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Heme oxygenase-1 mediates cytoprotective effects of immunostimulation in microglia

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ABSTRACT

Microglia are brain-resident immune cells playing a pivotal role in the neuroinflammation. Previously, it has been shown that immunostimulation protects microglial cells against nitric oxide toxicity. Herein, we report that heme oxygenase-1 (HO-1) mediates the protective effects of immunostimulation. Pro-inflammatory activation of BV-2 microglial cells with endotoxin lipopolysaccharide (LPS) conferred a protection against various cytotoxic stimuli, whereas anti-inflammatory cytokines such as IL-4 and IL-10 were without effects. The LPS-induced cytoprotection was accompanied by HO-1 induction. The cytoprotective effect of LPS treatment was significantly attenuated by co-treatment with a HO-1 inhibitor, zinc protoporphyrin. Adenoviral expression of HO-1 in microglial cells was similarly cytoprotective, indicating that HO-1 mediates the cytoprotective effects of pro-inflammatory stimulation. Additional experiments revealed the involvement of carbon monoxide (CO) and iron, products of HO-1-mediated heme degradation, in the cytoprotective effect of LPS. Taken together, our results suggest that immunostimulation of microglia with LPS provides cytoprotective effects via HO-1 induction followed by the generation of CO and iron.

1. Introduction

Microglia, the resident immune cells in the brain, are responsive to environmental stress or immunological challenges and have been implicated as the predominant cell type governing inflammation-mediated neuronal damage [1–4]. The microglial cells secrete inflammatory cytokines and toxic mediators, which may initiate or amplify the inflammatory responses in the central nervous system (CNS) [5,6], and activation of microglia has been observed during the development of neurodegenerative diseases such as Alzheimer's diseases and Parkinson's diseases [7,8]. BV-2 cells are immortalized microglial cell line that is widely used as a model of microglia *in vivo* [9]. BV-2 cells exhibit phenotypic and functional properties comparable to those of primary microglial cells [10]. Activated BV-2 microglial cells release inflammatory mediators, such as reactive oxygen species (ROS), tumor necrosis factor-α (TNF-α), interleukin-1, nitric oxide (NO), and arachidonic acid metabolites [11–13]. BV-2 cells were used in the current study to understand the role of immunostimulation in the microglial cell survival and death.

Lipopolysaccharide (LPS) is one of the most common inflammatogens that are used to investigate inflammatory responses of monocytes/macrophages system. The intracellular signaling mechanisms related to the effects of LPS have been well studied in several types of immune and inflammatory cells including macrophages, microglia, and astrocytes [14–16]. NO, as an inflammatory mediator produced by LPS-stimulated microglia, contributes to neural damage as well as their own demise [17]. It has been previously shown that stimulation of BV-2 microglia cells with LPS protects against NO-mediated apoptosis [18]. LPS has been reported to induce

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manganese-dependent superoxide dismutase (Mn-SOD) expression in BV-2 cells, and this was associated with accumulation of NO [19]. However, molecular mechanism underlying the protective effects of LPS stimulation in microglia is not known.

Heme oxygenase (HO) is a microsomal enzyme that oxidatively cleaves heme and produces biliverdin, carbon monoxide, and iron [20]. Three isoforms of HO (HO-1, HO-2 and HO-3) have been identified [21–23]. HO-1 is a member of the heat shock protein family and is induced by various stimuli, including heat shock, heme, metals, hormones, and oxidative stress [24–27]. In the CNS, this enzyme is induced in brain exposed to ischemic insults and intraparenchymal or subarachnoid hemorrhage [28–32]. HO-1 has been shown to exert cytoprotective and anti-inflammatory effects under various conditions [28–34]. Here, we report that LPS induces HO-1 expression in BV-2 microglial cells, and HO-1 mediates the cytoprotective effects of LPS stimulation. LPS-induced HO-1 also attenuated the NO production following inflammatory activation of microglia, which was in agreement with a previously reported anti-inflammatory role of HO-1 in macrophages [34]. Finally, carbon monoxide (CO) and iron appeared to play a central role in the cytoprotective action of HO-1.

2. Materials and methods

2.1. Reagents and cells

Lipopolysaccharide, sodium nitroprusside (SNP), etoposide, cisplatin, zinc(II) protoporphyrin IX (ZnPP), deferoxamine mesylate (DFO), tricarbonyldichlororuthenium(II) dimer (RuCO), and ferric citrate (FC) were obtained from Sigma Chemical Co. (St. Louis, MO). Recombinant mouse IFN-γ, IL-4, and IL-10 were purchased from R&D Systems (Minneapolis, MN). Staurosporine was from Calbiochem (La Jolla, CA). All other chemicals were obtained from Sigma Chemical Co., unless stated otherwise. BV-2 mouse microglial cells [9,10] were maintained in Dulbecco's modified Eagle medium (DMEM) supplemented with 5% fetal bovine serum (FBS), 2 mM glutamine, and penicillin-streptomycin (Gibco-BRL, Gaithersburg, MD).

2.2. Assessment of cytotoxicity by MTT assay

The cells were treated with stimuli in 96-well plates, and culture medium was removed after the treatment, and then 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT; 0.5 mg/ml) was added, followed by incubation at 37 °C for 2 h in CO_2 incubator. After insoluble crystals were completely dissolved in dimethylsulfoxide (DMSO), absorbance at 570 nm was measured with a microplate reader (Anthos Labtec Instruments GmbH; Salzburg, Austria).

2.3. Nitrite quantification

The cells were treated with stimuli in 96-well plates, and then NO_2^- in culture supernatants was measured to assess NO production. Fifty microliters of sample aliquots were mixed with 50 μl of Griess reagent (1% sulfanilamide/0.1% naphthylethylene diamine dihydrochloride/2% phosphoric acid) in 96-well plate and incubated at 25 °C for 10 min. The absorbance at 540 nm was measured with a microplate reader (Anthos Labtec Instruments GmbH). $NaNO_2$ was used as the standard to calculate NO_2^- concentrations.

2.4. Reverse transcription-polymerase chain reaction (RT-PCR)

Total RNA was extracted from BV-2 cells by TRIZOL reagent according to the manufacturer's protocol. Reverse transcription (RT)-PCR amplification using specific primer sets for HO-1 was carried out at 55 °C annealing temperature for 35 cycles. PCR for β-actin was carried out at 55 °C annealing temperature for 25 cycles. Nucleotide sequences of the primers were based on published cDNA sequences of mouse HO-1 and β-actin (HO-1 forward, 5′-AACAAGCAGAACCCAGTCTA-3; HO-1 reverse, 5′-CCTTCTGTGCAATCTTCTTC-3′; β-actin forward, ATCCTGAAAGACCTCTATGC; β-actin reverse, AACGCAGCTCAGTAACAGTC). The PCR reaction was performed with denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 1 min using a DNA Engine Tetrad Peltier Thermal Cycler (MJ Research, Waltham, MA). For the analysis of PCR products, 10 μl of each PCR reaction was electrophoresed on 1% agarose gel and detected under UV light. The β-actin was used as an internal control.

2.5. Virus and infection

Recombinant adenovirus expressing green fluorescent protein (GFP) and HO-1 was kindly provided by Dr. J. Park at Gyeongsang National University (Jinju, Korea) and Dr. T. Kwon at Keimyung University (Daegu, Korea), respectively. Adenovirus was amplified using HEK293A cell lines. Prior to infection, virions were semipurified from high-titer supernatants of infected HEK293A cells. Supernatants were clarified by centrifugation to eliminate cell debris and stored at −80 °C. BV-2 mouse microglial cells were infected with the adenovirus expressing GFP or HO-1 for 2 days, and cells were subsequently washed with phosphate buffered saline (PBS), and then cultured in complete medium.

2.6. Western blot analysis

The cells were washed with cold PBS and lysed in lysis buffer (50 mM Tris–HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 1% Nonidet P-40, 1 mg/ml aprotinin, 1 mM phenylmethylsulfonyl fluoride, 1 mM sodium metavanadate, 5 mM sodium fluoride) containing protease inhibitors ($NaVO_3$). The protein concentration in cell lysates was determined using a BCA protein assay reagent kit (Pierce, Rockford, IL). An equal amount of protein for each sample was separated by 12% SDS-polyacrylamide gel electrophoresis and transferred to PROTRAN nitrocellulose transfer membranes (Schleicher and Schuell, Germany). The membranes were blocked with 5% skim milk in Tris-buffered saline with Tween-20 (TBS-T; 20 mM Tris–HCl, pH 7.6, 137 mM NaCl, 0.05% Tween-20), and incubated sequentially with primary antibodies and with HRP-conjugated secondary antibodies (anti-mouse or -rabbit IgG; Pierce), followed by ECL detection (Amersham Biosciences). The

primary antibodies used were rabbit polyclonal anti-HO-1 antibody (Stressgen, Victoria, BC, Canada) or monoclonal anti-α-tubulin clone B-5-1-2 mouse ascites fluid (Sigma).

2.7. Statistical analysis

Statistical comparison between different treatments was done by Student's *t*-test. The $p < 0.01$ was considered statistically significant.

3. Results

3.1. Cytoprotective effects of pro-inflammatory stimulation in microglia

We first compared the effects of pro-inflammatory and anti-inflammatory stimulation on BV-2 microglial cell death. Pretreatment of BV-2 cells with endotoxin LPS, a representative pro-inflammatory stimulant, for 6 h protected the cells against cytotoxic agents such as etoposide, staurosporine, and NO donor SNP, and were also protective against the overactivation of the cells through the treatment with combination of LPS and IFN-γ (Fig. 1A). Overactivation of microglia is thought to induce apoptosis as a self-regulatory mechanism [35–37]. Anti-inflammatory cytokines such as IL-4 and IL-10, however, were without significant cytoprotective effects (Fig. 1B). Thus, pretreatment of microglia with a weak pro-inflammatory stimulant may protect the cells against the cytotoxic effects of excessive pro-inflammatory stimulation as well as exogenous toxic stimuli. When the cells were pretreated with LPS for 8 h, a similar cytoprotective effect was observed (Fig. 2). However, use of LPS at the same time or after exposure to cytotoxic agents significantly reduced the cytoprotection or did not confer the cytoprotection at all, respectively (Fig. 2), suggesting that the induction of LPS-dependent gene expression may be required for the cytoprotective effects of LPS. The cytoprotective effects of LPS pretreatment were observed at a wide range of concentrations of cytotoxic agents (data not shown), and 100 ng/ml of LPS gave an optimal result compared to 10 or 1000 ng/ml (data not shown).

3.2. Induction of heme oxygenase (HO)-1 by LPS in microglia

Previously, HO-1 has been shown to exert protective effects under stress conditions [33,38]. Thus, we hypothesized that the induction of HO-1 by LPS in microglia may be responsible for the cytoprotective effects of the LPS pretreatment. Western blot and RT-PCR analysis showed that the LPS treatment of BV-2 microglial cells for 6 h resulted in a strong increase in HO-1 protein levels (Fig. 3A) and mRNA levels (Fig. 3B). However, IL-4 and IL-10 did not significantly affect the HO-1 expression. These results indicate that HO-1 expression is specifically induced by LPS in microglia.

3.3. Role of HO-1 in the LPS-induced cytoprotective effect

The involvement of HO-1 in the cytoprotective action of the LPS pretreatment was tested using a specific HO-1 inhibitor, zinc(II) protoporphyrin IX (ZnPP). ZnPP at 100 nM partly abolished the LPS-induced protective effect (Fig. 4A), whereas ZnPP alone did not affect microglial cell viability (data not shown). Additionally, the LPS pretreatment reduced LPS/IFN-γ-induced NO production, which was completely abrogated by ZnPP pretreatment (Fig. 4B). These results indicate that HO-1 mediates the cytoprotective effects of LPS stimulation, and that HO-1 may also exert inhibitory effects on microglial NO

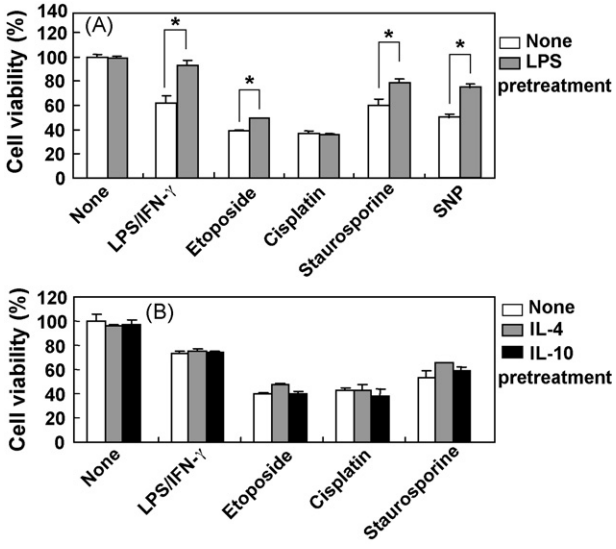

Fig. 1 – Cytoprotective effects of LPS pretreatment in microglia. BV-2 microglial cells were pretreated with LPS (100 ng/ml) (A; $^{*}p < 0.01$), IL-4 (20 ng/ml) or IL-10 (10 ng/ml) (B) for 6 h and then treated with LPS (100 ng/ml) plus IFN-γ (50 unit/ml), etoposide (10 μM), cisplatin (1 mM), staurosporine (50 nM), or SNP (0.5 mM) for 24 h. Afterwards, cell viability was assessed by MTT assay. Concentrations of IL-4 and IL-10 used in this study are previously known to induce biological effects. Bars represent mean ± S.D. values performed in triplicate and represent three independent experiments.

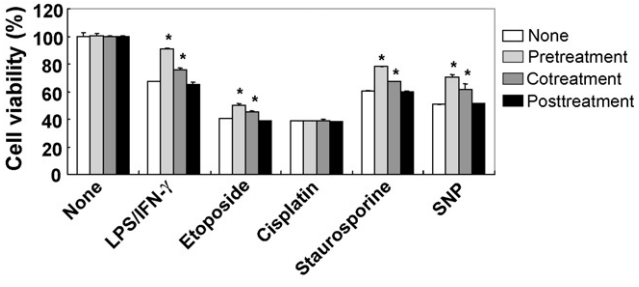

Fig. 2 – Kinetics of the LPS effects. BV-2 cells were either pretreated with LPS (100 ng/ml) for 8 h prior to the treatment with cytotoxic agents (of the same concentrations as in Fig. 1) (pretreatment) or co-treated with LPS and the cytotoxic agents at the same time (co-treatment). Alternatively, BV-2 cells were treated with LPS at 30 min after the treatment with the cytotoxic agents (posttreatment). Cell viability was assessed by MTT assay and analyzed in the same manner as in Fig. 1.

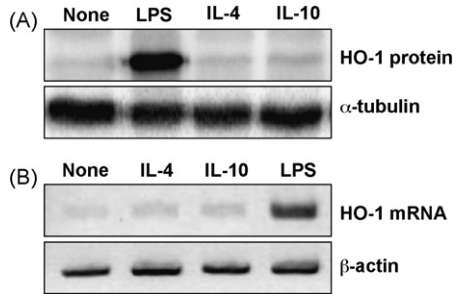

Fig. 3 – Induction of HO-1 expression by LPS in microglia. BV-2 microglial cells were treated with LPS (100 ng/ml), IL-4 (20 ng/ml), and IL-10 (10 ng/ml) for 6 h. HO-1 protein (A) or mRNA (B) was detected by Western blot analysis (A) or RT-PCR analysis (B). α-tubulin or β-actin was also detected to confirm the equal loading of the samples. The results are representative of three independent experiments.

production. These results were further confirmed by adenovirus-mediated overexpression of HO-1 in BV-2 cells (Fig. 5). Adenoviral expression of HO-1 provided cytoprotective (Fig. 5B) as well as NO-inhibiting effects (Fig. 5C) in a manner similar to the LPS pretreatment. Induction of HO-1 expression by adenoviral infection was confirmed by Western blot analysis (Fig. 5A). The adenoviral vector expressing GFP was used as a control, and it did not significantly influence HO-1 expression, BV-2 cell viability, or NO production.

3.4. *Cytoprotective effects of CO and iron*

HO-1 is an enzyme that oxidatively cleaves heme and produces iron, carbon monoxide and biliverdin [20]. Ferrous

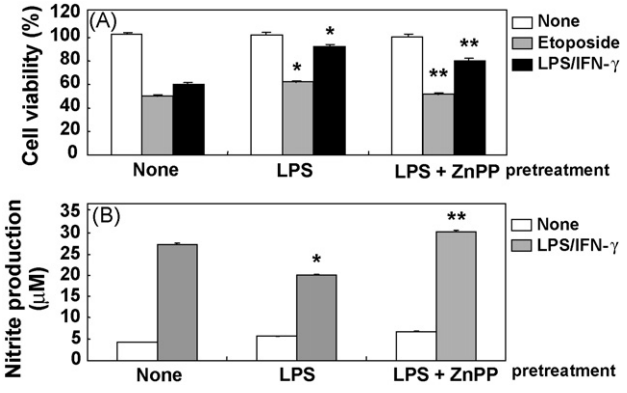

Fig. 4 – Role of HO-1 in the cytoprotection and NO production. BV-2 microglial cells were pretreated with LPS (100 ng/ml) alone or LPS in the presence of zinc protoporphyrin (ZnPP; 100 nM) for 6 h, and then treated with etoposide (10 μM) or LPS (100 ng/ml)/IFN-γ (50 unit/ml) for 24 h. Cell viability (A) or nitrite production (B) was assessed by MTT assay (A) or Griess reagent (B). Bars represent mean ± S.D. values performed in triplicate ($^{*}p < 0.01$ compared with no pretreatment; $^{**}p < 0.01$ compared with LPS-pretreatment) and represent three independent experiments.

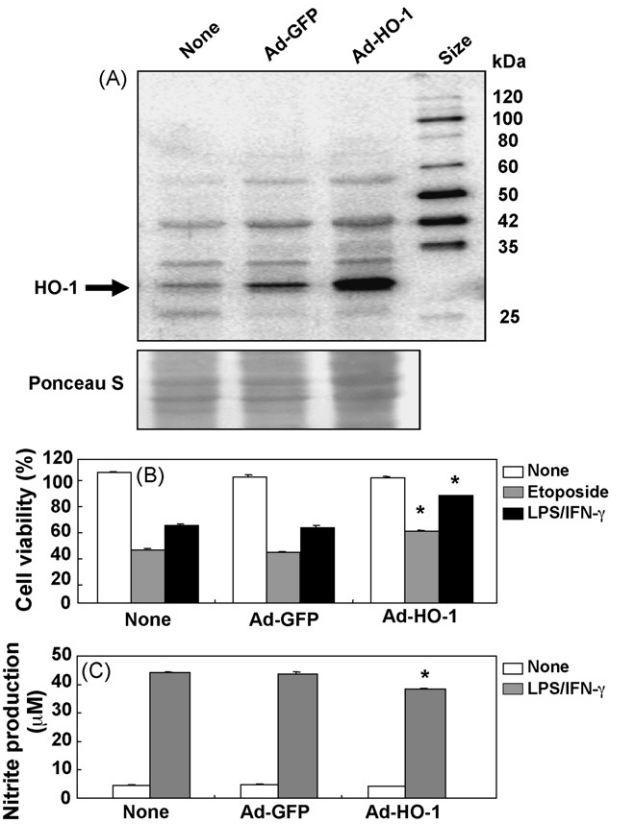

Fig. 5 – Cytoprotective role of HO-1 determined by adenoviral expression. BV-2 microglial cells were infected with adenoviral vectors expressing GFP (Ad-GFP) or HO-1 (Ad-HO-1) for 2 days, and then HO-1 protein was detected by Western blot analysis (A). Ponceau S staining was done to confirm the equal loading of the samples. Virus-infected cells were treated with etoposide (10 μM) or LPS (100 ng/ml)/IFN-γ (50 unit/ml) for 24 h, and then cell viability (B) or nitrite production (C) was assessed by MTT assay (B) or Griess reagent (C). Bars represent mean ± S.D. values performed in triplicate ($^{*}p < 0.01$ compared with Ad-GFP-infected cells) and represent three independent experiments.

iron released by HO-1 activity rapidly induces expression of ferritin, protecting cells under oxidizing conditions by sequestering free cytosolic iron [39]. CO acts as an activator of guanylyl cyclase in a manner similar to NO-like retrograde messenger [40] and carries out an anti-inflammatory and cytoprotective function [41]. To determine whether CO and iron released from heme degradation by HO-1 was responsible for the action of LPS, we examined the effects of tricarbonyldichlororuthenium(II) dimer (RuCO; a CO donor), deferoxamine (DFO; an iron chelator) and ferric citrate (FC; an iron donor) on the viability of BV-2 cells (Fig. 6). Pretreatment of BV-2 microglial cells with RuCO or FC conferred a cytoprotection, while DFO abolished the LPS effects (Fig. 6A and B). None of these reagents alone was cytotoxic (Fig. 6C). These results suggest that CO and iron derived from heme degradation mediate the LPS-induced cytoprotective effect in microglial cells.

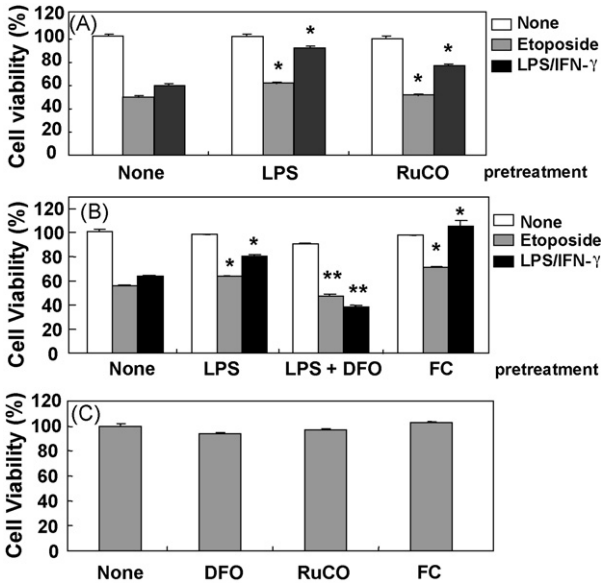

Fig. 6 – Cytoprotective effects of CO and iron. BV-2 microglial cells were pretreated with LPS (100 ng/ml) or CO donor tricarbonyldichlororuthenium(II) dimer (RuCO; 10 nM) for 6 h prior to the treatment with etoposide (10 μM) or LPS (100 ng/ml)/IFN-γ (50 unit/ml) for 24 h (A). Alternatively, BV-2 cells were pretreated with LPS or ferric citrate (FC; 50 μM) alone or LPS in the presence of desferrioxamine (DFO; 10 μM) for 6 h, and then treated with etoposide (10 μM) or LPS (100 ng/ml)/IFN-γ (50 unit/ml) for 24 h (B). BV-2 cells were also treated with DFO, RuCO, or FC alone for 24 h (C). Cell viability was assessed by MTT assay. Bars represent mean ± S.D. values performed in triplicate ($^{*}p < 0.01$ compared with no pretreatment; $^{**}p < 0.01$ compared with LPS-pretreatment). The results are representative of three independent experiments.

4. Discussion

In the CNS, microglial cells constitute the first line of defense against invading pathogens [1]. They are key immune cells that survey the brain parenchyma. During early onset of infection, microglia become activated and produce pro-inflammatory cytokines and chemokines. Production of these pro-inflammatory mediators may result in the infiltration of lymphocytes across the blood–brain barrier to sites of microbial infection [2]. Microglia are functionally very similar to macrophages in that they clear up dead neurons and other cell debris by phagocytosis [1,2]. Therefore, efficient immune functions by microglial cells are critical in protecting the CNS. Thus, microglial activation is generally considered as a physiological response aimed at protecting the neural tissue. There is now, however, growing evidence that toxic inflammatory mediators produced by activated microglia may also participate in the pathogenesis of neurodegenerative diseases such as Parkinson's diseases, Alzheimer's diseases, and HIV-associated dementia [42].

In addition to neurotoxic effects, overactivation of microglia leads to their own demise as well. Treatment of microglia with combination of LPS and IFN-γ has been shown to induce apoptosis of microglia. This has been proposed as an auto-regulatory mechanism whereby overactivated microglia can be eliminated *in vivo*. Now, our current work indicates that a prior stimulation of microglia with LPS induces HO-1 expression, which protects microglia against the overactivation-induced apoptosis. A similar protective mechanism also exists in astrocytes. NO at the high concentration induces apoptosis of astrocytes, whereas NO at the low concentration induces the expression of cytoprotective proteins that provide a resistance to NO toxicity [43]. The HO-1-induced cytoprotection was not limited to microglial activators, because LPS pretreatment rendered microglia resistant to cytotoxic agents such as etoposide and staurosporine. Furthermore, LPS-induced HO-1 showed an anti-inflammatory activity in microglia. Microglial NO production was decreased by HO-1 induction, which was reversed by HO-1 inhibitor. These results are in line with the anti-inflammatory role of HO-1 in macrophages [34]. It should be noted, however, that human microglia may produce no or minimal NO and have negligible inducible NO synthase [44]. Human astrocytes are probably the major cellular source of NO *in vivo*. Because the current study is based on the murine cell line, the results with respect to NO have to be interpreted with caution, and await further investigation.

Previously, IL-10 induced HO-1 expression in macrophages [34]. In the current study, however, IL-4 or IL-10 was without effect on the HO-1 expression in BV-2 microglial cells. Although the same concentration of recombinant IL-10 protein from the same commercial source was used in the current study compared to the previous report, HO-1 induction was not observed, indicating the differences in the gene regulatory mechanisms between macrophages and microglia. Signaling pathways underlying the LPS-dependent induction of HO-1 expression has been previously investigated. In one report, TNF-α has been implicated as a mediator of LPS-dependent HO-1 induction in liver [45]. In other studies, LPS-induced HO-1 expression was mediated via antioxidant response elements (ARE) of HO-1 gene promoter, which are activated through Nrf2 [46,47]. More recently, NF-κB and p38 mitogen-activated protein kinase signaling pathways have been shown to play an important regulatory role in the LPS-induced HO-1 expression in RAW264.7 cells [48]. Molecular mechanism of the LPS-induced HO-1 expression in microglia remains to be determined.

Three forms of HO have been identified: HO-1, HO-2, and HO-3 [21–23]. HO-1 can be induced by a variety of stimuli, including pro-inflammatory Th1 cytokines, ultraviolet irradiation, hypoxia and oxidative stress [24–27]. HO-2 and HO-3 have been reported as non-inducible genes. HO-1 is critical and indispensable to the survival of mammalians, and this paradigm is supported by observations of HO-1-deficient mice and humans. While HO-2-deficient mice can survive through their life span, HO-1-deficient mice frequently die in utero and only survive for less than 1 year, associated with marked splenomegaly and fibrosis [49,50]. All three forms of HO catalyze degradation of heme producing CO, iron, and biliverdin. Anti-inflammatory and cytoprotective effects of

these products have been previously investigated. Biliverdin reductase converts biliverdin into bilirubin, which alleviates oxidative damage at injury sites in experimental autoimmune encephalomyelitis models and [51,52]. CO attenuates bronchial inflammation induced by aeroallergen and prevents liver inflammation [40,41]. Finally, ferrous iron released by HO activity rapidly induces expression of ferritin, protecting cells under oxidizing conditions by sequestering free cytosolic iron [39]. Although we have not investigated the role of biliverdin in microglia, CO and iron released from heme degradation by HO-1 appeared to play an important role in the cytoprotective action of LPS pretreatment, based on the studies using CO donor, iron donor, and iron chelator.

In conclusion, we present evidence that HO-1 expression is induced by pro-inflammatory LPS treatment in BV-2 microglial cells, and HO-1 protects microglia against cytotoxic stimuli. The HO-1 protein and cytoprotective response were not, however, induced by anti-inflammatory cytokines like IL-4 and IL-10. Thus, pre-stimulation of microglia with inflammatogens may provide the cells with a protective phenotype that allows them to survive the later exposure to cytotoxic signals. Our results also suggest that pro-inflammatory stimuli play an important role in determining microglial survival and death as well as in regulating neuroinflammation and subsequent inflammation-mediated neurodegeneration.

Acknowledgments

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2007.06.016.

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