# Determining the neuroprotective effects of dextromethorphan in lipopolysaccharide-stimulated BV2 microglia

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**Abstract.** Microglial activation has been recognized as being vital in the pathogenesis of several neurodegenerative disorders. Therefore, the identification of therapeutic drugs to prevent microglial activation and thus protect against inflammation-mediated neuronal injury, is required. In the present study, dextromethorphan (DM), a compound widely used in antitussive remedies that has been demonstrated to possess neuroprotective effects, was shown to reduce proinflammatory mediator production in lipopolysaccharide (LPS)-stimulated BV2 mouse microglial cells. Western blot analysis revealed that DM markedly suppressed the activation of nuclear factor-κB (NFκB), caspase-3 signaling and the expression of another inflammation-inducing factor, heat shock protein 60 (HSP60) and heat shock factor-1, induced by LPS in BV2 cells. Results from ELISA assay demonstrated that DM reduced the release of HSP60, nitric oxide (NO), inducible NO synthase, tumor necrosis factor-α, interleukin (IL)-1β and IL-6 induced by LPS in BV2 microglia. These results were confirmed by immunofluorescence, suggesting that DM may exert a neuroprotective and anti-inflammatory effect by inhibiting microglial activation through the HSP60-NFκB signaling pathway. Therefore, DM may offer substantial therapeutic benefits in the treatment of neurodegenerative diseases that are accompanied by microglial activation.

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## Introduction

Microglial cells, resident brain macrophages of the central nervous system (CNS), are pivotal in the pathogenesis of several neurodegenerative diseases (1). Microglia are activated in response to stress, and are involved in innate and adaptive immune responses by inducing the production of various pro-inflammatory mediators, including nitric oxide (NO), inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β), IL-6, nuclear factor-κB (NFκB), caspase-3 and heat shock protein (HSP) 60 (2-6), all of which contribute to neurodegeneration (7,8). A number of microglia-targeted pharmacotherapies, such as protein kinase C inhibitors and microglia-inhibiting factors, have been proposed to suppress microglial activation and promote neuronal survival *in vivo* (9-11). However, the inability of these drugs to penetrate the blood-brain barrier and the complexity of current pharmacological agents, as well as the possible side effects, has halted long-term clinical use in the treatment and prevention of diseases of the CNS (12).

Dextromethorphan (DM), a derivative of morphinan, is one of the most widely used non-opioid cough suppressants, acting as the active ingredient in numerous antitussive formulations (13). As an antitussive, DM is superior to opioids used at antitussive doses in that DM lacks any gastrointestinal side effects, such as constipation, and produces a lower degree of depression of the CNS. DM is rapidly absorbed from the gastrointestinal tract, where it enters the bloodstream and crosses the blood-brain barrier (14). The anticonvulsant and neuroprotective properties of DM have been demonstrated, and treatment with DM has been shown to improve the cerebrovascular and functional consequences of global cerebral ischemia (14). However, the mechanisms underlying the neuroprotective effects of DM remain poorly understood.

Previous studies have demonstrated that naloxone, another analogue of morphinan, protects against lipopolysaccharide (LPS)-induced neurotoxicity *in vitro* and *in vivo* through the inhibition of the release of proinflammatory factors and free radicals (15-18). DM is structurally similar to naloxone and has been shown to protect against LPS-induced dopamine

neurodegeneration in mixed neuron-glia coculture through the inhibition of microglial overactivation, and the subsequent reduction in the levels of proinflammatory cytokines, free radicals and reactive oxygen species (19). In the present study, the DeltaVision Elite microscopy imaging system was used to analyze the effects of DM on the production of proinflammatory mediators.

## Materials and methods

*Materials*. The following reagents were used in this study: DM and LPS (Sigma-Aldrich, St. Louis, MO, USA); rabbit monoclonal antibodies against β-actin and NF-κB p65 (Abcam, Cambridge, MA, USA); mouse monoclonal anti-HSP60 and anti-heat shock factor 1 (HSF1) antibodies (Stressgen®, San Diego, CA, USA; Enzo Life Sciences, Inc., Farmingdale, NY, USA); rabbit polyclonal anti-caspase-3 antibody (Cell Signaling Technology, Inc., Beverly, MA, USA); proteinase inhibitor cocktails (Merck Chemicals, Whitehouse Station, NJ, USA); IL-6, IL-1β and TNF-α ELISA kits (eBioscience, San Diego, CA, USA); bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) kits (Pierce Biotechnology, Inc., Rockford, IL, USA); Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS; Gibco-BRL, Grand Island, NY, USA); Griess reagent and iNOS kits (Nanjing Jiancheng Bioengineering Institute, Nanjing, China); Cell Counting kit-8 (CCK-8; Beyotime Biotech, Jiangsu, China); and fluorescein isothiocyanate (FITC)-conjugated Affinipure goat anti-rabbit and tetramethylrhodamine (TRITC)-conjugated Affinipure goat anti-mouse antibodies (Abcam). All additional materials were purchased from ZSGB-BIO (Beijing, China), unless otherwise stated.

*Microglial cell culture*. BV2 mouse microglial cells (Shanghai Cell Bank, Shanghai, China) were cultured in DMEM supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 g/ml). The cultures were maintained at 37˚C in a humidified incubator with 95% $O_2$ and 5% $CO_2$. The cells were treated with 1 μg/ml LPS for 30 min, followed by administration of 10, 25, 50, 80 or 100 μM DM (dissolved in PBS) for 24 h.

*Cell viability assay*. Cell viability was measured using a CCK-8 assay. BV2 cells ($5x10^4$ cells in 100 μl/well) were seeded in 96-well plates. CCK-8 solution (10 μl) was added to each well and the cultures were incubated at 37˚C for 90 min. The absorbance at 450 nm was measured using a SmartSpec Plus Spectrophotometer (Bio-Rad, Beijing, China). The results are plotted as the means ± standard deviation (SD) of three separate experiments with four determinations per experiment for each experimental condition. The cell survival ratio was calculated, normalizing the results to the control group (without LPS+DM).

*ELISA*. The levels of IL-6, IL-1β, HSP60 and TNF-α present in the culture medium, were quantified according to the manufacturer's instructions for the respective ELISA kits. The absorbance was determined at 450 nm using a Model 680 microplate reader (Bio-Rad, Beijing, China).

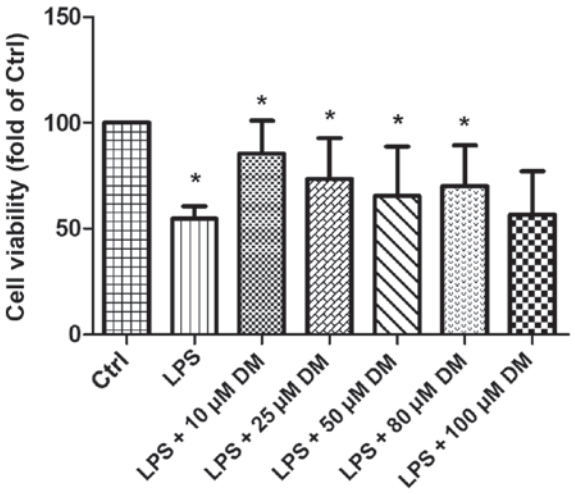


Figure 1. Dextromethorphan (DM) increases BV2 microglial cell viability. BV2 microglial cells were treated with lipopolysaccharide (LPS; 1 μg/ml) for 0.5 h, followed by treatment with various concentrations of DM (10, 25, 50, 80 or 100 μM) for 24 h. The viability of the untreated cells was set to 100. The viability of the LPS- and DM-treated cells is expressed as the percentage of that of the untreated control cells. The data represent the means ± standard error of the mean from three separate experiments, performed in triplicate. *P<0.05, LPS group as compared with the control group; DM groups as compared with the LPS group. Ctrl, control.

*Western blot analysis*. Western blotting was performed according to a standard method. Briefly, BV2 cells were washed with PBS three times and lyzed in radioimmunoprecipitation assay buffer. The protein concentration was determined using the BCA kit according to the manufacturer's instructions. Equal quantities of each protein sample were separated by SDS-PAGE and then transferred to a polyvinylidene difluoride membrane. The membranes were blocked with 5% milk powder and incubated with the respective primary antibodies (anti-P65 1;1,000; anti-caspase, 1:1,000; anti-HSP60, 1:1,000; anti-HSF-1, 1:200; and anti-β-actin, 1:5,000) in Tris-buffered saline with Tween-20 (TBST) overnight at 4˚C. Subsequent to rinsing in milk-TBST, the blots were incubated with horseradish peroxidase-conjugated secondary antibodies (1:5,000). The target proteins were detected by using the ECL detection system and X-ray films.

*Immunofluorescence*. BV2 cells ($5x10^4$ cells in 100 μl/well) were seeded on coverslips in 24-well plates. Following 24 h incubation, the cells were treated with LPS (1 μg/ml) for 1 h and then incubated with the indicated concentrations of DM for 24 h. Subsequently, the cells were washed twice with PBS, fixed with 4% paraformaldehyde in PBS for 15 min, washed three times with PBS and then permeabilized with 0.5% Triton X-100 in PBS for 20 min. The cells were again washed three times with PBS and blocked with 5% bovine serum albumin for 1 h at 37˚C. Following blocking, the cells were incubated with primary antibodies (anti-P65 1;100; anti-caspase, 1:100; anti-HSP60, 1:200; anti-HSF-1, 1:50; and anti-iNOS, 1:100) in PBS overnight at 4˚C, then rinsed with PBS, and incubated with the FITC- and TRITC-conjugated secondary antibodies (1:1,000) for 1 h at 37˚C. The cells were then washed with PBS, stained with DAPI and then mounted. Images were captured at magnification, x400 using

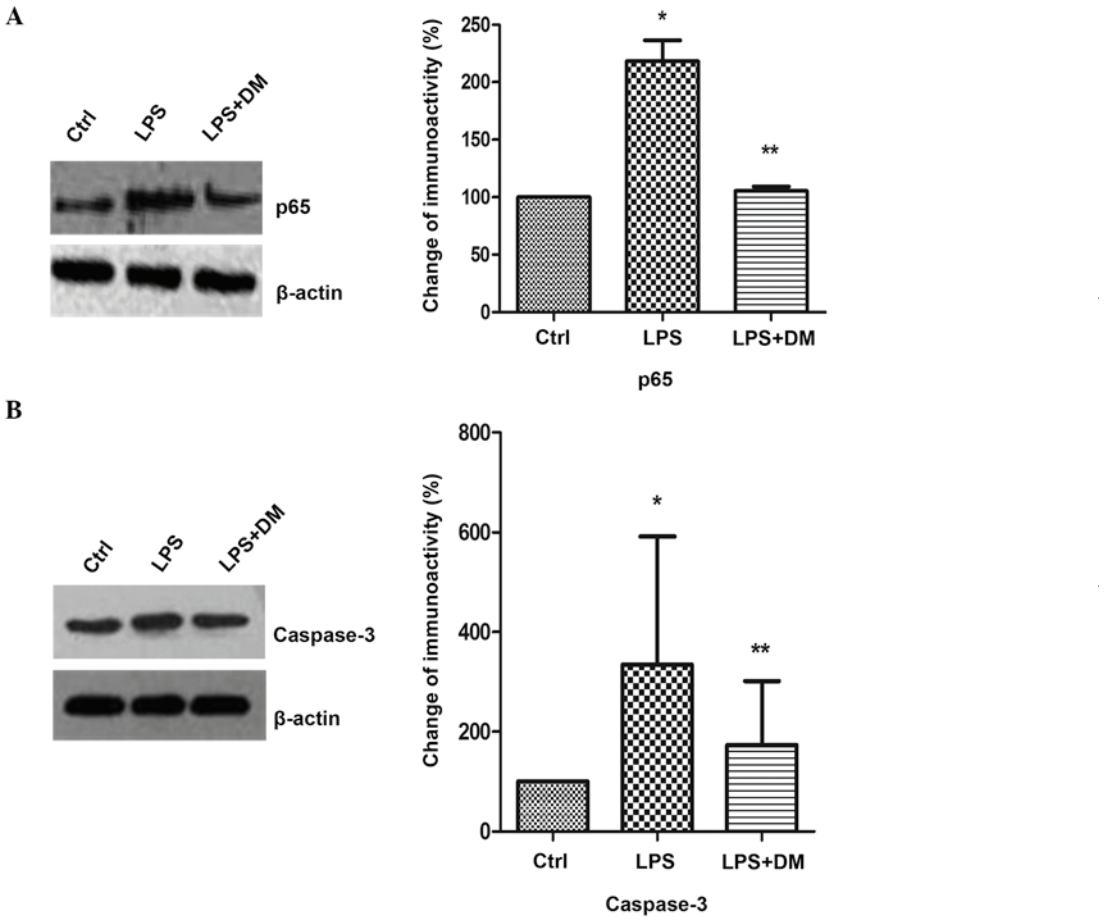


Figure 2. Dextromethorphan (DM) inhibits the increased expression of nuclear factor-κB (NFκB) p65 and caspase-3 in lipopolysaccharide (LPS)-stimulated BV2 microglia. The cells were pretreated with LPS for 0.5 h, followed by incubation with 10 $\mu$M DM for 24 h. The lysates were probed by immunoblotting with antibodies against NFκB p65 and β-actin (used as an internal loading control). (A) Analysis of p65 protein expression levels by western blotting. The right panel shows the ratio of p65/β-actin signal intensity in each group. (B) Analysis of the Caspase-3 protein expression levels by western blotting. The right panel shows the ratio of caspase-3/β-actin signal intensity in each group. Each result was derived from at least six independent cultures. The data represent the means ± standard error of the mean. *P<0.05, as compared with the control group. **P<0.05, as compared with the LPS group. Ctrl, control.

in LPS-stimulated BV2 microglia were investigated using western blotting. The levels of the p65 subunit of NFκB were significantly increased following LPS treatment, as compared with the control (P<0.05), but were markedly inhibited by DM, as compared with LPS treatment alone (P<0.05; Fig. 2A). Subsequent to LPS stimulation, caspase-3 expression was found to be significantly suppressed following DM treatment, as compared with LPS treatment only (P<0.05; Fig. 2B).

*DM inhibits HSP60 protein expression and release in LPS-stimulated BV2 microglia*. The levels of HSP60 expression and release were detected in activated BV2 cells. The western blotting results demonstrated that LPS significantly enhanced HSP60 expression levels, as compared with those of the control group (P<0.05), but DM significantly inhibited this increase, as compared with LPS treatment alone (P<0.05; Fig. 3A). HSF-1 has been shown to bind with the heat shock element on the HSP60 promoter to regulate HSP60 gene expression (20). Therefore, the HSF-1 expression levels were analyzed and were found to be significantly upregulated by LPS (P<0.05), but significantly downregulated by additional DM treatment (P<0.05). This indicated that HSP60 expression in the LPS group as compared with those in the DM group (Fig. 5A). The statistical analysis is shown in Fig. 5B and is consistent with the findings from the western blotting and ELISA. From these data, DM may be considered to effectively inhibit HSP60, HSF-1, NF-κB, caspase-3 and iNOS expression in LPS-stimulated microglia.

## Discussion

In the present study, treatment with 10 $\mu$M DM was demonstrated to effectively inhibit LPS-induced activation of microglia. DM reduced the expression levels of NFκB, caspase-3, HSP60, HSF-1 and iNOS in microglia, and effectively suppressed the release into the culture medium of HSP60, NO and several proinflammatory cytokines, including TNF-α, IL-6 and IL-1β, in microglia stimulated by LPS. Therefore, the data suggest that DM may be a useful therapeutic agent in the treatment of inflammatory diseases.

The activation of microglia is important in neural parenchymal defence against infectious diseases, as well as inflammation, trauma, ischaemia, brain tumors and neurodegeneration (22). The activated microglia secrete various proinflammatory and neurotoxic factors that are hypoth-

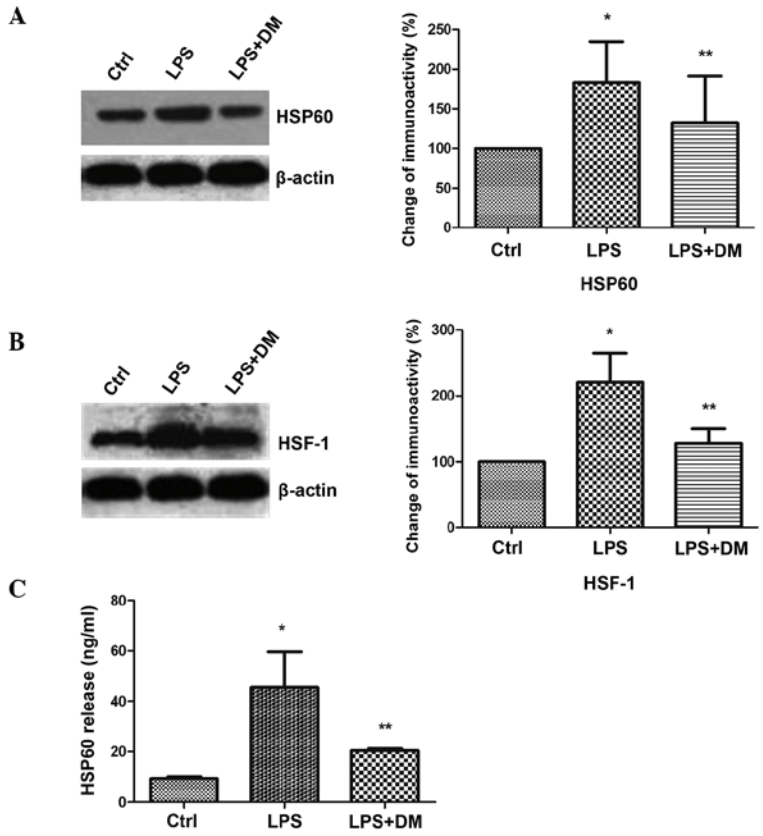


Figure 3. Dextromethorphan (DM) inhibits the increased expression and release of heat shock protein 60 (HSP60) and heat shock factor 1 (HSF-1) expression in lipopolysaccharide (LPS)-stimulated BV2 microglia. BV2 cells were pretreated with LPS for 0.5 h, followed by incubation with 10 $\mu$M DM for 24 h. (A) Analysis of protein expression by western blotting, using antibodies against HSP60 and β-actin (as an internal loading control). The right panel shows the ratio of HSP60/β-actin signal intensity. (B) The lysates were probed by immunoblotting with antibodies against HSF1 and β-actin. The right panel shows the ratio of HSF1/β-actin signal intensity. (C) Extracellular levels of HSP60 were detected by enzyme-linked immunosorbent assay. The data represent the means ± standard error of the mean from three independent experiments performed in triplicate. $^{*}$P<0.05, compared with the control group. $^{**}$P<0.05, as compared with the LPS group. Ctrl, control.

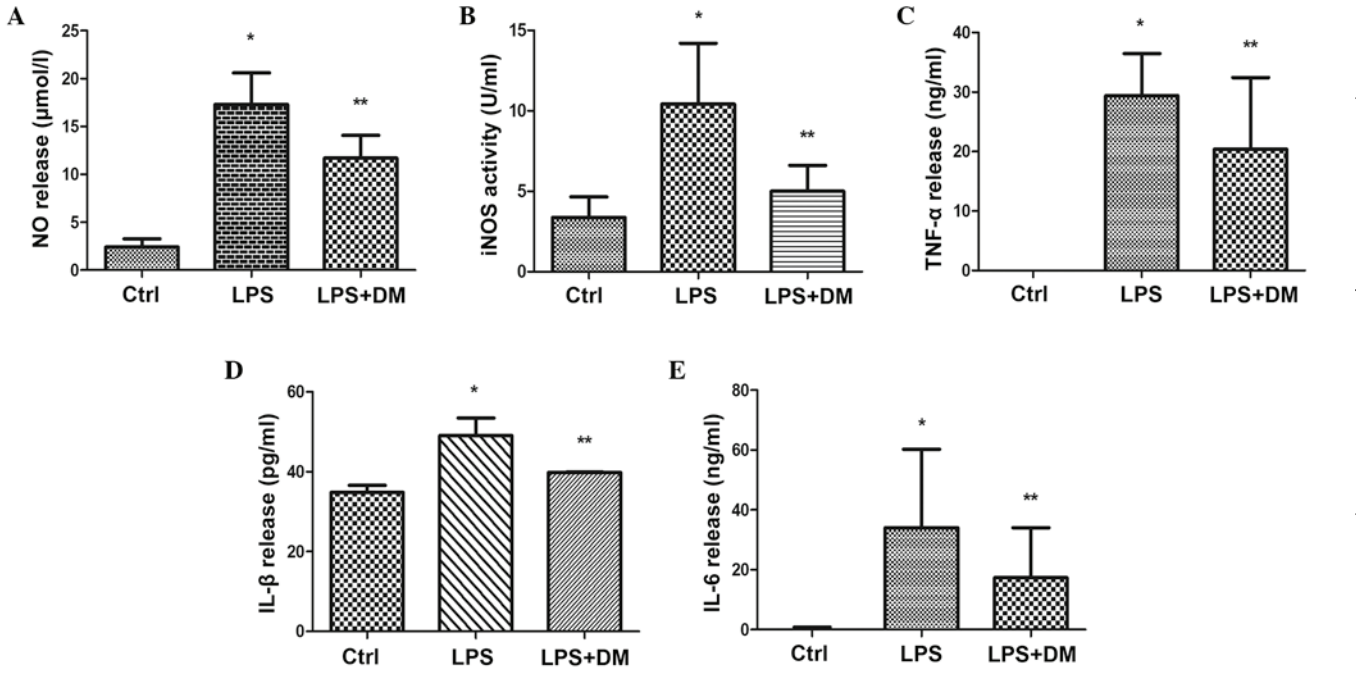


Figure 4. Dextromethorphan (DM) reduces the release of nitric oxide (NO), inducible NO synthase (iNOS), tumor necrosis factor-α (TNF-α), interleukin (IL)-6 and IL-1β in lipopolysaccharide (LPS)-stimulated BV2 microglia. The cells were pretreated with LPS for 0.5 h, followed by incubation with 10 $\mu$M DM for 24 h. Extracellular levels of (A) NO and (B) iNOS were assayed with Griess reagent and iNOS kits, and (C) TNF-α, (D) IL-1β and (E) IL-6 were measured by ELISA. The data represent the means ± standard error of the mean of three separate experiments performed in triplicate. $^{*}$P<0.05, as compared with the control group. $^{**}$P<0.05, as compared with the LPS group. Ctrl, control.

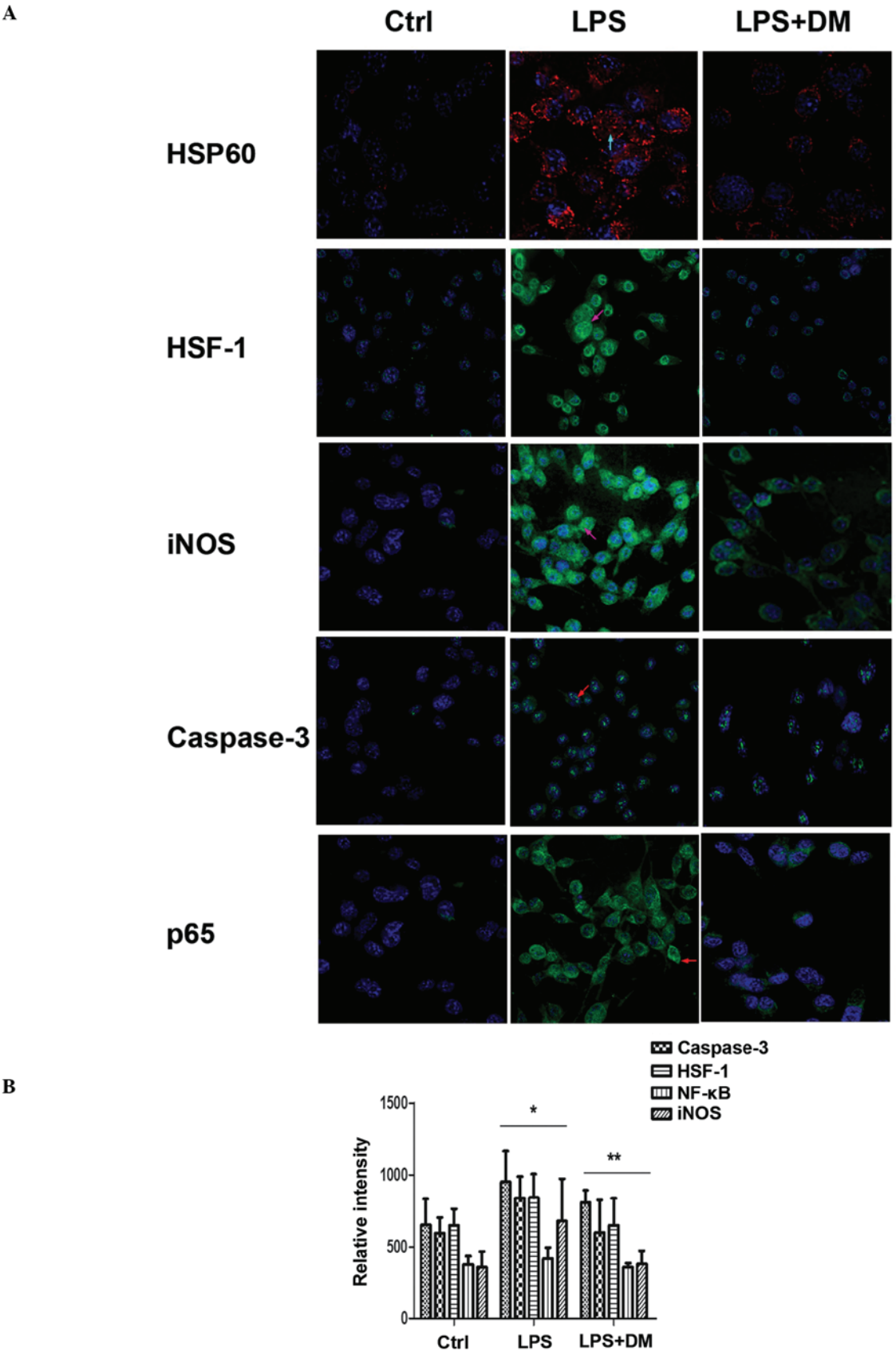


Figure 5. Dextromethorphan (DM) inhibits heat shock protein 60 (HSP60), heat shock factor 1 (HSF-1), nuclear factor-κB (NFκB), caspase-3 and inducible nitric oxide synthase (iNOS) expression in LPS-stimulated BV2 microglial cells, as detected by immunofluorescence. The cells were pretreated with lipopolysaccharide (LPS) for 0.5 h, followed by incubation with 10 $\mu$M DM for 24 h. (A) The expression fluorescence intensity of HSP60, HSF-1, iNOS, NFκB and caspase-3 varied between the control, LPS and LPS + DM groups. The arrows indicate the various subcellular localization of the respective proteins in the cells. All the images were produced by merging the cytoplasmic and nuclear images (digital image capture, magnification, x400). (B) Statistical analysis of the signal intensities of HSP60, HSF-1, NFκB, caspase-3 and iNOS. The data represent the means ± standard error of the mean of each separate experiment performed. $^{*}$P<0.05, as compared with the Ctrl group. $^{**}$P<0.05, as compared with the LPS group. Ctrl, control.

inhibited caspase-3 and the NFκB downstream mediator p65, suggesting that the anti-inflammatory effects of DM may be a result of inhibition of the NFκB signaling pathway.

HSP60 is primarily considered to be a mitochondrial protein, but may translocate to the plasma membrane, even being released extracellularly upon stress, which is a process that has been demonstrated to be induced by the NFκB-p65 cascade (21). Extracellular HSP60 may become toxic by targeting self-reactive T cells in inflammatory diseases (37). HSP60 gene expression is regulated by the corresponding transcription factor, HSF-1, which binds to the HSP60 gene promoter. NFκB has also been shown to initiate transcription of the HSP60 stress gene, which elicits a potent proinflammatory response in innate immune cells (38). TNF-α is a mediator of NFκB signaling and induces an increase in the expression levels of HSP60, which has been shown to be reversed by p65 inhibition (38). Therefore, in the present study, HSP60 expression in BV2 cells and extracellular release, as well as TNF-α levels in the culture medium were measured, following NFκB activation by LPS. The results reveal that DM suppressed HSP60 expression and release, and also reduced extracellular TNF-α levels.

Microglial activation is widely known to produce proinflammatory cytokines, NO and iNOS. This was confirmed in the present study by measuring the levels of IL-1β, IL-6, NO and iNOS in the culture medium of BV2 cells stimulated by LPS. The iNOS gene is under the transcriptional control of various inflammatory mediators, including cytokines and LPS. NO, a product of iNOS, has been found to be important as a signaling molecule in a number of areas of the body, as well as acting as a cytotoxic or regulatory effector molecule of the innate immune response (39). However, in the present study, following DM treatment, IL-1β, IL-6, NO and iNOS expression levels were markedly inhibited.

Combined with results from previous studies, the findings from the present study indicate that DM may exert neuroprotective action through the inhibition of the NFκB signaling pathway to prevent the overactivation of microglia.

## Acknowledgements

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