Rapid induction of heme oxygenase 1 mRNA and protein by hyperthermia in rat brain: Heme oxygenase 2 is not a heat shock protein

(heme oxygenase isozymes/cellular defense mechanisms/antioxidants/neurotoxicity)

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ABSTRACT Catalytic activity of heme oxygenase (heme, hydrogen-donor:oxygen oxidoreductase, EC 1.14.99.3) isozymes, HO-1 and HO-2, permits production of physiologic isomers of bile pigments. In turn, bile pigments biliverdin and bilirubin are effective antioxidants in biological systems. In the rat brain we have identified only the HO-1 isozyme of heme oxygenase as a heat shock protein and defined hyperthermia as a stimulus that causes an increase in brain HO-1 protein. Exposure of male rats to 42°C for 20 min caused a rapid and marked increase in brain 1.8-kilobase HO-1 mRNA. Specifically, a 33-fold increase in brain HO-1 mRNA was observed within 1 h and sustained for at least 6 h posttreatment. In contrast, the two HO-2 homologous transcripts (1.3 and 1.9 kilobases) did not respond to heat shock; neither the ratio nor the level of the two messages differed from that of the control when measured either at 1, 6, or 24 h after hyperthermia. The induction of a 1.8-kilobase HO-1 mRNA resulted in a pronounced increase in HO-1 protein 6 h after hyperthermia, as detected by both Western immunoblot and RIA. Immunocytochemistry of rat brain showed discrete localization of HO-1-like protein only in neurons of select brain regions. Six hours after heat shock, an intense increase in HO-1-like protein was observed in both Purkinje cells of the cerebellum and epithelial cells lining the cerebral aqueduct of the brain. We suggest that this increase in HO-1 protein, hence increased capacity to form bile pigments, represents a neuronal defense mechanism against heat shock stress.

The cells of all organisms respond to a stress, such as heat shock, by rapid and intense synthesis of a group of proteins, many of which are present in the organism at normal temperatures (reviewed in refs. 1–4). The major classes of heat shock proteins, hsp70, hsp90, and hsp110, have been rather extensively characterized with respect to inducers, organisms, and tissues. These proteins are postulated to protect, preserve, and recover function of various protein complexes (2). A number of other smaller proteins have also been reported to be heat shock proteins as determined by their enhanced rate of synthesis in response to this stimulus (5–8); included in this group is heme oxygenase. This enzyme has recently been identified in neoplastic cells in culture as a heat shock protein (9, 10).

Heme oxygenase, as is the case with many heat shock proteins (11) under normal conditions, has a defined cellular function, which is catalysis of the heme molecule to form bile pigments (12, 13). In the past, bile pigments had been viewed as waste products of cellular metabolism and a means for disposal of senescent heme compounds, such as hemoglobin, myoglobin, and the various cytochromes. Hence, the importance of heme degradation enzymes has been thought of in this context. Recently, however, it has become evident that bile pigments display potent antioxidant activity and thus may serve an important function in cellular defense against free radical-mediated injury (14, 15).

In mammals and certain fish, the heme molecule is ultimately converted to bilirubin IXα in the course of two sequential enzymatic steps involving catalytic activity of the microsomal heme oxygenase and biliverdin reductase (reviewed in ref. 16). Heme oxygenase activity is detected in tissues of all animal and plant species examined to date (16). Recent studies, however, have shown that the ability to form bile pigments in various mammalian systems, including human and rat, reflects the activity of two isozymes of heme oxygenase (heme, hydrogen-donor:oxygen oxidoreductase; EC 1.14.99.3), HO-1 and HO-2 (17, 18). The isozymes, which are products of two different genes (19, 20), differ in their pattern of tissue distribution and regulation as well as in number of their transcripts (21). With the exception of the brain, in all organs HO-1 is exquisitely sensitive to the regulating effect of environmental chemicals and endogenous factors (22–24). Furthermore, this isozyme is expressed at exceedingly low levels in the brain at all stages of development and maturation (18, 21). Indeed, earlier attempts to demonstrate its presence in the brain were unsuccessful (25). In contrast, the two HO-2 messages [1.3 and 1.9 kilobases (kb)] and encoded HO-2 protein are expressed at impressively high levels in this organ (18, 21).

To our knowledge, we report here for the first time on the induction of HO-1 mRNA and protein in the brain. Furthermore, we report on the identification of only the HO-1 form of heme oxygenase as a heat shock protein and show that this isozyme is selectively expressed in neurons located in discrete regions of the brain.

MATERIALS AND METHODS

Restriction enzymes were purchased from Boehringer Mannheim. [32P]dCTP and [125I] were purchased from Amersham and New England Nuclear, respectively. Two oligonucleotide primers, E2 (5'-TGCACATCCGTCAGAGAAT-3'), homologous to HO-1 cDNA nucleotides +71 to +90, and E5B (5'-AGGAAACTGAGTGTGAGGAC-3'), complementary to HO-1 cDNA nucleotides +833 and +814 (26), were obtained from Research Genetics (Huntsville, AL). Adult male Sprague–Dawley rats were used as tissue source for all experiments. AuroProbe, which was used for Western immunoblotting, was commercially obtained from Amersham. Fluorescein isothiocyanate-conjugated goat anti-rabbit IgG (FITC-GAR) was obtained from Organon Teknika–Cappel. Biliverdin reductase was purified as described (27). Hyperthermia was

Abbreviation: FITC-GAR, fluorescein isothiocyanate-conjugated goat anti-rabbit IgG.
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induced by exposure of animals to 42°C water for 20 min in a humidified atmosphere, as detailed by Nowak et al. (28). A core body temperature of 41°C–42°C was confirmed in heat-treated animals by measurement of rectal temperature. At either 1, 6, or 24 h posttreatment, rats were sacrificed, tissues were removed, and mRNA and microsomal fractions were prepared.

Probes. The HO-2 hybridization probe was the full-length (1300-base-pair) HO-2 cDNA recently purified by this laboratory from a rat testis cDNA library (20). An adaptation of the PCR technique (29), as previously described (21), was used to generate a cDNA fragment corresponding to HO-1 nucleotides +71 to +833 reported by Shibahara et al. (26). All probes used in this study, including mouse α-actin cDNA probes (30), were labeled by the random priming method according to the manufacturer's instruction (random primers DNA labeling system, BRL) and further purified by spin column chromatography using Sephadex G-50 as described (31).

RNA Preparation and Northern Blot Analysis. Total RNA was purified from rat brain by the guanidine isothiocyanate/cesium chloride method as described by Chirgwin et al. (32). Poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography (33), fractionated on a denaturing formaldehyde/agarose (1.2%) gel, and transferred to Nytran (33). Prehybridization, hybridization of the appropriate 32P-labeled cDNA, posthybridization treatment of filters, and autoradiography were performed essentially as described (21). Northern blots were quantified densitometrically using an UltraScan XL densitometer. Linearity of densitometric quantification was confirmed by three successive exposures of each filter.

RIA of HO-1. Quantification of HO-1 in rat brain microsomal preparations was performed essentially as described (21), except the microsomal preparations were resuspended in 0.05 M phosphate, 0.14 M sodium chloride at pH 7.5, prior to RIA.

Western Blot Analysis and Heme Oxygenase Activity Measurement. Protein samples were fractionated by SDS/polyacrylamide gel electrophoresis (34) and transferred to a Millipore Immobilon membrane using a Bio-Rad Trans-Blot transfer cell. Western analysis was carried out essentially as a modification of the procedure described (25). Antibody–antigen complexes were visualized using AuroProbe as recommended by the manufacturer. Brain microsomal protein was precipitated by the addition of 3 volumes of ice-cold acetone. The precipitate was processed and used for electrophoresis.

Microsomal heme oxygenase activity was measured as described (25). Purified rat liver biliverdin reductase was used to convert biliverdin to bilirubin. The protein concentration was determined by the method of Lowry et al. (35).

Immunohistochemistry of HO-1. Control or heat shocked rats were deeply anesthetized using a mixture of chlorohydrate and pentobarbital and perfused through the heart with 0.9% saline, followed by 4% (vol/vol) paraformaldehyde. The brain was dissected and then allowed to fully equilibrate successively with 10% (wt/vol), 20% (wt/vol), and 30% (wt/vol) sucrose solutions at 4°C prior to sectioning. The brains were frozen and cut on a sliding micromtome at 30 or 50 μm. A modified peroxidase-antiperoxidase technique of Sternberger (36), as described by Haber and Watson (37) or as modified by Hsu et al. (38, 39), was used. Fluorescent labeling of sections was obtained using FITC-GAR (1:100,000 dilution) as a secondary antibody. After incubation with primary antiserum (1:1000 dilution), sections were incubated with FITC-GAR (16 h, 4°C) and washed extensively with 0.1 M phosphate buffer at pH 7.5, prior to visualization by fluorescent microscopy.

Antibody Preparations Used in RIA, Western Immunoblotting, and Immunocytochemistry. Rat liver HO-1 and rat testis HO-2 were purified to homogeneity as described in detail (17, 40). The final preparations of HO-1 and HO-2 had specific activities of 6200 units/mg and 5700 units/mg of protein, respectively; 1 unit represents the amount of enzyme that catalyzes the formation of 1 nmol of bilirubin per h. These enzyme preparations were used to raise antibody in male New Zealand White rabbits as detailed before (18). Affinity-purified anti-HO-1 antibody was prepared by preadsorbing the antibody with purified HO-1. HO-1 and HO-2 greatly differ in their molecular properties and do not share similar antigenic epitopes (40).

RESULTS

The vivid difference in response to heat shock of HO-1 and HO-2 in rat brain is shown in Fig. 1. Heat shock resulted in a rapid and dramatic increase in HO-1 mRNA levels (1.8 kb, Fig. 1A). At 1 h (Fig. 1A, lane 2), the mRNA level measured 33-fold higher than the control value (Fig. 1A, lane 1). The elevation of HO-1 mRNA, however, was transient and began to decline by 6 h posttreatment (Fig. 1A, lane 3); it approached control levels by 24 h (Fig. 1A, lane 4). In contrast, both the levels and the ratios of 1.3- and 1.9-kb HO-2 homologous transcripts were refractory to hyperthermia (Fig. 1B). The induction response of HO-1 mRNA was further examined by assessing transcript levels 10 min after hyperthermia, and at this time nearly a 3-fold increase in the mRNA level was detected. Again no change in either the level or the ratio of the two HO-2 homologous transcripts was detected within 10 min (data not shown).

Induction of brain HO-1 was not only at the transcriptional level but was also observed at the protein level. Low constitutive levels of brain HO-1 protein are below the detection limit of Western blot analysis (refs. 21 and 25; Fig. 2, lane 2); however, the amount of HO-1 protein present in brain microsomes was increased to a detectable level 6 h after heat shock (Fig. 2, lane 3). Induction of HO-1 protein in response to heat shock was further confirmed by RIA of HO-1, whereby a 4-fold increase was detected in the level of immunoreactive HO-1-like material in brain at 6 h (430 ng/mg of protein compared to the control value of 120 ng/mg of brain microsomes) (Fig. 2, lane 3).
Fig. 2. Western immunoblot analysis of brain HO-1 protein levels following heat shock. Microsomes were isolated from rat brain 6 h after hyperthermic treatment (42°C, 20 min). The microsomal proteins were acetone precipitated and subjected to Western immunoblot analysis. The blot was probed with rabbit anti-rat HO-1 antisera. Lane 1, 0.5 μg of purified rat liver HO-1 protein. Lanes 2 and 3, 250 μg of microsomal protein obtained from brain of rats 0 and 6 h, respectively, after hyperthermic treatment.

The HO-2 protein concentration in the brain, as assessed by Western immunoblotting, did not change in response to hyperthermia (data not shown). The increase in HO-1 protein, however, did not result in an increase overall brain microsomal heme oxygenase activity; the activity in the brain of control animals measured 4.5 ± 0.4 nmol of bilirubin per mg of protein vs. 4.4 ± 0.8 nmol of bilirubin per mg of protein (mean ± SD) for the heat shock-treated rats. This observation most likely reflects the relatively greater degree of heat lability of HO-2 activity than that of HO-1 (17, 40), as well as the relative abundance of heme oxygenase isozymes in the brain (18, 25). HO-2-dependent activity is significantly decreased (15–20%) on exposure to 42°C, whereas HO-1-dependent heme degradation is not affected by such treatment (17, 40).

Assessment of HO-1 immunoreactive elements in control brain by immunocytochemistry (Fig. 3) revealed discrete localization of HO-1 staining in specific neuronal cell groups such as those found in the dentate gyrus of the hippocampal complex (Fig. 3A). Close examination of cells within the dentate gyrus (Fig. 3B) shows that both neuronal cell bodies and their dendritic processes were densely stained with the HO-1 antisera. Further, dendrites of many cells (>20 cells) within this region were labeled for more than 100 μm. Purkinje cells of the cerebellum (Fig. 3C) were also stained and dendritic arborizations were clearly outlined. Discrete localization of HO-1-immunoreactive elements was detected in other regions of the central nervous system including the ventral portion of the tuber cinereum adjacent to the median eminence and the dorsomedial nucleus of the hypothalamus, as well as the medial geniculate nucleus, the brachial nucleus of the inferior colliculus, and both the lateral and superior vestibular nuclei of the brain stem (data not shown).

Two observations should be pointed out: (i) Although HO-1-staining neurons were confined to discrete areas, differences in the intensity and frequency of cells staining HO-1 positive were seen within neurons comprising a given brain region. Although the basis for this observation is not understood, these patterns are suggestive of further specificity among neurons. (ii) The high abundance of HO-1-like immunostaining elements present in a subclass(es) of cells accounts for their detection using immunocytochemical techniques. Dilution of this immunoreactive material during preparation of microsomal fraction from whole brain is likely the basis for the difficulty in detecting HO-1 by Western immunoblotting in brain of control animals (Fig. 2, lane 2).

Profound changes in the amount of HO-1-like protein in the brain were observed following heat shock. Among neuronal cell populations, this is exemplified by the dramatic response of Purkinje cells in the cerebellum 6 h following heat shock treatment [Fig. 4A (control) vs. B (6-h heat shock)]. Heat shock induction of HO-1-like protein, however, was not observed exclusively in neuronal cells. Following heat shock,
increased HO-1-like protein was observed in glial cells dispersed throughout brain tissue, including those found in the granular layer of the cerebellum (Fig. 4B). Furthermore, epithelial cells of the choroid plexus(es) and ependymal cells, such as those lining the cerebral aqueduct, also showed a marked increase in HO-1-like immunoreactive elements 6 h following heat shock treatment; this was demonstrated by using immunofluorescent labeling [Fig. 4 C (control) vs. D (6-h heat shock)]. Although not shown here, similar induction of HO-1-like protein by heat treatment was observed throughout the lateral third and fourth ventricles where these epithelial cells were present. Heat shock induction of HO-1 protein in epithelial cells comprising the choroid plexus(es) suggests an intimate relationship between this cell population, the composition of the cerebral spinal fluid (which they replenish), and possibly the extensive capillary network that innervates this layer.

It should be noted that the specificity of all antibody–antigen complexes observed in this study was confirmed by using affinity purified anti-HO-1 antiserum. Except for nonspecific immunostain in present on the outer edge of some sections (“edge effect”), incubation of brain tissue with preadsorbed anti-HO-1 antiserum completely abolished the staining.

**DISCUSSION**

To our knowledge, the findings presented in this communication demonstrate for the first time the induction of HO-1 protein and its transcript in the brain of intact rat following hyperthermia. The results show that response to hyperthermia is unique to only one molecular form of heme oxygenase, HO-1, and that the regulatory mechanism for HO-2 is not sensitive to hyperthermia.

In their lack of response to hyperthermia, the 1.3- and 1.9-kb mRNA transcripts for HO-2 resembled one another, suggesting the similarity of their regulatory mechanism. Although we have established that the 1.3-kb message is more efficiently translated than the larger message (21) and most likely encodes HO-2 protein, at this time we have not established whether the two mRNAs are transcribed from the same or different genes. Nonetheless, the present findings plus our previous observations with the developmental patterns of these messages in the brain and liver (21, 41) suggest the coordinated regulation of these transcripts and indicate that neither transcript is under control of a heat shock promoter. The observed refractory response of HO-2 homologous transcripts to hyperthermia and the failure to date to identify an inducer for the isozyme allows the proposal that HO-2 is a housekeeping enzyme. The ubiquitous occurrence of a high level of expression of its protein (16) and mRNA (unpublished data) in mammalian tissues reinforces this proposal. On the other hand, the response of HO-1 mRNA to hyperthermia suggests that, like neoplastic cells in culture (9), a functional heat shock regulating element (42) for expression of HO-1 is present in the normal brain. It follows that the rapidity of the response may reflect the activation of a preexisting transcription factor (or, conversely, the release of an inhibitory factor) that prompts the rapid transcriptional activation of HO-1. Alternatively, it may reflect stabilization of a rapidly turning over HO-1 mRNA.

with rabbit anti-rat HO-1 antiserum (note the modest staining of the Purkinje cell layer). (B) Comparable section of adult rat cerebellum 6 h following heat shock (42°C, 20 min) and stained with the same antiserum (note the heavily stained Purkinje cells). (C and D) Fluorescence technique. (C) Cerebral aqueduct of control rat stained with rabbit anti-rat HO-1 antiserum. (D) Comparable section of cerebral aqueduct 6 h following heat shock and stained as before (note the heavily stained epithelial cells).
It is unlikely that in the brain as a whole the heat inducibility of HO-1 mRNA and protein reflects an increased cellular demand for an augmented heme degradation activity, since the organ already possesses high levels of such activity; rather, the presence of a response to hyperthermia of HO-1 protein in highly select regions and only in certain neuronal populations suggests the physiological importance of the protein in special cell populations. Furthermore, the specific response of these components to hyperthermia distinguishes HO-1 from other heat shock proteins that respond to hyperthermia in the mammalian brain (4). For instance, increases in HO-1 protein in both the ependyma lining the ventricles and Purkinje cells of the cerebellum clearly distinguish the HO-1 isoform from another well-characterized heat shock protein, hsp70; the level of heat-inducible hsp70 protein increases in rat glial cells and cerebellar granulocytes, but the constitutive pattern does not change, 6 h following hyperthermic treatment similar to that used in the present investigation (4, 43). Furthermore, the differential expression of two heat shock proteins may reflect the presence of tissue-specific factors that modulate heat shock transcription factor(s).

To elaborate on the suggested importance of HO-1 to the function of select brain regions and neurons, it is plausible that the low levels of glutathione and ascorbic acid in neurons would render bile pigments important contributors to neuronal antioxidant defense mechanisms. Although glutathione is the major antioxidant in most cells, it is of low abundance in neurons (44, 45).

At this time, however, the possibility that the heat shock response of HO-1 reflects cellular demands other than production of bile pigments cannot be dismissed. For instance, considering that in the case of hyperthermia most proteins (including hemoproteins) are denatured, the induction of HO-1 could reflect a mechanism by which disposition of heme of denatured hemoproteins is accelerated, thus preventing oxygen free radical formation by the heme molecule. Catalysis of denatured hemoproteins by heme oxygenase has been demonstrated (46), and the ability of the heme molecule to generate oxygen free radicals is well known (47). Furthermore, the possibility exists that HO-1 induction reflects a compensatory response of discrete brain regions and neuronal populations to heat inactivation of HO-2. In comparison to HO-1, HO-2 is more susceptible to heat inactivation (17, 40). When exposed at 65°C (10 min), 70% of HO-1 activity was retained; however, nearly 80% of HO-2 activity was lost (40). Moreover, although HO-1 and HO-2 isozymes have similar catalytic activity toward oxidation of heme molecules, which is binding the molecule in a specific orientation hence permitting specific cleavage of the a-methylene carbon bridge, the possibility exists that HO-1 may perform additional specialized function(s) in the cell separate from its heme oxygenase activity. It is conceivable, for instance, that the enzyme participates in regulation of various gene expressions by heme, thereby functioning as a binding and transport protein for the heme molecule.

In conclusion, we have attempted to provide plausible explanations for the biological significance for induction of HO-1 among neuronal as well as nonneuronal cell types in the brain; however, the exact function(s) of the heat shock response of HO-1 remains to be elucidated. This lack of full understanding, however, is not particular to HO-1, but is shared with other heat shock proteins (1).

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