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Induction of Heme Oxygenase-1 in the Rat Brain by Kainic Acid-Mediated Excitotoxicity: The Dissociation of mRNA and Protein Expression in Hippocampus

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Heme oxygenase-1 (HO-1) is induced under various stresses. Here we report the induction and localization of HO-1 in the rat brain by intraperitoneal administration of kainic acid (KA). Both mRNA and protein of HO-1 were markedly induced by KA treatment, and each maximal induction was observed 24 h after KA administration. *In situ* hybridization analysis showed that HO-1 mRNA appeared predominantly in glial cells, and confined neurons were positive in the cerebral cortex, basal ganglia, and hippocampal pyramidal cell layer. Immunohistochemical analysis showed that the positive cells in the cerebral cortex and hippocampus were mainly astrocytes and microglia, whereas neurons in the basal ganglia showed intense immunoreactivity. We also demonstrate the dissociation between HO-1 mRNA and protein level in the hippocampal pyramidal neurons, which is known to be vulnerable against excitotoxicity, and discuss the correlation between this dissociation and the vulnerability of hippocampal pyramidal neurons. © 1999 Academic Press

Mammalian cells produce various proteins as stress response during stress. In order to clarify the mechanisms underlying the stress responses against oxidative stress, we have been investigating several stress proteins induced by oxidative stress. We have previously reported two novel stress-inducible proteins termed MSP23 (1, 2) and A170 (3, 4, 5), which were isolated from murine peritoneal macrophages. Furthermore, we demonstrated that A170 was induced in the rat brain by kainic acid(KA)-mediated excitotoxicity (6).

Heme oxygenase-1 (HO-1)/HSP32 has been reported to be induced with various stresses including oxidative stress (7, 9, 10, 11, 12). Also, intracerebroventricular injection of KA induces HO-1 protein in the rat brain (8). These reports suggest that HO-1 plays an important role for protective action during stress. HO-1 catalyses the rate limiting step in the conversion of heme via biliverdin and carbon monoxide to bilirubin, one of the free radical scavengers (13). Such antioxidant activity of HO-1 is considered to be important for cells under oxidative stress (12).

On the other hand, KA binds to and activates a subtype of ionotropic glutamete receptors (14, 15) and causes excitotoxicity in the brain. KA triggers epileptiform activity and subsequent brain injury (14, 18) that is considered to result, in part, from oxidative stress (8, 14, 15, 16, 17, 18, 19).

Hippocampal pyramidal neurons, which show the vulnerability against ischemic stress, are also vulnerable against excitotoxicity and show progressive neuronal death after KA administration (14, 18). It is well known that protein synthesis in hippocampal neurons diminishes under ischemic condition, in spite of high expression level of mRNA (20). This dissociation between mRNA and protein level means the lack of indispensable protein synthesis, and this phenomenon is regarded as one of the reasons why hippocampal pyramidal neurons exhibit the vulnerability (21, 22). Accordingly, it is conseivable that the protein synthesis may also be inhibited under KA-mediated excitotoxicity, because similar neuronal death is sometimes observed. However, the camparison between mRNA and protein level of HO-1 under KA-mediated excitotoxicity has not been reported.

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The aim of this study is to clarify the induction process of HO-1 mRNA and protein, to localize them and compare with each other, and to examine the significance of this induction under KA-mediated excitotoxicity.

MATERIALS AND METHODS

Animal model. Male Wistar rats (280-320 g) were obtained from Japan SLC, INC. (Hamamatsu, Japan) and housed in an air-conditioned room (25°C and about 60% relative humidity) with free access to food and water. The rats were taken care of in accordance with the Guideline for Animal Experimentation of Tottori University.

KA (Wako) was dissolved in PBS and injected into rats intraperitoneally (10 mg/kg) during the daytime. After the time period indicated in the figure legends, rats were decapitated and their brains were divided into three parts (cerebral cortex, hippocampus, and basal ganglia).

Rats that showed no epileptic behavior 2 h after KA administration were excluded.

Preparation of probes for Northern blot analysis and in situ hybridization analysis. The cDNA of mouse HO-1 was obtained from the macrophage cDNA library (4), and that of mouse β-actin was kindly provided by Dr. S. Sakiyama.

cDNA fragment digested with required enzymes was prepared as a probe for Northern blot analysis. Antisense-cRNA probe for *in situ* hybridization analysis was produced through transcriptional reactions catalyzed by the promoter specific bacteriophage T3 RNA polymerase using linealized plasmid vector inserted mouse HO-1 cDNA. *In vitro* transcription was performed at 37°C for 2 h in the solution containing 40 mM Tris-HCl, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine, 10 mM dithiothreitol, 1 U/μl human placental ribonuclease inhibitor, 2 U/μl T3 RNA polymerase, and nucleotide mixture containing fluorescein-11-UTP. cRNA probe was hydrolyzed with alkali (80 mM NaHCO3, 120 mM Na2CO3) to approximately 250 b fragment.

Northern blot analysis. Total RNAs were prepared with RNAzolTM B (TEL-TEST, INC) from fresh rat brain. The RNAs (20 μg) were denatured at 65°C for 5 min in a solution that contains 400 mM 3-(N-morpholino) propanesulfonic acid, pH 7.0, 100 mM sodium acetate, 20 mM EDTA, 50% formamide, and 16% formaldehyde. Then denatured RNAs were separated electrophoretically on 1.2% formaldehyde-agarose gel and transferred onto the Zeta Probe Membrane (BioRad). After UV cross-linking, the RNAs blotted onto the membrane were hybridized with hybridization buffer (50% formamide, 125 mM Na2HPO4, pH 7.2, 250 mM NaCl, 7% SDS, 100 μg/ml salmon sperm DNA) containing 32P-labeled cDNA of mouse HO-1 at 42°C for 12 h. The hybridized membranes were then washed in 1×SSC/0.1% SDS and 0.1×SSC/0.1% SDS several times at 42°C and exposed to Hyperfilm-MP (Amersham) at −80°C for 48 h. In order to correct differences in RNA loading, the RNAs were rehybridized with cDNA probe of mouse β-actin by the same method described above.

Western blot analysis. Fresh brain tissue was homogenized in lysis buffer (50 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 2 mM EDTA, 1 mM alpha-phenylmethanesulfonyl fluoride). After denaturation by boiling at 100°C for 5 min, specimens were stored at −20°C until use. Dye and 2-mercaptoethanol were added to the specimens, and mixture was then boiled just before SDS-PAGE. Thirty μg of proteins were applied on each well for SDS-PAGE. After separating with 13% polyacrilamide gel, the proteins were transferred onto Immobilone-P membrane (Millipore) and hybridized with anti-rat HO-1 antiserum (Stressgen) in PBS containing 0.1% Tween 20. An immunoreactive signal was detected using horseradish peroxidase-conjugated anti-rabbit IgG (Amersham) antibody and ECL detection reagents (Amersham). Total protein content was determined with BCA protein assay reagents (Pierce).

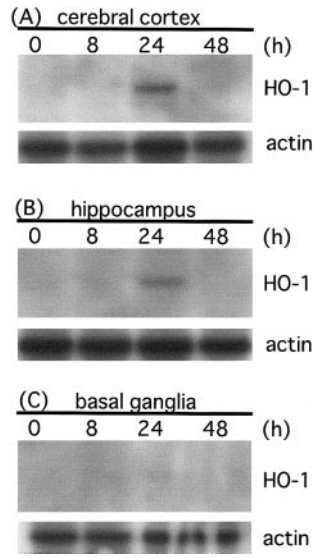

FIG. 1. Northern blot analysis of HO-1 mRNA expression following KA-mediated excitotoxicity. Total RNA was isolated from each part of the rat brain 0 h (without KA-injection), 8 h, 24 h, and 48 h after KA administration. Twenty micrograms of RNA was apllied on each well.

In situ hybridization and immunohistochemical analyses. For *in situ* hybridization, the brain fixed with 4% paraformaldehyde was embedded in paraffin, and 12 μm-sections were prepared. The sections were incubated in 20 mM HCl for 5 min and then in PBS with 0.1% Triton-X100 for 1 min. After washing with PBS several times, the sections were incubated in TE buffer containing 100 μg/ml proteinase K at 37°C for 10 min. The sections were incubated in PBS containing with 2 mg/ml glycine for 3 min, and in cold 20% acetic acid for 15 sec, then washed again several times and left to air dry prior to hybridization. Using antisence-cRNA probe including fluorescein-11-UTP molecule, hybridization was performed according to the manufacturer's protocol (RNA color kit, Amersham) with some modifications.

For immunohistochemical analysis, the brain fixed with 4% paraformaldehyde was embedded in paraffin. Then 6 μm sections were subjected to avidin-biotin peroxidase complex (ABC) method with a Vectastein ABC kit (Vector Laboratories). After the deparaffinizing, the sections were incubated with anti-HO-1 serum overnight at 4°C, followed by the biotinylated anti-rabbit IgG antibody for 1 h and then with ABC for 1 h. The sections were subjected to the peroxidase reaction using freshly prepared 0.02% 3,3-diaminobenzidine-tetrahydrochloride and 0.005% H2O2 in 50 mM Tris-HCl, pH 7.6 at room temperature for 10 min. To determine which glial cells were positive to HO-1, glial fibrillary acidic protein(GFAP)-staining was also performed.

RESULTS

Induction of HO-1 mRNA by KA-mediated excitotoxicity. In a Northern blot analysis, mRNA of rat HO-1 was detected at about 1.5 kb (Fig. 1). The basal expression of HO-1 mRNA was quite low, while the brain that was exposed to the KA-mediated excitotoxicity showed the marked increase of HO-1 mRNA, with maximal levels observed at 24 h, especially in the cerebral cortex

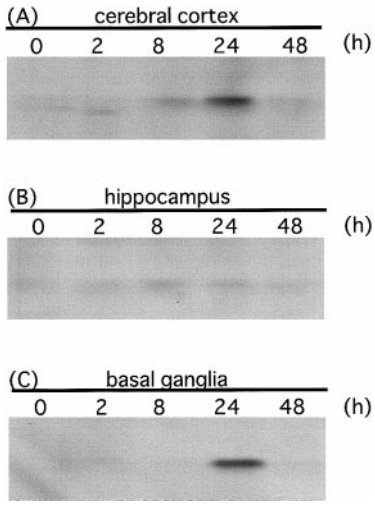

FIG. 2. The time course experiment for the induction of HO-1 protein. Protein was solubilized from each part of the rat brain 0 h (without KA injection), 2 h, 8 h, 24 h, and 48 h after the administration of KA. Thirty micrograms of total protein was applied on each well.

(Fig. 1A) and hippocampus (Fig. 1B). The increase in the basal ganglia was mild (Fig. 1C). During the next 24 hours, the expression of HO-1 decreased to almost basal level (Fig. 1A-C).

Induction of HO-1 protein by KA-mediated excitotoxicity. As shown in Fig. 2, the induction of HO-1 protein under KA-mediated excitotoxicity was clearly demonstrated in a Western analysis. Although HO-1 protein was expressed at a considerably low level in each part of the brain during 0-2 h, the expression level of HO-1 protein increased markedly during 8-24 h, and then decreased during the next 24 h. The induction of HO-1 protein was observed predominantly in the cerebral cortex (Fig. 2A) and basal ganglia (Fig. 2C), while the hippocampus showed relatively low expression level of HO-1 protein in spite of high expression level of mRNA (Fig. 2B).

Localization of HO-1 mRNA and protein. In order to determine the localization of HO-1 mRNA and protein, histological studies were performed. *In situ* hybridization study showed the localization of HO-1 mRNA (Fig. 3). Positive cells were hardly observed in the sections of non-stressed (0 h) rat brain (data not shown), while many positive cells were observed in the brain 24 h after KA administration (Fig. 3A-C). In the cerebral cortex and hippocampus, positive cells were observed predominantly in glia-like cells and also in some neuron-like cells (Fig. 3A,B). The cerebral cortex was studded with many positive cells, but special cluster of positive cells was not observed. In the hippocam-

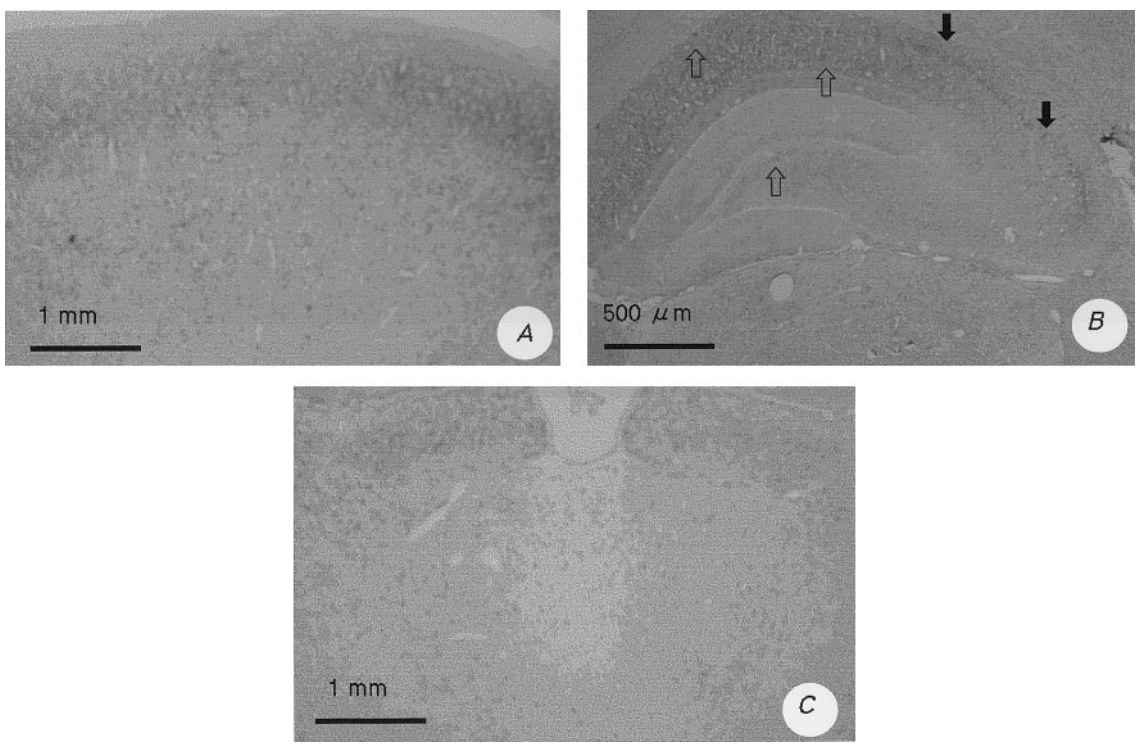

FIG. 3. *In situ* hybridization analysis using mouse antisence HO-1 cRNA shows the localization of cells expressing HO-1 mRNA in the rat brain 24 h after KA administration. (A) Cerebral cortex. Positive cells are observed both in neuron-like cells and in glia-like cells. (B) Hippocampus. Some glia-like cells (⇧) around pyramidal neurons and CA1-CA3 neurons (⬇) are positive for HO-1 mRNA. (C) Basal ganglia. Neuron-like cells are positive, and glia-like cell are almost negative.

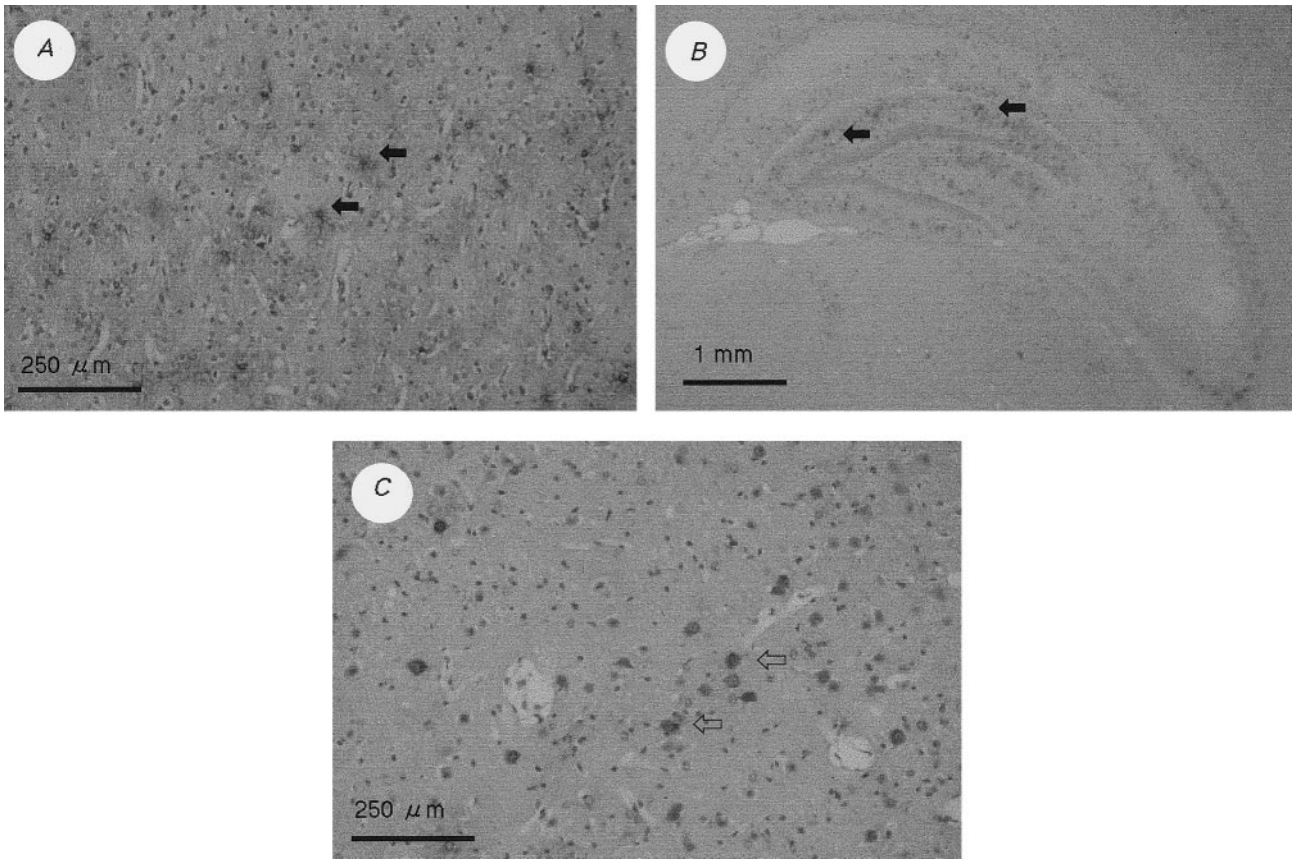

FIG. 4. Localization of HO-1 protein in the rat brain 24 h after the KA administration. (A) Cerebral cortex. Astrocytes (⬅) show the immunoreactivity to anti-HO-1 serum, and some neurons are also positive. (B) Astrocytes (⬅) and some microglia are positive, but not pyramidal neurons, in the hippocampus. (C) Neurons (⇦) in a part of basal ganglia show the intense immunoreactivity, while glial cells show less immunoreactivity. Counter staining is also performed.

pus, glia-like cells around CA1 subfield and pyramidal neurons of CA1-CA3 subfield were HO-1 mRNA positive, but most of the glia-like cells around CA2-CA3 and dentate gyrus were weakly positive (Fig. 3B). The granule neurons in the dentate gyrus were weakly positive (Fig. 3B). The neuron-like cells in the basal ganglia were considerably positive (Fig. 3C).

In order to determine which type of cells induces HO-1 protein under KA-mediated excitotoxicity in the brain, immunohistochemical analysis using anti-HO-1 serum was performed (Fig. 4). In the cerebral cortex (Fig. 4A) and hippocampus (Fig. 4B), astrocytes and some GFAP-negative glial cells, probably microglia, showed immunoreactivity to anti-HO-1 serum 24 h after KA administration. In the hippocampus, pyramidal neurons hardly showed immunoreactivity in spite of high expression level of mRNA, while astrocytes around pyramidal neurons clearly showed it (Fig. 4B). However, astrocytes around CA1 subfield expressed less HO-1 protein than those in other area in the hippocampus. In the basal ganglia, neurons showed intense immunoreactivity to anti-HO-1 serum 24 h after KA administration, but glial cells showed weak immunoreactivity (Fig. 4C).

DISCUSSION

In this study, we demonstrated marked induction of mRNA (Fig. 1) and protein (Fig. 2) of HO-1. The maximal induction of HO-1 mRNA and protein was observed 24 h after KA administration in each part of the brain (Figs. 1 and 2), and this induction was seizure-dependent (data not shown). The mRNA and protein of HO-1 induced by KA-mediated excitotoxicity decreased during the next 24 h (Figs. 1 and 2). These evidences suggest that HO-1 is transiently induced on transriptional level, and function only under a stressed condition in the rat brain.

Our results of *in situ* hibridization analysis and immunohistochemical analysis showed that HO-1 was distributed not only in neurons but also in glial cells 24 h after KA treatment (Figs. 3 and 4). Interestingly, the HO-1-positive cells in the cerebral cortex and hippocampus were different from those in the cerebral

basal ganglia. Briefly, for the most part in the cerebral cortex and hippocampus, numerous astrocytes and some microglia expressed HO-1, but in the basal ganglia neurons did (Fig. 4A-C). Although it has been reported that the induction of HO-1 was observed predominantly in major histocompatibility complex class-II positive microglia after intracerebroventricular injection of KA (8), HO-1 was expressed mainly in astrocytes rather than microglia in our study. Moreover, it has been reported that oligodendrocytes are also vulnerable against AMPA/kainate receptor-mediated excitotoxicity (23, 24), however, oligodendrocytes were not positive in this study.

The pyramidal neurons in the hippocampus, one of the most vulnerable parts against excitotoxicity, did not show the expression of HO-1 protein in spite of the high expression of HO-1 mRNA (Figs. 3B and 4B). The hippocampus also showed quantitative dissociation between mRNA and protein of HO-1 as indicated by Northern and Western analyses. Possibly the inhibition of protein synthesis may coexisted in the hippocampus under KA-mediated excitotoxicity. Such dissociation between mRNA and protein level of HSP70 in hippocampal CA1 neurons has been reported in the case of transient ischemia, and it is considered that this dissociation may cause a vulnerability in a part of the hippocampus (21, 22). As various stresses cause the degeneration of protein molecule, protein synthesis and repair of intracellular enviroment may be necessary for cellular recovery (20). Therefore, this dissociation between mRNA and protein levels of HO-1, or shortage of the indispensable protein synthesis, may also possibly cause a vulnerability against KA-mediated progressive damage in the brain.

KA-mediated excitotoxicity induces some metabolic disorders and causes severe brain damage (14, 25, 26). This pathogenesis may be associated, in part, with oxidative stress, because KA-mediated excitotoxicity is considered to induce leakage of superoxide anion from hypoxantine/xantine oxidase system (27). An increasing number of evidences suggest that KA may cause oxidative stress both *in vitro* and *in vivo* (8, 14, 15, 16, 17, 18, 19). Therefore, induction of HO-1 mRNA and protein in this study may be a result of KA-mediated oxidative stress.

HO-1 is important for mammalian iron homeostasis (28) and for rapid protection of cells from potential oxidative damage during stress (29). It has also been reported that cells which overexpress HO-1 increase resistance to oxidative stress (30, 31). It is considered that this protective activity against oxidative stress is owing to production of antioxidant bilirubin (12), decrease of oxidant heme (32), and evaluation of intracellular free iron levels to facilitate ferritin up-regulation (10, 33). Also, HO-1 produces carbon monoxide, a putative neuronal messenger (34), and may be related to the signal transduction under oxidative stress. Therefore, the disorder of HO-1 protein synthesis in cells, such as pyramidal neurons of CA1 subfield in the hippocampus, may be critical for cell survival.

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