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Induction of Heme Oxygenase-1 Is Involved in CO Mediated Central Cardiovascular Regulation

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Running Title: HO-1 expression in the NTS of rats

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ABBREVIATIONS: CO, carbon monoxide; NTS, nucleus tractus solitarii; HO, heme oxygenase; HO-1, heme oxygenase-1; HO-2, heme oxygenase-2; SD rat, Sprague-Dawley rat; ZnPPIX, zinc protoporphyrin IX; ZnDPBG, zinc deuteroporphyrin 2,4-bis glycol; NO, nitric oxide; cGMP, cyclic guanosine monophosphate; BP, blood pressure; HR, heart rate; ANOVA, analysis of variance; GFAP, anti-glial fibrillary acidic protein; NeuN, neuronal nuclei

Abstract

Carbon monoxide (CO) has been identified as an endogenous biological messenger in the brain. Heme oxygenase (HO) catalyzes the metabolism of heme to CO and biliverdin. Previously, we have shown the involvement of CO in central cardiovascular regulation, baroreflex modulation, and glutaminergic neurotransmission in the nucleus tractus solitarii (NTS) of rats. In this study, we examined which HO isoform could be induced after hemin injection in the NTS. We also investigated their in-situ distributions in the NTS after induction. Male Sprague-Dawley (SD) rats were anesthetized with urethane, and blood pressure was monitored intra-arterially. Unilateral microinjection of hemin (1 nmol), a heme molecule cleaved by HO to yield CO, produced significant decrease in blood pressure and heart rate. These cardiovascular effects of hemin were attenuated by prior administration of HO inhibitor zinc protoporphyrin IX (ZnPPIX). Microinjection of hemin into NTS resulted in significant induction of HO-1 protein expression in-situ. Pretreatment of ZnPPIX significantly inhibited the HO-1 induction after hemin injection. No significant changes of HO-2 expressions were found after hemin injection and ZnPPIX pretreatment. The in-situ inductions of the HO-1 protein expression were further confirmed to be in glial cells and neurons after hemin injections into the NTS. These results indicated HO-1 but not HO-2 might be responsible for the generation of CO and contribute to central control of cardiovascular effects.

Introduction

Heme oxygenase (HO) is the rate-limiting enzyme responsible for the catabolism of heme and subsequent production of carbon monoxide (CO), biliverdin and iron (Maines, 1997; Maines et al., 1998). To date, three isoforms of HO have been identified. HO-1, a 32-kDa protein, induced by heme and numerous oxidative stressors that is enriched in spleen and liver. HO-2, a 36-kDa protein, is present abundantly in the brain and testis as a constitutive enzyme (Maines, 1997; McCoubrey et al., 1997). HO-3 is a 33-kDa protein that shares about 90% homology with HO-2 but exhibits a relatively low catalytic activity (McCoubrey et al., 1997). HO and its metabolic products have been implicated in the regulation of numerous biological processes (Maines, 1997; Maines et al., 1998). Among them, CO derived from HO activity has been shown to function as a neurotransmitter (Verma et al., 1993), a vasodilator (Furchgott and Jothianandan, 1991), and an endogenous modulator of the NO-cGMP signaling in brain (Ingi et al., 1996).

HO-1 has been demonstrated to provide cytoprotection in various in vitro and in vivo systems. The activation of HO-1 gene has been considered to be an adaptive cellular defense mechanism (Poss and Tonegawa, 1997). In addition, either acute (Sacerdoti et al., 1989) or chronic (Escalante et al., 1991) administration of an inducer of HO-1 to spontaneously hypertensive rats led to a normalization of blood pressure. Other inducers of HO-1 or HO substrates have also been shown to decrease blood pressure in hypertensive rats (Levere et al., 1990; Martasek et al., 1991; Johnson et al., 1996). Moreover, treatment of normal (Johnson et al., 1995) or endotoxemic (Yet et al., 1997) rats with inhibitors of HO (metalloporphyrins) has been shown to produce an increase in systemic arterial pressure. Because biliverdin itself has not been

associated with the regulation of blood pressure (Johnson et al., 1995), these studies provided evidence that CO via the HO activity may contribute to the regulation of blood pressure.

HO is widely expressed in brain and is responsible for the CO-generating ability of the brain, including brainstem (Ewing and Maines, 1991; Maines et al., 1993). In the central nervous system, the nucleus tractus solitarii (NTS) is the site where afferent fibers arising from the arterial and cardiopulmonary baroreceptors make the first central synapse, and thus play an important role in the integration of autonomic control of cardiovascular system (Reis, 1984). It has been reported that CO formed within the NTS subserves a vasodepressor mechanism that is tonically active in awake rats (Johnson et al., 1997). Furthermore, we have shown that unilateral microinjection of hemin or hematin, a heme molecule cleaved by HO to yield CO, into the NTS produce dose-related depressor and bradycardic effects (Lo et al., 2000; Lin et al., 2003). On the other hand, either systemic administration (Johnson et al., 1997) or direct microinjection of HO inhibitor, zinc deuteroporphyrin 2,4-bis glycol (ZnDPBG) into the NTS attenuates the baroreceptor reflex (Lo et al., 2000). Taken together, these findings suggested that CO within the NTS might play an important role in the regulation of cardiovascular function. However, it is not clear whether HO actually exists in the NTS.

The present study investigated whether hemin injection into the NTS induced different HO isoform (HO-1 and HO-2) expression in addition to their in-situ localizations after induction. Our results showed that both HO-1 and HO-2 exist in the NTS but only HO-1 level is significantly elevated upon hemin microinjection.

These results suggested that HO-1 might play a role in central cardiovascular regulation.

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Materials and Methods

Materials. Experimental drugs such as urethane, and L-glutamate were purchased from Sigma Chemical Co. (St. Louis, MO, USA). ZnPPIX was obtained from Tocris Cookson Ltd. (UK). ZnPPIX was dissolved in 50 mmol/l Na_2CO_3 (pH 8.8 to 9.4) immediately before use. Hemin (1 nmole/60 nL, ICN Biochemicals Inc., USA) was dissolved in 30% 0.1 N NaOH (pH 8.6 to 9). All other drugs were dissolved in normal saline on the day of the experiment.

Animal procedures and tissue preparation. Male Sprague–Dawley rats (250 to 350 g) were obtained from National Science Council Animal Facility and housed in the animal room of Kaohsiung Veterans General Hospital (Kaohsiung, Taiwan). The rats were kept in individual cages in a room in which lighting was controlled (12 hours on/12 hours off), and temperature was maintained at 23 to 24^{o}C. The rats were given Purina Laboratory Chow and tap water ad libitum.

All animal protocols have been approved by the Research Animal Facility Committee at Kaohsiung Veterans General Hospital. Humane treatment is administered at all times. Rats were anesthetized with urethane (1.0 g/kg i.p. and 300 mg/kg i.v. if necessary). The preparation of animals for intra-NTS microinjection and the methods used in the localization of the NTS have been described previously (Tseng et al., 1996). For microinjections into the NTS, a glass cannula was filled with L-glutamate (0.154 nmol/60 nL) to functionally identify the NTS. A specific decrease in blood pressure (BP) and heart rate (HR) ($\geqq$-35 mm Hg and -50 bpm) was demonstrated after microinjection of L-glutamate in the NTS. After that the BP and HR were observed through microinjection of hemin (1 nmol/60nL) before and 10

minutes after intra-NTS administration with HO inhibitor ZnPPIX (1 nmol/60 nL) or vehicle alone (50 mmol/L Na_2CO_3, 60 nL)

After completion of the experiments, rats were perfused intracardially with saline followed sequentially by a solution of 4% formaldehyde. Paraffin embedding was performed. Five μm sections of the brainstem were stained with H&E. The proper placement of the pipette tip in the NTS was verified by histological examination. Maps and coordinates (from Bregma) are taken from the atlas of Paxinos and Watson. For total RNA and protein extractions, the NTS was removed immediately following the completion of the experiments without formaldehyde perfusion and cryopreserved for further extraction.

Western Blot Analysis. At the time points 10 min or 4 hour after microinjection of hemin or pretreatment with ZnPPIX into the NTS, the NTS tissue was separated carefully under the microscopy examination from individual rats. Briefly, tissues on both sides of the dorsomedial part of the medulla oblongata at the level of NTS (1 mm rostral or caudal from the obex) were collected by micropunches made with a stainless steel bore (1 mm i.d.) (Chan et al., 2004). Subsequentially, these individual NTS tissues were used for western blot. Western blot was used to determine the HO-1 and HO-2 protein expression levels in the NTS. The procedures were as previously described with minor modifications (Huang et al., 2004). Briefly, total protein were prepared by homogenized NTS in lysis buffer containing 20 mM Imidiazole-HCl (pH 6.8), 100 mM KCl, 2 mM MgCl, 20 mM EGTA (pH 7.0), 300 mM sucrose, 1 mM NaF, 1 mM Na-vanadate, 1 mM Na molybetadate, 0.2% Triton X-100, and proteinase inhibitor cocktail (Roche, USA) for 1 hour at 4^{o}C. Equal amounts (30 μg/sample

assessed by BSA protein assay, Pierce Chemical Co., IL) of protein were separated on 12.5% SDS-Tris glycine gel electrophoresis and transferred to a nitrocellulose membrane (Amersham, USA). The membrane was blocked with 5 % non-fat milk in TBST buffer [10 mM Tris (pH 7.5), 100 mM NaCl, 0.1% Tween 20] and incubated with rabbit anti-rat HO-1 polyclonal antibody (1:4,000, StressGen, Victoria, BC, Canada), rabbit anti-rat HO-2 polyclonal antibody (1:4,000, StressGen, Victoria, BC, Canada) (Ndisang et al., 2002), mouse anti-α tubulin antibody (1:10,000, Sigma, USA) (Huang et al., 2004) in TBST with bovine serum albumin and incubated for 1 hour at room temperature. Peroxidase conjugated anti-mouse or rabbit antibody (1:5,000 with HO-1, 1:15,000 with HO-2, and 1:10,000 with α-tubulin, Jackson Laboratory, USA) was then applied. The membrane was developed using ECL-Plus protein detection kit (Amersham, England). The protein expression levels were then determined using NIH-Image 1.61 software (USA).

Immunohistochemistry Analysis. Immunohistochemical staining was performed according to procedures as described (Huang et al., 2004). Briefly, rat brain was fixed with 4 % formaldehyde. Paraffin embedded serial sections was cut at 5 μm thickness. The sections were deparaffinized, biotin blocked (Biotin blocking system, DAKO), microwaved (0.01 M citric buffer, pH 6.4), quenched (3 % H_2O_2/methanol), blocked (3 % goat serum), and incubated in IHC-specific rabbit anti-rat HO-1 antibody, rabbit anti-rat HO-2 antibody (1:500, StressGen, Victoria, BC, Canada), rabbit anti-GFAP antibody (1: 500, DAKO, UK), or mouse anti-NeuN antibody (1: 500, Chemicon, USA) at 4°C overnight. After incubating the sections with biotinylated secondary antibody (1:200, Vector Laboratories, CA, USA) for 1 hour and then AB complex (1:100) for 30 minutes at room temperature. The sections

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were visualized using DAB substrate for 5 minutes (Vector Laboratories, CA, USA) and counterstained with hematoxylin. The sections were then photographed with an Olympus microscope equipped with CCD imaging system (Japan).

Quantitative real time RT-PCR (qRT-PCR). Total RNA from NTS before and after hemin treatment was extracted using TRI reagent according to manufacturer's protocols (Molecular Research Center, CA, USA). Five μg of total RNA was used to synthesize first strand cDNA using 50 U of StrataScript reverse transcriptase and 500 ng of oligo-dT primer (Stratagene, CA, USA). One twentieth of reverse-transcription products were used as template for qRT-PCR in LightCycler technology (Roche Molecular Biochemicals) using a SYBR green assay. PCR reaction was performed in 50 μl SYBR Green PCR Master Mix (Applied Biosystems; Foster City, CA) containing 10 μM forward primers and reverse primers, and approximately 30 ng cDNA. Amplification and detection were performed by: 1 cycle of 95 □ for 10 min, 40 cycles of 95 □ for 15 s, and 62 □ for 20 s, and 72 □ for 15 s. After completion, a final melting curve was performed by denaturation at 95 □ for 15 s and then was recorded by cooling to 60 □ and then heating slowly until 95 □ for 20 min according to the dissociation protocol of ABI7700 instrument. The primer sequences for HO-1: forward primer 5'- AGCTCTATCGTGCTCGC-3', reverse primer 5'-GTGTTCCTCTGTCAGCAGT-3', which amplified a 110 bp of HO-1 cDNA fragment. The β-actin mRNA level was determined using: forward primer 5'-TCACCCACACTGTGCCCATCTACGA-3' and reverse primer 5'-CAGCGGAAC CGCTCATTGCCAATGG -3', which amplified a 295-bp β-actin cDNA fragment.

Statistical analysis. All data were expressed as mean ± S.E.M. A paired *t* test

(before and after pre-treatments), unpaired *t*-test (for control and study group comparisons), or repeated-measures analysis of variance (ANOVA) followed by Dunnett's test for significant differences was applied to compare group differences. Differences with a *P* value of less than 0.05 were considered significant.

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Results

Cardiovascular Effects of Hemin in the NTS. In agreement with our previous finding, intra-NTS microinjection of hemin (1 nmol), a heme molecule cleaved by HO to yield CO, resulted in hypotension and bradycardia (Lo et al., 2000; Lin et al., 2003). The administration of vehicle (30% 0.1 N NaOH) itself elicited only slight cardiovascular effects, and the pattern of response was different from that of hemin. After pretreatment with a HO inhibitor, ZnPPIX (1 nmol), for 10 minutes the depressor and bradycardiac response to hemin were attenuated significantly (from -45±2 mm Hg and -64±7 bpm to -26±3 mm Hg and -28±5 bpm, respectively, $P<0.05$, paired *t*-test) (Figure 1 A, B). Pretreatment with the vehicle of ZnPPIX did not affect the cardiovascular responses to hemin (from -43±3 mm Hg and -59±7 bpm to -41±3 mm Hg and -65±6 bpm, respectively, $P>0.05$, paired *t*-test). In order to exclude the possibility of the hypotension and bradycardia was caused by biliverdin or iron, we have tested the cardiovascular effects of biliverdin and iron by the NTS microinjection. Unlike hemin, neither of them had cardiovascular effect when microinjection of biliverdin or ion even at the high dosage of 1 mole/60nL into NTS. These findings suggested that CO in the NTS might play an important role in the regulation of cardiovascular function.

HO-1 protein induced by hemin microinjection into the NTS. The roles of HO in central cardiovascular control remained undetermined. Two types of heme oxygenases were thought to be responsible for the HO functions in controlling cardiovascular effects: the inducible heme oxygenase 1 (HO-1) and the constitutive heme oxygenase 2 (HO-2). To determine the roles of these two enzymes in central cardiovascular control, we determined whether HO-1 and HO-2 protein expression

were induced in the NTS after microinjection of hemin. Western blot analysis revealed a significant increase in HO-1 protein expression (Figure 2A and 2B, 1.77±0.07-fold, $P<0.05$, n=6) after hemin microinjection into the NTS compared with that of control (Figure 2A, lane 4 versus lane 3). In order to identify the successful placement of the NTS, the L-glutamate response was taken as the control group. Furthermore, pretreatment with ZnPPIX attenuated hemin induced increase in HO-1 expression (Figure 2A, lane 5; 1.18±0.17-fold, $P<0.05$). In contrast, HO-2 protein expression showed no significant changes after hemin injection or ZnPPIX pretreatment compared to control (Figure 2C, 1.19±0.27-fold, 0.83±0.08-fold, respectively). These results suggested the HO-1 elevation might play a role in hemin mediated central cardiovascular effects.

Hemin induced HO-1 in-situ protein expression in the NTS. We then determined the in-situ protein expression levels and localizations of HO-1 and HO-2 after microinjection of hemin and pretreatment with ZnPPIX. Figure 3 and 4 showed immunohistochemical analysis of HO-1 and HO-2 expression in the NTS, respectively. Relatively low to mild-levels of HO-1 expression were observed in very few cells in the NTS of the control group (L-glutamate injection only) (Figure 3A and 3B). However, intensive immunostaining of the HO-1 in many cells with different morphology was noted after injecting hemin into the NTS (Fig. 3D and 3G). Qualitative and quantitative analysis of HO-1 protein expression was performed after hemin microinjection and ZnPPIX pre-treatment according the methods described in materials and methods. Figure 3E showed significant induction of HO-1 protein in the right hemisphere of NTS after hemin injection compared with the control (L-glutamate injection only) in the left hemisphere (26.7±0.9 % vs 10.3±0.5 %, n = 32,

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P<0.01). The potency of hemin induced HO-1 expression did not differ in the anatomical localization of NTS as equal induction of HO-1 was found after injecting hemin into either left or right hemisphere of NTS (Figure 3C-D vs 3F-G). Pretreatment of ZnPPIX significantly inhibited the induction of HO-1 in the NTS compared to control (L-glutamate injection only) (Figure 3J, 18.6±0.7% vs 26.9±0.9%, n = 32, *P*<0.01). HO-2 protein expression was then examined after hemin injection with or without pretreatment of ZnPPIX. Figure 4 demonstrated in-situ HO-2 expression in NTS after hemin injection and pretreatment of ZnPPIX. Quantitative analysis showed no significant changes of HO-2 positive cells in the NTS after hemin injection into right hemisphere of NTS compared with control (L-glutamate injection only) in the left hemisphere (Figure 4E, 35.4±1.2% vs 36.3±1.0%, n =32). Pretreatment with ZnPPIX did not significantly affect the HO-2 expression in the right hemisphere of NTS compared to that of control (L-glutamate injection only) in the left hemisphere (Figure 4J, 32.9±0.9% vs 35.0±0.9%, n = 32). It is surprising that the HO inhibitor blocked the hemin-induced HO-1 induction. This suggests that a HO products, carbon monoxide, biliverdin or iron, is responsible for the induction. The immunohistochemistry experiments were performed to investigate whether biliverdin or $FeCl_2$ solution elevates the HO-1 protein level in the NTS. Neither biliverdin nor $FeCl_2$ solution can elevate the HO-1 protein level in the NTS (data not shown). Taken together, we can exclude the possibility that biliverdin or iron is responsible for the HO-1 induction when microinjection of hemin into the NTS. CO can not be directly microinjected into the NTS, but by exclusion CO is highly suspected to be responsible for the HO-1 elevation.

Hemin microinjection induced HO-1 protein expression in NTS neurons and

glial cells. While examining the in-situ protein expressions of HO-1 in the NTS after hemin injection, we observed at least two major cell types stained positive for HO-1 antibody. One cell type was with smaller nucleus with multiple fine protrusions (Figure 5C, arrowhead). The other cell type was with larger nucleus with flat and polygonal morphology (Figure 5C, arrow). These cells were further confirmed to be of glial and neuronal origin by staining positive for GFAP and NeuN antibody, respectively (Figure 5D and 5H). The cells in the NTS that were found stained positive for HO-2 antibody were primarily large cells with scanty cytoplasm with flat and polygonal morphology (Figure 5G, arrow). They were further identified to be of neuronal origin since they stained positive with NeuN antibody. These results indicated that the protein expression of HO-1 in NTS neurons and glial cells might be involved in central controls of cardiovascular effects.

Induction of HO-1 mRNA expression in the NTS after hemin microinjection. We further determined whether HO-1 mRNA expression levels were altered after microinjection of hemin into the NTS. The quantitative Real-Time PCR was performed to examine the level of HO mRNA. The results showed that elevated HO-1 mRNA level was observed at the 10 min (26% increase) and 4 hours (121% increase) after hemin microinjection (Figure 6). These results indicate that HO-1 protein induction in the NTS after hemin injection may be mediated through transcriptional modification at the RNA level.

Discussion

In the present study, we demonstrated that microinjection of hemin, a precursor for CO production, into the NTS induced depressor and bradycardic effects. These effects of hemin required HO because prior administration of a HO inhibitor (ZnPPIX) significantly suppressed the cardiovascular effects of intra-NTS microinjection with hemin (Figure 1A and 1B). These results were similar to our previous findings (Lo et al., 2000; Lin et al., 2003). It has been shown that administration of an HO inducer such as heme-L-arginate or heme-L-lysinate causes a marked decrease in BP in spontaneously hypertensive rats (SHR) (Johnson et al., 1996). Intracardiac injection of high-titer retrovirus encoding HO-1 gene into SHR has been shown to decrease blood pressure (Sabaawy et al., 2001). In addition, HO inhibitors has been shown to increase BP and peripheral resistance and suggest that endogenous CO subserves a tonic vasodepressor function (Johnson et al., 1995). Direct microinjection into the NTS of heme oxygenase inhibitor, ZnDPBG or ZnPPIX, attenuates the baroreceptor reflex (Johnson et al., 1997; Lo et al., 2000). It has been indicated that CO apparently acts in the NTS promoting changes in glutamatergic neurotransmission and lowering blood pressure (Johnson et al., 1999). We have reported that metabotropic glutamate receptors may couple to the activation of HO via the liberation of CO to participate in central cardiovascular regulation (Lo et al., 2002). Taken together with our results that neither biliverdin nor iron, two products form hemin showed cardiovascular effect when microinjected into NTS, these findings suggest that the HO/CO system may be involved in the regulation of various neural and cardiovascular functions.

The nucleus tractus solitarii (NTS) is a nexus of nerve endings for cardiovascular chemo- and baroreceptor to regulate cardiovascular functions (Spyer, 1981; Reis,

1984). To date, no systematic study has been carried out to correlate the actual expression levels of HO-1 and HO-2 protein in the NTS. In our study, we examined the pattern of expression of HO-1 and HO-2 in the NTS by Western blot and immunohistochemical analysis. Our results showed the expression of HO-1 protein was significantly enhanced after hemin microinjection into the NTS in comparison to that of control. However, hemin treatment did not increase the expression of HO-2 protein in the NTS. On the other hand, HO-2 protein expression levels were not different between hemin treatment and ZnPPIX pretreatment groups (Figure 2). Moreover, Immunohistochemical staining also showed quantitative increase in HO-1 protein expression after hemin treatment. Pretreatment with ZnPPIX reduced hemin-induced HO-1 protein expression in the NTS. The expression of HO-2 protein was not different among the hemin treated groups and not affected by ZnPPIX pretreatment (Figure 3 and Figure 4). The quantitative Real-Time PCR was used to examine the level of HO mRNA (Figure 6) and the results showed that HO-1 mRNA level was elevated at the 10 min and 4 hours after hemin microinjection. These results supported our results that HO-1 protein level increased after hemin injection and indicated that HO-1 protein induction may be mediated through transcriptional modification at the RNA level. Together, these results implied that hemin induced the expression of HO-1, leading to the increase HO activity and enhanced CO production might be contributed to the central cardiovascular regulation.

Under normal conditions, HO-1 is present in the whole brain at the limit of detection by radioimmunoassay (Sun et al., 1990) and Western blot analysis (Trakshel et al., 1988). However, it has been shown that under normal conditions the HO-1 isozyme is expressed in very few select neuronal and non-neuronal cell populations

(Ewing and Maines, 1991; Ewing et al., 1992). It is usually considered that induction of HO-1 protects cells from oxidative stress and has an important role in antioxidant defense responses (Applegate et al., 1991; Chen et al., 2000). Given that prior HO-1 induction protects against insults in some systems, possible neuroprotective therapies based on inducing overexpression of HO-1 have been suggested (Panahian et al., 1999). In addition, it has been demonstrated that either acute (Sacerdoti et al., 1989) or chronic (Escalante et al., 1991) administration of an inducer of HO-1 (stannous chloride) to spontaneously hypertensive rats led to a normalization of blood pressure. Other inducers of HO-1 or HO substrates have also shown to decrease blood pressure in hypertensive rats (Levere et al., 1990; Martasek et al., 1991; Johnson et al., 1996). In the present study, we have demonstrated that CO via HO-mediated hemin metabolism can significantly regulate the central cardiovascular effects. Similarly, we have previously reported that the nitric oxide (NO) was involved in the NTS regulation of blood pressure and that NO synthase inhibitor attenuated baroreflex activation (Lo et al., 1996; Tseng et al., 1996). Other report has showed CO shares some of the chemical and biological properties of NO (Marks et al., 1991). Endogenous CO production could lead to cGMP synthesis through activation of guanylyl cyclase (Marks et al., 1991; Verma et al., 1993). The enzyme HO could act as a source of CO in neurons. Two forms of this enzyme are found in the brain. HO-1 normally shows only a limited distribution, but its synthesis can be selectively increased in certain neurons and glial cells through activation of heat shock elements by diverse stimuli. Conversely, HO-2 is widely distributed in the brain under all conditions (Maines et al., 1998).

Furthermore, we observed the in-situ protein expressions of HO-1 in the NTS

after hemin microinjection, we observed at least two major cell types stained positive for HO-1 antibody. These cell were further confirmed to be of glial and neuronal origin by staining positive for GFAP and NeuN antibody, respectively. HO-1 protein expression was predominantly noted in neurons of the NTS and glial cells were sparsely distributed in the NTS. However, HO-2 positive cells were found localized mostly in neurons (Figure 5). It has been suggested that excitotoxin-induced toxicity is partially mediated by oxidative stress (MacGregor et al., 1996; Matsuoka et al., 1998), and the glial cells in which HO-1 protein was induced may be resistant to oxidative stress. Overexpression of HO-1 may contribute to the resistance of glial cells to oxidative stress. Moreover, recent report has suggested that overexpression of HO-1 might exerted cytoprotective and antiapoptotic effects on glial cells (Munoz et al., 2005). At the same time, glial cells overexpressing HO-1 may be involved in repairing the lesioned area and reconstruction of the neuronal functions and/or scavenging dead neurons and/or terminals. HO-1 overexpression in NTS neurons might also elicit additional protection toward stress induced by Hemin. The actual roles of HO-1 expressing glial cells and neurons in NTS mediated central control of cardiovascular effects remained to be determined.

HO-2 was found concentrated in the brain and testes, accounting for the great majority of HO activity in the brain (Maines, 1997). Evidence has suggested that HO-2, localized to selective neuronal populations (Verma et al., 1993; Maines, 1997), plays a major role in neuromodulatory activities, with CO participating as a putative neurotransmitter. Thus, HO-2 mRNA and protein are selectively concentrated in discrete neuronal populations, although most, if not all, neurons do possess HO-2. The abundant expression of HO-2 has been associated with neuronal protection against

oxidative stress injury by quenching free radicals (Dore et al., 1999). In our experiments, we found HO-2 expressions in NTS neurons. Even though we did not detect any significant changes of HO-2 expression after hemin injection or pretreatment with ZnPPIX, the possibility of increased enzyme activity due to post-translational modification can not be excluded. Similar finding has been shown previously by Ndisang et al. In their study, the expression of HO-2 protein was not found different among all animal groups tested and not affected by hemin treatment (Ndisang et al., 2002). Although we have showed hemin induces HO-1expression that suggests HO-1 might be responsible for generation of CO in the NTS, it is still possible that the more abundant HO-2 also plays a role in CO generation in the NTS. The phosphorylation and activation of HO-2 remains unclear that will be investigated in the future to distinct the roles of HO-1 and HO-2 in the NTS.

Perspectives. The HO-CO system as already proved to be a potential regulator of various neural and cardiovascular functions. Results from this study show that HO-1 and HO-2 expression occurred in the NTS and we provide a broad array of evidence that HO-1 rather than HO-2, is implicated in the NTS effect. Furthermore, we are the first group to demonstrate the enhanced HO-1 protein expressions in-situ in the NTS after hemin injection. We further observed at least two major cell types stained positive for HO-1 antibody. We also suggested that microinjection of hemin into the NTS may induce the activation of HO-1 via the liberation of CO to participate in central cardiovascular regulation. The identification of the actual roles of HO-1 expression glial cells and neurons in the NTS will be matter of future research.

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Footnotes

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Legends for Figures

Figure 1. The cardiovascular effects of microinjection of hemin into the NTS before and after ZnPPIX. A, Tracings show cardiovascular effects of microinjection of hemin (1 nmol) into the NTS before and after ZnPPIX (1 nmol) in anesthetized rats. Hemin and ZnPPIX were injected at the indicated time points. BP, MBP, and HR recordings were made at a paper speed of 3 mm/min. Horizontal bar represents time period of 5 minutes. B, Comparative MBP and HR effects of hemin (1 nmol) by the HO inhibitor ZnPPIX on intra-NTS administration of the substances. Hemin was injected in the absence (control), presence of vehicle or ZnPPIX. Vertical bars represent SEM change from baseline values, which were 93±3 mm Hg for MBP and 348±5 bpm for HR. Each point represents the average of 8 rats. *****Significant different from vehicle control hemin response (*****$p<0.05$).

Figure 2. Western blot analysis of HO-1 and HO-2 protein expression in the NTS after hemin and HO inhibitor injections. **A**. Westen blot picture showed HO-1, HO-2 and α-tubulin protein expressions. Lane 1. rat spleen was used as a positive control for HO-1 protein expression. Lane 2. rat testis was used as a positive control for either HO-1 or HO-2 protein expression. Lane 3. HO-1 and HO-2 protein expression in the NTS after injecting L-gluatmate (control). Note low levels of HO-1 and abundant HO-2 protein. Lane 4. effects of Hemin on HO-1 and HO-2 protein expressions in the NTS. Note increased HO-1 but not HO-2 protein expression after hemin microinjection. Lane 5 effects of HO inhibotor ZnPPIX on hemin-induced expression of HO-1 and HO-2 in the NTS. Note decreased expression of HO-1 but not HO-2 after ZnPPIX microinjection. **B** and **C.** Densitometric analysis of HO-1 (B) and

HO-2 (C) level before and after hemin and ZnPPIX injections. Bars are the mean ± S.E. of four experiments. *****P<0.05, compared with lane 3, ＃P<0.05, compared with lane 4.

Figure 3. In-situ qualitative and quantitative analysis of HO-1 expressed cells in the NTS after hemin and HO inhibitor ZnPPIX microinjections. **A**. **C**. low power-field views of a SD rat NTS left and right hemispheres after L-glutamate (control) and hemin microinjection respectively (100X). **F**. **H**. low power-field views of a SD rat NTS left and right hemispheres after hemin and ZnPPIX + hemin microinjection respectively (100X). **B**. **D**. **G**. **I**. high power-field view of left and right hemisphere of NTS showing cytoplasmic HO-1 protein expression after hemin and ZnPPIX injections (400X). Arrowheads showed the HO-1 positive staining cells. Note increased numbers of HO-1 positive cells in the NTS after hemin injection (**D**. and **G**.) compared to control (**B**.). Also note reduced HO-1 positive cells after HO-1 inhibitor ZnPPIX pretreatment (**G**.). **E**. **J**. Graphs depicted quantitative analysis of in-situ HO-1 expressed cells in SD rat NTS after injecting L-glutamate (control), hemin, and ZnPPIX + hemin. Percent HO-1 positive cells were determined by counting HO-1 expressing cells in each hemisphere of the NTS at 100X powerfield divided by all the cells in the same paraffin section. Statistical analysis was determined by paired *t* test. ***** $P < 0.01$ (n = 32). Note a significant increase in in-situ HO-1 positive cells after injecting hemin in the NTS. Also note ZnPPIX pretreatment significantly decreased HO-1 positive cells in the NTS.

Figure 4. In-situ qualitative and quantitative analysis of HO-2 expressed cells in the NTS after hemin and HO inhibitor ZnPPIX microinjections. **A**. **C**. low power-field

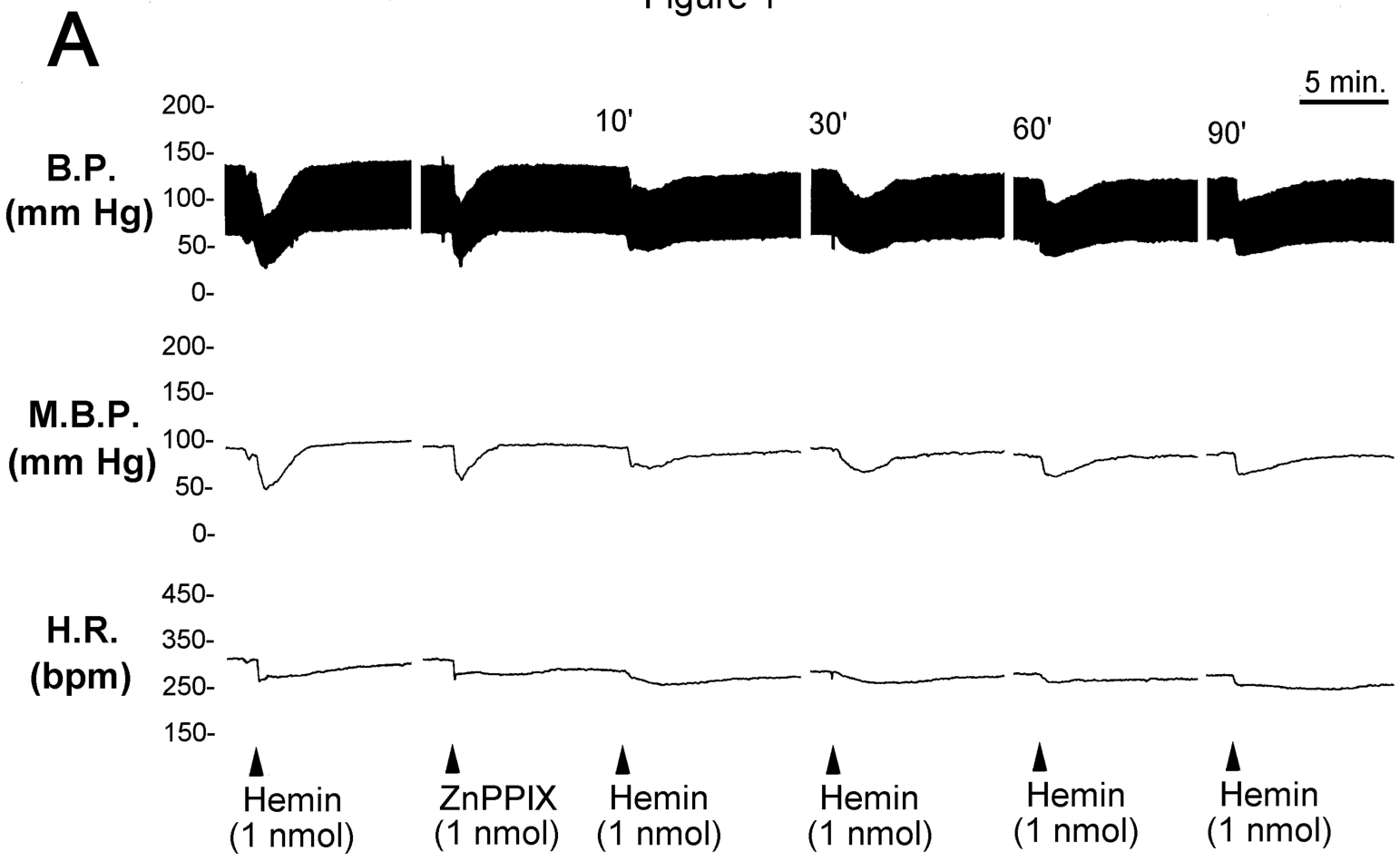
views of a SD rat NTS left and right hemispheres after L-glutamate (control) and hemin microinjection respectively (100X). **F**. **H**. low power-field views of a SD rat NTS left and right hemispheres after hemin and ZnPPIX + hemin microinjection respectively (100X). **B**. **D**. **G**. **I**. high power-field view of left and right hemisphere of the NTS showing cytoplasmic HO-2 protein expression after hemin and ZnPPIX injection (400X). Arrowheads showed the HO-2 positive staining cells. **E**. **J**. Graphs depicted quantitative analysis of in-situ HO-2 expressed cells in the NTS after injecting L-glutamine (control), hemin, and ZnPPIX + hemin. Percent HO-2 positive cells were determined by counting HO-2 expressing cells in each hemisphere of the NTS at high power-field (200X) divided by all the cells in the same paraffin section. Statistical analysis was determined by paired *t* test (n = 32). Note no significant differences of HO-2 positive cells were found after injecting hemin and pretreatment of ZnPPIX.

Figure 5. Immunohistochemical analysis of HO-1 and HO-2 protein expression in the NTS after hemin injection. **A**. SD rat spleen expressed high levels of endogenous HO-1 protein in interstitial lymphocytes and was used as a positive control for HO-1 protein expression in paraffin sections (400X). **B**. Left-hemisphere of the NTS showed HO-1 expression after hemin microinjection (100X). **C**. high power-field view of HO-1 expression in the left hemisphere of the NTS (400X). Note at least two types of cells expressed HO-1 protein after hemin injection. Arrowheads showed astrocytic cells expressed HO-1 in the cytoplasm. Arrows showed larger cells with scanty cytoplasm expressed HO-1 protein. **E**. SD rat testis expressed high levels of endogenous HO-2 protein was used as a positive control for HO-2 protein expression in paraffin sections (400X). **F**. left-hemisphere of the NTS showed HO-2 expression

after hemin microinjection (100X). **G**. high power-field view of HO-2 expression in the left hemisphere of the NTS (400X). Arrows showed large cells with scanty cytoplasm expressed HO-2 protein. **D** and **H**. serial sections of the NTS left hemisphere as **C**. stained with GFAP and NeuN antibody respectively (400X). Note in **D**. the astrocytic cells in **C**. were stained positive for glial cell marker GFAP. Note in **H**. the larger cells in **C**. and **G**. were stained positive for neuronal marker NeuN.

Figure 6. Quantification of HO-1 mRNA expression by real time RT-PCR.

Figure 1



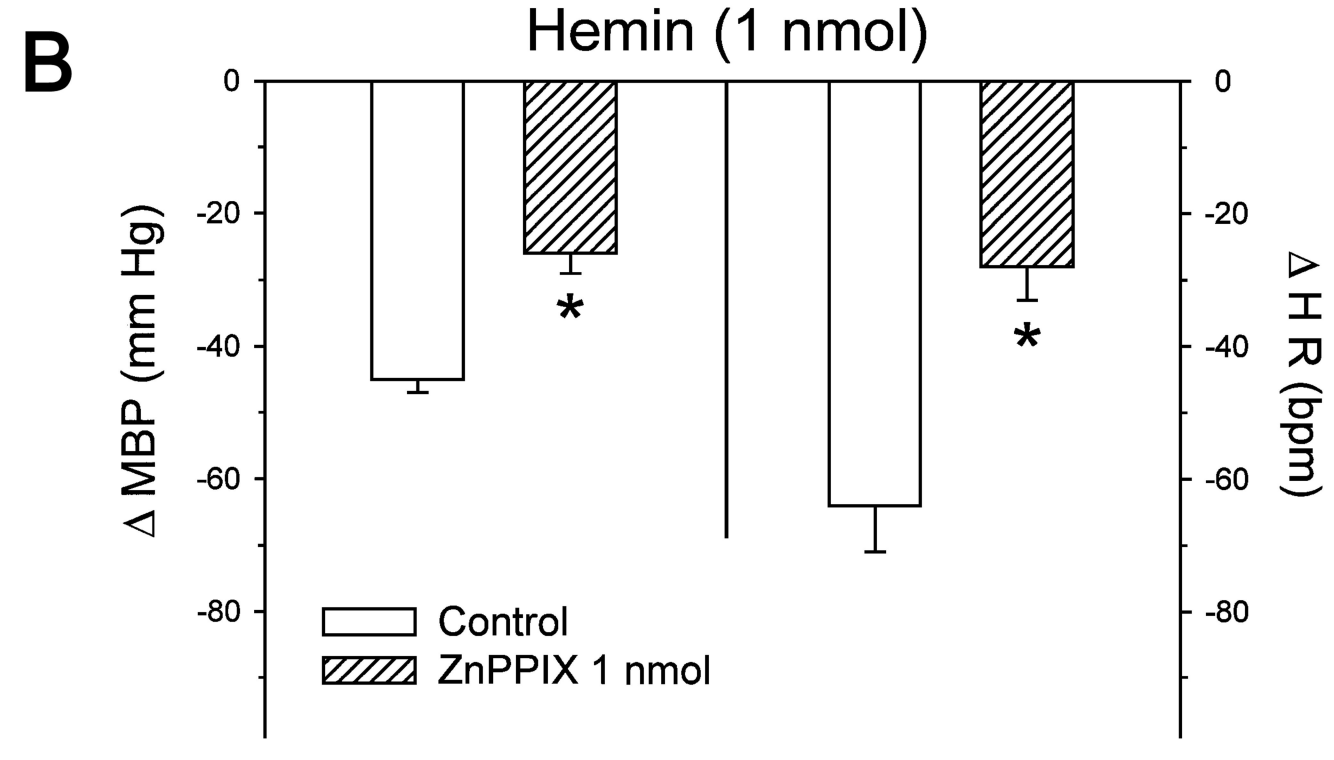
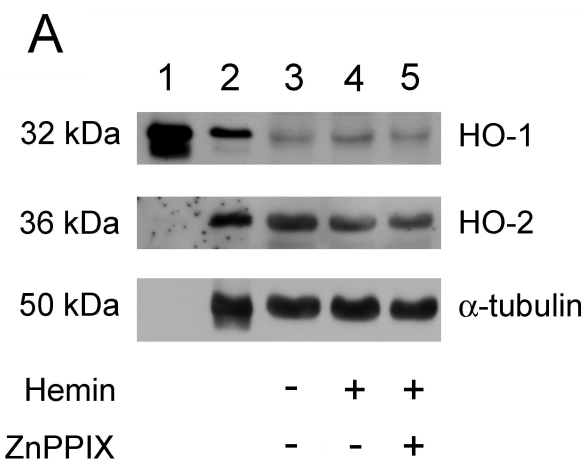


Figure 2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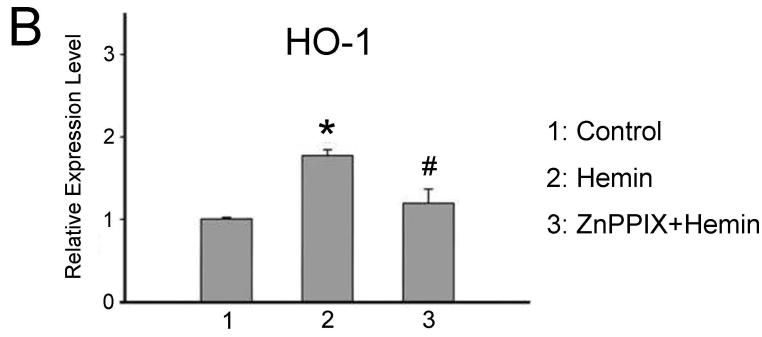
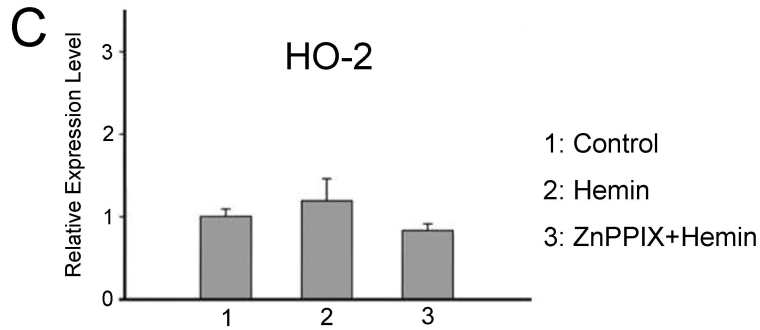
32 kDa
HO-1
36 kDa
HO-2
50 kDa
α-tubulin
Hemin - + +
ZnPPIX - - +


B
HO-1
Relative Expression Level
0
1
2
3
*
#
1 2 3
1: Control
2: Hemin
3: ZnPPIX+Hemin


C
HO-2
Relative Expression Level
0
1
2
3
1 2 3
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2: Hemin
3: ZnPPIX+Hemin

Figure 3

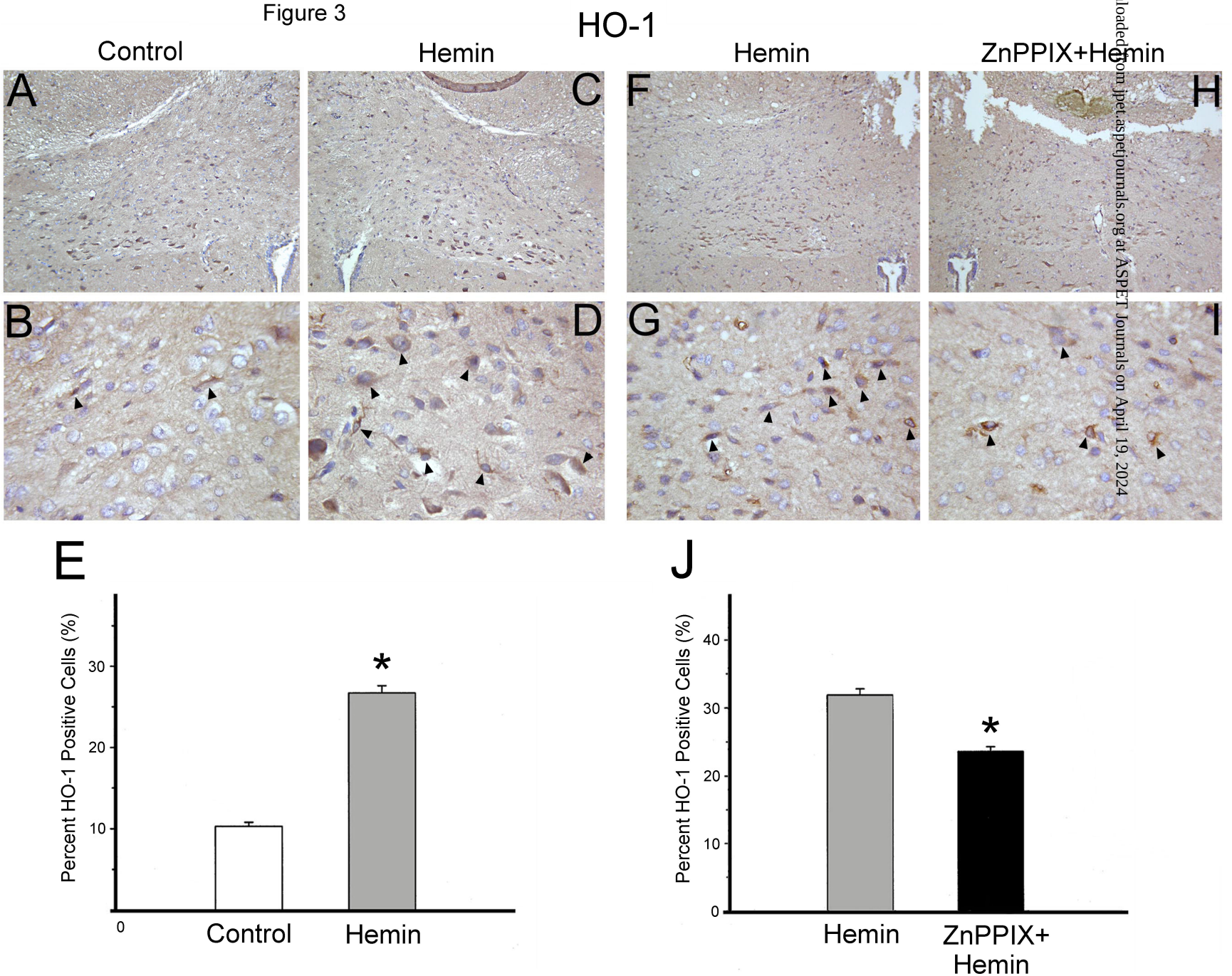

Figure 4

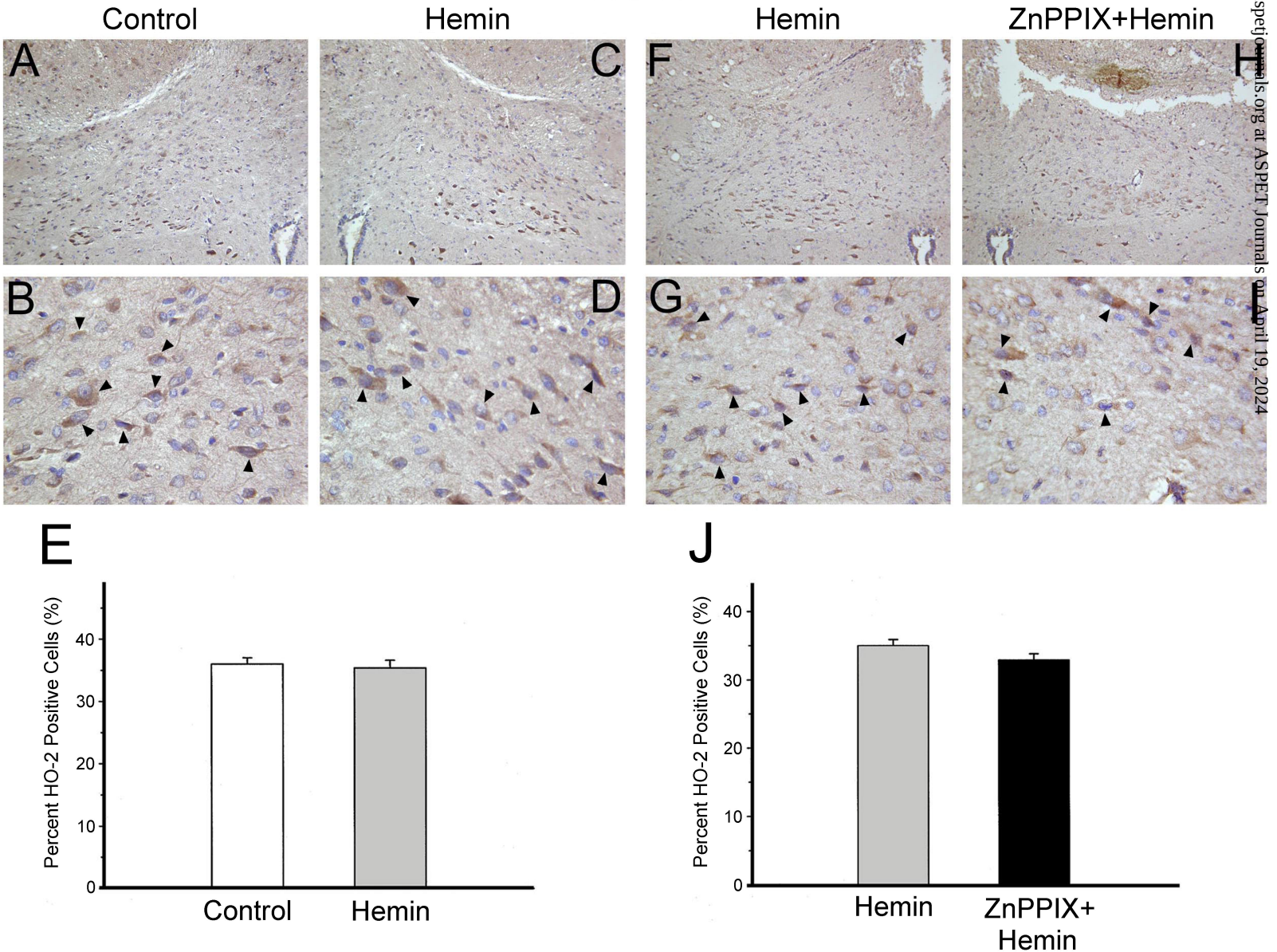

Figure 5

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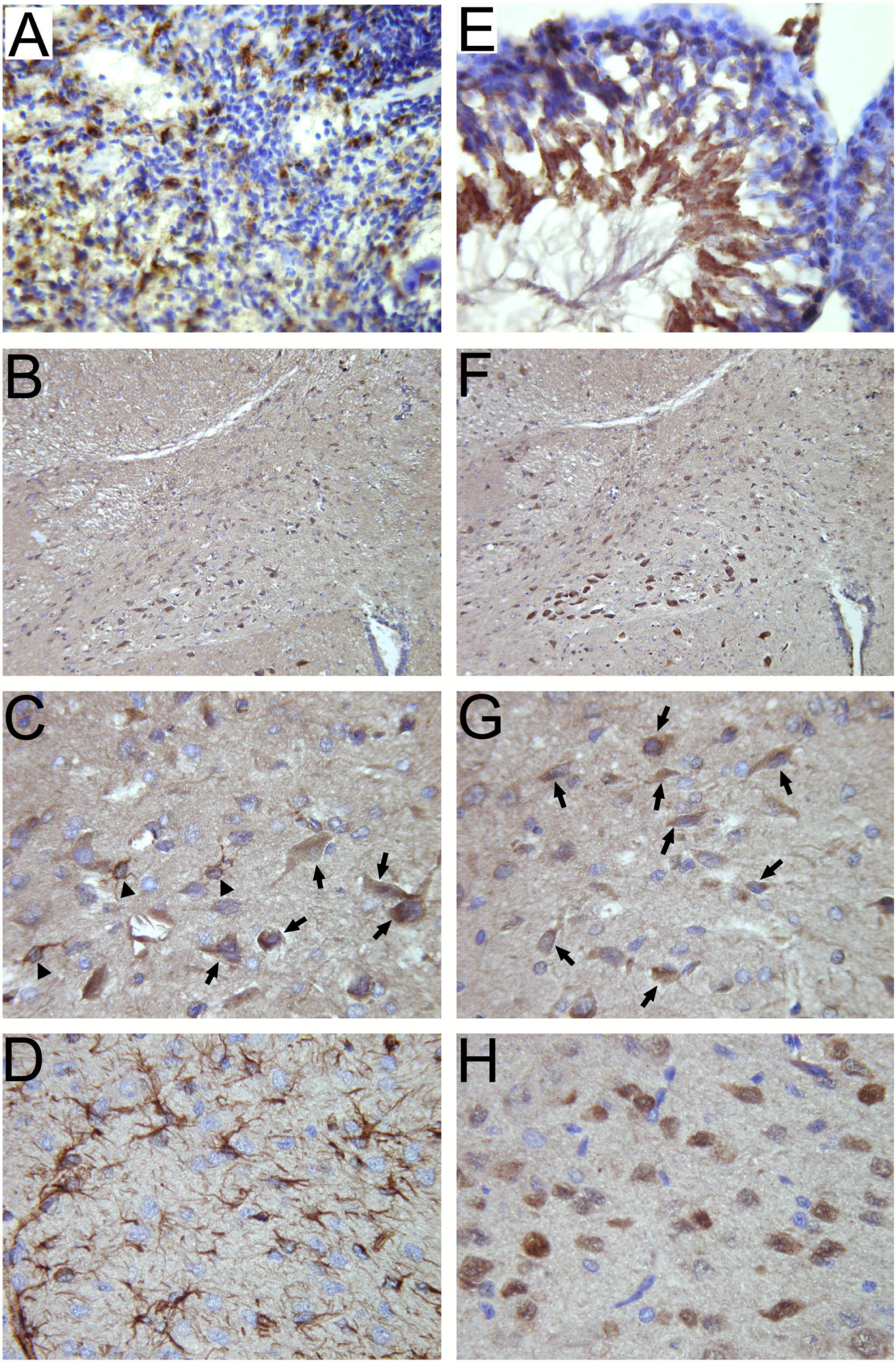
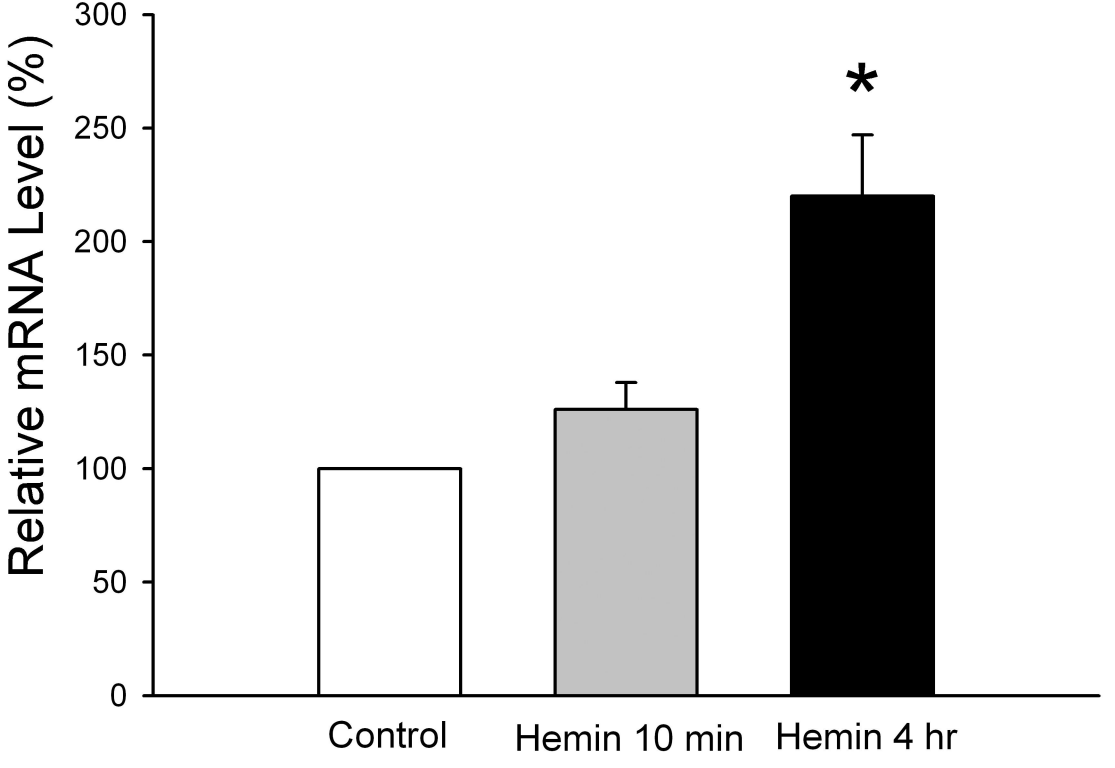


Figure 6


Relative mRNA Level (%)
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250
200
150
100
50
0
*
Control
Hemin 10 min
Hemin 4 hr