

The protective roles of nitric oxide and superoxide dismutase in adriamycin-induced cardiotoxicity

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Abstract

Objective: Treatment with adriamycin (ADR) is associated with cardiotoxicity mediated through the generation of superoxide ($O_2^{\cdot-}$). Because nitric oxide (*NO) reacts with $O_2^{\cdot-}$, generating peroxynitrite, we hypothesized that decreased *NO production would lead to protection in acute cardiac injury.

Methods: We investigated the role of decreased *NO levels in exacerbation of ADR-induced cardiotoxicity in vivo using iNOS ($-/-$) mice. Pathology, biochemical injury markers, and cardiac function were used to assess ADR-induced cardiac injury.

Results: Ultrastructural analysis demonstrated that iNOS ($-/-$) mice exhibited extensive cytoplasmic swelling and degeneration of mitochondria when compared to wildtype mice following treatment with ADR. Mice lacking iNOS exhibited a decrease in resting indices of cardiac function as well as an impairment in the positive inotropic actions of isoproterenol following treatment with ADR compared to nTg mice. Cardiac troponin, creatine phosphokinase, and lactate dehydrogenase levels were significantly increased after treatment in iNOS ($-/-$) mice as compared to controls and wildtype mice.

Conclusions: These results indicate that a lack of *NO production by iNOS caused significantly enhanced cardiac injury. However, when iNOS ($-/-$) mice were crossed with manganese superoxide dismutase (MnSOD)-overexpressing animals, mitochondrial injury was ameliorated to the level of the wild type. These findings suggest that reduction of *NO levels mediated by ADR treatment leads to increased cardiac mitochondrial injury that can be attenuated by a compensatory increase in MnSOD.

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1. Introduction

Adriamycin (ADR) is used to treat a wide range of malignant tumors. One limitation of this drug is an associated dose-dependent cardiac toxicity. Olson and Capen first showed cardiac damage in mitochondria and endoplasmic reticulum following ADR treatment using

electron microscopy [1,2]. Although it is perhaps one of the mechanisms of ADR's efficacy, a free radical mediated theory has been proposed to explain ADR-induced cardiac injury [3]. The quinone moiety of this compound is reduced through enzymatic and non-enzymatic mechanisms with concurrent generation of $O_2^{\cdot-}$ [4].

Previously we demonstrated that transgenic mice overexpressing human MnSOD (hMnSOD) were protected against ADR-induced myocardial injury when compared to nTg mice [5]. This finding suggests that $O_2^{\cdot-}$ radicals formed in mitochondria are related to ADR-induced cardiac

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injury. We have also reported that inactivation of mitochondrial complex I by ADR-induced $O_2^{\cdot-}$ production was prevented by overexpression of hMnSOD [7].

Nitric oxide synthase (NOS) catalyzes the oxidation of L-arginine through a five electron reduction, ending with formation of L-citrulline and subsequent production of *NO . Under certain circumstances, such as arginine and essential co-factor deficiencies, as well as the binding of ADR to the reductase domain of NOS III, uncoupling of the enzyme occurs, allowing for production of $O_2^{\cdot-}$ [8]. Inducible NOS has been shown to be expressed in all nucleated cells; this enzyme has been identified in the cardiovascular system and its level is regulated by different stimuli [9]. Inducible NOS can be up-regulated through inflammatory cytokines such as interferon gamma or tumor necrosis factors, and oxidative stress [10]. It has been suggested that regulation of iNOS occurs predominantly at transcriptional levels, but studies report that both post-transcriptional and post-translational regulation is possible [11].

Physiologic responses to *NO in the cardiovascular system include increase in [or modulation of] excitation–contraction coupling, myocardial relaxation and diastolic function, the Frank–Starling response, heart rate, β -adrenergic inotropic response, and myocardial energetics and substrate metabolism [12]. Aside from the essential role as a modulator of physiological myocardial function, *NO is known to exhibit antioxidant-like actions in lipid bilayers [13–16]. The limitation of diffusion of *NO in a lipid bilayer is dependent upon its reaction with cellular antioxidants, oxidants, and other free radicals [17].

Because iNOS is thought to be involved in the pathophysiological response to acute tissue injury, in the present study, we used an acute model to study the mechanism of ADR-induced heart injury. We hypothesized that iNOS deficiency would reduce ADR-induced cardiac injury. Unexpectedly, we found that the absence of iNOS caused an exacerbation of cardiac injury as demonstrated by both ultrastructural pathology and biochemical criteria. Moreover, this injury was circumvented by increasing the endogenous level of MnSOD.

2. Methods

2.1. Generation of mice deficient in iNOS and overexpressing MnSOD (iNOS $(-/-)$ and iNOS $(-/-)$ -TgM $(+/+)$)

All animal experiments in this study were performed with the approval of the University of Kentucky Animal Care and Use Committee and in accordance with the National Institute of Health's *Guide for the Care and Use of Laboratory Animals*. Inducible NOS knock-out mice purchased from Jackson Laboratories (Bar Harbor, ME) in the C57BL/6 background were bred into the B6C3 background further than 10th generation. The medium express-

ing line (TgM $(+/-)$) [5] was bred to obtain mice with homozygous MnSOD overexpression (TgM $(+/+)$). The protein and activity levels of MnSOD are increased in transgenic mice as previously demonstrated [5,6]. The iNOS $(-/-)$ -TgM $(+/+)$ cross was generated by sequential selection and back-crossing between iNOS $(-/-)$ and TgM $(+/+)$ mice as described by Chaiswing et al. [6].

2.2. Southern blot analysis of iNOS $(-/-)$ and iNOS $(-/-)$ -TgM $(+/+)$ mice

Genomic DNA was isolated from mouse tail, digested (*Bam*HI or *Pst*I), and separated on 0.7% or 0.9% agarose as previously described [18]. Following transfer, blots were hybridized using ^{32}P labeled iNOS or hMnSOD cDNA as previously described [5].

2.3. Electron microscopy

Heart tissue was isolated in ice cold phosphate buffered saline (PBS), cut into small 1 mm³ pieces, and immediately fixed in half strength Karnovskys fixative and analyzed as previously described [5].

2.4. Animal treatment and tissue preparation

Male mice, ages 8–10 weeks old, received one intraperitoneal injection (i.p.) (20 mg/kg) of ADRIAMYCIN PFS® (Pharmacia and Upjohn, Kalamazoo, MI) or saline. After 3 days, mice were anesthetized using Nembutal® sodium solution (65 mg/kg) (Abbott Laboratories, North Chicago, IL). The heart was excised, washed in ice cold PBS, and immediately frozen in liquid nitrogen. Frozen tissue was ground using a mortar and pestle prior to homogenization.

2.5. Measurement of cardiac function in intact heart preparations

Cardiac contractility was determined using an isolated perfused heart preparation with Krebs–Hensleit buffer as previously described, 3 days following treatment with ADR [19].

2.6. Creatine phosphokinase (CPK) and lactate dehydrogenase activity (LDH)

CPK and LDH activity in serum was analyzed as previously described [5].

2.7. Western blot analysis

Heart tissue homogenate (100 μ g) was electrophoresed using SDS-PAGE method as previously described [20]. An affinity purified rabbit anti-GAPDH antibody (1:3000) was purchased from Trevigen (Gaithersburg, MD) and

monoclonal mouse anti-cardiac troponin I (TnI) antibody was obtained from Advanced ImmunoChemical (Long Beach, CA).

2.8. Griess Assay

Serum nitrate levels were determined as previously described [6].

2.9. Real time PCR

Total tissue RNA, 6 h following ADR treatment, was isolated with Trizol (1 heart/1 mL) and further purified using the RNeasy Mini, RNA isolation kit (Qiagen, Valencia, CA). Complimentary DNA was obtained using oligo(dT) primers (Clontech Laboratories, Palo Alto, CA) and was stored at -80°C . Primers are listed below:

Annealing number of				
Gene	Temp., $^{\circ}\text{C}$	Cycles	Primer	Sequence
iNOS	55	40	5'-CTGATGGTCAAGATCCAGAGGTCT-3'	3'-CTGCATGTGCTTCATGAAGGACTCT-5'
GAPDH	50	20	5'-TGAAGGTCGGTGTGAACGGATTTGGC-3'	3'-CATGTAGGCCATGAGGTCCACCAC-5'

LightCycler-DNA Master SYBR Green I (Roche, Basel, Switzerland) was used with LightCycler 3.5.3 software to analyze results. Gene expression was normalized with mRNA expression of GAPDH. Samples were analyzed in triplicate using $N=3$ for each murine genotype and treatment.

2.10. Statistical analysis

Multiple comparisons for each variable were performed using analysis of variance (ANOVA) with the post hoc Fisher's or Tukey's test. When applicable, the unpaired t -test was used to test the significance of each pre-planned comparison. Data was considered statistically significant for $P \leq 0.05$. All data is presented as mean \pm SEM.

3. Results

3.1. Ultrastructural analysis

Previous studies have established that treatment with ADR induced mitochondrial injury [1,2,5]. To investigate the role of iNOS in ADR-induced injury, mice were treated with a single injection (20 mg/kg) of ADR and heart tissues were examined following a 5-day period. Ultrastructural pathology (Fig. 1) revealed that cardiac muscle from C57BL/6 mice (Fig. 1a) and nTg (B6C3) mice (Fig. 1c) treated with saline had normal morphology with normal mitochondria and prominent myofilaments. Heart tissues

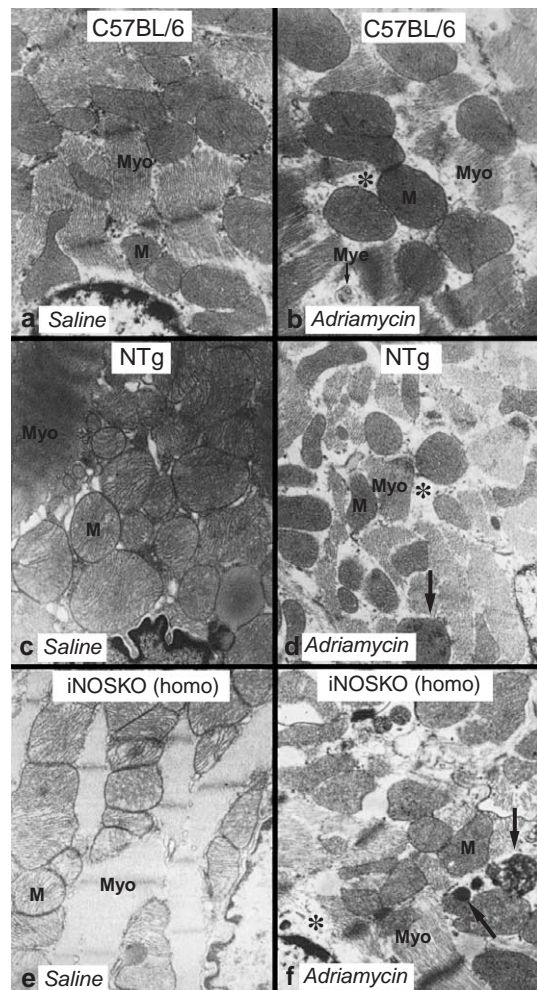


Fig. 1. Inducible NOS ($-/-$) mice demonstrated increased ultrastructural damage following ADR treatment compared to nTg. (a, b) C57BL/6 mice. Cardiac muscle from mice treated with saline (a) showed normal morphology with unremarkable mitochondria (M) and myofilaments (Myo). Cardiac muscle from mice treated with ADR (b) demonstrated mild cytoplasmic swelling (asterisk), myelin figures (Mye), mitochondria (M) with focal loss of cristae, and focal disorganization of myofilaments (Myo). (c, d) nTg (wildtype B6C3) mice. Cardiac muscle from mice treated with saline (c) showed normal morphology with unremarkable mitochondria (M) and myofilaments (Myo). Cardiac muscle from mice treated with ADR (d) exhibited mild cytoplasmic swelling (asterisk), mitochondria (M) with focal loss of cristae, focal disorganization of myofilaments (Myo), and some mitochondria with loss of subcellular structure (arrow). (e, f) Homozygous iNOS knockout (C57BL/6) mice. Cardiac muscle from mice treated with saline (e) showed normal morphology with unremarkable mitochondria (M) and myofilaments (Myo). Cardiac muscle from mice treated with ADR (f) demonstrated extensive cytoplasmic swelling (asterisk), focal disruption of mitochondria (M) and myofilaments (Myo), as well as extensive degradation of mitochondria (arrows).

from C57BL/6 (Fig. 1b) and nTg (B6C3) mice (Fig. 1d) treated with ADR demonstrated mild cytoplasmic swelling, myelin figures, mitochondria with focal loss of cristae, and focal disorganization of myofilaments. Additionally, nTg (B6C3) mice treated with ADR showed some mitochondria with loss of subcellular structure. Cardiac muscle from homozygous iNOS knockout mice (C57BL/6) treated with

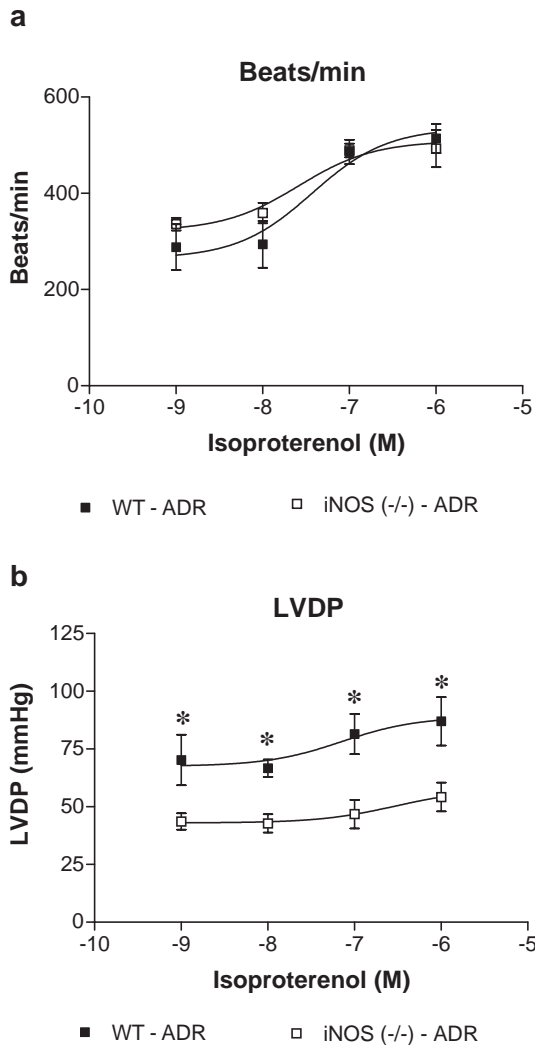


Fig. 2. The ability of isoproterenol to increase contractile force was significantly decreased in hearts from iNOS (-/-) mice as compared to nTg following ADR treatment, with no difference in heart rate. (a) Beats/min in 8–10 weeks old nTg (■) and iNOS (-/-) (□) mice treated with ADR ($n \geq 4$ mice in each group). Data are expressed as mean \pm SEM. (b) Left ventricular developed pressure (LVDP) in 8–10 weeks old nTg (■) and iNOS (-/-) (□) mice treated with ADR ($n \geq 4$ mice in each group). Data are expressed as mean \pm SEM. * $P < 0.05$ for nTg-ADR versus iNOS (-/-)-ADR for dose of isoproterenol.

saline (Fig. 1e) showed normal morphology with typical mitochondria and myofilaments. However, cardiac tissues from iNOS (-/-) mice (C57BL/6) treated with ADR (Fig. 1f) demonstrated extensive cytoplasmic swelling, focal disruption of mitochondria and myofilaments, as well as extensive degeneration of mitochondria; injury changes observed were more extensive than those observed in nTg mice.

3.2. Cardiac function in response to isoproterenol following *in vivo* treatment with ADR

To determine if these structural changes resulted in an impairment of cardiac function, contractile activity was

assessed in the isolated perfused heart. Neither the resting heart rate nor the positive chronotropic actions of isoproterenol were significantly altered in ADR treated

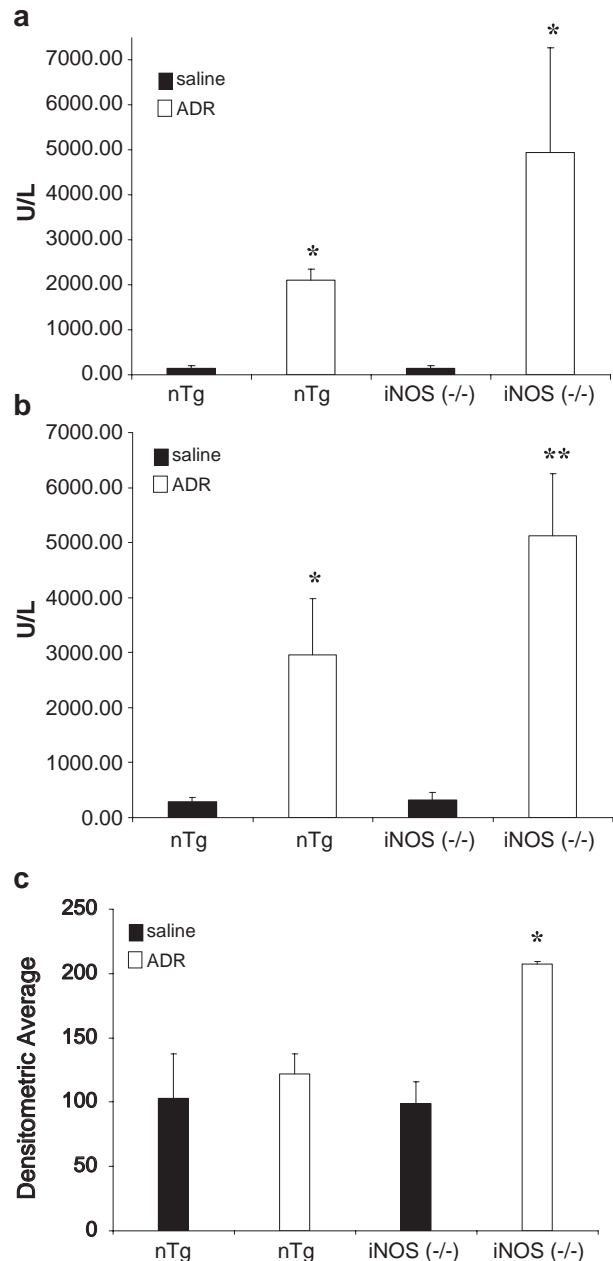


Fig. 3. CPK, LDH, and cTnI were more significantly elevated in iNOS (-/-) mice as compared to nTg following ADR treatment. (a) Serum creatine phosphokinase (CPK) in 8–10 weeks old wt (nTg) and iNOS (-/-) mice treated with ADR ($n = 4$ mice in each group). Data are expressed as mean \pm SEM. * $P \leq 0.0001$ versus saline for mice of the same genotype. (b) Serum lactate dehydrogenase (LDH) in 8–10 weeks old wt (nTg) and iNOS (-/-) mice treated with ADR ($n = 4$ mice in each group). Data are expressed as mean \pm SEM. * $P \leq 0.01$ versus saline for wt (nTg) mice. ** $P \leq 0.001$ versus saline for iNOS (-/-) mice. (c) Serum cardiac troponin in 8–10 weeks old wt (nTg) and iNOS (-/-) mice treated with ADR for 3 d ($n = 4$ mice in each group). Data are expressed as densitometric mean \pm SEM. * $P \leq 0.0001$ versus saline for iNOS (-/-) mice.

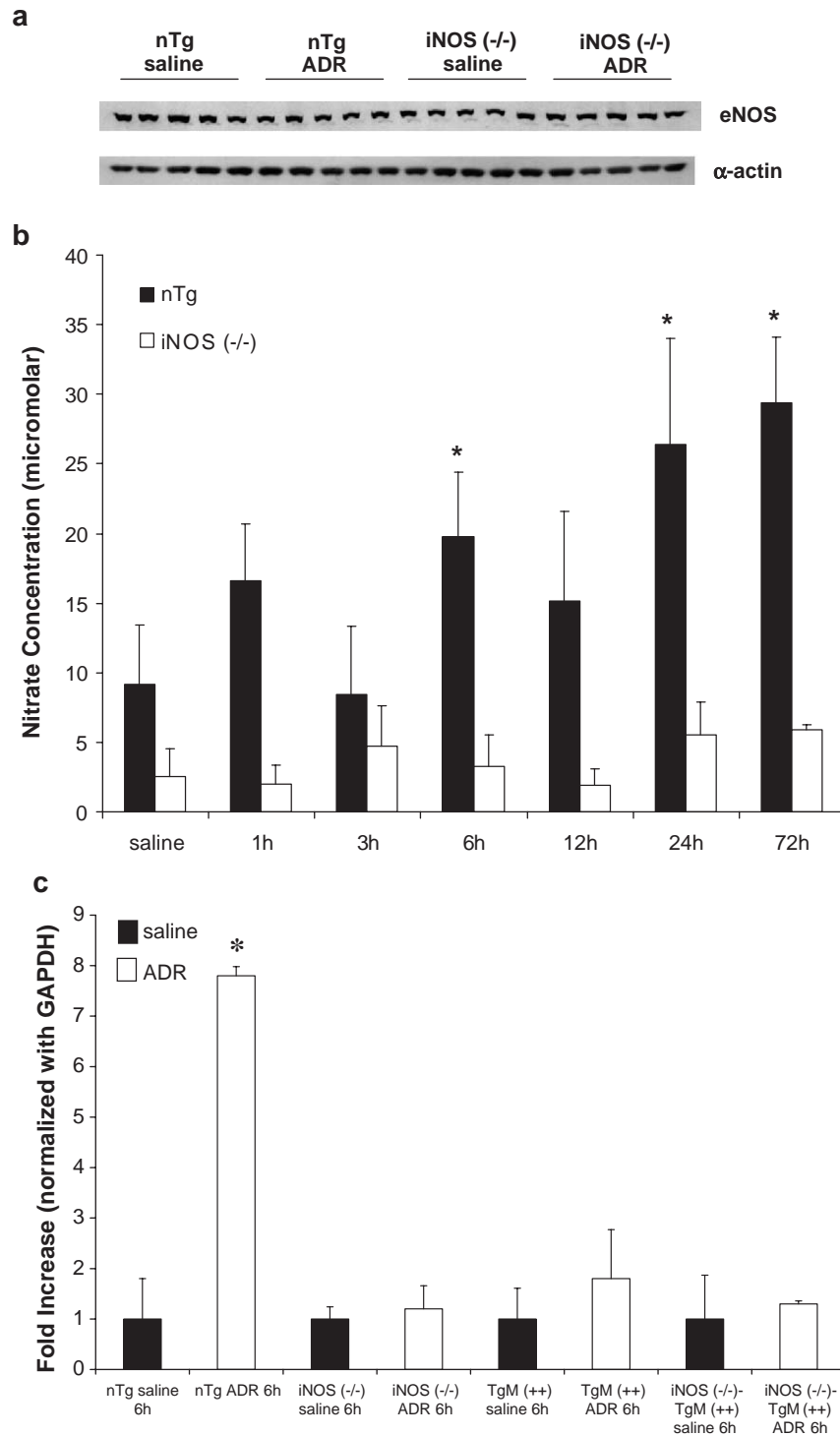


Fig. 4. Serum nitrate levels and iNOS mRNA expression were significantly elevated in nTg, but not iNOS (-/-) mice, with no compensatory increase in eNOS protein after treatment with ADR. (a) Protein extracts isolated from both nTg (wildtype) and iNOS (-/-) mice were analyzed by Western blot using an eNOS antibody three days after ADR treatment ($n=5$ mice in each group). Quantitation did not reveal any significant differences (data not shown). (b) Serum nitrate concentration (μM) following time course treatment with ADR ($n=6$ mice for each time point) was determined. nTg (wildtype) mice are represented by the black bars and iNOS (-/-) mice are represented by the white bars. Values are expressed as mean \pm SEM. $*P \leq 0.05$ versus saline for wt (nTg) mice. (c) Transcript levels of iNOS 6 h following treatment with ADR ($n=3$ mice for each group) was determined. Mice receiving saline or ADR are represented by the black or white bars, respectively. Values are expressed as fold increase from saline control after normalization with GAPDH transcript levels. $*P \leq 0.0001$ versus saline for wt (nTg) mice.

iNOS ($-/-$) and nTg mice (Fig. 2a). Wildtype mice treated with ADR showed expected increases in measures of contractile function. Increases in LVDP were observed following infusion of increasing amounts of isoproterenol in nTg control hearts. However, in iNOS deficient mice, a decrease in resting LVDP was observed. Additionally, the ability of isoproterenol to promote increases in these recorded parameters was significantly reduced in iNOS ($-/-$) mice (Fig. 2b).

3.3. CPK, LDH, and troponin serum levels

To verify the consequence of iNOS deficiency on ADR-induced cardiac injury, the levels of biochemical markers including CPK, LDH, and cTnI were assessed. Fig. 3 shows that increased levels of CPK, LDH, and cTnI were detected in serum 3 days following ADR treatment in nTg and iNOS ($-/-$) mice ($P \leq 0.0001$). Serum CPK was increased to a larger degree in iNOS ($-/-$) mice treated with ADR than nTg mice ($P \leq 0.05$)

(Fig. 3a). Compared to their respective controls, nTg and iNOS ($-/-$) mice had significant increases in serum LDH after ADR ($P \leq 0.01$ and $P \leq .001$) (Fig. 3b). Inducible NOS ($-/-$) mice demonstrated a greater serum LDH release following treatment with ADR as compared to nTg treated mice ($P \leq 0.01$). Using an antibody raised against cTnI, the levels were measured in the serum. Western analysis revealed that nTg mice treated with ADR had a slight increase in cTnI, but they were not found to be statistically significant. Serum from iNOS ($-/-$) mice treated with ADR showed a statistically significant increase in cTnI ($P \leq 0.0001$) as compared to saline control (Fig. 3c).

3.4. eNOS protein, serum nitrate levels, and iNOS mRNA gene expression

Adriamycin has been reported to induce eNOS transcription; therefore, Western blot analysis was used to determine whether treatment leads to a compensatory

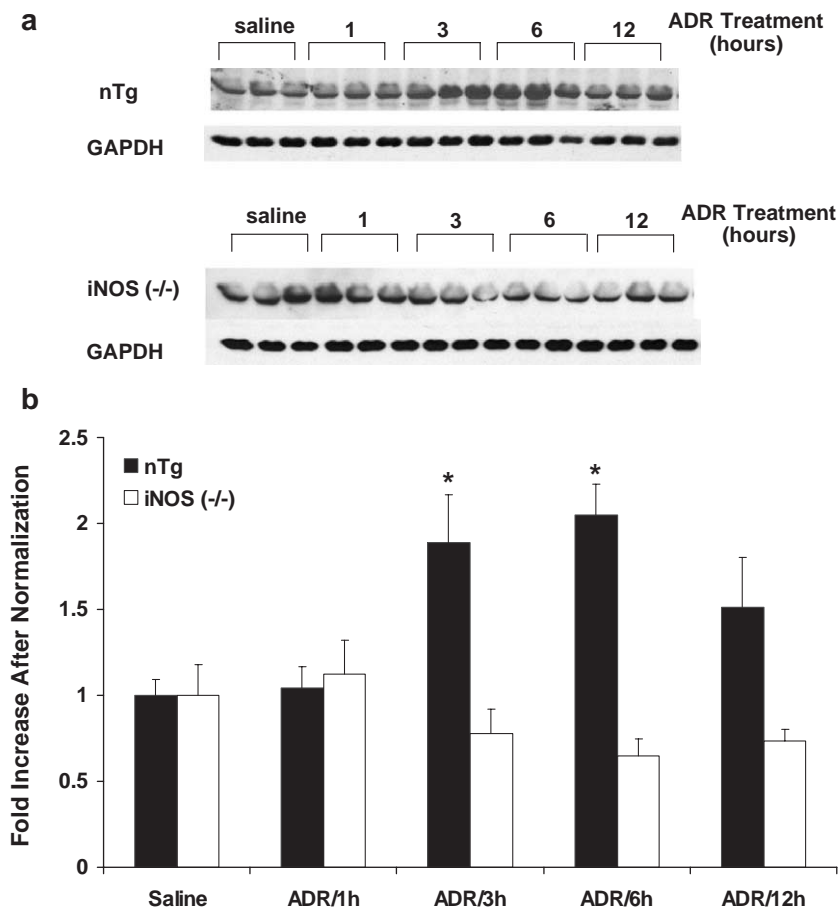


Fig. 5. Nitrotyrosine protein adducts increased in nTg, but not iNOS ($-/-$) mice after ADR treatment. (a) Protein extracts analyzed by Western blot using an antibody detecting nitrotyrosine ($n=3$ mice for each time point). Protein extracts were co-hybridized with an antibody detecting GAPDH to assess equal loading of the samples. (b) The autoradiographs of nitrotyrosine levels (a) were quantitated and normalized to GAPDH. nTg (wildtype) mice are represented by the black bars and iNOS ($-/-$) mice are represented by the white bars. Values are expressed as fold increases from respective saline control. * $P \leq 0.05$ versus saline for wt (nTg) mice.

increase in eNOS levels in nTg and mice lacking iNOS [21]. After normalization with actin, there was no change in the level of eNOS protein in nTg or iNOS ($-/-$) mice at 1, 3, 6, 12, 24, or 72 h following ADR treatment. Fig. 4a shows the typical results at 72 h.

The Griess reaction was used to assess the levels of total nitrate and nitrite generated after treatment with ADR. Using a time course study, serum nitrate levels were increased by 1 h following ADR treatment in nTg mice. Significant increases ($P \leq 0.05$) at 6, 24, and 72 h following treatment in nTg mice were observed (Fig. 4b). There was no increase at any time point in the level of serum nitrates in iNOS ($-/-$) mice.

The mRNA level of iNOS was assessed 6 h following treatment with ADR. There was a significant increase ($P \leq 0.0001$) in iNOS transcript levels in nTg mice following treatment with ADR as compared to saline-nTg mice (Fig. 4c). There were no significant differences in iNOS mRNA levels in iNOS ($-/-$), TgM (+) (mice transgenic for MnSOD), and iNOS ($-/-$)-TgM (+) mice after ADR compared to their respective controls. Both iNOS ($-/-$) and iNOS ($-/-$)-TgM (+) mice lacked appreciable iNOS mRNA expression compared to nTg and TgM (+) mice.

3.5. Assessment of reactive oxygen species (ROS)/reactive nitrogen species (RNS) modified proteins

The increases in serum nitrate levels suggest that ADR treatment can lead to an increase in $\cdot\text{NO}$ levels, therefore we detected a product of $\cdot\text{NO}$ with $\text{O}_2^{\cdot-}$, ONOO^- , which reacts with protein tyrosine residues. Time course studies using Western blot analysis revealed that there was an increase in nitrotyrosine adducted proteins (~ 40 kDa, one band) between 1 and 3 h following ADR treatment in the nTg mice (Fig. 5a). These increases seen in nTg mice were absent in ADR-treated iNOS ($-/-$) mice. Quantitation of nitrotyrosine adducted proteins revealed that nTg mice had significant increases at 3, 6, and 12 h (Fig. 5b).

3.6. Expression of MnSOD ameliorated ADR-induced cardiotoxicity in iNOS ($-/-$) mice

Following a 5-day period after receiving one 20 mg/kg dose of ADR, heart tissues from all four genotypes were analyzed for ultrastructural changes (Fig. 6). No pathological changes were found in the heart tissues of saline treated nTg mice (Fig. 6a), overexpressing MnSOD mice (TgM (+/+)) (Fig. 6c), iNOS ($-/-$) (Fig. 6e), or the cross between MnSOD transgenic and iNOS ($-/-$) mice (TgM+iNOS) (Fig. 6g). Cardiac tissues from nTg, iNOS ($-/-$) and TgM+iNOS mice treated with ADR showed increased myocardial damage (Fig. 6b, f, h) compared to saline controls, with changes consisting of mitochondria with myelin figures, mitochondria with loss of cristae,

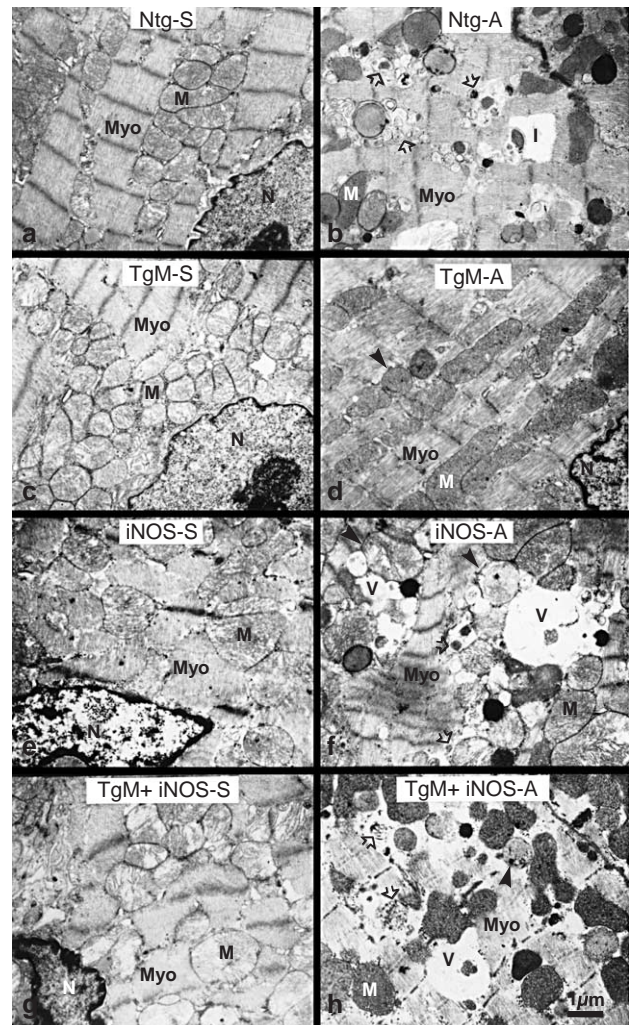


Fig. 6. Overexpression of MnSOD ameliorated ADR-induced cardiotoxicity in iNOS ($-/-$) mice. nTg (wildtype B6C3) mice treated with saline or ADR (a, b), overexpression MnSOD mice (TgM B6C3) treated with saline or ADR (c, d), iNOS ($-/-$) (B6C3) treated with saline or ADR (e, f), cross breed between overexpression MnSOD and iNOS knockout mice (TgM+iNOS) (B6C3) treated with saline or ADR (g, h). M indicates normal mitochondria, N indicates nucleus, Myo indicates myofibril, I indicates intracytoplasmic edema, V indicates intracytoplasmic vacuolization, arrow indicates mitochondria with myelin figures, and arrowhead indicates mitochondria with the loss of cristae. No pathological changes were found in the heart tissues of experimental mice that were treated with saline (a, c, e, and g). nTg, iNOS and TgM+iNOS mice showed increased myocardial damage (b, f, h), with changes consisting of mitochondria with myelin figures, mitochondria with loss of cristae, intracytoplasmic vacuolization, and edema. Inducible NOS mice treated with ADR (f) showed more extensive ultrastructural changes than other groups. In contrast, TgM mice (d) had only mild ultrastructural changes (loss of cristae) compared to the other ADR treated mouse groups.

intracytoplasmic vacuolization, and edema. Inducible NOS ($-/-$) mice treated with ADR (Fig. 6f) showed more extensive ultrastructural changes than other groups. In contrast, TgM (+/+) mice (Fig. 6d) had only mild ultrastructural changes compared to the other ADR treated mouse groups.

3.7. MnSOD overexpression enhances cardiac contractility in response to isoproterenol

In order to determine if cardiac function correlated with ultrastructural findings, LVDP was determined as a response to isoproterenol. Fig. 7a and b demonstrates that heart rate and LVDP was not altered in control animals (saline). Treatment with ADR did not alter heart rate in response to isoproterenol (Fig. 7c). However, in mice lacking iNOS there was a decrease in LVDP (Fig. 8d). Importantly, overexpression of MnSOD in mice lacking

iNOS restored cardiac function as a measure of LVDP (Fig. 7d).

3.8. Overexpression of MnSOD attenuates serum LDH and cardiac troponin levels after ADR treatment

To verify the cardioprotective effect of MnSOD in the iNOS (–/–) mice, we compared LDH and cardiac troponin serum levels. Analysis of LDH release in serum following treatment with ADR demonstrated that iNOS (–/–) mice had elevated levels compared to nTg mice

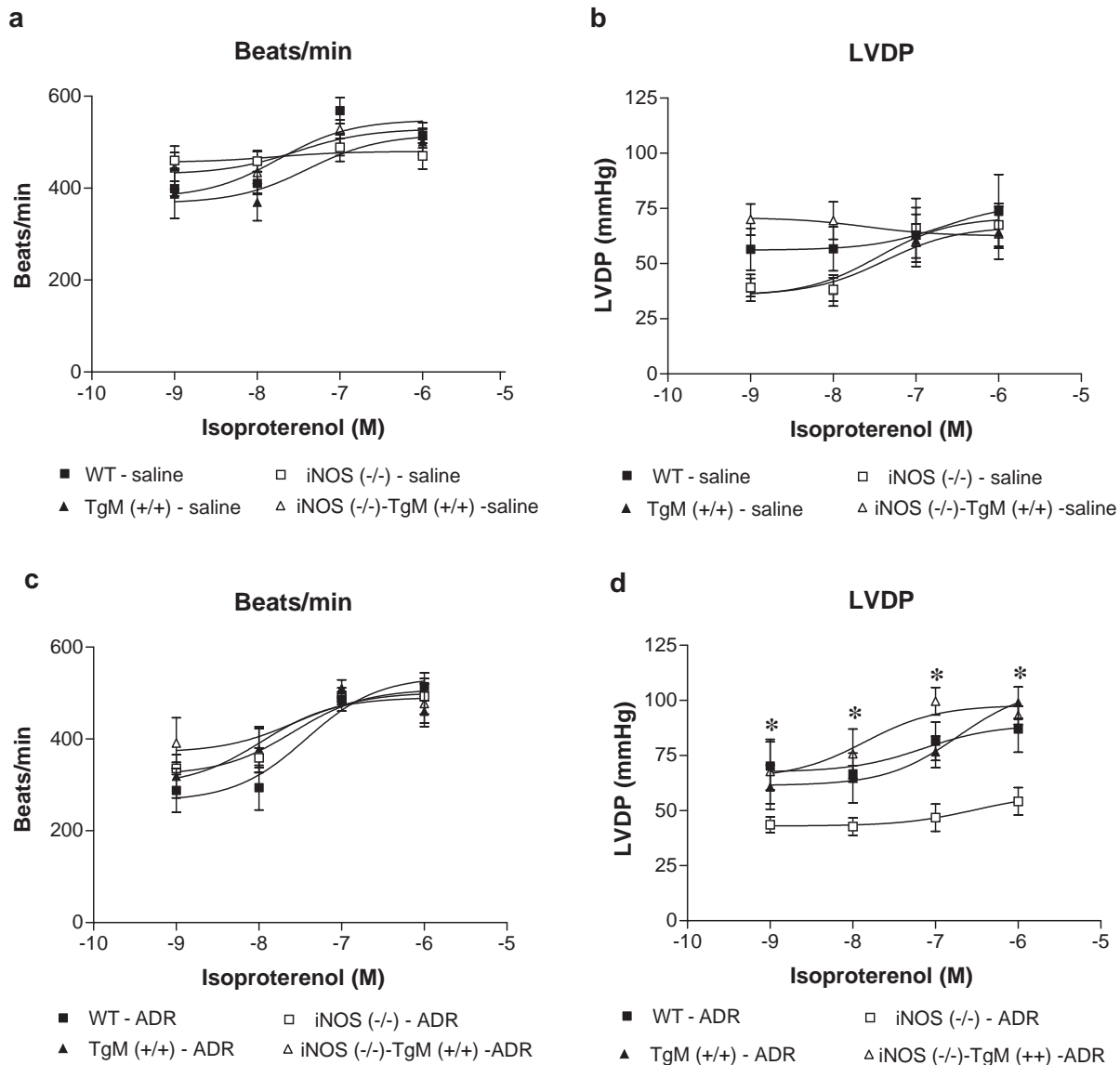


Fig. 7. The ability of isoproterenol to increase contractile force was significantly improved in hearts from mice lacking iNOS which overexpress MnSOD following ADR treatment, with no difference in heart rate. a Beats/min in 8–10 weeks old nTg (■), iNOS (–/–) (□), TgM (+/+) (▲), and iNOS (–/–)-TgM (+/+) (△) control mice (saline) ($n \geq 3$ mice in each group). Data are expressed as mean \pm SEM. b. Left ventricular developed pressure (LVDP) in 8–10 weeks old nTg (■) and iNOS (–/–) (□), TgM (+/+) (▲), and iNOS (–/–)-TgM (+/+) (△) control mice (saline) ($n \geq 3$ mice in each group). Data are expressed as mean \pm SEM. c. Beats/min in 8–10 weeks old nTg (■), iNOS (–/–) (□), TgM (+/+) (▲), and iNOS (–/–)-TgM (+/+) (△) mice treated with ADR ($n > 4$ mice in each group). Data are expressed as mean \pm SEM. d. Left ventricular developed pressure (LVDP) in 8–10 weeks old nTg (■) and iNOS (–/–) (□), TgM (+/+) (▲), and iNOS (–/–)-TgM (+/+) (△) mice treated with ADR ($n > 4$ mice in each group). Data are expressed as mean \pm SEM. * $P < 0.05$ for iNOS (–/–)-ADR versus nTg, TgM (+/+) and iNOS (–/–)-TgM (+/+) -ADR for dose of isoproterenol.

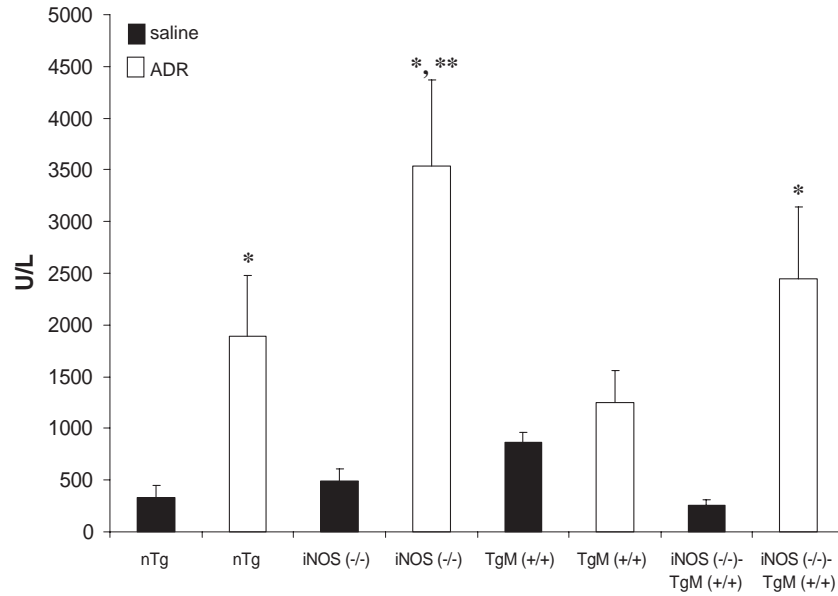


Fig. 8. MnSOD overexpression attenuated serum LDH levels after ADR treatment. Serum lactate dehydrogenase (LDH) in 8–10 weeks old wt (nTg), iNOS (-/-), TgM (+/+) and iNOS (-/-)-TgM (+/+) mice treated with ADR ($n=10$ mice in each group). Data are expressed as mean \pm SEM. * $P \leq 0.0001$ versus saline of mice for the same genotype. *** $P \leq 0.0001$ versus wt (nTg) and iNOS-TgM (+/+) ADR treated mice.

($P \leq 0.0001$) (Fig. 8). Mice transgenic for MnSOD (TgM (+/+)) demonstrated no significant change in LDH levels after treatment with ADR. Levels of LDH release were attenuated in iNOS (-/-)-TgM (+) mice, comparable to LDH levels after treatment with ADR in

nTg mice ($P > 0.05$). Fig. 9 shows an attenuation of cardiac troponin levels in serum with overexpression of MnSOD in mice that lack iNOS ($P \leq 0.0001$) following ADR treatment when compared to iNOS deficient mice.

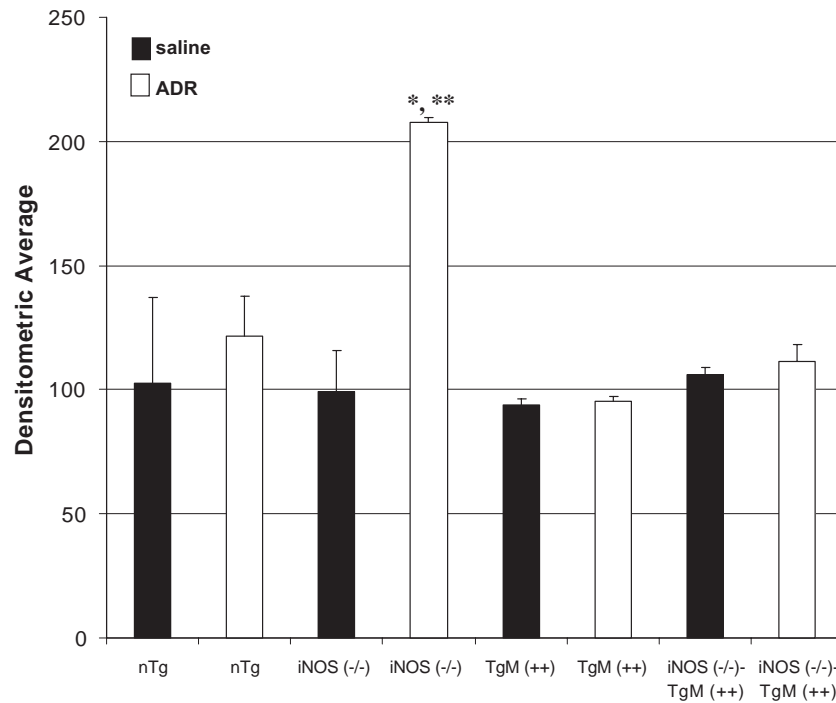


Fig. 9. Cardiac troponin was significantly decreased in iNOS (-/-) mice overexpressing MnSOD as compared to iNOS (-/-) mice. Serum cardiac troponin in 8–10 weeks old wt (nTg), iNOS (-/-), TgM (+/+), and iNOS (-/-)-TgM (+/+) mice treated with ADR for three days ($n=4-5$ mice in each group). Data are expressed as densitometric mean \pm SEM. * $P \leq 0.0001$ versus saline treated mice. *** $P \leq 0.0001$ versus wt (nTg) and iNOS-TgM (+/+) ADR treated mice.

4. Discussion

Adriamycin is one of the most frequently used chemotherapeutic drugs in the treatment of cancer. However, the clinical usefulness of ADR is limited by a dose-related cardiac toxicity. Mitochondria have been identified as one of the targets in ADR-induced subcellular damage in heart tissues [1]. Previously, our laboratory has shown that ventricular tissues isolated from wildtype mice treated with ADR exhibited mitochondrial damage in a dose-dependent manner [5]. The present study confirmed and extended our previous results and showed that iNOS null mice had more extensive mitochondrial damage when treated with ADR as compared to wildtype mice. This is the first report to demonstrate that NO produced by iNOS contributes to protection of normal tissue injury by ADR *in vivo*. These ultrastructural results are consistent with measurements of cardiac function and further supported by biochemical parameters analyzed.

Cardiac dysfunction as a response to ADR treatment has been shown previously by Kang et al. [22]. Using an *in vivo* method, Pacher et al. observed that iNOS ($-/-$) mice had slightly better cardiac function compared to wildtype 5 days following ADR treatment [23]. One additional difference, other than the obvious difference in methods of *ex vivo* versus *in vivo*, is that the background of the mice are different. Mice used for hemodynamic study by Pacher et al. are in the C57BL/6 background, whereas mice used in our experiments are bred more than 10th generation in B6C3 background. It is possible that differences in strains of mice used may contribute to the differences in some parameters measured. Since our functional data are in agreement with the ultrastructural pathology and biochemical data in the same genetic background, we are confident that the observed effect of iNOS is real in our animals. Limitations exist for both *in vivo* and *ex vivo* cardiac function measurements, including but not limited to, systemic and/or neurohumoral modulation such as CNS and blood volume, variables extrinsic of the heart. However, cardiac function determined *ex vivo* provides an indication of cardiac status independent of other systolic factors. Herein, using the isolated perfused heart to assess cardiac function, we observed no change in the resting heart rate in iNOS ($-/-$) ADR-treated mice when compared to nTg animals. Similarly we noted no difference in the ability of the β -adrenergic receptor agonist, isoproterenol, to promote increases in heart rate. In contrast, resting contractile force and the ability of isoproterenol to increase indices of contractile function was significantly decreased in mice lacking iNOS as compared to nTg mouse heart following treatment with ADR. This result demonstrates and confirms an enhancement of cardiac damage in iNOS ($-/-$) mice.

Serum CPK and LDH activities are used clinically to evaluate and diagnose cardiac dysfunction and disease [24]. Fig. 3 shows serum CPK and LDH were significantly

elevated in nTg and iNOS ($-/-$) mice 3 days following treatment with ADR. However, iNOS ($-/-$) mice treated with ADR had more significant increases in both CPK and LDH as compared to nTg treated mice. This finding directly correlates with the increased ultrastructural damage and cardiac contractility dysfunction exhibited in the iNOS ($-/-$) mice following ADR treatment as compared to nTg mice. Because serum CPK and LDH can be elevated in many conditions and may not be specific markers for cardiac injury, we chose to probe for a more specific biomarker of cardiac injury, cTnI [25,26]. The levels of cTnI in serum have been found to be directly correlated to infarct magnitude and more specifically to ADR-induced damage [26,27]. Inducible NOS ($-/-$) mice had higher elevations of cTnI following treatment with ADR as compared to nTg mice (Fig. 3c). These findings are also consistent with the biochemical analysis of CPK and LDH, as well as the ultrastructural results. Specifically, levels of cTnI correlate more closely with the ultrastructural results, indicating that cardiac injury in mice deficient in iNOS is attenuated after expression of MnSOD. Taken together, these results suggest that ADR-induced NO production from iNOS may play a role in preventing cardiac injury. The contribution of iNOS to NO production in cardiac tissue is supported by the following data: first, iNOS transcript levels and nitrate levels increased in nTg but not in iNOS ($-/-$) mice; second, eNOS levels in both nTg and iNOS ($-/-$) mice were unchanged before and after treatment with ADR, and third, nitrotyrosine in nTg, but not in iNOS ($-/-$) mice, an adduct of tyrosine modification, was increased.

Consistent with our results, Weinstein et al. demonstrated immunohistochemical staining of iNOS and of 3-nitrotyrosine in myocardial tissue after treatment with ADR [28]. Increases in nitrotyrosine can be directly linked to increases in RNS, rather than exclusively ONOO⁻ production [29]. Our results indicated that iNOS transcript levels are increased in nTg mice 6 h following treatment with ADR. Significant nitrate levels were found as early as 6 h and continued to increase afterward and the levels of nitrated proteins, as a measure of nitrotyrosine formation, also increased in nTg mice after ADR (Fig. 5). However, cardiac tissue from iNOS ($-/-$), TgM ($+/+$), and iNOS ($-/-$)-TgM ($+/+$) ADR-treated mice did not express nitrated proteins, consistent with the lack of increase in iNOS mRNA and nitrotyrosine. These results suggest that overexpression of MnSOD protects against ADR-induced iNOS expression. Taken together, this data would suggest that the increases in nitrotyrosine formation in nTg mice following ADR were dependent mainly on the iNOS activity. Further, the absence of nitrotyrosine formation in iNOS ($-/-$) mice would indicate that increases in nitrating species found in nTg mice were not involved in causing direct tissue injury or cytotoxic signaling. The clinical significance of these findings is that inhibition of iNOS as a therapeutic attempt to prevent deleterious effects of NO (but not necessarily other ROS) in both physiological and

pathological circumstances may actually prove to be deleterious.

It is well documented that $\cdot\text{NO}$ can react with $\text{O}_2^{\cdot-}$, therefore the level of $\cdot\text{NO}$ can alter the fate of $\text{O}_2^{\cdot-}$ in vivo [30]. Nitric oxide may aid SOD in the removal of excess $\text{O}_2^{\cdot-}$, to prevent generation of hydroxyl radical. Based upon the results reported herein and our previous findings demonstrating protection against ADR-induced injury with overexpression of hMnSOD, we postulated that increased MnSOD may compensate for the lack of iNOS and hence iNOS-mediated $\cdot\text{NO}$ production. Ultrastructural analysis of cardiac tissues from all four genotypes of mice following treatment with ADR indicated that TgM (+/+) mice exhibited the least cardiac damage (Fig. 6). Protection against ADR-induced cardiac injury in TgM (+/+) mice as demonstrated by ultrastructural analysis did not manifest into enhanced cardiac function. However, overexpression of MnSOD restored cardiac function in mice lacking iNOS (Fig. 7b). Importantly, the exacerbation of injury as seen in the iNOS (–/–) mice was attenuated in iNOS (–/–)–TgM (+/+) mice. The level of cardiac injury in MnSOD transgenic mice lacking iNOS was comparable to wildtype mice following treatment, further demonstrating that ADR-induced $\cdot\text{NO}$ production plays a cytoprotective role.

Taken together, the findings reported here demonstrate for the first time that cardiac injury associated with ADR treatment is exacerbated in iNOS (–/–) as compared to nTg mice; this conclusion was supported by: 1) ultrastructural analysis, assessment of cardiac contractile function, and biochemical markers for cardiac injury, 2) the enhanced cardiac injury induced by ADR in iNOS (–/–) mice appears to be $\text{O}_2^{\cdot-}$ mediated, a finding consistent with the protection effect observed in mice deficient in iNOS, but overexpressing MnSOD, 3) in contrast to iNOS (–/–) mice, nTg mice exhibited the ability to generate endogenous nitrate and increased iNOS mRNA expression, perhaps providing cytoprotective cell signaling events, and 4) overexpression of MnSOD can compensate for the lack of cytoprotective mechanisms resulting from the absence of iNOS. These results signify the intricate relationship between $\cdot\text{NO}$ and SOD in the prevention of cardiac tissue injury induced by cancer chemotherapy.

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