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News & Views

Mitochondrial and Nuclear p53 Localization in Cardiomyocytes: Redox Modulation by Doxorubicin (Adriamycin)?

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ABSTRACT

Reactive oxygen (ROS) and nitrogen species (RNS) generation have been proposed to be an important mechanism of doxorubicin (Adriamycin; ADR)-induced cardiotoxicity and cardiomyocyte apoptosis, processes that may be mediated by p53 protein. We note that ADR treatment resulted in increased levels of p53 protein in cardiomyocyte mitochondria and nuclei. Modulation of the cardiomyocyte redox state in genetically engineered mice by modulation of enzymes involved in metabolism of ROS/RNS, manganese superoxide dismutase (MnSOD), or inducible nitric oxide synthase (iNOS), or a combination of these, regulated levels of mitochondrial/nuclear p53 in cardiomyocytes after ADR administration. These observations led to the hypothesis that mitochondrial/nuclear p53 localization and function in the cardiomyocyte response to ADR may be regulated through redox-dependent mechanism(s). *Antioxid. Redox Signal.* 9, 1001–1008.

Doxorubicin (Adriamycin; ADR), a quinone-containing anthracycline antibiotic, is used in treatment of a broad spectrum of human cancers. However, clinical application of ADR is limited by its dose-dependent cardiotoxicity. The precise mechanism of ADR-induced cardiotoxicity is not completely understood; however, ROS generation during intracellular metabolism of ADR and subsequent oxidative stress has been proposed to be important mechanism(s) for its cardiac toxic side effects (12, 23). More important, ADR-induced cardiomyocyte apoptosis has been proposed to play an important role in its cardiotoxicity that is linked to the formation of ROS derived from redox cycling of ADR (20, 30), and apoptosis of cardiomyocytes is, in turn, dependent on the induction of the p53 tumor-suppressor protein (32).

p53 is a transcription factor that plays a central role in the cellular response to DNA damage by genotoxic agents such as UV, ROS, and certain anticancer drugs, including ADR. Depending on the cell type and the extent of DNA damage, p53 can modulate many cellular functions, including cell-cycle arrest, DNA repair, and apoptosis. Apoptosis mediated by p53 may reflect multiple links between ROS generation, DNA damage response, and transcription activation of p53 proapoptotic targets such as Bax (24). However, increasing evidence suggests that p53 can function independent of its transcription activation by directly acting on mitochondria, being involved either in mitochondrial DNA repair or in mitochondrial-driven apoptosis or both (2, 11, 14). These observations indicate the complexity of p53 in the DNA-damage response and induction of apoptosis at the subcellular level, processes that may be regulated through ROS or cellular redox status or both.

ROS/RNS AND REDOX BALANCE IN MODULATION OF P53 PROTEIN

Many lines of evidence suggest that ROS and RNS work together in biologic systems to achieve optimal signaling and redox balance. Cell signaling controlled by ROS/RNS is

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involved in the regulation of protein expression and function through modification of cysteine residues by redox-sensitive mechanisms. The site-specific DNA-binding activity of p53 contains a zinc ion coordinated on cysteine residues that is sensitive to the redox state, and p53 DNA-binding activity has been demonstrated to be regulated by intracellular redox status (16). ROS and RNS are not only involved in regulation of p53 through redox modulation mechanisms, but are also involved in the DNA damage response. ROS, nitric oxide (NO), and related molecules such as peroxynitrite ($ONOO^-$) have been demonstrated to activate upregulation of p53 protein through induction of DNA damage (1, 22). However, no clear evidence demonstrates whether p53 is increased through the DNA damage response or undergoes direct, redox-dependent modification(s) during the process of p53 protein activation.

A previous study demonstrated that apoptosis triggered by p53 was dependent on an increase of ROS levels and the release of apoptotic factors resulting from mitochondrial damage (28). In addition, overexpression of an antioxidant enzyme, MnSOD, has been shown to suppress p53-mediated induction of apoptosis (13). Moreover, NO has been documented to be involved in p53 nuclear accumulation and apoptosis induction (5, 22). These observations strongly support the involvement of ROS/RNS in regulation of apoptosis mediated by p53. However, in the presence of ROS, NO can be a powerful antioxidant, having the ability to suppress ROS-mediated oxidative stress and activation of p53 (34, 36). Evidence that $ONOO^-$, resulting from the reaction of superoxide ($O_2^{\bullet -}$) and NO, inactivates p53 transcription activity, and downstream expression (9) also supports the latter hypothesis.

We recently demonstrated increased levels of p53 in mitochondria and nuclei of cardiomyocytes in response to ADR, and provide evidence that mitochondrial p53 localization in cardiomyocytes is related to mitochondrial DNA (mtDNA) damage, whereas p53 nuclear accumulation is postulated to play a role in ADR-induced cardiotoxicity and cardiomyocyte apoptosis (25). However, the signal(s) mediating mitochondrial/nuclear localization of p53 in the cardiomyocyte response to ADR has not been documented. Evidence of ROS generation by redox cycling of ADR and NO generation through ADR-induced expression of inducible nitric oxide synthase (iNOS) (3, 20, 30) in cardiomyocytes focused considerable attention on the possible role of free radical formation and interactions in regulation of p53, a redox-sensitive protein.

MODULATION OF CARDIOMYOCYTE REDOX STATE ALTERED ADR-INDUCED OXIDATIVE DNA DAMAGE AND INCREASED LEVELS OF p53 PROTEIN

In the present study, we used genetically engineered MnSOD transgenic or iNOS knockout mice (or both; Appendix, note 1) to determine the involvement of ROS/RNS generation and redox balance in the induction of DNA damage and regulation of p53 protein in the response of cardiomyocytes to ADR. Levels and localization of 8-hydroxy-2'-deoxyguanosine (8-OHdG), a biomarker of oxidative DNA damage, and p53 were measured by using immunogold ultrastructural analysis techniques with specific antibodies; this method is a very sensitive and specific tool to detect target molecules in particular subcellular organelles *in vivo* (Appendix, note 2, and details on statistical analysis; see Appendix, note 3). This approach allowed us to evaluate the consequence(s) of altered ROS/RNS on the levels of oxidative DNA damage and p53 protein in cardiomyocyte mitochondria and nuclei, two important DNA-containing organelles in which p53 has been shown to be involved in both organelle-orchestrated DNA repair and induction of apoptosis.

A time-course study demonstrated that ADR induced an increase of 8-OHdG levels in cardiomyocyte mitochondria of nontransgenic [Ntg] mice at 3 and 24 h, correlating with the levels of p53, which were significantly increased in cardiomyocyte mitochondria at the same times. P53 protein levels in cardiomyocyte nuclei were significantly increased throughout the time-course analysis, whereas nuclear 8-OHdG did not significantly change after ADR treatment (Fig. 1A). In contrast, mouse hearts overexpressing MnSOD [$TgM^{(+/+)}$] did not show a significant increase in levels of 8-OHdG or p53 protein levels in either cardiomyocyte mitochondria or nuclei at any times after ADR treatment, and the levels were lower when compared with those of Ntg littermates (Fig. 1B). Similar to data from cardiomyocytes of $TgM^{(+/+)}$ mice, no significant alterations of p53 protein levels were observed in either cardiomyocyte mitochondria or nuclei of mice in which the iNOS gene had been inactivated [$iNOSKO^{(-/-)}$]. However, cardiomyocytes from $iNOSKO^{(-/-)}$ mice demonstrated a significant increase in levels of 8-OHdG in cardiomyocyte nuclei at 24 h after ADR treatment without a significant increase in levels of 8-OHdG in mitochondria (Fig. 1C). Cardiomyocytes from the crossbreed between these two strains of mice [$TgM^{(+/+)}$/$iNOSKO^{(-/-)}$] did not show any significant changes in levels of 8-OHdG and p53 protein in either mitochondria or nuclei throughout the time course of ADR treatment (Fig. 1D). These results indicated that modulation of the cardiomyocyte redox state by overexpression of MnSOD to reduce $O_2^{\bullet -}$ generation or inactivation of iNOS (or both) to prevent NO formation altered levels of oxidative DNA damage and p53 protein in specific subcellular site(s) in cardiomyocytes after exposure to ADR.

MODULATION OF CARDIOMYOCYTE REDOX STATE BY MnSOD AND/OR iNOS ENZYMES ALTERED THE MAGNITUDE OF CYTOCHROME *C* RELEASE FROM CARDIOMYOCYTE MITOCHONDRIA AFTER EXPOSURE TO ADR

Cytochrome *c* release from mitochondria into cytosol is a marker of mitochondrial injury and a downstream event allowing evaluation of apoptotic cell death. It is one of the characteristics of cardiomyocytes undergoing apoptosis that coordinates with upregulation of p53, increased expression of proapoptotic proteins such as Bax, and caspase activation (35). Analysis of

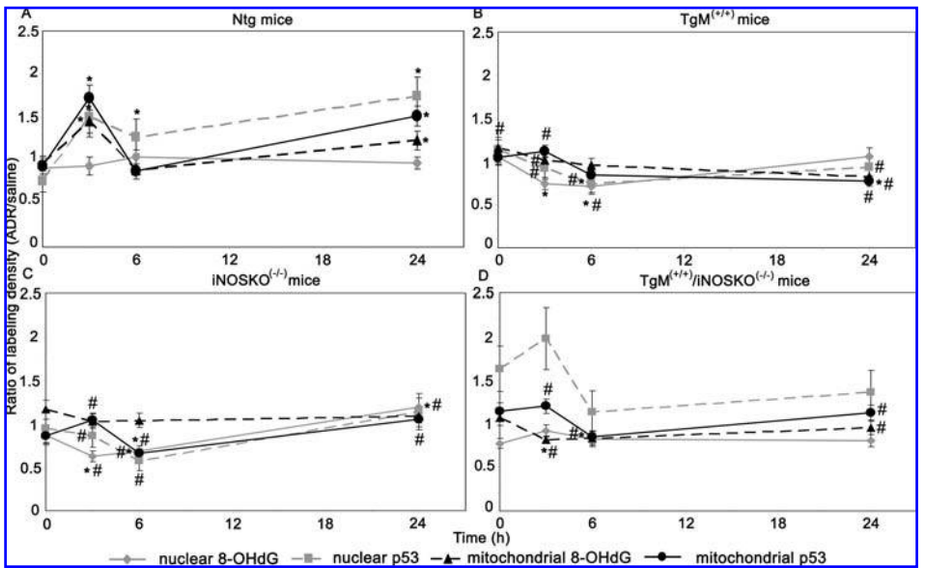

FIG. 1. Levels of oxidized DNA (8-OHdG) and p53 protein in cardiomyocyte mitochondria and nuclei of Ntg and genetically engineered mice after ADR treatment. Mice were treated with either saline or ADR and then were killed at 0, 3, 6, and 24 h, as indicated. Left ventricles were prepared for immunogold ultrastructural techniques. Data show the ratio of 8-OHdG and p53 labeling density (ADR/saline) in each compartment. *p value < 0.05 when compared with 0 h. #p value < 0.05 when compared with Ntg mice at the same time.

ultrastructural damage from our previous studies (6, 7) demonstrated that ADR-induced cardiomyocyte injury in Ntg mice, primarily in mitochondria, and overexpression of MnSOD [TgM$^{(+/+)}$] protected against ADR-induced mitochondrial injury. In contrast, a dramatic increase in mitochondrial injury after ADR treatment was shown in mice with inactivation of the iNOS enzyme [iNOSKO$^{(-/-)}$]. However, overexpression of MnSOD was demonstrated to reverse mitochondrial injury found in iNOSKO$^{(-/-)}$ mice [TgM$^{(+/+)}$/iNOSKO$^{(-/-)}$]. In the present study, we used cytochrome *c* release from mitochondria as a marker to determine whether increased/decreased levels of mitochondrial injury documented in our previous study correlated with upregulation/downregulation of p53 in the same mouse hearts, resulting in cardiomyocyte apoptosis. Cytochrome *c* protein in cardiomyocyte mitochondria and cytoplasm was examined by using immunogold ultrastructural analysis techniques (Appendix, notes 2 and 3).

Cytochrome *c* release, as determined by the ratio of cytosolic cytochrome *c*/mitochondrial cytochrome *c*, was significantly increased ~35% in cardiomyocytes of ADR-treated Ntg mice relative to their control counterparts at 24 h after treatment (Fig. 2A). Conversely, ADR treatment had no effects on cytochrome *c* release in cardiomyocytes of TgM$^{(+/+)}$ or TgM$^{(+/+)}$/iNOSKO$^{(-/-)}$ mice relative to their saline controls at any time (Fig. 2B and D). Dramatic cytochrome *c* release was observed in cardiomyocytes from iNOSKO$^{(-/-)}$ mice, ~90% increased at 6 h and 200% increased at 24 h relative to their corresponding controls (Fig. 2C). These results are consistent with mitochondrial injury data obtained previously from our laboratory and indicate that free radical generation by ADR may be a major mechanism contributing to cardiomyocyte injury and apoptosis, which may be involved in the function of p53 protein.

REDOX REGULATION OF p53 PROTEIN MITOCHONDRIAL/NUCLEAR LOCALIZATION AND FUNCTION DURING ADR-INDUCED CARDIOTOXICITY

Mitochondria are intracellular organelles that play a major role in maintenance of normal cell metabolic function and control of apoptosis. Therefore, oxidant-mediated mitochondrial damage can lead to severe cell injury and possibly cell death. Many previous studies suggest mitochondria as the primary subcellular organelle for the generation of ROS, as well as the major sites of intracellular injury in cardiomyocytes after exposure to ADR (12, 37). This hypothesis was confirmed by a previous study from our laboratory using the same set of mice used in the present study, and biomarkers of oxidative/nitrative damage demonstrated increased levels of 4-hydroxy-2-nonenal (4-HNE) and 3-nitrotyrosine (3-NT) protein adducts, primarily in cardiomyocyte mitochondria; these results documented ROS/RNS generation and alteration of cardiomyocyte mitochondrial redox state after ADR administration (6) (Fig. 3A). 4-HNE protein adducts are biomarkers of oxidative stress, and oxidative damage results from accumulation of various ROS, including $O_2^{\bullet-}$, hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) that are not neutralized by the antioxidant defense system. 3-NT is a biomarker for both oxidative and nitrative stresses from reaction

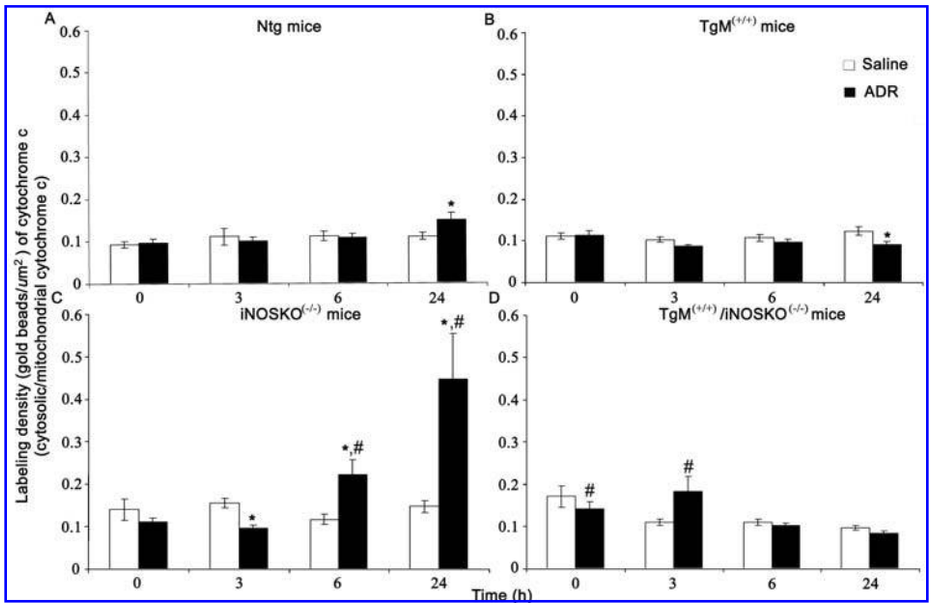

FIG. 2. Cytochrome *c* release from cardiomyocyte mitochondria of Ntg and genetically engineered mice after ADR treatment. Mice were treated with either saline or ADR and then were killed at 0, 3, 6, and 24 h, as indicated. Left ventricles were prepared for immunogold ultrastructural techniques. Data show ratio of cytosolic/mitochondrial cytochrome *c* in saline versus ADR treated-mice. **p* value < 0.05 when compared with saline control, #*p* value < 0.05 when compared with ADR-treated Ntg mice at the same time.

of $O_2^{\bullet-}$ and NO to form a biologic molecule, peroxynitrite ($ONOO^-$), which may react with tyrosine residues in proteins.

In the present study, we found that mtDNA was more vulnerable to oxidative damage than nuclear DNA (nDNA) in cardiomyocytes after ADR treatment. Hydroxyl radical and $ONOO^-$ are proposed to be involved in the formation of oxidized DNA (8-OHdG) (18). Additionally, we reported that increased mitochondrial p53 correlated with the levels of oxidative mtDNA damage, whereas increased levels of p53 in nuclei were observed without significant nDNA damage (25). We postulated that mitochondria may serve as a redox regulator of the cell, in which mitochondrial ROS/RNS generation and alteration of the mitochondrial redox state could regulate levels of p53 mitochondrial/nuclear localization and function through mtDNA-damage response and redox signaling pathways. Regulation of DNA binding of redox-dependent transcription factors such as NF-κB, AP-1, and p53 is one of the mechanisms by which cells transduce oxidative stress redox signaling into the induction of target genes involved in cellular processes, including apoptosis (4, 33). We have documented induction of p53 proapoptotic target proteins including PUMA and Bax, cytochrome *c* release, and subsequent caspase-3 activation in cardiomyocytes after ADR administration (25).

MnSOD is a primary antioxidant enzyme in mitochondria that dismutates $O_2^{\bullet-}$ to H_2O_2. We previously showed that overexpression of MnSOD prevented an increase of 4-HNE and subsequent 3-NT protein adducts levels observed in Ntg mice and suggested a critical role of MnSOD in regulation of ROS and RNS levels in cardiomyocytes exposed to ADR (7) (Fig. 3A). Therefore, overexpression of MnSOD has been postulated as one possible mechanism of protection from oxidative damage during pathologic conditions. In the present study, we documented that overexpression of MnSOD prevents increased oxidative mtDNA damage, increased levels of mitochondrial/nuclear p53 protein, and cytochrome *c* release from mitochondria observed in Ntg littermates. Consequently, these results indicate an involvement of ROS in regulation of p53 and support our hypothesis that the redox state regulates p53 mitochondrial/nuclear function in cardiomyocytes after ADR treatment. Our results are consistent with a previous study demonstrating that MnSOD protected against cell death by p53-mediated induction of apoptosis (13).

The exact function of NO in the response of cardiomyocytes to stress signals remains unclear, because NO has been shown to be involved in both pro- and anti-apoptotic effects. Evidence from our previous study illustrated that inactivation of the iNOS gene to prevent NO production resulted in potentiation of mitochondrial injury in cardiomyocytes after ADR treatment (7) (Fig. 3A). These results suggest a protective role of NO in the cardiomyocyte response to stress. Wink *et al.* (36) postulated that the antioxidant properties of NO function in suppression of biologic mechanisms associated with ROS generation and oxidative stress; thus, NO deficiency may result in increased ROS levels. In support of this hypothesis is the fact that 4-HNE protein adducts were dramatically increased in cardiomyocyte mitochondria of $iNOSKO^{(-/-)}$ mice early after treatment with ADR (7) (Fig. 3A). Further analysis in our present study demonstrated increased levels of oxidative nDNA damage and cytochrome *c* release in $iNOSKO^{(-/-)}$ cardiomyocytes at later times after ADR treatment but failed to detect increased levels of p53 protein.

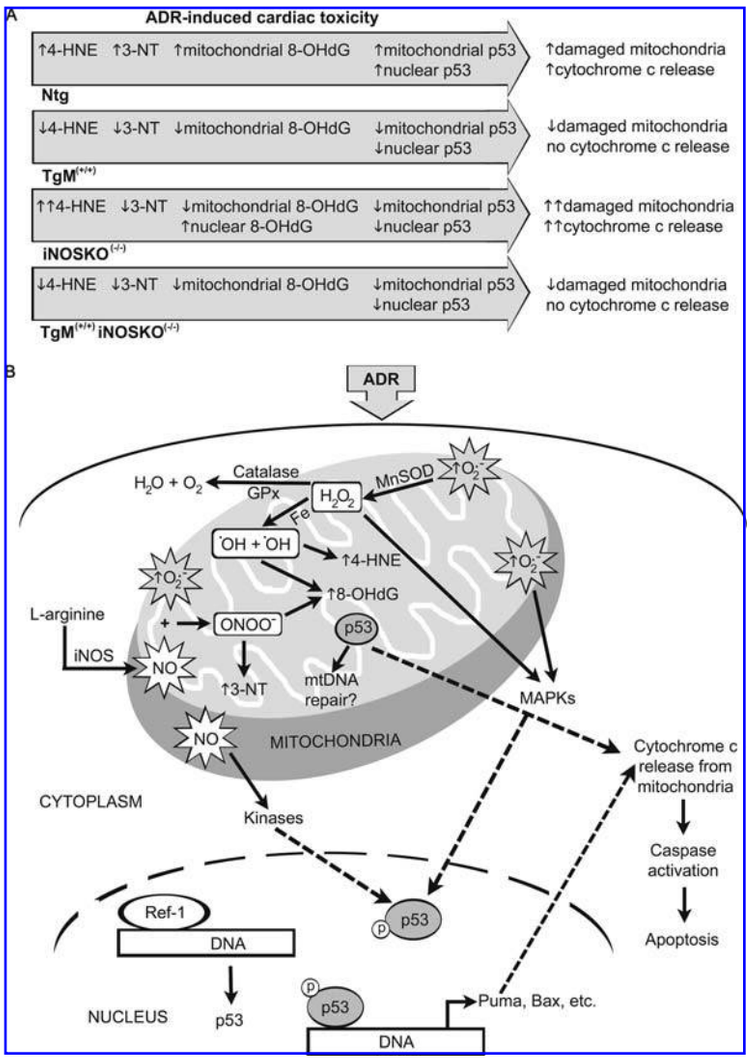

FIG. 3. The involvement of ROS/RNS and redox regulation of p53 during ADR-induced cardiotoxicity. (**A**) Data from previous studies (4-HNE, 3-NT, and mitochondrial injury) and the current study (8-OHdG, p53, cytochrome *c* release) are summarized. (**B**) Schematic illustration showing the proposed mechanism of ROS/RNS regulation of p53 protein levels, mitochondrial/nuclear localization, and function(s) in cardiomyocyte stress response to ADR. For detailed information, see Results and Discussion. 4-HNE, 4-hydroxy-2-nonenal protein adducts; 3-NT, 3-nitrotyrosine; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; MnSOD, manganese superoxide dismutase; iNOS, inducible nitric oxide synthase; Gpx, glutathione peroxidase; $O_2^{\bullet-}$, superoxide anion; H_2O_2, hydrogen peroxide; $^{\bullet}OH$, hydroxyl radical; NO, nitric oxide; $ONOO^-$, peroxynitrate; O_2, oxygen; MAPKs, mitogen-activated protein kinases; Ref-1, redox effector factor-1.

These results indicated that NO and RNS may be involved in the regulation of p53 protein induction and function. Evidence from iNOS knockout macrophages that failed to demonstrate nuclear p53 localization after *in vivo* bleomycin exposure also supports this latter hypothesis (10).

Notably, increased nDNA damage in iNOSKO$^{(-/-)}$ mouse heart at later times may be caused not only by ADR-induced ROS generation but also by lack of functional nuclear DNA repair enzymes. A previous study demonstrated that 4-HNE inhibited nuclear DNA-repair capacity through its direct interaction with proteins involved in DNA repair (15). Although we were not able to detect increased levels of p53 protein in response to nDNA damage, we could not rule out the possibility that p53 DNA-binding activity may be increased in cardiomyocytes of iNOSKO$^{(-/-)}$ mice. Additional studies are needed to evaluate the mechanism of cardiomyocyte apoptosis in mice deficient in NO in response to ADR. However, our data suggest that NO deficiency may shift the redox-state balance, resulting in cardiomyocyte apoptosis independent of p53 after ADR administration. A previous study demonstrated that exposure of cells to a high level of 4HNE directly induced cytochrome *c* release and apoptosis, independent of p53 protein accumulation (17).

Our data from TgM$^{(+/+)}$/iNOSKO$^{(-/-)}$ mice provide evidence for redox regulation by alteration of both ROS and RNS levels, in which overexpression of MnSOD overcame the effects observed in iNOSKO$^{(-/-)}$ mouse heart after ADR treatment (summary in Fig. 3A). These results indicate that NO deficiency may be compensated by increased levels of MnSOD to reduce high oxidative conditions and suggest a relation between ROS/RNS in regulation of redox balance and cell-signaling pathways in the cellular stress response.

MECHANISM OF REDOX MODULATION OF p53 PROTEIN IN CARDIOMYOCYTES DURING ADR-INDUCED CARDIOTOXICITY

The mechanism of redox regulation of p53 is not completely understood; however, it has been proposed that the intracellular redox state controls all levels of p53 induction and

function. p53 has been demonstrated to be regulated by the redox state at both transcriptional and posttranslational levels. At the transcriptional level, p53 expression has been shown to be induced by Ref-1, a redox/repair protein (19). Redox regulation at a posttranslational level often occurs through oxidation–reduction of cysteine residues of p53, which modulate conformation and DNA-binding activity (27). It is uncertain whether the redox state influences posttranslational modifications of p53. Recent studies have demonstrated that $O_2^{\bullet-}$ and NO directly regulate p53 signaling pathways through posttranslational modifications such as phosphorylation and acetylation (34, 38). Although our present study did not establish the mechanism of p53 regulation by redox modulation of ADR, evidence from previous studies demonstrated that p53 was stabilized in the cardiomyocyte response to ADR through activation of mitogen-activated protein kinases (MAPKs), resulting in phosphorylation of p53 at serine 15, and p53-mediated induction of cardiomyocyte apoptosis (8). Probucol, an antioxidant, has been shown to suppress the activation of MAPKs, indicating activation of MAPKs by ROS and oxidative stress in the cardiomyocyte exposure to ADR (21).

In summary, mitochondria are the most redox-active organelle and indispensable for cells to initiate or inhibit the apoptosis processes. Therefore, regulation of mitochondrial function and the mitochondrial redox state is important in determining cell survival and cell death; both processes at least partially involve functioning of the redox-sensitive p53 protein. Our study using MnSOD overexpressing mice or mice lacking the iNOS enzyme (or both) provide important evidence that p53 mitochondrial/nuclear localization and functions in cardiomyocytes after ADR administration may be regulated through redox-dependent mechanism(s) (Fig. 3B). In addition to MnSOD, the mitochondrial redox state has been shown to be controlled by the thioredoxin system, especially mitochondria-specific thioredoxin-2 (Trx-2), and peroxiredoxins are suggested to regulate cytochrome *c* release from mitochondria, which is a critical step in apoptotic pathways. Additionally, it has been shown that overexpression of thioredoxin-1 attenuates ADR-induced cardiotoxicity (31), whereas overexpression of Trx has been demonstrated to enhance anthracycline-mediated cancer cell apoptosis in a p53-dependent pathway (29). It will be interesting to examine the role of these antioxidant systems in ADR-induced cardiotoxicity to determine the relevance to p53 protein functions in nondividing cardiomyocytes versus dividing cancer cells.

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ABBREVIATIONS

ADR, Adriamycin; BSA-C, acetylated bovine serum albumin; H_2O_2, hydrogen peroxide; 4-HNE, 4-hydroxy-2-nonenal; iNOS, inducible nitric oxide synthase; iNOSKO$^{(-/-)}$, homozygous iNOS knockout mice; MAPKs, mitogen-activated protein kinases; MnSOD, manganese superoxide dismutase; mtDNA, mitochondrial DNA; NO, nitric oxide; 3-NT, 3-nitrotyrosine; Ntg, nontransgenic mice; nDNA, nuclear DNA; $^{\bullet}OH$, hydroxyl radical; 8-OHdG, 8-hydroxy-2'-deoxyguanosine; $ONOO^-$, peroxynitrite; RNS, reactive nitrogen species; ROS, reactive oxygen species; $O_2^{\bullet-}$, superoxide anion; TBS, Tris-buffered saline; TgM$^{(+/+)}$, homozygous MnSOD-overexpressing mice; TgM$^{(+/+)}$/iNOSKO$^{(-/-)}$, crossbreed of MnSOD-overexpressing mice and iNOS knockout.

APPENDIX

1. Generation of genetically engineered mice used in the present study has been previously described in detail (7). In brief, inducible NOS knockout mice (iNOSKO$^{(-/-)}$) purchased from Jackson Laboratories (Bar Harbor, ME) in the B57CL/6 background were bred into the B6C3 background for >10 generations. MnSOD-overexpressing mice expressing medium levels of activity (TgM$^{(+/-)}$) in the B6C3 background were bred to obtain homozygous MnSOD-overexpressing mice (TgM$^{(+/+)}$). The crossbreed TgM$^{(+/+)}$/iNOSKO$^{(-/-)}$ was generated by sequential selection and back-crossing between iNOSKO$^{(-/-)}$ and TgM$^{(+/+)}$ mice. All animal study protocols were approved by the Animal Care and Use Committee of the University of Kentucky in accordance with the National Institutes of Health guidelines for the use of laboratory animals.

Male mice, age 10–13 weeks, and weight, 22–28 g, were used in this study. Mice were randomly injected intraperitoneally with a single dose of either ADR (Pharmacia and Upjohn, Inc., Kalamazoo, MI) at 20 mg/kg (n = 2 mice for each time point) or the same volume of saline (2.9% sodium chloride solution, n = 1 mouse for each time point) as a control. Mice were killed with an i.p. injection of 20 mg/kg pentobarbital at four different time points (0, 3, 6, and 24 h), and heart tissues were subsequently collected and processed for immunogold ultrastructural analysis.

2. Heart tissues from the left ventricle were cut into 1-mm^3 cubes, fixed, embedded, and processed for immunogold electron microscopy, as described previously in detail (26), with minor modifications as described later in which the ultrasmall gold and silver enhancement technique was used to optimize the previously described immunogold labeling technique. Two embedded blocks from each heart for each mouse were sectioned and transferred to nickel grids. Grids were rinsed with TBS, blocked with 0.5% BSA-C (Aurion, Netherlands), and then washed with PBS. The grids were incubated with primary antibodies [anti-p53 (Santa Cruz Biotechnology Inc., Santa Cruz, CA) diluted 1:60, anti-8-OHdG [N45.1] (Nikken SEIL Corporation, JaICA, Shizuoka, Japan) diluted 1:100, and anti-cytochrome *c* antibody [clone 7H8.2C12] (PharMingen, San Diego, CA) diluted 1:40] at 4°C overnight in a humidified chamber. The grids were then washed and incubated with gold-conjugated secondary antibody [ultrasmall gold-conjugated F(ab′)$_2$ fragments of goat anti-rabbit IgG diluted 1:100 (Aurion, Netherlands) or 15-nm gold conjugated F(ab′)$_2$ fragments of goat anti-mouse IgG diluted 1:50 or goat anti-rabbit IgG diluted 1:75 (BB International Cardiff, UK)] for 90 min at room temperature. Grids were rinsed in TBS followed by ddH_2O.

Experimental grids stained with anti-p53 polyclonal antibody and ultrasmall gold conjugated secondary antibody were then incubated with R-gent SE-EM electron microscope grade silver-enhancement reagent (Aurion, Netherlands) for 20 min to enhance the visualization of gold particles. Grids were counterstained with uranyl acetate, observed, and photographed with an electron microscope (Hitachi H-600) operated at 75 kV.

In studies using the anti-8-OHdG antibody, the rinsing step, blocking step, and incubation with primary antibody were performed under vacuum to protect against false-positive results from the reaction of atmospheric oxygen and DNA in dried tissues.

As a control, normal rabbit or mouse serum (1:1,000, DAKO Inc., Carpinteria, CA) and antibody diluent (ScyTek, Logan, Utah) were used in place of primary antibody; these controls resulted in trace background labeling (data not shown).

The specificity of antibodies used in the present study was described previously in detail (25).

For relative quantification of an immunoreactive protein (p53 and cytochrome *c*) or 8-OHdG in an experimental group versus a control group, all sections were stained simultaneously under the same conditions. Random sampling was achieved by scanning the grid at low magnification so that immunogold beads could not be seen. Grids were scanned systematically from top to bottom and from left to right, and then photographs of entire cardiomyocyte cells were taken at × 8,000 magnification every eight to 10 grid fields.

Photographs of 30 cardiomyocyte cells were taken from each group. The area of each compartment (mitochondria, nucleus, and cytoplasm) was outlined and measured by image-analysis software, Scion Image Beta 4.02 (Scion Corporation, Frederick, MD). Gold beads within specific subcellular compartments were then counted per group. The mean density of gold beads/μm^2 area was expressed as mean value ± SEM of 30 cardiomyocyte cells. Data of 8-OHdG and p53 were shown as the ratio of labeling density (ADR/saline). For analysis of cytochrome *c*, data were expressed as mean value ± SEM of the ratio of cytosolic cytochrome *c*/mitochondrial cytochrome *c* from 30 cardiomyocytes.

3. Data were expressed as mean ± SEM. Statistical evaluation was performed with SPSS11 for Windows program (SPSS Inc., Chicago, IL). For comparison between ADR-treated mice versus control mice, independent Student's *t* test was used. For multiple comparisons of the 0 hour versus other time points, one-way ANOVA followed by post hoc test (LSD) was used.

The differences of ratio values between time points (0 h vs. other time points) and mouse groups (Ntg vs. genetically engineered mice) were compared by using bootstrap analysis. The statistical evaluations were performed by the R project for statistical computing software (www.r-project.org). Standard errors for ratio data were calculated by using propagation of error theory. A p value < 0.05 was considered significant, as indicated in the Results and Figures.

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