

# HSP60 is involved in the neuroprotective effects of naloxone

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**Abstract.** Heat shock protein (HSP)60 is primarily a mitochondrial protein. Previous experiments have found that changes in the location of intracellular HSP60 have been associated with apoptosis. Extracellular HSP60 mediates apoptosis via its ligand, Toll-like receptor (TLR)-4. TLR-4 is an important factor expressed on microglia, with a central role in generating neuroimmune responses in the pathogenesis of neurodegenerative disorders. Naloxone is a highly effective nonselective opioid receptor antagonist, and has been reported to be pharmacologically beneficial for the treatment of brain diseases through inhibiting microglia activation. However, the mechanisms underlying these beneficial effects of naloxone remain poorly understood. The present study aimed to investigate the role of HSP60 in the neuroprotective effects of naloxone on the production of proinflammatory mediators in lipopolysaccharide (LPS)-stimulated BV2 murine microglial cells and the possible signaling pathways involved. The results demonstrated that naloxone significantly inhibited the expression and release of HSP60 in BV2 cells. The expression levels of heat shock factor (HSF)-1 were upregulated in LPS-activated BV2 cells, which indicated that the increased expression of HSP60 was driven by HSF-1 activation. However, increased HSF-1 levels may be downregulated by naloxone. The levels of TLR-4 were elevated in activated

BV2 cells, and then inhibited by naloxone. Activation of TLR-4 is characterized by activation of nuclear factor- $\kappa$ B (NF- $\kappa$ B) followed by the production of various proinflammatory and neurotoxic factors. Data from the present study demonstrated that naloxone reduced the expression levels of NF- $\kappa$ B and its upstream protein caspase-3, and reduced the LPS-induced production of nitric oxide, inducible nitric oxide synthase, tumor necrosis factor  $\alpha$ , interleukin-1 $\beta$  and interleukin-6 in BV2 microglia. In light of this data, it was concluded that naloxone may exert its neuroprotective and anti-inflammatory effects by inhibiting microglia activation through a HSP60-TLR-4-NF- $\kappa$ B signaling pathway.

## Introduction

The activation of microglia, the primary immune cells of the central nervous system (CNS), occurs in almost all neurological disorders (1) and is often associated with the increased production of various pro-inflammatory mediators, including nitric oxide (NO), inducible nitric oxide synthase (iNOS), tumor necrosis factor (TNF)- $\alpha$ , interleukin (IL)-1 $\beta$ , nuclear factor- $\kappa$ B (NF- $\kappa$ B), caspase-3 and heat shock protein (HSP)60 (2-6), which all contribute to neurodegeneration (7,8). The importance of microglial activation in neurodegeneration has prompted speculation that the inhibition of microglial activation, in particular the control of neurotoxic factor production, may be an effective therapeutic option for neurodegenerative diseases. Numerous microglia-targeted pharmacotherapies, including protein kinase C inhibitors, microglia inhibiting factor, and various Chinese medicinal herb extracts, have been proposed to inhibit the activation of microglia and to promote neuronal survival *in vivo* (9-11). However, the inability of these drugs to penetrate the blood-brain barrier, in addition to side-effects that they may produce, limit their long-term use in the clinical setting.

Naloxone is a structural analog of morphine and an effective antagonist of the classic opioid receptors that are widely expressed on cells of the central and peripheral nervous systems (12). Administration of naloxone has been demonstrated to be beneficial in the treatment of experimental models

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of stroke, myocardial and brain ischemia, brain trauma, spinal cord injuries and septic shock (13-15). Naloxone has been demonstrated to attenuate the degeneration of dopaminergic neurons by inhibition of  $\beta$ -amyloid peptide(1-42)-induced microglial activation and degeneration of cortical and mesencephalic neurons, suggesting that naloxone may have potential therapeutic efficacy for the treatment of Parkinson's and Alzheimer's disease (16,17). These studies raise the possibility that naloxone binds to sites other than opioid receptors and exerts activity that does not involve the opioid receptor system. Compared with other microglial inhibitors, naloxone has several advantages, including its ability to penetrate the CNS and that it produces fewer side-effects, presenting a potential novel therapeutic option for neuroprotection.

Although naloxone has been demonstrated to inhibit lipopolysaccharide (LPS)-induced microglial activation in the CNS, the underlying mechanism is not well understood. HSP60 was demonstrated to be released extracellularly in cardiac myocytes during heart failure, and induces apoptosis by binding to Toll-like receptor (TLR)-4 (18,19). The aim of the present study was to examine whether HSP60 is involved in the neuroprotective effects of naloxone in LPS-induced inflammatory injury in BV2 microglia.

## Materials and methods

**Chemicals.** Naloxone and LPS were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies against  $\beta$ -actin, NF- $\kappa$ B and TLR-4 (Abcam, Cambridge, MA, USA), anti-HSP60 and anti-heat shock factor (HSF)1 antibodies (Stressgen, San Diego, CA, USA) and an anti-caspase-3 antibody (Cell Signaling Technology, Inc., Beverly, MA, USA) were acquired. Proteinase inhibitor cocktails were purchased from Merck Chemicals (Whitehouse Station, NJ, USA). IL-6, IL-1 $\beta$  and TNF- $\alpha$  ELISA kits were from eBioscience (eBioscience, CA, USA). Bicinchoninic acid (BCA) and enhanced chemiluminescence (ECL) kits were acquired from Pierce (Rockford, IL, USA). Dulbecco's modified Eagle's medium (DMEM) and fetal bovine serum (FBS) came from Gibco (Grand Island, NY, USA). Griess reagent (for identifying NO) and nitric oxide synthase (iNOS) kits were from Jiancheng Bioengineering Institute (Nanjing, China). The Cell Counting Kit-8 (CCK-8) was obtained from Beyotime (Nanjing, China).

**Microglial cell culture.** BV2 mouse microglia (Cell Bank, Shanghai, China) were cultured in DMEM supplemented with 10% FBS, penicillin (100 U/ml) and streptomycin (100 g/ml). Cultures were maintained at 37°C in a humidified incubator gassed with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Naloxone was dissolved in phosphate-buffered saline (PBS). Cells were treated with the indicated concentrations of naloxone for 24 h following the administration of LPS (200 ng/ml) for the indicated time period.

**Cell viability assay.** Cell viability was measured using the CCK-8. Cells ( $7.5 \times 10^3$  cells in 100  $\mu$ l culture medium/well) were seeded in 96-well plates. CCK-8 solution (10  $\mu$ l) was added to each well and the cultures were incubated at 37°C for 90 min. Absorbance at 450 nm was measured using an immunoreader (Bio-Rad, Beijing, China). The results were plotted as the mean  $\pm$  standard deviation of three separate experiments

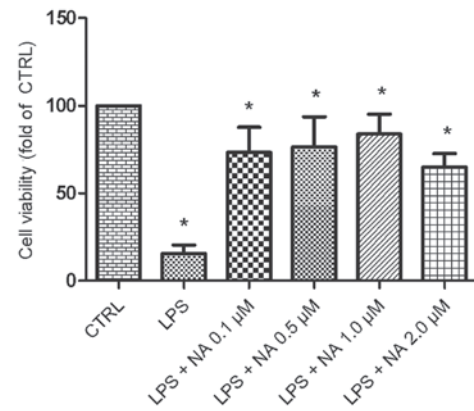


Figure 1. NA increased the cell viability of BV2 microglia. BV2 cells were treated with LPS (200 ng/ml) for 1 h, followed by treatment with various concentrations of NA (0, 0.1, 0.5, 1.0 or 2.0  $\mu$ M) for 24 h. The viability of untreated cells was set to 100. The viability of LPS- and NA-treated cells was expressed as a percentage of the viability of the untreated cells (CTRL). The results are presented as the mean  $\pm$  standard error of three separate experiments performed in triplicate, \* $P < 0.05$  (LPS group vs. CTRL or NA groups vs. LPS group). CTRL, control; LPS, lipopolysaccharide; NA, naloxone.

having four determinations per experiment for each experimental condition. The cell survival ratio was calculated by normalization to control.

**Enzyme-linked immunosorbent assay (ELISA).** The levels of IL-6, IL-1 $\beta$  and TNF- $\alpha$  in culture medium were quantified according to the manufacturer's instructions. Absorbance was determined at 450 nm using a microplate reader (Bio-Rad).

**Western blotting.** Following treatment, BV2 cells were washed with PBS three times and lysed with radioimmunoprecipitation assay buffer (Sigma-Aldrich). The protein concentration was determined with the BCA kit according to the manufacturer's instructions. Equal quantities of protein were loaded and run on SDS/polyacrylamide gels and then transferred to a polyvinylidene fluoride membrane. Membranes were blocked with 5% dried milk and incubated with primary antibodies in Tris-buffered saline with Tween 20 overnight at 4°C. After being rinsed in milk-TBST, blots were incubated in the horseradish peroxidase-conjugated secondary antibodies. The target proteins were detected using the ECL kit and X-ray films (Kodak, Shanghai, China).

**Statistical analysis.** Statistical significance was determined using one-way analysis of variance.  $P < 0.05$  was considered to indicate a statistically significant difference. All data are presented as the mean  $\pm$  standard error of the mean.

## Results

**Naloxone promotes the viability of BV2 microglia.** To determine whether naloxone has an effect on the apoptosis of LPS-stimulated BV2 cells, CCK-8 assay was performed. The results indicated that treatment of microglia with 0.1-2.0  $\mu$ M naloxone for up to 24 h significantly increased the viability of LPS-stimulated BV2 cells, compared with that of the LPS group (Fig. 1). Naloxone at a concentration of 1.0  $\mu$ M exhibited the maximal protection, so this concentration was selected for

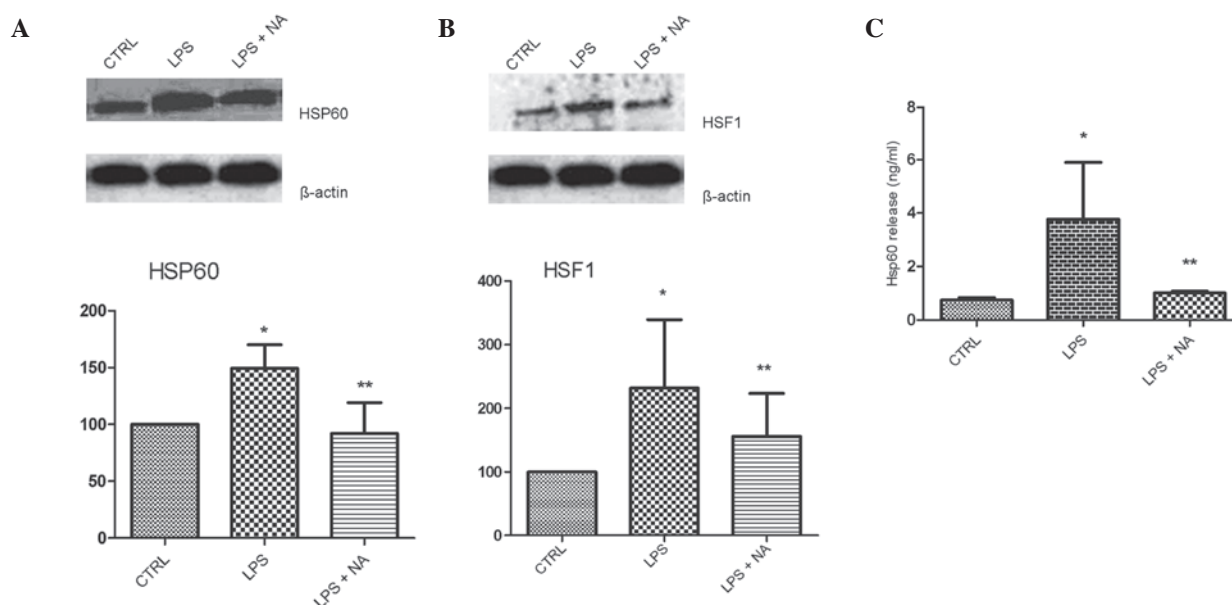


Figure 2. NA inhibited the increased expression and release of HSP60 and HSF-1 in LPS-stimulated BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by an incubation with 1.0  $\mu$ M NA for 24 h. The lysates were probed by immunoblotting with antibodies against HSP60, HSF-1 and  $\beta$ -actin. The graphs display ratios of the signal intensities of (A) HSP60/ $\beta$ -actin and (B) HSF1/ $\beta$ -actin. (C) Extracellular levels of HSP60 were detected by ELISA. Results are presented as the mean  $\pm$  standard error of three independent experiments performed in triplicate. \* $P$ <0.05 vs. CTRL, \*\* $P$ <0.05 vs. LPS group. CTRL, control; LPS, lipopolysaccharide; NA, naloxone; HSP, heat shock protein; HSF, heat shock factor.

the subsequent experiments. The results indicated that naloxone has a positive effect on the viability of LPS-stimulated BV2 microglia.

**Naloxone inhibits HSP60 protein expression and release in LPS-stimulated BV2 microglia.** Levels of HSP60 expression and release were measured in activated BV2 cells. Western blot results indicated that LPS induced an increase in the expression levels of HSP60 compared with levels in controls, and that naloxone significantly inhibited this increase (Fig. 2A). The HSP60 promoter has been reported to have a heat shock element that is the binding site for HSF-1, which regulates HSP60 gene expression (20). Therefore, HSF-1 levels were investigated, and demonstrated to exhibit the same expression pattern as that of HSP60, indicating that HSP60 expression is driven by HSF-1 (Fig. 2B). HSP60 has been reported to translocate extracellularly upon stress in order to exert injury effects (11). ELISA results demonstrated that HSP60 was released upon LPS-induced activation of BV2 cells, and this increased extracellular HSP60 may be suppressed by naloxone (Fig. 2C).

**Naloxone inhibits TLR-4, NF- $\kappa$ B and caspase-3 expression.** TLR-4 mediates inflammatory responses in activated microglia. In the present study, it was demonstrated that TLR-4 expression levels in LPS-stimulated BV2 cells were reduced by naloxone (Fig. 3A). The expression levels of the p65 subunit of NF- $\kappa$ B, which is a pivotal factor in the TLR-4 pathway, increased following LPS stimulation, but this effect was markedly inhibited by the addition of naloxone (Fig. 3B). Caspase-3 is upstream of NF- $\kappa$ B in this signaling pathway, and inhibition of caspase-3 has been demonstrated to prevent neuronal loss in brain diseases involving activated microglia. Thus, in the current study, the effects of naloxone on caspase-3 expression

were examined (Fig. 3C). Caspase-3 expression was suppressed in LPS-stimulated BV2 cells following naloxone exposure.

**Naloxone inhibits the production of proinflammatory factors.** Using ELISA assay, naloxone suppression of the release of proinflammatory factors, including NO, iNOS, TNF- $\alpha$ , IL-1 $\beta$  and IL-6, was investigated in LPS-stimulated BV2 cells. As shown in Fig. 4, 24-h naloxone treatment of LPS-stimulated BV2 cells resulted in significant reductions in the levels of the aforementioned factors in culture media compared those following LPS stimulation alone. These results implied that naloxone effectively suppresses the production of neurotoxic factors in activated microglia.

## Discussion

HSP60 is primarily considered to be a mitochondrial protein, but a number of studies have established that HSP60 is also involved in apoptosis. When HSP60 is in the cytosol or mitochondria, it is antiapoptotic and protective. However, when HSP60 is in the plasma membrane or extracellular space, it is associated with apoptosis. Previous studies detected HSP60 expression on the exofacial surface of myocytes, where it was a potential antibody target or innate immune system ligand of TLR-4 (18,19). TLR-4 has been shown to be present in microglia (21), and HSP60 is a ligand for TLR-4 in the immune system (22).

A number of studies have identified neuroprotective effects of naloxone, and it was demonstrated to inhibit LPS-induced microglial activation in the CNS (16,17). However, the mechanisms involved remain unclear. In the present study, it was hypothesized that LPS triggered HSP60 release from microglia, and extracellular HSP60 binds to TLR-4 on the surface of microglia, inducing apoptosis by

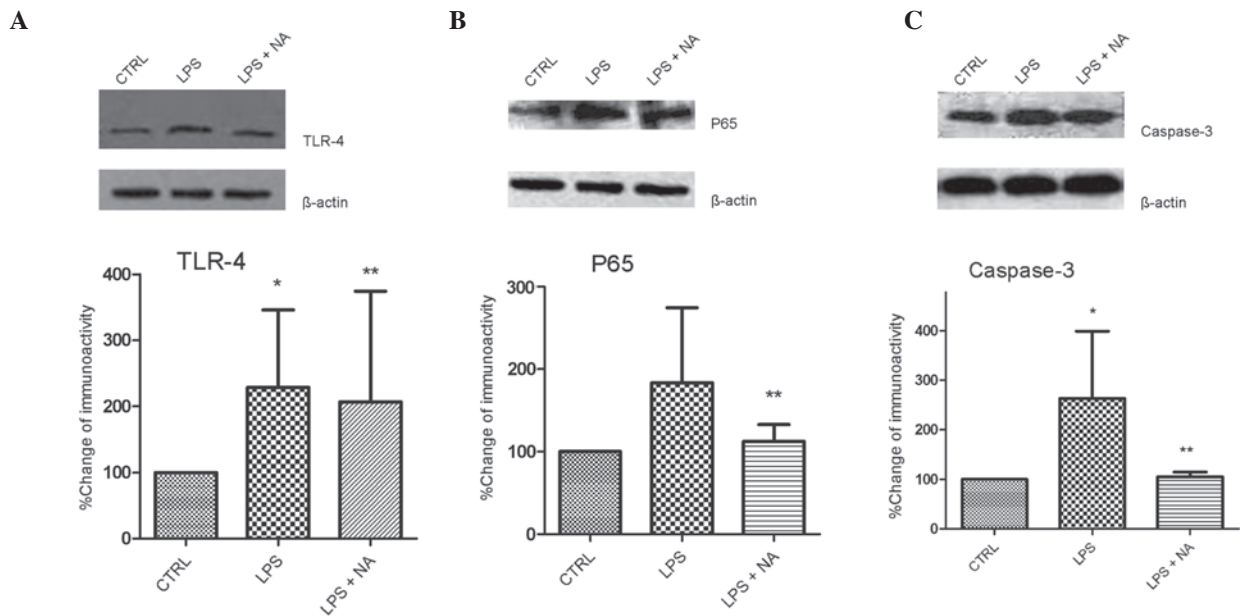


Figure 3. NA inhibited the increased expression of TLR-4, NF- $\kappa$ B and caspase-3 in LPS-stimulated BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by an incubation with 1.0  $\mu$ M naloxone for 24 h. The lysates were probed by immunoblotting with antibodies against TLR-4, p65, caspase-3 and  $\beta$ -actin. The graphs display ratios of the signal intensities of (A) TLR-4/ $\beta$ -actin, (B) p65/ $\beta$ -actin and (C) caspase-3/ $\beta$ -actin. Each experiment was derived from at least 6 independent cultures. \* $P < 0.05$  vs. CTRL, \*\* $P < 0.05$  vs. LPS group. CTRL, control; LPS, lipopolysaccharide; NA, naloxone; TLR, Toll-like receptor; NF- $\kappa$ B, nuclear factor- $\kappa$ B.

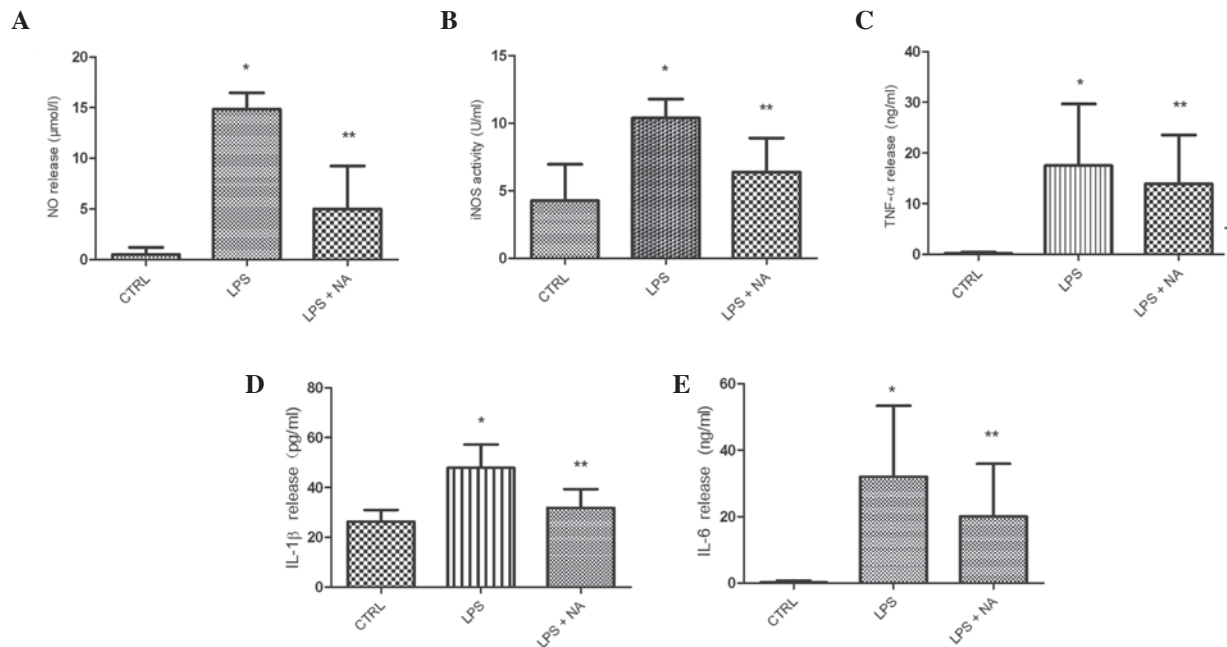


Figure 4. NA reduced the release of NO, iNOS, TNF- $\alpha$ , IL-6 and IL-1 $\beta$  in LPS-stimulated BV2 microglia. Cells were pretreated with LPS for 0.5 h, followed by an incubation with 1.0  $\mu$ M NA for 24 h. Extracellular levels of (A) NO and (B) iNOS were assayed with Griess reagent and iNOS kits, respectively, and levels of (C) TNF- $\alpha$ , (D) IL-1 $\beta$  and (E) IL-6 were measured using ELISA assay. The results are presented as the mean  $\pm$  standard error of three separate experiments performed in triplicate. \* $P < 0.05$  vs. CTRL, \*\* $P < 0.05$  vs. LPS group. NO, nitric oxide; CTRL, control; LPS, lipopolysaccharide; NA, naloxone; iNOS, inducible nitric oxide synthase; TNF, tumor necrosis factor; IL, interleukin; ELISA, enzyme-linked immunosorbent assay.

activating the NF- $\kappa$ B pathway. The results supported these hypotheses. A pretreatment of 1  $\mu$ M naloxone effectively suppressed the activation of microglia. While LPS stimulation increased the expression and release of HSP60, naloxone significantly suppressed these effects. HSP expression is

regulated by HSFs. In the current study, HSF-1 expression levels increased as did those of HSP60 when triggered by LPS and then were inhibited by naloxone. Thus, the inhibitory effect of naloxone on HSP60 may occur through inhibition of its transcription factor HSF-1. The overexpressed HSP60



released extracellularly may act as an innate immune signal to further activate microglia.

TLR-4-mediated microglial activation induced cell death, which is a mechanism by which activated immune cells are eliminated. Kim *et al* (18) demonstrated that extracellular HSP60 activated TLR-4 by binding to it at a different site to that which LPS binds, leading to cytokine production and cardiac myocytes apoptosis. Release of TNF- $\alpha$  may then lead to further myocyte apoptosis and increased HSP60 expression through activation of NF- $\kappa$ B (23). In the present study, it was demonstrated that TLR-4 may be activated on microglia not only by LPS but also by extracellular HSP60, and that activated TLR-4 was effectively inhibited by naloxone. LPS and HSP60 binding to TLR-4 may trigger a cascade of signaling events resulting in the activation of downstream effector molecules such as NF- $\kappa$ B and caspase 3 and culminating in the production of proinflammatory immune mediators, including TNF- $\alpha$ , IL-1 $\beta$ , IL-6 and IL-8. Release of NO and chemokines by these cells has also been reported (24). NF- $\kappa$ B is the major transcription factor in the induction of the transcription of pro-inflammatory genes. The activation of NF- $\kappa$ B has been demonstrated to lead to ischemia-induced neuronal injury (25). Caspase-3 is crucial for apoptosis and CNS inflammation (26) and when caspase-3/7 is blocked, activated microglia are non-toxic to neighboring neurons (27). Activation of the NF- $\kappa$ B-p65 cascade induces HSP60 production and release following oxidative stress (19) possibly by the binding of NF- $\kappa$ B to the HSP60 gene promoter (23). TNF- $\alpha$  is also a mediator of NF- $\kappa$ B signaling and drives the increased expression of HSP60, which can be reversed by p65 inhibition (23). Levels of HSP60, NF- $\kappa$ B and TNF- $\alpha$  are simultaneously decreased by naloxone. By demonstrating the marked inhibition of the expression of caspase-3 and the NF- $\kappa$ B downstream mediator p65, and production of NO, iNOS, TNF- $\alpha$ , IL-1 $\beta$  and IL-6 by naloxone treatment, the findings of the present study suggest that the neuroprotective and anti-inflammatory effects of naloxone may be due to inhibition of the HSP60-TLR-4-NF- $\kappa$ B pathway.

In summary, to the best of our knowledge, the results of the present study indicated for the first time that naloxone may exert its neuroprotective action through HSP60-TLR-4-NF- $\kappa$ B inhibition to prevent the overactivation of microglia.

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