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Hypoxia-Mediated Induction of Heme Oxygenase Type I and Carbon Monoxide Release from Astrocytes Protects Nearby Cerebral Neurons from Hypoxia-Mediated Apoptosis

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ABSTRACT

To study a putative paracellular protective mechanism of astrocytes for neurons, immunohistochemical analysis was performed in ischemic rat brain, which colocalized with the expression of heme oxygase-1 (HO-1) in astroglias surrounding dying TUNEL-positive neurons. As an *in vitro* paradigm for ischemia, cultured astrocytes were exposed to normobaric hypoxia (pO_2 ≈10 torr), which triggered marked increase in the expression of a 33 kDa stress protein, identified as HO-1. Induction of HO-1 message was observed within 4 h of hypoxia and peaked at 12 h, accompanied by an accelerated transcription of HO-1 message. Consistent with the induction of HO-1, a platelet bioassay revealed production of carbon monoxide by reoxygenated astrocytes. The presence of CO in the medium decelerated the hypoxia-mediated apoptotic type of cell death in cultured cerebral neurons via lowering the activity of caspase-3, a key enzyme regulating apoptotic cell death. This protection against apoptosis was likely mediated by CO-mediated increases in intracellular cGMP, because exposure of hypoxic neurons to CO increased intracellular cGMP levels, and addition of cGMP analogue to hypoxic neuronal cultures suppressed caspase-3 activity and promoted neuronal survival. These data describe a potentially important paracellular pathway through which astrocytes may rescue nearby neurons from ischemic death. *Antioxid. Redox Signal.* 9, 543–552.

INTRODUCTION

ASTROCYTES are strategically situated to perform neurotrophic functions in the central nervous system, even in pathological conditions such as those associated with ischemic cerebrovascular diseases (37). In order for astrocytes to perform these functions under stress conditions, astrocytes possess specific biochemical machinery which protects their own subcellular organelles, so that they can survive and respond to situations which neurons cannot (14, 39). A critical component of the adaptive response of astrocytes to environmental stress are a group of stress proteins, which are induced by environmental stress and which support cellular biosynthetic functions to promote cell survival (2).

In contrast to this resistant phenotype of astrocytes, neurons, especially when isolated under conditions of cell culture, are quite vulnerable to environmental alterations such as glucose deprivation (27), withdrawal of trophic factors (8), or oxygen deprivation. Neuronal death in these situations is likely to be via apoptosis (11). The exposure of neurons to hypoxia, for instance, accelerates the apoptosis of neurons via the downregulation of bcl2 antigen, a potent endogenous negative regulator of apoptosis (18). The extreme vulnerability of neurons to apoptotic death induced by diverse

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stimuli suggests that apoptosis is a default phenotype for neurons undergoing environmental stress. However, neurons may be able to evade apoptotic death by dint of trophic factors produced by nearby cells. Studies of experimental brain ischemia have demonstrated the inducible expression of a number of neurotrophic factors derived from astrocytes, including basic fibroblast growth factor (33), interleukin-1, and tumor necrosis factor (38). In this context, we have demonstrated the production of a neurotrophic cytokine, interleukin-6 (IL-6) from cultured astrocytes after reoxygenation (22) and shown that a 78 kDa glucose-regulated protein (GRP78/BiP) induced in hypoxic astrocytes participates in the elaboration of this cytokine as a molecular chaperone (13).

In the current manuscript, we define a new paracellular mechanism by which astrocytes may protect adjacent neurons subjected to ischemia, the induction of HO-1 in cultured astrocytes by hypoxia. While HO-2, another isozyme of HO-1 constituently expressed in neurons, functions as intrinsic protector in neurons (9), HO-1 induced in astrocytes may highlight their neuroprotective role in ischemic milieu. The carbon monoxide (CO) thereby released suppresses a critical pathway by which neurons would otherwise undergo apoptosis.

MATERIALS AND METHODS

Animal preparation and immunohistochemistry

Transient focal cerebral ischemia was performed in adult male Sprague–Dawley rats (weighing 280–300 g) as described previously (25). In brief, after rats were anesthetized, a monofilament nylon suture (100 μm in diameter) with blunted tip was threaded through the external carotid artery, stump into the internal carotid artery up to the stem of the middle cerebral artery (MCA), and left for 2 h. The operated rats were allowed to recover for 24 h. During the operation, rectal temperature was maintained at 37°C with a heating pad. Mean arterial blood pressure (MABP), pH, pO_2, and pCO_2 were serially monitored.

Rats were then sacrificed and perfused with paraformaldehyde (PFA, 4%) in phosphate-buffered saline (PBS, 0.1 *M*, pH 7.4). Their brains were paraffin embedded and sectioned 40 μm thick. Immunohistochemistry was performed using the avidin-biotin-horseradish peroxidase technique, with an anti-HO-1 antibody (StressGen, San Diego, CA) as described previously (25). Adjacent sections prepared from the same brain were also subjected to immunohistochemical analysis with either anti-microtubular associated protein II (MAPII) or anti-glial fibrillary acidic protein (GFAP), or analyzed by TUNEL method (16).

Cell culture, chemicals, and hypoxic exposure

Astrocytes were obtained from neonatal rat brains by the method described previously (16). In brief, cerebral hemispheres were harvested from neonatal Sprague–Dawley rats within 24 h of birth, and brain tissue was digested at 37°C using Dispase II (3 mg/ml; Boehringer–Mannheim, Tokyo, Japan). The mixture was plated in 175 cm^2 culture flasks (two brains/flask) and cells were grown in minimal essential medium supplemented with fetal calf serum (FCS; 10%; CellGrow, Boston, MA). After 10 days, culture flasks were incubated for 48 h with cytosine arabinofuranoside (10 mg/ml; Wako Chemicals, Osaka, Japan) to prevent fibroblast overgrowth, agitated on a shaking platform (Bioshaker, BR-30L, Taitek, Tokyo, Japan) to separate astrocytes from remaining microglia and oligodendroglia, and the adherent cell population was then identified by morphologic and immunohistochemical criteria (detection of glial fibrillary acidic protein). Cells were then replated at a density of 5 x 10^4 cells/cm^2 in the above medium.

Primary cerebral neurons were also obtained from the neonatal rat brain about 4–5 days after birth using the method described by Hori (12) with minor modifications. Briefly, cerebral hemispheres were harvested from neonatal Sprague–Dawley rats and meninges were carefully removed. Brain tissue was digested at 37°C for 1 h in pH 7.4 phosphate buffered saline (PBS) containing 10 m*M* collagenase (Boehringer Mannheim), resuspended in every 10 min and filtered through a 100 μm filter (Falcon, Tokyo, Japan). Cells harvested by centrifugation at 1,200 r.p.m. for 8 min were resuspended and grown in MEM (30 m*M* glucose) supplemented with fetal calf serum (FCS; 10%; CellGrow). Cells were seeded in poly L-lysine coated 100-mm plates, followed by the exchange of the medium 24 h after the plantation. Cerebral neurons were identified by morphological and immunohistochemical analysis using microtubular associated protein II (MAP II). Cultured cells were then exposed to hypoxia (H) using an incubator attached to a hypoxia chamber (Coy Laboratory Products, Ann Arbor, MI) which maintained a humidified atmosphere with low oxygen tension, as described previously (28). In experiments in which the cells were under hypoxia and carbon monoxide (H + CO), an airproof desiccator was used. The gas inlet was provided through a rubber tubing connected to a closed water trap in the bottom of the desiccator. Experimental gas containing CO (2.5–5%), CO_2 (5%), and the balance N_2 (Neriki Gas, Osaka, Japan) were hydrated at 37°C and flowed at a rate of 50 ml/min for 10 min into the desiccator in which the cell culture plates were placed. After clamping the gas inlet and outlet, the desiccator was set in a 37°C constant-temperature cell incubator. pO_2 and pCO_2 were monitored by collecting the outflow gas in a glass syringe and injecting the gas into an automated blood gas analyzer (28). The cerebral neurons under normoxia (N) were incubated in a humidified 5% CO_2/95% air incubator at 37°C. Where indicated, the cerebral neurons were incubated in the presence of either cGMP analogues (8-bromo-cGMP, or chlorophenylthio-cGMP; both reagents were purchased from Sigma, St. Louis, MO) or phosphodiestrease inhibitor (dipyridamole; Sigma) under hypoxia, and in the presence of a guanylate cyclase inhibitor (LY83583, BIOMOL) under H + CO.

Western and Northern blot analysis

Western blot analysis of HO-1 antigen was performed according to a method described by Towbin (40). In brief, cultured astrocytes (about 5 x 10^6 cells) were exposed to hypoxia, followed by reoxygenation, as described. At the indicated time points, cells were washed three times with ice-cold PBS and lysed in the presence of PBS (200 μl) containing NP-40 (1%), EDTA (5 m*M*), and PMSF (1 m*M*). After centrifugation (5,000 *g* for 5 min at 4°C), the supernatant (about 1 μg of protein) was applied to SDS-PAGE (12%), transferred to PVDF paper, and stained with anti-HO-1 IgG (500:1 dilution, StressGen). To examine whether exposure of astrocytes to environmental

stresses other than hypoxia could induce HO-1 expression, cultured astrocytes maintained in normoxia were either subjected to heat shock (43°C for 3 h) or hydrogen peroxide (5 μ*M* for 10 min), and subsequently incubated for 6 h prior to harvest. In other experiments, astrocytes were incubated under normoxic conditions and exposed to either cobalt chloride (100 μ*M*), 2-deoxyglucose (25 m*M*), or sodium azide (1 m*M*) for 24 h. Cells were then harvested and subjected to Western blotting using the anti-HO-1 antibody. In each experiment, samples were also immunoblotted with anti-HSP72 monoclonal antibody (Amersham, Tokyo, Japan) at the recommended concentration. Northern blot analysis using HO-1 probe was performed as described previously (39). Briefly, about 5 μg of total RNA was extracted from astrocytes exposed to hypoxia or hypoxia/reoxygenation for the indicated periods. Where indicated, astrocytes were exposed to hypoxia in the presence of cycloheximide (5 μg/ml). Then total RNA was purified by the AGPC method (5), separated by electrophoresis on 1.0% agarose/formamide gels, and transferred overnight onto Biodyne B paper (Pall BioSupport, Portsmouth, UK). The membrane was prehybridized for 3 h at 42°C in hybridization buffer (0.9 *M* NaCl; 90 m*M* sodium citrate, pH 7.0) containing 5X Denhardt's solution, SDS (0.5%), and heat-denatured salmon sperm DNA (100 μg/ml). Partial HO-1 cDNA (generously provided by Professor Shibahara, Tohoku University, Sendai, Japan) was radiolabeled with [^{32}P]*d*CTP (NZ522, New England Nuclear, Boston MA) by the random hexamer procedure (10). After hybridization overnight at 42°C in hybridization buffer containing radiolabeled cDNA probe (5 ng/ml), filters were washed twice with 2X SSC/0.5% SDS and 0.2X SSC/0.5% SDS for 30 min at 52°C, exposed to X-ray film (Fuji Photo Film, Tokyo, Japan), and subjected to autoradiography. In other experiments, astrocytes were also exposed to heat shock (43°C) for 3 h in the presence or absence of cycloheximide (5 μg/ml), followed by Northern blotting. In each case, the level of HO-1 mRNA was evaluated by the comparison with beta-actin mRNA.

Nuclear run-off analysis

To demonstrate an increase of HO-1 transcription in hypoxic astrocytes, nuclear run-off analysis was performed as described previously (22). In brief, nuclear suspension (0.2 ml), obtained from about 2 x 108 astrocytes prepared under the indicated conditions, was incubated with CTP, ATP, and GTP (0.5 m*M* of each) in the presence of ^{32}P-alpha-*d*UTP (250 μCi, 3,000 Ci/mmol; New England Nuclear). Samples were subjected to phenol/chloroform extraction, and RNA was precipitated and resuspended in hybridization buffer. Hybridization to denatured rat HO-1 and rat beta-actin (control) probes (20 μg in each case) dot-blotted onto nylon membranes was performed at 42°C for 2 days. Filters were washed, dried, and exposed to Fuji X-ray film.

Expression of HO-1 antigen in other cell types

To examine the expression of HO-1 antigen in the central nervous system, cultured rat microglia, brain microvessel endothelial cells, and cortical neurons were exposed to hypoxia for 24 h. Cells were lysed in PBS containing NP-40 (1%) and about 1 μg of protein extract was subjected to Western blotting by using the anti-HO-1 IgG.

Measurement of cGMP in cultured cerebral neurons

The levels of cGMP in cultured cerebral neurons were measured by a method described previously with a minor modification (29). In brief, cultured cerebral neurons (2 x 10^5 cells) exposed to either normoxia, hypoxia or H + CO for the indicated period were washed three times with ice-cold PBS and scraped into ice-cold 7% trichloroacetic acid (TCA, 500 μl), followed by the incubation for 30 min on ice. The cell lysates were then centrifuged (5,000 *g* for 5 min at 4°C), and the supernatant was subjected to ether extraction to remove TCA, dried, and reconstituted with 400 μl of reaction mixture containing PBS (pH 7.4), BSA (0.1 mg/ml), NP-40 (0.5%), and sodium azide (0.1%). Pellets were saved for the determination of protein content. Then the cGMP level in the reconstituent was determined by radioimmunoassay (New England Nuclear), according to manufacturer's instructions.

Detection of CO released from reoxygenated astrocytes by platelet

CO generated from reoxygenated astrocytes was detected by an increase in platelet cGMP levels (6). Human platelets obtained from healthy volunteers were pretreated with IBMX (0.1 m*M*, Sigma), resuspended in 2 ml of Tyrode's buffer (10^8 platelet/mL) containing *N*-methyl-L-arginine (L-NMA; 100 μ*M*), and maintained 4 h at 37°C. The effect of CO on platelet cGMP levels was confirmed by assay of platelet cGMP, after further incubating them in the presence or absence of CO (5%) for 4 h. Cultured rat astrocytes were plated onto a membrane insert (diameter 23.4 mm, for 6-well plate, Falcon) at the density of 2 x 10^5 cells/well, followed by the exposure to either normoxic or hypoxic condition for 24 h. Then, hypoxic astrocytes on the insert were placed to either hypoxic platelet suspension (hypoxic sample) or normoxic platelet suspension (reoxygenated sample). The normoxic astrocytes in the insert were also placed onto the normoxic platelet suspension to obtain the normoxic sample. After 4 h contact to the astrocytes, platelets were collected in ice-cold TCA (7%), followed by the determination of cGMP content as described above. Where indicated, astrocytes were reoxygenated in the presence of either L-NMA, an inhibitor of NO synthetase (100 μ*M*), or zinc-protoporphyrin IX (ZnPP-IX, 10 μ*M*), an inhibitor of HO-1. The preparation of hypoxic sample was performed inside the hypoxic chamber.

Measurement of caspase-3 activity

Cerebral neurons (2 x 10^6 cells) were incubated for 12 h in the presence of either cobalt chloride (Co, 100 μ*M*) or nickel chloride (Ni, 300 μ*M*) in normoxic condition. Cultured cerebral neurons were also exposed to hypoxia in the presence of CO (0–5%). At the indicated time point, activity of these proteases was assessed colorimetrically by the leavage of enzyme substrate, Ac-DEVD-MCA as described previously (17).

Quantitiative measurement of apoptotic type of cell death

Apoptotic type of cell death in hypoxic neurons was assessed by the quantitative measurement of intranucleosomal fragmented DNA. In brief, about 2 x 10^5 cerebral neurons in

6-well plates were exposed to hypoxia for indicated periods in the presence of CO (0–5%). Fragmented DNA was then measured by a kit commercially available (Cell Death Detection ELISA, Boehringer Mannheim, Tokyo) according to manufacturer's instructions. The increase of fragmented DNA was expressed by the fold increase of OD_{495nm} to that of the initial culture before the exposure to stresses.

Statistical analysis

Statistical analysis was performed either by nonpaired T analysis or by the multiple comparison method (Fisher's PLSD analysis), followed by ANOVA. Where indicated, Fisher's PLSD analysis was performed as a post-hoc test, followed by the two-way ANOVA. Means ± SD are presented. A $p < 0.05$ was considered statistically significant.

RESULTS

Expression of HO-1 antigen in ischemic brain

Immunohistochemical analysis using anti-HO-1 antibody revealed a marked increase in the expression of HO-1 antigen in ischemic hemisphere (Fig. 1A). This induction of HO-1 was observed in the area which surrounds degenerated neuronal cells, characterized by the positive TUNEL signal (Fig. 1C) and the loss of MAP-II immunogenicity (Fig. 1B). The induction of HO-1 or the degeneration of neuronal cells was not observed in the control nonischemic hemisphere (Fig. 1A, B, and C) or in control sections taken from nonmanipulated rat brains (data not shown). Immunohistochemical analysis of adjacent sections using an anti-GFAP primary antibody, a marker for astrocytes, indicated that HO-1 positive cells were identical to cells with a positive signal for GFAP, (Fig. 1D-I and D-II). These data suggested that the HO-1 antigen induced in the astroglia of the ischemic region may participate in the regulation of apoptotic neuronal cell death. To determine the mechanism of HO-1 induction and the role of this stress protein in the setting of brain ischemia, an experimental system using cultured astrocytes was employed.

Cell viability of cultured rat astrocytes under hypoxia

Oxygen tension in the medium fell to about 8 torr within 8 h after cultures were transferred to the hypoxia chamber. Viability of astrocytes was maintained throughout hypoxia and following replacement of cultures back to normoxia (reoxygenation; R), based on lack of LDH release into the culture supernatant, continued trypan blue exclusion, adherence of cells to the culture substrate, and unchanged morphologic features.

Induction of inducible type of heme oxygenase in cultured rat astrocytes

Western blot analysis with anti-HO-1 antibody detected a single band corresponding to MW about 33 kDa, whose induction became apparent within 4 h after the exposure to hypoxia and reached maximum within 8 h after the onset of

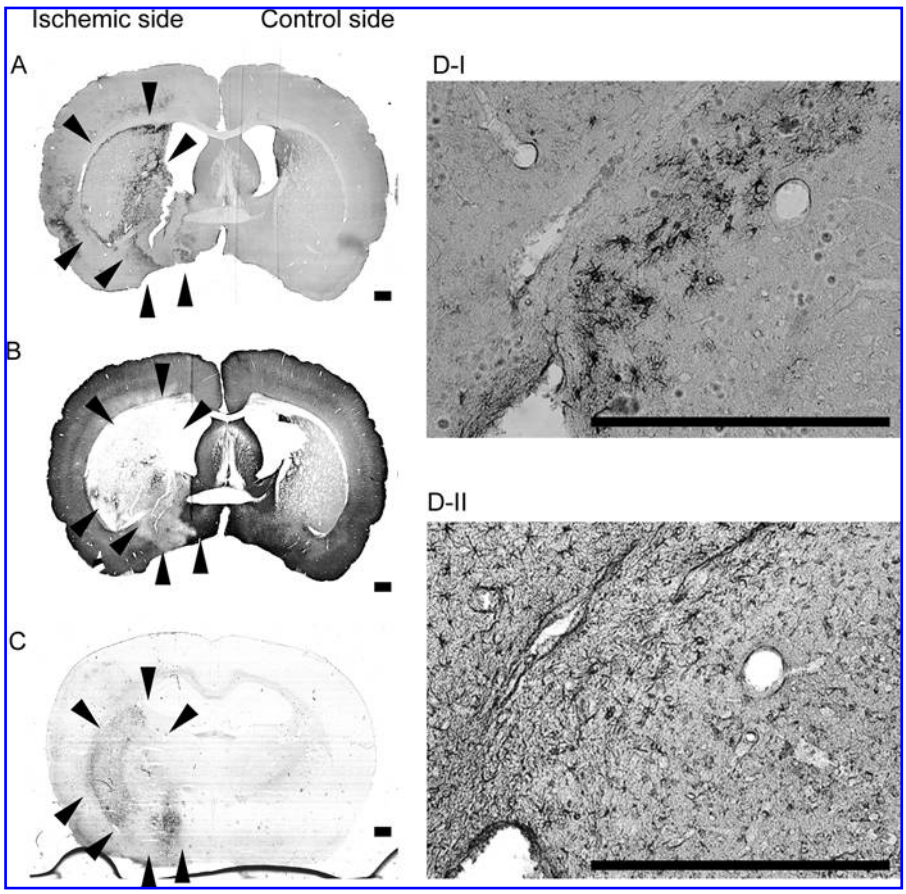

FIG. 1. Expression of HO-1 antigen in ischemic brain. Brain ischemia was introduced by a unilateral occlusion of middle cerebral artery for 2 h, as described in text. Animals were sacrificed 24 h after reperfusion, and brain slices were subjected to HO-1 immunostaining (**A**), MAPII immunostaining (**B**), or TUNEL analysis (**C**). (**D**) adjacent sections prepared from ischemic hemisphere was stained with either anti HO-1 antibody (**D-I**) or anti GFAP antibody (**D-II**). Signals are indicated by *arrowheads*. *Closed bars* represent 200 μm.

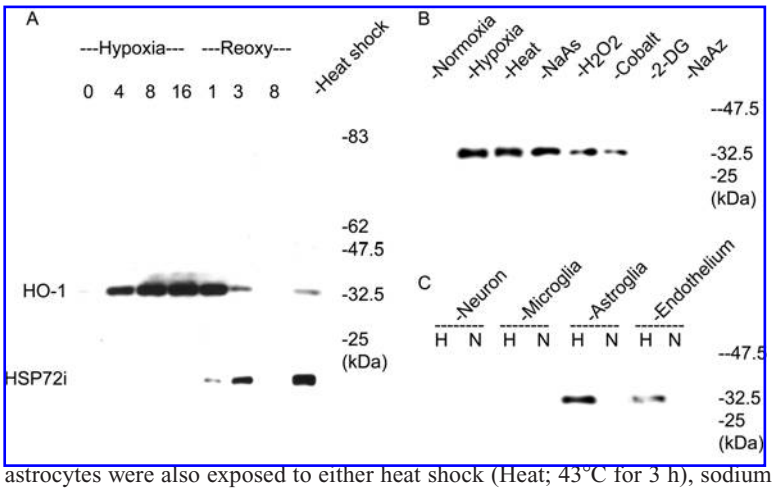

FIG. 2. Expression of HO-1 antigen in astrocytes by hypoxia, chemical stresses, and in other cell types of the central nervous system. (**A**) Cultured rat astrocytes (5 x 10^6 cells) were exposed to hypoxia (Hypoxia; 0–24 h) followed by reoxygenation (Reoxy; 2–12 h). At the indicated time point, cells were harvested and protein extract by NP-40 (1 μg in each lane) was subjected to SDS-PAGE (8%), followed by the Western blotting with either anti-HO-1 antibody (*upper panel*) or anti-HSP72 antibody (*lower panel*). Astrocytes were also subjected to heat shock (43°C for 3 h) and protein extract was subjected to Western blot as described above. (**B**) Astrocytes were maintained under either normoxic (Normoxia) or hypoxic condition (Hypoxia) for 8 h. Normoxic astrocytes were also exposed to either heat shock (Heat; 43°C for 3 h), sodium arsenite (NaAs; 0.1 m*M* for 3 h), cobalt chloride (Cobalt; 100 μ*M* for 12 h), 2-deoxyglucose (2-DG; 25 m*M* for 12 h), or sodium azide (NaAz; 1 m*M* for 12 h). Normoxic astrocytes were also exposed to hydrogen peroxide (H2O2; 5 μ*M* for 10 min) and maintained in normoxic condition for 6 h. (**C**) Astrocytes (AST), microglia (MCG), brain microvessel endothelial cells (BMEC), and cortical neurons (NRN) were separated, cultured, and exposed to either hypoxia (H) or normoxia(N) for 12 h (about 5 x 10^6 cells in each case). Protein extracts (1 μg in each lane) were subjected to SDS-PAGE (8%), followed by the Western blotting with anti-HO-1 antibody. In each panel, representative image of five repeated experiments is shown. Migration of molecular weight marker is shown on the *right side of the gel* [β-lactoglobulin-A (25 kDa), triosephosphate isomerase (32.5 kDa), aldolase (47.5 kDa), glutamic dehydrogenase (62 kDa), and fusion protein of maltose binding protein and paramyosin (83 kDa)].

hypoxia. The induction of this 33 kDa peptide returned to baseline very quickly after reoxygenation (Fig. 2A). The induction of the 33 kDa peptide was also detected in the normoxic culture following exposure to several stimuli known to induce expression of stress proteins, including heat shock, sodium arsenite, hydrogen peroxide, and cobalt chloride (Fig. 2B), suggesting that the 33 kDa peptide induced by hypoxia in astrocytes is identical to the inducible type of heme oxygenase (HO-1; ECC1.14.99.3). In contrast, expression of the 70 kDa heat shock protein (HSP70) was induced in astrocytes only after reoxygenation (Fig. 2A).

To examine whether other components of the central nervous system can induce HO-1 by hypoxia, cultured rat neurons, microglia, and brain microvessel endothelial cells were exposed to hypoxia, followed by the analysis by Western blot (Fig. 2C). After exposure to hypoxia for 12 h, brain microvessel endothelial cells showed a moderate increase of HO-1 antigen. In contrast, microglia and cortical neurons failed to induce HO-1 after hypoxic exposure (Fig. 2C). These data are consistent with the immunohistochemical analysis, where astroglia were identified as a main source of HO-1 in ischemic brain (Fig. 1D-1 and D-2).

Using a labeled cDNA probe which can detect HO-1 transcripts, the induction of HO-1 message was also demonstrated in hypoxic astrocytes (Fig. 3A). This induction of HO-1 message occurred in parallel to induction of antigen. Furthermore, the addition of cycloheximide, which suppressed protein synthesis to 10% that of the nontreated culture (evaluated by the

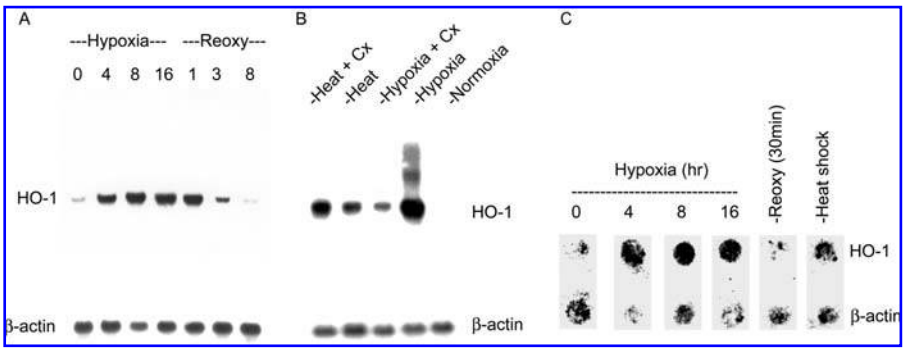

FIG. 3. Induction of HO-1 transcripts in cultured rat astrocytes by hypoxia, the effect of cycloheximide, and the effect of hypoxia and hypoxia/reoxygenation on the rate of transcription. (**A**) Cultured rat astrocytes (5 x 10^7 cells) were exposed to hypoxia/reoxygenation. At the indicated time point, total RNA (5 μg in each lane) was purified from the culture and subjected to Northern blot analysis with either rat HO-1 (*upper panel*) or beta-actin (*lower panel*) cDNA probe. (**B**) Astrocytes were exposed to either heat shock (43°C for 3 h) or hypoxia (Hypoxia; 8 h) in the absence or presence of cycloheximide (+Cx; 5 μg/ml). Astrocytes were also maintained under normoxic condition (Normoxia) for 12 h. Total RNA (5 μg in each lane) extracted from each culture was subjected to Northern blot as described above. (**C**) Nuclei were prepared from about 2 x 10^8 astrocytes exposed to hypoxia (Hypoxia; 0–12 h) or hypoxia followed by reoxygenation (Reoxy) for 30 min, incubated with CTP, ATP, and GTP in the presence of ^{32}P-α-UTP, RNA was extracted, and hybridized to denatured rat HO-1 or rat beta-actin (control) probes (20 μg) dot-blotted onto nylon membranes. Filters were then, washed, dried, and subjected to autoradiography.

incorporation of ^{3}H-leucine into the TCA precipitable fraction), inhibited the induction of HO-1 transcription by hypoxia. In contrast, heat-shock-mediated induction of HO-1 message was even enhanced by cycloheximide (Fig. 3B). These data, showing disparate stimuli (hypoxia *vs*. reoxygenation) which induce HO-1 or HSP70, differing transcriptional response of HO-1 to hypoxia *vs*. heat shock when a general inhibitor of protein synthesis is added, suggest that the hypoxia-mediated induction of HO-1 is distinct from that mediated by heat shock. Furthermore, the cycloheximide data confirm the requirement for *de novo* protein synthesis for HO-1 induction. As an additional experiment to determine if hypoxic induction of HO-1 is transcriptionally mediated, a nuclear run-off assay was performed in the nuclear extract prepared form hypoxic or normoxic astrocytes. The nuclear run-off analysis demonstrated a remarkable acceleration of the HO-1 transcription, which reached maximal within 4 h from the onset of hypoxia and returned to baseline within 30 min after reoxygenation (Fig. 3C).

Elaboration of carbon monoxide (CO) from astrocytes after reoxygenation

Heme oxygenase functions as a key enzyme in the catabolism of heme to produce biliverdin and carbon monoxide in the presence of oxygen. To confirm enzymatic activity of HO-1, CO produced from hypoxic astrocytes was detected by using a platelet bioassay (6). First, the presence of CO (5%) in the atmosphere caused an apparent increase of cGMP content in the platelet suspension (Fig. 4A), an experiment which was used as a positive control for the assay system. Under experimental conditions of 24 h hypoxia and 6 h reoxygenation, astrocytes elaborated a freely diffusible activity which increased the cGMP level in human platelets across a permeable membrane; this increase was blocked by the presence of ZnPP-IX, not by L-NMA, suggesting that reoxygenated astrocytes produce CO into the culture supernatant (Fig. 4B). This effect of reoxygenated astrocytes to increase cGMP in nearby platelets was only observed following reoxygenation; CO was not detected in culture supernatants from either normoxic or hypoxic astrocytes, where the induction of HO-1 reached maximal. These data indicate that HO-1 can be activated only after the replacement of hypoxic culture into an oxygen rich environment, which is not surprising, given the stoichiometric requirement of HO-1 for molecular oxygen to carry out the catabolism of heme. Since HO-1 protein and mRNA are still detectable 3 h after reoxygenation (Figs. 2A and 3A), it is plausible that CO could be generated residual HO-1 activity 4 h after reoxygenation (Fig. 4).

Neuroprotective properties of CO

To understand a potential protective role for CO in the ischemic brain, the effect of CO on neuronal survival was examined. Consistent with our previous report (39), exposure of cerebral neuronal cells to hypoxia greatly accelerated their cell death, which was partially blocked by the presence of CO in a dose-dependent manner (Fig. 5A and B). This line of experiments suggests that CO can function as a neurotrophic mediator in the ischemic brain.

To further determine the mechanism by which CO can protect against neuronal death, we examined the enzymatic activity of caspase-3, a key enzyme which promotes the apoptotic cell death of hypoxic neurons. Exposure of cultured cerebral neurons to hypoxia for 12 h upregulated the enzymatic activity and this increase was suppressed in the presence of CO in the hypoxic environment (Fig. 5C). The elevation of caspase-3 activity was not simulated in the normoxic culture by the addition of either cobalt or nickel ion (Fig. 5C), indicating that the increase of caspase-3 activity in hypoxic neurons is not likely to be mediated by a heme-containing protein.

In contrast, exposure of cerebral neurons to hypoxia resulted in a marked decrease of cGMP content, which was reversed by the presence of CO in a dose-dependent manner (Fig. 6A). Neuronal cell death was also diminished in a dose-dependent manner by the presence of the membrane-permeable cGMP analogues, 8-bromo-cGMP or chlorophenylthio-cGMP (Fig. 6B and C). The timing of this protection coincided with the time during which CPP32/Yama-like protease activity was suppressed by these same cGMP analogues (Fig. 6D). To further demonstrate the need for induction of cGMP in neuronal protection by CO, the neuroprotective effect of CO was shown to be blocked by LY83583, an inhibitor of guanylate cyclase. Furthermore,

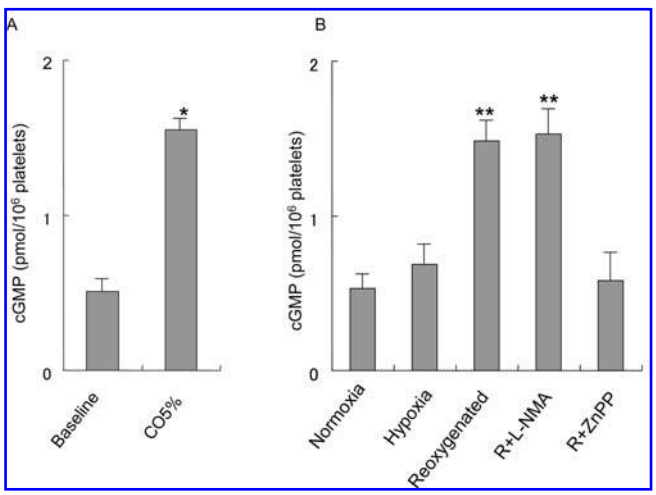

FIG. 4. Elaboration of carbon monoxide (CO) from reoxygenated astrocytes. (**A**) Platelets obtained from peripheral blood were incubated at 37°C in either the absence (Baseline) or presence of 5% CO (CO 5%) for 4 h. Platelet cGMP was then extracted by TCA (7.5%) and measured by radioimmunoassay as described in text. *denotes $p < 0.01$ by nonpaired T-test. (**B**) Platelets were contacted and co-incubated with cultured astrocytes (5 x 10^5 cells) for 4 h, which were previously cultured on the membrane insert under either normoxic (Normoxia), hypoxic (Hypoxia), or reoxygenated (Reoxygenated) condition. Then the concentrations of cGMP in the platelets were measured by radioimmunoassay. Astrocytes were also reoxygenated in the presence of L-NMA (100 μ*M*; R+L-NMA) or ZnPP-IX (10 μ*M*; R+ZnPP) before the co-incubation with human platelets. **denotes $p < 0.05$ by multiple comparison analysis followed by ANOVA. In each experiment, mean ± S.D. is shown ($n = 12$).

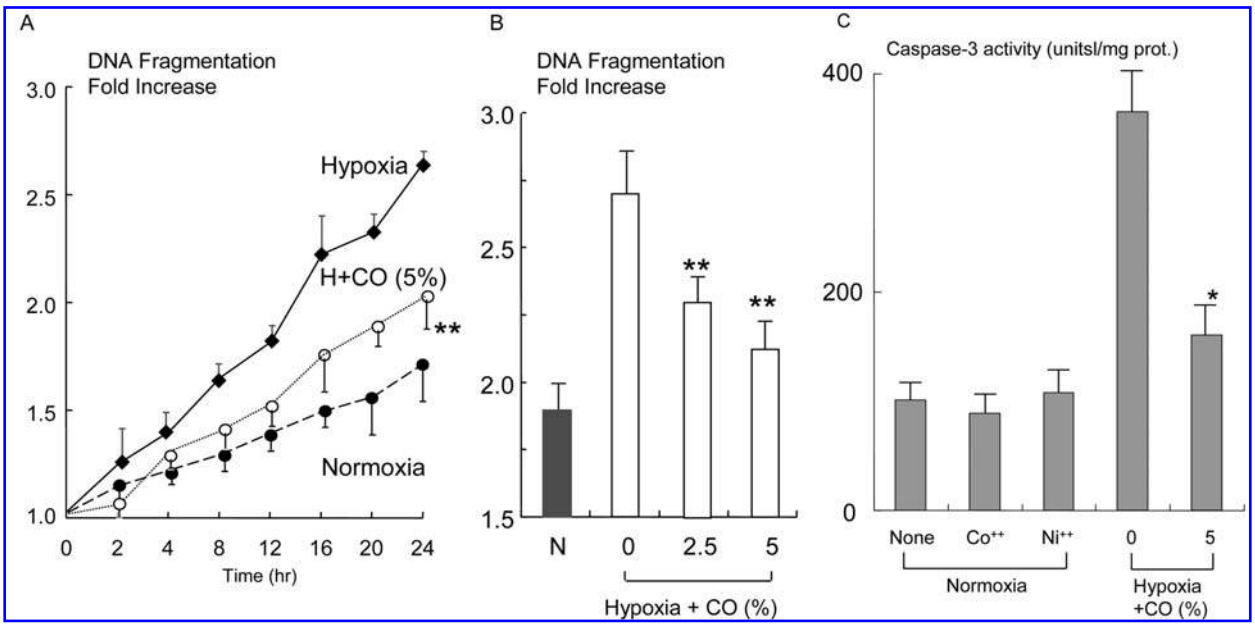

Fig. 5. Effect of CO on the viability of cultured neurons (A and B) and on the activity of CPP32/Yama like protease (C). (**A**) Cerebral neuronal cells cultured on 6-well plate (2 x 10^5 cells/well) were exposed to hypoxia either in the presence (H + CO) or absence (Hypoxia) of CO (5%). Cultured neurons were also maintained in normoxic atmosphere (Normoxia). At the indicated time point, fragmented DNA was quantitatively measured by ELISA as described in text. **denotes $p < 0.05$ compared with hypoxic cultures by multiple contrast analysis followed by two-way ANOVA. (**B**) Cerebral neurons were exposed to either normoxia (N) or hypoxia (H) with indicated CO concentration (0–5%) for 16 h. Cell viability was assessed as described above. In both panels, increase in fragmented DNA was expressed by the ratio of OD_{495nm} with that of initial cultures. **denotes $p < 0.05$ compared with hypoxia + CO (0%) by multiple comparison analysis followed by ANOVA. (**C**) Cerebral neurons (2 x 10^6 cells) were cultured in normoxic condition in the absence (None) or presence of cobalt chloride (100 μ*M*; Co++) or nickel chloride (300 μ*M*; Ni++) for 12 h. Neurons were also exposed to hypoxia in the presence of CO at the indicated concentration (Hypoxia + CO) for 12 h. Cells were then harvested and CPP32/Yama-like protease activity was assayed as described in text. *denotes $p < 0.01$ compared with hypoxia + CO (0%) by nonpaired T-test. In each experiment, mean ± S.D. is shown ($n = 6$).

addition of the phosphodiesterase inhibitor, dipyridamole, was accompanied by the maintenance of cGMP levels (data not shown) and suppressed hypoxia-mediated neuronal cell death (Fig. 6C). These data indicate that CO may exert neurotrophic functions by elevating cellular cGMP content, eventually leading to the suppression of caspase-3 activity.

DISCUSSION

Astrocytes, the most abundant cell type in the central nervous system, play an important role in maintaining neuronal functional homeostasis, especially in response to environmentally stressful situations that threaten neuronal survival. The stress response of astrocytes to ischemia is manifested by the triggering of a wide variety of neurotrophic behaviors, including the elaboration of neurotrophic mediators (22), uptake of excitatory amino acids, and a proliferative reaction which leads to tissue repair (37). Expression of stress proteins in this cell type induced by the exposure to hypoxia/reoxygenation may provide a principal basis by which astrocytes exert neurotrophic properties under stress.

The expression of an inducible type of 70 kDa heat shock protein (HSP72i) in astrocytes during the course of hypoxia/-reoxygenation represents one aspect of the stress response of astrocytes. More specifically, the induction of HSP72i, which is mediated by the energy depletion occurring after reoxygenation, supports increased protein synthesis synchronized with energy failure (15). In contrast, HO-1, which is also upregulated in response to heat shock (23), showed a different time course of induction in cultured astrocytes. Further, addition of cycloheximide to the culture, which caused a superinduction of HO-1 message mediated by heat shock, suppressed the hypoxia-mediated induction of HO-1 message in cultured astrocytes (Fig. 3B). This data suggest that the hypoxia-mediated induction of HO-1 is likely to employ a different signal transduction system from that responsible for HSP70 induction and different from that induced by heat shock. One possible mechanism is that HO-1 is induced in hypoxic astrocytes via hypoxia-inducible factor (20). Another mechanism which may induce HO-1 in astrocytes may be operated by a stress in the endoplasmic reticulum (ER). Astrocytes induces stress proteins in the ER (13, 19), suggesting a key role of ER stress proteins by which astrocytes may survive under deep hypoxic condition. Consistently, HO-1 can be induced by ER-stress in other cell types (21).

The induction of HO-1 as a cellular self-defense mechanism against environmental stress is reminiscent of its role in other conditions of stress (1), as well as brain ischemia (31). The formation of bilirubin by HO-1 and the fixation of free ion to stable ferritin describe a function of HO-1 as an antioxidant enzyme (3, 4). Our preliminary experiments demonstrate that astrocytes pre-exposed to hypoxia remain relatively impervious to reactive oxygen intermediates, substantiating an antioxidant

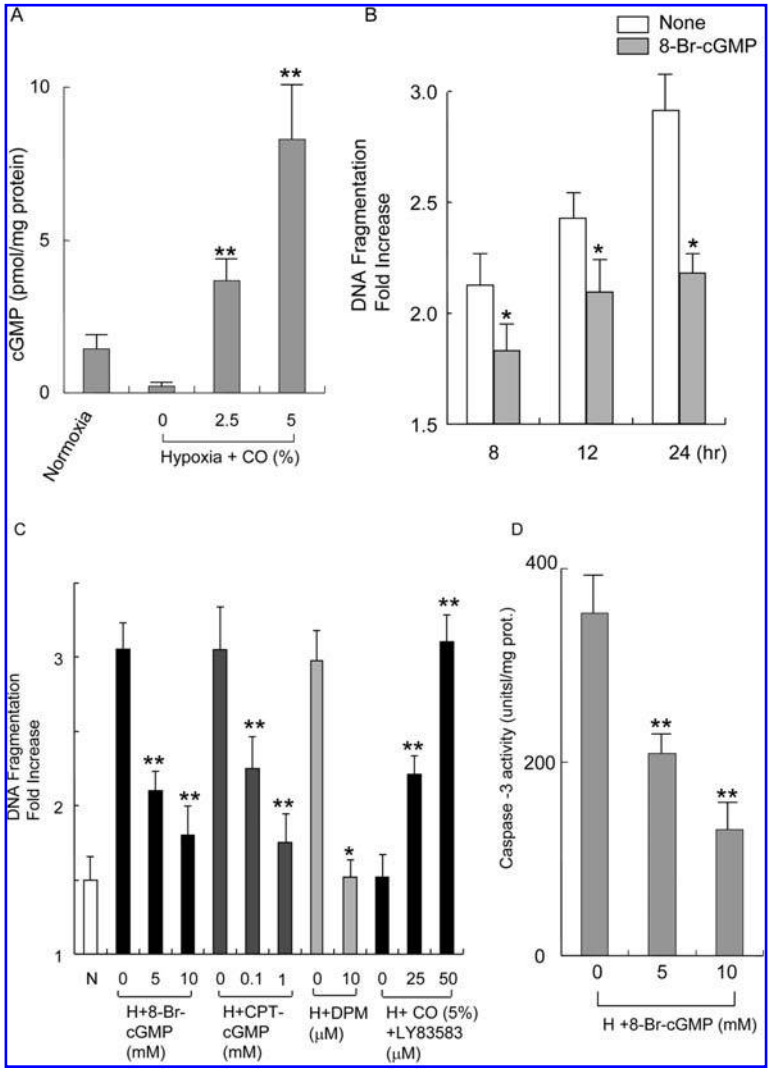

FIG. 6. Relationship between cellular cGMP levels and viability of cerebral neurons under hypoxia. (**A**) Cerebral neurons (2 x 10^5 cells) were cultured under either normoxic (Normoxia) or hypoxic condition (Hypoxia) in the presence of CO at indicated concentrations (0–5%) for 12 h. **denotes $p < 0.01$ compared with hypoxia + CO (0%) by Neuman–Kuehl's analysis followed by ANOVA. (**B**) Cerebral neurons (2 x 10^5 cells) were exposed to hypoxia either in the absence (*open bars*) or presence (*closed bars*) of 8-bromo-cGMP (8-Br-cGMP; 10 m*M*). At the indicated time point, intranucleosomal fragmented DNA was assessed by ELISA as described in text. *denotes $p < 0.05$ compared with cultures without 8-Br-cGMP by multiple comparison analysis, followed by two-way ANOVA. (**C**) Cerebral neurons were maintained either normoxic condition (N) or hypoxic condition (H) in the presence of either 8-Br-cGMP (0–10 m*M*), chlorophenylthio-cGMP (CPT-cGMP; 0–1 m*M*), or dipyridamol (DPM; 10 μ*M*). Neuron culture was also incubated under hypoxic condition in the presence of both CO (5%) and LY83583 (0–50 μ*M*). After 16 h, fragmented DNA was quantitatively measured by ELISA as described in text. * and **denote $p < 0.01$ compared with nontreated cultures by nonpaired *t*-test, and by multiple comparison analysis followed by ANOVA, respectively. (**D**) Cerebral neurons (2 x 10^6 cells) were maintained in the hypoxic condition (Hypoxia) in the presence of 8-Br-cGMP at the indicated concentration (0–10 m*M*) for 12 h. Cells were then harvested and CPP32/Yama-like protease activity was determined as described in text. **denotes $p < 0.05$ compared with nontreated cultures by multiple comparison analysis, followed by ANOVA. In each experiment, mean ± S.D. is shown ($n = 6$).

role for HO-1 among its possible portfolio of functions. In line with this theory, HO-1 is expressed in multiple conditions known to be associated with increased oxidant stress, such as expression of HO-1 in components of the central nervous system in cerebral ischemia (32) and Alzheimer's disease (35). In addition to this function of HO-1 as an intrinsic defense mechanism, our current study portrays another protective function of this enzyme in relation to ischemic insults of the central nervous system. HO-1 induced in hypoxic astrocytes can produce and elaborate a detectable amount of CO into the culture supernatant after reoxygenation, as demonstrated by the increase of cellular cGMP content in platelets (6), which then can then induce cGMP formation in target neurons and establish a preconditioned, protected state.

Because of its triple bond structure and lack of an unpaired electron, CO is far more stable and less reactive than another diatomic gas, nitric oxide (NO), which itself is an established gas transmitter involved in signal transduction in the brain (26). This stability of CO suggests its unique potential as a gas mediator. CO also participates in various signal transduction systems, including long-term potentiation in the hippocampus (36), modulation of stress response in the brain (24), and the regulation of vascular smooth muscle cell growth/survival (21, 34). Our current study also highlights a novel concept regarding the function of CO as a neurotrophic mediator in the setting of central nervous system ischemia via the downregulation of the activity of caspase-3, one of the key caspases which promotes apoptotic death of neurons (17).

These actions of CO in the central nervous system can be contrasted with those of nitric oxide, which also may act as a gaseous neurotransmitter, has the ability to directly injure neurons through activation of poly (ADP-ribose) synthetase (30). Therefore, although both CO and NO bind to heme proteins, their actions in terms of the effect on neuronal death are diametrically opposite. CO appears to mediate neuronal protection by elevating cGMP levels in neurons, which eventually leads to the suppression of apoptosis (7). This contrasts with the neuronal death-promoting activities of NO as a reactive species in cerebral ischemia, although NO also elevates cGMP levels in neurons via the activation of guanylate cyclase (29).

Taken together, our present study suggests that hypoxia-mediated induction of HO-1 in cultured astrocytes may play a neuroprotective role in the setting of ischemia/reperfusion of the brain. This novel function of CO, which can be released at

the site of reperfusion, underscores the critical role of astrocytes in the maintenance of neuronal viability in ischemic cerebrovascular disease.

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ABBREVIATIONS

FCS, fetal calf serum; GMP, cyclic guanosine 3',5'-monophosphate; MEM, minimum essential medium; NO, nitric oxide; PBS, phosphate buffered saline; TCA, trichloroacetic acid.

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