

# Manganese superoxide dismutase and inducible nitric oxide synthase modify early oxidative events in acute Adriamycin-induced mitochondrial toxicity

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## Abstract

In the present study, we used genetically engineered B6C3 mice [mice overexpressing manganese superoxide dismutase (TgM<sup>+/+</sup>), mice in which inducible nitric oxide synthase had been inactivated (iNOSKO<sup>-/-</sup>), and crosses of these two genotypes] to study the role of manganese superoxide dismutase (MnSOD) and inducible nitric oxide synthase (iNOS) in the development of acute Adriamycin-induced cardiotoxicity. Both nontransgenic and genetically engineered mice were treated with 20 mg/kg Adriamycin and cardiac left ventricular tissues studied at 0, 3, 6, and 24 hours. Ultrastructural damage and levels of 4-hydroxy-2-nonenal (4HNE) protein adducts and 3-nitrotyrosine (3NT) were determined in cardiomyocytes using immunogold ultrastructural techniques. Our previous results showed that Adriamycin caused mitochondrial injury without significant nuclear or cytoplasmic damage at early time points. Interestingly, overexpression of MnSOD protected against acute mitochondrial injury, whereas deficiency in iNOS potentiated mitochondrial injury in comparison with levels of injury present in cardiomyocyte mitochondria of nontransgenic mice. In TgM<sup>+/+</sup> mice,

there was a significant inverse correlation between mitochondrial injury and 4HNE/3NT levels at all time points analyzed, suggesting that reactive oxygen species/reactive nitrogen species damage products directly regulated acute Adriamycin-induced mitochondrial injury in these mice. The present studies are the first to directly quantify the effects of MnSOD and iNOS on mitochondrial injury during acute Adriamycin-induced cardiotoxicity and show extensive and specific patterns of posttranslational modifications of mitochondrial proteins following Adriamycin treatment. [Mol Cancer Ther 2005;4(7):1056–64]

## Introduction

The anthracycline antibiotic Adriamycin, or doxorubicin, is one of the most effective antitumor agents successfully used to treat human malignancies. However, treatment with Adriamycin is limited by cardiotoxicity. Many mechanisms have been proposed to explain Adriamycin-induced cardiotoxicity, including free radical production. Several *in vitro* studies have shown that reactive oxygen species (ROS)/reactive nitrogen species (RNS) are produced by Adriamycin treatment. Because of its quinone structure, Adriamycin can produce ROS/RNS by one-electron reduction reactions through metabolic activation catalyzed by several endogenous enzymes, including cytochrome P450, NADH dehydrogenase, and nitric oxide synthase (NOS; refs. 1–4).

Manganese superoxide dismutase (MnSOD) is the primary antioxidant enzyme that scavenges superoxide radicals (O<sub>2</sub><sup>•-</sup>) in mitochondria. Studies from other laboratories showed that MnSOD knockout mice died within 1 to 18 days after birth from neurodegeneration or dilated cardiomyopathy (5, 6). These studies proved that MnSOD plays a very important role in aerobic life, with lack of this enzyme resulting in death. Consistent with the important role of MnSOD, previous studies in our laboratory (7, 8) showed that overexpression of MnSOD in mouse cardiac mitochondria protected against Adriamycin-induced inhibition of mitochondrial respiratory complex I activity and reduced ultrastructural injury observed in cardiac mitochondria. These previous morphologic studies were only semiquantitative; without morphometric quantitation, it is not possible to determine the exact magnitude of the effects of MnSOD on Adriamycin-induced cardiac mitochondrial injury. Previous studies did not study the effects of inducible NOS (iNOS) on Adriamycin-induced cardiac injury.

Recently, the role of RNS in Adriamycin-induced cardiotoxicity has also been shown but has not been clearly

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defined. Mihm et al. (9) have shown 3-nitrotyrosine (3NT) accumulation in hearts of Adriamycin-treated mice. Aldieri et al. (10) showed that Adriamycin modulated iNOS expression by increasing both mRNA and protein levels with resultant increase in nitrate production. An increase in RNS formation may increase the level of oxidative/nitrative stress by generation of highly cytotoxic oxidant(s) such as peroxynitrite ( $\text{ONOO}^-$ ); alternatively, it may decrease the level of oxidative stress by neutralizing the toxic superoxide radical.

The present study was designed to analyze the role of MnSOD and iNOS enzymes in Adriamycin-induced acute toxicity by quantitation of the amount of mitochondrial injury and measuring levels of ROS/RNS damage products. We hypothesized that (a) MnSOD protects against cell injury by neutralization of the toxicity of superoxide radical, (b) the generation of nitric oxide ( $\text{*NO}$ ) by iNOS modulates Adriamycin-induced cardiac injury by regulation of ROS/RNS levels, and (c) the balance between activities of MnSOD and iNOS plays an important role in modulation of injury following Adriamycin treatment. The present studies are of especial relevance because both MnSOD and  $\text{*NO}$  can react with  $\text{O}_2^{\cdot-}$ , making prediction of biological outcome following modulation of MnSOD and iNOS difficult. The present study reports for the first time that the absence of iNOS potentiated Adriamycin-induced mitochondrial injury.

To test our hypotheses, we developed genetically engineered mice [mice overexpressing MnSOD because of increased levels of *sod2* gene ( $\text{TgM}^{+/+}$ ), mice in which the gene for inducible nitric oxide synthase (*nos2*) had been inactivated ( $\text{iNOSKO}^{-/-}$ ), and crosses between these two strains ( $\text{TgM}^{+/+}/\text{iNOSKO}^{-/-}$ )]; these mouse lines provide powerful tools to study mechanisms of Adriamycin-induced cardiotoxicity, particularly because these mouse models provide information from *in vivo* rather than *in vitro* systems. We analyzed cardiac mitochondrial injury and measured levels of mitochondrial oxidative [4-hydroxy-2-nonenal (4HNE) protein adducts, the result of reaction of free 4HNE with proteins] and nitrative [3NT, a marker of the production of reactive nitrogen-centered oxidants and their subsequent interaction with proteins] damage products by using immunogold ultrastructural techniques. Previous studies have measured ROS/RNS and/or oxidative/nitrative damage product levels in tissue homogenates (11–13); these assays measure total cellular ROS/RNS levels and/or damage products, but disruption of normal tissue three-dimensional architectures and specific subcellular localization may introduce numerous artifacts. Furthermore, disruption of tissues makes analysis of ROS/RNS levels in specific subcellular compartments impossible. It is possible that biochemical changes in reduction-oxidation (redox) chemistry occur even if redox is measured in purified isolated subcellular organelles. The use of immunogold techniques with specific antibodies to oxidative/nitrative damage products not only allows

for identification of intracellular sites where injury has occurred but also permits relative quantitation of ROS/RNS damage products at the site(s) of injury without disruption of tissue.

Data from the present study provides strong direct evidence supporting the hypothesis that significant mitochondrial injury and oxidative/nitrative protein modifications are early events preceding extensive cell injury following Adriamycin treatment. Most importantly, this study is the first to show that both MnSOD and iNOS are important enzymes involved in regulation of mitochondrial oxidative stress-mediated cardiac toxicity and posttranslational modifications of mitochondrial proteins following Adriamycin treatment.

## Materials and Methods

### Animals and Treatment

Mice were from The Jackson Laboratory (Bar Harbor, ME) and housed at the animal care facility of the University of Kentucky. The animal study protocols described here were approved by the animal care committee of University of Kentucky and conform to NIH guidelines for animal care. Males from inbred B6C3 mice, ages 10 to 13 weeks and weight 22 to 28 g, were used for this study. For quantitative ultrastructural and immunogold analysis, mice were injected i.p. with one dose of either Adriamycin (Pharmacia & Upjohn, Kalamazoo, MI) at 20 mg/kg (a dose which causes heart injury during the first five days but shows low mortality rate;  $n = 2$  mice for each time point) or the same volume of saline (0.9% sodium chloride solution;  $n = 1$  for each time point) as a control. Mice were euthanized by injection with 20 mg/kg of pentobarbital (Abbott Laboratories, North Chicago, IL) i.p. at different time points (for ultrastructural pathology and immunogold analysis: 0, 3, 6, and 24 hours and for Griess assay: 0, 12, and 24 hours). Heart tissues from the left ventricle were then cut into 1-mm<sup>3</sup> cubes and processed for further studies. The time period from euthanasia to placement of tissues into fixative was ~15 minutes. Thus, the time point labeled “zero” was actually ~15 minutes after the initial Adriamycin dose; this lag period was present for each time point studied in the text.

### Generation of Genetically Engineered Mice

iNOS knockout mice were purchased from The Jackson Laboratory in the C57BL/6 background. The  $\text{iNOS}^{-/-}$  mice were bred into the B6C3 background and the colony was maintained at the University of Kentucky. Three lines of MnSOD-overexpressing mice in the B6C3 background were generated using human MnSOD cDNA (low, medium, and high expression; ref. 8). The human  $\beta$ -actin 5' flanking sequence and promoter was used to target mRNA expression at significantly increased levels in heart tissues, as described by Yen et al. (8). The medium expressing line ( $\text{TgM}^{+/-}$ ) was bred to obtain homozygous MnSOD-overexpressing mice ( $\text{TgM}^{+/+}$ ). The  $\text{TgM}^{+/+}/\text{iNOSKO}^{-/-}$  cross was generated by sequential selection and backcrossing between  $\text{iNOSKO}^{-/-}$  and  $\text{TgM}^{+/+}$  mice. Homozygosity was confirmed by backcrossing to nontransgenic

mice. After >10 generations of backcrossing, 8- to 10-week-old male mice were used for experiments. Age- and gender-matched nontransgenic mice were bred and maintained in the B6C3 background to serve as controls. All genetically engineered mice showed normal phenotypes, and no pathologic lesions were observed during the life span of these genetically engineered mice.

#### **Southern Blot Analysis of Genetically Engineered Mice**

Genomic DNA was isolated from mouse tail, digested with *Pst*I, separated on 0.7% or 0.9% agarose gels and transferred to a nytran membrane (Schleicher & Schuell, Keene, NH) as previously described (8). Using a vacuum oven prewarmed to 80°C, the blot was baked for 2 hours. The blot was prehybridized, hybridized with <sup>32</sup>P labeled mouse iNOS or human MnSOD cDNA, and subsequently washed under high stringency conditions as previously described (8). The blot was then exposed to Kodak X-ray film at -80°C overnight.

#### **Western Blot Analysis**

Protein concentrations of heart tissue homogenates were determined using a Bradford dye binding assay (Bio-Rad Laboratories, Hercules, CA). Whole tissue homogenates were electrophoresed using standard SDS-PAGE techniques and used to detect MnSOD and CuZnSOD immunoreactive proteins by Western blot assay as previously described (14). Primary rabbit anti-MnSOD antibody (1:5,000) was purchased from Upstate Biotechnology (Lake Placid, NY) and primary sheep anti-CuZnSOD antibody (1:1,000) was purchased from Calbiochem (San Diego, CA). Secondary goat anti-rabbit (1:3,000) and rabbit anti-sheep (1:10,000) IgG antibodies conjugated to horseradish peroxidase were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Protein bands were visualized using the enhanced chemiluminescence detection system from Amersham Pharmacia Biotech (Buckinghamshire, England).

#### **Griess Assay of Serum from Genetically Engineered Mice**

Serum was centrifuged using ULTRAFREE-0.5 Biomax-30 membrane tubes at 10,000 × *g* for 30 minutes. A final volume of 200 μL, including 40 μL of sample, 40 μL of nitrate/nitrite assay buffer, and 10 μL each of nitrate reductase cofactor and nitrate reductase enzyme preparation, was incubated covered at room temperature for 3 hours. After the addition of 50 μL each of Griess reagents R1 and R2, the samples were incubated uncovered for 10 minutes at room temperature and the formation of a deep purple azo compound was detected using a spectrophotometer at 540 nm. All reagents were obtained from Cayman Chemical (Ann Arbor, MI).

#### **Morphometric Quantification by Electron Microscopy**

Heart tissues from left ventricle were fixed, embedded, and processed for routine electron microscopy as previously described (8). Three embedded blocks from each heart for each mouse were sectioned and transferred to copper grids. Only longitudinal sections of cardiac muscle were used for analysis. Grids were observed in an electron microscope (Hitachi H-600) operated at 75 kV. Random

sampling was achieved by scanning the grid at low magnification so that cell injury was not apparent, yet gross sample artifacts (folds in tissues, dust particles, etc.) could be avoided. Grids were systematically scanned from top to bottom and from left to right so that photographs of entire cardiomyocytes were taken at 10,000× magnification every 10 to 15 grid fields. Thirty cardiomyocytes were photographed for each mouse group.

Mitochondria displaying any or several of the following ultrastructural criteria were used for determination of *area involved by mitochondrial damage*: mitochondrial swelling, perimitochondrial swelling, mitochondria with the presence of myelin figures, mitochondria with loss of cristae, degeneration of mitochondria with disorganized electron dense cristae, lysosomal degradation of mitochondria, vacuolization in mitochondria, and mitochondrial membrane disruption. A previous article has illustrated these morphologic lesions in detail (15). The data for mitochondrial damage were presented as the average of the area involved by mitochondrial damage divided by the total area of mitochondria analyzed from 30 cardiomyocytes in each group at each time point.

Mitochondrial damage and areas were measured in μm<sup>2</sup> using image analysis software Scion Image Beta 4.02 (Scion Corp., Frederick, MD) with a PC computer (Dell OptiPlex GX200) as previously described (16). All ultrastructural data described in the present study were from cardiomyocytes and not other cell types present in heart.

#### **Ultrastructural Localization and Relative Quantification of 4HNE Protein Adducts, 3NT, and β-Actin**

Heart tissues from the left ventricle were fixed, embedded, and processed for immunogold electron microscopy as previously described in detail (16). Tight correlation between biochemical measurement of lipid peroxidation products and analysis of 4HNE protein adducts by immunogold electron microscopy techniques has been previously shown (17). Two embedded blocks from each mouse heart were sectioned and transferred to nickel grids. Only longitudinal sections of cardiac muscle were used for analysis. Grids were rinsed with TBS [0.05 mol/L Tris, 0.9% NaCl (pH 7.6)], blocked with acetylated bovine serum albumin (Aurion, AA Wageningen, The Netherlands), and washed with TBS/acetylated bovine serum albumin (1:10 dilution of acetylated bovine serum albumin solution in TBS). The grids were incubated with primary antibodies [rabbit anti-4HNE protein adducts antibody (obtained from Dr. Luke Szewda, Oklahoma Medical Research Foundation, Oklahoma City, OK) diluted 1:80, rabbit anti-3NT antibody (Upstate Biotechnology) diluted 1:400, or rabbit anti-β actin antibody (Santa Cruz Biotechnology) diluted 1:80] at 4°C overnight in a humidified chamber. The grids were incubated with diluted (1:75) gold conjugated secondary antibody (15-nm gold-conjugated goat anti-rabbit IgG H+L; BB International Cardiss, United Kingdom) for 90 minutes at room temperature. Grids were then washed in TBS, counterstained with uranyl acetate, observed, and photographed with an electron microscope (Hitachi H-600)

operated at 75 kV. A previous article has illustrated immunogold labeling of cardiomyocyte mitochondria from Adriamycin- or saline-treated mice using anti-4HNE protein adducts or 3NT antibodies (15).

For experimental grids stained with rabbit anti-3NT antibody, the rinsing step, blocking step, and incubation with the primary antibody were done under a vacuum system to protect against the reactions of ROS/RNS with atmospheric oxygen and nitrogen. The reaction of  $\cdot\text{NO}$  with oxygen ( $\text{O}_2$ ) leads to nitrogen dioxide ( $\text{NO}_2$ ) production, which is known to nitrate tyrosine residues with  $k_m = 2.9 \times 10^7 \text{ (mol/L)}^{-1}\text{s}^{-1}$  *in vivo* (18, 19). To protect against false positive results, the grid samples were kept under vacuum conditions until after staining with the primary antibody. Samples not treated in this fashion showed increasing nonspecific labeling as a function of time after exposure to air (data not shown).

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

For relative quantification of the immunoreactive protein of interest (4HNE modified proteins, 3NT, and  $\beta$ -actin) in an experimental group versus a control group, all of the sections were stained simultaneously under the same conditions. For successful analysis of immunogold studies, all tissues must be treated the same, which for technical

reasons limited the number of mice that could be simultaneously evaluated and thus was a factor in determining the number of mice used for this analysis. Random sampling was achieved by scanning the grid at low magnification so that immunogold beads could not be seen, yet gross sample artifacts (folds in tissues, dust particles, etc.) could be avoided. Grids were systematically scanned from top to bottom and from left to right, and then photographs of entire cardiomyocytes were taken at 12,300 $\times$  magnification every 10 to 15 grid fields.

Photographs of 30 cardiomyocytes were taken from each mouse group. Mitochondria were outlined and measured by image analysis software as previously described (20). Gold beads within mitochondria were then counted manually; over 1,000 gold beads were counted per group. The mean density of gold beads/ $\mu\text{m}^2$  area was expressed as mean value  $\pm$  SE of 30 cardiomyocytes.

#### Antibody Specificity

The specificity of antibodies used in present study has been previously described in detail (15).

#### Statistical Analysis

Quantitative results were expressed as means  $\pm$  SE. Statistical analysis of normalized values (Adriamycin/saline) and ratio values (Adriamycin/saline) between mouse groups (nontransgenic versus genetically engineered mice) and time points (0 hour versus other time points) was done using contrast analysis and bootstrap analysis respectively. These statistical evaluations were done by the R project for statistical computing software.<sup>5</sup> SEs for ratio data were calculated using propagation of error theory.

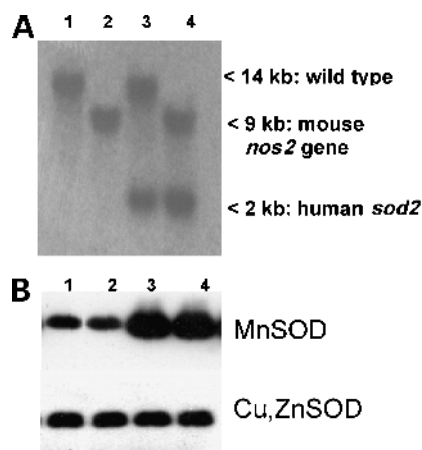
In cases of multiple comparisons (0 hour versus other time points or nontransgenic versus genetically engineered mice), one-way ANOVA followed by a post hoc test (LSD) were used. Linear regression analysis was used for correlation coefficient analysis (4HNE/3NT versus injury). These statistical evaluations were done by SPSS11 for windows program (SPSS, Inc., Chicago, IL).

$P \leq 0.05$  was considered significant as indicated in Results and figures.

## Results

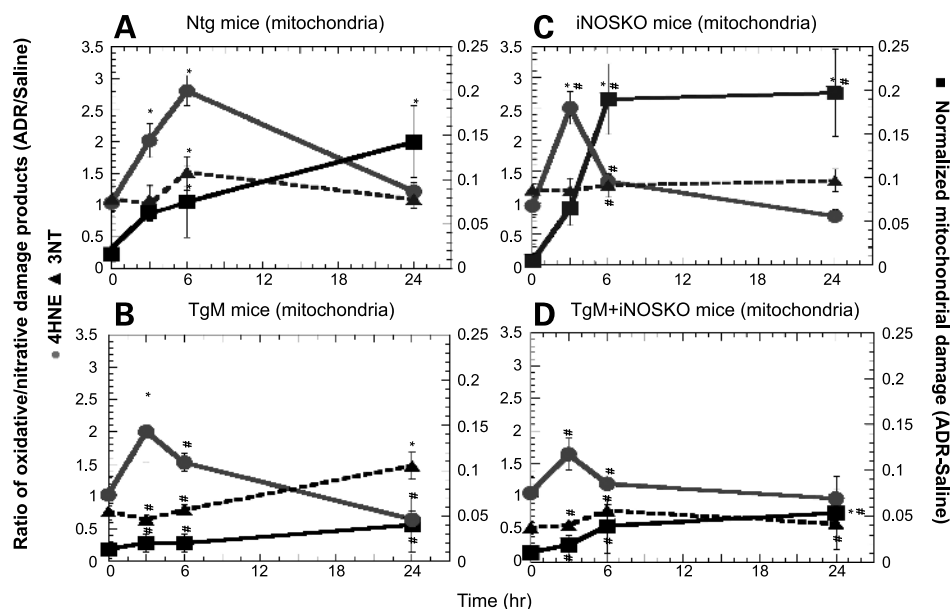
### Southern Blot and Western Blot Analyses of Genetically Engineered Mice

Mice that were homozygous and overexpressed human MnSOD (B6C3 background) were selected to cross with *iNOSKO*<sup>-/-</sup> mice propagated in the B6C3 background as described in Materials and Methods. Several founder mice that were homozygous for *nos2* deficiency but overexpressed the *sod2* gene were identified using Southern blot analysis following many rounds of backcrossing. The homozygosity of the transgene and knockout mice were further verified by the generation of 100% *sod2* transgene and *nos2*-deficient progenies when crossed with nontransgenic mice (data not shown). Figure 1A depicts a



**Figure 1.** Generation of genetically engineered mice. **A**, mouse tail DNA was analyzed for *nos2* and *sod2* expression by Southern analysis. Blots were cohybridized with mouse *iNOS* and human MnSOD cDNA. The 14-kb band represents the wild-type mouse *nos2* gene; the 9-kb band represents the *nos2* knockout gene; the 2-kb band represents the human *sod2* gene. Nontransgenic mice expressed only the wild-type *nos2* band (14 kb), whereas *iNOSKO*<sup>-/-</sup> mice expressed only the *nos2* knockout band (9 kb). *TgM*<sup>+/+</sup> mice expressed both wild-type mouse *nos2* and human *sod2*-overexpressing bands (14 and 2 kb), whereas *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice expressed mouse *nos2* knockout and human *sod2*-overexpressing bands (9 and 2 kb). Lane 1, nontransgenic mice; lane 2, *iNOSKO*<sup>-/-</sup> mice; lane 3, *TgM*<sup>+/+</sup> mice; lane 4, *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice. **B**, Western blot analysis of cardiac tissues for MnSOD immunoreactive protein expression, further verifying the overexpression of MnSOD in both *TgM*<sup>+/+</sup> and *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice. CuZnSOD protein expression was used for documentation of equal protein loading. 50  $\mu\text{g}$  of protein was loaded per well. Lanes are the same as described for (A).

<sup>5</sup> <http://www.r-project.org>.



**Figure 2.** Summary of time course of biochemical and ultrastructural changes in mitochondria after treatment with Adriamycin (ADR). **A**, nontransgenic (*Ntg*) mice showed an increase in 4HNE protein adducts levels at 3 h, which preceded an increase in 3NT levels and mitochondrial injury at 6 h. At 24 h, the levels of 4HNE protein adducts and 3NT were decreased, whereas mitochondrial injury increased as a function of time. **B**, *TgM*<sup>+/+</sup> mice showed a small increase in 4HNE protein adducts level at 3 h. 3NT formation was low and delayed to 24 h. Mitochondrial injury was minor and present only at 24 h after treatment with Adriamycin. **C**, *iNOSKO*<sup>-/-</sup> mice showed increased mitochondrial injury and accelerated time of maximal injury (6 h) in comparison with nontransgenic mice (maximal at 24 h). The maximal increase in 4HNE protein adducts levels occurred at 3 h, but 4HNE-protein adducts levels were only elevated for a short time. There were no significant increases in 3NT formation. **D**, *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice showed a minimal increase in 4HNE protein adducts and mitochondrial injury. 3NT levels were low and there was no significant change in 3NT formation in mitochondria as a function of time. \*,  $P \leq 0.05$  versus saline for nontransgenic mice; #,  $P \leq 0.05$  when compared with nontransgenic mice treated with Adriamycin at the same time points.

representative Southern blot analysis in which tail DNA from mice was analyzed for mouse *nos2* and human *sod2* expression. Blots were cohybridized with radiolabeled mouse iNOS and human MnSOD cDNA. As shown in Fig. 1A, nontransgenic mice expressed only wild-type *nos2* band at 14 kb, whereas *iNOSKO*<sup>-/-</sup> mice expressed only an *nos2* knockout band at 9 kb. *TgM*<sup>+/+</sup> mice expressed both wild-type mouse *nos2* and human *sod2*-overexpressing bands at 14 and 2 kb, respectively, whereas *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice expressed *nos2* knockout and human *sod2*-overexpressing bands at 9 and 2 kb, respectively.

Western blot analysis was also used to confirm levels of MnSOD immunoreactive protein expression in all four genotypes of mice. As shown in Fig. 1B, MnSOD immunoreactive protein levels were increased in *TgM*<sup>+/+</sup> and *TgM*<sup>+/+</sup>/*iNOSKO*<sup>-/-</sup> mice. MnSOD immunoreactive protein expression in *iNOSKO*<sup>-/-</sup> mice was unchanged from that of nontransgenic controls. Immunoreactive protein levels of CuZnSOD were not changed in all four genotypes.

#### Genetically Engineered Mice with Modulation of MnSOD and/or iNOS Levels Showed Alterations of Early Events in Adriamycin-Induced Mitochondrial Toxicity and Oxidative/Nitrative Stress

In the present studies, we used genetically engineered mice compared with nontransgenic mice (the latter results reprinted with permission; ref. 15) to study the role of MnSOD and iNOS in Adriamycin-induced cardiotoxicity.

All the data presented herein were obtained from cardiomyocytes only. The same set of mice used in the ultrastructural analysis was also used for 4HNE protein adducts and 3NT immunogold studies. At these early time points examined, there was minor cytoplasmic and nuclear damage (data not shown). Therefore, subsequent analysis was focused on mitochondria only. Changes in mitochondrial biochemistry and ultrastructural damage in each mouse line treated with Adriamycin are summarized in Fig. 2. Mitochondrial biochemistry data (4HNE/3NT) were presented as ratios (Adriamycin-saline). Mitochondrial ultrastructural damage data were presented as normalized values (Adriamycin/saline; data were not expressed as ratios because several values derived from saline-treated mice were zero). All raw data are presented in Supplementary Figs. i-iii.<sup>6</sup>

As shown in Fig. 2A, nontransgenic mice showed a significant increase in 4HNE protein adducts levels at 3 hours that preceded a significant increase in 3NT levels and mitochondrial injury at 6 hours. At 24 hours, levels of 4HNE protein adducts and 3NT were decreased, whereas mitochondrial injury increased as a function of time. As illustrated in Fig. 2B, transgenic mouse heart overexpressing MnSOD showed a dramatically significant decrease in

<sup>6</sup>Supplementary material for this article is available at Molecular Cancer Therapeutics Online (<http://mct.aacrjournals.org/>).

mitochondrial injury as early as 3 hours compared with nontransgenic mice (4-fold decrease). However, TgM<sup>+/+</sup> mice also showed an increase in 4HNE protein adducts levels at 3 hours, but at 6 hours, 4HNE protein adducts levels were decreased in comparison to levels in nontransgenic mice. In contrast, TgM<sup>+/+</sup> mice did not show a significant increase in immunoreactive 3NT protein levels until 24 hours after treatment with Adriamycin. Interestingly, cardiomyocytes from mice deficient in the *nos2* gene showed a significant increase in mitochondrial injury (about 2-fold increase) and showed an acceleration in the appearance of maximal injury (at 6 hours) in comparison to nontransgenic mice (maximal at 24 hours; Fig. 2C). The maximal increase in 4HNE protein adducts levels in iNOSKO<sup>-/-</sup> mitochondria occurred at 3 hours (25% higher when compared with nontransgenic at the same time point) and were elevated for only a short time in iNOSKO<sup>-/-</sup> mice (decreased at 6 and 24 hours). There were no significant changes in 3NT formation in iNOSKO<sup>-/-</sup> mitochondria at any time points. Finally, TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice, in which the metabolism of ROS and/or RNS have presumably been altered, also showed a decrease in mitochondrial injury as early as 3 hours (4-fold decrease) compared with nontransgenic mice (Fig. 2D). TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice showed a minimal increase in 4HNE protein adducts compared with the other mouse lines. There was no significant change in 3NT formation in mitochondria as a function of time in this latter group of mice.

#### Serum Nitrate Levels in Genetically Engineered Mice

The Griess reaction was used to assess the levels of total nitrate and nitrite generated in serum of genetically engineered mice after treatment with Adriamycin (0, 12, and 24 hours). There were no significant changes in serum nitrate levels of saline-treated mice. As shown in Fig. 3, nontransgenic mice showed an early slight increase and a subsequent large increase in serum nitrate levels after Adriamycin treatment at 12 and 24 hours, respectively. In contrast, serum nitrate levels in TgM<sup>+/+</sup>, iNOSKO<sup>-/-</sup>, and TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice were not significantly different after treatment with Adriamycin at any time points. Serum nitrate levels in these genetically engineered mice were lower than in serum from nontransgenic mice after treatment with Adriamycin.

#### Early Reduction in the Ratio of Mitochondrial 4HNE/3NT Levels in MnSOD-Overexpressing Mice Inversely Correlated with Mitochondrial Injury following Adriamycin Treatment

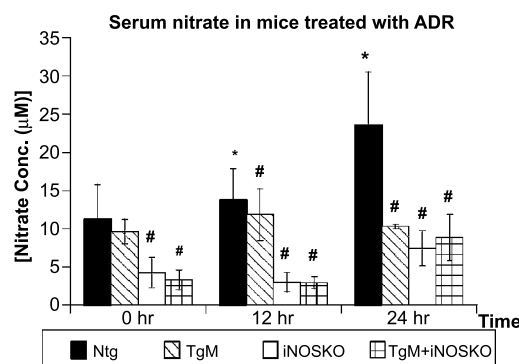
Correlation analysis between the ratio of mitochondrial 4HNE/3NT levels and degree of injury using linear regression analysis was done. As shown in Fig. 4, correlation analysis clearly showed an inverse relationship between the ratio of mitochondrial 4HNE/3NT levels and injury in MnSOD-overexpressing mice throughout the time course of treatment with Adriamycin ( $R = 0.91$ ,  $P = 0.045$ ). Similar correlation analysis in nontransgenic, iNOSKO<sup>-/-</sup>, and TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice did not show direct relationships between mitochondrial injury and 4HNE/3NT levels at all time points examined (data not shown).

#### Effect of Adriamycin on the Housekeeping Protein $\beta$ -Actin

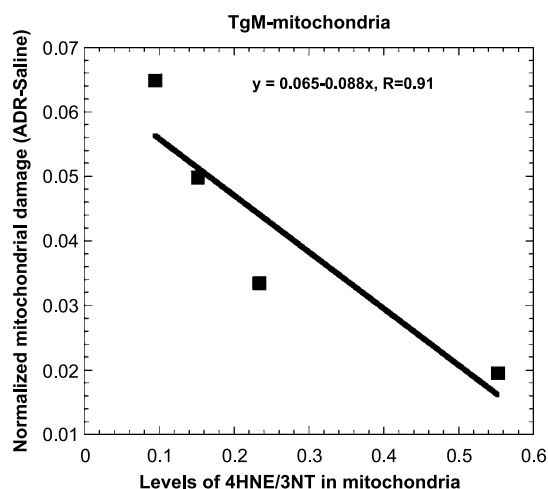
We measured  $\beta$ -actin immunoreactive protein levels by using specific antibody with immunogold labeling and image analysis techniques to confirm the fact that the alterations in our modified proteins of interest (4HNE protein adducts and 3NT) were real and not due to changes in overall protein expression induced by Adriamycin. The same mouse tissues used for 4HNE protein adducts and 3NT studies were analyzed. There were no significant differences in  $\beta$ -actin immunoreactive protein levels in cardiomyocytes from all mouse groups at any time points (data not shown). These data confirmed the specificity of our oxidative/nitrative damage products expression data.

#### Discussion

In the present studies, we used genetically engineered transgenic and knockout mice to study possible mechanism(s) by which MnSOD and iNOS modulate early events in Adriamycin-induced cardiotoxicity. One of the prominent theories of the mechanism of injury in Adriamycin-induced cardiotoxicity is oxidative/nitrative damage. In TgM<sup>+/+</sup> mice, we observed a decrease of mitochondrial 4HNE protein adducts levels following Adriamycin treatment in comparison with results obtained from nontransgenic mice. One possible mechanism by which MnSOD may decrease 4HNE protein adduct levels is by increasing the dismutation of  $O_2^{\bullet-}$  in mitochondria and reducing mitochondrial  $O_2^{\bullet-}$  levels hence reducing hydroxyl radical and subsequent 4HNE formation. Moreover, we observed a delay in mitochondrial 3NT formation in TgM<sup>+/+</sup> mice. We postulated that the delay in 3NT levels may be due to a decrease in  $O_2^{\bullet-}$  concentration thereby resulting in reduced reaction of  $^{\bullet}NO$  with  $O_2^{\bullet-}$ . Our results are in agreement with the hypothesis of Liochev and Fridovich (21) that MnSOD provides  $^{\bullet}NO$  with a longer half-life by sparing it from



**Figure 3.** Serum nitrate levels were significantly elevated in nontransgenic (Ntg) but not iNOSKO<sup>-/-</sup> mice. Serum nitrate concentration ( $\mu\text{mol/L}$ ) as a function of time following treatment with Adriamycin (ADR,  $n = 6$  mice for each time point) was determined. \*,  $P \leq 0.05$  versus saline for nontransgenic mice; #,  $P \leq 0.05$  when compared with nontransgenic mice treated with Adriamycin at the same time points.



**Figure 4.** Correlation coefficient analysis of mitochondrial 4HNE/3NT ratios and mitochondrial injury in TgM<sup>+/+</sup> mice after treatment with Adriamycin (ADR).

reaction with  $O_2^{\bullet-}$  thus allowing  $\bullet NO$  to carry out its multiple physiologic functions. Moreover, MnSOD together with hydrogen peroxide ( $H_2O_2$ ) has been shown to cause rapid breakdown of  $\bullet NO$  and production of  $ONOO^-$  *in vitro* (22). This latter reaction might also explain the increase in 3NT levels observed in TgM<sup>+/+</sup> mice at later time points.

MnSOD scavenges  $O_2^{\bullet-}$  and produces  $H_2O_2$  in mitochondria. Thus, there is the possibility that TgM<sup>+/+</sup> mice could overexpress  $H_2O_2$ . Many studies have shown that high levels of  $H_2O_2$  cause injury and may generate more highly reactive free radical species in the presence of iron. However, our control mice (TgM<sup>+/+</sup> treated with saline at 0 hour) did not show any significant cell injury and showed low levels of 4HNE protein adducts and 3NT (Supplementary Figs. i-iii).<sup>6</sup> Thus, even if  $H_2O_2$  was produced at significant levels in mice overexpressing MnSOD, some adaptive response must have neutralized the increased  $H_2O_2$ . In fact, catalase and glutathione peroxidase enzyme activities have been shown to be unchanged in TgM<sup>+/+</sup> mice (7); because these enzymes are induced by  $H_2O_2$ , the absence of increased enzyme activities suggests  $H_2O_2$  levels were not elevated.

It is still unclear whether  $\bullet NO$  is primarily protective or injurious following Adriamycin treatment, because  $\bullet NO$  has been shown to play a key role in many pathophysiologic processes. Moreover, RNS have been postulated to regulate ROS and cellular redox homeostasis (23). The present study showed that Adriamycin induced  $\bullet NO$  production because we observed increases in serum nitrate and nitrate damage products after Adriamycin treatment. We observed that deficiency of cardiac iNOS resulted in reduced cardiomyocyte mitochondrial 4HNE protein adducts in relation to levels found in nontransgenic mice, but cardiomyocytes showed increased susceptibility of mitochondria to Adriamycin-induced injury. The smaller in-

crease in mitochondrial 4HNE protein adducts levels in iNOSKO<sup>-/-</sup> mice occurred at the same time as the first appearance of mitochondrial injury (3 hours), which are results similar to those observed in nontransgenic mice. It is possible that mitochondrial 4HNE protein adducts may actually first appear between 0 and 3 hours in iNOSKO<sup>-/-</sup> mice, because we did not measure 4HNE protein adducts at these earlier time points. Importantly, iNOS deficiency with presumed reduction of  $\bullet NO$  levels (because 3NT levels were low and the *nos2* gene was inactivated) was associated with increased Adriamycin-induced cardiac injury, perhaps because of the reduced reaction of  $\bullet NO$  with  $O_2^{\bullet-}$ . The rate constant for the reaction of  $\bullet NO$  with  $O_2^{\bullet-}$  to yield  $ONOO^-$  [ $1.9 \times 10^{10}$  (mol/L/L)<sup>-1</sup>s<sup>-1</sup>] is more than three times faster than that of the enzymatic dismutation of  $O_2^{\bullet-}$  catalyzed by SOD ( $k_{SOD} = 2 \times 10^9$  (mol/L/L)<sup>-1</sup>s<sup>-1</sup>; ref. 24). Therefore, mitochondria with inadequate  $\bullet NO$  levels may be more sensitive to injury than normal mitochondria. The injury in iNOSKO<sup>-/-</sup> mice was increased throughout the time course of the experiment, whereas 4HNE protein adducts levels were decreased after 3 hours, presumably due to degradation by the proteasome and/or lysosome systems (25); one could also postulate the existence of a previously unidentified protein repair system that enzymatically removes 4HNE from protein (26), although no evidence of such a system has been reported to date.

Mikkelsen and Wardman (23) suggested that all of the following factors determine whether  $\bullet NO$  enhances or ameliorates cell injury: the relative rate of production, steady-state concentrations, cellular site(s) of production of  $\bullet NO$  with  $O_2^{\bullet-}$ , and the dominant mechanisms of oxidant damage in various tissues at the time of  $\bullet NO$  and  $O_2^{\bullet-}$  production. Herein, we postulated from our data that iNOS-derived  $\bullet NO$  is an important molecule in the protection of cardiomyocytes from Adriamycin-induced mitochondrial toxicity at early time points, because deficiency of  $\bullet NO$  enhanced mitochondrial toxicity and mitochondrial oxidative damage was prominent (occurring as early as 3 hours). Mikkelsen and Wardman (23) also proposed that RNS is a redox messenger that is activated by oxidative stress. Conversions of oxidative events to nitrate events are postulated to provide an amplification mechanism that activates redox signal transduction pathways regulating biological responses. Moreover, Liochev and Fridovich (27) have proposed that  $O_2^{\bullet-}$  in one subcellular compartment can be converted to a membrane-permeable product ( $ONOO^-$ ), which can then diffuse into an adjacent subcellular compartment and exert biological effects. We postulate that balancing mitochondrial redox state following Adriamycin treatment requires the presence of mitochondrial RNS.

By measuring 4HNE and 3NT levels in TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice, we have provided indirect evidence that both ROS and RNS participated in Adriamycin-induced mitochondrial toxicity and provided new direct evidence that MnSOD and iNOS modulate cardiomyocyte mitochondrial injury and specific patterns and levels of mitochondrial oxidative/nitrate damage products

following Adriamycin treatment. TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice showed only a slight increase in mitochondrial 4HNE protein adducts and injury following Adriamycin treatment. In this mouse line, the decrease in levels of mitochondrial 4HNE protein adducts may result in protection from Adriamycin-induced injury. Mitochondrial 3NT levels were low in TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice and did not significantly change as a function of time after Adriamycin treatment. Therefore, cardiac protection by overexpression of MnSOD may reduce the need for \*NO produced by iNOS. However, longer treatment with Adriamycin (5 days) resulted in less injury in TgM<sup>+/+</sup> mice with an intact *nos2* gene than observed in TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice (data not shown). Therefore, adequate levels of iNOS may still be required for protection of the heart under pathologic conditions.

Remarkably, our correlation analysis showed that the ratio of mitochondrial 4HNE/3NT inversely correlated with mitochondrial injury in TgM<sup>+/+</sup> mice ( $R = 0.91$ ), which indicated that overexpression of MnSOD attenuated mitochondrial injury by decreasing mitochondrial oxidative/nitrative products formation at early time points after treatment with Adriamycin. The results showed in TgM<sup>+/+</sup>/iNOSKO<sup>-/-</sup> mice also directly suggest the possibility that mitochondrial superoxide scavengers reduce Adriamycin-induced cardiotoxicity in the absence of a compensatory response by iNOS. This hypothesis will be directly tested in our laboratory using low molecular weight SOD mimics.

Together, our data suggest that (a) Mitochondria were the subcellular organelle most extensively injured in Adriamycin-induced cardiac toxicity (15). (b) Oxidative/nitrative modified proteins appeared at early time points following Adriamycin treatment. (c) MnSOD protected against cell injury, with one possible mechanism being neutralization of the toxicity of superoxide radical. (d) iNOS deficiency increased Adriamycin-induced injury, with one possible mechanism being lack of nitric oxide protection against ROS toxicity. (e) The proper balance between levels of 4HNE protein adducts and 3NT played an important role in cardiomyocyte injury following Adriamycin treatment.

Our results satisfy the criteria proposed by Halliwell and Whiteman to define whether tissue damage induced by an agent (Adriamycin) is at least partially due to ROS (28). These criteria are (a) Oxidative damage should always be demonstrable at the site of injury. (b) The time course of formation of the oxidative damage should be consistent with the time course of tissue injury, preceding or accompanying it. (c) Direct application of the ROS (in this case, Adriamycin induced) over a relevant time course (point b above) to the tissue at concentrations within the range found *in vivo* should reproduce most or all of the tissue injury and oxidative damage observed. (d) Removing the oxidative damage or inhibiting its formation should diminish the tissue injury (TgM<sup>+/+</sup> data). Our results suggest that modulation of mitochondrial ROS levels should be tested in animal models in an

attempt to selectively reduce Adriamycin-induced cardiac injury without interfering with the potent anticancer effect of Adriamycin. Such studies are currently planned in our laboratory.

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