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The Nitrone Spin Trap 5,5-dimethyl-1-pyrroline *N*-oxide Affects Stress Response and Fate of Lipopolysaccharide-primed RAW 264.7 Macrophage Cells

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Abstract

The nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) is commonly used to study free radicals. Due to its free radical trapping properties, DMPO is thought to reduce free radial-mediated oxidative damage and other related cellular responses. The purpose of this study was to assess the effect of DMPO on lipopolysaccharide (LPS)-induced inflammation, endoplasmic reticulum (ER)-stress, and apoptosis in RAW 264.7 cells. The results showed that DMPO at 50 mM inhibited inducible nitric oxide synthase expression when added shortly after LPS treatment (≤3 h). Interestingly, DMPO increased anti-inflammatory heme oxygenase-1 (HO-1) expression and reversed LPS-induced decrease in HO-1 expression. LPS could increase cellular ER stress as indicated by CHOP induction; DMPO reduced LPS effect on CHOP expression. Unexpectedly, DMPO had a synergistic effect with LPS on increased caspase-3 activity. Overall, DMPO harbors multiple modulating effects, but may induce apoptosis in LPS-stressed cells when given at 50 mM, an effective dose for its anti-inflammatory activity *in vitro*. Our data provide clues for further understanding of DMPO, the old nitrone spin trap with therapeutic potential.

Keywords

apoptosis; 5,5-dimethyl-1-pyrroline *N*-oxide; ER stress; inflammation; lipopolysaccharide

INTRODUCTION

Reactive chemical species including free radicals and non-free radical species have a significant role in cell signaling and tissue damage [1]. Nitrone spin traps are a group of

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CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest.

synthetic chemicals initially designed for stabilizing free radicals to allow their detection by electron spin resonance (ESR) spectroscopy [2]. The nitrone spin trap 5,5-dimethyl-1-pyrroline *N*-oxide (DMPO) was first introduced by Janzen and his colleagues about 40 years ago [3]. Since then, DMPO has been widely used in the ESR spin trapping technique and is becoming the most popular nitrone spin trap in free radical research [4, 5]. DMPO received extra attention during the last 10 years due to the development of an anti-DMPO antibody, which has largely expanded the application of DMPO in the study of free radicals in biological systems ([6] and references cited therein). Today, traditional immunoassays (e.g., immunofluorenscence and Western blot) in combination with anti-DMPO antibody can be used as an alternative and convenient methodology to detect free radicals *in vitro* and also *in vivo* [6–9].

Free radicals are highly reactive and therefore very short half-lived. In order to overcome the radical decay mechanism, high concentrations (e.g., 200–500 mM) of DMPO are added immediately before or during the formation of free radicals to trap them and allow the accumulation of DMPO-radical adducts [6]. Free radicals and other reactive oxygen species (ROS) are ubiquitous in the living cells and are involved in various cellular processes. Consequently, it is likely that high concentrations of DMPO used to trap free radicals in cell systems can alter cell physiology and cell response to stress. On the other hand, overproduction of free radicals and other ROS is known to cause oxidative stress, which is associated with inflammation, endoplasmic reticulum (ER) stress, and apoptosis that affect cell homeostasis and fate [10]. Therefore, by trapping free radicals and blocking free radical chain reactions, DMPO, at appropriate doses, may reduce free radical-mediated oxidative stress and cell damage [11]. In this regard, nitrone spin straps have been actively studied for their potential therapeutic values as anti-oxidant and anti-inflammatory agents. Notable examples of this kind of spin traps include the phenyl-*N*-*tert*-butylnitrone (PBN) and its derivatives, which produced several protective effects in animal models of disease and injury, including endotoxic shock and intracerebral glioma [12, 13]. Besides, PBN was shown to significantly slow down the aging process in animals by reducing age-associated oxidative damage [14].

The evidence for the pharmacological activities of DMPO is relatively limited. It has been reported that DMPO reduces the mortality resulting from endotoxic shock in a rat model of sepsis [15], and protects against reperfusion-induced injury or arrhythmias in isolated rat heart models [16, 17]. We recently demonstrated that DMPO inhibited lipopolysaccharide (LPS)-induced ROS production and inflammatory responses, and thereby protected cells from LPS-induced cell damage in a macrophage cell line [18, 19]. However, the effects of DMPO on other LPS-related cellular responses such as ER stress and apoptosis are unknown. Therefore, the main purpose of this study was to investigate the effects of DMPO on LPS-induced cellular stress responses, such as ER stress and apoptosis.

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% CO_2 incubator. Cells less than 25 passages were used in this study.

LPS and DMPO Treatments

LPS (*Escherichia coli* serotype 055:B5, L2637) was from Sigma (St. Louis, MO). DMPO was from Alexis Biochemicals (San Diego, CA). DMPO stock aliquots (10 M) were stored under an argon atmosphere at −80°C until use. Cells were allowed to attach to the culture ware for at least 2 h before exposure to DMPO and/or LPS.

Nitric Oxide Assay

RAW 264.7 cells (1.6×10^6 per well) were treated with 1 ng/ml LPS and/or 50 mM DMPO for 24 h. Culture medium was collected for the determination of both nitrite and nitrate as indicators of nitric oxide (˙NO) production using a commercial Nitric Oxide Colorimetric Assay kit from BioVision (Mountain View, CA). In a second set of experiments, RAW 264.7 cells were pretreated with 50 mM DMPO for 4 h, then washed with phosphate buffered saline (PBS) to remove extracellular DMPO, and LPS (1 ng/ml) was added and cells continued incubation for another 20 h. Nitrite accumulation in the culture medium was determined using the classical Griess reaction.

Western Blot Analysis

Following treatments with LPS and/or DMPO for the indicated times, RAW 264.7 cells were rinsed with PBS, cell lysates were prepared, and Western blot analysis was performed as described previously [18]. The following primary antibodies were used: pro-caspase-3, cleaved caspase-3 (Asp175), protein kinase-like endoplasmic reticulum kinase (PERK), inositol-requiring enzyme 1-alpha (IRE1α), binding immunoglobulin protein (BiP), protein disulfide isomerase (PDI) (Cell Signaling Technology, Danvers, MA), heme oxygenase-1 (HO-1), 14-3-3 (Epitomics, Burlingame, CA), iNOS, β-actin, α-tubulin (Sigma), Bcl-2 (Abcam, Cambridge, MA), and GADD13 (CHOP) (Santa Cruz Biotechnology, Santa Cruz, CA). All primary antibodies were diluted at 1:1000 except for anti-CHOP at 1:500.

Confocal Imaging

Following LPS and/or DMPO treatment, cells were washed and stained with ER-Tracker Red dye (Molecular Probes, Eugene, OR) in HBSS for 20 min at 37°C. Stained cells were fixed in 4% paraformaldehyde and then serially incubated with rabbit anti-DMPO antibody (Alexis Biochemicals) and goat anti-rabbit Alexa Fluor 488 (Invitrogen, Carlsbad, CA). Finally, cells were rinsed and mounted on cover glasses with Prolong Gold antifade reagent with 4′-6-diamidino-2-phenylindole (DAPI, Invitrogen), and the immunostaining examined by confocal imaging using a Leica SP2 MP Confocal Microscopy.

Caspase-3 Activity Assay

After treated with LPS and/or DMPO for 24 h, RAW 264.7 cells were lysed for 20 min in the chilled cell lysis buffer provided with the Caspase-3/CPP32 Colorimetric Assay kit (BioVision). Following spinning at 12,000 g for 10 min at 4°C, the cytosolic fraction was tested for caspase-3 activity using the commercial assay kit.

Annexin V Staining

Following treatments with LPS and/or DMPO overnight, RAW 264.7 cells were incubated in accutase-enzyme cell detachment medium (eBioscience, San Diego, CA) for 10 min. Then, cells were serially stained with fluorochrome-conjugated Annexin V and propidium iodide (PI) according to the Annexin V Apoptosis Detection kit's instruction (eBioscience). Apoptotic cells were analyzed on the BD FACSAria flow cytometer (BD Biosciences, San Jose, CA) within 1 h.

Protein Assay

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

Statistical Analysis

Results are expressed as mean values of 2–4 assays ± SEM. Differences between the treatment groups were tested by two-way analysis of variance (group×experiment) using the Statistix software (Analytical software, Tallahassee, FL). A difference between the groups with a $P<0.05$ was considered as significant.

RESULTS

Previous studies have shown that DMPO at 50 mM had no remarkable cytotoxicity, and provided maximal protection from LPS-induced cell damage [19]. Based on those data, in the present study we used a similar condition to assess the effect of DMPO on LPS-induced cellular stress response in RAW 264.7 cells.

DMPO Reduces LPS-induced ·NO Production and iNOS Expression

LPS activates RAW 264.7 macrophages to produce ROS and inflammatory mediators including ·NO [20, 21]. ·NO is rapidly oxidized to nitrite and nitrate, which were measured in the culture medium following treatment of RAW 264.7 cells with 1 ng/ml LPS and/or 50 mM DMPO for 24 h. In agreement with a previous report [22], we found that LPS induced nitrite and nitrate accumulation in culture medium at a ratio of approximately 3:2. DMPO reduced the production of nitrite and nitrate, but did not affect the nitrite/nitrate ratio (Fig. 1A). To further test the effect of DMPO on LPS-induced ·NO production, RAW 264.7 cells were pre-incubated with DMPO for 4 h and then rinsed to remove DMPO. LPS was subsequently added to stimulate cells for another 20 h. The results showed that DMPO pre-incubation significantly reduced LPS-induced ·NO production (Fig. 1B). To test the most effective time point for addition of DMPO in the regulation of iNOS expression, RAW 264.7 cells were treated with 1 ng/ml LPS for 24 h. In these experiments, DMPO was either

added simultaneously with LPS or up to 24 h after addition of LPS. Western blot analysis of iNOS showed that DMPO caused a strong inhibition of iNOS expression when it was added within 3 h after LPS was added. DMPO showed little effect when added 6 h after LPS stimulation (Fig. 1C), suggesting that DMPO inhibits LPS-induced iNOS gene induction.

DMPO Regulates HO-1 Expression

The iNOS expression is controlled mainly by the redox-sensitive transcriptional factor NF-κB, however, it can also be regulated by other mechanisms as well, including c-Jun N-terminal kinase (JNK) and HO-1 [23, 24]. JNK is rapidly phosphorylated following LPS stimulation. We demonstrated that DMPO at 25 mM inhibited LPS-induced JNK phosphorylation with a similar effectiveness as SP600125 (20 μM, Sigma), a pharmacological inhibitor of JNK (data not shown). HO-1 is an inducible anti-inflammatory molecule by LPS in mouse peritoneal macrophages and RAW 264.7 cells [24, 25]. HO-1 is an important feedback negative regulatory mechanism following LPS treatment. To determine the effect of DMPO on HO-1 expression, RAW 264.7 cells were treated with LPS and/or DMPO for 24 h. We found that treatment of the cells with LPS for 24 h caused low expression of HO-1. Interestingly, DMPO blocked the LPS effect on HO-1 expression, and profoundly increased HO-1 expression when it was added alone (Fig, 1D), consistent with the anti-inflammatory property of DMPO. To investigate the causes for the decreased expression of HO-1 by LPS, we treated RAW 264.7 cells with LPS for different time points, and HO-1 protein expression was analyzed by Western blot. The results showed that HO-1 expression was variable, with a decrease at 3–9 h, an increase at 12–18 h, and again a decrease at 24 h (Fig. 1E), confirming the observation in Fig. 1D.

DMPO Affects LPS-induced ER Stress

LPS is known to induce ER stress in macrophages as exemplified by the induction of the transcription factor CHOP [26]. Our previous study suggested that ER might be an important cellular compartment for the generation of protein-centered radicals following LPS treatment [19]. Therefore, we determined the co-location of ER and protein-centered radicals using ER-Tracker dye and immunostaining with anti-DMPO antibody (Fig 2A). The appearance of nitrone adducts inside cells treated with LPS and DMPO indicates increased oxidative damage and possible ER stress following LPS treatment. To determine the effect of DMPO on LPS-induced ER stress, RAW 264.7 cells were treated with LPS and/or DMPO for 24 h, and several ER stress-associated proteins including PERK, BiP, IRE1α, PDI and CHOP were measured by Western blot (Fig. 2B). Our results showed that treatment with DMPO reduced the baseline expression levels of CHOP and IRE1α. LPS decreased PERK expression, but, as expected, it induced CHOP synthesis. DMPO partially prevented LPS-induced decrease in PERK expression and significantly inhibited LPS induced CHOP synthesis (Fig. 2C). Interestingly, although DMPO caused a slight decrease in the basal expression of IRE1α, it enhanced LPS-induced IRE1α expression. The chaperone PDI and BiP were not affected by any of the treatments with respect to the no treatment control.

DMPO Inhibits LPS Effect on 14-3-3 and Bcl-2 Expression

It has been suggested that LPS-induced oxidative stress, inflammation, and ER stress may lead to apoptosis, a process characterized by an imbalance of the expression of pro- and anti-

apoptotic proteins [27]. Therefore, we examined two anti-apoptotic proteins, 14-3-3-and Bcl-2, in LPS-treated cells. As shown in Fig. 3A, LPS decreased the expression of both anti-apoptotic proteins, however, the expression levels of these two proteins was restored to the control levels when DMPO was present in the culture medium.

DMPO Increases LPS Effect on Capase-3 Activity and Apoptosis

Caspase-3 is an important executer of the apoptotic program [28]. Therefore, we examined the cleavage of pro-caspase-3 and the formation of active caspase-3 in cells treated with LPS and/or DMPO for 24 h. As shown in Fig. 3B, LPS increased the cleaved caspase-3 with a consequent diminution of pro-caspase-3. Curiously, DMPO enhanced LPS effect on caspase-3 activation. To confirm this observation, we further determined the caspase-3 enzyme activity. As expected, our data were consistent with a synergistic effect of DMPO on LPS-induced caspase-3 activation (Fig. 3C). Finally, cell death was analyzed by using the Annexin V and PI staining. Generally, the number of the Annexin V positive/PI negative cells and that of the Annexin V positive/PI positive cells showed a similar increasing trend following DMPO and LPS exposure. However, since the number of the Annexin V positive/PI negative cells was less than one tenth of that of the Annexin V positive/PI positive cells in all treatment groups, apoptosis was no longer separated and only the data of Annexin V positive cells are shown (Fig. 3D). It was found that both LPS and DMPO induced cells to undergo the apoptotic program. However, their combined effect did not reach significance.

DISCUSSION

LPS priming of macrophages activates the redox-sensitive NF-κB [21, 29], leading to the transcription of various genes encoding inflammatory mediators and enzymes (e.g., TNF-α and iNOS). These mediators and enzymes may amplify inflammatory signaling and cause cell damage via autocrine and paracrine routes. Western blot analysis of iNOS expression in cells to which DMPO was added at different time points after LPS stimulation supports the notion that DMPO disturbs LPS-initiated early signaling, which is the basis for its anti-inflammatory activities including the decreased TNF-α and ·NO production.

HO-1 is the key enzyme in the degradation of heme into biliverdin, iron, and carbon monoxide (CO). HO-1 and its byproducts are known to participate in the negative feedback regulation of inflammatory responses [30, 31]. Increasing evidence support that HO-1 induction is closely related to the negative control of iNOS expression and ·NO production in LPS-activated macrophages [24, 25, 32]. The inhibition of iNOS by HO-1 is thought to go through several regulatory mechanisms including degrading heme needed for iNOS synthesis and CO-binding to iNOS leading to the blockage of ·NO production [25]. The upregulation of HO-1 function has been found to be a common action mechanism of many anti-inflammatory agents and natural products [32, 33]. In this regard, DMPO anti-inflammatory effects may operate by increasing the baseline HO-1 expression in RAW 264.7 cells and inhibiting LPS-mediated decrease in HO-1 expression. The decreased HO-1 expression in RAW 264.7 cells exposed to LPS for 24 h was unexpected because LPS was seen to induce HO-1 in both primary macrophages and RAW 264.7 cells [24, 25, 32]. These

observations are in apparent discrepancy with our data. Consequently, we determined the HO-1 expression in RAW 264.7 cells treated with LPS for different time points, and found that HO-1 expression was highly variable in a time-dependent pattern (Fig. 3B). This observation is in agreement with previous reports [24, 25] and may explain the apparent discrepancy as we mentioned before. For instance, Srisook et al. [25] reported that LPS induced HO-1 protein expression in a biphasic manner, a small peak at 1–2 h and a marked peak between 10–24 h following LPS treatment. These authors suggested that superoxide radical anion produced shortly upon LPS stimulation is responsible for the first phase HO-1 expression and ·NO whose production starts at 6 h is responsible for the significant second-phase HO-1 expression [25]. Therefore, HO-1 induction reflects altered oxidative status and cell response to oxidative damage [34].

ER is the site for folding and refolding of newly synthesized proteins to be secreted. Any condition that interferes with the normal ER functions may lead to accumulation of toxic protein aggregates that cause ER stress. For example, activation of macrophages with LPS causes ER stress [35]. To deal with the ER stress, the cells activate the unfolded protein response pathway mediated through ER-transmembrane protein factors including PERK and IRE1α [10]. BiP, an ER chaperone, negatively regulates PERK and IRE1α by binding and stabilizing them in their inactive state. In ER stress, PDI is induced to assist protein post-translational folding events. Activation and/or upregulation of these ER stress molecules lead to the expression of pro-apoptotic proteins (e.g., CHOP) that trigger inflammation and apoptosis [26, 36]. We found that DMPO and LPS had different effects on these ER stress markers. DMPO alone decreased IRE1α and CHOP expression, suggesting that DMPO may modulate baseline ER stress markers (Fig. 2B). On the other hand, we found that LPS significantly reduced PERK expression, but this effect was inhibited by DMPO. Although DMPO enhanced LPS-induced IRE1α expression, it decreased CHOP expression, indicating that DMPO at 50 mM consistently protected cells from LPS-induced ER stress. It should be noted that to gain more understanding of the modulating effects of DMPO on the ER stress, it would be necessary to further determine the phosphorylation levels of ER stress markers and other pathways that contribute to CHOP induction.

LPS-initiated inflammatory signaling and CHOP induction modulate Bcl-2 expression and induce apoptosis [37, 38]. In the apoptosis cascades, caspase-3 is an executioner of apoptosis. Proteolytic cleavage of procaspase-3 leads to caspase-3 activation, which cleaves over hundred different cellular proteins that ultimately result in apoptotic cell death [28]. We found that DMPO reversed LPS-induced decrease in the expression of 14-3-3 and Bcl-2, suggesting an anti-apoptotic effect of DMPO. However, DMPO increased caspase-3 activation and the number of the Annexin V-positive cells, and had a synergistic effect with LPS-induced caspase-3 activity. These evidences suggest that DMPO, under the conditions used in our experiments, was somewhat pro-apoptotic. Since DMPO was simultaneously added with LPS, our apoptosis data more likely reflect an early effect of DMPO on the secretion and further autocrine/paracrine pro-apoptotic effect of TNF-α, rather than the late NO pro-apoptotic effect, which would require iNOS expression. Notably, the protective effects of DMPO against LPS-induced cell death have been assessed by different methods such as MTT reduction, Trypan blue exclusion, and lactate dehydrogenase release [18, 19]. It is interesting in the future to clarify why DMPO protected cells from LPS-induced death

while it synergistically induced caspase-3 activation. It is possible that DMPO activates some potent survival signaling pathways in the meantime that outcompete the apoptosis mechanisms.

Taken together, our data indicate that the nitrone spin trap DMPO modulates a wide range of LPS-induced cellular responses, putatively through trapping free radicals and interfering with ROS production. DMPO has been suggested to be robustly anti-inflammatory, but it may be pro-apoptotic, especially when used at high doses.

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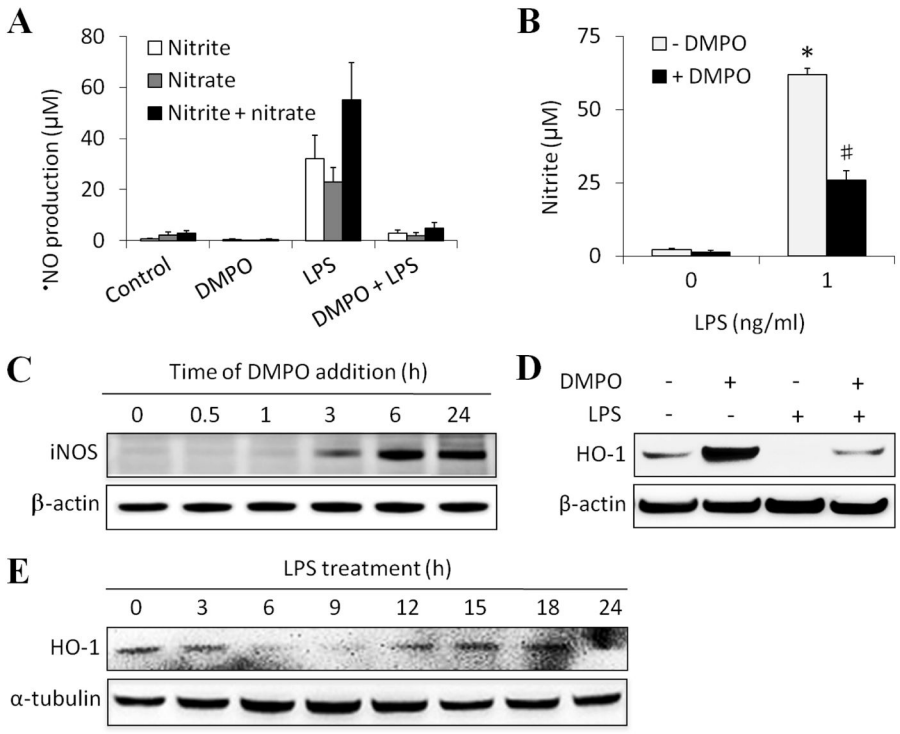

Fig. 1. DMPO modulates iNOS and HO-1 expression in LPS-treated RAW 164.7 cells

(A) RAW 264.7 cells were incubated in 6-well plates with LPS (1 ng/ml) and/or DMPO (50 mM) for 24 h, then culture supernatants were collected for the determination of nitrite and nitrate by using a colorimetric assay. (B) The cells were pre-incubated with 50 mM DMPO for 4 h in 96-well plates, then rinsed to remove DMPO and subsequently incubated with 1ng/ml LPS for another 20 h. Supernatants were collected for nitrite measurement by using the Griess assay. (C) Western blot analysis of iNOS expression in cells incubated with 1 ng/ml LPS and/or 50 mM DMPO. DMPO was added simultaneously with LPS (0 h) or at different times after LPS addition (0.5, 1, 3, 6 or 24 h). After incubation for total 24 h following LPS treatment, cells were harvested and the iNOS expression was assessed in the cell homogenates. Note that the addition of DMPO at 0, 0.5, or 1 h after LPS addition (or 24, 23.5, 23 h before harvesting the cells) resulted in no expression of iNOS, whereas DMPO added 3 or 6 h after LPS treatment (or 21 or 18 h before harvesting the cells) resulted in the decreased or no effect on iNOS expression. (D) RAW 264.7 cells were treated with LPS (1 ng/ml) and/or DMPO (50 mM) for 24 h, HO-1 expression was assessed by Western blot analysis with β-actin as a loading control. (E) Western blot analysis of HO-1 expression in cells treated with 1ng/ml LPS for different times. HO-1 expression was estimated with α-tubulin as a loading control. Data are shown as mean values ± SEM of 2–4 experiments run in duplicate. **P*<0.05 vs baseline control; #P<0.05 vs LPS treated group.

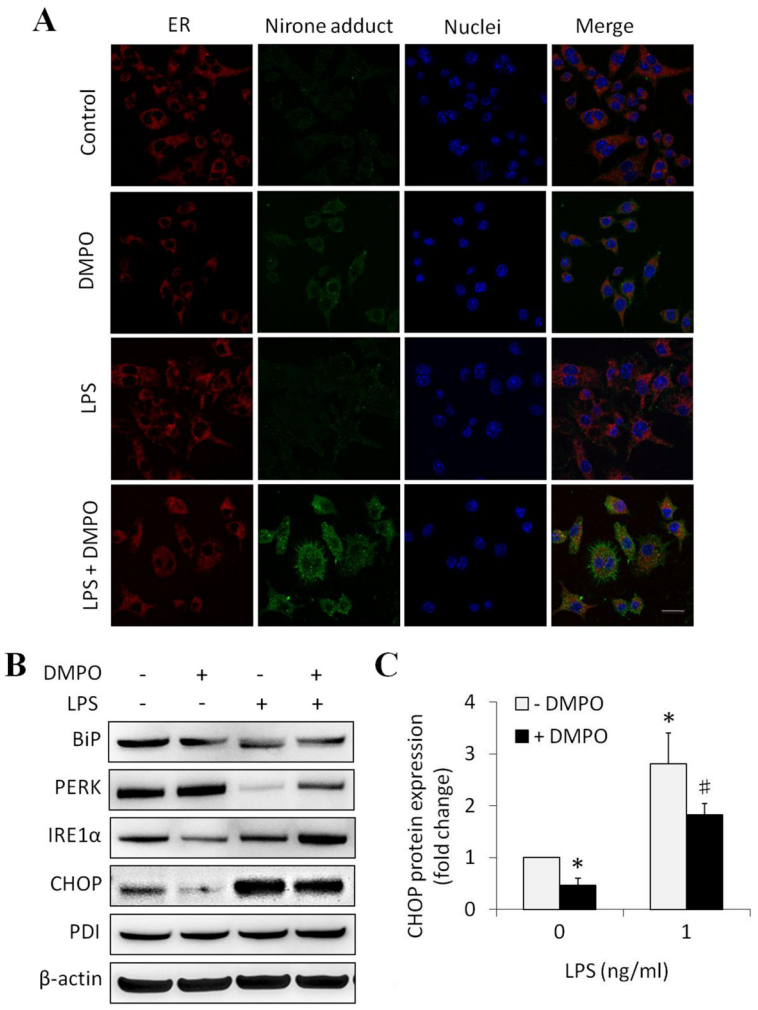

Fig. 2. DMPO modulates LPS-induced ER stress in RAW 264.7 cells

(A) Following treatments with LPS and/or DMPO for 24 h, living cells were stained with ER-Tracker red dye (red) and then fixed. Fixed cells were visualized by confocal imaging of cells immunostained for nucleus and nitrone adducts using DAPI (blue) and Alexa Fluor 488 secondary antibody conjugates (green), respectively. Scale bar: 20 μm. (B) Cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 24 h. Equal amounts of cell lysates were prepared and analyzed by Western blot for ER stress markers, i.e., BiP, PERK, IRE1a, CHOP, and PDI. Protein expression of these ER stress markers was estimated using β-actin as a loading control. (C) The CHOP expression was digitally quantitated using the ImageJ software. Data are shown as mean values ± SEM of 4 experiments. **P*<0.05 vs baseline control; #P<0.05 vs LPS treated group.

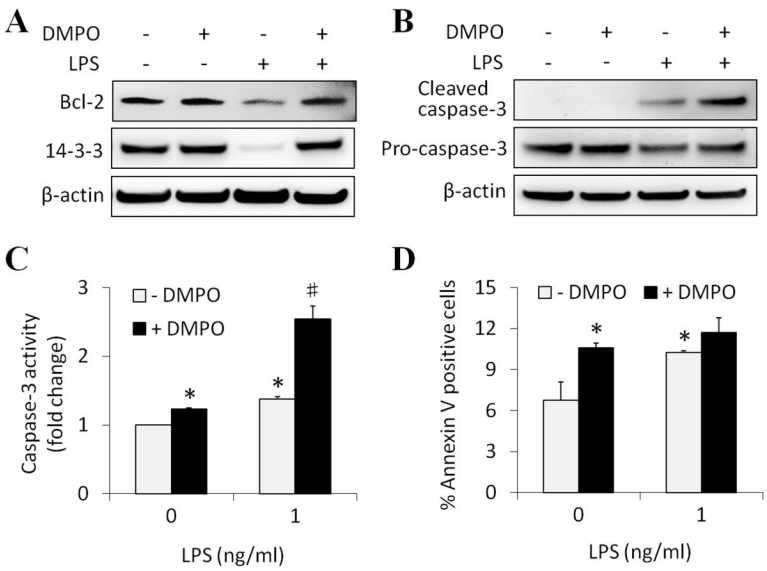

Fig. 3. Effect of DMPO on anti-apoptotic and apoptotic markers in LPS-treated macrophages

RAW 264.7 cells were treated with LPS (1 ng/ml) and DMPO (50 mM) for 24 h. (A) Equal amounts of cell lysates were analyzed by Western blot for the anti-apoptotic markers 14-3-3 and Bcl-2. (B) Caspase -3 cleavage was analyzed in cell lysates by Western blotting and estimated with β-actin as a loading control. (C) Caspase-3 activity was measured by using a colorimetric assay. (D) Cell death was assessed by Annexin V staining and analyzed by flow cytometry. Data are shown as mean values ± SEM of 2–3 independent experiments run in triplicate. **P*<0.05 vs baseline control; #*P*<0.05 vs LPS treatment control.