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HEME OXYGENASE-1 PROTECTS BRAIN FROM ACUTE EXCITOTOXICITY

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Abstract—Heme oxygenase is a rate-limiting enzyme that degrades heme, a pro-oxidant, into carbon monoxide, iron, and bilirubin. Heme oxygenase has two active isoforms: heme oxygenase-1 and heme oxygenase-2. Heme oxygenase-1 can be induced by various insults. Several investigators have postulated that it has cytoprotective activities, although its role in the nervous system is not fully understood, especially considering that normally heme oxygenase-2 accounts for the vast majority of heme oxygenase activity in the brain. Here, the basal effect of heme oxygenase-1 was investigated in acute glutamatergic excitotoxicity to test the hypothesis that *N*-methyl-D-aspartate-induced acute toxicity in brain is attenuated by heme oxygenase-1. *N*-methyl-D-aspartate was unilaterally injected into the striatum of wildtype and heme oxygenase-1 knockout mice. After 48 h, brains were harvested, sectioned, and stained with Cresyl Violet to measure the lesion size. Lesion volume was significantly ($P<0.05$) greater in brains of heme oxygenase-1 knockout mice (15.2 ± 3.1 mm^3; $n=10$) than in those of wildtype mice (6.2 ± 1.5 mm^3; $n=11$). In addition, Western blot analysis indicated no detectable differences between wildtype and heme oxygenase-1 knockout mouse brains in the levels of the glutamate or *N*-methyl-D-aspartate receptors studied. To test whether heme oxygenase-1 could specifically protect neurons, mouse primary neuronal cell cultures of wildtype and heme oxygenase-1 knockout mice were treated with or without *N*-methyl-D-aspartate. Cell viability of the heme oxygenase-1 knockout neurons was significantly less than that of wildtype neurons at each of the *N*-methyl-D-aspartate concentrations tested ($12.8\pm1.3\%$, $16.0\pm1.4\%$, and $18.4\pm1.8\%$ at 30, 100, and 300 μM *N*-methyl-D-aspartate, respectively). These results indicate that heme oxygenase-1 provides neuroprotection against acute excitotoxicity and suggest that potential intervention that can increase heme oxygenase-1 activity within the brain should be considered as a therapeutic target in acute and potentially chronic neurological disorders. **© 2006 IBRO. Published by Elsevier Ltd. All rights reserved.**

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Abbreviations: CO, carbon monoxide; Fe^{2+}, iron; GluR1, glutamate receptor 1; HO, heme oxygenase; HO-$1^{-/-}$, heme oxygenase-1 knockout; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide; NMDA, *N*-methyl-D-aspartate; NR1, *N*-methyl-D-aspartate receptor 1; WT, wildtype.

Key words: cultures, glutamate, mouse, neurodegeneration, NMDA.

Rapid synaptic excitation is mediated by one or more of the three classes of glutamate-gated ion channel receptors (AMPA, kainate, and *N*-methyl-D-aspartate (NMDA)). NMDA receptors, the most abundant of the three, are universally distributed throughout the brain. This receptor is implicated mainly in excitatory neurotransmission and is considered to play a vital role in normal CNS function (Lipton and Rosenberg, 1994). Abnormally high synaptic levels of NMDA or glutamate activate NMDA receptors and cause excitotoxicity. During this process, the elevated levels of synaptic glutamate overexcite the cells, rendering them unable to respond properly, and ultimately leading to cell death (Arundine and Tymianski, 2003).

Under excitotoxic conditions, free heme, a pro-oxidant, is generated from hemoproteins released by dying cells (Lindquist and Craig, 1988; Sass et al., 2003; Takahashi et al., 2004; Tang et al., 2004). Heme oxygenase (HO) oxidizes free heme in the presence of NADPH-cytochrome P_{450} reductase and then cleaves it specifically into three end products: biliverdin, carbon monoxide (CO), and iron (Fe^{2+}) (Maines and Panahian, 2001; Otterbein et al., 2003b). Biliverdin is rapidly reduced by biliverdin reductase to bilirubin, which has antioxidant activity (Stocker et al., 1987; Llesuy and Tomaro, 1994; Doré and Snyder, 1999). Of the two active isoforms of HO that have been cloned and characterized, HO-2 is constitutively expressed, whereas HO-1 is inducible. HO-2 is enriched in the brain, and under normal conditions accounts for the vast majority of HO activity in that organ (Ewing and Maines, 1997; Doré, 2002). HO-1 is most highly expressed in the spleen and liver, and normally is barely detectable in the brain; however, its expression is increased under various stress-inducing pathological conditions (Camhi et al., 1995; Choi et al., 1996; Nimura et al., 1996; Doré et al., 1999a; Maines and Gibbs, 2005). Although HO-1 is clearly induced in microglia, its presence in neurons is still controversial, but some reports have shown immunoreactivity of HO-1 in neurons, endothelial cells, and astrocytes in various models of neurodegeneration (Fukuda et al., 1996; Koistinaho et al., 1996; Panahian et al., 1999; Lu and Ong, 2001; Munoz et al., 2005).

Kainic acid, an excitatory amino acid, activates kainate receptors and has been reported to induce HO-1 expression in microglial cells, supporting a role for HO-1 in excitotoxicity and potentially also oxidative stress injury (Matsuoka et al., 1998; Lu and Ong, 2001). A recent report indicated that HO-1 induction by cobalt protoporphyrin pro-

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tects cerebral vascular endothelial cells of newborn pigs from glutamate-induced cell death (Parfenova et al., 2005). The authors proposed that the toxic effect of glutamate caused oxidative stress by increasing the intracellular level of reactive oxygen species; the toxic effect was decreased by an increase in HO activity.

Several research groups, including ours, have studied the role of HO-2 in neuroprotection by manipulating various cellular processes (Doré et al., 1999a, 2000; Chang et al., 2003). Although it has been shown that HO-1 prevents various kinds of toxicity in cell culture (Ferris et al., 1999; Kim et al., 2005), its role in neurologic conditions *in vivo* is still not clear, and there is a need to explore the possible neuroprotective role of HO-1 in acute excitotoxicity. We hypothesized that HO-1 would afford neuroprotection in *in vivo* and *in vitro* models of acute excitotoxicity. To determine the neuroprotective potential of HO-1, wildtype (WT) and heme oxygenase-1 knockout (HO-$1^{-/-}$) mice were given unilateral injections of NMDA intrastriatally, and lesion volumes were compared. To further investigate whether protection was mediated through neurons, mouse primary embryonic neuronal cell cultures derived from WT and HO-$1^{-/-}$ mice were treated with NMDA, and cell viability was evaluated. The overall objective was to determine whether acute excitotoxicity is modulated by HO-1 in naïve animals.

EXPERIMENTAL PROCEDURES

Chemicals

Unless stated otherwise, all chemicals were purchased from Sigma Co. (St. Louis, MO, USA), and materials used for cell cultures were obtained from Invitrogen (Carlsbad, CA, USA).

Mice

Adult male HO-$1^{-/-}$ mice and WT littermates (20–25 g) were used in this study, which was conducted in accordance with National Institutes of Health guidelines for the use of experimental animals. The animal protocols were approved by the Institutional Animal Care and Use Committee of Johns Hopkins University. In accordance with the guidelines set by the National Institutes of Health, the number of animals used was kept to the minimum required to establish statistical significance. Also, every attempt was made to minimize the pain and suffering of animals during and after surgery.

Treatment of mice

Weight and rectal temperature of each mouse were recorded before the surgical procedure. Anesthesia was induced with 3.0% halothane and thereafter maintained at 1.0% halothane. Each mouse was mounted on a stereotaxic frame (Stoelting Co., Wood Dale, IL, USA), and 0.3 μl of NMDA (67 mM, pH, 7.2), prepared in phosphate-buffered saline, was injected into the right striatum over a 2-min period as described previously (Ayata et al., 1997; Ahmad et al., 2006); the needle was left *in situ* for an additional 5 min to prevent backflow. WT (n=11) and HO-$1^{-/-}$ (n=10) mice were treated identically; sham control mice (WT, n=4; HO-$1^{-/-}$, n=4) underwent the same procedures but received saline only. After injections, mice were placed in a humidified, thermoregulated chamber maintained at 31 °C and were returned to their cages after full recovery from anesthesia. Throughout the experimental procedure, mouse rectal temperature was monitored and maintained at 37.0±0.5 °C.

Assessment of lesion volume

Forty-eight hours after injection, weight and rectal temperature were recorded, and brains were harvested and immediately frozen in 2-methylbutane (pre-cooled over dry ice); 20-μm sections were cut on a cryostat and stained with Cresyl Violet to measure lesion volume. Brain sections were photographed and analyzed with SigmaScan Pro 5.0 (Systat, Inc., Point Richmond, CA, USA).

Primary cultures of mouse embryonic neuronal cells

Cultures of corticostriatal neurons were isolated from 17-day embryos of pregnant WT and HO-$1^{-/-}$ mice. Cultures were prepared in serum-free conditions. Neurons were plated onto poly-D-lysine-coated, 24-well dishes at a density of 1×10^6 cells/well in B27 supplemented with HEPES-buffered, high glucose Neurobasal medium, as described (Doré et al., 2000). Cells were incubated in growth medium at 37 °C in a 95% air/5% CO_2-humidified atmosphere until the day of experiment. Half of the initial medium was removed at day 4 and replaced with fresh medium.

Treatment of mouse primary embryonic neuronal cell cultures

After 14 days in culture, cells were incubated in fresh medium containing NMDA. Experimental treatments with different doses of NMDA (0, 30, 100, and 300 μM) were conducted in the Neurobasal medium B27 minus antioxidant supplement (B27-AO™).

Assessment of cell survival

Neurons were maintained for a period of 24 h, and their survival was assessed by phase-contrast microscopy with Trypan Blue exclusion assay and quantified with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) colorimetric assay. After 1.5 h incubation at 37 °C, living cells containing MTT formazan crystals were solubilized in a solution of anhydrous isopropanol, 0.1 N HCl, and 0.1% Triton X-100. The optical density was determined at 570 nm. Experiments were performed in triplicate, and repeated with at least three separate batches of cultures.

Expression of glutamate and NMDA receptors

Cortex and striatum were dissected from mouse brains and homogenized for Western blot analysis as described earlier (Doré et al., 2000). Proteins were quantified by the BCA assay (Pierce, Rockford, IL, USA) according to the manufacturer's directions. Western blots were performed on 12% SDS/PAGE gels (Invitrogen), and proteins were transferred to nitrocellulose membranes (Bio-Rad, Hercules, CA, USA). Membranes were stained with Ponceau S solution (Sigma) to verify that equal amounts of protein were loaded and transferred. Membranes then were blocked for 1 h at room temperature with 5% skim milk in PBS with 0.1% Tween 20 before incubation at 4 °C overnight with primary antibodies to *N*-methyl-D-aspartate receptor 1 (NR1; rabbit anti-NR1, 1:2000, Sigma), NR2A (rabbit anti-NR2A; Chemicon, Temecula, CA, USA, 1 μg/ml), NR2B (rabbit anti-NR2B, Chemicon, 1 μg/ml), glutamate receptor 1 (GluR1; rabbit anti-GluR1, Chemicon, 1 μg/ml), and GluR2 (rabbit anti-GluR2, Chemicon, 1 μg/ml). The blots were also probed for beta-tubulin (Promega, Madison, WI, USA) as a control. Blots were washed and incubated with secondary antibodies (Amersham Biosciences, Piscataway, NJ, USA) for 1 h at room temperature and then developed with ECL (Amersham).

Statistical analysis

Data were analyzed with SigmaStat 2.0 (Systat), and significance level was set at $P<0.05$. Statistical analysis was performed by Student's t-test. All data are reported as mean±S.D.

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