

Article

## Brain Activity of Thioctic Acid Enantiomers: *In Vitro* and *In Vivo* Studies in an Animal Model of Cerebrovascular Injury

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**Abstract:** Oxidative stress is an imbalance between the production of free radicals and antioxidant defense mechanisms, potentially leading to tissue damage. Oxidative stress has a key role in the development of cerebrovascular and/or neurodegenerative diseases. This phenomenon is mainly mediated by an enhanced superoxide production by the vascular endothelium with its consequent dysfunction. Thioctic, also known as alpha-lipoic acid (1,2-dithiolane-3-pentanoic acid), is a naturally occurring antioxidant that neutralizes free radicals in the fatty and watery regions of cells. Both the reduced and oxidized forms of the compound possess antioxidant ability. Thioctic acid has two optical isomers designated as (+)- and (−)-thioctic acid. Naturally occurring thioctic acid is the (+)-thioctic acid form, but the synthetic compound largely used in the market for stability reasons is a mixture of (+)- and (−)-thioctic acid. The present study was designed to compare the antioxidant activity of the two enantiomers *versus* the racemic form of thioctic acid on

hydrogen peroxide-induced apoptosis in a rat pheochromocytoma PC12 cell line. Cell viability was evaluated by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay and free oxygen radical species (ROS) production was assessed by flow cytometry. Antioxidant activity of the two enantiomers and the racemic form of thioctic acid was also evaluated in spontaneously hypertensive rats (SHR) used as an *in vivo* model of increased oxidative stress. A 3-h exposure of PC12 cells to hydrogen peroxide ( $H_2O_2$ ) significantly decreased cell viability and increased levels of intracellular ROS production. Pre-treatment with racemic thioctic acid or (+)-enantiomer significantly inhibited  $H_2O_2$ -induced decrease in cell viability from the concentration of 50  $\mu\text{mol/L}$  and 20  $\mu\text{mol/L}$ , respectively. Racemic thioctic acid and (+)-salt decreased levels of intracellular ROS, which were unaffected by (–)-thioctic acid. In the brain of SHR, the occurrence of astrogliosis and neuronal damage, with a decreased expression of neurofilament 200 kDa were observed. Treatment of SHR for 30 days with (+)-thioctic acid reduced the size of astrocytes and increased the neurofilament immunoreaction. The above findings could contribute to clarify the role played by thioctic acid in central nervous system injury related to oxidative stress. The more pronounced effect of (+)-thioctic acid observed in this study may have practical therapeutic implications worthy of being investigated in further preclinical and clinical studies.

**Keywords:** oxidative stress; thioctic acid; brain; spontaneously hypertensive rats; PC12 cells

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## 1. Introduction

Free radical (e.g.,  $\cdot\text{OH}$ ) generation following stroke and traumatic brain injury has been documented [1–3]. Reactive oxygen species (ROS) such as  $\cdot\text{OH}$  can initiate free radical chain reactions which can lead to oxidation of cellular constituents and, ultimately, to cell death. The occurrence of lipid peroxidation was shown in animal models of both global and focal ischemia [4], in which an increase of conjugated dienes and aldehydes derived from oxidized lipids have been reported [5,6]. Pentane evolved from lipid peroxidation has been also detected in the expired breath of gerbils following global ischemia [7].

Free radical involvement in neurodegeneration following stroke is also facilitated by antioxidant defenses breakdown. Both ischemic [8] and traumatic [9] central nervous system (CNS) injuries result in the loss of  $\alpha$ -tocopherol. Decreases in both ascorbic acid and glutathione have been described in animal models of CNS injury. Antioxidants of natural and/or synthetic origin were also proposed as neuroprotective agents in CNS [10–12].

Thioctic or alpha-lipoic acid, (1,2-dithiolane-3-pentanoic acid), is a naturally occurring dithiol compound synthesized enzymatically in the mitochondrion from octanoic acid. The compound is a necessary cofactor for mitochondrial  $\alpha$ -ketoacid dehydrogenases, and therefore has a critical role in mitochondrial energy metabolism. It is synthesized in the organism and is adsorbed intact from dietary sources. After adsorption, it accumulates transiently in many tissues. There is growing evidence that orally supplied thioctic acid may not be used as a metabolic cofactor but instead, it elicits a unique set

of biochemical activities with potential therapeutic value against different pathophysiological insults. Thiocctic acid has been proposed as a potent biological antioxidant and a detoxification agent for treating diabetic neuropathy, improving age-associated cardiovascular, cognitive, and neuromuscular deficits, and as a modulator of various inflammatory signaling pathways [13]. The pharmacology of thiocctic acid and its role as a biological antioxidant and neuroprotectant and its function in liver metabolism and disease were reviewed [14–17].

Due to the presence of an asymmetric carbon C3, thiocctic acid exists in two enantiomers, namely (+)- and (–)-thiocctic acid. Analysis of bacterial and mammalian pyruvate dehydrogenase (PDH) complexes has shown that the natural cofactor of the complex is the (+)-enantiomer. Moreover, (–)-thiocctic acid acts either as a poor substrate or as an inhibitor of (+)-thiocctic acid when it interacts with 2-oxoacid dehydrogenase multienzyme complexes. Both (+)- and (–)-thiocctic acid are reduced intracellularly via two enzymatic pathways. (+)-thiocctic acid is reduced by dihydrolipoamide dehydrogenase (the E3 enzyme in the PDH complex), whereas (–)-thiocctic acid is reduced by glutathione reductase [18,19].

An interesting *in vitro* model of oxidative stress and its pharmacologic treatment is given by PC12 cells: a rat pheochromocytoma cell line as a model of dopaminergic neurons, which produces catecholamines [20].

Rat strains with genetically inherited hypertension were developed 50 years ago. The spontaneously hypertensive rat (SHR) is probably the model most extensively studied. It has specific and uniform genetic predisposition to develop arterial hypertension [21], allowing to study causes, mechanisms, pathology and behavioral consequences of the disease. Arterial hypertension also represents an important cause of oxidative stress. In fact, it could be considered as a free radical generating source [22] and, therefore, SHR can be used as an animal model of oxidative stress. Hypertension-dependent organ damage was demonstrated [21,23–25]. These studies clearly evidenced that different organs of SHR (e.g., kidney, heart and brain), undergo to hypertension-related ROS-dependent damage [26]. Hence, SHR may be useful to study the effect of any antioxidant compound.

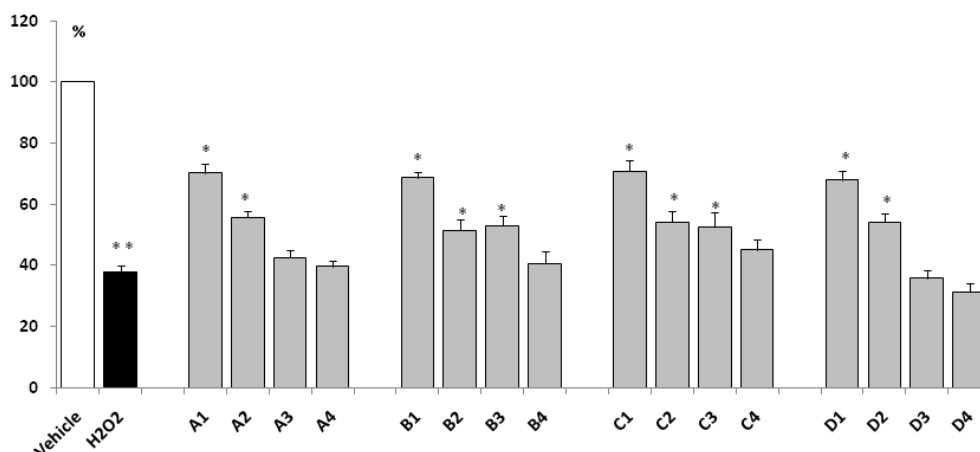
This study was designed to investigate the possible neuroprotective effect of thiocctic acid and its enantiomers both *in vitro* and *in vivo* using different techniques such as flow cytometry, immunohistochemistry, and morphologic analysis.

## 2. Results

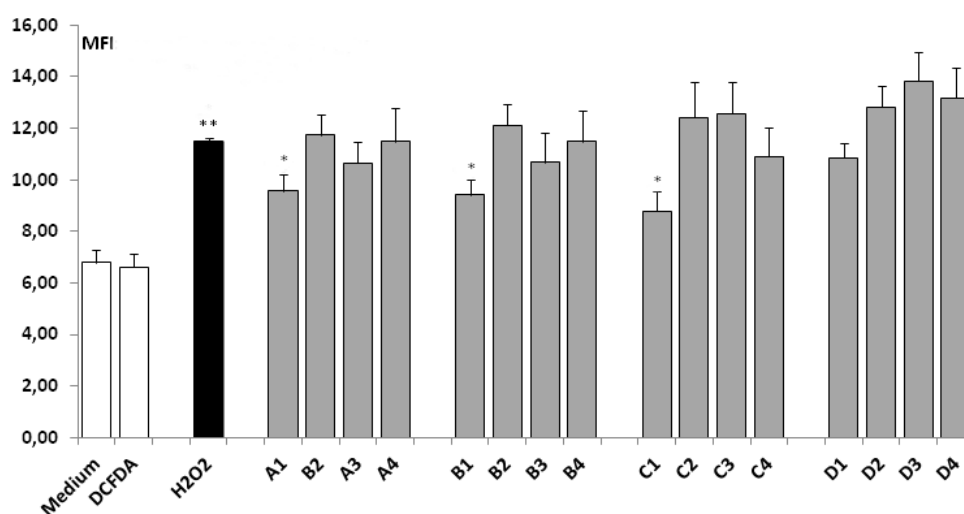
### 2.1. In Vitro Experiments

As evaluated by the MTT test, treatment with H<sub>2</sub>O<sub>2</sub> decreased the cell viability of PC12 cells by approximately 60%. Pretreatment with (+)-thiocctic acid increased cell viability at the 20 µmol/L concentration. The same parameter was sensitive to (+/–)-thiocctic acid concentrations from 50 to 100 µmol/L (Figure 1). The ability of thiocctic acid to reduce ROS was measured in PC12 cells treated with 200 µmol/L H<sub>2</sub>O<sub>2</sub> using DCFDA and flow cytometric analysis. As expected, H<sub>2</sub>O<sub>2</sub> induced a twofold increase of DCFDA fluorescence after 3 h. Pre-treatment with 100 µmol/L (+/–)-thiocctic acid or (+)-thiocctic acid for 18 h strongly decreased H<sub>2</sub>O<sub>2</sub>-induced DCFDA fluorescence intensity. No effects were found using (–)-thiocctic acid (Figure 2) or vehicle (data not shown).

**Figure 1.** MTT analysis of PC12 cell viability after different treatments: Vehicle (H<sub>2</sub>O or DMSO), H<sub>2</sub>O<sub>2</sub> (hydrogen peroxide, 200 μmol/L), **A** ((+/-)-thioctic acid), **B** ((+)-thioctic acid, piperazine salt), **C** ((+)-thioctic acid, lysine salt), and **D** ((-)-thioctic acid). The Arabic numbers (1, 2, 3 and 4) close to the letters represent the different concentrations (100, 50, 20 and 10 μmol/L, respectively) for every treatment. The data were expressed as the percentage of viable cells and were the mean of ± SE of three different experiments. \*\* *p* < 0.05 vs. vehicle alone ; \* *p* < 0.05 vs. H<sub>2</sub>O<sub>2</sub> only.



**Figure 2.** MTT analysis of PC12 cell viability after different treatments: Medium, DCFDA (2',7'-dichlorofluorescein diacetate) H<sub>2</sub>O<sub>2</sub> (200 μmol/L), **A** ((+/-)-thioctic acid), **B** ((+)-thioctic acid, piperazine salt), **C** ((+)-thioctic acid, lysine salt), and **D** ((-)-Thioctic acid). The Arabic numbers (1, 2, 3 and 4) close to the letters represent the different concentrations (100, 50, 20 and 10 μmol/L, respectively) for every treatment. The data were expressed as the mean of fluorescence intensity and were the mean of ± SE of three different experiments. \*\* *p* < 0.05 vs. medium or DCFDA alone; \* *p* < 0.05 vs. H<sub>2</sub>O<sub>2</sub> only.



## 2.2. In Vivo Experiments

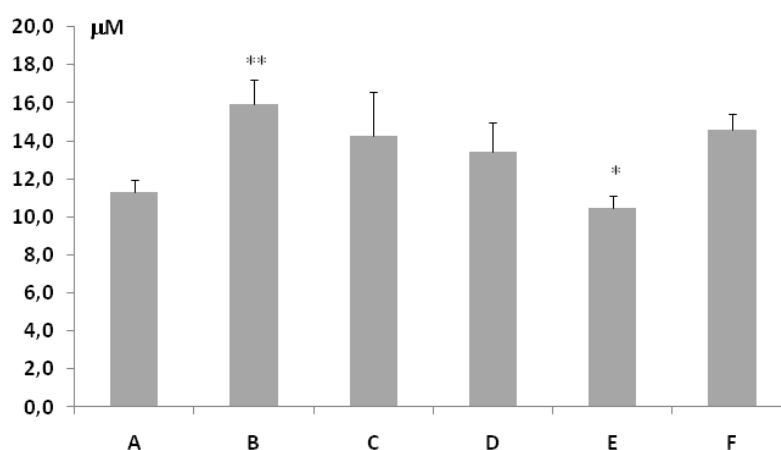
Body weight values were similar in normotensive WKY or SHR, both in the control or those treated with different formulations of thioctic acid. Brain weight values were lower in SHR for both controls

and those treated compared to normotensive WKY rats (data not shown). Systolic blood pressure values averaged  $156 \pm 6$  mmHg in WKY rats ( $n = 6$ ) and  $209 \pm 7$  mmHg in control SHR ( $n = 6$ ,  $p < 0.01$  vs. WKY rats). Treatment with any formulation of thiocctic acid did not affect significantly blood pressure values in SHR (data not shown).

### 2.3. Plasma Analysis

Thiobarbituric acid reactive substances (TBARS) levels increased in the plasma of SHR rats compared to WKY rats, thereby indicating an increase of oxidative stress in this animal model (Figure 3). Only (+)-thiocctic acid treatment decreased the TBARS value significantly.

**Figure 3.** TBRAS levels in plasma, expressed as  $\mu\text{M}$  of malonyldialdeide, of WKY control rats (A), control SHRs (B), SHR treated with (+/-)-thiocctic acid 250  $\mu\text{mol/kg/day}$  (C), SHR treated with (+/-)-thiocctic acid 125  $\mu\text{mol/kg/day}$  (D), SHR treated with (+)-thiocctic acid 125  $\mu\text{mol/kg/day}$  (E), and SHR treated with (-)-thiocctic acid 125  $\mu\text{mol/kg/day}$  (F). \*\*  $p < 0.05$  vs. WKY control rats; \*  $p < 0.05$  vs. SHR control rats.



### 2.4. Cerebral Cortex and Hippocampus Immunohistochemistry

The results of image analysis of frontal cortex and hippocampus are summarized in Table 1. In control SHR, a significant increase in the size of glial fibrillary acidic protein (GFAP) immunoreactive astrocytes was observed (Table 1, Figures 4 and 5). This phenomenon was more pronounced in the CA1 subfield and in dentate gyrus and, to a lesser extent, in the frontal cortex and in the CA3 subfield in the order (Table 1). In WKY rats, astrocytes were apparently normal and only few hypertrophic elements were observed (Figures 3A and 4A). In SHR, the presence of hypertrophic elements characterized by hyper-reactive astrocytes (H/R) and hypertrophic/ hyper-immunoreactive astrocytes (H/H) were observed (Figures 4B and 5B). In the frontal cortex, clusters of H/R and H/H elements were observed in zone IV near the corpus callosum, where the astrocytes presented a higher number and length of cellular processes compared to WKY rats (Figure 4B). In the hippocampus of SHR, there was a higher number of H/H elements evident compared to WKY (Figure 5B). Astrocytes displayed a marked GFAP immunoreaction in the cell body and an increased number of arborizations.

**Table 1.** Size of GFAP-immunoreactive astrocytes in the frontal cortex and hippocampus of the different animal groups investigated.

	Frontal Cortex Grey matter	Frontal cortex White matter	Hippocampus CA1 subfield	Hippocampus CA3 subfield	Dentate gyrus
Mean of immunoreaction areas of astrocytes					
WKY Control	100.2 ± 11.6	75.9 ± 7.8	126.3 ± 16.9	149.9 ± 9.9	102.1 ± 2.8
SHR Control	129.1 ± 5.1 †	98.8 ± 5.8 †	172.3 ± 9.5 †	145.3 ± 5.7	161.6 ± 11.4 †
SHR (+/-)	132.4 ± 5.2	83.2 ± 3.3 ‡	153.8 ± 15.9 ‡	153.4 ± 14.1	177.4 ± 4.3
250 µmol/kg/day					
SHR (+/-)	141.6 ± 11.6	98.5 ± 6.4	210.1 ± 13.4	150.5 ± 12.6	168.4 ± 19.5
125 µmol/kg/day					
SHR (+)	118.9 ± 9.3 ‡	88.6 ± 5.9 ‡	130.2 ± 5.9 ‡	154.6 ± 14.1	173.6 ± 18.4
125 µmol/kg/day					
SHR (-)	134.1 ± 8.1	130.6 ± 11.6	178.6 ± 12.1	150.1 ± 13.8	175.9 ± 9.7
125 µmol/kg/day					

Mean of immunoreactions areas of astrocytes is expressed in micron square meter ( $\mu\text{m}^2$ ). Data are mean  $\pm$  SE; †  $p < 0.05$  vs. WKY control rats; ‡  $p < 0.05$  vs. SHR control rats.

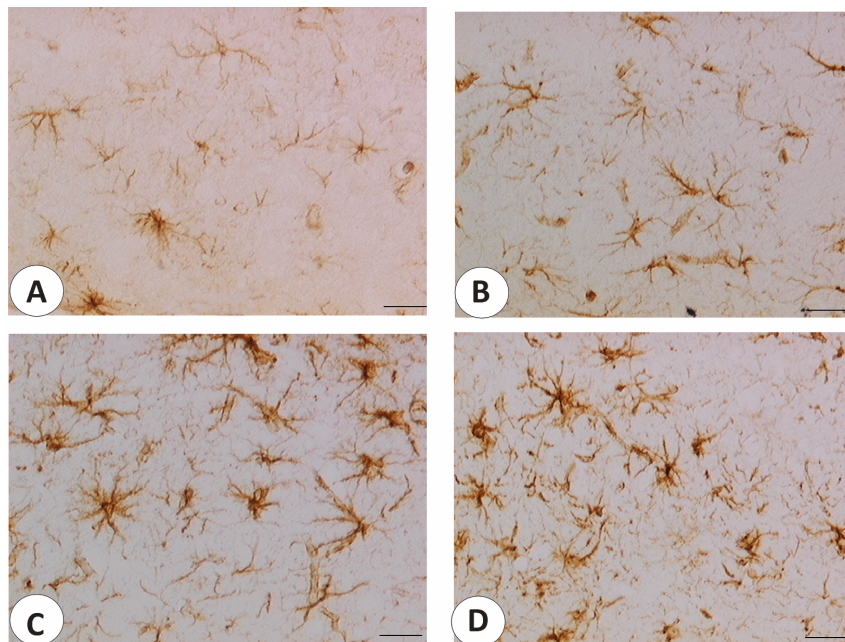
**Figure 4.** Sections of the frontal cortex (grey matter) processed for the immunohistochemical demonstration of glial fibrillary acidic protein (GFAP) in control WKY rats (A), control SHRs (B), SHR treated with (+/-)-thioctic acid 125 µmol/kg/day (C), SHR treated with (+/-)-thioctic acid 250 µmol/kg/day (D), SHR treated with (+)-thioctic acid 125 µmol/kg/day (E), and SHR treated with (-)-thioctic acid 125 µmol/kg/day (F). Calibration bar: 25 µm.

Figure 4. Cont.

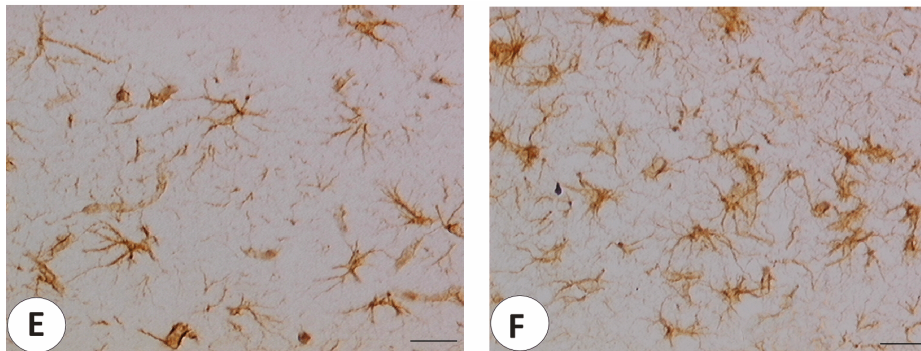
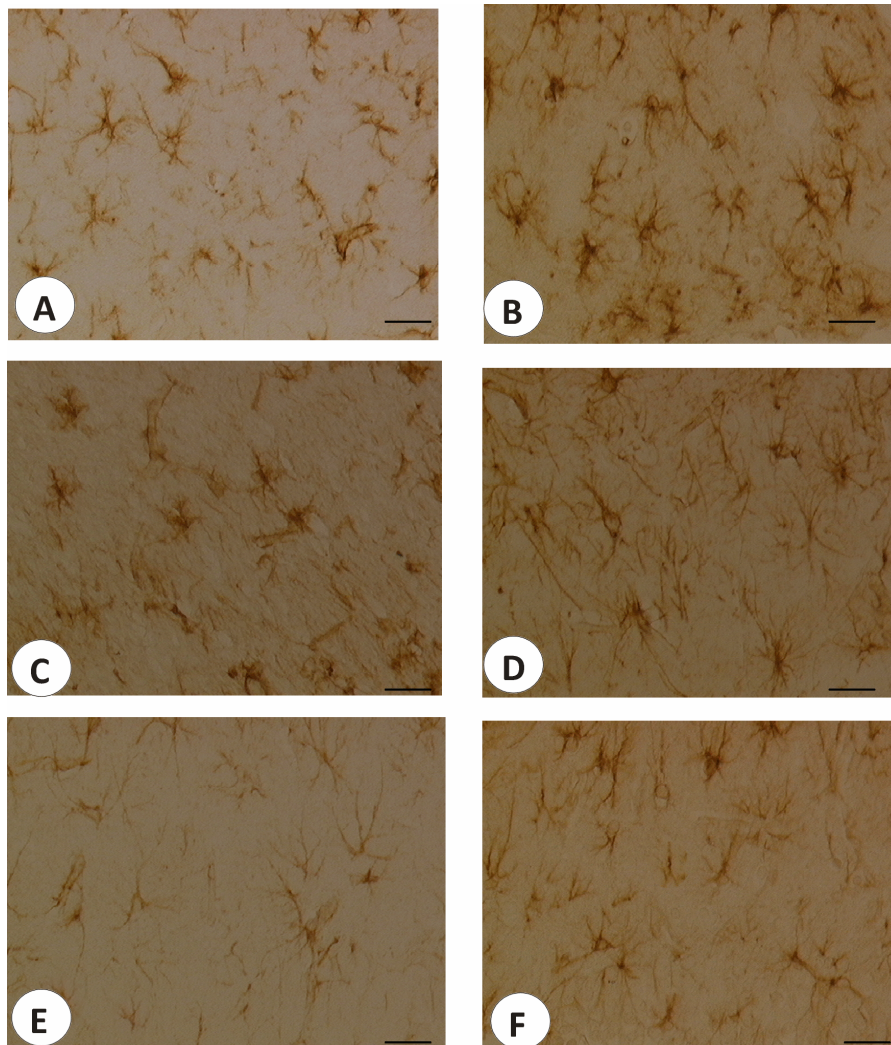
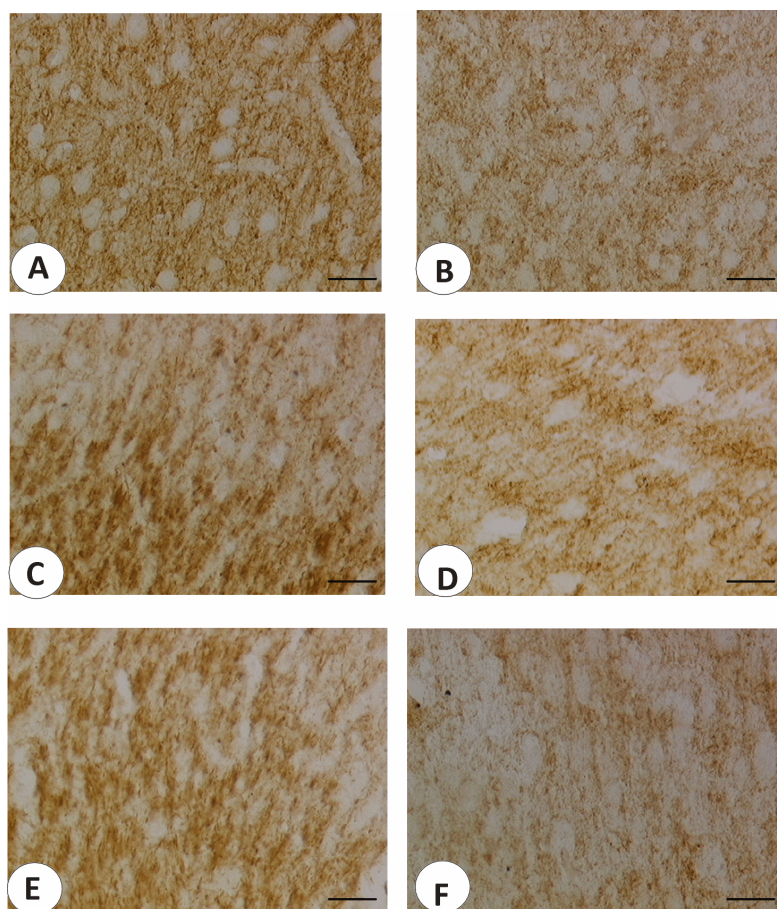


Figure 5. Sections of the CA1 subfield (stratum radiatum) processed for the immunohistochemical demonstration of glial fibrillary acidic protein (GFAP) in control WKY rats (A), control SHRs (B), SHR treated with (+/-)-thioctic acid 125 µmol/kg/day (C), SHR treated with (+/-)-thioctic acid 250 µmol/kg/day (D), SHR treated with (+)-thioctic acid 125 µmol/kg/day (E), and SHR treated with (-)-thioctic acid 125 µmol/kg/day (F). Calibration bar: 25 µm.



Treatment with (+/-)-thioctic acid (250  $\mu\text{mol/kg/day}$ ) (Table 1, Figures 4D and 5D) and, to a greater extent, with (+)-thioctic acid (125  $\mu\text{mol/kg/day}$ ) (Table 1, Figures 4E and 5E) countered the volume increase of GFAP-immunoreactive astrocytes. (-)-Thioctic acid (125  $\mu\text{mol/kg/day}$ ) did not affect astroglial reaction (Table 1, Figures 3F and 4F). Treatment with (+)-thioctic acid (125  $\mu\text{mol/kg/day}$ ) (Figures 4E and 5E) but not with (+/-)-thioctic acid at the same concentration (Figures 4C and 5C) decreased the number of H/R and H/H elements, both in the frontal cortex and in the CA1 subfield. Neurofilament 200 kDa protein (NFP) immunoreactivity was localized in nerve fiber-like structures within the frontal cortex (Figure 6) and hippocampus (data not shown). Quantitative image analysis revealed in the frontal cortex and, to a lesser extent, in the hippocampus a decrease of NFP-immunoreactive structures in SHR, as compared to WKY rats (Figure 6 and Table 2). This loss was countered by treatment with (+)-thioctic acid (125  $\mu\text{mol/kg/day}$ ) but not by treatment with other forms of thioctic acid used (Figure 6 and Table 2).

**Figure 6.** Sections of the frontal cortex (Zone 2) processed for the immunohistochemical demonstration of neurofilament phosphorylated 200 kDa in control WKY rats (**A**), control SHRs (**B**), SHR treated with (+/-)-thioctic acid 125  $\mu\text{mol/kg/day}$  (**C**), SHR treated with (+/-)-thioctic acid 250  $\mu\text{mol/kg/day}$  (**D**), SHR treated with (+)-thioctic acid 125  $\mu\text{mol/kg/day}$  (**E**), and SHR treated with (-)-thioctic acid 125  $\mu\text{mol/kg/day}$  (**F**). Calibration bar: 25  $\mu\text{m}$ .





**Table 2.** Neurofilament 200 kDa immunoreactivity, in the frontal cortex and hippocampus of the different animal groups investigated.

	Frontal cortex Zone 1	Frontal cortex Zone 2	Hippocampus CA1 subfield	Hippocampus CA3 subfield	Dentate gyrus
NFP200 kDa immunoreaction density					
WKY Control	29.2 ± 2.3	45.6 ± 2.1	40.3 ± 2.8	37.4 ± 1.9	41.5 ± 1.3
SHR Control	15.1 ± 0.4 †	28.84 ± 0.3 †	30.3 ± 1.3 †	36.5 ± 2.3	31.8 ± 1.1 †
SHR (+/–) 250 µmol/kg/day	25.8 ± 1.3 ‡	31.4 ± 0.7 ‡	35.3 ± 2.1 ‡	33.5 ± 1.9	36.8 ± 2.1 ‡
SHR (+/–) 125 µmol/kg/day	18.9 ± 0.7	24.1 ± 1.2	29.3 ± 2.1	35.5 ± 2.2	30.8 ± 2.1
SHR (+) 125 µmol/kg/day	23.9 ± 1.5 ‡	39.2 ± 0.8 ‡	36.3 ± 1.9 ‡	38.5 ± 2.1	38.2 ± 2.1 ‡
SHR (–) 125 µmol/kg/day	16.8 ± 1.3	25.9 ± 1.2	31.3 ± 2.3	37.5 ± 2.9	29.8 ± 1.7

Neurofilament immunoreactivity. Values are expressed in arbitrary units calculated microdensitometrically as detailed in the materials and methods section. Data are mean ± SE; †  $p < 0.05$  vs. WKY control rats; ‡  $p < 0.05$  vs. SHR control rats.

### 3. Discussion

Oxidative stress is caused by an imbalance in the redox state of the cell, either by overproduction of the reactive oxygen species, or by dysfunction of the antioxidant systems. There are many different varieties of partially reduced ROS, including superoxide ( $O_2^{\bullet-}$ ), hydrogen peroxide ( $H_2O_2$ ), and the hydroxyl radical ( $OH^{\bullet}$ ). The current use of the term “ROS” includes both oxygen radicals and non-radicals that are easily converted into free radicals ( $O_3$ ,  $H_2O_2$ ,  $1O_2$ ) [27]. ROS have different degrees of reactivity, the hydroxyl radical  $OH^{\bullet}$  being one of the most reactive ROS. Due to their high reactive activity, ROS chemically interact with biological molecules, thus leading to changes in cell function and, ultimately, cell death. As a result, oxygen has the potential to be poisonous, and aerobic organisms can afford this potential damage because of the existence of antioxidant defenses [28].

Neurons and astrocytes, are largely responsible for the brain’s massive consumption of  $O_2$  and glucose. Although brain represents only ~2% of the total body weight, it accounts for more than 20% of the total consumption of oxygen [29]. Despite the essentiality of oxygen for living organisms, hyperoxia causes toxicity and neurotoxicity of nervous tissue [30]. Oxidative stress has been detected in several neurodegenerative diseases, and emerging evidence from *in vitro* and *in vivo* disease models suggests that oxidative stress may play a role in the pathophysiology of a variety of neurological disorders.

Pathophysiology of arterial hypertension involves complex interactions of multiple vascular effectors, including the activation of the sympathetic nervous system, of the renin-angiotensin-aldosterone system, and of inflammation mediators. Oxidative stress and endothelial dysfunction are commonly observed in hypertensive individuals [31], but increasing evidence suggests that they also have a causal role in the molecular processes leading to hypertension. ROS may directly alter vascular function or cause changes in vascular tone by several mechanisms, including altered nitric oxide (NO) bioavailability or

signaling [31]. ROS-producing enzymes involved in the increased vascular oxidative stress observed during hypertension include the NADPH oxidase, xanthine oxidase, the mitochondrial respiratory chain and an uncoupled endothelial NO synthase. Hypertension is also considered as a risk factor for the development of cognitive dysfunction, for its negative effects on cerebral vasculature and on blood flow [32]. In the elderly, it is a major risk factor for vascular cognitive impairment and vascular dementia [33–35].

SHR were developed as an animal model of genetic hypertension and are largely used for investigating causes, mechanisms and pathology of hypertension, as well as the influence of pharmacological treatments on the development and course of arterial hypertension [21,23,24]. This genetic model of hypertension exhibits enhanced NAD(P)H oxidase-mediated O<sub>2</sub> generation in resistance arteries (mesenteric), conduit vessels (aorta), and kidneys [26]. 8-Hydroxy-2'-deoxyguanosine, a marker for oxidative stress-induced DNA damage, and protein carbonylation, a marker for oxidation status of proteins, are overexpressed in the aorta, heart, and kidney of SHR compared to their normotensive cohorts [26]. The enhanced oxidative stress occurring in SHR in addition to a brain vascular injury [23] make SHR a reasonable model for investigating the potential neuroprotective activity of antioxidant treatment. On the other hand, oxidative stress plays a relevant role in ischemia–reperfusion injury [36–38]. Thiocctic acid was chosen as an antioxidant as increasing evidence indicates neuroprotective activity of the compound in nervous system disorders characterized by vascular injury [13,39]. The antioxidant activity of the compound was assigned to the (+)-enantiomer [13], although there is no general agreement on it as of yet [40].

In our *in vitro* experiments on PC-12 cell cultures treated with H<sub>2</sub>O<sub>2</sub>, only (+)-thiocctic acid and (+/-)-thiocctic acid decreased ROS levels, whereas no effect was observed after treatment with the (–)-enantiomer. As demonstrated by MTT assay, the (+)-enantiomer was the most active compound able to inhibit the H<sub>2</sub>O<sub>2</sub>-induced reduction in cell viability, displaying its effects at a lower concentration compared with the other isoform investigated. These findings documenting a higher activity of (+)-thiocctic acid are consistent with those of a previous study demonstrating a greater activity of it [41].

The goal of the *in vivo* experiments, was to assess in the model of brain vascular injury represented by SHR the influence of treatment with antioxidants on astroglial reaction and on neurofilament expression in the primary motor cortex and in a key area for learning and memory, such as hippocampus. The findings that the different thiocctic acid formulations did not affect blood pressure levels in SHR indicate that any activity observed in SHR brain is not related to changes in blood pressure. Astrocytes play an active role in maintaining the structure, metabolism and function of the brain [42] and become hypertrophic in response to diverse brain injuries. Depending on their activation status, they are also referred to as reactive and/or activated astrocytes [43–45]. Reactive astrocytes are recognized by their increased size, upregulation of GFAP expression and immunoreactivity [46], and that arterial hypertension increases astrocyte activation [23–25]. Oxidative stress likely causes a brain suffering status accompanied by increased astrocyte immunoreaction. Treatment with thiocctic acid decreases the area occupied by glial cells and therefore counters brain injury accompanied by increased oxidative stress. These data, similar to those found in another animal model of brain injury [47], indicate a more pronounced activity of (+)-thiocctic acid compared with (+/-)-thiocctic acid, whereas (–)-thiocctic acid was almost inactive. Similar results were obtained by analysis of

cytoskeleton expression. (+)-Thioctic acid was more active in countering cytoskeletal breakdown, whereas (+/-)-thioctic acid and (-)-enantiomer were less active or ineffective, respectively. A comparative analysis of the results obtained in *in vivo* experiments with (+)- and with a double dose of (+/-)-thioctic acid suggests that the lower activity of the racemic antioxidant is probably due to a negative influence of (-)-thioctic acid on the biological activity of the compound.

Preclinical and clinical studies have suggested that thioctic acid, alone or in association with other antioxidant molecules, may represent a neuroprotective agent in cognitive decline [39,48–50]. Our *in vivo* studies showing that systemic administration of thioctic acid has a neuroprotective activity in brain areas with a key role in motor and cognitive functions support indirectly the above findings and indicate that the compound has a brain tropism.

In summation, thioctic acid has a neuroprotective effect on microanatomical changes typical of a model of brain vascular injury and, therefore, merits further investigations in hypertensive patients with brains at risk. Moreover, the more pronounced effect of (+)-thioctic acid demonstrated in this study and the lack of activity of (-)-thioctic acid may have practical consequences worth investigating in further studies.

## 4. Materials and Methods

### 4.1. In Vitro Experiments

#### 4.1.1. Cell Cultures

Rat pheochromocytoma (PC12) cells (American Type Culture Collection, ATCC, Rockville, MD, USA), were maintained in HAM'S-F12 medium with L-glutamine (Lonza, Basel, Switzerland) supplemented with 15% heat-inactivated horse serum and 2.5% fetal bovine serum (Lonza), 100 IU/mL penicillin and 100 mg/mL streptomycin (Lonza) at 37 °C, 5% CO<sub>2</sub> and 95% humidity.

#### 4.1.2. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide Assay (MTT)

Cell viability was measured by MTT. PC12 cells were plated at a density of  $1 \times 10^4$  cells/well in 96-well plates and then cultured with different concentrations of (+)-thioctic acid (lysine and piperazine salts), (-)-thioctic acid and (+/-)-thioctic acid. Cell cultures were treated for 18 h at different concentrations of the above derivatives of thioctic acid (10, 20, 50 and 100 µmol/L) or the respective vehicles (DMSO or H<sub>2</sub>O) and then incubated with 200 µmol/L H<sub>2</sub>O<sub>2</sub> for 3 h. The percentage of cytotoxicity was measured by MTT assay. At the end of treatment, MTT was added to the media at the final concentration of 0.8 mg/mL and incubated for 3 h. Supernatants were discarded, and colored formazan crystals were dissolved with 100 µL of dimethyl sulfoxide (DMSO) and read by an enzyme-linked immunoabsorbent assay (ELISA) reader (BioTek Instruments, Winooski, VT, USA). Four replicates were used for each treatment. Sample data are represented as the mean ± SD of at least three separate experiments.

#### 4.1.3. Measurement of ROS Production

PC12 cells were seeded in 24-well plates at a density of  $3 \times 10^5$  cells/well. After 24 h, cells were pulsed with 10  $\mu\text{g}/\text{mL}$  2',7'-dichlorofluorescein diacetate (DCFDA, Sigma Aldrich, St. Louis, CA, USA) for 20 min at 37 °C 5% CO<sub>2</sub>, treated with (+)-thioctic acid (lysine and piperazine salts), (-)-thioctic acid and (+/-)-thioctic acid (10, 20, 50 and 100  $\mu\text{mol}/\text{L}$ ) or DMSO for 18 h, and then incubated with 200  $\mu\text{mol}/\text{L}$  H<sub>2</sub>O<sub>2</sub> for 3 h. Cells were then washed with PBS, detached from wells and quickly analyzed on a FACScan flow cytometer (Becton Dickinson, San José, CA, USA) and the Cell Quest software (version 3.1f; Becton Dickinson; San José, CA, USA; 1995).

#### 4.2. In Vivo Experiments

##### 4.2.1. Animals and Tissue Treatment

Twenty-week-old male SHR ( $n = 30$ ) and age-matched WKY rats were treated for 30 days with intraperitoneal injection of 250  $\mu\text{mol}/\text{kg}/\text{day}$  of (+/-)-thioctic acid ( $n = 6$ ); 125  $\mu\text{mol}/\text{kg}/\text{day}$  of (+/-)-thioctic acid ( $n = 6$ ); 125  $\mu\text{mol}/\text{kg}/\text{day}$  of (+)-thioctic acid lysine salt ( $n = 6$ ) and 25 mM/kg/day of (-)-thioctic acid ( $n = 6$ ).

Control SHR and WKY rats received the same amounts of vehicle. Rats were handled according to internationally accepted principles for care of laboratory animals (European Community Council Directive 86/609, O.J. n° L358, 18 December, 1986). Blood pressure values were measured once a week by an indirect tail-cuff method in conscious rats. Before killing, animals were anaesthetized with pentobarbital sodium (50 mg/kg, i.p.), 5 mL of blood samples were collected by intracardiac withdrawal, and then decapitated. In the blood samples, levels of TBARS were measured using commercial kits (Cayman Chemical Company, Cat. No. 10009055). The brain was removed from the skull, then washed, weighed and divided into the two hemispheres. The left hemisphere was fixed in a HistoChoice solution, and embedded in a semi-synthetic paraffin. Serial consecutive 8- $\mu\text{m}$  thick sections were stained with Nissl's method (cresyl violet 1.5%) for morphometric analysis and with hematoxylin and eosin for assessing the occurrence of relevant microanatomical changes. The right hemisphere was embedded in a cryoprotectant medium and stored at -80 °C until ready for use. Serial consecutive 12- $\mu\text{m}$  thick sections were cut using a microtome cryostat and processed for immunohistochemistry as detailed below.

##### 4.2.2. Immunohistochemistry

Paraffin-embedded coronal sections of the brain (12- $\mu\text{m}$  thick) were processed for the immunohistochemical detection of GFAP, and NFP. The 1st, 4th, 7th, 10th, and 13th consecutive sections were processed for GFAP immunohistochemistry using a mouse serum against GFAP (Chemicon, Millipore, Cat. No. 3402) diluted 1:500 with 0.3% PBS-Triton X 100. The 2nd, 5th, 8th, 11th and 14th consecutive sections were processed for NFP 200 kDa immunohistochemistry by exposing them to a mouse monoclonal antibody raised against NFP 200 kDa (clone RT97, Chemicon, Millipore, Cat. No. 5262) diluted 1:500. The 3rd, 6th, 9th, 12th and 15th were used as controls and exposed to a non-immune IgG instead of the primary antibody. For immunohistochemistry, sections

were exposed overnight in a moist chamber at 4 °C to primary antibodies and then for 30 min at 25 °C to corresponding secondary biotinylated antibodies (mouse-antirabbit IgGs or goat-antimouse IgGs) diluted to 1:200. The product of immune reaction was revealed using 3,3'-diaminobenzidine as a chromogen.

#### 4.2.3. Image Analysis

Nissl's stained sections were viewed under a light microscope at a final magnification of  $\times 160$ . Via a TV connection, images were transferred from the microscope to the screen of an IAS 2000 image analyzer and used as a microanatomy reference for quantitative immunohistochemistry. The cell body area of astrocytes, considered as cells displaying a dark-brown GFAP immunoreactivity, were assessed using an overlap function of the IAS 2000 image analyzer. Morphometric data was then analyzed according to the protocol described in an earlier paper of our group [51]. The density of immunoreaction area occupied by NFP was measured by image analysis in the frontal cortex and hippocampus (subfields CA1, CA3 and dentate gyrus) by protocol detailed elsewhere [52]. The intensity of NFP immunostaining developed in the neuropil of the frontal cortex and hippocampus was assessed microdensitometrically by calibrating the image analyzer taking as "zero" the background developed in sections incubated with a non-immune serum, and "250" as the conventional value of the maximum intensity of staining.

#### 4.2.4. Data Analysis

Means of different parameters investigated were calculated from single animal data, and group means  $\pm$  SEM were then derived from single animal values. The significance of differences between means was analyzed by analysis of variance (ANOVA) followed by the Newman–Keuls multiple range test.

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