Original Articles

Superoxide induces protein oxidation in plasma and TNF-α elevation in macrophage culture: Insights into mechanisms of neurotoxicity following doxorubicin chemotherapy

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ABSTRACT
Chemotherapy-induced cognitive impairment (CICI) is a quality of life-altering consequence of chemotherapy experienced by a large percentage of cancer survivors. Approximately half of FDA-approved anti-cancer drugs are known to produce ROS. Doxorubicin (Dox), a prototypical ROS-generating chemotherapeutic agent, generates superoxide (O2•-) via redox cycling. Our group previously demonstrated that Dox, which does not cross the BBB, induced oxidative damage to plasma proteins leading to TNF-α elevation in the periphery and, subsequently, in brain following cancer chemotherapy. We hypothesize that such processes play a central role in CICI. The current study tested the notion that O2•- is involved and likely responsible for Dox-induced plasma protein oxidation and TNF-α release. Addition of O2•- as the potassium salt (KO2) to plasma resulted in significantly increased oxidative damage to proteins, indexed by protein carbonyl (PC) and protein-bound HNE levels. We then adapted this protocol for use in cell culture. Incubation of J774A.1 macrophage culture using this KO2-18crown6 protocol with 1 and 10 μM KO2 resulted in dramatically increased levels of TNF-α produced. These findings, together with our prior results, provide strong evidence that O2•- and its resulting reactive species are critically involved in Dox-induced plasma protein oxidation and TNF-α release.

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Introduction

More than half of the FDA approved anti-cancer drugs are known to cause reactive oxygen species (ROS) production [1]. Doxorubicin (Dox) is a quinone containing antineoplastic anthracycline used commonly in multi-drug chemotherapy regimens primarily to treat solid tumors [2]. Dox, a prototypical ROS-producing chemotherapeutic agent, in the presence of molecular oxygen, generates the reactive superoxide radical anion (O2•-) via redox cycling of the quinone moiety [3–7]. Our group has demonstrated that Dox-induced oxidative damage to plasma proteins in vivo induces the elevation of the inflammatory cytokine, tumor necrosis factor-alpha (TNF-α), in the periphery [1,2,8]. TNF-α crosses the blood–brain barrier (BBB) via receptor-mediated endocytosis resulting in central nervous system toxicities including further TNF-α elevation in brain, oxidative and nitrosative damage to key biomolecules, mitochondrial dysfunction, and neuronal death [8–13].

O2•- is considered a key reactive radical generated within the cell leading to protein oxidation, lipid peroxidation, and hydrogen peroxide (H2O2) and hydroxyl radical (•OH) production that can further damage biomolecules [14–17]. The goal of this study was to determine if O2•- produces oxidative protein damages in plasma and TNF-α elevation in macrophages similar to that observed following Dox administration in order to further elucidate the mechanisms...
by which Dox may cause chemotherapy-induced cognitive impairment (CICI), despite the inability of Dox or its major metabolite to cross the BBB. Previously, our laboratory demonstrated that Dox-induced oxidation of apolipoprotein A-1 (ApoA-1) in J774.41 macrophage culture led to increased TNF-α production [2].

To accomplish this goal, O₂⁻• was added to plasma samples from wild-type (WT) mice in the form of potassium superoxide salt (KO₂) in an appropriate solvent including an 18crown6 stabilized [14,18,19], and oxidative stress parameters, protein carbonyl (PC) and protein-bound 4-hydroxy-2-trans-nonenal (HNE), were measured. PC levels serve as a measure of protein oxidation, while protein-bound HNE is a lipid peroxidation product that damages proteins [20,21]. The O₂⁻• protocol we developed for our plasma experiments was then adapted for use in macrophage cell culture to determine if O₂⁻• induces Dox-like TNF-α consequences.

### Methods and materials

#### Chemicals

Chemicals, proteases, protease inhibitors, and antibodies used in this study were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise noted. Precision Plus Protein™ All Blue Standards, BCA reagents, and nitrocellulose membranes were purchased from Bio-RAD (Hercules, CA, USA).

#### Statistical analysis

All data are presented as mean ± SEM. Statistical analyses were performed using ANOVA (and Bonferroni’s multiple comparison post-test) followed by a two-tailed Student’s t-test to make individual comparisons between groups where appropriate, with p < 0.05 considered significant. Normality of data sets was tested using the D’Agostino & Pearson omnibus normality test where appropriate.

#### Animals

All procedures using animals were performed according to the protocols approved by the University of Kentucky Animal Care and Use Committee. Wild-type, male, SKH1 hairless, albino mice (2–3 months old) were purchased from the Jackson Laboratory. Mice were kept under standard conditions housed in the University of Kentucky Animal Facility, and all experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Kentucky. These animals were euthanized and blood and tissues collected for molecular or biochemical analysis. Whole blood collected by cardiac puncture was immediately collected in EDTA tubes and plasma immediately separated by centrifugation.

#### Sample preparation

Protein estimation was performed using the bicinchoninic acid (BCA, Pierce) assay. Homogenized plasma samples were diluted according to initial protein estimation results using 20 μg sample in isolation buffer [0.32 M sucrose, 2 mM EDTA, 2 mM EGTA, and 20 mM HEPES pH 7.4 with protease inhibitors, 0.2 mM PMSF, 20 μg/mL trypsin inhibitor, 4 μg/mL leupeptin, 4 μg/mL pepstatin A, and 5 μg/mL aprotinin].

#### Slot blot assay

The slot-blot method was used to determine levels of protein carbonyl and protein-bound HNE in plasma as previously described [2,22]. For protein carbonyl determination, samples were derivatized with 2,4-dinitrophenylhydrazine (DNPH). For protein-bound HNE, samples were solubilized in Laemmli buffer. Protein (250 ng) from each sample was loaded onto a nitrocellulose membrane in respective wells of a slot-blot apparatus (Bio-Rad) under vacuum. Nitrocellulose membranes were used to enhance solubility and stability [19].

**Solvent selection and potassium superoxide solution preparation**

KO₂ is a yellow solid that reacts readily with water and decomposes if exposed to water vapor or carbon dioxide in air. To avoid this, a saturated solution of KO₂ was prepared fresh, according to the method previously described [14,18,19], in a solvent of anhydrous dithethyl sulfoxide (DMSO) containing 200 mM crown ether (18crown6) to aid in solubility. To the prepared solvent, excess KO₂ was added and the KO₂ concentration estimated by UV-vis absorbance and using Beer’s law [18,23]. A saturated solution of KO₂ was approximately 250 μM under the stated conditions. Serial dilutions of this saturated solution were performed using the DMSO+18crown6 solvent to obtain the desired O₂⁻• concentrations.

**Plasma oxidation with potassium superoxide**

KO₂ in a solvent of DMSO. A saturated solution containing 18crown6 was added to plasma from WT mice (2–3 months old WT, male, SKH1 hairless, albino mice purchased from the Jackson Laboratory) and incubated at 37°C for 0, 15, 30, and 90 min. Concentrations of 0, 0.1, 1.0, or 10 μM KO₂ made using serial dilution were added to plasma to broadly encompass KO₂ concentrations used in previous studies. The solvent, DMSO containing 18crown6, was added to all control incubations.

**Macrophage stimulation with potassium superoxide**

Cell culture experiments were carried out using mouse BALB/c monocyte macrophage cell line (J774A.1) collected from murine blood. The mouse macrophage cell line J774A.1 (American Type Culture Collection) was cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, streptomycin (100 μg/mL), and penicillin (100 U/mL). All cultures were incubated at 37°C in a humidified atmosphere with 5% CO₂, J774A.1 macrophage cells were plated at a density of 5 × 10⁵ cells/well in 48-well plates. J774A.1 macrophages were seeded onto a 48-well plate at 5 × 10⁵ cells/well and allowed to grow overnight under standard culture conditions. KO₂ was prepared as described above. Preincubation of solvent, lipopolysaccharide (LPS; 1 μg/mL), KO₂ (0.1 μM; 1 μM; 10 μM) for 1 h was performed before their addition to J774A.1 macrophages. Lipopolysaccharide (LPS; 1 μg/mL) or KO₂ (0.1 μM; 1 μM; 10 μM) was added and the cells were incubated for 24 h and compared with cells incubated in media only and cells incubated in media containing DMSO + 18crown6 vehicle. The supernatant was collected and levels of TNF-α (pg/mL) were determined with a specific ELISA kit for mouse TNF-α (R&D Systems).

**Results**

### Protocol development

A variety of solvents and combinations were explored to make a stable solution of KO₂. KO₂ releases O₂⁻• upon addition to an aqueous environment [14]. O₂⁻• then reacts rapidly with water present in any solvent combination forming H₂O₂. In this study, the reaction of KO₂ with water was more rapid and vigorous than expected. In fact, KO₂ reacted with water vapor in the air during any attempt at weighing KO₂, contrary to some methods describing standard weighing preparation or preparation in a water-based solution [24,25]. Transition metals are known to influence the reactivity of dioxygen radicals including those present in O₂⁻• [26]. Chelex removal of metal ions did not prevent this problem [27], and prior addition of catalase to the solvent only accelerated the reaction of KO₂ with water presumably by reacting away the formed H₂O₂ and shifting the reaction equilibrium toward product [28–30]. This prompted the pursuit of a suitable anhydrous solvent for KO₂. KO₂ is slightly soluble in anhydrous DMSO [31]. A crown ether, 18crown6, was used to enhance solubility and stability [19].

**Plasma oxidation from potassium superoxide**

Plasma samples were treated with 0, 0.1, 1.0, or 10 μM KO₂ for 0, 15, 30, and 90 min and analyzed via slot blot to determine relative levels of PC and protein-bound HNE as measures of protein oxidation and lipid peroxidation, respectively [20,21]. PC damage to protein in plasma following incubation with KO₂ was rapid. Using 10 μM KO₂, PC levels for each successive time point were significantly elevated over the previous one indicated by Bonferroni’s Multiple Comparison Test. After 15 min incubation at 37°C, significant increases in PC were observed at 0.1, 1.0 and 10 μM KO₂ (Fig. 1a, ***p < 0.005, **p < 0.005, and *p < 0.01, respectively). Significant increases in PC in plasma were also observed at each concentration, 0.1, 1.0 and 10 μM of KO₂ measured after 15 min incubation at 37°C (Fig. 1b, ***p < 0.005, **p < 0.005, and *p < 0.01, respectively). Similar experiments were performed to assess KO₂-induced protein-bound oxidation, with **protocol we developed for our plasma experiments was then adapted for use in macrophage cell culture to determine if O₂⁻• induces Dox-like TNF-α consequences.
HNE in plasma. Protein-bound HNE was significantly elevated after 30 and 90 min incubations with 10 μM KO₂ at 37 °C (*p < 0.05, #p < 0.001, respectively) (Fig. 1c). Protein-bound HNE levels were not significantly elevated at the KO₂ concentrations tested for the 15 min incubation at 37°C. Significant increases in protein-bound HNE levels were seen after 30 min incubation at 37 °C with the 10 μM KO₂ concentration (*p < 0.05) and after 90 min incubation at 37 °C with the 1 μM and 10 μM KO₂ concentrations (*p < 0.05, #p < 0.001, respectively) (Fig. 1d). Decisions for moving forward were based on these preliminary KO₂ dose–response results with varied incubation times.

**Superoxide induces TNF-α elevation in macrophage culture similar to that seen following doxorubicin administration**

Previously, we reported TNF-α elevation in plasma or macrophage culture following Dox treatment and O₂⁻• produced through redox cycling of Dox as the likely cause [2,10]. Our group also demonstrated in a cross-over human clinical study that TNF-α and soluble TNF-α receptor levels are elevated in human plasma following i.v. Dox administration [32]. Macrophages are a principal source of TNF-α production in vivo [33–35], and microglial activation in brain following Dox-induced TNF-α elevation leads to the previously mentioned central nervous system toxicities [24,33]. Here, we test our hypothesis that O₂⁻•, administered as KO₂, will lead to TNF-α elevation in macrophage culture similar to that observed following Dox administration [2]. Significantly increased TNF-α elevation in J774.A1 macrophage culture after incubation with KO₂ for 24 h was observed. TNF-α was increased in these cell lines following incubation with 1 and 10 μM KO₂ (***p < 0.0001) (Fig. 2). Incubation of these macrophages with the 10 μM KO₂ concentration resulted in TNF-α greater than treatment of the cells with lipopolysaccharide (LPS; 1 μg/mL), a known initiator of TNF-α transcription via nuclear factor κ-light-chain enhancer of activated B cells (NF-κB) (Fig. 2) [36–41].
Higher concentrations of KO₂ and longer incubation times tested were required to reach significant increases in protein-bound HNE following KO₂ addition (Fig. 1c) which might reflect the negative charge of O₂⁻⁻ being slow to enter a hydrophobic environment.

In biological systems, O₂⁻⁻ is produced enzymatically during reactions catalyzed by oxidases and non-enzymatically during inefficient actions of the mitochondrial electron transport chain. The damaging effects of O₂⁻⁻ to biomolecules may be limited due to the rapid rate of radical–radical reactions, the limited reactivity of O₂⁻⁻ with non-free radical targets, and the diffusion–limited efficiency of superoxide dismutase (SOD) enzymes [43–45]. Reaction of O₂⁻⁻ with SOD produces a less reactive H₂O₂ that can be converted to water and molecular oxygen by peroxidase enzymes. However, when the chemotherapeutic agent Dox is present in vivo, continued redox cycling of the quinone moiety creates a continued source of O₂⁻⁻ as Dox travels into the cell and into the nucleus [2,32]. O₂⁻⁻ reacts with other free radicals including nitric oxide (NO⁻) rapidly, at approximately diffusion–limited rates. The reaction of O₂⁻⁻ with NO⁻ produces the even more reactive peroxynitrite (ONOO⁻) which, through downstream reactivities damages SOD, thereby limiting natural defenses against these free radicals [5,46]. O₂⁻⁻ has been shown to be highly reactive with iron–sulfur clusters producing H₂O₂ and, in a subsequent reaction, iron (II (Fe²⁺)). Increased production of H₂O₂ produced via the actions of SOD or reaction of O₂⁻⁻ with iron–sulfur clusters, through Fenton Chemistry can lead to the production of the highly reactive •OH, the strongest oxidant in biological systems [47]. Reactive oxygen species (ROS), including O₂⁻⁻, NO⁻, •OH, H₂O₂, and reactive aldehydes (i.e., HNE) can oxidize intracellular proteins and other biomolecules [48]. In a series of reactions outlined by Stadtmann, 1997 under conditions where only O₂⁻⁻ and •OH were formed, radical–mediated protein oxidation leads to oxidation of amino acid side chains, fragmentation of the peptide backbone, and protein–protein cross links [48]. These provide supporting evidence for a plausible chemical mechanism for the oxidation of proteins by O₂⁻⁻ and its reaction products.

This study directly addresses a critical aspect of our proposed mechanism of CICI, the question of whether superoxide, produced via redox cycling of Dox, is an oxidant capable of inducing oxidative damage to plasma protein and TNF-α elevation in macrophages, the proposed cytokine culprit of CICI [2,32]. These results are consistent with our previous results demonstrating increased protein oxidation and lipid peroxidation markers in plasma and subsequent TNF-α elevation following Dox administration in vivo and in macrophage culture. These results demonstrate that O₂⁻⁻, when added to plasma in the form of KO₂ salt, stabilized by the molecular cage of a crown ether, 18crown6, and incubated at physiologic 37 °C, results to protein oxidation in plasma and TNF-α elevation in macrophage culture similar to that observed following Dox administration in vivo. Together, these results are compelling evidence supporting the notion that O₂⁻⁻ production as a result of Dox administration is the likely initiating event in the neurotoxicity associated with Dox and provides useful insights into our hypothesized mechanism of CICI caused by cancer chemotherapy with ROS-producing chemotherapeutic agents like Dox.

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Conflict of interest

There was no conflict of interest in this work.