CHAPTER 3

Adriamycin-induced iNOS expression and nitration of manganese superoxide dismutase: Insights into the mechanism of chemobrain

Abstract

Adriamycin (ADR), the potent antitumor agents, produces reactive oxygen species (ROS) in cardiac tissue. Treatment with ADR is dose limited by cardiotoxicity. However, the effect of ADR in the other tissues, including the brain, is unclear because ADR did not pass the blood brain barrier. However, some cancer patients receiving ADR treatment develop a transient memory loss. We previously demonstrated that ADR causes CNS toxicity, in part, via systemic release of cytokines and subsequent generation of reactive oxygen and nitrogen species (RONS) in the brain. Here, we demonstrated that treatment with ADR led to an increased circulating level of tumor necrosis factor alpha in wild-type mice and in mice deficient in the inducible form of nitric oxide (iNOSKO). However, the decline in mitochondrial respiration and mitochondrial protein nitration after ADR treatment were observed only in wild-type mice, but not in the iNOSKO mice. Importantly, the activity of a major mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD), was reduced and the protein was nitrated. Together, these results suggest that NO is an important mediator coupling the effect of ADR with cytokine production and subsequent activation of iNOS expression. Our results also identified mitochondria as important targets of ADR-induced NO-mediated CNS injury.

Introduction

Adriamycin (ADR), a prominent member of the anthracycline family, is an important therapeutic agent that has exhibited activity against a wide spectrum of human and experimental animal tumors. It is well established that ADR leads to generation of free radicals, which account for some of the normal tissue damage resulting from cancer treatment (Meredith and Reed 1983; Licinio 1997; Singal et al. 2000). This process may result from the redox cycling capability of anthracycline, its potential to bind nitric oxide (Vasquez-Vivar et al. 1999; Weinstein et al. 2000; Kalivendi et al. 2001; Kalyanaraman et al. 2002), or induced cytokine production (Ujhazy et al. 2003; Usta et al. 2004). The clinical use of ADR is compromised by an unusual and potentially lethal cardiac toxicity (Singal et al. 2000; Weinstein et al. 2000). However, its potential toxicity to other normal tissue, including the central nervous system (CNS), has recently been reported (Joshi et al. 2005) and is a topic of considerable discussion. Recent studies in breast cancer survivors have shown persistent changes in cognitive function, including memory loss, distractibility, and difficulty in performing multiple tasks, following treatment with chemotherapy including ADR (Ahles and Saykin 2002; Ahles et al. 2002).

Although the biochemical basis for ADR-induced CNS injury is unknown, it has been demonstrated that cancer therapeutic agents such as ADR can modulate endogenous levels of cytokines such as tumor necrosis factor alpha (TNF) (Ujhazy et al. 2003; Usta et al. 2004). Systemicly released TNF signals the complex interaction of the immune-neuroendocrine system which affects the CNS (Gutierrez et al. 1993; Licinio and Wong 1997; Mandi et al. 1998; Osburg et al. 2002). In support of the role of TNF in ADR-induced CNS injury, we recently demonstrated that intra-peritoneal injection of a neutralizing antibody against TNF eliminated ADR-induced brain mitochondrial dysfunction (Tangpong et al. 2006, in press)

Among potential down-stream effects of TNF is an increase in generation of reactive nitrogen species (RNS) (Szelenyi 2001). An RNS is nitric oxide, which is known to play a key role in many physiological and pathological conditions. Nitric oxide is synthesized from L-arginine through two distinct enzyme-catalyzed pathways. The first pathway is a tightly regulated, constitutively expressed nitric oxide synthase found mainly in endothelial and brain cells (Vasquez-Vivar et al.

1999; Kalivendi et al. 2001). This potency regulates vascular tone, blood flow, and blood pressure (Kaufmann et al. 2004; Wilkerson et al. 2005). The second pathway is a calcium-independent, cytokine-inducible form of nitric oxide synthase (iNOS) (Zeng et al. 2005). This enzyme, once expressed, stays active as long as substrates are available.

Within the CNS, microglia and astrocyte can generate NO radicals from iNOS activation (Marcus et al. 2003; Candolfi et al. 2004). Nitric oxide reacts rapidly with superoxide radicals, which limits NO lifetime. NO reacts with superoxide radicals to form peroxynitrite ONOO-), potent biological oxidant that has been implicated in diverse forms of free radical-induced tissue injury (Ste-Marie et al. 2001; Choi et al. 2002; Rubbo et al. 2002). Peroxynitrite is capable of directly oxidizing protein and nonprotein sulfhydryls. Peroxynitrite can also be protonated to peroxynitrous acid (ONOOH), which exhibits both unique and hydroxyl radical-like reactions. Thus, NO can potentiate many aspects of superoxide-mediated tissue injury via ONOO- formation (Ste-Marie et al. 2001). Inhibition of mitochondrial electron transport and inactivation of FeS-containing enzymes by NO have also been demonstrated (MacMillan-Crow et al. 1996; Radi 2004). Thus, it is possible that increased production of NO may participate in the development of neurotoxicity by generating peroxynitrite and inactivating key components of the mitochondrial defense system.

ADR treatment led to an increase in NO in breast cancer cells in vitro, and cytotoxicity to the cells in vivo (Ozen et al. 2001; Gyorffy et al. 2005). The mechanism by which these agents stimulate NO production is currently unclear. It has been shown that NF-kB enhancer elements regulate cytokine-mediated induction of the inducible NOS gene (Kim et al. 1999; Akama and Van Eldik 2000). NF-kB is a redox-sensitive transcription factor that has been shown to be activated by oxidizing agents such as hydrogen peroxide and ionizing radiation (Schreck et al. 1992; Snyder and Morgan 2005; Santoro et al. 2003). Thus, it is possible that increased NO production by ADR is a result of TNF-mediated NF-kB activation of iNOS expression. Since nitric oxide and superoxide radicals can both be generated in mitochondria, it is reasonable to hypothesize that both radicals and the resulting reactive species are, in part, responsible for normal tissue injury during cancer therapy.

An important primary antioxidant defense in the mitochondria is the manganese containing superoxide dismutase (MnSOD). Inactivation of the MnSOD gene in mammals yielded

detrimental effects. Thus, lack of MnSOD activity can severely impair mitochondria and affect the brain, which have high demands for oxidative metabolism. Overexpression of MnSOD protects against numerous agents and conditions that cause oxidative stress and/or neuronal injury (Keller et al. 1998; Li et al. 1998; Gonzalez-Zulueta et al.1998). Thus, a high level MnSOD activity is needed for protection of neuronal cells in conditions where overproduction of ROS or RNS is involved.

In this report, we demonstrated that NO is an important mediator in ADR-associated CNS toxicity using wild type and iNOSKO mice. We found that ADR-induced brain mitochondrial injury occurred only in the wild-type but not in the iNOSKO mice. The levels of nitrotyrosine in brain proteins were increased in the wild-type but not in iNOSKO mice. Interestingly, in the wild-type mouse brains, MnSOD protein was nitrated and its enzymatic activity was reduced without a reduction in the protein level. These results support the role of NO in ADR-mediated CNS injury and suggest the link between cytokine production and iNOS induction in brain tissues.

Materials and methods

Eight-week-old male wild-type, B6C3, and iNOSKO mice (25-30g) were kept under standard conditions and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. Mice were injected in a single intra-peritoneal (i.p.) treatment with adriamycin (Doxorubicin hydrochloride, 20 mg/kg, Gensia Sicor Phamaceuticals, Irvine, CA), TNF-α (4 ng/kg, Sigma, St Louis, MO), anti-mouse TNF-antibody (40 ng/kg, R&D Systems, Minneapolis, MN), dipropylenetriamine NONOate (DPTA NONOate, 4 mM, Alexis biochemicals, San Diego, CA), lipopolysacchacride (LPS, 1 mg/kg, Sigma, St Louis, MO), or preimmune IgG (Santa Cruze, CA). The mouse enzyme-linked immunosorbent assay was purchased from R&D Systems (mouse TNF-α/TNFSF1A immunoassay, R&D Systems, Minneapolis, MN).

Enzyme-linked Immunosorbent assay (ELISA)

Mice were treated with ADR, TNF-α, anti-mouse TNF-antibody followed by ADR, DPTA NONOate, or preimmune IgG, as well as saline as a control. Blood samples were collected at 3 h after ADR treatment and allowed to clot at 2-8^{O}C overnight. Serum samples were used to measure TNF levels, according to the mouse ELISA, following the manufacturer's instructions (mouse TNF-α/TNFSF1A immunoassay, R&D Systems, Minneapolis, MN). The TNF concentration in the sample was calculated from the recombinant mouse TNF standard curve, with a minimum detected limit typically less than 5.1 pg/mL.

Mitochondrial Isolation and Purification

Mice were perfused via cardiac puncture with cold mitochondrial isolation buffer, the brain promptly removed, the cerebellum dissected away, and mitochondria immediately isolated from the freshly obtained brain by a modification of the method described by Mattiazzi et al. (2002). Brain mitochondria were isolated in cold mitochondrial isolation buffer, containing 0.07 M sucrose, 0.22 M mannitol, 20 mM HEPES, 1 mM EGTA, and 1% bovine serum albumin, pH 7.2. Tissues were

homogenized with a Dounce homogenizer and centrifuged at 1,500xg at 4^{O}C for 3 min before transferring the supernatant. The pellets were resuspended and centrifuged at 1,500xg at 4^{O}C for 3 min. The supernatants were combined and centrifuged at 1,500xg at 4^{O}C for 3 min. The supernatants were centrifuged at 13,500xg at 4^{O}C for 10 min. Mitochondrial pellets were washed twice and finally resuspended in 50-100 µL cold isolation buffer. Protein concentration of isolated mitochondria was determined by the Bradford assay (Bradford 1976).

Mitochondrial Respiration

Mitochondrial respiration was determined using Clark-type polarographic oxygen sensors (Hansatech Instruments, UK) to measure the rate of oxygen consumption. Freshly isolated mitochondria were suspended in respiration buffer at a concentration of 0.5 mg mitochondrial protein per mL of respiration buffer, which consists of 0.25 M sucrose, 50 mM HEPES, 1 mM EGTA, 10 mM KH_2PO_4, and 2 mM $MgCl_2$, 0.2% bovine serum albumin, pH 7.4. Oxygen consumption was measured with either pyruvate (10 mM) plus malate (10 mM) as substrates for respiration from complex I in the absence of exogenous ADP (state II) and after addition of 300 mM ADP (state III respiration). The ATPase inhibitor oligomycin (100 µg/mL) was added to inhibit mitochondrial respiration such that state IV respiration was similar to the state II respiration rate. FCCP (1 µM), an uncoupling agent, was added as a control of respiration. Respiration control ratios (RCR) were calculated as the ratios of state III and state II respiration. The unit for state II rate and state III rate is nmole/min/mg protein.

Preparation of brain homogenate

Brains were perfused and dissected from mice treated with i.p. ADR 20 mg/kg or saline treated control for 3 h, and placed in 50 mM phosphate buffered saline (PBS), pH 7.4, containing protease inhibitors (4 µg leupeptin, 4 µg pepstatin, 5 µg aprotinin). Brain tissues were washed and chopped in ice-cold PBS containing these protease inhibitors. Tissues were homogenized

with a Potter-Elvehjem glass homogenizer with a loose-fit Teflon pestle, and protein concentration was determined by the Bradford assay (Bradford 1976).

MnSOD activity assay

MnSOD activities in the brain homogenate were measured by the nitroblue tetrazolium (NBT)-bathocuproin sulfonate (BCS) reduction inhibition method described by (Spitz and Oberley 1989). Sodium cyanide (2 mM) was used to inhibit Cu/ZnSOD activity. MnSOD activity was expressed in units per milligram of protein.

Western Blot Analysis

Brain homogenate proteins were size-separated through denaturing polyacrylamide gel electrophoresis (SDS-PAGE). Proteins (50 μg) were electrophoresed on 12.5 % SDS-PAGE and transferred to a nitrocellulose membrane. The membrane was blocked for 1 h at room temperature in blocking solution consisting of 5% non-fat dried milk, Tris-buffered saline (TBST), [10mM Tris-HC, 150mM NaCl, 0.05% Tween 20], pH 7.9. After blocking, the membrane was incubated overnight at 40C with primary antibodies against MnSOD (Upstate, Lake Placid, New York), Cu/ZnSOD (Calbiochem, San Diego, CA), and β-actin (Sigma, St Louis, MO) in blocking solution. The membrane was washed twice in TBST and incubated for 1 h with horseradish peroxidase-conjugated secondary antibodies in blocking solution. After incubation with secondary antibodies, the membrane was washed twice with TBST and once in TBS (TBS without 0.05% Tween-20). Immunoreactivities of the protein bands were detected by enhanced chemiluminescence autoradiography (ECL, Amersham Phamacia Biotech, Arlington Heights, IL) as described by the manufacturer.

Immunoprecipitation Assays

Solubilized isolated mitochondrial proteins (500 μg) in 500 μL RIPA buffer (9.1 mM Na_2HPO_4, 1.7 mM NaH_2PO_4, 150 mM NaCl, 0.5% sodium deoxycholate, 1% v/v Nonidet P40,

0.1% sodium dodecyl sulfate, pH 7.2) were incubated 16 h at 4^{O}C with 10 µg/ mL of anti-nitrotyrosine antibody (Cayman Chemical, Ann Arbor, MI). Immune complexes were precipitated with 50 µL of protein A/G-Agarose. Immunocomplexes were collected by centrifugation at 1,500xg for 5 min at 4^{O}C, and then washed four times with RIPA buffer. Immunoprecipitated samples were recovered by resuspending in 50 µL of 2x sample loading buffer, heated to 95^{O}C for 5 min, and immediately fractionated by reducing SDS/PAGE in 12.5 % gels. Isolated mitochondria sample from mouse brains were treated with 20 µM ONOO- as positive controls (provided by Dr. Timothy R. Miller, Graduate Center for Toxicology, University of Kentucky, Lexington, Kemtucky) and the sample was incubated with 10 µg/ mL of rabbit IgG as negative controls, respectively. Immunoreactivities of the MnSOD nitrated protein bands were detected by enhanced chemiluminescence autoradiography (ECL, Amersham Phamacia Biotech, Arlington Heights, IL) as described by the manufacturer and evaluated by densitometric analysis using a Bio-RAD densitometer (Bio-RAD Laboratory, Hercules, CA).

Slot Blot Analysis

3-Nitrotyrosine levels, a peroxynitrite marker, were determined as described previously (Lauderback et al. 2001). Briefly, 5 µg of isolated mitochondrial proteins were incubated with Laemmli sample buffer (0.125 M Trisma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min. Then 250 ng of protein were blotted onto the nitrocellulose membrane using a slot-blot apparatus. The membrane was rinsed with TBST buffer blocked by incubation in the presence of 5% BSA, followed by incubation with rabbit polyclonal anti-nitrotyrosine antibody as primary antibody for 1 h. The membranes were washed with TBST buffer and further incubated with goat-anti rabbit horseradish peroxidase-conjugated as the secondary antibody for 1 h. After incubation with secondary antibodies, the membrane was washed twice with TBST and once in TBS (TBS without 0.05% Tween-20). Immunoreactivities of the protein bands were detected by enhanced chemiluminescence autoradiography (ECL, Amersham Phamacia Biotech, Arlington Heights, IL) as described by the manufacturer and scanned by photoshop and then quantified by densitometry (Bio-Rad Laboratories, Inc. Hercules, CA).

RT-PCR Analysis

At 3 h after treatment, mice were anesthetized and perfused via cardiac puncture with 0.1 M PBS, pH 7.4. The mRNA was isolated using Micro-FastTrackTM 2.0 Kit (Invitrogen, Grand Island, New York) according to the manufacturer's instructions. The purified mRNA (5 μg) was subjected to reverse-transcription into first strand cDNA in each 20 μL of reaction mixture using SuperScriptTM III First-Strand Synthesis System for RT-PCR (Invitrogen) according to the manufacturer's instruction. A 25 μL PCR reaction contained 5 μL first strand cDNA, 0.1 units Taq DNA polymerase (Invitrogen), 10 mM 10xPCR buffer, 1.5 mM $MgCl_2$, 2.5 mM dNTP, and 10 pmol of each specific primer. PCR samples were subjected to the following conditions: 35 cycles for iNOS (94^{O}C: 1 min; 65^{O}C: 1 min; and 72^{O}C:1 min), 32 cycles for β-actin (94^{O}C: 30 sec; 55^{O}C: 30 sec; and 72^{O}C: 10 min) on a thermocycler (Perkin-Elmer). Primer sequences for iNOS was 5'-CTGATGGT-CAAGATCCAGAGGT CT-3'(forward) and 5'-CTGCATGTGCTTCATGAAGGACTCT-3' (reverse), and the primer for β-actin was 5'-TGTTACCAACTG GGACGACA-3' (forward) and 5'-CTGGGTCATCTTTTCACC GT-3' (reverse). After amplification, PCR products were subjected to 1% agarose gel electrophoresis, and visualized by ethidium bromide staining. The relative density of bands was analyzed under ultraviolet light (Bio-RAD Laboratory, Hercules,CA). Experiments were repeated three times for reproducibility.

Statistical Analysis

Statistical comparisons were made using one-way ANOVA followed by Newman-Keuls multiple comparisons test. Data are expressed as mean ± SEM.

Results

Adriamycin-induced circulating TNF levels

TNF levels in serum were significantly higher in mice treated with ADR for 3 h ($p < 0.001$, Fig 3.1A). The increased circulating TNF levels were detectable in both wild-type and iNOSKO mice. Directed i.p. injection of TNF increased systemic TNF in both wild-type and iNOSKO mice ($p<0.001$, Fig 3.1B, C). ADR treatment followed by anti-TNF antibody treatment prevented a detectable increase in serum TNF (Fig 3.1B). DPTA NONOate was used to generate NO at the same level as TNF-induced murine macrophage produce NO (Espey et al. 2000), and was used to prove that exogenous NO can cause CNS toxicity in iNOSKO mice. The results show that DPTA NONOate directly injected to iNOSKO mice did not lead to changes in serum TNF levels (Fig 3.1C).

Adriamycin-induced TNF-mediated iNOS mRNA in brain tissues

The possibility that ADR caused elevation of circulating TNF-mediated increased NO production in brain tissues was investigated. We determined iNOS mRNA in wild-type mice. As shown in Figure 3.2, the levels of iNOS mRNA was significantly increased in TNF-, ADR-, and LPS-treated mice compared to saline controls. Neutralizing antibody against circulating TNF prevented TNF-induced iNOS mRNA expression and increasing of NO levels in brain tissues.

Adriamycin-induced mitochondrial dysfunction

To investigate the effect of ADR on brain mitochondrial function, mitochondrial respiration using pyruvate plus malate as the substrates was measured. The data are presented as the RCR of each treatment group and the saline-treated control group from each set of experiments. The values of RCR in wild-type mice, but not in iNOSKO mice, were significantly decreased in the ADR treated group compared to the control saline group ($p<0.05$, Fig. 3.3). Treatment of wild-type mice with TNF caused a mitochondrial respiration decline similar to that observed by ADR treatment (Fig.

3.4). Injection of neutralizing antibody to TNF followed by ADR abolished the reduced brain mitochondrial respiration (Fig. 3.4). Importantly, in iNOSKO mice, treatment with TNF or ADR had no effect on the RCR, while treatment with DPTA NONOate, a NO generator, significantly lowered the RCR in iNOSKO mice ($p<0.05$, Fig. 3.5). Taken together, these results suggest that ADR-induced circulating TNF levels subsequently increased brain levels of TNF, which may activate iNOS to produce NO resulting in the inhibition of the NAD-linked state III respiration rate.

ADR-induced brain protein nitration

Since reactive products of NO can cause protein nitration (Butterfield and Stadtman. 1995; Castegna et al. 2003), nitrotyrosine adducted proteins from mitochondria isolated from brain treated-mice with ADR 20 mg/kg for 3 h were determined. The levels of tyrosine-nitrated proteins were significantly increased in wild-type, but not iNOSKO mice, after treatment with ADR for 3 h ($p<0.01$, Fig. 3.6).

Adriamycin-induced MnSOD nitration/inactivation in brain tissues

An increased NO production within the mitochondria could lead to nitration of MnSOD. Immunoprecipitation of mitochondrial protein with anti-nitrotyrosine antibody was used to pull down nitrotyrosine-MnSOD immunocomplex. The immunoprecipitated protein demonstrated an increase of nitrated MnSOD in wild-type mice ($p<0.01$, Fig. 3.7), but not in iNOSKO mice. Since MnSOD is known to be sensitive to peroxynitrite-induced inactivation, MnSOD activity after treatment with ADR 20 mg/kg for 3 h was determined. ADR significantly decreased MnSOD activity in brain tissue homogenates of wild-type mice, but not in iNOSKO mice ($p<0.01$, Fig 3.8).

MnSOD and CuZnSOD level in brain tissues

There is a possibility that the decline in MnSOD activity in ADR-treatment in iNOSKO mice brains following ADR is due to a reduced MnSOD protein level. Accordingly, we

determined the MnSOD and CuZnSOD levels in brain tissue homogenates by Western blot analysis, following administration of ADR. The results indicate no change in both wild-type and iNOSKO mice (Fig 3.9).

Discussion

Adriamycin, a quinine-containing anthracycline anticancer drug, which generates reactive oxygen species (ROS), is well documented to produce ROS in normal tissues (Joshi et al. 2005; Kalayanaraman et al. 2002; Singal et al. 2000). We have recently reported that brain tissue damage is mediated, at least in part, by the systemic release of the cytokine, TNF, which accumulates in neurons. The increasing of TNF in the brain tissue correlated with mitochondrial respiration dysfunction, cytochrome c released, activated caspase 3 and TUNEL apoptotic cell death (Tangpong et al. 2006, in press). The results in the present studies demonstrate that TNF production led to subsequent generation of reactive oxygen and nitrogen species (RONS) in the brain. Although ADR-induced circulating levels of tumor necrosis factor alpha increased in both wild-type mice and iNOSKO mice, the decline in mitochondrial respiration after ADR treatment was observed only in wild-type mice, suggesting a role for NO in ADR-induced, TNF-mediated mitochondrial dysfunction. Consistent with this finding, ADR treatment led to brain protein nitration in the wild-type mice but not in the iNOSKO mice.

Peripheral TNF can act on the brain to regulate neural-immune interactions as well as other brain functions. Peripheral TNF can enter the CNS and thus act directly on brain parenchyma by crossing the blood-brain barrier, either by active transport or passive diffusion in the circumventricular organs, the area outside the blood-brain barrier (Sternberg 1997). TNF can also be generated within the CNS, and all neuronal cell types are thought to contain TNF-receptors and be capable of synthesizing TNF in brain tissues (Sternberg 1997; Madrigal et al. 2002; Perry et al. 2002). Enhanced circulating TNF can initiate local TNF production via activation of glia cells leading to production of RONS (Sternberg 1997; Szelenyi 2001). Our results, which indicate that the iNOS mRNA is increased in brain tissues when animals were directly injected with TNF, LPS, or ADR, support the role of TNF in mediating the ADR effects in the brain.

It is important to distinguish the reversible inhibition of the respiratory chain by low concentrations of NO at complex IV and the irreversible inhibition resulting from damage to iron-sulphur centers in the respiration chain and mitochondria aconitase. Irreversible inhibition has been suggested to contribute to the cytotoxicity of high concentrations or long-term exposure to NO

(Moncada and Higgs 1993; Radi 2004). The irreversible inhibition of the respiratory chain by NO may be the result of the reaction of superoxide with nitric oxide to generate peroxynitrite, since NO may be converted to peroxynitrite by mitochondrial-generated superoxide (Radi 2004). Peroxynitrite has been shown to inhibit mitochondrial respiration at complexes I, II, and possibly III, but not complex IV in contrast to NO alone (Cooper and Brown 1994; Cooper et al. 2003). This possibility is strongly supported by the following: 1) Our previous studies, which demonstrated that transgenic mice overexpressing MnSOD are protected from ADR-induced complex I inactivation in cardiac tissues (Yen et al. 1996; Yen et al. 1999); 2) The present result from the study, which indicates that ADR, TNF, or LPS induced a diminution in brain mitochondrial respiration via complex I in wild-type mice; 3) The NO generator, DPTA NONOate, in iNOSKO mice caused a decline in brain mitochondrial respiration via complex I; and 4) The levels of nitrotyrosine-adducted proteins from brain mitochondrial tissues increased in wild-type mice, but not in iNOSKO mice.

Two independent lines of MnSOD mutant mice, in which the MnSOD gene was selectively inactivated by recombinant technology, suffer neonatal lethality (Li et al. 1995; Lebovitz et al. 1996). The MnSOD homozygous knockout mice were apparently healthy at birth, but grew poorly and died within weeks (Li et al. 1995). Autopsies of the first line of homozygous MnSOD knockout mice revealed dilated cardiomyopathy, lipid accumulation in the liver and skeletal muscle, and metabolic acidosis. They also showed a severe loss of succinate dehydrogenase and aconitase, two key mitochondrial enzymes (Li et al. 1995.; Huang et al. 1999). The second line of homozygous knockout mice also exhibited extensive mitochondrial injury within degenerative neurons (Libovitz et al. 1996). The heterozygous knockout mice developed normally and did not appear to have any life-threatening disorders, but were highly susceptible to agents that cause oxidative stress. For example, brief substrate deprivation resulted in production of superoxide and high mortality of neurons in culture (Saez et al. 1987); transfection of the MnSOD gene into cultured neuronal cells prevented cell death caused by treatment with amyloid beta peptides and iron (Keller et al. 1998); reduced expression of MnSOD by selective inactivation of the MnSOD gene sensitized cultured mouse cortical neurons to glutamate-induced neurotoxicity (Li et al. 1998); and overexpression of MnSOD provided dramatic protection against N-methyl-D-aspartate (NMDA) and NO toxicity in cortical culture (Gonzalez-Zulueta et al. 1998).

Consistent with these findings, the activity of a major mitochondrial antioxidant enzyme, manganese superoxide dismutase (MnSOD), was reduced and the protein was nitrated. MnSOD inactivation is linked to oxidative modification of MnSOD in vivo via nitration and hydroxylation, protein modifications promoted, respectively, by NO-derived oxidants (peroxynitrite or peroxidase-catalyzed reactions) and hydroxyl radical-like oxidants such as those generated by redox active transition metal ions during Fenton and Haber-Weiss oxidation chemistry (Halliwell et at., 1998). Taken together, the present studies provide evidence of ongoing profound oxidative and nitrosative stress in brain tissues with downstream consequences of SOD inactivation and nitration. Consistent with this notion, we recently reported elevated oxidative and nitrosative stress in brains of ADR-treated mice (Joshi et al. 2005).

Mitochondrial alterations have been reported in neurodegenerative diseases, such as Alzheimer's (AD) and multiple sclerosis (MLS), which also have inflammatory components involving activation of brain astrocytes and microglia to express iNOS and high levels of NO (Smith et al. 1997, Castegna et al. 2003; Calabesse et al. 2004; Sultana et al. 2006). Peroxynitrite-induced nitration of tyrosine residues in MnSOD has been reported (MacMillan-Crow et al. 1996; Yamakura et al. 1998; Chaiswing et al. 2004; Radi 2004). Generation of peroxynitrite can amplify the increased cycle of oxidative stress by tyrosine nitration and inactivation of MnSOD progressively which further enhances peroxynitrite production and subsequent mitochondrial damage during oxidant stress (MacMillan-Crow et al. 1996). Thus, neurotoxicity of ADR-induced TNF production resembles the free radical-mediated level in an AD brain (Butterfield and Landerbauk 2002).

In conclusion, ADR treatment i.p. led to an increased circulating level of TNF in wild-type mice and in iNOSKO mice. Circulating TNF, in turn, mediated iNOS induction to produce NO in brain tissues that subsequently led to a decline in mitochondrial respiration after ADR treatment. ADR caused protein nitration and led to nitrated MnSOD, which was inactivated in wild-type mice. These results are consistent with the notion that nitric oxide is a mediator coupling the effect of ADR with cytokine production and subsequent inactivation of MnSOD in the brain. Thus, prevention of MnSOD inactivation by neutralizing elevated systemic TNF or removal of NO production conceivably could be an effective means for the prevention of ADR-induced CNS toxicity. Studies to test this notion are in progress.

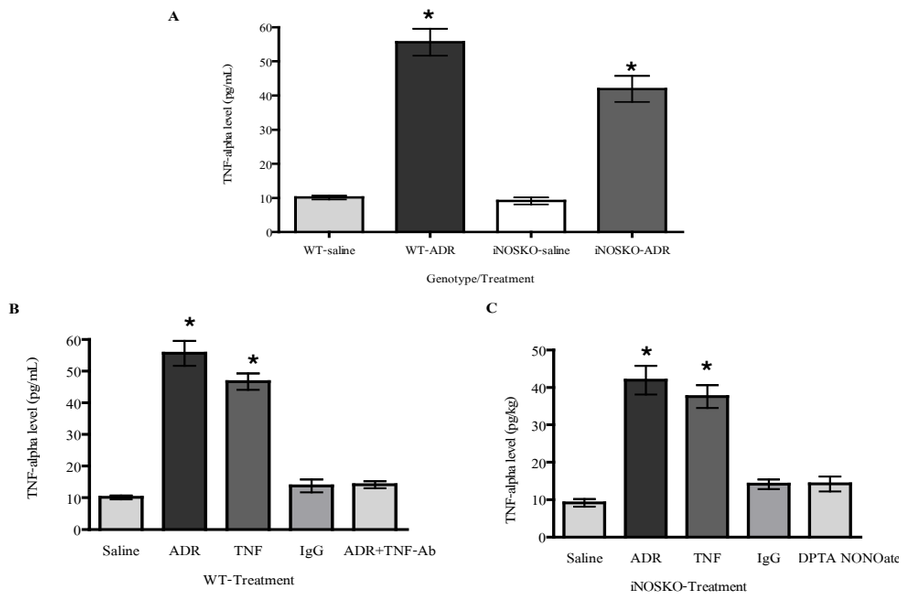

Figure 3.1: Adriamycin increased circulating TNF. TNF levels are significantly elevated in both wild type and iNOSKO mice 3 h after 20 mg/kg ADR compared with saline control (*$p < 0.001$, A). Exogenous TNF injected i.p. increased the circulating TNF level (*$p < 0.001$, B, C). Neutralizing antibody against circulating TNF abolished serum increased in anti-TNF antibody followed by ADR treatment (B). TNF or DPTA NONOate had no effect on the circulating TNF levels (C). Preimmune IgG was injected as a negative control.

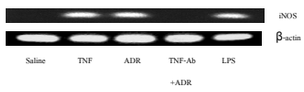

Figure 3.2: Adriamycin-induced iNOS mRNA expression in wild-type mice. Representative RT-PCR product of ADR induced iNOS mRNA and NO production in brain tissues after treatment with ADR, TNF, and LPS for 3 h compared with saline control. Blocking TNF from circulating TNF with anti-TNF antibody prevented ADR-mediated iNOS increase in the brain tissues.

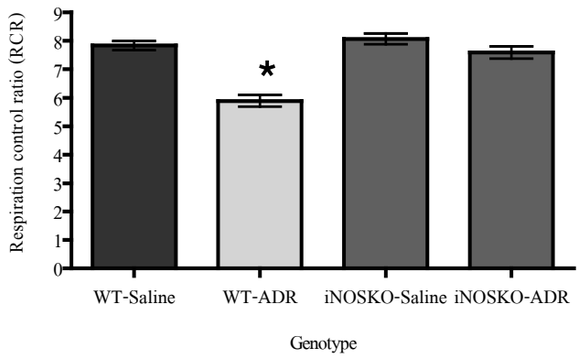

Figure 3.3: Adriamycin-mediated TNF elevation leads to mitochondrial dysfunction. Brain mitochondrial respiration was determined using pyruvate+malate as substrates. Mitochondrial respiration complex I, pyruvate+malate as substrates, was significantly decreased in wild-type mice 3 h after treatment with ADR (20 mg/kg) (*$p<0.05$) compared to all other groups. Data represent the mean $\pm$ SEM of three independent experiments.

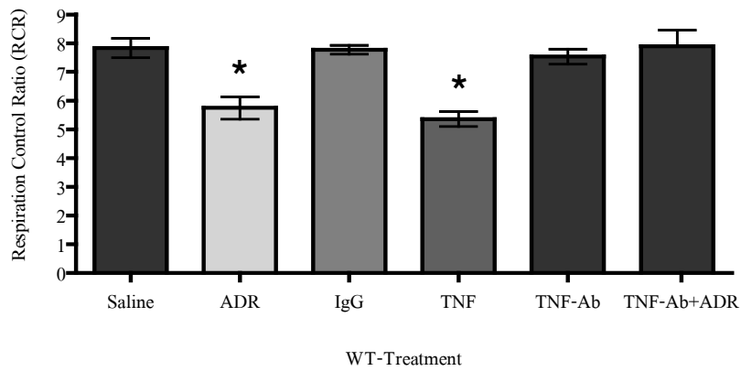

Figure 3.4: Adriamycin-mediated TNF elevation leads to mitochondrial dysfunction in wild-type mice. Brain mitochondrial respiration was determined using pyruvate+malate as substrates in mitochondria from mice treated with saline, ADR or TNF. The results show mitochondrial respiration complex I, pyruvate+malate as substrates, was significantly decreased (*$p<0.05$ compared to saline or IgG groups) 3 h after treatment with ADR or TNF, and the mitochondrial respiration decline was blocked by anti-TNF antibody. Data represent the mean ± SEM of three independent experiments.

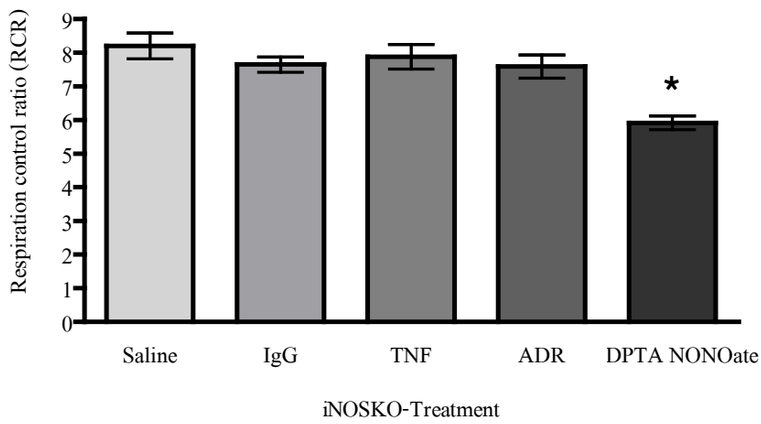

Figure 3.5: DPTA NONOate causes mitochondrial dysfunction in iNOSKO mice. Brain mitochondrial respiration was determined using pyruvate+malate as substrates in mitochondria from iNOSKO mice treated with saline, IgG, ADR, TNF, or DPTA NONOate. Mitochondrial respiration complex I, pyruvate+malate as substrates, was significantly decreased 3 h after treatment with DPTA NONOate (*$p<0.05$), ADR or TNF had no effect on mitochondrial respiration in iNOSKO mice. Data represent the mean ± SEM of three independent experiments.

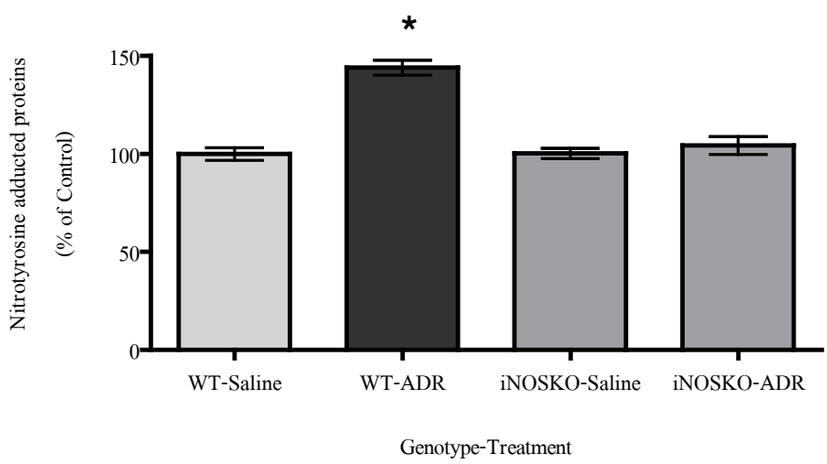

Figure 3.6: ADR-induced protein nitration. Representative slot blot of immunoreactive levels of nitrotyrosine adducted proteins. Nitrotyrosine adducted protein from isolated brain mitochondria of mice 3 h after treatment with ADR 20 mg/kg or saline control. Result are reported as mean + SEM of nitrotyrosine adducted protein density of three independent experiments and shown significantly increased in ADR in wild-type mice, but not different in iNOSKO mice (*$p < 0.01$)

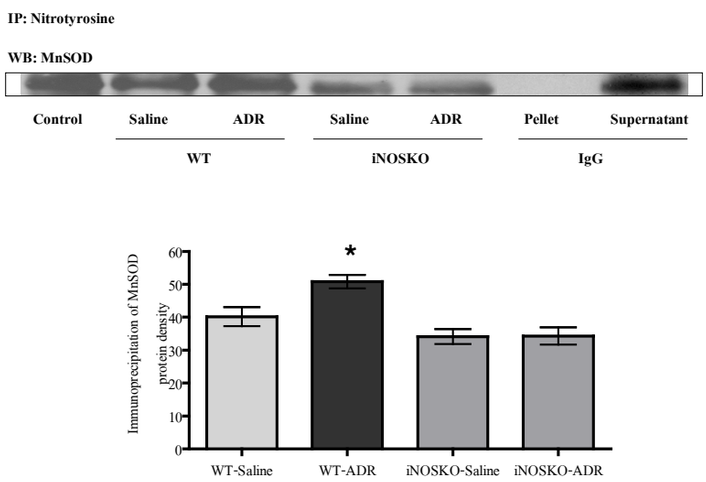

Figure 3.7: ADR-induced MnSOD Nitration. Immunoprecipitation coupled to Western analysis show the level of nitrotyrosine containing an MnSOD immunocomplex significantly increased 3 h after 20 mg/kg of ADR treatment in wild-type mice, but not in iNOSKO mice similarly treated (*$p<0.01$, A). Brain isolated mitochondria treated with 20 μM ONOO- was used as positive control and IgG was used as negative control. **Top**, Representative of the nitrated MnSOD by Western blots. **Bottom**, The quantitative analysis of nitrated MnSOD protein density. Data represent the mean ± SEM of three independent experiments.

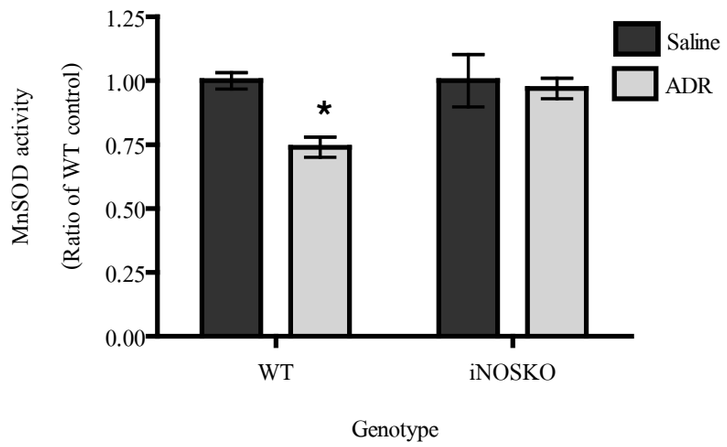

Figure 3.8: ADR induced MnSOD inactivation. Brain homogenates were used for SOD activity assay. MnSOD activity was significantly decreased in wild-type mice after treatment with ADR 20 mg/kg for 3 h (*$p < 0.05$). The MnSOD was not changed in iNOSKO mice similarly treated. Data represent the mean ± SEM of three independent experiments.

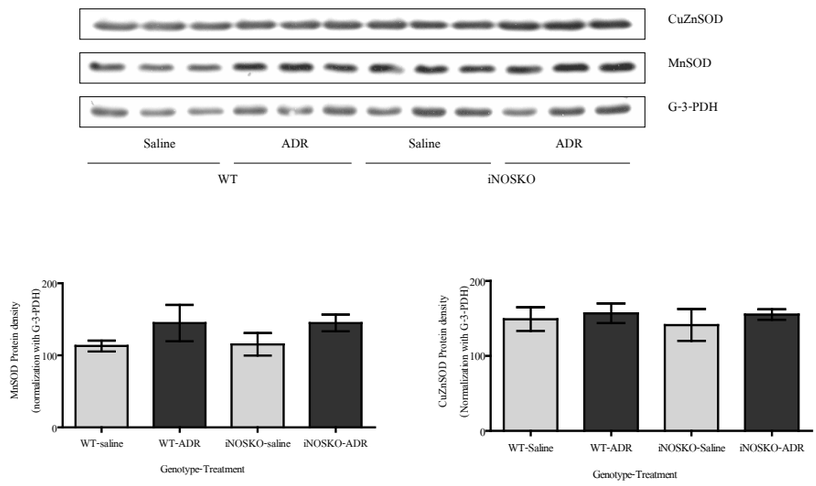

Figure 3.9: ADR does not alter levels of MnSOD and CuZnSOD. Representative Western blots of brain homogenate proteins from mice 3 h after treatment with ADR 20 mg/kg or saline. Immunodetection with the polyclonal anti-MnSOD and anti-Cu/ZnSOD antibody exhibited no change in protein density levels of MnSOD or CuZnSOD in both wild type and iNOSKO mice after treatment with ADR or saline. Data represent the mean ± SEM of three independent experiments. **Top**, representative Western blot showed MnSOD, CuZnSOD, and G-3-PDH was used as normalization of protein loading. **Bottom**, representative protein density of MnSOD and CuZnSOD after normalized with G-3-PDH.