Education, and Welfare, Department of Health and Human Services, NIH Publication 85-23). One to two day old Sprague Dawley rats were euthanized by hypothermia and placed in a glove finger into ice water until they became unresponsive. Ventricles were dissected free after rapid heart excision. NRCMs were isolated with the Neonatal Cardiomyocyte Isolation System from Worthington (Lakewood, NJ) according to the manufacturer's instruction based on Simpson and Savion's method [17]. Briefly, ventricles were minced in petri dishes with D-Hank's balanced salt solution (HBSS; in g/L: 8.00 NaCl, 0.4 KCl, 0.06 KH₂PO₄, 0.35 NaHCO₃, 0.09 Na₂HPO₄·7H₂O, and 0.05% trypsin), and then trypsinized at 4°C overnight. Cells were centrifuged at 1000× g for 5 min after 45 min 0.2% collagenase (type I) digestion at 37°C, then preplated into dishes (Falcon, Swedesboro, NJ) in DMEM medium for 1 hour to remove fibroblasts and other non-cardiomyocyte cells. The cells still in suspension were seeded at a density of 5×10⁶ cells/ml with F-12 medium containing 0.1 mmol/L bromodeoxyuridine. In this culture condition, no significant DNA replication or mitotic activity was observed in NRCMs as reported by investigators who developed this method [17]. After 24 hours of culture, NRCMs were treated with different doses of H₂O₂ for 30

min or exposed to 200 $\mu\text{mol/L}$ H_2O_2 for 5 to 60 min or for 30 min and then allowed to recover at 37°C for 30 min up to 72 hours by replacing H_2O_2 -containing medium with fresh medium.

Antibodies

Primary antibodies were purchased as following: 53BP1, pATR Ser428, ATM, ATR, H2AX and γ -H2AX were purchased from Cell Signaling Technology (Beverly, MA). pATM Ser1981 and another γ -H2AX were from Millipore (Billerica, MA). Secondary antibodies against rabbit and mouse IgGs with Dylight 488, Dylight 550, and horseradish peroxidase (HRP) were purchased from Thermo Fisher Scientific Inc (Rockford, IL). Alexa647-phalloidin was obtained from life technologies (Grand Island, NY).

SDS gel electrophoresis and Western blotting

Cultured NRCMs were collected and extracted with RIPA buffer. An equal amount of total protein, determined with the Bio-Rad Coomassie Protein Assay (Bio-Rad Laboratories, Hercules, CA), was loaded to each lane. NuPAGE® Tris-Acetate Pre-Cast gels (Invitrogen, Grand Island, NY) were used to separate high molecular weight proteins over 200 kD. Proteins smaller than 200kD were separated by 4–12% NuPAGE® Bis-Tris Pre-Cast gels or 12% SDS-polyacrylamide gels. Target proteins were detected with specific antibodies using SuperSignal™ West Pico Chemiluminescent Substrate (Thermo, Rockford, IL). Protein band intensity was quantified with NIH Image J software (<http://rsb.info.nih.gov/ij/>) using relative densitometric values on the duplicates of three independent experiments from each group.

Immunofluorescent labeling and confocal microscopy

Immunolabeling and confocal microscopy were performed as described previously[18]. The NRCMs were cultured on 25×25mm glass coverslips in 6-well plates. On each experimental time point, cultured cells were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 10 min. After quenching paraformaldehyde with 0.1 mol/L glycine in PBS for 25 min, cell membranes were permeabilized with 1% Triton X-100 for 20 minutes, and washed with PBS. To block nonspecific binding, coverslips were immersed in 10% normal goat serum for 60 min and then incubated with primary antibodies for overnight at 4°C. After washing 6 times in PBS, secondary antibody was added. Nuclear counterstain was performed with 4', 6-Diamidino-2-Phenylindole (DAPI, Sigma-Aldrich, St. Louis, MO). Sarcomeres were revealed with Alexa647-phalloidin. Confocal sections were collected with Olympus FV1000 confocal microscope (Olympus America Inc., Melville, NY) under uniform settings. Negative controls were incubated with the omission or substitution of primary antibodies with rabbit serum under the same conditions.

DDR foci count and colocalization

Confocal images were collected at the same conditions for control and treatment groups. The total nuclear foci number and area of γ -H2AX, pATM, pATR, and 53BP1 were automatically measured with NIH image J (<http://rsb.info.nih.gov/ij/>) in at least 25 nuclei from 3 experiments for each group. Foci numbers were also counted visually, which gave

identical results to NIH image J. A pannuclear stain was considered when the majority (75%) of a nucleus was occupied by foci. Colocalization of two interested signals was analyzed by Coloc 2 of image J and Manders coefficients [19] were used to characterize the degree of their overlap between images. Manders coefficients represented proportion of green channel signal coincident with the red signal.

Statistic analysis

Data were presented as mean $\pm$ standard error (SE) of the mean. Differences among the group means were analyzed with SPSS 13.0 software (IBM Corporation, Armonk, NY) using One-Way ANOVA. If a statistical significance was detected among groups, Bonferroni test was performed between group means when equal variances were present. If equal variances were not assumed, Tamhane's T2 test was used for multiple comparison tests.

Results

Histone H2AX is quickly phosphorylated at serine 139 when DSBs are induced in various cell types [4, 20]. This modified histone H2AX is concentrated at sites of DSBs, forming γ -H2AX foci that can be used as surrogate markers for DSBs [5, 6]. ATM and ATR are two main kinases that mediate the phosphorylation of H2AX and other DNA damage repair factors [1]. The temporal dynamics and subcellular movement of these molecules have been investigated in many cell lines after DSB induction by different agents [1, 5]. Whether CMs have similar DDR to these cell lines is unknown. H_2O_2 , a critical reactive oxygen radical, can elicit DSB and initiate DDR by activating ATM and ATR dependent on cell types, the duration and dosage of H_2O_2 exposure [20, 21]. To determine whether H_2O_2 can induce DSB and activate DDR in CMs, we empirically treated NRCMs with an intermediate dose of H_2O_2 at 200 μ mol/L for 5, 10, 15, 20, 30, and 60 min. This pilot study revealed that ATM was activated at 5 min while γ -H2AX showed an obvious increase at 20 min after the H_2O_2 treatment (Fig. 1A), indicating ATM was activated before H2AX phosphorylation. pATR levels, however, showed no significant change and revealed no correlation with either γ -H2AX or pATM levels. Although γ -H2AX continued to elevate at 60 min, no further increase in pATM was observed from 30 to 60 min, indicating ATM was maximally activated by 30 min of 200 μ mol/L H_2O_2 exposure. These findings indicate that H_2O_2 can induce DSB and activate DDR in NRCMs and 30 min is an optimal H_2O_2 exposure time to investigate DDR in NRCMs.

γ -H2AX foci formation in response to different doses of H_2O_2

To determine dosage response of DSB induction to H_2O_2 in NRCMs, we treated them with 50 to 800 μ mol/L H_2O_2 for 30 min and investigated γ -H2AX levels and foci formation immediately after H_2O_2 treatment. Using two specific antibodies against γ -H2AX, we found that γ -H2AX levels correlated positively to H_2O_2 dosages by Western blotting (Fig. 1B) and the number of γ -H2AX foci detected by immunofluorescent labeling also increased proportionally to H_2O_2 doses (Fig. 2). Discrete γ -H2AX foci were seen 30 min after 50 μ mol/L H_2O_2 treatment, but γ -H2AX became predominantly pannuclear when H_2O_2 was at 400 and 800 μ mol/L (Fig. 2A and 2B). The average number of γ -H2AX foci per nucleus was

10.8±0.6, 23.5±1.4, and 32.8±1.4 in 50 µmol/L, 100 µmol/L and 200 µmol/L H₂O₂ groups respectively compared to only 4.9±1.3 in the control group without H₂O₂ treatment (Fig. 2C). The total area occupied by γ-H2AX foci per nucleus was also increased proportionally to H₂O₂ concentrations (Fig. 2C).

Time courses of γ-H2AX foci formation and disappearance after 30 min, 200 µmol/L H₂O₂ exposure

To explore the temporal DSB repair dynamics, NRCMs were treated with 200 µmol/L H₂O₂ for 30 min and then allowed to recover for up to 72 hours. γ-H2AX levels reached to the highest level at first hour and then started to decline after treatment (Fig. 1C and 1E and Fig. 3). The average number of γ-H2AX foci was 32.1±1.4 at 0 min and started to decline slightly at 4 hours after the completion of 30 minute treatment (Fig. 3B). γ-H2AX foci remained slightly elevated in contrast to untreated controls (Fig. 3), even when γ-H2AX levels returned to the basal level at 48 and 72 hours (Fig. 1C). This suggests that counting γ-H2AX foci is more sensitive to detect DSBs than measuring total γ-H2AX levels by Western blotting.

Activation of ATM and ATR by H₂O₂

ATM is activated by phosphorylation at serine 1981 upon the induction of DSBs while ATR is mainly activated by SSD preceding HR or during DNA replication through phosphorylation at serine 428 [1]. pATM, but not ATM increased with H₂O₂ dosages until 200 µmol/L with 30 min treatment (Fig. 1B). After the completion of 30 min, 200 µmol/L H₂O₂ treatment, pATM, but not ATM was significantly increased at 0 min similar to γ-H2AX, started to decrease at 2 hours, but remained elevated for up to 48 hours (Fig. 1C and 1E). Interestingly, pATM foci number and area per nucleus (Fig. 4) demonstrated a slower decline than pATM levels by Western blots (Fig. 1C and 1E).

In contrast to pATM and γ-H2AX, pATR remained relatively stable at different doses of H₂O₂ treatment (Fig. 1B). Both ATR and pATR showed mild increase during recovery after 30 min, 200 µmol/L H₂O₂ treatment (Fig. 1C and 1F). However, pATR/ATR ratio revealed less than 40% changes among different time points (Fig. 1C and 1E). Nuclear pATR was increased and formed discrete dots that were smaller than pATM and γ-H2AX foci (Fig. 3). These pATR dots showed much less changes in size compared to pATM and γ-H2AX foci during the recovery after H₂O₂ treatment (Fig. 3, Fig. 4, and Fig 5A).

Rapid recruitment of DNA damage response proteins to DSBs

ATM and ATR can be recruited to DSBs after their activation. Our immunolabeling with specific antibodies and confocal microscopy revealed that nuclear pATM was significantly increased and formed distinct foci that colocalized with γ-H2AX foci (Fig. 2 and Fig. 4). Although nuclear pATR dots were notably increased, they revealed much less change in their size than pATM and γ-H2AX foci did during the recovery after H₂O₂ treatment (Fig. 3 and Fig. 5A). Most pATR dots were less than 40 pixels in size with or without H₂O₂ treatment (Fig. 5A). Although pATM and γ-H2AX foci were also often less than 40 pixels in size before H₂O₂ treatment, a significant portion of them was larger than 40 pixels in size after H₂O₂ treatment (Fig. 4 and Fig. 5A). Moreover, pATR dots revealed no colocalization

with γ -H2AX or pATM foci (Fig. 3 and Fig. 5B). As pATR is recruited to DSBs following the formation of SSD after DNA end resection, the lack of pATR in pATM and γ -H2AX foci indicates there is no extensive DNA end resection in NRCMs following DSB induction by H_2O_2 treatment.

DNA damage detection and mediator proteins, such as 53BP1, are recruited to DSBs at the γ -H2AX foci [1]. 53BP1 can bind to γ -H2AX, but not H2AX, marking DSBs [22]. Similar to other cells with DSBs, 53BP1 expression levels showed no significant changes in response to different doses of H_2O_2 or during 72 hour recovery following 30 min, 200 μ mol/L H_2O_2 treatment in NRCMs (Fig. 1C). However, nuclear 53BP1 distribution demonstrated dramatic change. In normal cells, 53BP1 was diffusely distributed over the nucleus with one or two bright dots occasionally observed (Fig. 4). Distinct 53BP1 foci were observed immediately after H_2O_2 treatment. Initially, 53BP1 foci only partially colocalized with γ -H2AX foci (Fig. 5B). Over time, more 53BP1 co-distributed with γ -H2AX and eventually near all γ -H2AX foci contained 53BP1. The number of 53BP1 foci also positively correlated with H_2O_2 dosages similar to γ -H2AX foci (Fig. 2B and 2C).

Discussion

Oxidative stress is increased in a variety of heart diseases and impairs cardiac function [12, 13, 23–25]. ROS can modify and damage macromolecules including lipids, proteins, and DNA [24, 26]. Alleviating oxidative stress can prevent abnormal cardiac remodeling [27, 28]. H_2O_2 , an important ROS, has been shown to induce DSBs dependent on cell types and dosage [1, 20, 21, 29]. It is not clear whether it can also elicit DSBs in CMs. Nonetheless, γ -H2AX, a marker of DNA damage is elevated in end-stage failing human hearts compare to healthy controls [10]. During DDR, γ -H2AX accumulates at DSBs, but not other forms of DNA lesions [5, 6]. However, it is not known if γ -H2AX forms distinct nuclear foci to mark DSBs in CMs during oxidative stress. To reveal foci formation of DNA repair proteins, fluorescence-based techniques are essential to localize DSBs. In this study, we assessed and quantified DSB detection and repair dynamics in CMs after H_2O_2 treatment. Our results revealed that H_2O_2 induced DSBs and invoked a robust DDR in CMs.

DSB repair dynamics has been well documented in immortalized and transformed cell lines. In hamster CHO and human IMR90 cells, γ -H2AX levels rapidly increase and γ -H2AX foci become apparent within min after DSB induction [5, 20]. After γ -H2AX levels peak at 10–30 min, they start to slowly decrease over 90 min. γ -H2AX foci, however, persist over 15–60 min and only decrease in number at 180 min [5]. Our data showed that there was no significant change in γ -H2AX levels until 15 min of H_2O_2 treatment, but they remained at highest levels during first hour and then started to decline after H_2O_2 treatment. γ -H2AX foci, however, only showed a mild decrease at 4 hour after H_2O_2 treatment. These findings indicate that CMs may have a slower DSB repair dynamics than established cell lines. Similarly, DNA damage is repaired slower *in vivo* in the heart than other organs after radiation exposure [11].

Generation and release of ROS are significantly increased in a variety of cardiac disorders [12, 13, 23–25]. These reactive molecules can activate specific signaling pathways to

modulate cardiac remodeling and CM survival dependent on their dosages [30, 31]. H_2O_2 is a stable ROS and mainly modifies DNA bases and causes SSBs in low doses. Mitochondrion is the main source of ROS production and is subjected to oxidative DNA damage. In mitofusion 2 deficient hearts, cardiac dysfunction is associated with increased mitochondrial DNA double strand breaks [32]. In SF268 anaplastic astrocytoma cells, 30 min treatments of 10 and 50 $\mu\text{mol/L}$ of H_2O_2 do not cause DSBs and thus no $\gamma\text{-H2AX}$ is detected [20]. However, in normal foreskin fibroblasts, 2 mmol/L H_2O_2 for 30 min can induce $\gamma\text{-H2AX}$ focus formation [21]. Interestingly, even high dose of H_2O_2 cannot induce DSBs in Epstein-Barr virus-immortalized normal human lymphoblasts [29]. Our results demonstrated that $\gamma\text{-H2AX}$ levels and foci showed dose-related changes in CMs. Even though $\gamma\text{-H2AX}$ levels only showed mild increase at 50 $\mu\text{mol/L}$ H_2O_2 , distinct $\gamma\text{-H2AX}$ foci were visible at this dosage. Furthermore, $\gamma\text{-H2AX}$ levels and foci increased proportionally to H_2O_2 up to 200 $\mu\text{mol/L}$. As $\gamma\text{-H2AX}$ levels continued to increase in higher doses, $\gamma\text{-H2AX}$ became diffuse in the nucleus as pannuclear. This dose dependent response of CMs to H_2O_2 suggests that CMs are very sensitive to changes in oxidant levels and a mild increase in H_2O_2 can induce DSBs. Thus enhancing DSB repair can be explored to protect the heart from oxidative damage.

H_2O_2 can act as a signal molecule to activate several kinases in a dose-dependent manner. Ten $\mu\text{mol/L}$ of H_2O_2 has been shown to activate ERK1/2, but not JNK, p38 kinase or Akt [30]. Higher doses of H_2O_2 increase the activity of JNK, p38 kinase and Akt, and ERK1/2 [30, 31, 33]. DNA damage transducing kinases, ATM and ATR, can be activated by ROS dependent on cell types and the intensity of oxidative stress. In Epstein-Barr virus-immortalized normal human lymphoblasts, even in 5 mM of H_2O_2 could not activate ATM [29]. On the other hand, Guo et al have recently shown that H_2O_2 could induce ATM activation at 250 $\mu\text{mol/L}$ without detectable DSBs [34]. In human fibroblast and lymphoblastoid cell lines, 2 mmol/L of H_2O_2 can induce both DSBs and ATM activation [21]. Here we showed that ATM activation by H_2O_2 in CMs was rapid and dosage dependent up to 200 $\mu\text{mol/L}$. In addition, its activation preceded $\gamma\text{-H2AX}$ elevation and was associated the formation of pATM foci that corresponded to $\gamma\text{-H2AX}$ foci. Therefore, ATM activation is directly related to DSBs induced by H_2O_2 .

Doxorubicin has cumulative dose-dependent cardiotoxicity by inducing ROS and DDR [8, 9]. In cultured HL-1 atrial-derived cells, doxorubicin can induce DSB with $\gamma\text{-H2AX}$ foci [35]. It is not clear whether DSBs form or how they are repaired in primary cultured cardiomyocytes and in intact hearts after doxorubicin treatment. Further investigation with dynamic monitoring of DSB markers will provide new strategy to prevent doxorubicin cardiac toxicity. Blocking ATM activation has been shown to prevent p53 accumulation and protect the heart from doxorubicin-induced cardiac damage and dysfunction [8, 36]. On the other hand, global deficiency of ATM accentuates functional deterioration, cardiac fibrosis, and hypertrophy induced by $\beta\text{-AR}$ agonist, isoprenaline [7]. This difference indicates that ATM deficiency may affect cardiac development and CM maturation. It is also possible that ATM has differential effects on CMs, fibroblasts, endothelial and smooth muscle cells. The duration and the nature of insults have to be considered in the interpretation as well. Therefore, elucidating the differential effects of ATM on cardiac protection in different injuries in CM is critical to explore this pathway for the prevention and treatment cardiac

dysfunction and myocardial remodeling in cardiac diseases. ATR is activated by SSD during DNA replication stress or DNA resection preceding HR. Total and phosphorylated ATR levels as well as nuclear pATR dots were mildly increased after H₂O₂ treatment, indicating that ATR can be activated and translocated to the nucleus during oxidative stress. However, we did not observe significant colocalization of pATR dots with pATM, γ -H2AX or 53BP1 foci. Furthermore, pATR dots showed no significant size change in comparison to dramatic increase of pATM, γ -H2AX or 53BP1 foci after H₂O₂ treatment. During DDR to DSBs, DNA damage foci with sensor and repair proteins often spread along both sides of DSBs [5, 6]. Our findings indicate that pATR is not recruited to DSBs in CMs. As ATR interacts with SSD and is critical to HR, its absence in DDR foci suggests that there is no significant DNA end resection and thus SSD formation and HR activity in CMs.

53BP1 was originally identified as p53 binding protein using the yeast two-hybrid system [37]. It binds to the DNA-binding domain of p53 and enhances p53-mediated transcriptional activation. ATM and ATR can phosphorylate SQ/TQ consensus phosphorylation sites at the N-terminal domain of 53 BP1, promoting its recruitment of DSBs and 53BP1 foci formation. 53BP1 is critical for DSB detection and repair. Its persistence in DNA damage sites indicates unrepaired DSBs. Our data showed that 53BP1 formed distinct nuclear foci and colocalized with γ -H2AX and ATM, but not ATR. Interestingly, rare large foci persisted up to 72 hours, indicating some DSBs cannot be repaired in CMs following DSB induction.

Understanding how CMs repair their DNA damage, especially DSBs induced by different agents, is essential to protect the heart from various insults. Many factors including microRNA can modulate DDR in CMs [38]. Our study characterized the dynamics of DSB detection and repair in CMs under oxidative stress based on the phosphorylation and nuclear foci formation of ATM and H2AX. These results indicate that novel methods developed to assess DDR in other cell systems can be explored to investigate oxidative DSBs in CMs.

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Highlights

1. H₂O₂ induces DNA double strand breaks (DSBs) in cultured neonatal rat CMs.
2. CMs respond to DSBs and forms detection and repair foci with γ -H2AX, ATM and 53BP1.
3. ATM is the main kinase to mediate the response of cardiac myocytes to DSBs.

Figure 1. H2AX, ATM, and ATR phosphorylation in NRCMs treated with H₂O₂

A: Representative Western blots from 2 pilot experiments with continuous 200 μmol/L H₂O₂ treatment for up to 60 min demonstrated no significant change in total H2AX, ATM, and ATR in NRCMs. pATM started to increase significantly at 5 min, but showed no further increase after 30 min. γ-H2AX showed only mild increase before 15 min, elevated significantly at 20 min, and became stronger with time. pATR demonstrated subtle variations at different time point and its change was not as obvious as γ-H2AX and pATM.

B: NRCMs were treated with 50, 100, 200, 400, and 800 μmol/L H₂O₂ for 30 min. H2AX and ATM did not change with H₂O₂ dosages, but γ-H2AX and pATM levels started to elevate at 50 μmol/L of H₂O₂, kept increasing with higher H₂O₂ dosages, reaching maximal level at 200 μmol/L of H₂O₂. ATR and pATR remained relatively stable.

C: Temporal changes of γ-H2AX, pATM, pATR at 0 min to 72 hours following the completion of 30 min, 200 μmol/L H₂O₂ treatment. H2AX and ATM showed no significant change, but γ-H2AX and pATM levels were elevated immediately (0 min) and started to decline at 2 hours after the treatment. γ-H2AX and pATM returned to basal levels at 48 and 72 hours respectively. Both ATR and pATR showed mild increase during recovery.

D: Fold changes in densitometric ratios of γ-H2AX/H2AX, pATM/ATM, and pATR/ATR relative to controls in response to different doses of H₂O₂ for 30 min. ***P*<0.01 versus control; †*P*<0.05 versus 50 μmol/L group; ††*P*<0.01 versus 50 μmol/L group; ‡*P*<0.05 versus 100 μmol/L group.

E:

Fold changes in densitometric ratios of γ -H2AX/H2AX, pATM/ATM, and pATR/ATR relative to controls at different time points of recovery up to 72 hours following 30 min, 200 μ mol/L H₂O₂. ** P <0.01 versus control; †† P <0.01 versus immediately (0 hour) after the completion of H₂O₂ treatment. F: Fold changes of phosphorylated (upper) and total (lower) H2AX, ATM and ATR during recovery after 30 min, 200 μ mol/L H₂O₂ treatment. There were six independent experiments in each group in B to F. 53BP1, and β -tubulin showed no change with times or dosages and were used as loading controls.

Figure 2. Dosage response of γ -H2AX, pATM, and 53BP1 foci to H_2O_2
NRCMs were treated with 50, 100, 200, 400, and 800 $\mu\text{mol/L}$ H_2O_2 for 30 min and labeled with γ -H2AX and pATM (A) or 53BP1 (B). Nuclei were stained with DAPI (blue) and sarcomeres were revealed with Alexa647-phalloidin (grey). The number of γ -H2AX, pATM, and 53BP1 foci increased with H_2O_2 dosage. γ -H2AX foci were bigger than pATM foci, but there were more pATM foci than γ -H2AX foci. At 400 $\mu\text{mol/L}$ H_2O_2 or higher, both γ -H2AX and pATM became predominantly pannuclear and 53BP1 foci showed no further increase. Scale bars, 5 μm C: Bar graphs showed numbers and total pixel areas of γ -H2AX, pATM and 53BP1 foci per nucleus. $**P<0.01$ versus control; $\dagger\dagger P<0.01$ versus 50 $\mu\text{mol/L}$ group; $\ddagger\dagger P<0.01$ versus 100 $\mu\text{mol/L}$ group.

Figure 3. pATR formed fine nuclear dots that increased in number, but did not increase in size or show colocalization with γ -H2AX

A: NRCMs were stained with γ -H2AX, pATR, DAPI (blue), and Alexa647-phalloidin (grey) at different times after recovery from the completion of 30 min, 200 μ mol/L H_2O_2 treatment. pATR demonstrated a fine nuclear dots in both control and treatment groups. Although nuclear pATR levels were increased, its size revealed no obvious increase. Additionally, pATR showed only occasional overlap, but no colocalization with γ -H2AX foci at any time point. Scale bars in B, 5 μ m. B: Bar graphs summarized the number and total pixel area of γ -H2AX and pATR foci.

Figure 4. γ -H2AX foci colocalized with pATM and 53BP1
A: NRCMs were stained with γ -H2AX, DAPI (blue), Alexa647-phalloidin (grey) and pATM (upper panel) or 53BP1 (lower panel) at different time points after 30 min, 200 μ mol/L H_2O_2 exposure. γ -H2AX foci increased dramatically immediately after the H_2O_2 exposure and then decreased slowly during the recovery. pATM had a diffuse granular distribution in control nuclei, increased after the H_2O_2 treatment, and formed larger nuclear spots with time. γ -H2AX foci colocalized with larger pATM foci. 53BP1 foci increased significantly following H_2O_2 treatment, but started to decline 4 and 8 hours after H_2O_2 treatment

respectively. γ -H2AX became more colocalized with 53BP1 over time during the recovery. Scale bars, 5 μ m. Bar graphs demonstrated the number and total pixel area of pATM foci (B) and 53BP1 foci (C) per nucleus.

Figure 5.
A: Size distribution of nuclear pATR dots as well as γ -H2AX, pATM, and 53BP1 foci in control and at 0, 4 and 8 hours after the completion of 30 min, 200 μ mol/L H_2O_2 treatment.
B: Manders colocalization coefficient of γ -H2AX with pATM, pATR, and 53BP1 during the recovery up to 72 hours following 200 μ mol/L H_2O_2 .