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Activated Microglia Are Less Vulnerable to Hemin Toxicity due to Nitric Oxide-Dependent Inhibition of JNK and p38 MAPK Activation

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In intracerebral hemorrhage, microglia become rapidly activated and remove the deposited blood and cellular debris. To survive in a harmful hemorrhagic or posthemorrhagic condition, activated microglia must be equipped with appropriate self-defensive mechanism(s) to resist the toxicity of hemin, a component released from damaged RBCs. In the current study, we found that activation of microglia by pretreatment with LPS markedly reduced their vulnerability to hemin toxicity in vitro. Similarly, intracorpus callosum microinjection of LPS prior to hemin treatment reduced the brain tissue damage caused by hemin and increased microglial density in the penumbra in rats. LPS induced the expressions of inducible NO synthase (iNOS) and heme oxygenase (HO)-1, the rate-limiting enzyme in heme degradation in microglia. The preventive effect by LPS was significantly diminished by an iNOS inhibitor, L-N^6-(1-iminoethyl)lysine, whereas it was mimicked by a NO donor, diethylamine-NONOate, both suggesting the crucial role of NO in the modulation of hemin-induced toxicity in activated microglia. We further found that NO reduced hemin toxicity via inhibition of hemin-induced activation of JNK and p38 MAPK pathways in microglia. Whereas HO-1 expression in LPS-stimulated microglia was markedly blocked by L-N^6-(1-iminoethyl)lysine, the HO-1 inhibitor, tin protoporphyrin, increased iNOS expression and decreased the susceptibility of LPS-activated microglia to hemin toxicity. The data indicate that the mutual interaction between NO and HO-1 plays a critical role in modulating the adaptive response of activated microglia to hemin toxicity. Better understanding of the survival mechanism of activated microglia may provide a therapeutic strategy to attenuate the devastating intracerebral hemorrhagic injury. *The Journal of Immunology, 2011, 187: 000–000.

Intracerebral hemorrhage (ICH) only accounts for 10–15% of all cases of stroke, but its devastating neurologic outcome is closely associated with the highest mortality rate >50–60% (1, 2). To cope with this formidable challenge, considerable research work on its pathologic process has been carried out. Recently, the importance of damage caused by mechanical effects of hematoma has been replaced by newly emerging interest in mechanisms of secondary brain injury intrigued by the observation that many patients deteriorate clinically even without rehemorrhage (3). This secondary brain tissue damage may result primarily from the toxicity of high amounts of hemin (10 mM) liberated from hemoglobin in a hematoma (4).

Microglia, as the first line of defense guards in brain pathologies, are rapidly activated, possibly within minutes after the onset of ICH (5). Activated microglia are thought to act like a double-edged sword in ICH. On one hand, the primary role for activated microglia is to exert its phagocytic ability of engulfing the deposited blood, damaged and dead cell debris, providing a nurturing environment for tissue reconstruction. In contrast, activated microglia ignite inflammatory responses by releasing a variety of cytokines, chemokines, free radicals, NO, and other potentially toxic molecules (6). Recently, Wang and Doré (7) reported that in ICH activated condition of microglia persisted for 3–4 wk. Thus, activated microglia must be equipped with some exquisite mechanisms to survive in harmful hemorrhagic or posthemorrhagic conditions.

The hemorrhagic brain tissue damage is dependent on the degree of the deposited intraparenchymal blood. Hemin, a breakdown cytotoxic product of hemoglobin, has been well known to elicit the secondary brain damage in ICH (1, 8). There is ~2.5 mM hemoglobin in blood, which when broken down will yield 10 mM hemin (4). Hematogenous phagocytes or activated microglia gradually clear hemin over weeks or months (2). Thus, activated microglia must be equipped with the self-defensive mechanism(s) against hemin toxicity. In the current study, to investigate how activated microglia overcome the hemin toxicity, a strong immunostimulant, LPS was used to activate microglia. We found that...
activated microglia became much less susceptible to hemin toxicity via self-production of NO and consequent inactivation of JNK and p38 signaling pathways.

Materials and Methods
Reagents and Abs
LPS, hemin, L-Aβ4-(1-minoethyllysine (L-NIL), diethylamine-NONOate (DEA-NONOate), and aminoguanidine were obtained from Sigma-Aldrich (St. Louis, MO), and tin protoporphyrin IX (SnPP) IX from Tocris (Ellisville, MO). SB203580 and SP600125 were purchased from Calbiochem (Darmstadt, Germany).

Cell cultures
Primary cortical neuronal cultures were prepared from embryonic 17–18-d-old fetal Sprague–Dawley rats, as described before (9). In brief, meninges-free brain tissues were dissociated by triturating through a Pasteur pipette. Neuronal cells (1.5 × 10^6 cells/mm²) were seeded onto plates precoated with poly-o-lysine/laminin and maintained in 10% FBS (Hyclone)-supplemented DMEM in humidified 95% air/5% CO₂ at 37 °C. Three days later, 5 μM cytosine arabinoside was added for 1 d to reduce the proliferation of nonneuronal cells. Subsequent media replacement was carried out every 3 d. Experiments were performed at 10 d after initial plating.

Mixed glial cells were prepared from the prefrontal cortices of 1-d-old Sprague–Dawley rat pups. Meninges-free cerebral tissues were dissected and softly triturated through a Pasteur pipette. Cells were then plated onto poly-o-lysine (1 μg/ml)-coated 75-cm² T-flask and kept for 1 wk in MEM supplemented with 10% heat-inactivated FBS and penicillin (100 U/ml)/streptomycin (100 μg/ml).

Pure microglia or astrocyte cultures were prepared from the mixed glial cell cultures. To obtain microglial cells, the flasks were shaken at 200 rpm for 1 min at 37 °C on day 8 of the mixed glia cultures. The medium containing detached microglial cells was collected and centrifuged at 2000 rpm for 20 s. The medium after centrifuge was taken as microglial-conditioned medium. Cells were resuspended, counted, and plated at a density of 1.05 × 10^5 cells/mm² in 48- or 6-well plates in conditioned medium. After 12 h, microglia were used for experiments. To obtain astrocytes, the flasks were shaken at 200 rpm overnight at 37 °C on day 8 of the mixed glia cultures. The medium containing detached microglial cells and oligodendrocytes were removed. The remaining astrocytes were trypsinized, washed, and replated at a density of 3.6 × 10^5 cells/mm² in the growth medium onto poly-o-lysine (10 μg/ml)-coated 48-well plates. Cells were used for experiments 4 or 5 d after plating.

LPS and hemin treatments and cell death determination
All treatments with LPS and hemin were prepared in serum-free MEM supplemented with 10% microglial-conditioned medium. To test the effect of LPS on hemin toxicity, cells were pretreated with LPS for 12 h, and then added with hemin. Six hours later, cell injury or death was morphologically observed under phase-contrast microscopy and quantified by measuring the release of lactate dehydrogenase (LDH) into the culture medium. In brief, stock solutions of sterile 100 mM potassium phosphate buffer and 23 mM sodium pyruvate in potassium phosphate buffer (pH 7.4) were prepared in advance and stored at 4 °C until use. A solution of NaN₃ (0.5 mM/ml in phosphate buffer) was freshly prepared. A 50-μl sample of medium was removed from each culture and was placed in a 96-well assay plate. Ten minutes after the addition of 125 μl NaN₃ solution into each well, 25 μl pyruvate was rapidly added using an Eppendorf repeater pipette with 8-well adapter. The absorbance of the reaction mixture at 340 nm was determined at 1-s intervals for 5 min, using a kinetic plate reader (Molecular Devices, Sunnyvale, CA). Cell viability was expressed as percentage of total LDH, which was measured in sister cultures frozen and thawed after those experiments. Cell injury or death was also assessed by measuring the uptake of cell membrane-impermeable fluorescent dye propidium iodide (PI). In brief, cells were fixed for 15 min with 4% paraformaldehyde at room temperature, then washed twice with PBS and stained with PI (2 μg/ml) for 20 min at room temperature. After three washes with PBS, fluorescence images were acquired using a fluorescence microscope (Leica DMIL) equipped with a Leica camera (DFC 420C). Cell death was quantified by counting the PI-positive cells.

Nitrite assay
NO production was determined by measuring the formation of nitrite, a stable oxidation product of NO. An aliquot of the conditioned medium was mixed with an equal volume of 0.1% N-1-naphthylethenediamine dihydrochloride and 1% sulfanilamide in 5% phosphoric acid. Absorbance at 550 nm was determined using a microplate reader (Molecular Devices).

Western blot analysis
Cells were washed twice with PBS and then lysed for 10 min at 4 °C in a lysis buffer containing 25 mM HEPES-NaOH (pH 7.4), 2 mM EGTA, 1% Triton X-100, 10% glycerol, 1 mM Na₂VO₃, 5 mM NaF, 1 mM-4-(2-aminoethyl)benzenesulfonyl fluoride, 10 μg/ml aprotinin, and 10 μg/ml leupeptin. The lysate was centrifuged at 15,000 × g for 10 min, and the protein concentration of the resulting supernatant was measured with the BCA Protein Assay Kit. For SDS-PAGE, the lysate was diluted 1:5 with 5× sample buffer (10% SDS, 12.5% 2-ME, 300 mM Tris [pH 6.8], 0.05% bromphenol blue, and 50% glycerol) and heated at 95 °C for 5 min before resolution on SDS-PAGE gels. Proteins were transferred onto polyvinylidene difluoride membranes. Membranes were blocked with 5% nonfat milk in TBST (25 mM Tris [pH 8], 150 mM NaCl, and 0.1% Tween 20) and then incubated at 4 °C overnight with 1:5000 dilution of rabbit anti–β-actin (NeoMarkers, Fremont, CA), 1:30,000 dilution of mouse anti-heme oxygenase (HO-1) (Stressgen Bioreagents, Victoria, BC, Canada), 1:1000 dilution of mouse anti-inducible NO synthase (iNOS; BD Transduction Laboratories, Lexington, KY), or 1:1000 dilution of primary Abs that recognize the phosphorylated or total forms of p38 MAPK and stress-activated protein kinase/JNK (Cell Signaling Technology, Beverly, MA) in 5% milk in TBST. Reporter bands were visualized by HRP-conjugated secondary Abs (Amersham Biosciences, Buckinghamshire, U.K.: 1:3000 dilution in 5% milk in TBST) and chemiluminescence using Western Lightning ECL (Amersham Biosciences).

Animals
Male Sprague–Dawley rats were purchased from Charles River Laboratories (Seoul, Korea), maintained under a 12-h light/dark cycle, and housed under conditions of controlled temperature (23 ± 1 °C) and humidity (55 ± 2%). Animals had access to rat chow and water ad libitum, and were used for experiments at 8 wk of age. All experimental procedures using animals were in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and were approved by the Committee of Korea University, College of Medicine.

Microinjection of LPS into corpus callosum
Rats were anesthetized with an i.p. injection of ketamine/xylazine hydrochloride (75/10 mg/kg, respectively) and placed in a stereotaxic frame (David Kopf Instruments, Tujunga, CA). Microinjection of LPS (Escherichia coli serotype 055:B5; Sigma-Aldrich) into the corpus callosum was performed with a 30-gauge needle through a dentist’s burr hole. To perform the injection into the corpus callosum, the following coordinates were used: 1.8 mm lateral from the sagittal suture, and 3.2 mm below the dura mater. LPS (5 μg/μl) or vehicle (sterile saline) injection was performed at a rate of 0.5 μl/min with 25 μl Hamilton Syringe operated by a microinjection pump (PDH 2000; Harvard Apparatus, Norwell, MA).

Microinjection of hemin into striatum
One day after LPS or vehicle microinjection into corpus callosum, microinjection of hemin into striatum was performed with the following coordinates: 2.7 mm lateral from the sagittal suture, and 4.7 mm below the dura mater. Each rat was microinjected with 15 μl hemin (0.8 mM) at a rate of 1 μl/min.

iNOS inhibitor treatment
To study whether iNOS expression contributes to the effect of LPS pretreatment on hemin toxicity in brain, rats were i.p. administered with the iNOS inhibitor, aminoguanidine (Sigma-Aldrich; 200 mg/kg) twice immediately before and 4 h after LPS microinjection.

Measurement of brain lesion area
Rats were sacrificed 3 d after hemin microinjection, and their brains were dissected out, fixed overnight in 4% paraformaldehyde in 0.1 M PBS, and placed in a 30% sucrose solution for cryopreservation. Serial 30-μm-thick coronal sections were prepared using a freezing microtome (Leica, Nussloch, Germany) and stained with cresyl violet for determination of lesion area. A digitized image of each cresyl violet-stained brain was obtained under a light microscope (Olympus BX 51; Olympus), and hemin-induced brain lesion area was determined using a computer-assisted image analysis program (OPTIMAS 5.1; Optimas, Edmonds, WA).
Rats were sacrificed 1 or 3 d after hemin microinjection and perfused transcardially with 4% paraformaldehyde in 0.1 M phosphate buffer. Brains were removed and postfixed overnight in 4% paraformaldehyde, followed by a 30% sucrose solution for cryoprotection. Serial 30-μm-thick coronal sections were prepared by a freezing microtome (Leica) and stored in PBS at 4°C as free-floating sections. Sections were incubated with 0.3% H2O2 in PBS for 30 min to quench endogenous peroxidase activity and then incubated in blocking buffer (10% normal horse serum-supplemented PBS) for 30 min. Sections were incubated overnight at room temperature with anti-ionized calcium-binding adaptor molecule 1 (Iba1, diluted 1:100; Wako, Osaka, Japan) or anti-HO-1 (1:400; Stressgen Bioreagents) Abs in PBS containing 0.3% Triton X-100 and 1% normal horse serum. After washing with PBS, immunoperoxidase histochemistry was performed using ABC method (Vector Laboratories, Burlingame, CA). A digitalized image of each section was obtained under a light microscope equipped with a charge-coupled device camera (Olympus BX 51; Olympus) and analyzed by using a cell counter plugin of ImageJ image processing and analysis program (National Institutes of Health). For quantification of microglial cells with Iba1 immunoreactivity, four nonoverlapping sections (971 × 729 μm^2) were randomly, but systematically selected from the ipsilateral penumbra near hemin infusion site, and cell counts were performed using ImageJ (National Institutes of Health) in a blind manner. Although we did not classify cell numbers depending on morphologies of Iba1-positive cells, there is no detectable resting or quiescent cell in ipsilateral penumbra sections examined for cell counts.

**Confocal immunofluorescence microscopy**

Sections were permeabilized and blocked in blocking buffer, as described above. Sections were then incubated overnight at room temperature with anti-phospho-p38 MAPK (Thr180/Tyr182) (clone 12F8; 1:50; Cell Signaling, Danvers, MA) and anti-Iba1 (1:10; Abcam, Cambridge, U.K.) Abs. After washing with PBS, the sections were incubated with a mixture of Alexa 488 donkey anti-goat IgG (1 μg/ml; Molecular Probes, Eugene, OR) and Cy3-conjugated donkey anti-rabbit IgG (2.0 μg/ml; The Jackson Laboratory, Bar Harbor, ME). After washing with PBS, the sections were counterstained with Hoechst staining and mounted with fluorescence mounting medium. Immunoreactivity was then observed under a confocal laser microscope (Zeiss LSM510; Zeiss, Oberkochen, Germany) with 488 and 543 nm laser lines for excitation, and 505–530 nm bandpass and 560 nm long-pass filters for emission. For quantification of nuclei-positive, Iba1/phospho-p38 MAPK double-immunolabeled cells, three nonoverlapping optical sections (450 × 450 μm^2, 5.0 μm thickness) were randomly, but systematically selected from the ipsilateral penumbra around hemin-damaged tissue core, and cell counts were performed using ImageJ (National Institutes of Health) in a blind manner and repeated by two independent counts by different persons. For high-power z-projection images, a single scan was taken at 1 μm thickness and further processed with Zeiss LSM510 image browser (ver. 4.10; Zeiss) and ImageJ (National Institutes of Health).

**Statistical analysis**

Data are expressed as mean ± SD or SEM, and analyzed for statistical significance using Student’s t tests or ANOVA, followed by Scheffe’s test for multiple comparisons. A P value <0.05 was considered significant.

**Results**

First, we examined the cell type-dependent hemin toxicity. Regardless of cell types, neurons, astrocytes, and microglia were all injured by hemin, and the order of vulnerability was microglia > neurons > astrocytes (Fig. 1A). Interestingly, pretreatment of LPS attenuated the hemin toxicity only in microglia (Fig. 1A). Concomitant treatment of LPS with hemin, however, did not diminish the hemin toxicity in microglia (Fig. 1B), suggesting that the preventive effect of LPS from hemin toxicity may not be direct, but involves the expression and/or activation of downstream mediators or signaling molecules.

NO has been shown to regulate the uptake and metabolism of hemin in vascular endothelial cells (10). Thus, a selective iNOS inhibitor, L-NIL, and a NO donor, DEA-NONOate, were respectively applied to address whether NO is involved in the reduction of hemin toxicity observed in activated microglia. Application of L-NIL to microglia for 1 h prior to LPS treatment completely blocked microglial NO production (Fig. 2A) and abolished the resistance of LPS-activated microglia to hemin toxicity (Fig. 2B, 2C). Expectedly, a NO donor, DEA-NONOate, attenuated hemin toxicity in a concentration-dependent manner (Fig. 2D), suggesting the putative role of NO in the resistance of activated microglia to hemin-induced toxicity.

Previously, HO, the rate-limiting enzyme in heme degradation, has been shown to closely interact with NO in the control of cellular homeostasis in vascular endothelial cells (11) and macrophages (12). Although activated microglia in hemorrhagic cerebral stroke induce both iNOS and HO-1 expressions (13, 14), little study has been done on their interaction. Thus, we further studied the possible interaction between HO-1 and iNOS in LPS-stimulated microglia. The expression of both HO-1 and iNOS increased in LPS-treated microglia in a time-dependent manner (Fig. 3A). LPS-induced expression of HO-1 in microglia was largely blocked by pretreatment with the iNOS inhibitor L-NIL. Furthermore, the NO donor, DEA-NONOate, mimicked LPS treatment in microglia (Fig. 3B), implying that NO regulates the expression of HO-1. In contrast, pretreatment with a HO-1 inhibitor, SnPP, significantly increased both LPS-induced iNOS expression and NO production (Fig. 4A, 4B). Whereas LPS induced the expressions of both iNOS and HO-1, hemin did that of HO-1 only, not iNOS (Fig. 4C). Interestingly, hemin downregulated the LPS-induced expression of iNOS, whereas it up-regulated that of HO-1 (Fig. 4C). In addition, inhibition of HO-1 activity by SnPP increased the resistance of LPS-activated microglia to hemin toxicity (Fig. 4D).

MAPK signaling pathways have been associated with cell survival/death processes in response to extracellular stress stimuli (15). Because hemin was previously reported to upregulate MAPKs (16), we further investigated whether MAPKs were involved in the toxicity of hemin and the cytoprotective effect of NO. Of MAPKs, JNK and p38 MAPKs were significantly phosphorylated by 1-h treatment of hemin (Fig. 5). The phosphorylation of both kinases was also induced by LPS treatment as early as 30 min (data not shown), but returned to their basal levels 12 h later. Interestingly, LPS pretreatment led to >50% decrease in hemin-elicted prompt phosphorylation of JNK and p38 MAPKs, whereas this inhibition could be totally abolished by an iNOS inhibitor, L-NIL. To confirm whether JNK and p38 MAPKs were involved in hemin toxicity, corresponding specific inhibitors SP600125 (JNK inhibitor) and SB203580 (p38 MAPK inhibitor) were applied (Fig. 6). As expected, hemin-induced LDH release in microglia was significantly reduced by those inhibitors.

**FIGURE 1.** Cell type-dependent hemin toxicity. A. Primary cultures of cortical neurons, astrocytes, and microglia were pretreated with 10 ng/ml LPS for 12 h and then treated with 40 μM hemin for 6 h. B. Microglia were cotreated with 10 ng/ml LPS and 40 μM hemin for 6 h. Cell injury or death was determined by measuring the extent of LDH release at the end of incubation with hemin. Data are expressed as percentage of total LDH and represent means ± SEM of four separate experiments. **p < 0.01, significantly different from hemin alone-treated group.
To further confirm the crucial role of NO in hemin toxicity in vivo, we microinjected hemin into the striatum of rat brain. The area of lesion reached to a maximum size at 3 d after microinjection of hemin. Intracerebral delivery of LPS leads to a strong microglial activation and has been widely used as a model to investigate the role of microglia in vivo (17–19). Previously, we reported that microinjection of LPS into rat corpus callosum highly activated microglial cells in the ipsilateral hemisphere, and maximum activation of microglial cells was observed as early as 1 d after LPS microinjection (20). Starting from 1 d after injection, the density of microglial cells in ipsilateral striatum was gradually increased over 7 d, possibly due to proliferation and/or recruitment (data not shown). As expected, intracorpus callosum microinjection of LPS significantly decreased the hemin-induced brain damage, whereas the reduced hemin toxicity by LPS was blocked by concomitant injection of an iNOS inhibitor aminoguanidine with LPS (Fig. 7).

To examine the vulnerability of activated microglia to hemin toxicity in vivo, we examined the morphology and the density of microglia in hemin-injected lesion area. Hemin strongly activated microglial cells and increased expression of HO-1 in ipsilateral hemisphere (Fig. 8A). LPS injection prior to hemin treatment significantly increased the density of microglial cells in the ipsilateral penumbra around hemin-injected site, where decreased density of microglial cells by hemin was observed (Fig. 8). Double immunolabeling and confocal images demonstrated that hemin induced strong microglial activation and phosphorylation of p38 MAPKs in reactive microglia at 1 d after hemin injection (Fig. 9). Consistent with in vitro study, LPS pretreatment led to modest,
but significant decrease in hemin-elicited phosphorylation of p38 MAPKs, as shown in the decreased number of phospho-p38 MAPK-labeled microglia in the ipsilateral penumbra (Fig. 9). Quiescent microglia in the contralateral striatum was neither significantly different in density before and after hemin or LPS injection (Fig. 8), nor immunostained with anti–phospho-p38 MAPK Abs (Fig. 9A).

Discussion

Hemoglobin oxidation leads to the sustained generation of cytotoxic by-products within the parenchyma for weeks after a brain hemorrhage, causing extensive brain injury (21, 22). One such product, hemin, is a potent oxidant that accumulates in intracranial hematomas at high concentrations and is toxic to multiple cell types. Hemoglobin- or hemin-mediated oxidative injury has been investigated in astrocytes (23, 24) and neurons (25, 26), but there is no relevant report for microglia to date. Furthermore, although much evidence has been accumulated for the association of activated microglia with hemorrhagic brain injury in animal experimental models (27, 28), little is known about their survival during or after hemorrhagic insult. To our knowledge, this is the first study to demonstrate that LPS-activated microglia become more resistant to the toxicity of hemin. The reduced vulnerability of activated microglia to hemin must be helpful for them to exert this primary role, such as elimination of hematoma even under injurious hemorrhagic or posthemorrhagic environments. Our in vitro and in vivo studies showed that iNOS expression was critically associated with cell survival from hemin toxicity. Of cell types tested, only activated microglia show less vulnerability to hemin toxicity. The cell type-dependent resistance appears to be related with the capability of NO production. In this study, the iNOS inhibitor, L-NIL, blocked NO production in activated microglia and abolished their resistance to hemin toxicity. Furthermore, the preventative effect caused by LPS treatment was mimicked by a NO donor, DEA-NONOate. In the
present experimental condition (i.e., 12-h treatment with LPS), notable NO production was observed only in microglial cells, but neither in cortical neurons nor astrocytes (data not shown). These results may not be fully consistent with previous studies, in which LPS treatment was shown to stimulate the expression of iNOS in rodent astrocytes (29–31). However, some researchers insist that LPS-induced NO production in cultured astroglial cells may be due to the coexisting microglia (32). In fact, LPS treatment did not provoke NO production in our pure astrocyte cultures. Another possibility is that the duration (i.e., 12 h) of LPS treatment used in our study was not sufficient to stimulate the NO production in astrocytes.

In the current study, we found that NO increased the protein expression of HO-1 in microglia. Because the expression of HO-1 in LPS-treated microglia was blocked by L-NIL, NO is thought to be the upstream regulator of HO-1 expression. Such NO-mediated HO-1 expression was similarly observed in many other cell types, such as macrophage and vascular endothelial cells (12, 33). In contrast, the LPS-induced activity of iNOS was increased by HO-1 inhibitor SnPP, and its expression was remarkably inhibited by HO-1 inducer hemin, suggesting that the enhanced expression of HO-1 by LPS and hemin may result in negative feedback inhibition of iNOS activity. Similar feedback inhibition was also reported in macrophage (12). Furthermore, we found that treatment with SnPP led to increased resistance of LPS-activated microglia to hemin toxicity, possibly due to recovered NO production. The present results may in part explain the previous findings showing that administration of HO-1 inhibitor reduced volumes of hematoma and edema, and improved neurologic deficits after ICH (34–36). Interactive regulation between NO and HO-1 may adjust the cellular level of each other within a physiologically endurable range to keep microglial homeostasis against pathological threats in cerebral hemorrhagic lesion.

MAPKs are well known to be activated by various environmental stresses and associated with their cytotoxicities (37). In the current study, we showed that activation of JNK and p38 MAPKs was implicated in the cytotoxicity of hemin in microglia (Figs. 5, 6, 9). Treatment of microglia with hemin dramatically increased JNK and p38 MAPK phosphorylation, which was attenuated by LPS pretreatment. The reverse of hemin-induced JNK/p38 MAPK phosphorylation by L-NIL implies that iNOS expression in activated microglia might attenuate hemin toxicity by reducing the activities of JNK and p38 MAPKs. In this study, cotreatment of both JNK and p38 MAPK inhibitors did not show any additive or synergistic inhibition of hemin toxicity, suggesting that the activation of both JNK and p38 MAPK pathways may act on common downstream death signaling pathways and/or is required for full execution of hemin-induced microglial cell death. Similarly, we recently reported that inhibition of only one of the MAPK pathways is sufficient to block the activated microglia-induced neuronal cell death (38). On the contrary to JNK and p38 MAPKs, ERK might not be involved in hemin toxicity, because its activity was not altered by hemin (data not shown). The activation of ERK, however, was previously reported to potentiate hemin toxicity in astrocyte and neuron cultures (23, 26). Further studies are needed to delineate the cell type-dependent role of ERK in hemin toxicity.

FIGURE 6. Reduced hemin toxicity by JNK and p38 inhibitors. A, Microglia were pretreated with SP600125 (SP, 20 μM) or SB203580 (SB, 20 μM) for 2 h, and then stimulated by hemin (40 μM) for 1 h. Levels of the phosphorylated forms of MAPKs were normalized with respect to the total forms of kinases and expressed as relative fold changes in comparison with hemin alone-treated group, which were arbitrarily set to 1.0. n = 3. B, Microglia were pretreated with SP600125 or SB203580 for 2 h, followed by hemin treatment for 6 h. Data are expressed as percentage of total LDH and represent means ± SEM. n = 4. *p < 0.05, **p < 0.01, significantly different from hemin alone-treated group.

FIGURE 7. Reduced hemin toxicity in LPS-pretreated rat brain. A, Coronal sections were stained with cresyl violet 3 d after intrastriatal infusion of 15 μl hemin into rat striatum. Brain lesion area indicated by line was determined using a computer-assisted image analysis program (a, original magnification ×100). B, The size of brain lesion area 3 d after hemin infusion. Values are mean ± SD. n = 10. **p < 0.01, significantly different between the indicated groups.
What is the molecular mechanism regulating hemin-induced JNK and p38 MAPK activation and NO-mediated inhibition of the activation of both signaling pathways? One of the most plausible explanations is that hemin induces oxidative injury either on its own or by releasing catalytic iron, which may activate JNK and p38 MAPKs. Because NO has high affinity against heme and iron–sulfur proteins, it might be postulated that excess NO may nitrosylate hemin to inhibit its oxidative potential. In fact, it has been demonstrated that cellular heme readily combines with NO intracellularly to form nitrosyl-heme, which inhibits the reactivity of heme moiety and prevents catabolism of the heme ring and release of iron as well as hydrogen peroxide-induced cell death in a series of elegant experiments using cultured human and porcine endothelial cells (10). Considering the hydrophobic nature of this reaction, we might imagine the situation that the concentration of released NO from microglia is not high enough to meet the required stoichiometry inside neighboring neurons and astrocytes. It might explain the microglia-specific resistance against hemin toxicity.

Under certain circumstances, prestimulation of microglia with inflammatogens may allow them to survive the later exposure to cytotoxic signals. Accordingly, previous studies have shown that proinflammatory activation of BV-2 murine microglial cells with LPS conferred a protection against cytotoxic agents, such as NO, etoposide, and staurosporine (39, 40). Another study demonstrated that activation of microglia with the neuron-derived chemokine fractalkine reduced Fas-induced cell death (41), and fractalkine-deficient mice are less susceptible to transient focal cerebral ischemia (42). In the current study, we found that LPS treatment...
Disclosures

The authors have no financial conflicts of interest.

References


