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Hemeoxygenase expression after reversible ischemia of rat brain

Wulf Paschen*, Akira Uto, Bogdan Djuricic, Justina Schmitt

Max-Planck-Institute for Neurological Research, Department of experimental Neurology, Gleuelerstraße 50, 50931 Köln, Germany

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Abstract

Heme oxygenase (HO-1) gene expression was studied in the brains of rats subjected to 30 min global cerebral ischemia followed by recirculation of up to 24 h. Total RNA was isolated from the cerebral cortex, striatum and hippocampus and reverse-transcribed into cDNA. cDNA was taken as template for PCR using HO-1-specific primers. We found that, when PCR reactions were run for 22 cycles, the amount of PCR products correlated closely with the amount of cDNA. HO-1 gene expression was sharply increased after cerebral ischemia in all three brain structures studied. In the cortex and striatum, the HO-1 mRNA content increased constantly after cerebral ischemia up to 24 h of recovery, being 8- and 9-fold over control after 24 h of recirculation in the cortex and striatum, respectively. In the hippocampus, HO-1 mRNA levels peaked at 4 h after ischemia (9-fold over control) and declined thereafter to 4.5-fold over control 24 h after ischemia. Assuming that the observed increase in mRNA levels is paralled by increased HO-1 protein synthesis, formation of the products of HO reaction, biliverdin and carbon monoxide, is activated after ischemia. These products may produce different and divergent effects on the recovery from the metabolic stress produced by cerebral ischemia.

Key words: Antioxidant; Carbon monoxide; Gene expression; Global cerebral ischemia; Heme oxygenase; Rat

The only physiological role of the enzyme heme oxygenase (HO) was up to recently considered to be the degradation of the heme molecule. However, within the last few years the products of HO activity (bile pigments and carbon monoxide (CO)) have been identified as potent molecules exhibiting activities as antioxidant (bile pigments [19,20]) and activator of the enzyme guanylyl cyclase (CO; for a review, see Ref. 9). Similarly to nitric oxide (NO) CO is thought to play a physiological role (via the stimulation of cGMP formation) in the regulation of the tonus of vascular smooth muscle cells [4,5]. In the brain, a role for CO as a neuronal messenger and regulator of cGMP levels has been considered [25]. In olfactory neurons studied in vitro, cells were depleted of endogenous cGMP by inhibitors of HO whereas inhibitors of NO synthase (NOS) were without effect, thus, implying that in some brain regions CO may play a more prominent role than NO for cGMP formation [25].

Two different HO isoenzymes have been identified: constitutively expressed HO (HO-2) and HO-1 which is expressed at only low levels in control brain but expression is strongly activated as a response to various stumuli (for reviews, see Refs. 7 and 8). In fact, the stress protein heat-shock protein32 (HSP32) has been identified to be the enzyme HO-1 [6,16].

In the brain, HO-1 expression is sharply activated by heat shock or oxidative stress produced by glutathione depletion and it has been assumed that this is a protective response [10] since bile pigments (the products of HO activity) are potent antioxidants [19,20]. In addition, CO is produced during HO reaction and it has been discussed that the increase in HO-1 protein observed after glutathion depletion may compensate for the reduced cGMP formation due to depressed NOS activity [10]. Since it has been suggested that oxidative damage may participate in the manifestation of cell damage after cerebral ischemia, we studied changes in the levels of HO-1 mRNA induced by reversible global cerebral ischemia.

Global cerebral ischemia was produced in anesthetized rats by using the four-vessel occlusion model [14]. After 30 min of ischemia brains were recirculated for 2, 4, 8 or 24 h. At the end of the experiments, animals were reanesthetized and decapitated. Brains were taken out

*Corresponding author. Fax: (49) (221) 4726–298.

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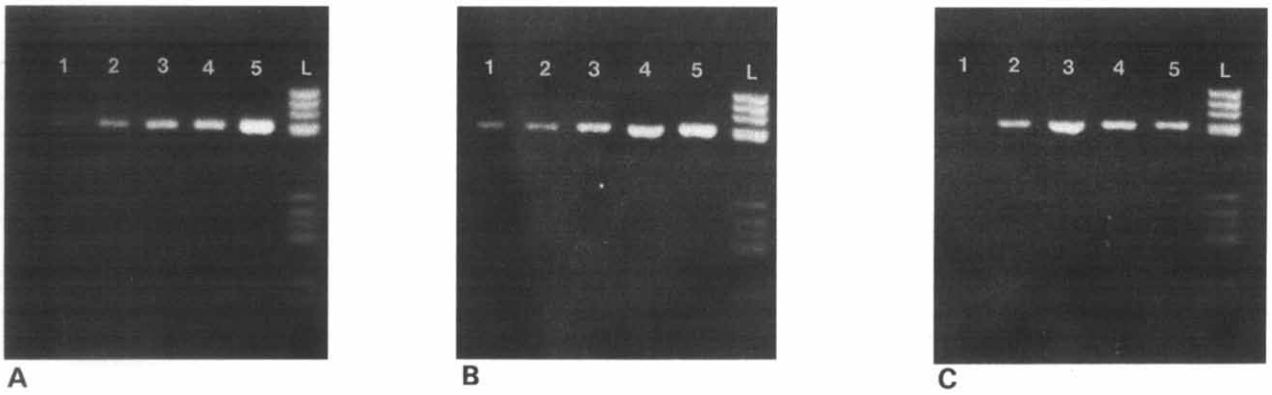

Fig. 1. Ischemia-induced changes in HO-1 mRNA levels. Animals were subjected to 30 min forebrain ischemia followed by 2, 4, 8 or 24 h of recirculation. RNA was isolated from the cortex (A), striatum (B) and hippocampus (C) and reverse-transcribed into cDNA which was used as template for PCR using HO-1-specific primers. PCR products were electrophoresed, gels were transilluminated with UV light and photographed. Lanes were loaded as follows: No. 1: control; Nos. 2–5: 2, 4, 8 and 24 h of recirculation. L: ladder V from Boehringer (Mannheim, Germany).

and put into prechilled phosphate buffer (0.1 M, pH 7.4). Total RNA was extracted from the cerebral cortex, striatum and hippocampus, according to the technique described by Chomczynski and Sacchi [2]. 5 μg of total RNA was reverse-transcribed into cDNA (in a total volume of 20 μl). 1 ml of this solution (equivalent to 250 ng of total RNA) was used as template for PCR. HO-1 cDNA was ampified by PCR using the appropriate primers [15]: upper strand primer: 5′-TGGAAGAGGAGATAGAGCGA-3′ lower strand primer: 5′-TGTTGAGCAGGAAGGCGGTC-3′, amplification product length: 451 bp; intrinsic lower strand primer: 5′-GAAGGCGGTCGTTTGAACTTGGTGGGGTTG-3′, amplification product length: 367 bp. Conditions for PCR were: after a first denaturation step at 94°C for 5 min cycling was done at 60°C, 1 min, 72°C, 1 min, 94°C, 1 min (22 cycles). The reaction was finished with 2 min at 60°C and a final synthesis step at 72°C for 10 min.

For analysis, PCR amplification products were mixed with 4 ml of electrophoresis dye and electrophoresed on a 2% agarose gel supplemented with ethidium bromide. Gels were transilluminated with UV light and fluorescent bands were photographed with standard films. Films were scanned with a rotating microdensitometer (Scandig 3, Joyce Loebl, Gateshead, UK). Optical density of bands were quantified using an image-analysis system run on a Macintosh computer (using the program of Wayne Rasband, NIH, Bethesda, MD). For semiquantitative assessment of data, the optical density of bands obtained from control rat brains were set to 100% and the relative increase in HO-1 gene expression produced by reversible cerebral ischemia was evaluated.

The PCR product produced with HO-1-specific primers had the expected size of 451 bp. To evaluate whether the PCR product had the expected sequence, we run a second PCR with the first PCR product as template and with a new intrinsic 3′-primer. The second PCR product was also of the expected size (367 bp, data not shown). To establish whether the content of PCR product reflects the amount of HO-1 cDNA, different amounts of a cDNA solution produced from total RNA isolated from control brain (relativ amount 0.1–1.0) were processed (PCR, electrophoresis and image analysis) and the optical density of bands related to the amount of cDNA. A close linear relationship was obtained when the amount of cDNA was related to the optical density of bands (correlation coefficient $r = 0.95$, $P < 0.05$). In fact, it has been shown recently that the technique used in the present study to analyse ischemia-induced changes in HO-1 mRNA levels, including reverse transcriptase reaction, PCR and electrophoresis, is suitable to be used as a semiquantitative approach [1].

Ischemia-induced changes in HO-1 gene expression are illustrated in Fig. 1 and results are summarized in Fig. 2. As shown in Fig. 1, HO-1 gene expression was sharply increased after cerebral ischemia and a marked increase in the amount of the HO-1 mRNA was present already 2 h after onset of recirculation in all three brain structures studied. There were, however, regional differences in the time course of H0–1 expression. In the cerebral cortex and striatum, HO-1 mRNA levels increased with time up to 24 h of recirculation (8.2- and 9.2-fold over control levels after 24 h of recirculation in the cortex and striatum, respectively). In the hippocampus, highest HO-1 mRNA levels were reached after 4 h of recirculation (9-fold over control) and mRNA content declined to 4.5-fold over control after 24 h of recovery.

As illustrated by the results of the present study, HO-1 mRNA levels are sharply increased after transient global cerebral ischemia. A similar increase in HO-1 mRNA levels has been observed after ischemia of the kidney or the liver [11,21]. Thus, activation of HO-1 expression seems to be a response to the metabolic stress produced by ischemia. The molecular mechanisms leading to the observed increase in HO-1 mRNA levels have still to be elucidated, namely whether these changes are triggered

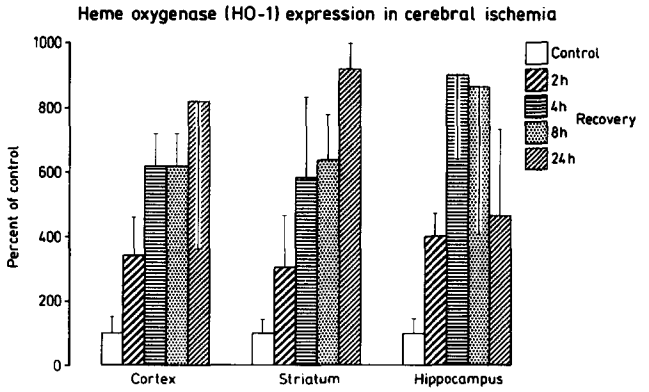

Fig. 2. Relative changes in HO-1 mRNA levels produced by reversible cerebral ischemia. Tissue samples were taken from control brains and brains of animals subjected to 30 min global forebrain ischemia followed by 2–24 h of recirculation, total RNA was isolated from these samples and was processed as described above (see also Fig. 1). Gels transilluminated with UV light were photographed. Film negatives were scanned and optical density of bands were evaluated by using an image-analysis system. For semiquantification of the postischemic increase in HO-1 gene expression, the optical density of bands obtained from control animals were set to 100% (n = 3–4 each group). Note that in the cortex and striatum the highest HO-1 mRNA levels were found after 24 h of recirculation whereas in the hippocampus HO-1 mRNA levels peaked at 4 h of recirculation and decline thereafter.

by any of the metabolic disturbances occurring during ischemia (depletion of high-energy phosphates, acidotic pH shift, sharp increase in intracellular calcium activity) or by free oxygen radicals which are suggested to be produced early during recirculation after ischemia.

It has yet to be proven whether HO-1 protein synthesis and activity is increased after cerebral ischemia in parallel with the observed increase in HO-1 mRNA levels. However, it has already been shown that the synthesis of other stress proteins, such as heat-shock protein70 (HSP70) [23] and the enzyme ornithine decarboxylase [13], is significantly activated after cerebral ischemia, even in the hippocampal CA1 subfield in which global protein synthesis is markedly depressed at the same time [22]. Another point of uncertainty is that it is not known whether the increase in HO-1 gene expression observed in the present study takes place in neurons or glial cells. Heat shock activates HO-1 gene expression and protein synthesis preferentially in glial cells throughout the forebrain and in cerebellar Purkinje cells [3]. However, it has to be considered that expression of the heat-shock gene HSP70 is particularly activated in glial cells after a heat shock as is the HO-1 gene [12] but after cerebral ischemia activation is observed in neurons [24]. Irrespective of the site of the possible increase in HO activity (neurons or glial cells) CO, the product of HO enzymatic activity, is a diffusible gas and has, therefore, the ability to influence neurons and blood vessels in the surrounding of its site of production.

An increase in HO activity may produce divergent effects on the tissue or cellular level during recovery from metabolic stress produced by transient ischemia, although the precise consequences have yet to be established. Any increase in the formation of bile pigments may be viewed protective since bile pigments have a strong antioxidant activity [19,20] as mentioned above. CO, the second product of the HO-catalyzed enzymatic reaction, causes an increase in blood flow due to its dilating effects. This relative increase in blood flow may be of particular importance after cerebral ischemia when the CO_2 reactivity is abolished.

The role of CO as neuronal messenger [25] is corroborated by the observation that the HO-catalysed CO formation plays a role in long-term potentiation (LTP) [18] and that HO inhibitors markedly reduce the depolarization-induced glutamate release from synaptosomes [17]. Assuming that CO production is increased after cerebral ischemia, depolarization-induced glutamate release may be enhanced, producing toxic effect to neurons sensitive to high extracellular glutamate levels. Thus, the postischemic increase in HO-1 gene expression may produce different and divergent effects. Whether HO-1 plays any role in the recovery of the brain tissue from the metabolic stress produced by ischemia or whether it is involved in the development of cell damage has to be established in further experiments.

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