ELSEVIER

Legal Medicine 5 (2003) S360–S366

LEGAL MEDICINE

www.elsevier.com/locate/legalmed

Age-associated increases in heme oxygenase-1 and ferritin immunoreactivity in the autopsied brain

Wataru Hirose, Kazuya Ikematsu*, Ryouichi Tsuda

Division of Forensic Pathology and Science, Department of Translational Medical Sciences, Course of Medical and Dental Sciences, Graduate School of Biochemical Sciences, Nagasaki University, Nagasaki City, Nagasaki, 852-8523, Japan

Abstract

Heme oxygenase-1 (HO-1) is a 32 kDa heat shock protein (HSP) that catalyzes heme to biliverdin, free iron and carbon monoxide in the brain. Furthermore, the release of free ferrous ion by HO-1 plays an essential role in ferritin synthesis, and ferritin stores iron either for intracellular utilization, or for detoxification. It is well known that HO-1 immunoreactivity is enhanced greatly in neurons and glia of the hippocampus and cerebral cortex in various pathophysiological conditions. The expression of HSP 70 is well known for the age-associated increase, but the expression modalities of HO-1 and ferritin associated with aging are still unknown. A study was therefore performed to examine the correlations in the expression of HO-1 and ferritin with age using immunohistochemistry. We investigated 31 autopsied brains (3–84-year-olds) without traumatic brain injury and neurodegenerative disease. The specimens were taken from the cerebral cortex and hippocampus. In the cerebral cortex, age (aging) had a statistically significant positive correlation with HO-1 ($r = 0.894$, $P < 0.01$) and ferritin ($r = 0.731$, $P < 0.01$). In the hippocampus, age had a significant positive correlation with only HO-1 ($r = 0.660$, $P < 0.01$). These results showed that HO-1 and ferritin underwent an age-related increase in human brain, especially in the cerebral cortex. Our results also indicate that various stress responses may persist in the aged human brain.

Keywords: Heme oxygenase-1; Heat shock protein; Ferritin; Aging

1. Introduction

Heme oxygenase (HO) is an enzyme implicated in the metabolism of heme to biliverdin, carbon monoxide (CO) and iron [1]. HO-1 is a family member of HO, a 32 kDa heat shock protein (HSP 32), which is induced by a variety of stress factors including oxidative stress [1], exposure to heavy metals, heat, ultraviolet radiation, focal cerebral ischemia (FCI) [2,3] and traumatic brain injury (TBI) [4,5]. HO-1 is known to be expressed in several mammalian organs including the central nervous system [1]. The enzyme is prevailingly expressed by microglia/macrophages, but also by astrocytes and neurons in FCI and TBI. It has been shown that the functional role of HO-1 includes the regulation of heme protein turnover and iron metabolism, the suppression of oxidative stress through the formation of the powerful antioxidants bilirubin and biliverdin, and possibly a contribution to CO modulation in the capillary endothelium. It has been revealed that the iron liberated by HO-1 can bind ferritin, known to be an iron storage protein, via the chaperone effects of HO-1 [6].

Recently, HO-1 expression was detected in neurodegenerative diseases including Parkinson's disease

* Corresponding author. Tel.: +81-95-849-7076; fax: +81-95-849-7078.

E-mail address: k-ikema@remus.dti.ne.jp (K. Ikematsu).

1344-6223/03/$ - see front matter © 2002 Elsevier Science Ireland Ltd. All rights reserved.
doi:10.1016/S1344-6223(02)00133-5

Table 1
Case profiles and number of HO-1 and ferritin positive cells

Case no.	Age (years)	Sex	Postmortem duration (h)	Cause of death	Number of HO-1 positive cells		Number of ferritin positive cells	
					POL[a]	HP	POL	HP
1	3	M	7	Drowning	6	8	19	13
2	6	M	48	Drowning	7	10	26	18
3	11	M	17	Ligature strangulation	13	18	25	33
4	16	M	15	Drowning	10	11	12	28
5	18	F	17	Ligature strangulation	14	28	25	30
6	22	M	10	Ligature strangulation	20	28	13	25
7	23	M	11	Drowning	22	21	21	20
8	34	M	24	Smothering	28	29	21	20
9	36	F	7	Ligature strangulation	19	33	26	26
10	37	F	72	Drowning	26	18	39	12
11	39	M	24	Drowning	17	22	24	20
12	39	F	6	Ligature strangulation	19	30	24	21
13	45	F	12	Hanging	21	23	46	26
14	48	M	18	Drowning	27	24	35	25
15	49	M	11	Drowning	21	21	39	15
16	52	F	24	Drowning	24	28	31	19
17	54	F	14	Drowning	28	24	39	15
18	55	M	14	Drowning	25	29	42	37
19	57	F	20	Drowning	34	28	23	9
20	57	M	13	Ligature strangulation	40	37	26	14
21	58	F	15	Drowning	33	24	29	10
22	58	M	7	Hanging	30	38	26	24
23	58	M	17	Choking	43	38	36	23
24	59	M	24	Drowning	51	42	39	18
25	61	F	8	Drowning	48	34	32	17
26	63	M	48	Drowning	47	23	41	33
27	65	M	13	Drowning	41	33	39	15
28	69	F	24	Ligature strangulation	50	30	42	37
29	74	M	24	Ligature strangulation	50	46	50	28
30	75	M	24	Drowning	54	27	50	18
31	84	F	24	Ligature strangulation	50	27	42	30

[a] POL, parieto-occipital lobe; HP, hippocampus.

and Alzheimer's disease [7]. It is well known that chaperones, including 70 kDa heat shock protein (HSP 70), increase in the brain [8] and kidney [9] in association with aging. However, the expression modalities of HO-1 associated with aging in humans are still unknown. Ferritin in the human brain is also present in astrocytes but is primarily confined to those in the gray matter in the old age group [10,11]. Although the iron liberated via HO-1 can bind ferritin, no report is available on the expression modalities of HO-1 and ferritin in association with aging in normal human brains.

The present study examined the possible correlation between aging and the expression amounts of both HO-1 and ferritin by immunohistochemistry, using brain specimens collected from forensic autopsies. The purpose of this study was to understand the pathophysiological role by which HO-1 and ferritin are regulated in aged human brains.

2. Materials and methods

2.1. Samples

We investigated brain specimens derived from patients who died after mechanical asphyxia ($n = 31$; age 3–84 years). Further clinical and autopsy data, including the postmortem period (6–72 h) and cause of death, are listed in Table 1. Paraffin-embedded samples from the brain lesion, parieto-occipital lobe and hippocampus were routinely stained by the hematoxylin and eosin (H&E) method and analyzed by light microscopy. In all cases the brains were neuropathologically examined and found to be normal.

2.2. Immunohistochemistry

Rabbit polyclonal antibodies directed against rat HO-1 (SPA-895) were purchased from Stress Gen Biotechnologies (Victoria, British Columbia) and diluted 1:800 in 0.3% bovine serum albumin (BSA) in 0.1 M phosphate-buffered saline (PBS) solution. The antisera was specifically labeled HO-1 in human and rat microsomes. Rabbit polyclonal antibodies directed against human ferritin were purchased from Dako Co. and diluted 1:100 in 0.3% BSA in 0.1 M PBS. The tissue sections were deparaffinized, washed with PBS, and incubated in 3% H_2O_2 for 30 min to eliminate endogenous peroxidase activity. The sections were then treated for 10 min with 3% BSA in PBS solution, and incubated with anti-HO-1 overnight at 4 °C, and anti-human ferritin for 30 min, respectively. The sections were subsequently processed using the labeled streptavidin biotin (LSAB) method (Dako, USA). Reaction products were visualized as a brown precipitate using diaminobenzidine (DAB)-H_2O_2 as chromogen. Following immunocytochemical staining, the sections were counterstained with methyl-green stain, dehydrated, and then coverslipped.

2.3. Morphometrical analysis

In each section, two microscopic fields were randomly selected under a hundred-fold magnification. The number of HO-1 and ferritin positive cells was counted in each field, and the average of the two fields was evaluated for the intensity of HO-1 and ferritin expression, respectively. Two different individuals carried out this analysis independently.

2.4. Statistical analysis

Pearson's correlation test was employed to statistically analyze the results. A value of $P < 0.05$ was considered significant.

3. Results

In any of these cases, macroscopic and neuro-histological findings revealed no obvious pathological alteration such as infarction or hemorrhage. However, we found physiological age-related changes such as deposition of senile plaque with core or granulovascular degeneration in some cases (data not shown). Figs. 1 and 2 show representative HO-1 immunoreactive cells (a) and ferritin positive cells (b) in the parieto-occipital lobe and hippocampus, respectively. HO-1 immunoreactivity was predominantly detectable in the glia of the parieto-occipital lobe (Fig. 1a), but was also found in the neurons as well as glia of the hippocampus (Fig. 2a). Ferritin staining positive cells were composed of only glia both in the cerebral cortex and hippocampus (Figs. 1b and 2b). HO-1 and ferritin immunoreactive cells were counted in layers II–VI of the parieto-occipital lobe.

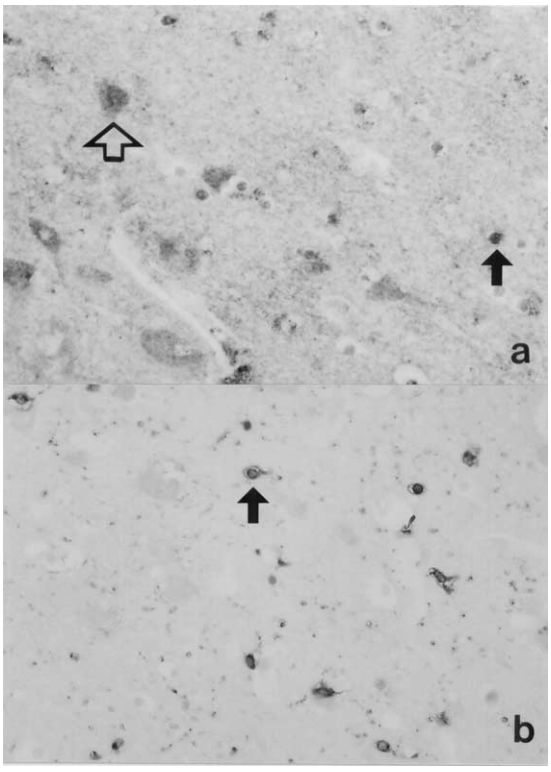

Fig. 1. HO-1 and ferritin immunoreactivity in parieto-occipital lobe in case 30. (a) HO-1 immunoreactivity. (b) Ferritin immunoreactivity. HO-1 and ferritin positive cells are shown (arrow) in parieto-occipital lobe (cerebral cortex layers II–IV). Neuron, open arrow; glial cell, black arrow.

In the hippocampus, HO-1 and ferritin immunoreactivity were also detected. Table 1 shows the counted number of HO-1 and ferritin positive cells in the respective cases.

We investigated a possible correlation between aging and the expression levels of HO-1 and ferritin. The correlations between aging and HO-1 positive cells were statistically significant in the parieto-occipital lobe (Fig. 3a, Pearson's test, $r = 0.894$, $P < 0.01$) and hippocampus (Fig. 3b, $r = 0.660$, $P < 0.01$). Furthermore, the assessment of the possible correlation between age and the number of ferritin positive cells showed the statistically significant positive correlation only in the parieto-occipital lobe (Fig. 3c, $r = 0.731$, $P < 0.01$). There was no significant correlation in the hippocampus (Fig. 3d, $r = 0.033$). When the number of HO-1 and ferritin positive cells was compared, the significant positive correlation was observed only in the parieto-occipital lobe (Fig. 4a, $r = 0.656$, $P < 0.01$), with no obvious correlation in the hippocampus (Fig. 4b, $r = 0.101$).

4. Discussion

HO-1 is an enzyme involved in the metabolism of heme to biliverdin, CO and iron [1]. The enzyme is known to be present in several mammalian tissues including the central nervous system. The HO-1 reaction is important since heme may contribute to vasospasm and increase oxidative stress in cells. Furthermore, HO-1 is induced by various stimuli, including heat shock, heme, metals, hormones, and oxidative stress [1]. On the other hand, ferritin is located in all the organs including the brain and stores iron either for eventual intracellular utilization, for potential use by other cells, or for detoxification. It

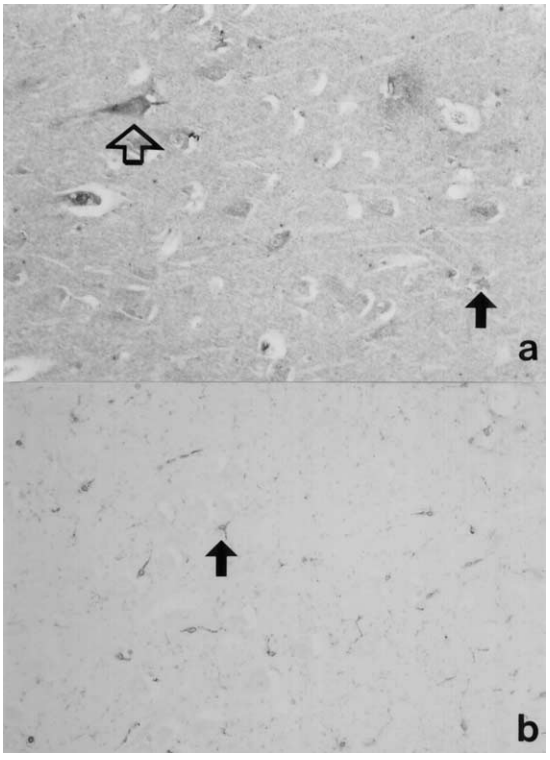

Fig. 2. HO-1 and ferritin immunoreactivity in hippocampus in case 16. (a) HO-1 immunoreactivity. (b) Ferritin immunoreactivity. HO-1 and ferritin positive cells are shown (arrow) in hippocampus (CA-1 area). Neuron, open arrow; glial cell, black arrow.

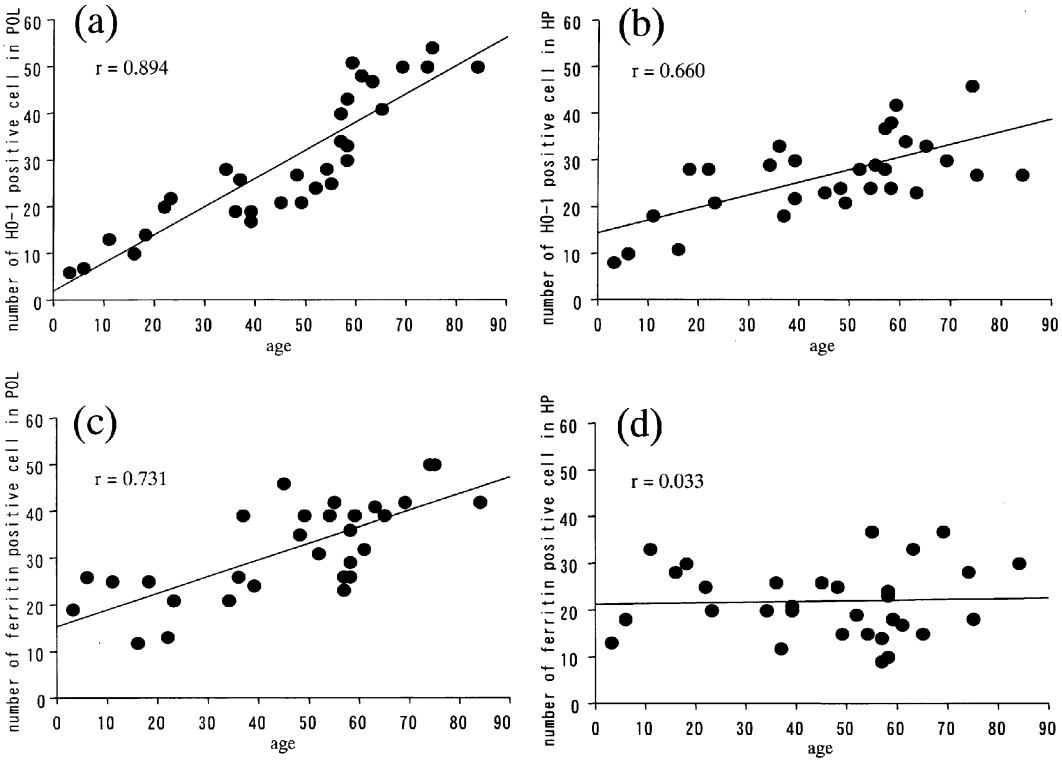

Fig. 3. Relationship between age and the number of HO-1 or ferritin positive cells in parieto-occipital lobe and hippocampus. (a) Relationship between age and the number of HO-1 positive cells in parieto-occipital lobe. (b) Relationship between age and the number of HO-1 positive cells in hippocampus. There is an age-related increase in HO-1 in parieto-occipital lobe and hippocampus and a straight relationship between age and the number of HO-1 positive cells in parieto-occipital lobe ($P < 0.01$) and HO-1 in the hippocampus ($P < 0.01$). (c) Relationship between age and the number of ferritin positive cells in parieto-occipital lobe. There is an age-related increase in ferritin in parieto-occipital lobe and a straight relationship between age and the number of ferritin positive cells ($P < 0.01$). (d) Relationship between age and the number of ferritin positive cells in hippocampus. There was no relationship ($r = 0.033$).

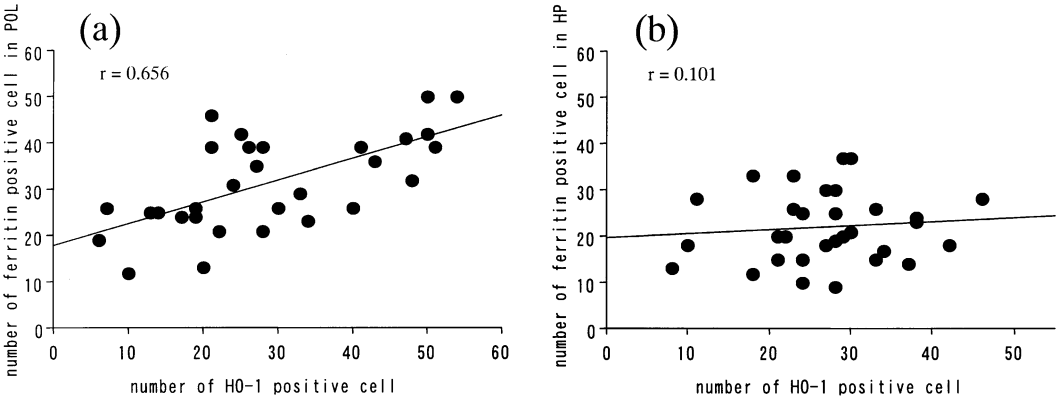

Fig. 4. Relationship between the number of human brain HO-1 and ferritin positive cells in parieto-occipital lobe (a) and hippocampus (b).

has also been shown that ferritin is implicated in iron metabolism in chaperones such as HO-1.

In the 31 human brains that were employed in the present study, there are no obvious pathological alterations but physiological age-related changes. Therefore, the brains are considered as normal. To the best of our knowledge, there is no report describing the distribution of HO-1 containing cells in the normal human brain from young to old, and there are few studies on brain ferritin [10,11]. Thus, we investigated the distribution of HO-1 and ferritin positive cells in normal human brains. This study shows that HO-1 is expressed primarily in glia in the cerebral cortex. This observation is consistent with the latest findings in animal experiments [2–5]. There are various reports available as to whether neuron or glia are responsible for inducing the expression of HO-1 in the rat hippocampus [2–5]. In this study, HO-1 positive cells appeared in both neurons and glia in the human hippocampus. Therefore, the expression modalities of HO-1 may not be completely consistent in the hippocampus of rats and humans. Our study also revealed that ferritin positive cells from the human brain comprised only glia both in the cerebral cortex and hippocampus. These results are similar to the reports by Connor et al. [10] and Ozawa et al. [11] for the human brain.

Incidentally, it is well known that chaperones, including HSP 70, increase in the brain [8] and kidney [9] in association with aging. Nevertheless, the expression modalities of HO-1 associated with aging in the human brain are still unknown. In the present study, we investigated the differences in the aging-induced alterations of HO-1 in the human brain using immunohistochemistry. We showed that there was a notably significant positive correlation between normal aging and the number of HO-1 positive cells in the cerebral cortex and hippocampus. These results suggested that HO-1 increases its expression depending on normal aging like other chaperones such as HSP 70 [12]. Other laboratories have shown the age-related alteration of HO-1 expression in the mouse liver and brain [13,14]. Barnes et al. [13] suspected that an age-related increase of liver HO-1 might contribute to increased susceptibility to oxidative stresses in association with aging. Accordingly, we suspected that the age-related increase of HO-1 in the human brain might contribute to increased susceptibility to oxidative stress in association with aging. Beschorner et al. [5] reported that HO-1 positive cells were only observed in brains with clinical or pathological evidence of neurological disease, but not in normal human brains. In contrast, our investigation showed that HO-1 immunoreactivity was consistently observed in all normal human brains. The difference in the HO-1 positive cell pattern may reflect the difference in immunoreactivity between our staining method and that employed by Beschorner et al. In their report, it is interesting to note that almost all the cases were elderly, whereas our study revealed an increase in the number of HO-1 positive cells in association with aging. Influences due to aging should be taken into account whenever histopathological changes in stress proteins such as HO-1 are to be demonstrated in brains with clinical or pathological evidence of neurological disease.

As the result of the correlation between aging and the number of ferritin positive cells, a significant positive correlation was found between age (aging) and the number of ferritin positive cells in the cerebral cortex, although there was no significant correlation in the hippocampus.

The mechanism of the increase in the number of ferritin positive cells in association with aging remains to be elucidated. It is well known that HO-1 plays an essential role in ferritin synthesis, and ferritin stores iron either for intracellular utilization, or for detoxification [1]. In the cerebral cortex, age (aging) had a statistically significant positive correlation with HO-1 and ferritin. Accordingly, the increased HO-1 expression associated with aging might be involved in the increase of ferritin along with aging. In this study, we demonstrated an increase in the expressions of HO-1 and ferritin in the human brain depending on age. Although various mechanisms (including oxidation stress) might contribute to the age-related increase of HO-1 and ferritin expression, this has not yet been completely clarified. Nevertheless, our findings provide important fundamental data in the investigation into the pathophysiological kinetics of HO-1 in human brains under various pathological conditions.

References

[1] Maines MD. Heme oxygenase: function, multiplicity, regulatory mechanisms, and clinical applications. Fed Am Soc Exp Biol J 1988;2:2557–2568.

[2] Nimura T, Weinstein PR, Massa SM, Panter S, Sharp FR. Heme oxygenase-1 (HO-1) protein induction in rat brain following focal ischemia. Mol Brain Res 1996;37:201–208.

[3] Turner CP, Panter SS, Sharp FR. Anti-oxidants prevent focal rat brain injury as assessed by induction of heat shock protein (HSP 70, HO-1/HSP32, HSP 47) following subarachnoid injections of lysed blood. Mol Brain Res 1999;65:87–102.

[4] Fukuda K, Richmon JD, Sato M, Sharp FR, Panter SS. Induction of heme oxygenase-1 (HO-1) in glia after traumatic brain injury. Brain Res 1996;736:68–75.

[5] Beschorner R, Adjodah D, Schwab JM, Mittelbronn M, Pedal I, Mattern R, Schluesener J, Meyermann R. Long-term expression of heme oxygenase-1 (HO-1, HSP-32) following focal cerebral infarction and traumatic brain injury in humans. Acta Neuropathol 2000;100:377–384.

[6] Turner CP, Bergeron M, Matz P, Zegna A, Noble L, Panter SS, Sharp FR. Heme oxygenase-1 is induced in glia throughout brain by subarachnoid hemoglobin. J Cereb Blood Flow Metab 1998;18:257–273.

[7] Schipper HM. Heme oxygenase-1: role in brain aging and neurodegeneration. Exp Gerontol 2000;35:821–830.

[8] Unno K, Asakura H, Shibuya Y, Kaiho M, Okada S, Oku N. Increase in basal level of Hsp70, consisting chiefly of constitutively expressed Hsp 70 (Hsc 70) in aged rat brain. J Gerontol Biol Sci 2000;55A:B329–B335.

[9] Maiello M, Boeri D, Sampietro L, Pronzato MA, Odetti P, Marinari UM. Basal synthesis of heat shock protein 70 increases with age in rat kidneys. Gerontology 1998;44:15–20.

[10] Connor JR, Menzies SL, Martin SM, Mufson EJ. Cellular distribution of transferrin, ferritin, and iron in normal and aged human brains. J Neurosci Res 1990;27:595–611.

[11] Ozawa H, Nishida A, Mito T, Takashima S. Development of ferritin-positive cells in cerebrum of human brain. Pediatr Neurol 1994;10:44–48.

[12] Tohgi H, Utsugisawa K, Yoshimura M, Yamagata M, Nagae Y. Heat-shock cognate 70 messenger RNA expression in postmortem human hippocampus: regional differences and age-related changes. Neurosci Lett 1995;196:89–92.

[13] Barnes CJ, Cameron IL, Puleo-Scheppke B, Lee M. Age alters expression and inducibility of heme oxygenase isozymes in mice. Age 1998;21:123–128.

[14] Iijima N, Tamada Y, Hayashi S, Tanaka M, Ishihara A, Hasegawa M, Ibata Y. Expanded expression of heme oxygenase-1 (HO-1) in the hypothalamic median eminence of aged as compared with young rats: an immunocytochemical study. Neurosci Lett 1999;271:113–116.