Neurobiology of Aging 24 (2003) 501–509

NEUROBIOLOGY OF AGING

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Glutathione *S*-transferase isoenzymatic response to aging in rat cerebral cortex and cerebellum

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Received 8 February 2002; received in revised form 7 June 2002; accepted 18 July 2002

Abstract

Aging is associated with increased oxidant generation. One mechanism involved in the defense of oxidative products is the family of glutathione transferases (GST). We have analyzed the activity, distribution and expression of GSTP1 and GSTA4 isoenzymes in the cerebral cortex and cerebellum of young, adult and aged rats. The total GST activity, measured with the universal substrate 1-chloro-2,4-dinitrobenzene (CDNB), increased only with the maturation process; however GSTA4 activity, using the specific substrate 4-hydroxynonenal (HNE), did show an age-dependent increase in both brain regions. Cellular location of GSTA4 in astrocytes was not changed except for young cerebral cortex and adult/aged cerebellum that also showed immunoreactivity in layer III pyramidal neurons and Bergman radial glia, respectively. Distribution of GSTP1 was similar among groups and only an increased number of positive oligodendrocytes was found in the Purkinje and granular layer of adult/aged cerebellum. The GSTA4 and GSTP1 expression increased from young to adult/aged brain and GSTA4 even augmented in the aged cerebral cortex. These results suggest a GST isoenzymatic response with aging, but above all with the maturation process.

Keywords: Aging; Glutathione transferase isoenzymes; Cerebral cortex; Cerebellum; Glia; Oxidative stress

1. Introduction

Aging is a multifactorial process involving neurodegenerative changes in cell morphology and biochemistry. The free radical or "oxidative stress" theory holds that oxidative reactions are the factors underlying these changes [45]. Highly reactive oxygen species (ROS) cause a wide spectrum of cell damage, including lipid peroxidation, inactivation of enzymes, alteration of intracellular oxidation–reduction state, and DNA damage [20,59].

Aerobic organisms are provided with non-enzymatic and enzymatic systems either to prevent the formation of radicals or to neutralize them after they have been produced. Although considerable progress has been made in identifying and understanding the mode of action of individual enzymes and antioxidative components, the complex network responsible for the coordination and integration of the various cellular defense systems is not well understood [59]. Indeed, activity of antioxidant enzymes has been reported either to increase or decrease, depending on the tissue, age and individual antioxidant enzyme [13,48,25,56,40], suggesting that some regulatory mechanisms must exist in senescent tissues to provide an efficient defense against oxygen-free radicals generated during aging.

One of the enzymatic mechanisms involved in this defense is the family of glutathione *S*-transferase (GST) enzymes, which play critical roles in providing protection against electrophilic xenobiotics (drugs, pesticides, carcinogens, etc.) and products of oxidative stress [35]. GSTs also catalyze the GSH conjugation of endogenous substrates, including cholesterol α-oxide [39], prostaglandin [10], leukotriene A4 [36,55], bile acids [4,30], steroid hormones [24,37] and neurotransmitters [1].

All known eukaryotic species possess multiple cytosolic and membrane-bound GST isoenzymes, each displaying distinct catalytic and non-catalytic binding properties [21]. Cytosolic GSTs exist as dimeric proteins consisting of two identical or closely-related subunits from the same class: Alpha, Mu, Pi [34], Theta [38], Kappa [46] and Zeta [8].

Evidence suggests that the level of GST expression is crucial in determining the sensitivity of cells to a broad spectrum of toxic chemicals. Moreover, enzymatic activity is

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PII: S0197-4580(02)00139-2

regulated at the transcriptional level by a variety of inducing compounds; in addition, post-translational regulation of GST by active oxygen species, protein kinase C or methylation has also been suggested [21].

The central nervous system is especially vulnerable to oxidative stress for various reasons: non-replicating cells, high metabolic rates in comparison to other tissues, relatively high levels of polyunsaturated fatty acids and relatively low levels of natural antioxidants and of protective enzymes. Consequently, a relatively uniform functional decline of CNS cells takes place with age [31].

The regional, cellular and subcellular distribution of GSTs in brain has been studied in detail, showing an isoenzymatic-specific pattern [1,11,53,26]. In rat and mice, the Mu-class GST isoenzymes have been immunocytochemically located in astrocytes, subventricular zone cells, ependymal cells, and tanicytes [1]; Pi-class GST have been found in oligodendrocytes and associated with myelin [11,53]; Alpha-class GSTs have been detected in astrocytes, choroid plexus, endothelial cells and/or astrocytic end feet associated with blood vessels, Purkinje cells and neurons [26]. The CNS glial cells, highly involved in the CNS defense [43,44], have the greatest abundance of GSH and might protect neurons, which are poor in GSH, against oxidative insults [51].

However, brain GST isoenzymatic expression has not been analyzed with regard to aging, either in relation to all stages of life, including senescent animals, or simultaneously in different brain zones. To elucidate the contribution of GST to the protection against oxidative stress in the CNS with aging, we propose the biochemical and immunocytochemical evaluation of two GST isoenzymes: GSTP1 and GSTA4 expressed in oligodendrocytes and astrocytes, respectively, in two brain zones, closely associated with the senescent process, such as the cerebral cortex and cerebellum from young to adult and aged rats.

2. Materials and methods

2.1. Animals

The study was performed on 10 young (2-month-old), 10 adult (4-month-old) and 10 aged (30-month-old) male albino Wistar rats, kept under standard conditions of light and temperature and allowed ad libitum access to commercial rat chow. All the experiments were carried out according to EU guidelines on the use of animals for biochemical research (86/609/EU).

2.2. Immunohistochemistry

Five rats from each group were deeply anaesthetized with Ketolar (Parke Davis, 15 mg/100 mg body weight) and then injected with heparin through the penis dorsal vein (Rovi, 500 IU/kg body weight) to avoid blood coagulation.

The animals were perfused through the left ventricle with 20–30 ml of 0.01 M phosphate-buffered saline (PBS; pH 7.4) gassed with 95% O_2/5% CO_2, and then with 100–120 ml of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). The brains from young, adult and aged animals were removed, cut into 4–5 mm coronal blocks, and then post-fixed for 4 h in the same fixative at room temperature. The blocks were then rinsed and cryoprotected by immersion overnight at 4 °C in 0.1 M PB containing 30% sucrose.

Afterwards, blocks of three rats from each group were embedded in OCT medium (Tissue-Tek/Sakura) and frozen under 2-methylbutane at liquid nitrogen temperature. Serial sections (40 μm thick) were cut using a cryostat (2800 Frigocut E, Reichert-Jung). The free-floating sections were then incubated overnight with rabbit polyclonal anti-rat GSTP1 (Biotrin, Dublin) and GSTA4 antisera (gift from Dr. J. Hayes) (1:500) in PBS containing 0.2% Triton X-100 at 4 °C. After several rinses in PBS, the sections were incubated with biotinylated anti-rabbit IgG produced in goat (1:100, Vector Laboratories Ltd.) followed by peroxidase-linked ABC (Vector Laboratories Ltd.). The peroxidase activity was demonstrated following the nickel-enhanced diamino-benzidine procedure [49]. Sections were then mounted on slides, dehydrated in ascending ethanol series, and covered using DPX (Fluka).

Blocks from another two animals of each group were frozen in liquid nitrogen and thawed in cold PB to improve antibody penetration. Sections of 40 μm thick were cut with a Vibratome (VT 1000S Leica) and immunostaining as previously described except that Triton X-100 was not included in the incubation solutions. Sections were routinely processed for electron microscopy and mounted on Durcupan ACM resin slides (Fluka) under a plastic cover slip and incubated for 3 days at 56 °C. Selected areas of the cerebral cortex and cerebellum were dissected out, and reembedded in Durcupan to prepare 2 μm thick semi-thin sections (Ultracut E Reichert-Jung).

2.3. Enzyme assays

For biochemical analysis, five rats of each age were killed by cervical dislocation and the brains were immediately removed. The cerebral cortex and cerebellum were dissected, rinsed in saline solution and stored at −80 °C until used. Brain tissues were homogenized in 1/3 (w/v) of 30 mM Tris–HCl, pH 7.4 containing 0.5 mM DTT, 1 mM EDTA, and protease inhibitors. The resulting homogenates were centrifuged for 1 h at 100,000 × g. All the operations were performed at 0–4 °C.

GST activities towards 1-chloro-2,4-dinitrobenzene (CDNB) and 4-hydroxynonenal (HNE) were measured in the supernatant as described by Habig et al. [19] and Alin et al. [3], respectively. Protein concentrations were determined by the Bradford method [9], with bovine serum albumin as the standard.

2.4. Western blot

Equal amounts (10 μg of protein) of the denatured supernatants, regardless of tissue or age, were loaded and separated on a 12% SDS–polyacrylamide gel (Mini Protean II, BioRad), as described by Laemmli [32]. Afterwards, proteins were transferred to a PVDF membrane (Immobilon P, Millipore). The membrane was blocked with 5% powdered non-fat milk in 25 mM Tris–HCl, pH 7.6; 137 mM NaCl, 2.6 mM KCl, 0.1% Tween-20, and incubated overnight at 4 °C with rabbit polyclonal anti-rat GSTP1 and GSTA4 antisera diluted 1:1000 in blocking solution. Bound antibody was revealed by means of an enhanced chemiluminescence kit (Amersham) according to the manufacturer's instructions. The relative amount of the proteins in each sample was quantified by densitometric scanning. After immunodetection, membranes were probed with anti α-tubulin (Sigma) as a loading control.

3. Results

3.1. Immunocytochemistry

The immunocytochemical analysis of GSTA4 in the cerebral cortex indicated that the immunoreactivity, located in astrocytes soma and feed end surrounding blood vessels, was homogeneously distributed throughout the different cortical layers regardless of age. A high number of immunoreactive astrocytes appeared in adult and aged rats; however in young rats, we also found, in the V and III cortical layers, some immunostained pyramidal neurons that

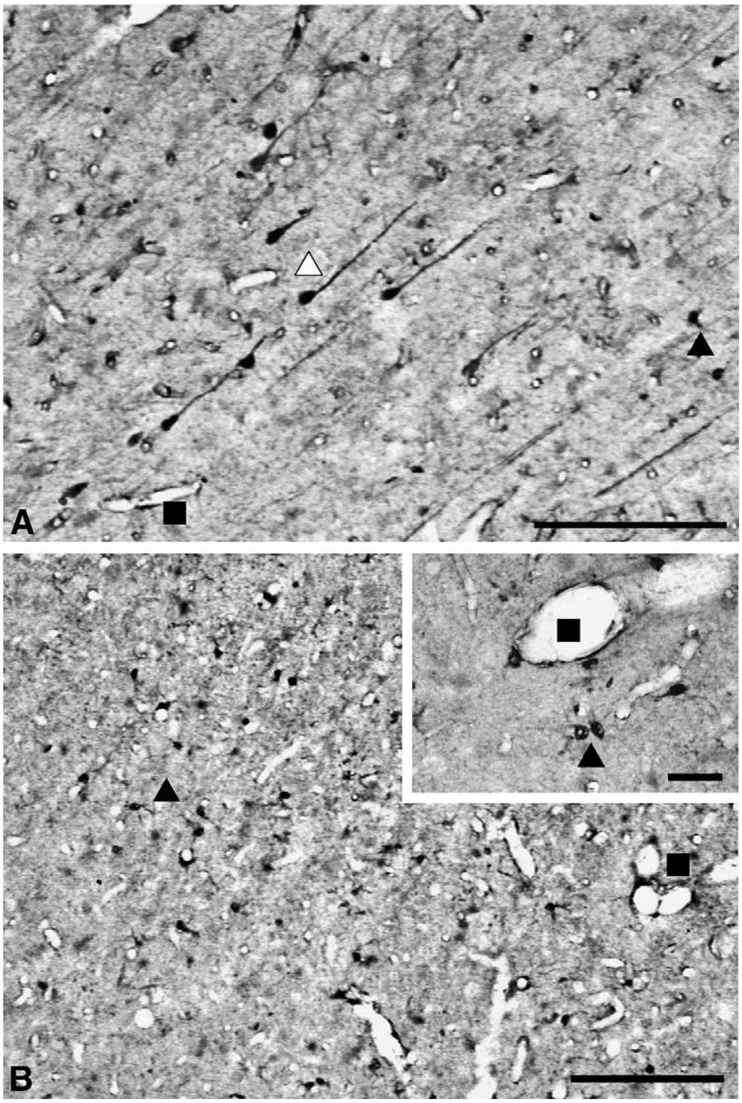

Fig. 1. GSTA4 immunoreactivity (IR) in the fronto-parietal cortex of young (A) and aged (B) rats. Insert in B shows a detail of GSTA4 immunostaining in astrocytes and structures surrounding blood vessels. Black triangles, GSTA4-IR astrocytes; white triangles, GSTA4-IR pyramidal neurons; black squares, GSTA4-IR structures surrounding blood vessels. Bar scale: A and B 100 μm, insert in B 20 μm.

send their immunoreactive non-ramified apical processes towards the superficial layers (Fig. 1).

In sections from cerebellum of all ages, GSTA4 was detected mainly in astrocytes located in Purkinje and granular layers of the cortex and in the white matter. Moreover, GSTA4-immunostained Bergmann radial glia cells were found around the Purkinje neurons but only in adult and aged rats. In the oldest animals, these glial cells presented irregular outlines and varicose processes (Fig. 2).

In all experimental groups, GSTP1-positive oligodendrocytes were detected throughout the thickness of the cortex although in layer I their number was very low. Immunoreactive

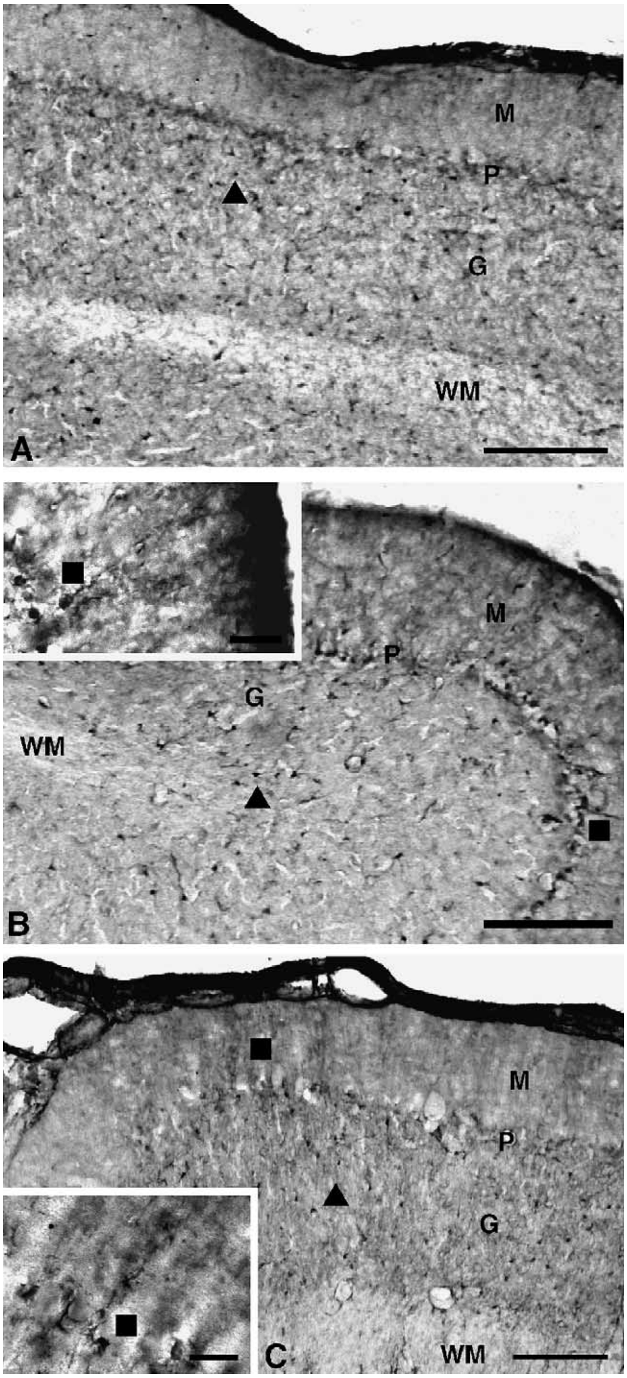

Fig. 2. GSTA4-IR in the cerebellum of young (A), adult (B) and aged (C) rats. Inserts in B and C show the GSTA4-IR Bergmann radial glia (BRG) in adult and aged rats, respectively. Black triangles, GSTA4-IR astrocytes; black squares, GSTA4-IR BRG; M, molecular layer; P, Purkinje layer; G, granular layer; WM, white matter. Bar scale: A, B and C 100 μm, inserts in B and C 20 μm.

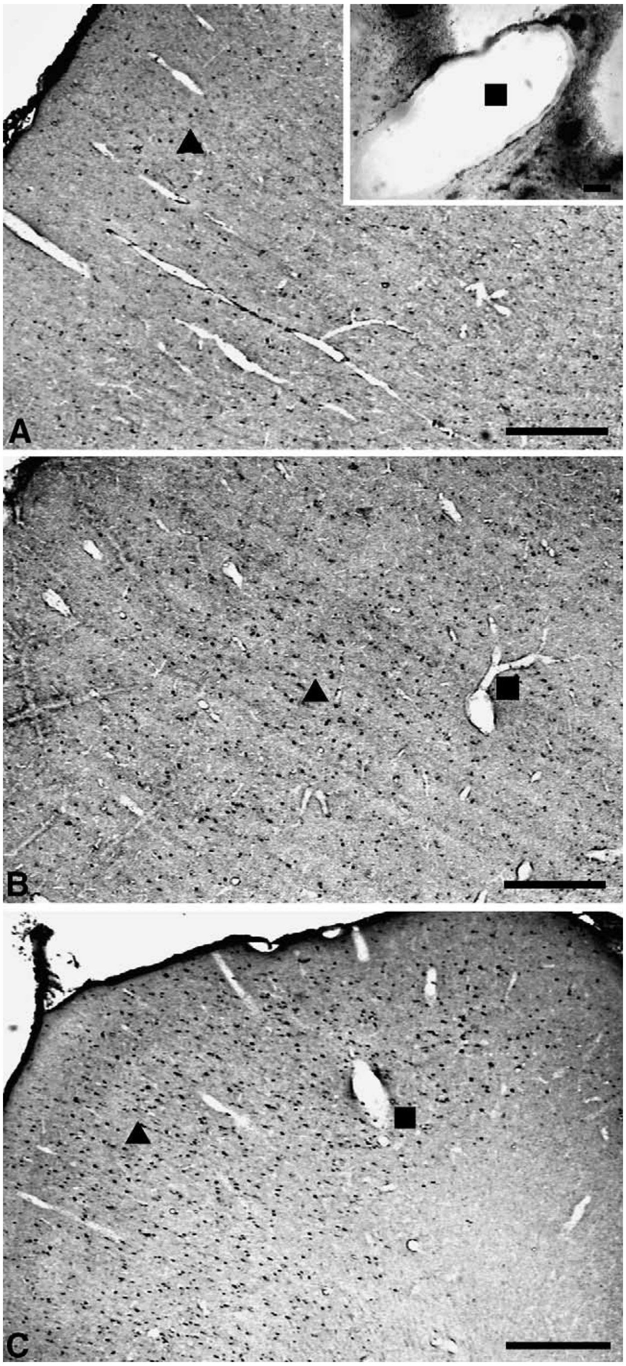

Fig. 3. GSTP1-IR in the frontal cortex of young (A), adult (B) and aged (C) rats. Insert in A corresponds to a semi-thin section showing perivascular GSTP1-IR cells. Black triangles, GSTP1-IR oligodendrocytes; black squares, GSTP1-IR perivascular cells. Bar scale: A, B and C 100 μm, insert in A 10 μm.

structures corresponding to perivascular cells also appeared in the blood vessels (Fig. 3). In addition, an apparent age-dependent increase in the number of immunoreactive structures became especially patent in the II–III cortical layers of the oldest animals (Fig. 3).

In the cerebellum, GSTP1 was detected in oligodendrocytes from Purkinje and granular layers of the cerebellar cortex as well as in white matter in all the experimental groups. In fact, the molecular layer only occasionally presented immunoreactive cells. Reactive fibers in the white matter, corresponding to oligodendrocyte sheets, were also detected in all experimental groups. In the oldest animals, the immunostaining was particularly predominant in the Purkinje cell layer (Fig. 4).

3.2. Enzymatic assays

The levels of GST activity in the cerebral cortex and cerebellum of young, adult and aged rats measured with the GST universal substrate CDNB, and the GSTA4 specific substrate HNE, are presented in Table 1. GSTP1 activity was not measured, due to the absence of a substrate specific enough for this isoenzyme. The GST activity towards CDNB was always significantly higher in adult and aged rats compared to young ones. Nevertheless, no statistical differences appeared in the comparison between adult and aged rats. Using HNE as the substrate, GST activity increased with age in the cerebral cortex. In the cerebellum, no significant variations in GST activity towards HNE

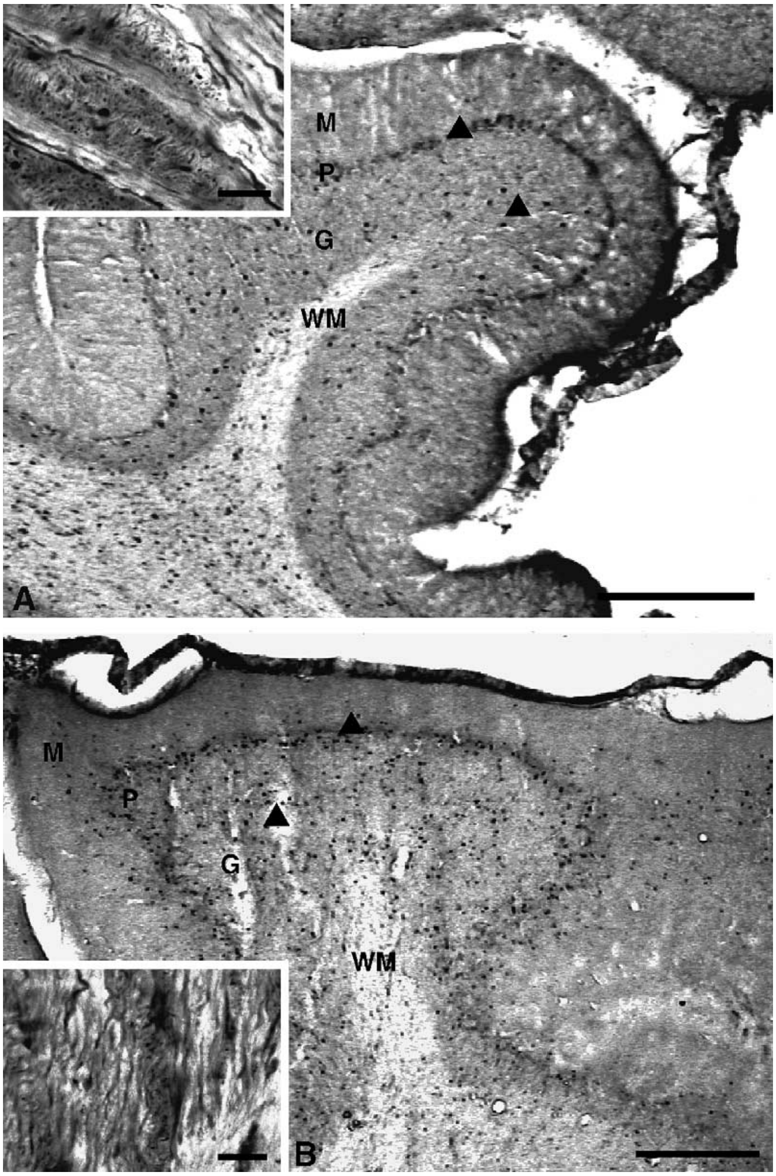

Fig. 4. GSTP1-IR in the cerebellum of young (A) and aged (B) rats. Inserts in A and B show GSTP1-IR fibers in the white matter. Black triangles, GSTP1-IR oligodendrocytes; M, molecular layer; P, Purkinje layer; G, granular layer; WM, white matter. Bar scale: A and B 200 μm, inserts in A and B 20 μm.

Table 1
Brain GST activities in young, adult and aged rats

Experimental group	GST specific activity (U/mg)			
	CDNB		HNE	
	Cerebral cortex	Cerebellum	Cerebral cortex	Cerebellum
Young	0.155 ± 0.016	0.143 ± 0.023	0.127 ± 0.012	0.1028 ± 0.060
Adult	0.271 ± 0.018[b]	0.186 ± 0.015[a]	0.190 ± 0.012[a]	0.1706 ± 0.033
Aged	0.247 ± 0.030[b]	0.190 ± 0.012[a]	0.263 ± 0.001[b,c]	0.1913 ± 0.012

One unit of enzyme activity is defined as 1 μmol of GSH–CDNB or GSH–HNE conjugate formed per min. Data are the means (±S.D.) of five determinations. *P*: *P*-value.

[a] Activity significantly greater than in young experimental group ($P < 0.01$).

[b] Activity significantly greater than in young experimental group ($P < 0.001$).

[c] Activity significantly greater than in adult experimental group ($P < 0.01$).

were found, although the activity tended to increase with age.

Comparing GST activity towards CDNB in the cerebral cortex versus cerebellum, we found no differences between the young groups, although this activity in the cerebral cortex of adult ($P < 0.001$) and aged ($P < 0.02$) rats was significantly higher than in the cerebellum; however, these differences were not detected in young rats. Moreover,

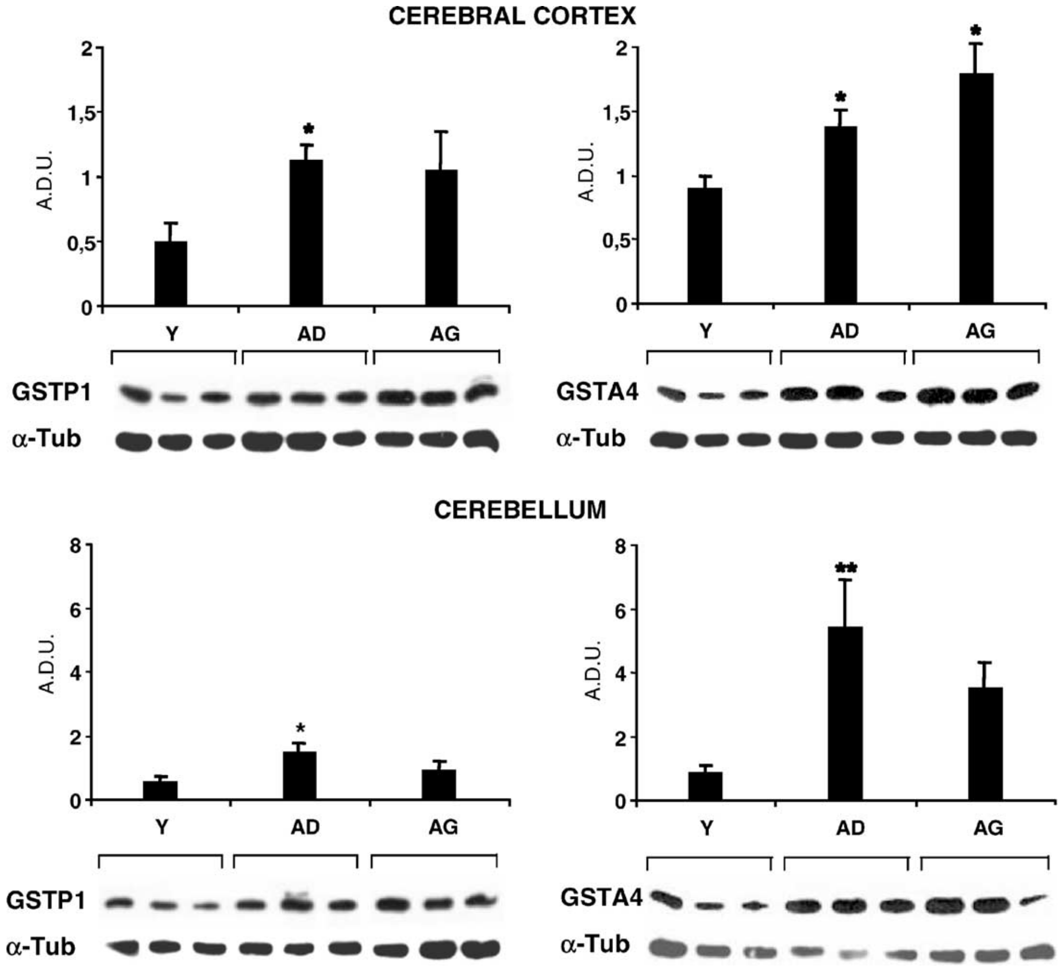

Fig. 5. GSTP1 and GSTA4 protein expression in cerebral cortex and cerebellum with aging. The top panel is a representative Western blot analysis of GSTP1 and GSTA4 protein in the cerebral cortex of 3 young (Y), 3 adult (AD) and 3 aged (AG) rats. The lower panel represents GSTP1 and GSTA4 protein expression in the cerebellum of the same experimental groups. Densitometric quantifications (A.D.U.) are shown above each blot; results are average values of five experimental animals. *Protein expression significantly greater than in young experimental group ($P < 0.01$). **Protein expression significantly greater than in young experimental group ($P < 0.02$).

HNE-conjugating activity exhibited this same behavior only in the oldest group ($P < 0.01$).

3.3. Western blot

Western blot analysis (Fig. 5) showed a significant increase in GSTA4 and GSTP1 protein level in the cerebral cortex of adult and aged rats compared to young animals. In addition, despite no significant statistical differences between GSTA4 expression in the cerebral cortex of adult and aged rats, a trend to increase appeared from the adult to the aged group.

A higher GSTA4 and GSTP1 protein level was found in the cerebellum (Fig. 5) of adult rats compared to the young ones. Nevertheless, the expression of both GSTs decreased in the cerebellum of aged rats, although not statistically significant, compared to the adult stage. Finally, the concentration of GSTA4 in the cerebellum was higher than in the cerebral cortex, regardless of the age of the animal. In contrast, no regional differences in the GSTP1 expression level were observed (results not shown).

4. Discussion

In the rat cerebral cortex and cerebellum, CDNB-conjugating assay indicated an increase in the total GST activity related to the maturation process, but no differences between the adult and aged stages were found. In this sense, although the present data imply age-related changes in the cerebrum, studies elsewhere are scant and irregular [15,18,29]. In fact, these studies showed rising GST activity until 12 months, followed by a decline with aging; however, experimental animals were not older than 24 months. Our results also support the contention that brain regions differ with respect to total cytosolic GST activity with age. In young animals (2–3 months old), in agreement with our results, no differences between cerebral cortex and cerebellum activities have been reported [15,26]. Nevertheless, aging promotes different regional changes, evident in our observations of greater GST activity in cerebral cortex compared to cerebellum of adult and aged animals but not in the young ones.

With the exception of Theta-class isoenzymes [21,22] all the other GSTs conjugate the substrate CDNB to GSH, so that the evaluation of GST activity exclusively with this substrate could mask possible changes in the activity of individual isoenzymes. In this way, we have examined not only the activity with CDNB but also with HNE as well as the expression and tissue distribution in cerebral cortex and cerebellum of two GST isoenzymes (GSTA4 and GSTP1) belonging to different GST classes expressed mainly in glial cells.

Glial cells contribute to the modulation and development of age-related degenerative or regenerative processes. In fact, in the CNS, not only neurons but also glia are continuously exposed to ROS, and each glial type differs in susceptibility to certain conditions believed to involve oxidative stress, such as aging [16,23].

Our GSTP1 Western blot and immunocytochemical analysis in cerebral cortex showed increasing expression levels of this isoenzyme from young to adult animals. In relation to GSTP1 cell location, as has been reported in young and adult animals [11,26,53], we found immunoreactivity in the oligodendrocytes irrespective of the age of the animals. In addition, we have also noted immunostained perivascular structures with similar patterns in all the experimental groups, suggesting that GST may control the uptake and release of various compounds as well as the removal of toxins and harmful metabolic by-products [1–33].

Among the glial cells, oligodendrocytes are extremely sensitive to oxidative stress [23], apparently due to their lower amount of GSH and high concentrations of iron [27,28]. GSTP1, located primarily in these glial cells, has been reported in young rats as the principal GST isoenzyme expressed in the CNS [26]; on the other hand, an unchanged number of oligodendrocytes has been described in the cerebral cortex of aged rats [42]. Consequently, taking into account the present data concerning the GSTP1 expression in the CNS of the oldest rats, we can conclude that this defense system is maintained with aging.

Our immunocytochemical observations in the cerebral cortex showed no strong age-dependent changes in the cellular distribution of GSTA4, a GST isoenzyme expressed mainly in astrocytes [26]. However, the protein expression and the activity towards HNE, a highly specific substrate for this isoenzyme, clearly increased in the adult and aged rats with respect to the young ones. In addition, both parameters increased in the oldest animals respect to the adult ones. Despite that in young cerebral cortex the expression level of GSTA4 isoenzyme proved lower than that of other GST isoenzymes [26], our results reveal that in all experimental groups, even in the young, this activity is considerably high. These results support the proposal that astrocytes help defend the brain against ROS, since they contain higher levels of various antioxidants than do the other nerve cell types [16]. After an oxidative process, the neuroprotective role of astrocytes may be accentuated because of increases in a number of their activities, such as the expression of antioxidant enzymes, transport and metabolism of glucose or synthesis of glutathione [57]. In addition, in the cerebral cortex of aged rats, greater numbers and more severe hypertrophy of astrocytes have been reported [42]. In fact, GSTA4 and, to a lesser extent, GSTP1 are involved in the detoxification of the highly cytotoxic endogenous α–β-unsaturated aldehydes (4-hydroxyalkenals) formed during lipid peroxidation [3,6,14,17,50,58]. Moreover, α–β-unsaturated aldehydes mediate the inducible expression of GSTA4 through an antioxidant response element (ARE) [54] and redox cycling compounds regulate the expression of GSTP1 in rat neurons and glia through an antioxidant/electrophile responsive element (ARE/EpRE) [2].

In the cerebellum, we have found GSTA4 positive astrocytes and GSTP1 immunoreactive oligodendrocytes predominantly in Purkinje and granular layers but also in the white matter. These locations are consistent with the distribution of GSH in the cerebellum in young rats [47]. However, we have discerned a change in the GSTP1 distribution pattern with aging, given that in the oldest rats the immunoreactive oligodendrocytes increased, particularly in the Purkinje cell layer. In relation to GSTA4, the only significant immunohistochemical difference with aging was the appearance of positive Bergmann radial glia next to Purkinje cells in adult and aged rats. The expression level of GSTP1 and GSTA4 quantified in cerebellum by Western blot exhibited a maturation-related increase followed by a non-significant statistical decline with aging. However, GSTA4 activity was similar in adult and aged rats, suggesting a higher activity of the GSTA4 protein in the cerebellum of the oldest animals.

Cerebellar Purkinje cells are highly sensitive to oxidative stress [5,12,41]. In fact, the major site affected by most degenerative cerebellar diseases is the Purkinje cells layer. Moreover, it should be noted that Purkinje cells soma are surrounded by basket cells, and their processes by stellate and granular cells, which are abundant in nitric oxide synthase enzyme. Nitric oxide (NO) production in the cerebellum has been strongly correlated with cerebellum degeneration [52]. In fact, in immunocytochemical studies, our group has found increasing NO production in the cerebellum with aging [7]. Consequently, strengthened defense mechanisms such as the GST system should be expected.

Thus, the present results support the proposal that GST undergoes age-dependent changes in its isoenzymatic expression pattern in the rat cerebral cortex and the cerebellum as part of an adaptive response to the aging phenomenon.

Acknowledgments

This work was supported by the Dirección General de Investigación Científica y Técnica (PM98-016-CO2-02) and Junta de Andalucía (CVI-0184). The authors are indebted to Dr. Hayes (University of Dundee) for providing the GSTA4 antibody.

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