*Journal of Neurochemistry*, 2007, **100**, 191–201 doi:10.1111/j.1471-4159.2006.04179.x

# Adriamycin-mediated nitration of manganese superoxide dismutase in the central nervous system: insight into the mechanism of chemobrain

Jitbanjong Tangpong,*,† Marsha P. Cole,* Rukhsana Sultana,‡ Steven Estus,§ Mary Vore,* William St. Clair,¶ Suvina Ratanachaiyavong,† Daret K. St. Clair* and D. Allan Butterfield‡

*Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky, USA
†Biomedical Sciences Faculty of Medicine, Prince of Songkla University, Songkhla, Thailand
‡Department of Chemistry, Center of Membrane Sciences, and Sanders-Brown Center on Aging, §Department of Physiology, College of Medicine, and ¶Department of Radiation Medicine, University of Kentucky, Lexington, Kentucky, USA

**Abstract**

Adriamycin (ADR), a potent anti-tumor agent, 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 does not pass the blood–brain barrier. Some cancer patients receiving ADR treatment develop a transient memory loss, inability to handle complex tasks etc., often referred to by patients as chemobrain. 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 demonstrate 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 was observed only in wild-type mice, 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. We also identified the mitochondrion as an important target of ADR-induced NO-mediated CNS injury.

**Keywords:** adriamycin-induced chemobrain, central nervous system toxicity, inducible nitric oxide synthase knockout mice, manganese superoxide dismutase, mitochondrial respiration, nitric oxide.

*J. Neurochem.* (2007) **100**, 191–201.

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

Received May 6, 2006; revised manuscript received June 18, 2006; accepted August 9, 2006.

Address correspondence and reprint request to D. Allan Butterfield, Professor of Chemistry, 121 Chemistry-Physics Building, University of Kentucky, KY 40536, USA. E-mail: dabcns@uky.edu

*Abbreviations used*: ADR, adriamycin; CuZnSOD, copper-zinc superoxide dismutase; DPTA NONOate, dipropylenetriamine NONOate; iNOS, inducible nitric oxide; iNOSKO, mice deficient in the inducible form of nitric oxide; L-NAME, NG-nitro-L-arginine-methyl ester; LPS, lipopolysaccharide; MnSOD, manganese superoxide dismutase; NO, nitric oxide; $ONOO^-$, peroxynitrite; RCR, respiration control ratio; RNS, reactive nitrogen species; RONS, reactive oxygen and nitrogen species; ROS, reactive oxygen species; TNF-α, tumor necrosis factor alpha.

 

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). Patients often refer to these CNS symptoms as 'chemobrain' (Schagen *et al*. 1999).

Although the biochemical basis for ADR-induced CNS injury is unknown, it has been demonstrated that cancer therapeutic agents like ADR can modulate endogenous levels of cytokines, such as tumor necrosis factor alpha (TNF-α) (Ujhazy *et al*. 2003; Usta *et al*. 2004). Systematically-released TNF-α 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-α in ADR-induced CNS injury, we recently demonstrated that intraperitoneal injection of a neutralizing antibody against TNF-α eliminated ADR-induced brain mitochondrial dysfunction (Tangpong *et al*. 2006).

Among potential downstream effects of TNF-α is an increase in generation of reactive nitrogen species (RNS) (Szelenyi 2001). One RNS is nitric oxide (NO), which is known to play a key role in many physiological and pathological conditions. NO 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 tightly 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 astrocytes can generate NO radicals from iNOS activation (Marcus *et al*. 2003; Candolfi *et al*. 2004). NO reacts rapidly with superoxide radicals, which limits the NO lifetime. NO reacts with superoxide radicals to form peroxynitrite ($ONOO^-$), a 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 oxidizing protein and non-protein sulfhydryls directly; it 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 peroxynitrite formation (Ste-Marie *et al*. 2001). Inhibition of mitochondrial electron transport and inactivation of iron-sulfur-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 some key components of the mitochondrial defense system.

ADR treatment led to an increase in NO in breast cancer cells *in vitro*, and is reportedly cytotoxic 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 nuclear factor-kappa B (NF-κB) 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; Santoro *et al*. 2003; Snyder and Morgan 2005). Thus, it is possible that increased NO production by ADR is a result of TNF-α-mediated NF-κB activation of iNOS expression. As NO 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 manganese-containing superoxide dismutase (MnSOD). Inactivation of the MnSOD gene in mammals yielded detrimental effects (Li *et al*. 1998). Thus, lack of MnSOD activity can severely impair mitochondria and affect the brain, both of which have high demands for oxidative metabolism. Overexpression of MnSOD protects against numerous agents and conditions that cause oxidative stress and/or neuronal injury (Gonzalez-Zulueta *et al*. 1998; Keller *et al*. 1998; Li *et al*. 1998). Therefore, a high level of MnSOD activity is needed for protection of neuronal cells in conditions where overproduction of ROS or RNS is involved.

In this report, we demonstrate that NO is an important mediator in ADR-associated CNS toxicity using wild-type and inducible nitric oxide synthase (iNOSKO) mice. We found that ADR-induced brain mitochondrial injury occurred only in the wild-type and not in the iNOSKO mice. The levels of nitrotyrosine in brain proteins were increased in the wild-type but not in the iNOSKO mice. Interestingly, in brains from ADR-treated wild-type mice, 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 a link between cytokine production and iNOS induction in brain tissues.

## Materials and methods

Eight-week-old male wild-type B6C3 and iNOSKO mice (25–30 g) 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 intraperitoneal (i.p.) treatment with adriamycin (doxorubicin hydrochloride, 20 mg/kg; Gensia Sicor Phamaceuticals, Irvine, CA, USA), TNF-α (4 ng/kg; Sigma, St Louis, MO, USA), anti-mouse

TNF-antibody (40 ng/kg; R & D Systems, Minneapolis, MN, USA), 1-hydroxy-2-oxo-3-di-(3-aminopropyl)-1-triaze (DPTA NONOate, 4 mM; Alexis biochemicals, San Diego, CA, USA), lipopolysaccharide (LPS, 1 mg/kg; Sigma), NG-nitro-L-arginine-methyl ester (L-NAME, 10 mg/kg; Sigma) or pre-immune IgG (Santa Cruz Biotecnology, Santa Cruz, CA, USA). The mouse enzyme-linked immunosorbent assay (ELISA) was purchased from R & D Systems (mouse TNF-α/TNFSF1A immunoassay).

### Enzyme-linked immunosorbent assay (ELISA)

Mice were treated with ADR, TNF-α or anti-mouse TNF-antibody, followed by ADR, DPTA NONOate or pre-immune 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°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). The TNF concentration in the sample was calculated from the recombinant mouse TNF standard curve, with a minimum detection limit typically less than 5.1 pg/mL.

### Mitochondrial isolation and purification

Mice were perfused via cardiac puncture with cold mitochondrial isolation buffer, then the brain was 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 (BSA), pH 7.2. Tissues were homogenized with a Dounce homogenizer (VWR, West Chester, PA, USA) and centrifuged at 1500 *g*, 4°C, for 3 min, before transferring the supernatant fluid. The pellets were resuspended and again centrifuged at 1500 *g*, 4°C, for 3 min. The supernatant fluids were combined and centrifuged at 1500 *g*, 4°C, for 3 min. These supernatant fluids were then centrifuged at 13 500 *g*, 4°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, King's Lynn, 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/mL respiration buffer, which consisted of 0.25 M sucrose, 50 mM HEPES, 1 mM EGTA, 10 mM $KH_2PO_4$, 2 mM $MgCl_2$ and 0.2% BSA, pH 7.4. Oxygen consumption was measured with either pyruvate (10 mM) or pyruvate 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. Carbonylcyanide-p-trifluoromethoxyphenylhydrazone (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 nanomoles per minute per milligram of protein.

### Preparation of brain homogenate

Brains were perfused, the cerebellum removed and the brain dissected from mice treated with i.p. ADR 20 mg/kg, or saline treated as a control, for 3 h. Brains were placed in ice-cold 50 mM phosphate-buffered saline (PBS), pH 7.4, containing the protease inhibitors 4 μg leupeptin, 4 μg pepstatin and 5 μg aprotinin. Then, brain tissues were washed and chopped in ice-cold PBS containing these protease inhibitors. Tissues were homogenized with a Potter-Elvehjem glass homogenizer (Bellco Glass, Inc., Vineland, NJ, USA) with a loose-fitting Teflon pestle, and protein concentration was determined by the Bradford assay (Bradford 1976).

### MnSOD activity assay

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

### Western blot analysis

Brain homogenate proteins were size-separated using denaturing sodium dodecyl sulfate–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 (22°C) in blocking solution consisting of 5% non-fat dried milk, Tris-buffered saline (TBST; 10 mM Tris-HC, 150 mM NaCl, 0.05% Tween 20), pH 7.9. After blocking, the membrane was incubated overnight at 4°C with primary antibodies against MnSOD (Upstate, Lake Placid, NY, USA), Cu/ZnSOD (Calbiochem, San Diego, CA, USA) and β-actin (Sigma) in blocking solution. The membrane was washed twice in TBST, then incubated for 1 h with horseradish peroxidase-conjugated secondary antibody in blocking solution. After incubation with secondary antibody, 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, USA) 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% SDS, pH 7.2) were incubated for 16 h at 4°C with 10 μg/mL anti-nitrotyrosine antibody (Cayman Chemical, Ann Arbor, MI, USA) or polyclonal anti-MnSOD antibody (Upstate Biotechnology). Immune complexes were precipitated with 50 μL protein A/G-Agarose (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA). Immunocomplexes were collected by centrifugation at 1500 *g* for 5 min at 4°C, and then washed four times with RIPA buffer. Immunoprecipitated samples were recovered by resuspending in 50 μL 2× sample loading buffer, heated to 95°C for 5 min, and immediately fractionated by reducing SDS–PAGE in 12.5% gels. Isolated

mitochondria samples from mouse brains were treated with 20 μM peroxynitrite as positive control (provided by Dr Timothy R. Miller, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky, USA) and the sample was incubated with 10 μg/mL IgG as negative control. Immunoreactivities of the MnSOD nitrated protein bands were detected by ECL (Amersham Phamacia Biotech) as described by the manufacturer. Nitration of MnSOD was quantified using an akaline phosphatase-linked secondary antibody (Sigma), as described previously (Sultana *et al*. 2004), and evaluated by densitometric analysis using a Bio-Rad densitometer (Bio-Rad Laboratories, Hercules, CA, USA).

### Two-dimensional (2D) gel electrophoresis

Supernatant fluids obtained from the control- and ADR-treated mitochondria, after immunoprecipitation of MnSOD, were dissolved in rehydration buffer containing 8 M urea, 2 M thiourea, 2% 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), 0.2% (v/v) biolytes, 50 mM dithiothreitol (DTT) and bromophenol blue. The samples were sonicated three times for 20 s on ice, and then applied to the IPG Readystrips (pH 3–10) from Bio-Rad to obtain gel maps (Levine *et al*. 1994). Isoelectric focusing was carried out at 20°C as follows: 800 V for 2 h linear gradient; 1200 V for 4 h of slow gradient; 8000 V for 8 h of linear gradient; 8000 V for 10 h of rapid gradient. The strips were stored at − 80°C until second dimension separation was performed. Gel strips were equilibrated, before second dimension separation, for 10 min in 50 mM Tris-HCl (pH 6.8) containing 6 M urea, 1% (m/v) SDS, 30% (v/v) glycerol and 0.5% DTT, followed by re-equilibrium for 10 min in the same buffer containing 4.5% iodoacetamide in place of DTT. The gel strips were then placed in the linear gradient precast Criterion Tris-HCl gels (8–16%, Bio-Rad) to perform the second dimension electrophoresis.

### Sypro ruby Staining

The gels were fixed in a solution containing 10% (v/v) methanol and 7% (v/v) acetic acid for 40 min, and then stained overnight at room temperature (22°C) with gentle agitation in 50 mL Sypro ruby gel stain (Bio-Rad). The gels were placed in deionized water overnight and scanned.

### Slot-blot analysis

3-Nitrotyrosine levels, a peroxynitrite reaction product marker, were determined as described previously (Lauderback *et al*. 2001). Briefly, 5 μg isolated mitochondrial proteins were incubated with Laemmli sample buffer (0.125 M Trisma base, pH 6.8, 4% SDS, 20% glycerol) for 20 min, and then 250 ng 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 secondary antibody for 1 h. After incubation with secondary antibody, 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 ECL (Amersham Phamacia Biotech) as described by the manufacturer. Blots were scanned into Adobe Photoshop and quantified by the densitometry method (Bio-Rad).

### 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 the Micro-FastTrack 2.0 Kit (Invitrogen, Groningen, Netherlands) 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 the SuperScript 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 U Taq DNA polymerase (Invitrogen), 10 mM 10× PCR 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°C: 1 min; 65°C: 1 min; 72°C: 1 min), 32 cycles for β-actin (94°C: 30 s; 55°C: 30 s; 72°C: 10 min), on a thermocycle (Perkin-Elmer, Wellesley, MA, USA). The primers with the target gene sequences were synthesized by Invitrogen. The primer sequence for iNOS was 5′-CTGATG-GTCAAGATCCAGA-GGTCT-3′ (forward) and 5′-CTGCATGTG-CTTCATGAAGGACTCT-3′ (reverse), and the primer for β-actin was 5′-TGTTACCAACTGGGACGACA-3′ (forward) and 5′-CTG-GGTCATCTTTTCACCGT-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). 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-α levels

TNF-α levels in serum were significantly higher in mice 3 h after treatment with ADR than those from controls ($p < 0.001$, Fig. 1a). The increased circulating TNF-α levels were detectable in both wild-type and iNOSKO mice. Direct i.p. injection of TNF-α increased systemic TNF-α both in wild-type and iNOSKO mice ($p < 0.001$, Figs 1b and c). ADR treatment followed by anti-TNF antibody treatment prevented the increase in serum TNF-α (Fig. 1b). DPTA NONOate was used to generate NO at the same level as that produced by TNF-α-induced murine macrophages (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 into iNOSKO mice did not lead to changes in serum TNF-α levels (Fig. 1c).

### Adriamycin-induced TNF-mediated iNOS mRNA in brain tissues

The possibility that ADR caused elevation of circulating TNF-induced iNOS expression in brain tissues was investigated. As shown in Fig. 2, the levels of iNOS mRNA were significantly increased in TNF-, ADR-, and LPS-treated mice

 

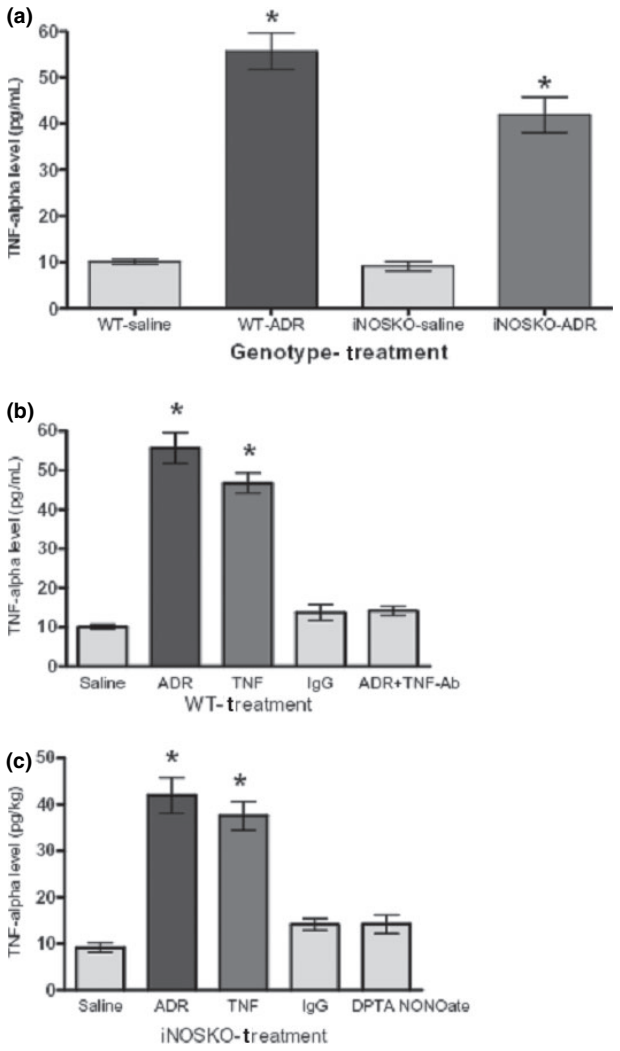


**Fig. 1** Adriamycin increased circulating TNF-α. (a) TNF-α levels are significantly elevated in both wild-type and iNOSKO mice after 20 mg/kg ADR for 3 h compared with saline control (*$p < 0.001$). (b, c) Exogenous injected TNF-α increased the circulating TNF-α level (*$p < 0.001$). (b) Neutralizing antibody against circulating TNF-α abolished increased TNF-α in serum 3 h after treatment with ADR. (c) DPTA NONOate had no effect on circulating TNF-α levels. Pre-immune IgG injection was used as a negative control.

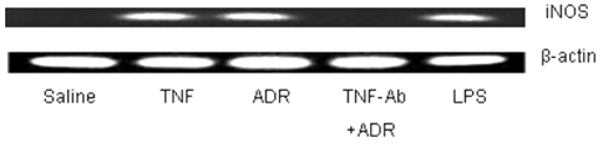


**Fig. 2** Adriamycin-induced iNOS mRNA expression in wild-type mice. Representative RT-PCR product of ADR-induced iNOS mRNA in brain tissues after treatment with ADR, TNF-α or LPS for 3 h compared with saline control. Blocking circulating TNF-α with anti-TNF-α antibody prevented ADR-mediated iNOS increase in the brain tissues.

compared with saline controls. Neutralizing antibody against circulating TNF prevented TNF-induced iNOS mRNA expression 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 mitochondria from wild-type mice, but not iNOSKO mice, were significantly decreased in the ADR-treated group compared with the control saline group ($p < 0.05$, Fig. 3a). Treatment of wild-type mice with TNF caused a mitochondrial respiration decline similar to that observed by ADR treatment (Fig. 3b). Injection of neutralizing antibody to TNF followed by ADR abolished the reduced brain mitochondrial respiration (Fig. 3b). 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. 3c). 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

As reactive products of NO can cause brain protein nitration (Butterfield and Stadtman 1997; Castegna *et al*. 2003; Sultana *et al*. 2006), nitrotyrosine adducted proteins from mitochondria, isolated from brain of mice 3 h after treatment with ADR at 20 mg/kg were determined. The levels of tyrosine-nitrated proteins were significantly increased in wild-type mice after treatment with ADR for 3 h ($p < 0.01$, Fig. 4a), but not in iNOSKO mice.

### Adriamycin-induced MnSOD nitration/inactivation in brain tissues

An increased NO level within the mitochondria could lead to nitration of MnSOD. Immunoprecipitation of mitochondrial protein with anti-nitrotyrosine antibody and anti-MnSOD antibody was used to pull down nitrotyrosine-MnSOD immunocomplex. The immunoprecipitated protein demonstrated an increase of nitrated MnSOD in wild-type mice ($p < 0.01$, Figs 4b and c), but not in iNOSKO mice. As MnSOD is known to be sensitive to peroxynitrite-induced inactivation, MnSOD activity after treatment with ADR at 20 mg/kg for 3 h was determined. MnSOD activity was significantly decreased by ADR in brain tissue homogenates of wild-type mice, but not in those of iNOSKO mice ($p < 0.01$, Fig. 5a). Treatment of mice with L-NAME, a non-selective NOS inhibitor, prevented MnSOD inactivation and led to increased MnSOD activity in brains compared with those of saline- or ADR-treated mice ($p < 0.001$, Fig. 5b).

 

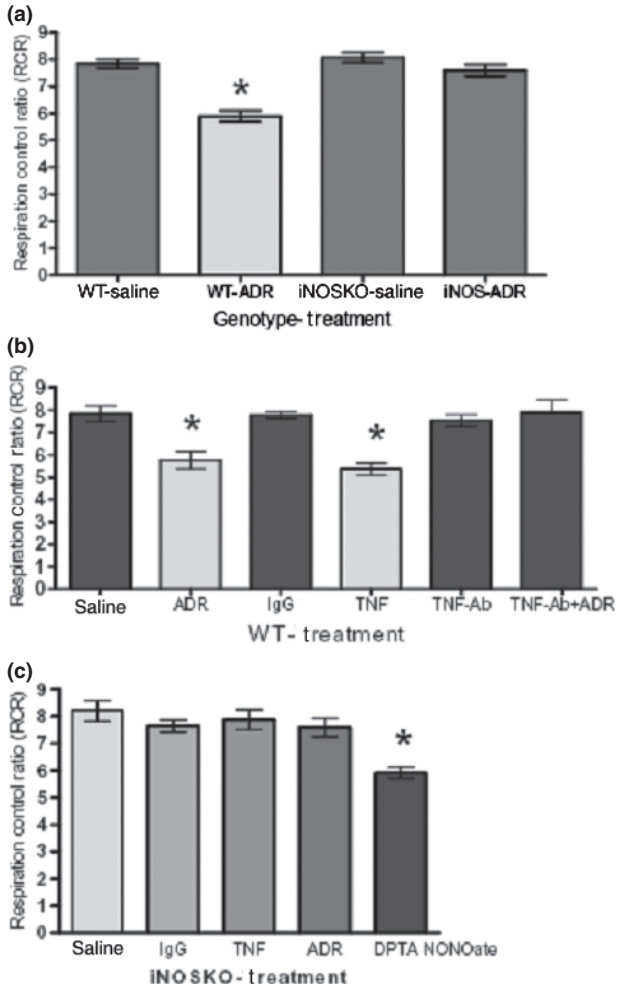


**Fig. 3** Adriamycin-mediated TNF-α elevation leads to mitochondrial dysfunction. Brain mitochondrial respiration was determined using pyruvate + malate as substrates. (a) Mitochondrial respiration complex I, pyruvate + malate as substrates, was significantly decreased in wild-type mice 3 h after treatment with ADR (20 mg/kg) compared with saline control (*$p < 0.05$), but not in iNOSKO mice. (b) The mitochondrial respiration decline was blocked by anti- TNF-α antibody. Exogenous TNF-α treatment for 3 h led to mitochondrial respiration complex I being significantly decreased compared with saline or IgG treatment groups in wild-type mice (*$p < 0.05$), but not in iNOSKO mice. (c) DPTA NONOate causes mitochondrial dysfunction in iNOSKO mice compared with the same genotype mice treated with saline, IgG, ADR or TNF-α (*$p < 0.05$). Data represent the mean ± SEM of three independent experiments.

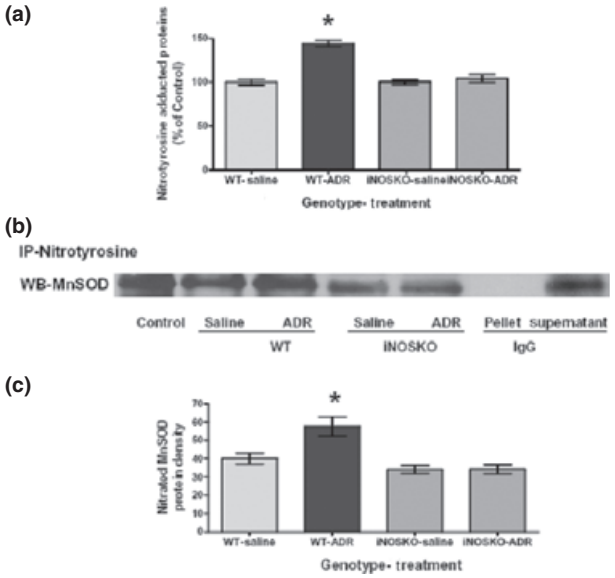


**Fig. 4** ADR-induced protein nitration. (a) Immunoreactive levels of nitrotyrosine-adducted proteins from isolated brain mitochondria of mice 3 h after treatment with ADR 20 mg/kg or saline control. The results show significantly increased protein nitration in ADR-treated wild-type mice, but no difference with controls in iNOSKO mice (*$p < 0.01$). (b, c) Immunoprecipitation coupled to western blot analyses showed the level of nitrotyrosine in MnSOD significantly increased 3 h after 20 mg/kg ADR treatment in wild-type mice, but not in iNOSKO mice similarly treated (*$p < 0.01$). Mitochondria, isolated from brain, treated with 20 μM peroxynitrite, was used as a positive control and IgG was used as negative control. (b) Representative western blots of the nitrated MnSOD. (c) The quantitative analysis of nitrated MnSOD protein density. Results are reported as mean ± SEM of nitrotyrosine-adducted MnSOD protein density from three independent experiments.

### MnSOD and CuZnSOD levels in brain tissues

There is a possibility that the decline in MnSOD activity in brains from ADR-treated wild-type mice 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 levels of either MinSOD or CuZnSOD in both wild-type and iNOSKO mouse brain (Figs 6a and b).

Further, to confirm the correct identification of MnSOD as a nitrated protein, we immunoprecipitated MnSOD protein from control and ADR-treated mitochondria and probed with anti-3-nitrotyrosine antibody. This showed a significant increase in nitration of MnSOD ($p < 0.04$) and no difference in expression (Figs 7a and b). In addition, the 2D map obtained from the supernatant fluid of the MnSOD immunoprecipitated sample (Fig. 8b) showed a missing spot corresponding to MnSOD (Figs 8a and b). These results confirm the nitration of MnSOD in ADR-treated mitochondria.

## Discussion

Adriamycin, a quinone-containing, anthracycline anti-cancer drug that generates ROS, is well documented to produce ROS in normal tissues (Singal *et al*. 2000; Kalyanaraman *et al*. 2002; Joshi *et al*. 2005). We have recently reported that

 

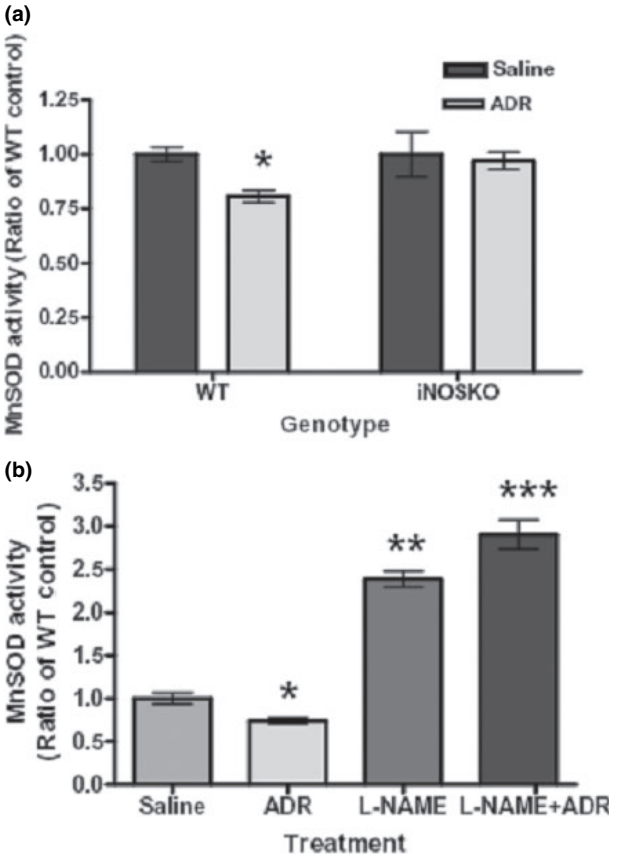


**Fig. 5** ADR-induced MnSOD inactivation. Brain homogenates were used for SOD activity assay. (a) MnSOD activity was significantly decreased in wild-type mice after treatment with ADR 20 mg/kg for 3 h (*$p < 0.05$). The MnSOD was not changed in iNOSKO mice similarly treated. (b) L-NAME, a NOS inhibitor, led to increased MnSOD activity compared with that from saline- or ADR-treated mice (*$p < 0.001$). L-NAME prevented MnSOD inactivation but increased enzymatic activity after treatment with ADR in wild-type mice (***$p < 0.001$). Data represent the mean + SEM of three independent experiments.

brain tissue damage is mediated, at least in part, by the systemic release of the cytokine, TNF-α, which accumulates in neurons. The increase of TNF-α in the brain tissue correlated with mitochondrial respiration dysfunction, cytochrome *c* release, caspase 3 activation and terminal transferase-mediated dUTP nick end labeling (TUNEL) staining, consistent with apoptotic cell death (Tangpong *et al*. 2006). The results in the present studies demonstrate that TNF-α production led to subsequent generation of reactive oxygen and nitrogen species (RONS) in the brain. Although ADR-induced circulating levels of TNF-α 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-α can act on the brain to regulate neural–immune interaction, as well as other brain functions. Peripheral TNF-α 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, an area outside the blood–brain barrier (Sternberg 1997). TNF-α can also be generated within the CNS, and all neuronal cell types are thought to contain TNF-receptors and be capable of synthesizing TNF-α in brain tissues (Sternberg 1997; Madrigal *et al*. 2002; Perry *et al*. 2002). Enhanced circulating TNF-α can initiate local TNF-α production via activation of glia cells, leading to production of RONS (Sternberg 1997; Szelenyi 2001). Our results, which indicate that iNOS mRNA is increased in brain tissues when animals are directly injected with TNF-α, lipopolysaccharide (LPS) or ADR, support the role of TNF-α 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-sulfur centers in the respiration chain and mitochondrial 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, as 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: (i) 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, 1999); (ii) the results from the present study, which indicate that ADR, TNF-α or LPS induced a diminution in brain mitochondrial respiration via complex I in wild-type mice; (iii) the NO generator, DPTA NONOate, in iNOSKO mice caused a decline in brain mitochondrial respiration via complex I; (iv) 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). These 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

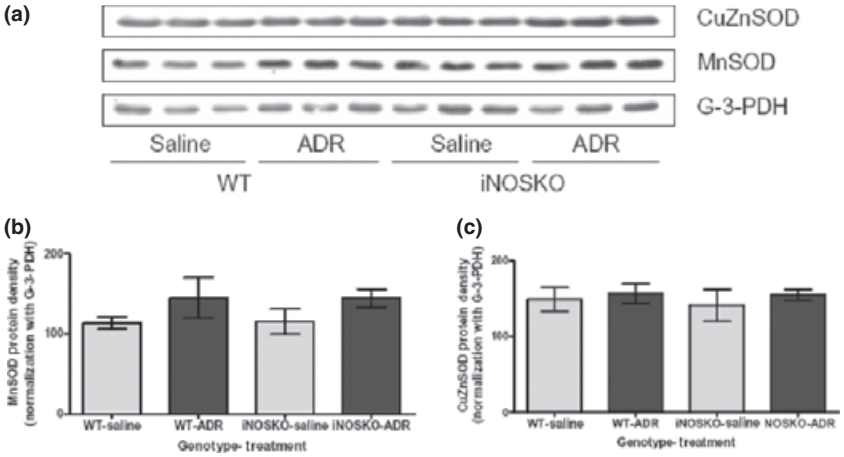


**Fig. 6** No change in MnSOD and CuZnSOD protein levels in ADR-treated mice. Representative western blots of brain homogenate proteins from mice 3 h after treatment with ADR 20 mg/kg or saline. Immunodetection with the polyclonal anti-MnSOD and anti-Cu/ZnSOD antibody exhibited no change in protein density 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. (a) representative western blot of MnSOD, CuZnSOD and G-3-PDH, the latter used for normalization of protein loading. (b) representative protein density of MnSOD, and representative protein density of CuZnSOD after normalization with G-3-PDH.

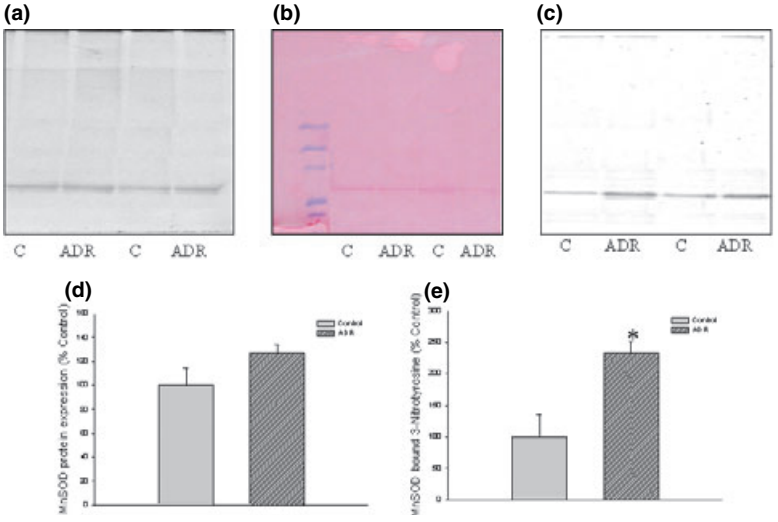


**Fig. 7** MnSOD protein nitration was quantified by immunoprecipitation using anti-MnSOD antibody, and probed with anti-3-nitrotyrosine antibody as described in Methods. (a) No significant difference was observed in the expression of proteins between control and ADR-treated brain mitochondria. (c) A significantly increased protein nitration was observed in ADR-treated mitochondrial sample compared with that of control ($p < 0.04$). (b) Ponceau-stained blot. (d) and (e) Histograms from (a) and (c), respectively.

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 (Lebovitz *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, 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-cata-

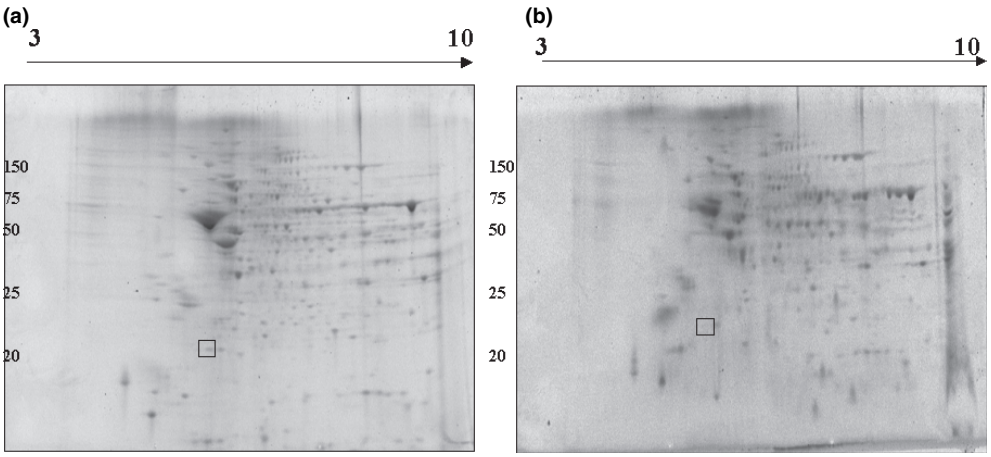


**Fig. 8** MnSOD protein was immunoprecipitated using anti-MnSOD antibody as described in Methods. Supernatant fluids were used to carry out 2D gel electrophoresis before (a) and after (b) immunoprecipitation, which showed a missing spot corresponding to MnSOD (indicated by box).

lyzed reactions) and hydroxyl radical-like oxidants such as those generated by redox active transition metal ions during Fenton and Haber-Weiss oxidation chemistry (Halliwell and Gutteridge 1999). 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 disease (AD) and multiple sclerosis (MS), 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; Calabrese *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-α production resembles the free radical-mediated level in an AD brain (Butterfield and Lauderback 2002).

In conclusion, ADR treatment i.p. led to an increased circulating level of TNF-α in wild-type mice and in iNOSKO mice. Circulating TNF-α, in turn, mediated iNOS induction to produce NO in brain tissues that subsequently led to a decline in mitochondrial respiration after ADR treatment, but not in iNOSKO mice. 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-α or removal of NO production could conceivably be an effective means for the prevention of ADR-induced CNS toxicity, which may underlie chemobrain. Studies to test this notion are in progress.

## Acknowledgements

This work was supported in part by NIH grants to DAB (AG-10836, AG-05119) and DSC (AG-05119, CA-80152 and CA-94853). JT is partially supported by The Ministry of University Affairs of Thailand under The Faculty Development Program. We thank Dr Timothy R. Miller, Graduate Center for Toxicology, University of Kentucky, Lexington, Kentucky, who provided the peroxynitrate used.

## References

Ahles T. A. and Saykin A. J. (2002) Breast cancer chemotherapy-related cognitive dysfunction. *Clin. Breast Cancer* **3**, 84–90.

Ahles T. A., Saykin A. J., Furstenberg C. T. *et al.* (2002) Neuropsychologic impact of standard-dose systemic chemotherapy in long-term survivors of breast cancer and lymphoma. *J. Clin. Oncol.* **20**, 485–493.

Akama K. T. and Van Eldik L. J. (2000) Beta-amyloid stimulation of inducible nitric-oxide synthase in astrocytes is interleukin-1beta- and tumor necrosis factor-alpha (TNFalpha)-dependent, and involves a TNFalpha receptor-associated factor- and NFkappaB-inducing kinase-dependent signaling mechanism. *J. Biol. Chem.* **275**, 7918–7924.

Bradford M. M. (1976) A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72**, 248–254.

Butterfield D. A. and Lauderback C. M. (2002) Lipid peroxidation and protein oxidation in Alzheimer's disease brain: Potential causes

and consequences involving amyloid b-peptide-associated free radical oxidative stress. *Free Radic. Biol. Med.* **32**, 1050–1060.

Butterfield D. A. and Stadtman E. R. (1997) Protein oxidation processes in aging brain. *Adv. Cell Aging Gerontol.* **2**, 161–191.

Calabrese V., Boyd-Kimball D., Scapagnini G. and Butterfield D. A. (2004) Nitric oxide and cellular stress response in brain aging and neurodegenerative disorders: the role of vitagenes. *In Vivo* **18**, 245–267.

Candolfi M., Jaita G., Zaldivar V., Zarate S., Pisera D. and Seilicovich A. (2004) Tumor necrosis factor-alpha-induced nitric oxide restrains the apoptotic response of anterior pituitary cells. *Neuroendocrinology* **80**, 83–91.

Castegna A., Thongboonkerd V., Klein J. B., Lynn B., Markesbery W. R. and Butterfield D. A. (2003) Proteomic identification of nitrated proteins in Alzheimer's disease brain. *J. Neurochem.* **85**, 1394–1401.

Chaiswing L., Cole M. P., St Clair D. K., Ittarat W., Szweda L. I. and Oberley T. D. (2004) Oxidative damage precedes nitrative damage in adriamycin-induced cardiac mitochondrial injury. *Toxicol. Pathol.* **32**, 536–547.

Choi J. S., Chung H. Y., Kang S. S., Jung M. J., Kim J. W., No J. K. and Jung H. A. (2002) The structure-activity relationship of flavonoids as scavengers of peroxynitrite. *Phytother. Res.* **16**, 232–235.

Cooper C. E. and Brown G. C. (1994) The interactions between nitric oxide and brain nerve terminals as studied by electron paramagnetic resonance. *FEBS Lett.* **356**, 295–298.

Cooper C. E., Davies N. A., Psychoulis M., Canevari L., Bates T. E., Dobbie M. S., Casley C. S. and Sharpe M. A. (2003) Nitric oxide and peroxynitrite cause irreversible increases in the K(m) for oxygen of mitochondrial cytochrome oxidase: *in vitro* and *in vivo* studies. *Biochem. Biophys. Acta* **17**, 27–34.

Espey M. G., Miranda K. M., Pluta R. M. and Wink D. A. (2000) Nitrosative capacity of macrophages is dependent on nitric-oxide synthase induction signals. *J. Biol. Chem.* **275**, 11 341–11 347.

Gonzalez-Zulueta M., Ensz L. M., Mukhina G., Lebovitz R. M., Zwacka R. M., Engelhardt J. F., Oberley L. W., Dawson V. L. and Dawson T. M. (1998) Manganese superoxide dismutase protects nNOS neurons from NMDA and nitric oxide-mediated neurotoxicity. *J. Neurosci.* **18**, 2040–2055.

Gutierrez E. G., Banks W. A. and Kastin A. J. (1993) Murine tumor necrosis factor alpha is transported from blood to brain in the mouse. *J. Neuroimmunol.* **47**, 169–176.

Gyorffy B., Serra V., Jurchott K., Abdul-Ghani R., Garber M., Stein U., Petersen I., Lage H., Dietel M. and Schafer R. (2005) Prediction of doxorubicin sensitivity in breast tumors based on gene expression profiles of drug-resistant cell lines correlates with patient survival. *Oncogene* **24**, 7542–7551.

Halliwell B. and Gutteridge J. M. C. (1999) Free Radicals In Biology and Medicine. Third Edition, Oxford University Press, UK.

Huang T. T., Carlson E. J., Raineri I., Gillespie A. M., Kozy H. and Epstein C. J. (1999) The use of transgenic and mutant mice to study oxygen free radical metabolism. *Ann. N Y Acad. Sci.* **893**, 95–112.

Joshi G., Sultana R., Tangpong J., Cole M. P., St. Clair D. K., Vore M., Estus S. and Butterfield D. A. (2005) Free radical mediated oxidative stress and toxic side effects in brain induced by the anti cancer drug adriamycin: insight into chemobrain. *Free Rad. Res.* **39**, 1147–1154.

Kalivendi S. V., Kotamraju S., Zhao H., Joseph J. and Kalyanaraman B. (2001) Doxorubicin-induced apoptosis is associated with increased transcription of endothelial nitric-oxide synthase. Effect of antiapoptotic antioxidants and calcium. *J. Biol. Chem.* **276**, 47 266–47 276.

Kalyanaraman B., Joseph J., Kalivendi S., Wang S., Konorev E. and Kotamraju S. (2002) Doxorubicin-induced apoptosis: implications in cardiotoxicity. *Mol. Cell Biochem.* **234–235**, 119–124.

Kaufmann P. A., Rimoldi O., Gnecchi-Ruscone T., Bonser R. S., Luscher T. F. and Camici P. G. (2004) Systemic inhibition of nitric oxide synthase unmasks neural constraint of maximal myocardial blood flow in humans. *Circulation* **110**, 1431–1436.

Keller J. N., Kindy M. S., Holtsberg F. W., St Clair D. K., Yen H. C., Germeyer A., Steiner S. M., Bruce-Keller A. J., Hutchins J. B. and Mattson M. P. (1998) Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxynitrite production, lipid peroxidation, and mitochondrial dysfunction. *J. Neurosci.* **18**, 687–697.

Kim J. I., Ju W. K., Choi J. H., Choi E., Carp R. I., Wisniewski H. M. and Kim Y. S. (1999) Expression of cytokine genes and increased nuclear factor-kappa B activity in the brains of scrapie-infected mice. *Brain Res. Mol. Brain Res.* **73**, 17–27.

Lauderback C. M., Hackett J. M., Keller J. N., Varadarajan S., Szweda L., Kindy M., Markesbery W. R. and Butterfield D. A. (2001) Vulnerability of synaptosomes from ApoE knock-out mice to structural and oxidative modifications induced by Aβ (1–40): implications for Alzheimer's disease. *Biochemistry* **40**, 2548–2554.

Lebovitz R. M., Zhang H., Vogel H., Cartwright J., Jr, Dionne L., Lu N., Huang S. and Matzuk M. M. (1996) Neurodegeneration, myocardial injury, and perinatal death in mitochondrial superoxide dismutase-deficient mice. *Proc. Natl Acad. Sci. USA* **93**, 9782–9787.

Levine R. L., Williams J. A., Stadtman E. R. and Shacter E. (1994) Carbonyl assays for determination of oxidatively modified proteins. *Meth. Enzymol.* **233**, 346–357.

Li Y., Huang T. T., Carlson E. J. *et al.* (1995) Dilated cardiomyopathy and neonatal lethality in mutant mice lacking manganese superoxide dismutase. *Nat. Genet.* **11**, 376–381.

Li Y., Copin J. C., Reola L. F., Calagui B., Gobbel G. T., Chen S. F., Sato S., Epstein C. J. and Chan P. H. (1998) Reduced mitochondrial manganese-superoxide dismutase activity exacerbates glutamate toxicity in cultured mouse cortical neurons. *Brain Res.* **814**, 164–170.

Licinio J. (1997) Central nervous system cytokines and their relevance for neurotoxicity and apoptosis. *J. Neural Transm.* **49(Suppl.)**, 169–175.

Licinio J. and Wong M. L. (1997) Pathways and mechanisms for cytokine signaling of the central nervous system. *J. Clin. Invest.* **100**, 2941–2947.

MacMillan-Crow L. A., Crow J. P., Kerby J. D., Beckman J. S. and Thompson J. A. (1996) Nitration and inactivation of manganese superoxide dismutase in chronic rejection of human renal allografts. *Proc. Natl Acad. Sci. USA* **93**, 11 853–11 858.

Madrigal J. L., Hurtado O., Moro M. A., Lizasoain I., Lorenzo P., Castrillo A., Bosca L. and Leza J. C. (2002) The increase in TNF-alpha levels is implicated in NF-kappaB activation and inducible nitric oxide synthase expression in brain cortex after immobilization stress. *Neuropsychopharmacology* **26**, 155–163.

Mandi Y., Ocsovszki I., Szabo D., Nagy Z., Nelson J. and Molnar J. (1998) Nitric oxide production and MDR expression by human brain endothelial cells. *Anticancer Res.* **18**, 3049–3052.

Marcus J. S., Karackattu S. L., Fleegal M. A. and Sumners C. (2003) Cytokine-stimulated inducible nitric oxide synthase expression in astroglia: role of Erk mitogen-activated protein kinase and NF-kappaB. *Glia* **41**, 152–160.

Mattiazzi M., D'Aurelio M., Gajewski C. D., Martushova K., Kiaei M., Beal M. F. and Manfredi G. (2002) Mutated human SOD1 causes

 

dysfunction of oxidative phosphorylation in mitochondria of transgenic mice. *J. Biol. Chem.* **277**, 29 626–29 633.

Meredith M. J. and Reed D. J. (1983) Depletion *in vitro* of mitochondrial glutathione in rat hepatocytes and enhancement of lipid peroxidation by adriamycin and 1,3-bis (2-chloroethyl)-1-nitrosourea (BCNU). *Biochem. Pharmacol.* **32**, 1383–1388.

Moncada S. and Higgs A. (1993) The L-arginine-nitric oxide pathway. *N. Engl. J. Med.* **329**, 2002–2012.

Osburg B., Peiser C., Domling D., Schomburg L., Ko Y. T., Voigt K. and Bickel U. (2002) Effect of endotoxin on expression of TNF receptors and transport of TNF-alpha at the blood–brain barrier of the rat. *Am. J. Physiol. Endocrinol. Metab.* **283**, E899–E908.

Ozen S., Usta Y., Sahin-Erdemli I. *et al.* (2001) Association of nitric oxide production and apoptosis in a model of experimental nephropathy. *Nephrol. Dial. Transplant.* **16**, 32–38.

Perry S. W., Dewhurst S., Bellizzi M. J. and Gelbard H. A. (2002) Tumor necrosis factor-alpha in normal and diseased brain: Conflicting effects via intraneuronal receptor crosstalk? *J. Neurovirol.* **8**, 611–624.

Radi R. (2004) Nitric oxide, oxidants, and protein tyrosine nitration. *Proc. Natl Acad. Sci. USA* **101**, 4003–4008.

Rubbo H., Trostchansky A., Botti H. and Batthyany C. (2002) Interactions of nitric oxide and peroxynitrite with low-density lipoprotein. *Biol. Chem.* **383**, 547–552.

Saez J. C., Kessler J. A., Bennett M. V. and Spray D. C. (1987) Superoxide dismutase protects cultured neurons against death by starvation. *Proc. Natl Acad. Sci. USA* **84**, 3056–3059.

Santoro M. G., Rossi A. and Amici C. (2003) NF-kappaB and virus infection: who controls whom. *EMBO J.* **22**, 2552–2560.

Schagen S. B., van Dam F. S., Muller J. J., Boogerd W., Lindeboom J. and Bruning P. F. (1999) Cognitive deficits after postoperative adjuvant chemotherapy for breast carcinoma. *Cancer* **85**, 640–650.

Schreck R., Albermann K. and Baeuerle P. A. (1992) Nuclear factor kappa B: an oxidative stress-responsive transcription factor of eukaryotic cells (a review). *Free Radic. Res. Commun.* **17**, 221–237.

Singal P. K., Li T., Kumar D., Danelisen I. and Iliskovic N. (2000) Adriamycin-induced heart failure: mechanism and modulation. *Mol. Cell Biochem.* **207**, 77–86.

Smith M. A., Richey Harris P. L., Sayre L. M., Beckman J. S. and Perry G. (1997) Widespread peroxynitrite-mediated damage in Alzheimer's disease. *J. Neurosci.* **17**, 2653–2657.

Snyder A. R. and Morgan W. F. (2005) Differential induction and activation of NF-kappaB transcription complexes in radiation-induced chromosomally unstable cell lines. *Environ. Mol. Mutagen.* **45**, 177–187.

Spitz D. R. and Oberley L. W. (1989) An assay for superoxide dismutase activity in mammalian tissue homogenates. *Anal. Biochem.* **179**, 8–18.

Ste-Marie L., Hazell A. S., Bemeur C., Butterworth R. and Montgomery J. (2001) Immunohistochemical detection of inducible nitric oxide synthase, nitrotyrosine and manganese superoxide dismutase following hyperglycemic focal cerebral ischemia. *Brain Res.* **918**, 10–19.

Sternberg E. M. (1997) Neural–immune interactions in health and disease. *J. Clin. Invest.* **100**, 2641–2647.

Sultana R. and Butterfield D. A. (2004) Oxidatively modified GST and MRP1 in Alzheimer's disease brain: implications for accumulation of reactive lipid peroxidation products. *Neurochem. Res.* **29**, 2215–2220.

Sultana R., Poon H. F., Cai J., Pierce W. M., Merchant M., Klein J. B., Markesbery W. R. and Butterfield D. A. (2006) Identification of nitrated hippocampal proteins in Alzheimer's disease brain using a redox proteomics approach. *Neurobiol. Dis.* **22**, 76–87.

Szelenyi J. (2001) Cytokines and the central nervous system. *Brain Res. Bull.* **54**, 329–338.

Tangpong J., Cole M. P., Sultana R., Joshi G., Estus S., Vore M., St Clair W., Ratanachaiyavong S., St Clair D. K. and Butterfield D. A. (2006) Adriamycin-induced, TNF-alpha-mediated central nervous system toxicity. *Neurobiol. Dis*. **23**, 127–139.

Ujhazy P., Zaleskis G., Mihich E., Ehrke M. J. and Berleth E. S. (2003) Doxorubicin induces specific immune functions and cytokine expression in peritoneal cells. *Cancer Immunol. Immunother.* **52**, 463–472.

Usta Y., Ismailoglu U. B., Bakkaloglu A., Orhan D., Besbas N., Sahin-Erdemli I. and Ozen S. (2004) Effects of pentoxifylline in adriamycin-induced renal disease in rats. *Pediatr. Nephrol.* **19**, 840–843.

Vasquez-Vivar J., Hogg N., Martasek P., Karoui H., Tordo P., Pritchard K. A., Jr, and Kalyanaraman B. (1999) Effect of redox-active drugs on superoxide generation from nitric oxide synthases: biological and toxicological implications. *Free Radic. Res.* **31**, 607–617.

Weinstein D. M., Mihm M. J. and Bauer J. A. (2000) Cardiac peroxynitrite formation and left ventricular dysfunction following doxorubicin treatment in mice. *J. Pharmacol. Exp. Ther.* **294**, 396–401.

Wilkerson M. K., Lesniewski L. A., Golding E. M., Bryan R. M., Jr, Amin A., Wilson E. and Delp M. D. (2005) Simulated microgravity enhances cerebral artery vasoconstriction and vascular resistance through endothelial nitric oxide mechanism. *Am. J. Physiol. Heart Circ. Physiol.* **288**, H1652–H1661.

Yamakura F., Taka H., Fujimura T. and Murayama K. (1998) Inactivation of human manganese-superoxide dismutase by peroxynitrite is caused by exclusive nitration of tyrosine 34 to 3-nitrotyrosine. *J. Biol. Chem.* **273**, 14 085–14 089.

Yen H. C., Oberley T. D., Vichitbandha S., Ho Y. S. and St Clair D. K. (1996) The protective role of manganese superoxide dismutase against adriamycin-induced acute cardiac toxicity in transgenic mice. *J. Clin. Invest.* **98**, 1253–1260.

Yen H. C., Oberley T. D., Gairola C. G., Szweda L. I. and St Clair D. K. (1999) Manganese superoxide dismutase protects mitochondrial complex I against adriamycin-induced cardiomyopathy in transgenic mice. *Arch. Biochem. Biophys.* **362**, 59–66.

Zeng C., Lee J. T., Chen H., Chen S., Hsu C. Y. and Xu J. (2005) Amyloid-beta peptide enhances tumor necrosis factor-alpha-induced iNOS through neutral sphingomyelinase/ceramide pathway in oligodendrocytes. *J. Neurochem.* **94**, 703–712.

 