Original Article

Protective Effect of Antioxidant Ebselen (PZ51) on the Cerebral Cortex of Stroke-Prone Spontaneously Hypertensive Rats

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An increase in reactive oxygen species has been shown to play a role in perpetuating hypertension and cerebral injury in stroke-prone spontaneously hypertensive rats (SHRsp). Lipid peroxidation in the cerebral cortex is much more intense in SHRsp after establishment of severe hypertension as compared to that in normotensive Wistar-Kyoto rats (WKY). Cortical neurons from SHRsp are more vulnerable to hypoxia and hyponutritional conditions. We sought to investigate whether long-term administration of seleno-glutathione peroxidase mimic ebselen (PZ51) would have a protective effect on cortical neurons in SHRsp, and, if so, the possible mechanisms of this effect. Twenty-two 8-week-old SHRsp were randomized into a PZ51 group and control group. Age-matched WKY were used as normal controls. We examined the levels of malonaldehyde (MDA) and nitric oxide (NO) in the cerebral cortex (CC) homogenate, detected the three isoforms of nitric oxide synthase (NOS) by Western blotting, and examined cortical neurons by transmission electron microscopy. The results showed that PZ51 treatment significantly decreased both MDA and NO in the CC, inhibited inducible nitric oxide synthase (iNOS) protein expression, and alleviated the damage to cortical neurons compared to the findings for the control group. In conclusion, the present study showed that PZ51 administration suppressed lipid peroxidation and inhibited iNOS protein expression in CC homogenate, and it was suggested that these mechanisms may play a role in the protective effects of PZ51 on cortical neurons of SHRsp. (*Hypertens Res* 2005; 28: 249–254)

Key Words: ebselen (PZ51), stroke-prone spontaneously hypertensive rats, cortical neuron, nitric oxide, inducible nitric oxide synthase

Introduction

Hypertension is one of the strongest risk factors for stroke. Over the past decade, it has become apparent that both endothelial cells and vascular smooth muscle cells are capable of producing an excess of reactive oxygen species (ROS) from a variety of enzymatic and non-enzymatic sources in a hypertensive state (1). ROS can lead to lipid peroxidation, endothelial dysfunction and vascular remodeling. Patients with essential hypertension have been shown to have high levels of ROS, impairment of anti-oxidant status, and increased

response to oxidative stress (2, 3). The content of polyunsaturated fatty acid in cerebral tissue is especially high, but the level of endogenous antioxidant is very low (4). Thus cerebral tissues, and especially neurons, are vulnerable to ROS (5). Accordingly, antioxidants may have the potential for stroke prevention and neuroprotective effects in stroke (6, 7).

The establishment of stroke-prone spontaneously hypertensive rats (SHRsp) has made an enormous contribution to basic research on hypertension-related deleterious complications and the prevention of stroke in humans. Oxidative stress is higher in the brains of SHRsp than in those of Wistar-Kyoto rats (WKY) and is involved in perpetuating hypertension and

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Table 1. BW, SBP, BWR, CC NO ($\mu\text{mol}/\text{mg}$ prot) and CC MDA (nmol/mg prot) of WKY and Two SHRsp Groups

Group	BW (g)	SBP (mmHg)	BWR ($[\text{g}/\text{g}] \times 1,000$)	CC NO	CC MDA
WKY	220 $\pm$ 26.8	128.0 $\pm$ 17.6	7.7 $\pm$ 0.6	0.64 $\pm$ 0.21	1.14 $\pm$ 0.38
PZ51	222.8 $\pm$ 30.3	217.0 $\pm$ 24.5 [†]	7.7 $\pm$ 0.7	0.82 $\pm$ 0.19 ^{*†}	2.09 $\pm$ 0.62 ^{*†}
Control	209.4 $\pm$ 33.0	225.1 $\pm$ 19.0 [†]	7.9 $\pm$ 1.3	1.24 $\pm$ 0.28 [†]	4.11 $\pm$ 0.45 [†]

^{*} $p < 0.01$ vs. control group; [†] $p < 0.01$ vs. WKY. BW, body weight; SBP, systolic blood pressure; BWR: brain weight ratio; CC, cerebral cortex; MDA, malonaldehyde; WKY, Wistar-Kyoto rats; SHRsp, stroke-prone spontaneously hypertensive rats; PZ51, ebselen.

cerebral injury in SHRsp (8–10). Almost all SHRsp suffer from cerebral edema and softening.

A newly developed antioxidant, ebselen (PZ51), 2-phenyl-1,2-benzisoselenazol-3[2H]-one, is a lipid-soluble seleno-organic compound that potently inhibits lipid peroxidation through a glutathione peroxidase (GPX)-like action (11, 12). This agent effectively inhibits both nonenzymatic (13) and enzymatic (the lipoxygenase pathway of the arachidonate cascade) (14) lipid peroxidation *in vitro*. Since the involvement of ROS in brain damage due to ischemia has been supported by several studies, PZ51 has been demonstrated to be beneficial in both experimental and clinical studies of acute cerebral ischemia (15–18). In these studies, PZ51 reduced infarct size in rats with transient middle cerebral artery occlusion (17) and improved the outcome of patients with acute ischemic stroke (15). However, there has been no investigation into the effects of PZ51 on the chronic process of hypertension-related cerebral damage. The purpose of the present study was to investigate whether long-term administration of PZ51 would exert a protective role on cortical neurons of SHRsp before stroke occurrence, and, if so, to explore the possible mechanisms of this effect.

Methods

Animals and Experimental Protocol

Treatment of the animals was based on the “Guide for the Care and Use of Laboratory Animals” published by the US National Institutes of Health (NIH publication NO. 85-23, revised 1985). Eight-week-old SHRsp weighing 160 $\pm$ 20 g were housed in a humidity and temperature-controlled semi-barrier space with 12-h light and dark cycles in the experimental animal department of the Cardiovascular Institute and Fuwai Hospital. The animals were given 1.0% saline water to accelerate the progression of hypertension and organ damage. Twenty-two SHRsp were randomized to a PZ51-treatment group or a control group. Body weight was measured every 2 days. Based on previous findings, PZ51 at a dose of 60 mg/kg/day was considered to be sufficient to exert an antioxidant activity in the brain of rats (15, 17, 19). Rats in the PZ51 group received PZ51 dissolved in polyethylene glycol at 60 mg/kg/day by gastric gavage for 6 weeks, while those in the control group received vehicle solution instead. Age-matched genetically normotensive WKY served as controls. Resting

blood pressure (tail-cuff method) was measured once weekly.

Preparation of Tissue Samples

At the end of 6 weeks, the rats were anesthetized by intraperitoneal injection of 50 mg/kg pentobarbital sodium and sacrificed. The brains were removed and weighed on an electronic scale and the brain weight ratio (BWR = brain weight [g] / body weight [g] $\times$ 1,000) was calculated. The cerebral cortex (CC) was carefully dissociated, and then about 1 mm³ of the left frontal CC was placed in 2.5% glutaraldehyde, about 0.3 g of CC was made into 10% homogenate for nitric oxide (NO) and malonaldehyde (MDA) detection, and the rest was promptly frozen in liquid nitrogen for the Western blotting analysis.

Measurement of the Level of NO, MDA, and Protein in CC Homogenate

The CC homogenate was divided into two parts. One part was centrifuged at 1,000 rpm for 5 min and the supernatant was used to detect NO concentration. The other part was centrifuged at 12,000 rpm for 20 min and the supernatant was used for MDA detection. The NO kit and MDA kit were purchased from Nanjing Jiancheng Biotechnology Co. (Nanjing, China).

We measured the concentration of NO in CC homogenate by the methods of Tracey *et al.* (20) and Ling (21). NO was determined by its oxidation products, nitrite and nitrate. Nitrate present in the samples is reduced to nitrite by reduced nicotinamide adenine dinucleotide phosphate in the presence of the enzyme nitrate reductase. The nitrite formed reacts with sulfanilamide and *N*-(1-naphtyl)-ethylenediamine dihydrochloride to give a red-violet diazo dye. Finally, the diazo dye is measured on the basis of its absorbance at 550 nm by a spectrophotometer.

The concentration of MDA in the CC was measured by a thiobarbituric acid reactive substance assay (TBARS) according to the method of Kato (22). Two hundred μl of 10% CC homogenate was pipetted into a 10-ml centrifuge tube and 10 μl of butylate-hydroxytoluene was added to the sample to prevent oxidation during the subsequent boiling step. Then, 200 μl of 8.1% sodium lauryl sulfate, 1,500 μl of 20% acetic acid (pH=3.5) and 1,500 μl of 0.8% thiobarbituric acid were added to the sample. The sample was then boiled for 1 h at 95°C and centrifuged at 3,000 $\times g$ for 10 min. The absorbance

Fig. 1. Western blot analysis of the protein expression of eNOS, nNOS, and iNOS in the CC of SHRsp. No significant differences in the protein levels of eNOS (A) or nNOS (B) were observed between WKY and SHRsp, or between the PZ51 group and control group. The level of iNOS (C) increased significantly in SHRsp compared with that in WKY. PZ51 decreased iNOS protein expression significantly. * $p < 0.01$ vs. WKY; † $p < 0.01$ vs. the control group.

of the supernatant was measured by a spectrophotometer at 532 nm comparing the absorbance to that of a standard consisting of 200 μ l of 1,3,3-tetramethoxy propane in place of the homogenate. CC homogenate protein was detected according to the Bradford method (23).

Western Blotting

The CC was homogenized with polytron in a lysis buffer (pH=7.6) consisting of 1 mol/l Tris-HCl, 0.01 mmol/l EDTA, and 0.01 μ g/ml PMSF. After the homogenate was centrifuged at $20,000 \times g$ for 30 min, the protein concentration was determined using the Bradford method. Fifty μ g/lane of protein were size-fractionated on 8% polyacrylamide gel by electrophoresis using a Mini-Protean Dual stab cell, then electrotransferred onto Hybond-ECL membranes (Amersham Life Science Inc., Arlington Heights, USA) in the glycine/methanol buffer. The membrane was prehybridized in 20 ml buffer A (10 mmol/l Tris-HCl, 100 mmol/l NaCl, 0.05% Tween-20 and nonfat milk powder) for 1 h and then hybridized in the same buffer containing anti-eNOS, anti-nNOS or anti-iNOS (NOS, NO synthase; e, endothelial; n, neuronal; i, inducible) polyclonal antibody (1:400) (Santa Cruz Biotechnology, Santa Cruz, USA) at 4°C overnight. The membrane was washed with washing buffer A without nonfat milk for 1 h in a shaking bath, and the buffer was changed every 10 min. The membrane was then incubated in buffer A plus horseradish peroxidase-conjugated anti-rabbit-IgG at 1:4,000 for 2 h. Finally, after washing for another hour, the membrane was developed with the ECL-plus chemiluminescence detection system by exposing it to roentgen-film in a dark room to visualize the bands. β -Actin was used as an internal control. The concentration of protein was obtained from the densitometry value of the corresponding band measured by TotalLab software (Nonlinear Dynamics, Durham, USA).

For electron microscopic examination, after fixation in glutaraldehyde, the CC sample was refixed in 1% osmium tetroxide, embedded in epoxy resin 812, sectioned at 0.1- μ m thickness, and double-stained with uranium acetate and lead citrate. The sections were observed with a transmission electron microscope (Hitachi-600, Hitachi, Tokyo, Japan).

Data Analysis

The results are presented as the mean $\pm$ SEM for each group of rats. Statistical analysis was performed with SPSS software, version 10.0 (SPSS, Chicago, USA). The normality of the distribution of the data was checked. Statistical analysis was carried out using one-way ANOVA. Values of $p < 0.05$ were considered statistically significant.

Results

Blood Pressure, Body Weight and Brain Weight Ratio

Systolic blood pressure (SBP) was significantly higher in SHRsp than in WKY, but was not different between the two SHRsp groups. There was no significant difference in body weight or the brain weight ratio among the three groups (Table 1).

MDA and NO Level in CC Homogenate

Table 1 also shows the concentration of MDA and NO in CC homogenate. MDA and NO content were significantly higher in SHRsp than in WKY rats ($p < 0.01$). Treatment with PZ51 led to a significant reduction of CC MDA and NO in SHRsp ($p < 0.01$).

Fig. 2. Transmission electron microscopic examination of cortical neurons. In the control group sample (A), the nuclear chromatin is condensed (as indicated by the black arrow). There is evident edema of the perinuclear area. In the cytoplasmic area, swollen mitochondria with disrupted and dissolved cristae are evident. There are vacuolated mitochondria (white triangle). PZ51 alleviated these neuronal injuries (B). The nuclear chromatin of the PZ51-treated sample is not condensed but dispersed regularly, the perinuclear area is not edematous, and there are several slightly dilated mitochondria ($\times 12,000$).

Effect of PZ51 on eNOS, nNOS and iNOS Expression in the CC as Detected by Western Blotting

Figure 1 shows the amounts of the eNOS, nNOS, and iNOS proteins based on Western blot analysis. No significant difference in the amount of eNOS or nNOS protein was observed between WKY and SHRsp, nor between the PZ51 group and the control group. The level of iNOS was very low compared with the levels of other constitutive NOS isoforms, but was increased significantly in SHRsp compared with that in WKY. PZ51 decreased iNOS protein expression significantly ($p < 0.01$).

Transmission Electron Microscopy Examination

Figure 2 shows the results of the transmission electron microscopic examination. In the control group sample, the nuclear chromatin is condensed. There is evident edema of the perinuclear area. In the cytoplasmic area, swollen mitochondria with deranged and disrupted or dissolved cristae are evident, and there are vacuolated mitochondria. Some of the rough endoplasmic reticulum is dilated. PZ51 alleviated these neuronal injuries. In the PZ51-treated sample, the nuclear chromatin is not condensed but dispersed regularly, and the perinuclear area is not edematous. There are several slightly dilated mito-

chondria, and the endoplasmic reticula are not dilated.

Discussion

The major findings of the present study are as follows: 1) administration of PZ51 to SHRsp for 6 weeks resulted in a significant decrease of MDA and NO in the CC; 2) PZ51 decreased CC iNOS protein expression significantly; and 3) electron microscopy showed that PZ51 had a protective effect on cortical neurons of SHRsp.

Endogenous GPX is considered to be one of the major antioxidants in the brain and has been demonstrated to be an important enzyme for neuronal defense against peroxide toxicity *in vitro* (24). In contrast to the endogenous enzymatic scavenger, PZ51 is a relatively small, lipophilic compound and may therefore prove to be of greater clinical utility against cerebral damage. PZ51 is rapidly absorbed from the gastrointestinal tract, maintains a stable plasma concentration, and can easily penetrate the brain blood barrier (BBB). It inhibits the peroxidation of membrane phospholipids, inhibits lipoyxygenase, and blocks the production of superoxide anions by activated leukocytes (25).

Recent studies have provided compelling evidence that increased levels of ROS are involved in the pathogenesis and maintenance of hypertension in SHR and SHRsp (26, 27).

The level of endogenous antioxidant in the blood and cerebral tissue of SHRsp is much lower than that of WKY, while the opposite is true of the levels of ROS in cerebral tissue (8, 28). ROS have been reported to play an important role in the pathogenesis of cerebral injury of SHRsp by promoting membrane lipid peroxidation and oxidative damage to DNA and protein. Lipid peroxidation in the cerebral cortex is much more intense in SHRsp after establishment of severe hypertension as compared to that in normotensive WKY (29). The attack on the cell membrane by free radicals leads to lipid peroxidation, the existence of which can be assessed by measuring tissue MDA (30). The tissue MDA assay has been reported to be a reliable and reproducible procedure for analyzing the level of lipid peroxides in various organs (31). Maulik *et al.* showed that PZ51 lowered oxidative stress in the ischaemic heart of rats by assaying MDA concentration (32). We assayed the level of CC MDA to monitor the oxidative stress and extent of lipid peroxidation. In the present study, the level of CC MDA in SHRsp was significantly higher than that in WKY, in agreement with a previous report (29). PZ51 administration decreased CC MDA significantly compared with that of the control group by virtue of its free radical-scavenging properties.

NO plays an important role in both the vascular function and pathophysiology of cardiovascular disorders. NO is produced endogenously by the conversion of L-arginine to citrulline by NOS. Three types of NOS have been identified, constitutive calcium/calmodulin-dependent neuronal and endothelial isoforms and an inducible calcium-independent isoform. eNOS-derived NO is beneficial in promoting collateral circulation and microvascular flow. The role of nNOS-derived NO has not been fully clarified. iNOS is calcium-independent, with high activity as long as it is formed. The level of NO production of this origin is several grades higher than that from constitutive NOS and is harmful in the pathogenesis of hypertension, endothelial injury and target organ damage (33).

It has been reported that iNOS is already activated in SHRsp prior to stroke occurrence (34). Our study was conducted in relatively young SHRsp in order to detect the effect of PZ51 on the CC before stroke occurrence. We found that iNOS protein expression was significantly higher in the CC of SHRsp than in that of WKY. PZ51 administration decreased the iNOS protein expression of SHRsp significantly. eNOS and nNOS concentrations were not different between WKY and SHRsp, nor between the two SHRsp groups. We speculated that the decreased level of NO in the CC of the PZ51 group was caused by the decreased iNOS expression. A previous study showed that PZ51 inhibits iNOS expression *in vitro* (11). In our study, this effect was verified *in vivo* for the first time. PZ51 can also inhibit iNOS activity by reacting with a critical thiol group of iNOS (35). As to the mechanism of iNOS inhibition against cerebral injury of SHRsp, Takasago *et al.* showed a number of neuroprotective and anti-oedema effects of iNOS inhibition on hypertensive cerebral

injury, including inhibition of leukocyte-endothelial cells adhesion and protection against endothelial injury (18).

There is accumulating evidence that cortical neurons from SHRsp are more vulnerable to hypoxia and hyponutritional conditions (5). In an *in vitro* model system, antioxidants were the most important agents for the reduction of oxygen-free radical damage in cortical neurons of SHRsp (36). In our study, although there were no differences in blood pressure or BWR between the control and PZ51 group, electronic microscopy showed that the injury to neurons was alleviated by PZ51 administration. The incipient protective effect of PZ51 on cortical neurons prior to stroke occurrence may be due its inhibition of lipid peroxidation and iNOS expression.

iNOS has been detected in the cells of several tissues under stimuli, including polymorphonuclear neutrophils, monocytes, astrocytes, and vascular smooth muscle cells. In the present study, we determined only that PZ51 decreased iNOS protein expression in the CC of SHRsp. Further studies will be needed to identify the types of cells in which iNOS-derived NO production is decreased.

The major conclusions drawn from this study are that the antioxidant property of PZ51 is associated with decreased iNOS protein expression, and therefore with generation of NO, in the CC of SHRsp. These findings should help to clarify the mechanism by which PZ51 protects the CC, in addition to the mechanism of its well-established free radical-scavenging effects.

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