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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 (O_2^{-}•) 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 O_2^{-}• is involved and likely responsible for Dox-induced plasma protein oxidation and TNF-α release. Addition of O_2^{-}• as the potassium salt (KO_2) 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 KO_2-18crown6 protocol with 1 and 10 μM KO_2 resulted in dramatically increased levels of TNF-α produced. These findings, together with our prior results,

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Conflict of interest
There was no conflict of interest in this work.

provide strong evidence that O_2^{-}• and its resulting reactive species are critically involved in Dox-induced plasma protein oxidation and TNF-α release.

Keywords

Superoxide free radical and doxorubicin induced oxidative stress; tumor necrosis factor-alpha; macrophage; chemotherapy induced cognitive impairment; cancer chemotherapy

1. 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 (O_2^{-}•) 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].

O_2^{-}• is considered a key reactive radical generated within the cell leading to protein oxidation, lipid peroxidation, and hydrogen peroxide (H_2O_2) and hydroxyl radical (•OH) production that can further damage biomolecules [14–17]. The goal of this study was to determine if O_2^{-}• 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-I (ApoA-I) in J774.A1 macrophage culture led to increased TNF-α production [2].

To accomplish this goal, O_2^{-}• was added to plasma samples from wild-type (WT) mice in the form of potassium superoxide salt (KO_2) in an appropriate solvent including an 18crown6 stabilizer [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 O_2^{-}• protocol we developed for our plasma experiments was then adapted for use in macrophage cell culture to determine if O_2^{-}• induces Dox-like TNF-α consequences.

2. Methods and Materials

2.1 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).

2.2 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.

2.3 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.

2.4 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 ug sample in isolation buffer [0.32 M sucrose, 2 mM EDTA, 2mM EGTA, and 20mM HEPES pH 7.4 with protease inhibitors, 0.2 mM PMSF, 20ug/mL trypsin inhibitor, 4 μg/ml leupeptin, 4 μg/ml pepstatin A, and 5 μg/ml aprotinin].

2.5 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 in a slot-blot apparatus (Bio-Rad) under vacuum. Nitrocellulose membranes were blocked in 3% bovine serum albumin (BSA) in PBS with 0.2% (v/v) Tween-20 for 1.5 h and then incubated in primary antibody (anti-dinitrophenylhydrazone primary or anti-protein-bound HNE, respectively, each produced in rabbit, Sigma-Aldrich) for 2 h, washed three times in PBS with 0.2% (v/v) Tween-20 and then incubated for 1 h with secondary antibody (goat anti-rabbit secondary linked to alkaline phosphatase). Nitrocellulose membranes were developed with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) dipotassium and nitro blue tetrazolium (NBT) chloride in alkaline phosphatase activity (ALP) buffer, dried, and scanned for analysis. Image analysis was performed using Scion Image (Scion Corporation, Frederick, MD).

2.6 Solvent selection and potassium superoxide solution preparation

KO_2 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 KO_2 was prepared fresh, according to the method previously described [14, 18, 19], in a solvent of anhydrous dimethyl sulfoxide (DMSO) containing 200mM crown ether (18crown6) to aid in solubility. To the prepared solvent, excess KO_2 was added and the KO_2 concentration estimated by UV-vis absorbance and using Beer's law [18, 23]. A saturated solution of KO_2 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 $O_2^{-}\bullet$ concentrations.

2.7 Plasma oxidation with potassium superoxide

KO_2 in a solvent of DMSO 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 KO_2 made using serial dilution were added to plasma to broadly encompass Dox concentrations used in previous studies. The solvent, DMSO containing 18crown6, was added to all control incubations.

2.8 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% (v/v) fetal bovine serum, streptomycin (100μg/ml), and penicillin (100U/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. J774.A1 macrophages were seeded onto a 48-well plate at 5×10^5 cells/well and allowed to grow overnight under standard culture conditions. KO_2 was prepared as described above. Preincubation of solvent, lipopolysaccharide (LPS; 1 μg/ml), KO_2 (0.1μM; 1μM; 10μM) for 1 h was performed before their addition to J774.A1 macrophages. Lipopolysaccharide (LPS; 1 μg/ml) or KO_2 (0.1μM; 1μM; 10μM) 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).

3. Results

3.1 Protocol development

A variety of solvents and combinations were explored to make a stable solution of KO_2. KO_2 releases $O_2^{-}\bullet$ upon addition to an aqueous environment [14]. $O_2^{-}\bullet$ then reacts rapidly with water present in any solvent combination forming H_2O_2. In this study, the reaction of KO_2 with water was more rapid and vigorous than expected. In fact, KO_2 reacted with water vapor in the air during any attempt at weighing KO_2, 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

O_2^{-}• [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 KO_2 with water presumably by reacting away the formed H_2O_2 and shifting the reaction equilibrium toward product [28–30]. This prompted the pursuit of a suitable anhydrous solvent for KO_2. KO_2 is slightly soluble in anhydrous DMSO [31]. A crown ether, 18crown6, was used to enhance solubility and stability [19].

3.2 Plasma oxidation from potassium superoxide

Plasma samples were treated with 0, 0.1, 1.0, or 10 μM KO_2 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 KO_2 was rapid. Using 10 μM KO_2, 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, and 10 μM KO_2 (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, and 10 μM, of KO_2 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 KO_2-induced protein-bound HNE in plasma. Protein-bound HNE was significantly elevated after 30 and 90 min incubations with 10 μM KO_2 at 37°C (*$p<0.05$, #$p<0.001$, respectively (Fig. 1c). Protein-bound HNE levels were not significantly elevated at the KO_2 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 KO_2 concentration (*$p<0.05$) and after 90 min incubation at 37°C with the 1 uM and 10 μM KO_2 concentrations (*$p<0.05$, #$p<0.001$, respectively) (Fig. 1d). The higher concentrations of KO_2 and longer incubation times were required to reach significant increases in protein-bound HNE following KO_2 addition (Fig. 1c, Fig. 1d). Decisions for moving forward were based on these preliminary KO_2 dose-response results with varied incubation times.

3.3 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 O_2^{-}• 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 O_2^{-}•, administered as KO_2, 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 KO_2 for 24 h was observed. TNF-α was increased in these cell lines following incubation with 1 and 10 μM KO_2 (p<*** 0.0001) (Fig. 2). Incubation of these macrophages with the 10 μM KO_2 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].

4. Discussion

Prior studies by our laboratory implicated O_2^{-}•, produced *in vivo* via the redox cycling of the chemotherapeutic agent Dox, in increased oxidative damage to plasma proteins, elevation of TNF-α in the periphery, followed by transfer of TNF-α to brain and further TNF-α elevation in the parenchyma. Subsequent CNS toxicity including mitochondrial dysfunction and neuronal death were observed, and we suggest such processes are involved in CICI [1, 2, 8–13, 42]. The quinone moiety within the molecular structure of Dox cycles between the quinone and semi-quinone, producing superoxide free radical from molecular oxygen as it cycles back to the quinone [2–5, 42]. The current study was undertaken to test the plausibility of our hypothesis that O_2^{-}• from Dox is responsible for oxidative protein damage, TNF-α elevation, and cognitive consequences observed following chemotherapy with ROS-producing anti-cancer drugs, like Dox, that do not cross the BBB but result in these unwanted clinical signs and symptoms.

In the current study, superoxide caused oxidative damage to plasma proteins *in vitro* rapidly and at small concentrations of KO_2, similar to damage caused by Dox. All incubation times tested, 15, 30, and 90 min, resulted in significantly increased plasma protein oxidative damage as indicated by PC elevation with PC at each successive time point significantly increased over the previous one (Fig. 1a) [20]. Significant PC elevation was observed in plasma even at the lowest concentrations of KO_2 tested (Fig. 1b). Evidence of lipid damage was also found in KO_2-treated plasma in the form of protein-bound HNE, a product of lipid peroxidation [21] (Fig. 1c). Significant increases in protein-bound HNE was observed after 30 min incubation at 10 uM KO_2 (Fig. 1c, Fig. 1d) and at 1 and 10 uM KO_2 (Fig. 1d). Higher concentrations of KO_2 and longer incubation times tested were required to reach significant increases in protein-bound HNE following KO_2 addition (Fig. 1c) which might reflect the negative charge of O_2^{-}• being slow to enter a hydrophobic environment.

In biological systems, O_2^{-}• is produced enzymatically during reactions catalyzed by oxidases and non-enzymatically during inefficient actions of the mitochondrial electron transport chain. The damaging effects of O_2^{-}• to biomolecules may be limited due to the rapid rate of radical-radical reactions, the limited reactivity of O_2^{-}• with non-free radical targets, and the diffusion-limited efficiency of superoxide dismutase (SOD) enzymes [43–45]. Reaction of O_2^{-}• with SOD produces a less reactive H_2O_2 that can be converted to water and molecular oxygen by peroxidase enzymes. Reaction of However, when the chemotherapeutic agent Dox is present *in viv*o, continued redox cycling of the quinone moiety creates a continued source of O_2^{-}• as Dox travels into the cell and into the nucleus [2, 32]. O_2^{-}• reacts with other free radicals including nitric oxide (NO•) rapidly, at approximately diffusion-limited rates. The reaction of O_2^{-}• with NO• produces the even more reactive, peroxynitrite ($ONOO^{-}$) whose downstream reactivity damages SOD, thereby limiting natural defenses against these free radicals [9, 46]. O_2^{-}• has been shown to be highly reactive with iron-sulfur clusters producing H_2O_2 and, in a subsequent reaction, iron II (Fe^{2+}). Increased production of H_2O_2, produced via the actions of SOD or reaction of

O_2^-• 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 O_2^-•, NO•, •OH, H_2O_2, and reactive aldehydes (i.e., HNE) can oxidize intracellular proteins and other biomolecules [48]. In a series of reactions outlined by Stadtman, 1997 under conditions where only O_2^-• and •OH were formed, radical-mediated protein oxidation lead 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 O_2^-• 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 O_2^-•, when added to plasma in the form of KO_2 salt, stabilized by the molecular cage of a crown ether, 18crown6, and incubated at physiologic 37°C, results 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 O_2^-• 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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Abbreviations

ROS	reactive oxygen species
Dox	doxorubicin
O_2^-•	superoxide
TNF-α	tumor necrosis factor-alpha
BBB	blood-brain barrier
H_2O_2	hydrogen peroxide
•OH	hydroxyl radical
CICI	chemotherapy-induced cognitive impairment
ApoA-I	apolipoprotein A-I

WT	wild-type
KO_2	potassium superoxide salt
PC	protein carbonyl
HNE	4-hydroxy-2-trans-nonenal
BCA	bicinchoninic acid
DNPH	2,4-dinitrophenylhydrazine
BSA	bovine serum albumin
BCIP	5-bromo-4-chloro-3-indolyl-phosphate dipotassium
NBT	nitro blue tetrazolium chloride
ALP	alkaline phosphatase activity buffer
DMSO	dimethylsulfoxide
18crown6	crown ether, 18-crown-6
LPS	lipopolysaccharide
NF-κB	nuclear factor κ-light-chain enhancer of activated B cells
SOD	superoxide dismutase
NO•	nitric oxide
ROS	reactive oxygen species

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HIGHLIGHTS

- We showed earlier doxorubicin produces $O_2^{-}\bullet$ and leads to TNF elevation in plasma
- We showed earlier that elevated plasma TNF translocated to brain to cause apoptosis
- Here KO_2 added to plasma or macrophages elevated oxidative stress and TNF, respectively
- We conclude that $O_2^{-}\bullet$ from Dox is involved in processes that lead to brain cell death

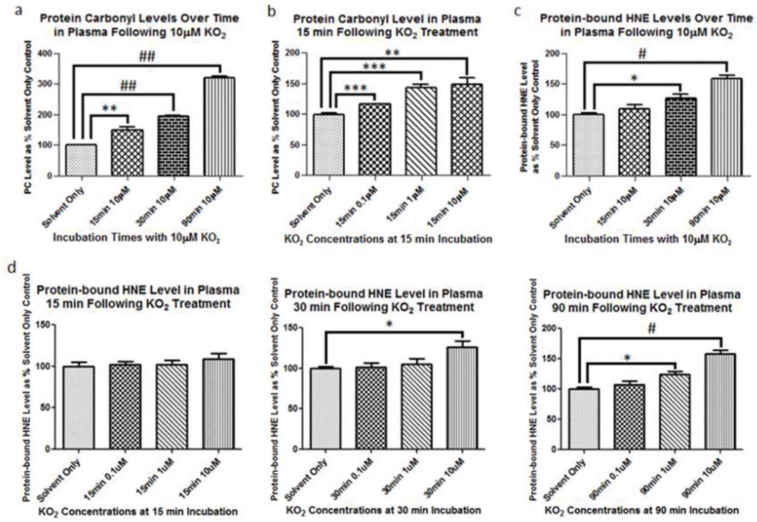

Fig. 1.

Protein carbonyl (PC) and protein-bound HNE (HNE) are measures of protein oxidation and lipid peroxidation. (a) PC levels were assessed at 15, 30, and 90 min incubations with 10 µM KO_2 at 37°C. All incubation times tested at 10 µM KO_2 and 37°C, 15, 30, and 90 min, resulted in significantly increased PC compared to solvent alone (**$p<0.01$, ##$p<0.0001$, ##$p<0.0001$, respectively). PC for each successive time point at 10 µM KO_2 was significantly elevated over the previous one indicated by Bonferroni's Multiple Comparison Test. (b), Significant increases in PC were observed at 0.1, 1, and 10 µM KO_2 after 15 min incubation at 37°C (***$p<0.005$, ***$p<0.005$, and **$p<0.01$, respectively). (c) Protein-bound HNE was significantly elevated after 30 and 90 min incubations with 10 µM KO_2 at 37°C (*$p<0.05$, #$p<0.001$, respectively). (d) Protein-bound HNE levels were not significantly elevated at the KO_2 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 KO_2 concentration (*$p<0.05$) and after 90 min incubation at 37°C with the 1 uM and 10 µM KO_2 concentrations (*$p<0.05$, #$p<0.001$, respectively).

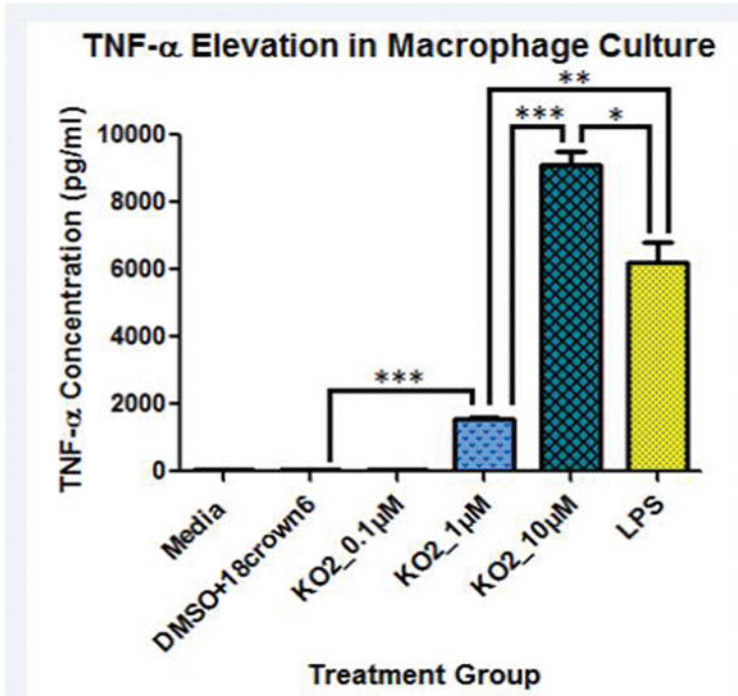

Fig. 2.

Superoxide (O_2^{-}•) 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 solvent, lipopolysaccharide (LPS; 1 μg/ml), KO_2 (0.1μM; 1μM; 10μM) for 1 h was performed before their addition to J774.A1 macrophages. Following 24 h incubation, supernatants were collected and analyzed for TNF-α concentration. Values are means±SEM (n=3). (*p<0.05, **p<0.005, ***p<0.0001 compared to solvent alone). One-way ANOVA (p<0.0001) with Bonferroni's Multiple Comparison Test also demonstrated these significant differences between groups.