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The spin trap 5,5-dimethyl-1-pyrroline *N*-oxide inhibits lipopolysaccharide-induced inflammatory response in RAW 264.7 cells

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Abstract

Aim—Exposure of macrophages to lipopolysaccharide (LPS) induces oxidative and inflammatory stresses, which cause cell damage. Antioxidant and anti-inflammatory properties have been attributed to the nitron spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO), commonly used in free radical analysis, but these aspects of DMPO have been little explored. In this study, we sought to establish the anti-inflammatory activity of DMPO, presumably by removing free radicals which otherwise help activate inflammatory response and damage cells.

Main methods—RAW 264.7 macrophages were treated with LPS and/or DMPO for different time points, cell damage, production of inflammatory mediators, inducible nitric oxide synthase (iNOS) expression, NF- κ B p65 activation, phosphorylation of MAPKs and Akt, and intracellular reactive oxygen species (ROS) were determined.

Key findings—After cells were treated with LPS and/or DMPO for 24 h, DMPO reduced the LPS-induced inflammatory response as indicated by downregulated iNOS expression and production of inflammatory mediators. Accordingly, DMPO protected cells from LPS-induced cytotoxicity. In order to understand the mechanistic basis of these DMPO effects, the NF- κ B p65 activation and the phosphorylation of MAPKs and Akt were examined. We found, by assaying cells treated with LPS and/or DMPO for 15–60 min, that DMPO inhibited the phosphorylation of MAPKs, Akt, and I κ B α , and reduced the NF- κ B p65 translocation. Furthermore, we demonstrated that DMPO inhibited LPS-induced ROS production.

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Conflict of interest The authors declare that there are no conflicts of interest.

Significance—DMPO showed the anti-inflammatory activity and attenuated LPS-induced cell damage, most likely by reducing ROS production and thus preventing the subsequent inflammatory activation and damage.

Keywords

5,5-dimethyl-1-pyrroline *N*-oxide; free radical; inflammation; lipopolysaccharide; macrophage, NF- κ B

Introduction

Nitrone spin traps are a class of synthetic chemicals specifically designed for trapping free radicals and making them more stable. Two commonly used nitrone spin traps are 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) and phenyl-*N*-*tert*-butylnitrone (PBN). Due to the oxygen free radical trapping capacity, PBN has been studied for its antioxidant and anti-inflammatory activities (Ahmed et al., 2003). It produces a diverse array of protective or beneficial effects in animal models of disease and injury that include endotoxin shock, ischemia-reperfusion injury, stroke, intracerebral glioma, and aging (Floyd et al., 2008; Kotake, 1999).

Given that both MPO and PBN detect free radicals, e.g., superoxide and hydroxyl radical, though they may have quite different reaction rate constants for a specific radical species, DMPO may behave in a similar way as PBN in terms of its antioxidant and anti-inflammatory activities. However, DMPO has been far less studied in this regard. Limited data demonstrated that preadministration of DMPO reduces the mortality associated with endotoxin shock in the rat (Hamburger and McCay, 1989) and protects against reperfusion-induced injury or arrhythmias in isolated rat heart models (Tosaki et al., 1992; Zuo et al., 2009). Recently, when we used DMPO to detect macromolecular free radicals in lipopolysaccharide (LPS)-activated RAW 264.7 macrophages (Gomez-Mejiba et al., 2010), we found that DMPO protects against LPS-induced cytotoxicity and, interestingly, the earlier the treatment of cells with DMPO after LPS induction, the better were the protective effects, suggesting that DMPO interferes with the LPS-triggered early inflammatory signaling. However, this observation needs to be refined.

Exposure of macrophages to LPS induces oxidative stress and an inflammatory response in which the transcription factor NF- κ B plays a central role (Bhattacharyya et al., 2004). Activation of NF- κ B by LPS depends on toll-like receptor 4-initiated signaling that is modulated by several mitogen-activated protein kinases (MAPKs) including ERK1/2, JNK, and p38 (Chan and Riches, 2001; Chung et al., 2006; Guha and Mackman, 2001). MAPKs are rapidly activated through phosphorylation in response to LPS and therefore have been used as a hallmark of LPS-induced signaling (Jang et al., 2006; Kim et al., 2010). The regulation of NF- κ B activity by MAPKs seems to be dependent on reactive oxygen species (ROS) and free radicals as messenger molecules (Chung et al., 2006). However, among them, it is relatively well established that hydrogen peroxide is involved in the cross talk between MAPKs and NF- κ B signaling (Gloire et al., 2006). It is not clear whether and which free radicals involved in LPS-elicited signaling are targets of DMPO.

The purpose of this study was to test the anti-inflammatory activity of DMPO in LPS-activated RAW 264.7 macrophages, presumably by targeting free radicals that act as cell signals in inflammatory response. We determined the inhibitory effect of DMPO on LPS-induced inflammatory response, cell damage, NF- κ B translocation and MAPK activation. Our results showed the experimental effectiveness of DMPO in controlling LPS-mediated inflammatory conditions, and suggest that its anti-inflammatory activity might be associated with the blocking of the upstream inflammatory signaling cascades including the free radical reactions.

Materials and methods

Cell culture

RAW 264.7 cells were obtained from American Type Culture Collection (TIB-71, Rockville, MD) and grown in DMEM supplemented with 10% fetal bovine serum at 37°C in a 5% CO₂ incubator. Cells between passages 5 and 20 were used in this study.

LPS and DMPO treatments

LPS (*Escherichia coli* serotype 055:B5, L2367) was from Sigma (St. Louis, MO). DMPO was from Alexis Biochemicals (San Diego, CA). Cells were cultivated on the indicated culture ware and allowed to attach for at least 2 h, then the medium was removed and replaced with the indicated medium with DMPO and/or LPS. According to our earlier studies, LPS at 1 ng/ml was a reasonable concentration that could induce RAW 264.7 cell activation but caused less cell damage. DMPO was mostly used at 50 mM in this study. The dose of DMPO was chosen because it could provide the effective protection against LPS (1 ng/ml)-induced cytotoxicity but itself showed no detectable toxicity based on the tetrazolium 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT)-based colorimetric assay.

Cell viability assay

The cytotoxicity of LPS and DMPO was assessed using the MTT assay. After treatments with LPS and DMPO in 96-well plates, the culture medium was replaced with 0.5 mg/ml of MTT (Amresco Inc., Solon, OH) in phosphate buffered saline (PBS) and cells incubated at 37°C for 1 h. After aspiration of MTT, the formazan crystals in viable cells were solubilized in dimethyl sulfoxide and quantified by reading absorbance at 570 nm with a 630 nm reference using an Infinite 200 microplate reader (Tecan, Research Triangle Park, NC).

Alternatively, the cytotoxicity of LPS and DMPO was determined by assaying the release of lactate dehydrogenase (LDH) into the culture medium using a QuantiChrom LDH kit (BioAssay Systems, Hayward, CA).

Cytokine assay

To measure the production of proinflammatory cytokines, RAW 264.7 cells were grown in 6-well plates and treated with LPS and/or DMPO for 24 h. The culture medium was collected for several cytokine assays. Tumor necrosis factor-alpha (TNF- α), interleukin (IL)-10, and granulocyte-macrophage colony-stimulating factor (GM-CSF) were assayed

using commercial DuoSet ELISA kits from R&D Systems (Minneapolis, MN). IL-1 β , IL-6, keratinocyte-derived chemokine (KC), and IL-12p70 were assayed using a MS6000 Mu ProInflammatory 7-Plex Ultra-Sensitive kit (Meso Scale Discovery, Gaithersburg, MD) according to the manufacturer's specifications, and an MSD Sector Imager 6000 was used to read the plates.

NO assay

Nitrite accumulation in culture medium was determined as an indicator of nitric oxide (NO) synthesis. RAW 264.7 cells were seeded in 96-well plates and stimulated with LPS in the presence or absence of DMPO for 24 h. At different time points after LPS treatment, the culture medium was collected for nitrite accumulation using the classical Griess reaction. In a second set of experiments, DMPO was either added simultaneously with LPS or up to 24 h after LPS addition. Culture supernatants were collected for the nitrite assay after cells were stimulated with LPS for 24 h.

ROS assay

Intracellular ROS production was determined using carboxy-H₂DCFDA, a fluorogenic probe for general ROS such as hydrogen peroxide, peroxynitrite, and hydroxyl radical. Cells were incubated in 96-well plates with 25 μ M of carboxy-H₂DCFDA (Invitrogen, Carlsbad, CA) in PBS for 30 min, then washed twice and treated with indicated concentrations of LPS and DMPO for another 30 min. Fluorescence intensity was measured at Ex/Em = 495/529 nm.

For superoxide detection, cells were serum starved overnight in 6-well plates. Then the medium was changed to PBS containing 10 μ M dihydroethidium (DHE, Invitrogen) with 50 mM DMPO, 1 μ M diphenyleneiodonium chloride (DPI, Sigma), or 100 μ M apocycin (CalBiochem, San Diego, CA) for 20 min at 37°C. Cells were activated with the addition of 1 ng/ml LPS plus 1% (v/v) fetal bovine serum in PBS for 30 min. In the presence of superoxide, cell permeable DHE is oxidized to ethidium, which intercalates with DNA with the emission of red fluorescence (Ex/Em = 488/610 nm). The change in fluorescent signal indicates the level of intracellular superoxide. Cells were scraped off and the fluorescence intensity was analyzed by FACS Calibur (BD Biosciences, San Jose, CA) (McAdams et al., 2006). For each sample, 20,000 events were collected and percent gated cell count was obtained based on the right shift of red fluorescent signal in the FL2 channel.

Preparation of cell lysates

Following treatments with LPS for the indicated times, RAW 264.7 cell activation was stopped by the removal of medium and addition of ice-cold PBS (Jang et al., 2006). In brief, whole cell lysates were prepared and used to detect proteins of interest. Cells were lysed with the CellLytic M lysis solution (Sigma) containing 1% (v/v) protease inhibitor cocktail (Amresco). Cell debris was removed by centrifugation at 12,000 g for 15 min at 4°C, and the resultant supernatants were stored at -80°C until use.

To assess NF- κ B p65 translocation, subcellular fractions were prepared (Jones et al., 2007; Terra et al., 2007). Cells were lysed in ice-cold buffer A (10 mM HEPES, pH 7.9, 1.5 mM

MgCl₂, 10 mM KCl, 0.25% Nonidet P-40, 0.5 mM dithiothreitol and 1% (v/v) protease inhibitors for 10 min. The cytoplasmic fraction was collected by centrifugation at 12,000 g for 3 min. The unlysed nuclei were gently resuspended in ice-cold buffer B (20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 420 mM NaCl, 25% glycerol and 1% (v/v) protease inhibitors) and left on ice for 20 min, then a 4-fold volume of buffer C (20 mM HEPES, pH 7.9, 50 mM KCl, 0.5 mM dithiothreitol, 0.2 mM EDTA and 1% (v/v) protease inhibitors) was added, and the nuclear fraction was collected by centrifugation at 12,000 g for 5 min.

The protein concentrations in cell lysates were determined using a BCA protein assay kit (Pierce Labs, Rockford, IL) with bovine serum albumin as standard.

Determination of protein nitration

Protein nitration was assessed as a marker for oxidative damage of proteins in LPS-treated RAW 264.7 cells. Cell lysates, diluted in 0.1 M bicarbonate buffer, pH 9.6, were incubated in the ELISA plates overnight at 4°C. Following washing with 0.05% Tween-20 in PBS and then blocking with 2.5% cold-water fish skin gelatin (Sigma) for 1 h, the plates were incubated with rabbit anti-nitrotyrosine (1:1,000 dilution, Sigma) at 37°C for 1 h. The immunocomplexes were detected using goat anti-rabbit IgG-HRP conjugate and VisiGlo Chemilu HRP substrate solution (Amresco), and read by a microplate reader.

Western blot analysis

Cell lysates were mixed with 4×SDS NuPAGE sample loading buffer (Invitrogen) plus 100 mM 2-mercaptoethanol. After heat denaturation, equal amounts of cellular proteins were separated on 4-12% reducing NuPAGE Bis-Tris Gels (Invitrogen), followed by electrotransfer onto a nitrocellulose membrane (0.2 μm pore size). After blocking with 5% non-fat milk in PBS, the immunoblot was performed by incubation with a primary antibody overnight at 4°C, and then HRP-conjugated goat anti-rabbit or goat anti-mouse IgG secondary antibody for 1 h at room temperature. The immunocomplexes were visualized using SuperSignal West Pico Chemiluminescent HRP Substrate (Thermo Fisher Scientific) and recorded with a FluorChem HD2 imager (Alpha Innotech Corp., San Leandro, CA). The following primary antibodies were used: anti-NF-κB p65, p44/42 MAPK (ERK1/2), SAPK/JNK, phospho-SAPK/JNK (Thr183/Tyr185), phospho-p38 (Thr180/Tyr182), p38 MAPK, phospho-Akt (Ser473) (Cell Signaling Technology, Inc., Danvers, MA), p53, checkpoint kinase Chk1, histone H2B, phospho-ERK1/p44 (pT202/Y204), Akt (Epitomics, Burlingame, CA), iNOS, β-actin (Sigma), phospho-histone H2AX (Ser139) (Active Motif, Carlsbad, CA), and IκBα (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Real time RT-PCR

RAW 264.7 cells were seeded at 1.6×10^6 cells per well in 6-well plates with LPS and/or DMPO for 6 h. Total RNA was isolated using a RNeasy Mini kit (Qiagen, Valencia, CA) with extra on-column DNase digestion before RNA cleanup using RNase-Free DNase Set (Qiagen). The integrity, quantity and purity of RNA were examined using NanoDrop 1000 Spectrophotometer (Thermo Scientific, Wilmington, DE). For each sample, 0.13 μg of total RNA was reverse transcribed using the SuperScript III first-strand synthesis system for RT-PCR (Invitrogen) with random hexamer primers. Real-time qRT-PCR was used to determine

the relative amount of iNOS mRNA. For a typical reaction, 12.5 μ l iTaq SYBR Green 2 $\times$ supermix, 10 μ M primer and 3 μ l of cDNA template were mixed and the final volume was adjusted to 25 μ l. ABI Prism 7000 Sequence Detection System (Applied Biosystems) and iTaqTM SYBR Green Supermix (Bio-Rad, CA) were used for detection and quantitation. The primers for mouse iNOS were 5'-GGC AGC CTG TGA GAC CTT TG-3' (forward) and 5'-GCA TTG GAA GTG AAG CGT TTC-3' (reverse). The primers for 18S rRNA were 5'-CGC GGT CCT ATT CCA TTA TTC-3' (forward) and 5'-CCC GAA GCG TTT ACT TTG AAA-3' (reverse). The default PCR conditions were as follows: initiation: 2 min at 50°C; hot start of the enzyme: 10 min at 95°C; amplification: denaturation at 95°C for 15 s, followed by annealing and extension at 60°C for 1 min; 40 cycles. The specificity of gene PCR product was evaluated by a melt dissociation curve. Relative quantification of gene expression was estimated using the C_t method. The relative expression of iNOS was normalized with 18S rRNA.

Statistical analysis

Results are expressed as mean value $\pm$ SEM. Effects were assessed using the Student's *t* test. A difference between treatment groups with $P < 0.05$ was considered statistically significant.

Results

DMPO reduces LPS-induced cytotoxicity

To investigate the cytotoxicity of LPS and DMPO, RAW 264.7 cells were incubated with 1 ng/ml LPS and 50 mM DMPO for 24 h. As shown in Fig. 1A, LPS decreased cell number as assessed using the MTT assay. DMPO alone did not affect cell viability, but it suppressed LPS toxicity. To evaluate LPS-induced protein oxidative damage, we determined protein nitration and found that DMPO significantly inhibited LPS-mediated production of protein nitration (Fig. 1B). To evaluate LPS-induced DNA damage, we determined histone H2AX phosphorylation and the expression levels of DNA damage sensor p53 and checkpoint Chk1 in cells exposed to LPS for 24 h. We found that LPS induced phosphorylation of histone H2AX, but decreased the expression of p53 and Chk1 (Fig. 1C). However, DMPO inhibited the LPS effect on the DNA damage parameters. Furthermore, LDH release into the culture medium was evaluated as an indicator of cell death. We found that DMPO reduced LPS-induced release of LDH into the culture medium (Fig. 1D). These data suggest that DMPO has a protective effect against LPS-induced cell damage.

DMPO reduces LPS-induced inflammatory cytokine production

To investigate the anti-inflammatory effect of DMPO, we determined the production of several typical inflammatory cytokines, TNF- α , IL-1 β , IL-6, KC, IL-10, IL-12, and GM-CSF, in RAW 264 cells incubated with or without 1 ng/ml LPS and/or 50 mM DMPO for 24 h. As shown in Fig. 2, LPS stimulated cells to secrete the inflammatory cytokines. DMPO alone had no effect, but inhibited LPS-induced production of these cytokines, suggesting that DMPO inhibits LPS-induced cell inflammatory response.

DMPO reduces LPS-induced NO production and iNOS expression

NO in combination with inflammatory cytokines is mostly responsible for LPS-mediated cell damage, therefore, we also determined the effect of DMPO on nitrite accumulation in the culture medium of cells treated with 1 ng/ml LPS and 50 mM DMPO for different time points (0-24 h). In agreement with previous reports (Seminara et al., 2007; Stuehr and Marletta, 1987), there was a lag phase of about 6 h followed by a linear increase in nitrite accumulation. However, the increasing nitrite production was inhibited when DMPO was present in the medium (Fig. 3A).

In LPS-activated macrophages, high levels of NO are produced through the induction of iNOS. The effect of DMPO on the iNOS induction was therefore determined. First we used an indirect method (Zhai et al., 2009). Once macrophages are activated, iNOS gene transcription is initiated at approximately 1 h, whereas its protein expression occurs some 3-4 h later. If DMPO-mediated decrease in NO production is through interference with the transcriptional induction process, earlier intervention with DMPO (i.e. 0.5 h after LPS stimulation) should have a more profound effect on NO production than adding DMPO later. Therefore, in this study, 50 mM DMPO was either added simultaneously with 1 ng/ml LPS or up to 24 h after addition of LPS. The results showed that after LPS treatment, the earlier addition of DMPO resulted in more inhibition of NO production (Fig. 3B), indicating that DMPO inhibits the transcriptional induction of iNOS. However, there were no remarkable differences in NO production for DMPO addition between 0-0.5 h after LPS stimulation.

The inhibitory effect of DMPO on the iNOS expression was further confirmed. RAW 264.7 cells were treated with LPS and DMPO for 6 h for iNOS mRNA expression or 24 h for its protein expression. The results showed that DMPO significantly reduced the expression of iNOS protein and mRNA (Fig. 3C and D).

DMPO reduces LPS-induced NF- κ B p65 translocation and I κ B α degradation

To determine whether the decreased iNOS expression is due to the blocking of NF- κ B activation by DMPO, we measured the expression levels of NF- κ B p65 in both cytosolic and nuclear fractions of RAW 264.7 cells treated with 1 ng/ml LPS and/or 50 mM DMPO for 1 h. As shown in Fig. 4A, LPS induced the translocation of NF- κ B p65 from the cytosol to nucleus, but the presence of DMPO inhibited the translocation of NF- κ B p65. To explain the inhibition of NF- κ B p65 nuclear translocation, we next investigated the effect of DMPO on the I κ B α degradation. As shown in Fig. 4B, I κ B α was almost completely degraded upon LPS stimulation for 15 min, but DMPO inhibited the degradation of I κ B α , suggesting that DMPO inhibits LPS-triggered upstream signaling of NF- κ B activation.

DMPO reduces LPS-induced phosphorylation of MAPKs and Akt

To further explore the molecular mechanism of DMPO, we determined its effect on the upstream signaling pathways of NF- κ B activation, that is, MAPKs and Akt. Because these kinases are activated through phosphorylation, their phosphorylation levels were evaluated. After RAW 264.7 cells were treated with 1 ng/ml LPS and/or 50 mM DMPO for 15 min, LPS induced the appearance of phosphorylated ERK1/2, JNK, p38, and Akt, but DMPO

showed inhibitory effects on the phosphorylation of these kinases, especially Akt (Fig. 4C). Either LPS or DMPO alone had no effect on any total kinase.

DMPO reduces LPS-induced intracellular ROS production

DMPO has free radical trapping properties that are the basis for its use in free radical research. Oxygen free radicals are suggested to be signaling messengers in LPS-mediated inflammatory response (Gloire et al., 2006). Therefore, we tested the effect of DMPO on LPS-induced intracellular ROS production. As shown in Fig. 5A, DMPO inhibited LPS-induced ROS production in a dose-dependent manner. Of the many ROS, superoxide is important because other oxidants can be derived from it. Superoxide was detected in LPS-treated cells by staining with a fluorescence dye DHE. Our data show that DMPO blocked any LPS-induced increase in superoxide production (Fig. 5B). In contrast, the NADPH oxidase inhibitors DPI and apocycin were only somewhat inhibitory. These data suggest that DMPO inhibits LPS-induced intracellular ROS production, and suppressing ROS production may weaken the downstream NF- κ B activity and accordingly reduce various biochemical events that otherwise cause excessive oxidative and inflammatory damage.

Discussion

Inflammation mediators are important for the host defense. However, improper upregulation of the inflammatory mediators is responsible for the origin and progression of many pathological conditions. For example, induced TNF- α and NO are known to be strong inducers of cell damage and apoptosis (Comalada et al., 2003; Gotoh et al., 2002; Xaus et al., 2000). In the present study, we first evaluated the protective effect of DMPO against LPS-induced cell damage using several methods, i.e., MTT-based mitochondrial enzyme activity, LDH release, protein nitration, and histone H2AX phosphorylation (Fig. 1).

Chk1 is an important checkpoint in the cell cycle and DNA damage response. In response to damaged DNA induced by genotoxic stress such as ROS, Chk1 is phosphorylated and activated, and peaks at about 90 min following LPS stimulation (Sharma et al., 2010). It has been reported that the Chk1 gene was down-regulated 3 h after LPS treatment in RAW 264.7 macrophages (Nemeth et al., 2003). Our observed decrease in Chk1 protein expression 24 h after LPS treatment may be due to the collective result of the downregulated gene expression and the degraded phosphorylated protein. In our study, LPS-induced DNA damage was also indicated by phosphorylation of the variant histone H2AX (Fig. 1C), one of the earliest biochemical events at the sites of DNA strand breaks (Celeste et al., 2003). Histone H2AX phosphorylation is an upstream signaling of Chk1 activation (Reinhardt and Yaffe, 2009). The data of Chk1 protein expression and histone H2AX phosphorylation in combination with other cell damage assays confirmed the protective effect of DMPO against LPS-induced cell damage.

The protective properties of DMPO in LPS-induced cell damage could be largely attributed to its inhibition of the overproduction of toxic inflammatory mediators including TNF- α , IL-1 β and NO (Fig. 2 and 3). However, the inhibitory effects of DMPO on all inflammatory mediators observed might just be secondary to the perturbation of common signaling pathways triggered by LPS. To explore the mechanistic basis of DMPO for its anti-

inflammatory activity, we first determined NO production in RAW 264.7 cells by adding DMPO at different time points after LPS treatment (Fig. 3B). The results showed that DMPO presented at the onset of LPS-induced iNOS expression (< 3h) resulted in a stronger inhibitory effect on NO production, suggesting that DMPO blocks iNOS gene induction. This reasoning was supported by the analysis of iNOS protein and mRNA expression (Fig. 3). The inhibition of iNOS expression by DMPO could be further extended to its ability to inhibit the upstream signaling as evidenced by the decreased NF- κ B p65 translocation and I κ B α degradation (Fig. 4).

NF- κ B plays a critical role in inflammatory conditions and, therefore, inhibition of NF- κ B activation has become an important anti-inflammatory pharmacological manipulation (Gupta et al., 2010; Uwe, 2008). NF- κ B activation is multifactorial in nature, involving a coordinated activation of many kinases (Jones et al., 2007), including MAPKs and Akt. Therefore, the inhibition of NF- κ B translocation by DMPO may not be a single-event consequence, but a cumulative result of inhibition of MAPKs, Akt, and some other signaling molecules. MAPKs and Akt are phosphorylated as early as 10 min following LPS stimulation and thus comprise an important part of the early signaling events of LPS-initiated macrophage activation (Sharma et al., 2010). The phosphorylated MAPKs and Akt regulate and activate the NF- κ B signaling pathway via phosphorylation of many possibly overlapping signaling molecules including IKK α / β and I κ B α (Madrid et al., 2001; Yang et al., 2000). Therefore, we investigated the effect of DMPO on the phosphorylation of MAPKs and Akt in RAW 264.7 cells stimulated with LPS for 15 min, and found that the phosphorylation and activation of these kinases were to some extent inhibited by DMPO (Fig. 4C). Although DMPO's inhibition of the signaling molecules of MAPKs and Akt seems to be unselective, it is likely involved in a common mechanistic pattern. Since free radicals are involved in the upstream signaling of these kinases (Asehnoune et al., 2004), it is proposed that DMPO functions through trapping free radicals that activate these kinases. We have confirmed the inhibitory effect of DMPO on intracellular ROS (Fig. 5A) and in particular superoxide production (Fig. 5B). It should be noted that DHE staining for superoxide was performed after serum withdrawal overnight in order to reduce the background levels of intracellular superoxide and to expectedly increase superoxide production upon addition of serum and LPS (Gurjar et al., 2001; Lim and Clement, 2007). Inhibited ROS production by DMPO will undoubtedly result in weakened downstream signaling activities, lower production of inflammatory mediators, and less cell damage.

NF- κ B signaling is redox-regulated (Chung et al., 2006; Gloire et al., 2006; Yao et al., 2007). In LPS-stimulated macrophages, superoxide may be the first ROS induced. Superoxide can be produced by NADPH oxidase whose activation is dependent on the phosphorylation of the cytosolic subunit p47^{phox}. It has been suggested that Akt is responsible for p47^{phox} phosphorylation (Hoyal et al., 2003). From superoxide, many other reactive oxygen and nitrogen species, e.g., hydrogen peroxide, peroxynitrite, hydroxyl radical, and hypochlorous acid, can be directly or indirectly derived. These oxidants are thought to be important mediators in LPS-triggered NF- κ B activation (Gloire et al., 2006). Although superoxide is produced early in response to LPS and inhibition of superoxide production by, for example, DPI, shows potential anti-inflammatory activity (Miesel et al., 1995; Qian et al., 2007), DMPO is a poor trap of superoxide with a rate constant of 10 M⁻¹

s⁻¹ (Finkelstein et al., 1979). In contrast, the reaction of hydroxyl radicals with DMPO is very fast, $k = 2 \times 10^9 \text{ M}^{-1} \text{ s}^{-1}$ (Finkelstein et al., 1979; Makino et al., 1991). As for NO, though its large production requires the induction of iNOS, macrophages contain constitutive eNOS to maintain low levels of NO (Buras et al., 2000; Connelly et al., 2003); however, it is known that DMPO is ineffective in trapping NO (Pou et al., 1994) and it cannot alter eNOS expression (data not shown). In addition to these small molecule reactive species, macromolecular free radicals may have a role in NF- κ B activation. It is possible that some kinases or kinase-interacting molecules may produce protein-centered radicals when phosphorylated and activated. Hydroxyl radical, peroxynitrite, and hypochlorous acid are known to oxidize proteins with the formation of protein-centered radicals as intermediates (Davies et al., 1991; Hawkins and Davies, 1998; Lopes de Menezes and Augusto, 2001). The precise mechanism of DMPO in blocking free radicals as cell signals warrants further studies.

Our previous data showed that 50 mM DMPO provided the most effective protection against LPS-induced cell damage but itself did not show detectable cell toxicity based on the MTT assay. Increased DMPO concentrations may produce cell damage and lose the protective effect against LPS action. For example, 100 mM DMPO generated a protective effect only commensurate with 12.5 mM DMPO. However, DMPO at 50 mM could not inhibit LPS-mediated macrophage activation completely as indicated by production of proinflammatory cytokines and NO, suggesting that there may be both ROS-dependent and ROS-independent LPS-induced signaling pathways. Chandel et al. had reported that LPS activates NF- κ B via a ROS-independent mechanism in the J774.1 macrophage cell line model (Chandel et al., 2000). Considering that DMPO is a spin trap, that is, DMPO has a potential to trap free radicals, its effect on ROS-independent signaling pathways is expected to be rather limited or ineffective, but could not be excluded.

Taken together, we demonstrated that DMPO reduces LPS-mediated inflammatory response and cell damage in a macrophage cell line. Based on our data we propose that the anti-inflammatory effect of DMPO is likely through trapping or inhibiting ROS that are upstream signaling molecules, thus preventing later inflammatory response and damage. Further studies will be needed to detect the free radicals trapped by DMPO in the early signaling events, to explore the possible alternative mechanisms of DMPO rather than spin trapping, and to evaluate its anti-inflammatory effectiveness in animal models. DMPO has been used as a spin trap for over 40 years (Taniguchi and Madden, 2000). The present study will help look at this “old” spin trap in new ways, including its anti-inflammatory use and mechanism of action.

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References

- Ahmed S, Rahman A, Hasnain A, Goldberg VM, Haqqi TM. Phenyl N-tert-butyl nitron down-regulates interleukin-1 beta-stimulated matrix metalloproteinase-13 gene expression in human chondrocytes: suppression of c-Jun NH2-terminal kinase, p38-mitogen-activated protein kinase and activating protein-1. *J Pharmacol Exp Ther*. 2003; 305:981–8. [PubMed: 12626640]
- Asehnoune K, Strassheim D, Mitra S, Kim JY, Abraham E. Involvement of reactive oxygen species in Toll-like receptor 4-dependent activation of NF-kappa B. *J Immunol*. 2004; 172:2522–9. [PubMed: 14764725]
- Bhattacharyya J, Biswas S, Datta AG. Mode of action of endotoxin: role of free radicals and antioxidants. *Curr Med Chem*. 2004; 11:359–68. [PubMed: 14965237]
- Buras JA, Stahl GL, Svoboda KK, Reenstra WR. Hyperbaric oxygen downregulates ICAM-1 expression induced by hypoxia and hypoglycemia: the role of NOS. *Am J Physiol Cell Physiol*. 2000; 278:C292–302. [PubMed: 10666024]
- Celeste A, Fernandez-Capetillo O, Kruhlak MJ, Pilch DR, Staudt DW, Lee A, et al. Histone H2AX phosphorylation is dispensable for the initial recognition of DNA breaks. *Nat Cell Biol*. 2003; 5:675–9. [PubMed: 12792649]
- Chan ED, Riches DW. IFN-gamma + LPS induction of iNOS is modulated by ERK, JNK/SAPK, and p38(mapk) in a mouse macrophage cell line. *Am J Physiol Cell Physiol*. 2001; 280:C441–50. [PubMed: 11171562]
- Chandel NS, Trzyna WC, McClintock DS, Schumacker PT. Role of oxidants in NF-kappa B activation and TNF-alpha gene transcription induced by hypoxia and endotoxin. *J Immunol*. 2000; 165:1013–21. [PubMed: 10878378]
- Chung HY, Sung B, Jung KJ, Zou Y, Yu BP. The molecular inflammatory process in aging. *Antioxid Redox Signal*. 2006; 8:572–81. [PubMed: 16677101]
- Comalada M, Xaus J, Valledor AF, Lopez-Lopez C, Pennington DJ, Celada A. PKC epsilon is involved in JNK activation that mediates LPS-induced TNF-alpha, which induces apoptosis in macrophages. *Am J Physiol Cell Physiol*. 2003; 285:C1235–45. [PubMed: 12867362]
- Connelly L, Jacobs AT, Palacios-Callender M, Moncada S, Hobbs AJ. Macrophage endothelial nitric-oxide synthase autoregulates cellular activation and pro-inflammatory protein expression. *J Biol Chem*. 2003; 278:26480–7. [PubMed: 12740377]
- Davies MJ, Gilbert BC, Haywood RM. Radical-induced damage to proteins: e.s.r. spin-trapping studies. *Free Radic Res Commun*. 1991; 15:111–27. [PubMed: 1661698]
- Finkelstein E, Rosen GM, Rauckman EJ, Paxton J. Spin trapping of superoxide. *Mol Pharmacol*. 1979; 16:676–85. [PubMed: 229403]
- Floyd RA, Kopke RD, Choi CH, Foster SB, Doblas S, Towner RA. Nitrones as therapeutics. *Free Radic Biol Med*. 2008; 45:1361–74. [PubMed: 18793715]
- Gloire G, Legrand-Poels S, Piette J. NF-kappaB activation by reactive oxygen species: fifteen years later. *Biochem Pharmacol*. 2006; 72:1493–505. [PubMed: 16723122]
- Gomez-Mejiba SE, Zhai Z, Gimenez MS, Ashby MT, Chilakapati J, Kitchin K, et al. Myeloperoxidase-induced genomic DNA-centered radicals. *J Biol Chem*. 2010; 285:20062–71. [PubMed: 20406811]
- Gotoh T, Oyadomari S, Mori K, Mori M. Nitric oxide-induced apoptosis in RAW 264.7 macrophages is mediated by endoplasmic reticulum stress pathway involving ATF6 and CHOP. *J Biol Chem*. 2002; 277:12343–50. [PubMed: 11805088]
- Guha M, Mackman N. LPS induction of gene expression in human monocytes. *Cell Signal*. 2001; 13:85–94. [PubMed: 11257452]
- Gupta SC, Sundaram C, Reuter S, Aggarwal BB. Inhibiting NF-kappaB activation by small molecules as a therapeutic strategy. *Biochim Biophys Acta*. 2010; 1799:775–87. [PubMed: 20493977]
- Gurjar MV, Deleon J, Sharma RV, Bhalla RC. Role of reactive oxygen species in IL-1 beta-stimulated sustained ERK activation and MMP-9 induction. *Am J Physiol Heart Circ Physiol*. 2001; 281:H2568–74. [PubMed: 11709424]
- Hamburger SA, McCay PB. Endotoxin-induced mortality in rats is reduced by nitrones. *Circ Shock*. 1989; 29:329–34. [PubMed: 2688966]

- Hawkins CL, Davies MJ. Hypochlorite-induced damage to proteins: formation of nitrogen-centred radicals from lysine residues and their role in protein fragmentation. *Biochem J.* 1998; 332(Pt 3): 617–25. [PubMed: 9620862]
- Hoyal CR, Gutierrez A, Young BM, Catz SD, Lin JH, Tsuchis PN, et al. Modulation of p47PHOX activity by site-specific phosphorylation: Akt-dependent activation of the NADPH oxidase. *Proc Natl Acad Sci U S A.* 2003; 100:5130–5. [PubMed: 12704229]
- Jang SI, Kim HJ, Kim YJ, Jeong SI, You YO. Tanshinone IIA inhibits LPS-induced NF-kappaB activation in RAW 264.7 cells: possible involvement of the NIK-IKK, ERK1/2, p38 and JNK pathways. *Eur J Pharmacol.* 2006; 542:1–7. [PubMed: 16797002]
- Jones E, Adcock IM, Ahmed BY, Punchard NA. Modulation of LPS stimulated NF-kappaB mediated Nitric Oxide production by PKCepsilon and JAK2 in RAW macrophages. *J Inflamm (Lond).* 2007; 4:23. [PubMed: 18036230]
- Kim JH, Bae CH, Park SY, Lee SJ, Kim Y. Uncaria rhynchophylla inhibits the production of nitric oxide and interleukin-1beta through blocking nuclear factor kappaB, Akt, and mitogen-activated protein kinase activation in macrophages. *J Med Food.* 2010; 13:1133–40. [PubMed: 20828308]
- Kotake Y. Pharmacologic properties of phenyl N-tert-butyl nitron. *Antioxid Redox Signal.* 1999; 1:481–99. [PubMed: 11233146]
- Lim S, Clement MV. Phosphorylation of the survival kinase Akt by superoxide is dependent on an ascorbate-reversible oxidation of PTEN. *Free Radic Biol Med.* 2007; 42:1178–92. [PubMed: 17382199]
- de Menezes, S Lopes; Augusto, O. EPR detection of glutathionyl and protein-tyrosyl radicals during the interaction of peroxynitrite with macrophages (J774). *J Biol Chem.* 2001; 276:39879–84. [PubMed: 11518708]
- Madrid LV, Mayo MW, Reuther JY, Baldwin AS Jr. Akt stimulates the transactivation potential of the RelA/p65 Subunit of NF-kappa B through utilization of the Ikappa B kinase and activation of the mitogen-activated protein kinase p38. *J Biol Chem.* 2001; 276:18934–40. [PubMed: 11259436]

Fig. 1.

DMPO reduces LPS-induced oxidative damage and cell death. RAW 264.7 cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 24 h. (A) Cell viability was assessed by the MTT assay. (B) Protein nitration as a marker of protein oxidative modification was determined by ELISA. (C) Equal amounts of whole cell lysates were prepared and analyzed by Western blot with antibodies against p53, Chk1, and phospho-histone H2AX. The detection of these proteins was estimated with histone H2B as a loading control. (D) As a marker of cell death, LDH release into culture medium was measured using a colorimetric assay. The data are representative or expressed as the mean $\pm$ SEM of two independent experiments run in triplicate. * $P < 0.05$ vs the baseline control; # $P < 0.05$ vs the LPS treatment group.

Fig. 2.

DMPO reduces LPS-induced production of inflammatory cytokines. RAW 264.7 cells were incubated with LPS (1 ng/ml) and/or DMPO (50 mM) for 24 h, then culture supernatants were collected for determination of TNF-α (A), IL-1β (B), IL-6 (C), KC (D), IL-10 (E), IL-12p70 (F), and GM-CSF (G) by commercial assay kits. The data are expressed as the mean $\pm$ SEM of four independent experiments run in duplicate. * $P < 0.05$ vs the baseline control; ** $P < 0.001$ vs the baseline control; # $P < 0.05$ vs the LPS treatment group.

Fig. 3. DMPO reduces LPS-induced NO production and iNOS protein and mRNA expression. (A) Time course of NO production. RAW 264.7 cells were treated with LPS (1 ng/ml) in the presence or absence of DMPO (50 mM) for different time points, and the nitrite accumulation in culture medium was determined by the Griess reaction. (B) Time course of inhibition of NO production by DMPO. Cells were incubated in the presence or absence of LPS (1 ng/ml) for 24 h. DMPO (50 mM) was either added simultaneously with LPS (0 h) or up to 24 h after addition of LPS. The nitrite accumulation in the culture medium was determined 24 h after LPS stimulation. (C) Cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 24 h, then whole cell lysates were assayed by Western blot for iNOS expression. (D) Cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 6 h. Total RNA was extracted and assayed for iNOS mRNA expression by real time RT-PCR analysis. The data are representative or expressed as the mean $\pm$ SEM of two or three independent experiments run in triplicate. * $P < 0.05$ vs the baseline control; # $P < 0.05$ vs the LPS treatment group.

Fig. 4. DMPO reduces LPS-induced nuclear translocation of NF-κB p65, degradation of IκBα, and phosphorylation of ERK1/2, JNK, p38, and Akt. (A) RAW 264.7 cells were incubated with LPS (1 ng/ml) and/or DMPO for 1 h, then cytosolic and nuclear fractions were isolated and assayed for NF-κB p65 by Western blot. (B) RAW 264.7 cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 15 min, then equal amounts of whole cell lysates were assayed for IκBα degradation by Western blot. (C) RAW 264.7 cells were incubated with LPS (1 ng/ml) and/or DMPO for 15 min, then whole cell lysates were prepared and subjected to Western blot analysis for MAPKs and Akt. Western blot detection of respective total kinase was considered to be a loading control. The data are representative of at least two experiments.

Fig. 5. DMPO reduces LPS-induced intracellular ROS and superoxide production. (A) Intracellular ROS production was determined by carboxy- H_2DCFDA , a fluorogenic probe for general ROS. (B) Superoxide production was determined by DHE staining and analyzed by flow cytometry. The data are expressed as the mean $\pm$ SEM of two independent experiments run in triplicate. * $P < 0.05$ vs the baseline control; # $P < 0.05$ vs the LPS treatment group.