



Original Contribution

2-Mercaptoethane sulfonate prevents doxorubicin-induced plasma protein oxidation and TNF- α release: Implications for the reactive oxygen species-mediated mechanisms of chemobrain

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ABSTRACT

Doxorubicin (DOX), an anthracycline used to treat a variety of cancers, is known to generate intracellular reactive oxygen species. Moreover, many patients who have undergone chemotherapy complain of cognitive dysfunction often lasting years after cessation of the chemotherapy. Previously, we reported that intraperitoneal administration of DOX led to elevated TNF- α and oxidative stress in the plasma and brain of mice. However, the mechanisms involved in nontargeted tissue damage remain unknown. In this study, we measured plasma oxidative stress and cytokine levels in patients treated with DOX. We observed increased plasma protein carbonylation and elevation of TNF- α 6 h after DOX administration in the context of multiagent chemotherapy regimens. Importantly, patients not treated coincidentally with 2-mercaptoethane sulfonate (MESNA) showed statistically significantly increased plasma protein-bound 4-hydroxynonenal, whereas those who had been coincidentally treated with MESNA as part of their multiagent chemotherapy regimen did not, suggesting that concomitant administration of the antioxidant MESNA with DOX prevents intravascular oxidative stress. We demonstrate in a murine model that MESNA suppressed DOX-induced increased plasma oxidative stress indexed by protein carbonyls and protein-bound HNE, and also suppressed DOX-induced increased peripheral TNF- α levels. A direct interaction between DOX and MESNA was demonstrated by MESNA suppression of DOX-induced DCF fluorescence. Using redox proteomics, we identified apolipoprotein A1 (APOA1) in both patients and mice after DOX administration as having increased specific carbonyl levels. Macrophage stimulation studies showed that oxidized APOA1 increased TNF- α levels and augmented TNF- α release by lipopolysaccharide, effects that were prevented by MESNA. This study is the first to demonstrate that DOX oxidizes plasma APOA1, that oxidized APOA1 enhances macrophage TNF- α release and thus could contribute to potential subsequent TNF- α -mediated toxicity, and that MESNA interacts with DOX to block this mechanism and suggests that MESNA could reduce systemic side effects of DOX.

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Doxorubicin (DOX) is an antineoplastic agent commonly used in multiagent chemotherapy regimens to treat solid tumors and leukemias. The mechanism of DOX action is proposed to be threefold, although the specific mechanism by which DOX is lethal to cancer cells remains elusive. DOX has been shown to intercalate into DNA in cells and halt cellular replication [1–3], inhibit topoisomerase II [4], and increase production of reactive oxygen species (ROS) [5,6].

The structure of DOX contains a quinone moiety, which is capable of undergoing one-electron redox reactions by redox cycling. In this process, DOX quinone is converted to DOX semiquinone by accepting an electron from an oxidant; in the presence of oxygen, this semiquinone is converted back to its native DOX quinone, producing superoxide (O_2^-) as a by-product [7,8]. O_2^- can damage biological components directly, as well as being converted to more reactive ROS/RNS (reactive nitrogen species). DOX-induced mitochondrial impairment has been well documented, particularly in heart, an organ rich in this organelle [9–11]. In fact, DOX-induced cardiotoxicity limits the dosage and frequency with which DOX can be administered.

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Consistent with other studies, our previous report showed that administration of DOX to rodents results in oxidative stress in blood plasma [12–18]. Administration of DOX also leads to increased plasma iron content, possibly exacerbating oxidative stress [19]. In addition to heart and plasma, oxidative stress as a result of DOX has also been observed in testes [20], kidneys [21,22], liver [21], and even brain [23–25], despite the inability of DOX or its metabolites to cross the blood–brain barrier (BBB). DOX increases levels of the proinflammatory cytokine tumor necrosis factor- α (TNF- α) in the periphery [23], which can migrate into tissues (including brain) and stimulate local inflammation and oxidative stress [23,24], eventually leading to cellular apoptosis [24] and, possibly, the appearance of side effects. Therefore, the actions of DOX in the blood are capable of causing adverse consequences for some organs apart from cellular penetration by the drug and redox cycling. Indeed, we have hypothesized that the peripheral elevation of TNF- α after DOX is the source of elevation of this cytokine in brain that leads to mitochondrial dysfunction, oxidative stress, and neuronal death, processes that may contribute to the cognitive dysfunction observed in a significant fraction of patients undergoing chemotherapy, i.e., chemobrain [23–25].

Because DOX-mediated extracellular protein oxidation may lead to toxicity independent of its intracellular antitumor activity, a method to prevent DOX-mediated extracellular oxidation may prevent toxicity without jeopardizing therapeutic goals. 2-Mercaptoethane sulfonate (MESNA) is a uroprotective sulfhydryl-containing antioxidant that is not taken up by cells. MESNA is frequently administered with ifosfamide to protect the bladder from acrolein, a toxic breakdown metabolite of ifosfamide, but also, coincidentally, a product of oxidative damage to lipids. MESNA localizes in the plasma before kidney uptake, allowing for the possibility of this molecule to protect plasma proteins from oxidative damage resulting from DOX administration.

Here, we report findings from analyses of oxidative stress and inflammation in plasma from patients treated with DOX. Unexpectedly, we observed a trend in the clinical data, which showed that DOX-treated patients who concomitantly and coincidentally received MESNA, for uroprotection in multiagent chemotherapy regimens, were partially protected from oxidative damage to plasma proteins. This serendipitous discovery led to testing the hypothesis that concomitant administration of MESNA with DOX would suppress inflammatory markers and oxidative stress in plasma. Furthermore, using redox proteomics and macrophage-stimulation experiments, we identified a possible mechanism for oxidative stress-induced elevation of TNF- α in the periphery as a result of DOX administration.

Materials and methods

Patient consent and clinical study

Human investigations were performed after approval by the University of Kentucky Institutional Review Board and Markey Cancer Center Protocol Review Committee and in accordance with an assurance filed with and approved by the Department of Health and Human Services. Informed consent was obtained from parents at the University of Kentucky Medical Center and assent was obtained when appropriate. All enrolled patients were receiving DOX as a part of their prescribed multiagent chemotherapy regimen and had vascular access devices for blood sample acquisition. Blood samples were obtained immediately before and 6 h after DOX administration.

Animals

Male B6C3 mice (2–3 months of age), approximately 30 g in size, housed in the University of Kentucky Central Animal Facility under 12-h light/dark conditions and fed standard Purina rodent laboratory chow ad libitum, were used. The animal protocols were approved by the University of Kentucky Animal Care and Use Committee. DOX (25 mg/kg) was administered to two groups of mice, and the other two groups

received saline injections. The DOX dose used was similar to the maximum single clinical therapeutic dose (60–75 mg/m² [26,27]) using the calculation according to the conversion factor described by Freireich et al. [28]. MESNA (150 mg/kg) was administered 15 min before DOX, as well as 3 and 6 h after DOX. Animals were anesthetized with sodium pentobarbital and blood was obtained via left ventricle puncture. Plasma for oxidative stress measurements and proteomics was prepared as previously described [12]. Serum for TNF measures was prepared by allowing blood to clot for 1 h on ice followed by centrifugation at 6000 rpm for 10 min. Aliquots of the serum were stored at –80 °C until needed.

Oxidative stress assays

The slot-blot method was used to determine levels of protein-bound 4-hydroxynonenal (HNE) and protein carbonyls in plasma [12]. For protein carbonyl determination, samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH); for protein-bound HNE assay, samples were solubilized in Laemmli buffer. Briefly, 500 ng of protein was loaded onto a nitrocellulose membrane under vacuum. Membranes were blocked overnight in 3% bovine serum albumin (BSA) and then incubated in primary antibody for 2 h (anti-dinitrophenylhydrazine primary or anti-protein-bound HNE, respectively). Membranes were washed three times in buffer and then incubated with secondary antibody (goat anti-rabbit secondary linked to alkaline phosphatase) for 1 h. Membranes were developed with Sigma Fast tablets, dried, and scanned for analysis. Image analysis was performed using Scion Image.

2,7-Dichlorofluorescein assay

The 2,7-dichlorofluorescein (DCF) assay was performed as previously described with minor alterations [29]. Dichlorofluorescein diacetate (DCFH-DA) was hydrolyzed with 2 N HCl and 2 N NaOH before incubation to yield DCFH. ROS can oxidize DCFH to the highly fluorescent DCF. DOX, MESNA, and PBS were incubated with DCFH for 2 h at 37 °C and fluorescence was measured at λ_{ex} 495 nm, λ_{em} 530 nm. To show that DOX and MESNA did not interfere with signal intensity, each was incubated in the absence of DCFH and analyzed simultaneously.

Cytokine assays

The TNF- α level of serum was determined using the Quantikine mouse TNF- α immunoassay kit (R&D Systems, Minneapolis, MN, USA) according to the supplier's instructions.

Redox proteomics

Sample preparation

Mice plasma protein (150 μ g) was incubated with 4 volumes of DNPH (20 mM in 2 N HCl) or 2 N HCl (for 2-D gel protein mapping and mass spectrometry analysis) at room temperature for 30 min and then mixed with ice-cold trichloroacetic acid (final concentration 15%) and incubated on ice for 10 min. Precipitates were centrifuged at 14,000 g at 4 °C for 2 min. The pellets were washed with 500 μ l of ethyl acetate/ethanol (1/1, v/v) three times. The final pellet was dissolved in 200 μ l of rehydration buffer containing 7 M urea, 2 M thiourea, 2% Chaps, 0.8% (v/v) ampholyte, pH 3–10, 1% zwittergent, 50 mM dithiothreitol (DTT), and a trace amount of bromophenol blue. Samples were then sonicated on ice for 20 s three times.

Two-dimensional gel electrophoresis

Immobilized pH gradient strips were actively rehydrated with 200 μ l of samples at 50 V and 20 °C for 16 h. Isoelectric focusing (IEF) was performed at 20 °C in a Protean IEF cell (Bio-Rad) as follows: 800 V for 2-h linear gradient, 1200 V for 4-h slow gradient, 8000 V for 8-h linear

gradient, and 8000 V for 10-h rapid gradient. Before the second-dimension separation, the strips were equilibrated in 0.375 M Tris–HCl (pH 8.8) containing 6 M urea, 2% sodium dodecyl sulfate, 20% (v/v) glycerol, and 0.5% DTT for 10 min, followed by a reequilibration in a similar buffer containing 4.5% iodoacetamide in place of DTT for 10 min. Strips were placed on Criterion precast gels and electrophoresed at 200 V for 65 min. Gels of HCl-treated samples were fixed in a solution containing 10% (v/v) methanol, 7% (v/v) acetic acid for 30 min and stained in SYPRO ruby gel stain (50 ml/gel) with agitation at room temperature overnight. Images of the stained gels were obtained using a fluorescence imager, Storm 860 (λ_{ex} 470 nm, λ_{em} 618 nm; Molecular Dynamics, Sunnyvale, CA, USA). Gels of DNPH-treated samples were transferred to a nitrocellulose membrane at 90 mA/gel for 2 h. Blots were blocked for 2 h in 3% BSA and then incubated with primary anti-DNPH antibody for 2 h. Membranes were washed in Wash Blot, incubated with secondary antibody for 1 h, and washed again to remove secondary antibody. Blots were developed with SigmaFast tablets.

Image analysis

PDQuest software (Bio-Rad) was used to analyze the gels and the blots and to compare protein and specific carbonyl contents in plasma samples of DOX-, saline-, and MESNA-treated mice. Densitometric intensity of SYPRO ruby-stained gels corresponded to protein levels on 2-D gels; specific carbonyl content was assessed by the spot intensity on 2-D Western blots divided by the corresponding protein level as assessed by 2-D gel quantitation. Spot intensities were normalized relative to total density of spots in the corresponding gel or blot to correct for slight differences in staining and/or loading, as previously described [25].

In-gel digestion

Samples were prepared according to the method described by Thongboonkerd et al. [30]. Briefly, the gel piece containing the protein of interest was cut out from the gel with a clean razor blade and transferred into a 1.5-ml microcentrifuge tube. The gel piece was incubated with 30 μl of 0.1 M NH_4HCO_3 at room temperature for 15 min, then for another 15 min after the addition of 30 μl of acetonitrile, and was air-dried for 30 min after the removal of the liquid. The gel piece was rehydrated with 20 μl of 20 mM DTT in 0.1 M NH_4HCO_3 at 56 °C for 45 min. Next, the gel piece was incubated with 20 μl of 55 mM iodoacetamide in 0.1 M NH_4HCO_3 in the dark at room temperature for 30 min. The gel plug was incubated with 30 μl of 50 mM NH_4HCO_3 at room temperature for 15 min, then for another 15 min after the addition of 30 μl of acetonitrile, and was air-dried for 30 min after the removal of the liquid. The gel piece was rehydrated with the addition of a minimal volume of 20 ng/ μl modified trypsin in 50 mM NH_4HCO_3 and was incubated with shaking at 37 °C overnight (18 h).

Mass spectrometry

Tryptic peptides were analyzed with an automated nanospray Nanomate Orbitrap XL tandem mass spectrometry (MS/MS) platform. The Orbitrap MS was operated in a data-dependent mode whereby the eight most intense parent ions measured in the FT at 60,000 resolution were selected for ion trap fragmentation under the following conditions: injection time 50 ms, 35% collision energy, MS/MS spectra measured in the FT at 7500 resolution, and dynamic exclusion set for 120 s. Each sample was acquired for a total of ~2.5 min. MS/MS spectra were searched against the ipi_Mouse Database using SEQUEST with the following criteria: Xcorr > 1.5, 2.0, 2.5, 3.0 for +1, +2, +3, and +4 charge states, respectively, and *P* value (protein and peptide) < 0.01. IPI accession numbers were

cross-correlated with SwissProt accession numbers for final protein identification.

Macrophage stimulation

The mouse macrophage cell line J774A.1 (American Type Culture Collection) was cultured in Dulbecco's modified Eagle's medium supplemented with 10% (v/v) fetal bovine serum, streptomycin (100 $\mu\text{g}/\text{ml}$), and penicillin (100 U/ml). All cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2 . J774A.1 macrophage cells were plated at a density of 5×10^5 cells/well in 48-well plates. Oxidized apolipoprotein A1 (oApoA1) was prepared by subjecting ApoA1 to $\text{Fe}^{2+}/\text{H}_2\text{O}_2$, a potent source of hydroxyl radicals. The reaction was terminated by the addition of catalase. Lipopolysaccharide (LPS; 1 $\mu\text{g}/\text{ml}$), oApoA1, or nonoxidized ApoA1 (10 $\mu\text{g}/\text{ml}$; 20 $\mu\text{g}/\text{ml}$) in complete medium was added and the cells were incubated for 4 h. Cells were then challenged with LPS and the supernatants were collected at 4 h. The supernatants were kept frozen until use. The levels of mouse TNF- α were determined with a specific ELISA kit for mouse TNF- α (R&D Systems).

Statistical analysis

For patient data, a paired *t* test was used to observe differences between plasma samples from the same patients before and after DOX. Proteomics data were analyzed using the Mann–Whitney *U* test, whereas multiple group analyses were performed using one-way ANOVA and Student Newman–Keuls test. *P* values less than 0.05 were considered significant. GraphPad Prism software was used to calculate statistical significance.

Results

DOX administration to patients leads to oxidative damage and inflammation in the plasma

We previously have shown that DOX causes increases in oxidative damage to plasma proteins in mice, as well as increases in the levels of the proinflammatory cytokine TNF- α [12,23]. Therefore, we sought to determine the effects of DOX administration on plasma oxidative stress and inflammation in patients undergoing chemotherapy. We collected and analyzed plasma samples from 12 patients before and 6 h after cessation of DOX infusion. We observed a significant increase in TNF- α in plasma after DOX administration compared to before DOX ($P < 0.05$; Fig. 1a). No significant differences were observed for the other cytokines measured (Fig. 1a). For analyses of oxidative stress, samples were analyzed for protein carbonyls, a marker of oxidative damage to proteins, as well as protein-bound HNE. This α,β -alkenal is a reactive product of oxidative damage to arachidonic acid, a vital component of cell membranes. Results of oxidative stress analyses revealed a significant increase in protein carbonyls in plasma after DOX compared to before DOX in these patients ($P < 0.05$; Fig. 1b). Interestingly, no significant differences were observed in plasma protein-bound HNE levels (Fig. 1c); however, analysis of concomitant medications revealed that patients who were also administered MESNA either during DOX infusion or after infusion ended but before the “Post DOX” sample was collected, showed a trend toward decreased plasma HNE levels in the Post sample compared to before DOX ($P = 0.087$; Fig. 1c). Patients not receiving DOX and MESNA concomitantly showed a significant increase in plasma protein-bound HNE levels after DOX ($P < 0.05$) (Fig. 1c).

MESNA is capable of scavenging DOX-derived free radicals in vitro

DOX is often administered in multiagent chemotherapy regimens that include the alkylating agent ifosfamide. MESNA is administered

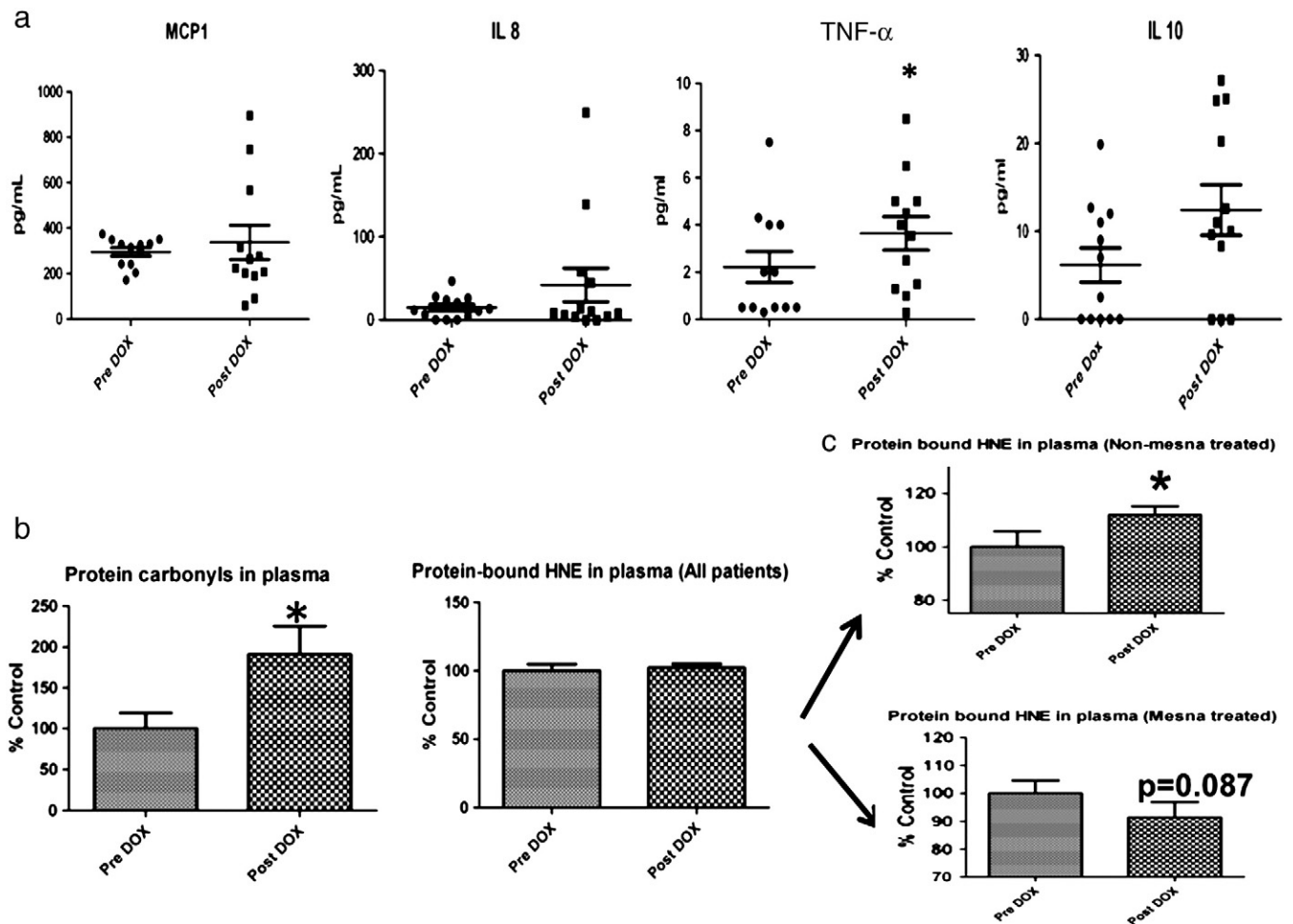


Fig. 1. (a) Cytokine analysis from patients treated with DOX. Samples of plasma were taken immediately before and 6 h after cessation of DOX infusion. No changes in the levels of MCP-1, IL-8, or IL-10 were observed as a result of DOX. Patients exhibited increased levels of TNF- α after DOX compared to before ($*P < 0.05$, $n = 12$ paired samples). (b) Oxidative stress analysis of plasma samples from patients treated with DOX. A significantly increased plasma protein carbonyl level was observed after DOX administration compared to before DOX ($*P < 0.05$, $n = 12$ paired samples). (c) Protein-bound HNE levels in plasma from patients treated with DOX. Overall, no changes were observed in global levels of protein-bound HNE as a result of DOX. However, we noticed a trend in the data that showed that patients also given MESNA either during DOX administration or after cessation of DOX administration but before the "Post DOX" sample was collected were protected from HNE damage to proteins ($P = 0.087$, $n = 5$). Patients who did not receive MESNA concomitantly had significantly increased protein-bound HNE levels after DOX compared to plasma levels before DOX ($*P < 0.05$, $n = 7$).

in ifosfamide-containing regimens because of its ability to detoxify the ifosfamide metabolite acrolein and prevent hemorrhagic cystitis. Because of both the clinical observations and the ability of MESNA to scavenge products of lipid peroxidation, we tested the hypothesis that MESNA is capable of scavenging reactive oxygen/nitrogen species as well. We observed significantly higher DCF fluorescence when DOX (250 nM) was added relative to control, indicative of increased production of ROS/RNS. Coadministration of MESNA suppressed this effect, relative to DOX alone (Fig. 2a), demonstrating a direct interaction between MESNA and DOX that heretofore was not suspected. Neither DOX nor MESNA interfered with or quenched the fluorescence intensity of the assay.

Coadministration of DOX and MESNA to mice suppresses oxidative damage and inflammation in the periphery

Because the data obtained from human subjects could be modulated by coincidental administration of additional agents, we sought to directly test the hypothesis that the antioxidant molecule MESNA can prevent oxidative stress and inflammation in the plasma caused by DOX. Free radical damage to proteins typically results in altered functionality. As shown in Fig. 2b, DOX administration to mice increased protein carbonyls and protein-bound HNE in plasma ($P < 0.05$). MESNA given

concomitantly with DOX significantly suppressed both markers of oxidative stress relative to DOX alone ($P < 0.05$; Fig. 2b).

DOX has also been implicated in the increase in TNF- α . Although DOX is incapable of crossing the BBB, TNF- α receptors allow this signaling molecule to infiltrate organs and initiate local inflammation and oxidative stress [23,31,32]. For instance, we previously showed that DOX administration led to an increase in peripheral levels of TNF- α , which migrates across the BBB and causes oxidative stress and inflammation in the brain parenchyma [23]. Therefore, TNF- α levels in the periphery can have downstream consequences for other organs, regardless of DOX tissue distribution. In our study, peripheral TNF- α levels were increased with DOX and suppressed with coadministration with MESNA ($P < 0.05$; Fig. 2b). MESNA alone caused no significant changes in TNF- α levels relative to treatment with saline (Fig. 2b).

Identification of APOA1 in plasma as a target of DOX-mediated oxidation that is prevented by coadministration of MESNA

Oxidative damage changes the 3-D conformational structure of proteins, generally hindering their activity. Papers from our laboratory have shown that increased specific carbonyl levels of proteins identified by redox proteomics result in their decreased activity in the brains of subjects with Alzheimer disease [33,34]. Therefore, we sought to investigate the plasma of mice treated ip with DOX for potentially

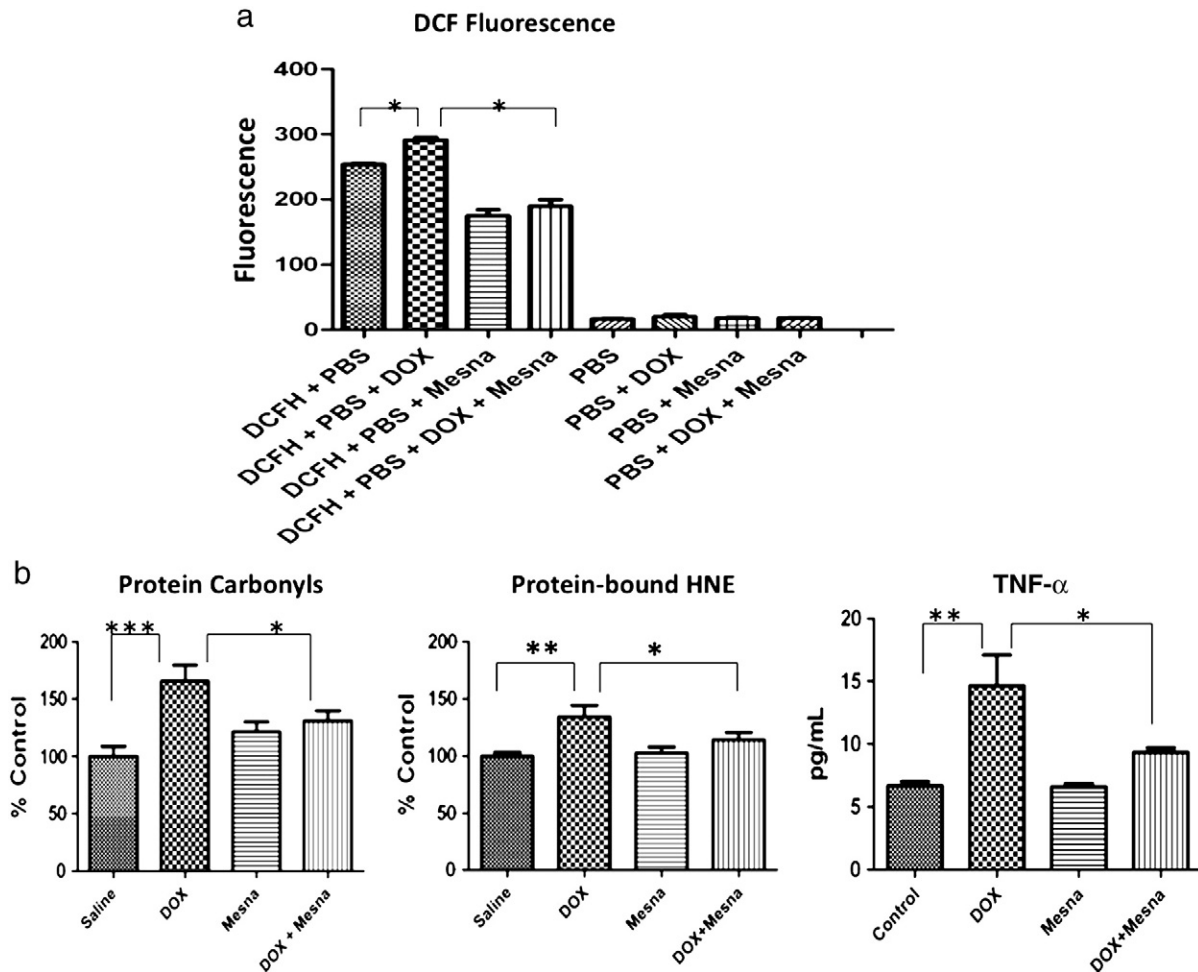


Fig. 2. (a) DCF fluorescence as a result of DOX and MESNA. DCF fluorescence measures free radical-generating capacity. Incubation with DOX significantly increased DCF fluorescence relative to control, with MESNA ameliorating this effect. Lower DCF fluorescence was measured when MESNA was incubated relative to control, possibly due to MESNA scavenging basal levels of free radicals. However, no fluorescence interference by either DOX or MESNA alone was observed ($*P < 0.0001$, $n = 3/\text{group}$). (b) Plasma oxidative stress and TNF levels in mice treated ip with DOX and/or MESNA. DOX administration caused significant increases in protein carbonyls and protein-bound HNE relative to saline-treated mice. Concomitant administration of MESNA with DOX suppressed both markers of oxidative stress relative to DOX alone. Furthermore, TNF levels were also elevated as a result of DOX relative to saline-treated mice; MESNA administration with DOX significantly suppressed this effect relative to DOX alone ($*P < 0.05$, $***P < 0.01$, $****P < 0.001$).

oxidized proteins using redox proteomics. Our analysis revealed a significant increase in the specific protein carbonyl level of APOA1. The oxidation of this protein was significantly lower in mice that were coadministered MESNA with DOX (Fig. 3). Redox proteomics studies in a patient treated with DOX also identified APOA1 to be oxidatively modified (Fig. 4). Because APOA1 has been implicated in the suppression of TNF- α levels [35], we investigated whether oxidative modification of this protein compromises this function.

Ability of APOA1 to block TNF- α elevation is impaired upon its oxidation

TNF- α levels in inflammatory diseases are strongly influenced by monocyte and T lymphocyte interaction. Hyka et al. showed that human serum displayed inhibitory activity toward monocytic activation by stimulated T cells and that APOA1 was the inhibitory factor [35]. Functional assays and flow cytometry analysis revealed that APOA1 limited contact-mediated activation of these two cell types, thus inhibiting production of TNF- α at both the protein and the messenger RNA levels [35]. Because oxidation of proteins affects structure and function, we sought to examine whether the TNF- α -suppressing properties of APOA1 are compromised upon its oxidation.

Macrophage stimulation experiments using J774.A1 cells showed that, as a positive control, LPS dramatically increased TNF- α release (or secretion) into the medium. The addition of various amounts of

oAPOA1 to macrophages in the absence of LPS also increased TNF- α levels relative to controls (Fig. 5a). Native APOA1 significantly lowered TNF- α levels relative to LPS, with 20 $\mu\text{g}/\text{ml}$ protein having the more pronounced suppressing effect (Fig. 5a). In contrast, oAPOA1 addition to stimulated macrophages significantly enhanced TNF- α levels relative to LPS alone (Fig. 5a). Furthermore, preincubation of APOA1 with DOX and MESNA affected TNF- α levels when incubated in macrophage culture. As shown in Fig. 5b, incubation of APOA1 with DOX significantly increased TNF- α levels relative to controls; this increase was significantly higher than DOX administration to macrophages alone, demonstrating that DOX-induced modification of APOA1 contributes more to TNF- α modulation than simply administration of the drug alone (Fig. 5b). However, incubation of MESNA with DOX and APOA1 and subsequent addition of this mixture to macrophages resulted in a significant decrease in TNF- α levels (Fig. 5b), most probably due to the protection of APOA1 by MESNA from DOX-induced ROS; this notion is further supported by the fact that TNF- α levels were not suppressed when APOA1 was incubated with DOX alone and added to the macrophage culture with later addition of MESNA (Fig. 5b).

Conceivably, the protective effects of MESNA against DOX-induced TNF- α release from macrophages could involve MESNA interacting with DOX to change its structure. To test this possibility, MESNA was mixed with DOX and the autofluorescence of DOX was compared to

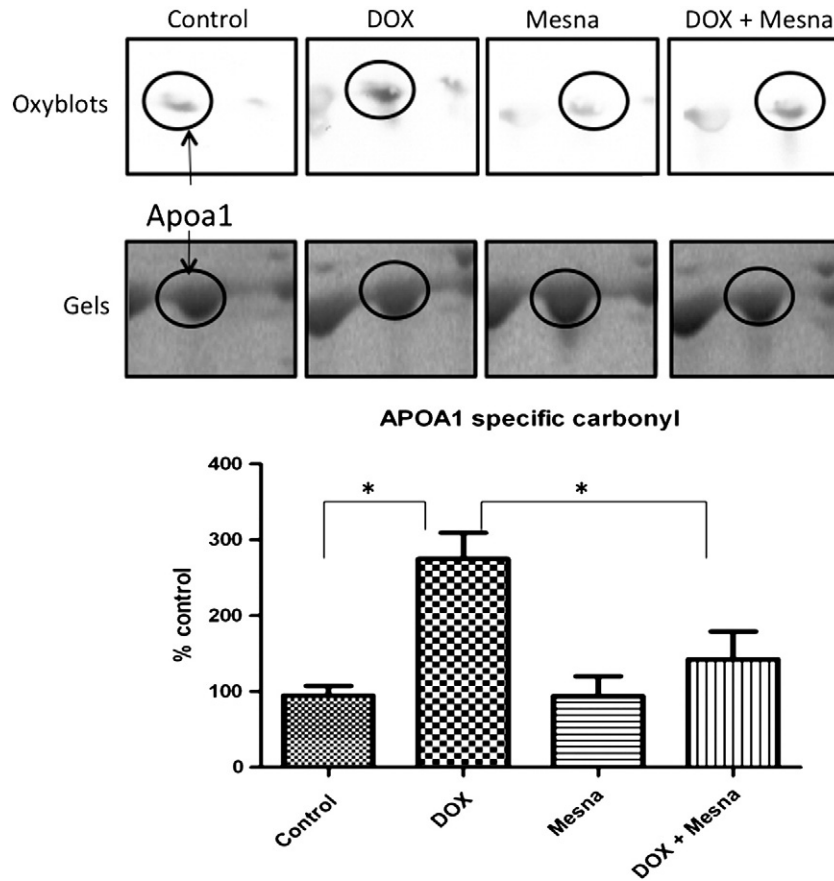


Fig. 3. Redox proteomics analysis of plasma from mice treated ip with DOX. Analysis of these samples revealed a significant increase in the oxidation level of a plasma protein from DOX-treated mice relative to saline-treated mice; this spot was identified by MS/MS and the SEQUEST database as apolipoprotein A1. Oxidation of this protein was significantly suppressed when MESNA was coadministered with DOX ($n = 7/\text{group}$, $*P < 0.05$). See the supplementary figure for identification information.

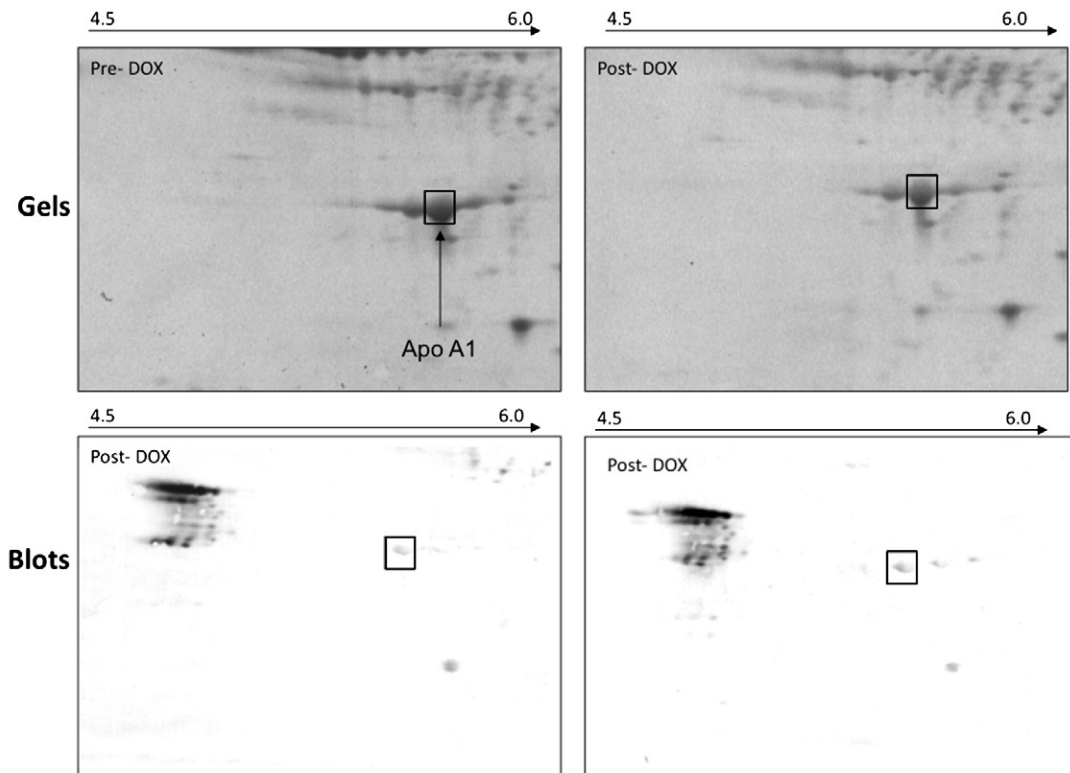


Fig. 4. Plasma from a human treated with DOX has oxidized APOA1.

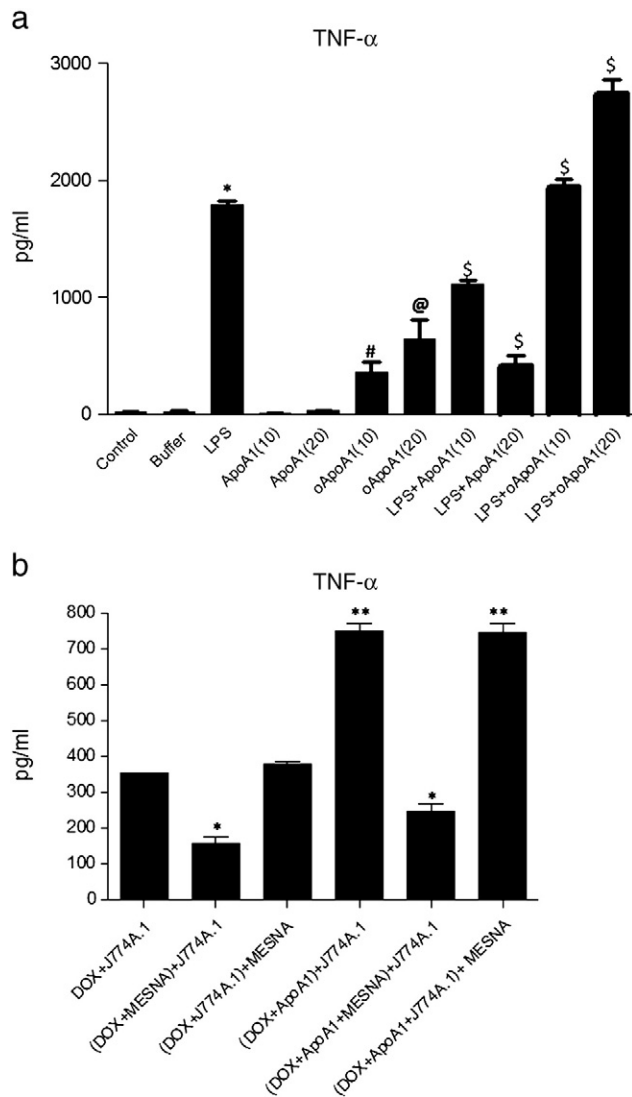


Fig. 5. (a) Macrophage stimulation experiment to test oxidation status of APOA1 on TNF suppression ability. J774.A1 macrophages were seeded onto a 48-well plate at 5×10^5 cells/well and allowed to grow overnight. The cells were treated with 10 and 20 $\mu\text{g/ml}$ oAPOA1 or native APOA1 for 20 min and then with LPS (1 $\mu\text{g/ml}$) for 4 h. Supernatants were collected and analyzed for TNF- α concentration. Values are means \pm SEM ($n = 3$). * $P < 0.001$ compared to all other groups; $^{\#}P < 0.001$ compared to LPS; $^{\textcircled{a}}P < 0.05$ and $^{\textcircled{b}}P < 0.001$ compared to control (medium alone). (b) DOX oxidizes APOA1 and induces TNF- α production in J774.A1 macrophages. J774.A1 macrophages were seeded onto a 48-well plate at 5×10^5 cells/well and allowed to grow overnight. Preincubation of 20 $\mu\text{g/ml}$ APOA1 along with DOX (1 μM) and MESNA (25 $\mu\text{g/ml}$) for 1 h was performed before their addition to J774.A1 macrophages as per the pretreatment with MESNA, and posttreatment was performed by incubating 20 $\mu\text{g/ml}$ APOA1 with DOX (1 μM) for 1 h, adding them first to the J774.A1 macrophages, and, after 4 h incubation, adding MESNA (25 $\mu\text{g/ml}$). Supernatants were collected and analyzed for TNF- α concentration. Values are means \pm SEM ($n = 3$). * $P < 0.01$, ** $P < 0.001$, compared to DOX + J774.A1 cells.

that of DOX alone at the same concentration. The results ($n = 3$ separate experiments) were that DOX plus MESNA yielded a mean of 645 fluorescence units, whereas DOX alone yielded a mean of 637 fluorescence units, essentially identical values that strongly suggest MESNA does not interact with DOX directly to change the structure or properties of DOX.

Discussion

Prior studies from our laboratory (and others) have shown that DOX administration to mice leads to oxidative damage to plasma proteins, as well as increases in peripheral TNF- α levels [12,14,23,36]. Both oxidative stress and increased TNF- α levels have been

implicated in the toxicity of DOX to heart [37], kidney [37], and brain [23,38]. Although DOX and its major metabolite doxorubicinol do not cross the BBB, increased blood TNF- α levels as a result of DOX have been shown to cross the BBB and induce oxidative stress and inflammation, providing a potential mechanism for DOX-mediated CNS toxicity [23–25]. Many patients treated with DOX and other ROS-associated chemotherapeutic agents complain of loss of cognition problems [39]. This includes loss of executive function, i.e., processes involved in multitasking, memory recall, and reasoning, which leads to a diminished quality of life for cancer survivors who experience what patients often term as “chemobrain” or “chemofog”.

In this study we investigated the effects of DOX on plasma oxidative stress and inflammation from patients undergoing treatment for various malignancies. We observed significant increases in the plasma levels of protein carbonyls and TNF- α 6 h after DOX compared to plasma from the same patients before DOX, in patients not receiving MESNA coincidentally as a part of a multiagent chemotherapy regimen. In addition, using redox proteomics, we found evidence that the abundant plasma protein APOA1 was oxidized in patient plasma after DOX exposure. Because this initial study potentially was confounded by many uncontrolled variables, we went back to the laboratory to examine the hypothesis that MESNA could prevent DOX-mediated plasma protein oxidation and TNF- α release. In controlled laboratory studies in mice, we found that DOX alone caused significant increases in plasma oxidative damage in the form of protein carbonyls and protein-bound HNE and that coadministration of MESNA with DOX significantly suppressed this effect, relative to DOX alone (Fig. 2).

Because of their extremely high content in plasma (~70 mg/ml), coupled to the ready availability of metal ions, proteins in this fluid are susceptible to oxidative attack. We hypothesize it is this oxidative damage to plasma proteins, specifically APOA1, via DOX administration that leads to increased levels of the proinflammatory cytokine TNF- α , as observed in this study.

Protein-bound HNE is formed via oxidative attack on arachidonic acid. As an α,β -unsaturated aldehyde, HNE can covalently attack protein residues via Michael addition, changing their 3-D structure [40]. Once MESNA enters the bloodstream, a large percentage of the drug is oxidized to MESNA disulfide, whereas a fraction remains in the reduced form. This oxidation is similar in nature to the actions of glutathione, which forms disulfide bonds to detoxify H_2O_2 . We hypothesize that the oxidation of this sulfhydryl group contributes to the antioxidant property of MESNA in the plasma. The free sulfhydryl group of MESNA allows quenching of free radicals by this drug, as shown here and elsewhere (Fig. 2a) [41]. The amount of free (reduced) MESNA in the blood increases with dosages above 30–100 mg/kg [42]. Therefore, protection from HNE damage of plasma proteins by MESNA could occur via two mechanisms: free radical scavenging, so as to prevent HNE from forming, or quenching HNE levels through Michael addition to the free-SH group, similar to its effects with acrolein in the bladder. Interestingly, in addition to suppressing oxidative stress in the plasma, MESNA was capable of suppressing DOX-induced increases in peripheral TNF- α levels relative to DOX alone.

Our redox proteomics analysis revealed that a highly abundant plasma protein, APOA1, is oxidized in mouse plasma as a result of DOX administration. A study by Szapacs et al. showed that APOA1 is among the most severely oxidized plasma proteins in the presence of electrophiles, such as HNE, in plasma [43]. Oxidation of this plasma protein in mice treated with DOX was ameliorated with coadministration of MESNA (Fig. 3). In addition to cholesterol transport, APOA1 has a secondary function as a TNF- α suppressor [35]. Activated T cells bind monocytes to initiate production of TNF- α at the onset of an inflammatory stimulus; APOA1 is capable of blocking this interaction and, therefore, suppressing TNF- α production [35]. Thus, in addition to complications of cholesterol transport, oxidation of APOA1 could contribute to the observed increase in peripheral TNF- α levels as a result of DOX-induced oxidative stress.

The addition of two different amounts of native APOA1 to an LPS-treated macrophage group was capable of significantly reducing TNF- α levels relative to LPS alone (Fig. 5a). However, similar additions of oxidized APOA1 protein to cells stimulated with LPS did not confer protection in the form of lower TNF- α levels; instead, an increase in TNF- α was observed for LPS plus 10 and 20 $\mu\text{g}/\text{ml}$ oxidized APOA1 relative to LPS alone. Therefore, the oxidation of this protein by DOX compromises its function as a TNF- α suppressor and also can specifically contribute to the observed increased TNF- α levels. In fact, we also observed a significant increase in the level of TNF- α in the oxidized APOA1 groups alone versus controls (no LPS in either group). In a separate experiment, incubation of native APOA1 with DOX (1 μM) before incubation with macrophages induced significantly higher TNF- α secretion compared with macrophages treated only with DOX, suggesting that DOX-induced oxidative modification of APOA1 contributes more to the observed TNF- α elevation than administration of DOX alone (Fig. 5b). When APOA1 was incubated with DOX and MESNA, however, TNF- α levels were suppressed, providing supporting evidence that MESNA can suppress TNF- α elevation by protecting APOA1 from oxidative damage.

Intracellular oxidative damage to biomolecules as a result of DOX is well documented, and antioxidants that are taken up by tissues and tumors may be protective to both. However, both the extracellular mechanism of toxicity described in these studies, APOA1 oxidation leading to TNF- α release, and the extracellular remedy, coadministration of MESNA, avoid the potential issue of tumor protection. Several aspects make the possible coadministration of MESNA with DOX a feasible clinical intervention: (1) MESNA was developed as an antioxidant that would be clinically useful as a uroprotectant specifically because it is cleared from the bloodstream with little tissue distribution and cellular uptake [42]; (2) MESNA has been shown not to interfere with DOX efficacy [44]; and (3) MESNA is already commonly given concomitantly with DOX in multiagent chemotherapy regimens to prevent hemorrhagic cystitis caused by oxazaphosphorine-induced production of acrolein, an α,β -unsaturated aldehyde, as is HNE.

Despite the significant side effects of DOX, this drug is still routinely used in standard chemotherapy regimens because of its potent antitumor activity. In addition to the acute side effects, chronic side effects resulting from DOX are also capable of negatively affecting the quality of life for cancer patients. Although intracellular oxidative stress may be one mechanism by which DOX acts as an anticancer agent, oxidative damage to plasma proteins and resulting increased levels of TNF- α may be partially responsible for the side effects of DOX. Because MESNA blocks this extracellular mechanism of action, coadministration of DOX with MESNA in a rational schedule of administration designed to exploit this drug interaction may prevent plasma protein oxidation, TNF- α release, and subsequent TNF- α -mediated host toxicity. Given that greater than 50% of FDA-approved anticancer drugs are known to cause ROS generation, the findings of this study have broad implications on cancer therapy.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.freeradbiomed.2011.03.009.

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