Res. Chem. Intermed., Vol. 26, No. 7,8, pp. 667-677 (2000)

EPC-K1 PROTECTS NEURONAL CELLS FROM PEROXYNITRITE-MEDIATED OXIDATIVE DAMAGE

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Received 3 March 2000; accepted 25 April 2000

Abstract—Protective effects of EPC-K1 (L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyldecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt, a difunctional derivative of vitamin C and vitamin E) on neuronal cell damage mediated by peroxynitrite were studied. Primary cultures of cerebellar granule cells were exposed to peroxynitrite by treatment with 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1), which generated nitric oxide and superoxide anion simultaneously upon decomposition. The results showed that SIN-1 treatment triggered time-dependent cell death, which was accompanied by the decrease in the cellular GSH level, the increase in the lipid peroxidation level, and the alteration of cell membrane biophysical characteristics. EPC-K1 showed only moderate scavenging effect on peroxynitrite, but could effectively protect neuronal cells from oxidative damage mediated by peroxynitrite.

INTRODUCTION

Nitric oxide is a diffusible free radical messenger that is known to exhibit a variety of biological activities such as vasodilation, host defense and neuronal communication [1]. Synthesis of nitric oxide from L-arginine by the enzyme nitric oxide synthase (NOS) occurs in several cell types, including endothelial cells, neurons, glial cells, and macrophages [2]. Nitric oxide reacts with superoxide anion at high rate ($k > 6.7 \times 10^9$ $M^{-1} \cdot s^{-1}$) to form peroxynitrite. Peroxynitrite (and its breakdown product, hydroxyl radical-like species) can oxidize biomacromolecules including membrane, protein and DNA, and cause oxidative injury in different cell types [3,4]. Under certain pathologic conditions (for example, ischemia/reperfusion injury), simultaneous production of superoxide and nitric oxide may occur in the central nervous system (CNS), potentially leading to the formation of peroxynitrite. It has been proposed that peroxynitrite-induced neuronal injury might be involved in different neuronal disorders such as brain ischemia, Alzheimer's disease, Parkinson's disease and multiple sclerosis [5,6,7,8].

In the present investigation several aspects of peroxynitrite-mediated

neuronal damage were studied using primary culture of rat cerebellar granule cells as a model. Cells were treated with peroxynitrite by incubation with 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1), which generated nitric oxide and superoxide anion simultaneously upon decomposition (Figure 1). The protective effect of EPC-K1 (L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyldecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt, a synthetic difunctional derivative of vitamin C and vitamin E) on neuronal cells from peroxynitrite-mediated neuronal injury was also studied.

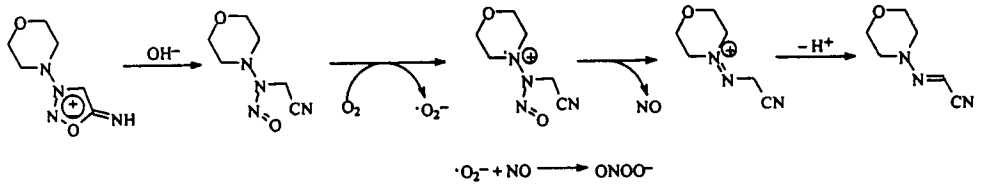

Figure 1. Structure and decomposition pathways of SIN-1.

EXPERIMENTAL

Materials

Seven-day-old Wistar rats were purchased from the Experimental Animal Center of Beijing Medical University, China. Cell culture plastic ware was purchased from Corning Costar (Acton, MA, USA). Dulbecco's modified Eagle Medium (DMEM), cell culture supplements, fetal bovine serum and trypsin (1:250) were products of Gibco BRL (Grand Island, NY, USA). Glutathione (GSH), poly-L-lysine, catalase, bovine serum albumin (BSA), 3-morpholinosydnonimine-*N*-ethylcarbamide (SIN-1), 5,5-dimethyl-1-pyrroline-*N*-oxide (DMPO), 5-doxyl-stearic acid (5-doxyl), 16-doxyl-stearic acid (16-doxyl) and 3-maleimidoproxyl (3-mal) were purchased from Sigma (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR123) was purchased form Molecular Probes (Eugene, OR, USA). L-Ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethytridecyl)-2H-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1) was a generous gift from Senju Pharmaceutical Co. Ltd., Japan. Other reagents were made in China.

Cell Culture and Peroxynitrite Treatment

Primary cultures of rat cerebellar granule cells were prepared following procedures described previously [9]. Briefly, cerebella from 7-day-old Wistar rats were dissected out. After removal of the meninges and blood vessels, cerebella were rinsed with Hanks' buffered salt solution (HBSS), and dissociated by mild trypsinization. Cells

were plated on poly-L-lysine-coated 35 mm Petri dishes at 4×10^6 cells/2 ml or 96-well plates at 2×10^5 cells/100 μl. Culture medium consisted of DMEM supplemented with KCl (19.6 mM), glutamine (2 mM), HEPES (10 mM) and fetal bovine serum (10 %, v/v). Cells were maintained at 37 °C in a humidified 5 % CO_2-95 % air atmosphere. Cytosine arabinoside (10 μM) was added to cells 24 h after plating to suppress the proliferation of non-neuronal cells.

After 10 d *in vitro*, cells were exposed to peroxynitrite by treatment with SIN-1, which generated nitric oxide and superoxide anion simultaneously upon decomposition. Briefly, cells were washed twice with HBSS and incubated in HBSS at 37 °C for 15 min. Aliquots of freshly prepared stock solution of SIN-1 were added to cells. After incubation at 37 °C for 1 h, cells were washed twice with HBSS, the original culture medium was restored, and cells were cultured for indicated time. In some experiments, cells were pre-incubated with 100 μM of EPC-K1 for 15 min, washed twice with HBSS, and then exposed to SIN-1.

Assessment of Cell Injury

The early cell injury was assessed by MTT assay [10]. Cells cultured in 96-well plates were treated with 1 mM SIN-1 for 1 h and cultured in fresh medium for indicated time. 10 μl of MTT solution (5 mg/ml in PBS) was added to each well and cells were incubated at 37 °C for 30 min. Then, 100 μl of solubilization solution (25 % dimethylformamide, 10 % SDS, 1 % acetyl acid, pH 3.5) was added to the wells followed by mixing for 10 min. The absorption at 570 nm was measured.

The loss of cell membrane integrity, which characterizes the irreversible damage of cell, was measured by the lactate dehydrogenase (LDH) released from the injured cells into the culture medium [11]. After treatment with 1 mM SIN-1 for 1 h and cultured in fresh medium for indicated time, the LDH activity in the culture supernatant was determined using an LDH clinical diagnosis kit (Zhongsheng Hightech Bioengineering Co., Beijing, China). The percentage of LDH released into the medium was defined as the ratio of LDH activity in the culture medium to the total LDH activity, where the total LDH activity represents the LDH activity in the cells and the medium. The total LDH activity was measured in sister cultures by freezing/thawing cells rapidly [12].

Biochemical Assays

Thiobarbituric acid (TBA) assay was employed to determine the lipid peroxidation level in cells [4]. After treatment with 1 mM SIN-1 for 1 h and cultured in fresh medium for indicated time, 10^7 cells were pelletted, washed twice, and resuspended in 0.4 ml PBS. The suspension was mixed with 0.4 ml of 10 % trichloroacetic acid

and 1 ml of 0.67 % thiobarbituric acid, and the mixtures were heated at 95 °C for 1 h. After extraction by n-butanol, the absorption at 532 nm was measured.

The cellular glutathione level was determined by a modified fluorescent method [12]. Briefly, cells cultured in 35 mm Petri dishes were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, washed twice with HBSS, and incubated with 50 μM monochlorobimane at 37 °C for 30 min. Then cells were washed twice with HBSS and lysed in 0.2 % Triton X-100. After centrifugation at 12000 × g for 10 min, the fluorescence of the supernatant was measured with excitation at 400 nm and emission at 480 nm. The concentration of GSH was calculated from standard curves and expressed as nmol/mg protein. The protein concentration was determined by Bradford method with BSA as standard.

Spin Labeling

The fluidity of cell membrane was determined by ESR using 5-doxyl and 16-doxyl as spin labels. The conformation alteration of cell membrane protein sulfhydryl groups was determined by ESR using 3-mal as spin label [13]. Briefly, after treatment with SIN-1 for 1 h and cultured in fresh medium for 8 h, cells were washed 3 times with PBS, mixed with 10 μM spin label (5-doxyl, 16-doxyl) or 100 μM spin label (3-mal) and incubated at 37 °C for 30 min (5-doxyl, 16-doxyl) or 3 h (3-mal). The labeled cells were washed 4 times with PBS, and then transferred into quartz capillaries for ESR measurement. The ESR spectra were recorded at room temperature (298 K) by a Varian E-109 spectrometer with measurement conditions as: X-band, central magnetic field 325 mT, sweep width 20 mT, microwave power 20 mW, frequency 100 kHz, modulation amplitude 0.2 mT, time constant 0.128 s. The methods of calculation for the order parameter (S), the rotational correlation time (τ_c), and the ratio of strongly immobilized component to the weakly immobilized component (s/w) were the same as previously described [14,15].

Determination of Peroxynitrite

The formation of peroxynitrite from the decomposition of SIN-1 was determined by the oxidation of DHR123 to rhodamine 123 [16]. The reaction mixture contained 25 μM SIN-1, 50 μM DHR123, 0.1 mM diethylenetriaminepentaacetic acid (DETEPAC), 100 U/ml catalase and various concentrations of EPC-K1. After incubation at 37 °C for 1 h, the absorption at 500 nm was measured.

Statistical Analysis

Each experiment was performed at least three times and results are presented as mean

± SD. Statistical analyses were performed using one-way ANOVA or Student's *t*-test. A probability of < 0.05 was considered significant.

RESULTS

Scavenging Effect of EPC-K1 on Peroxynitrite

Non-fluorescent DHR123 can be oxidized to fluorescent Rh123 by peroxynitrite as well as by hydrogen peroxide. In the presence of catalase and DETAPAC, hydrogen peroxide-dependent oxidation of DHR123 is suppressed and thus the amount of Rh123 formed is correlated with the concentration of peroxynitrite. Addition of EPC-K1 inhibits the formation of Rh123 dose-dependently, suggesting that EPC-K1 could scavenge peroxynitrite formed from the decomposition of SIN-1 (Figure 2).

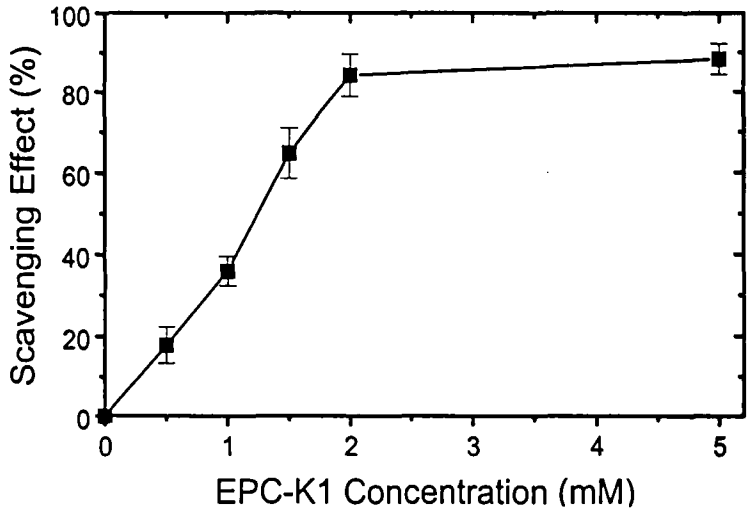

Figure 2. Scavenging effect EPC-K1 on peroxynitrite. Data are means ± SD of 4 experiments.

Neurotoxicity Induced by Peroxynitrite

Tetrazolium salt MTT can be reduced to formazan by mitochondrial respiratory enzymes. The amount of formazan formed indicates the cell viability accurately. Results of MTT assay shows SIN-1 treatment causes time-dependent cell death. Pre-treatment with 50 μM of EPC-K1 significantly protected cells from peroxynitrite-induced injury (Figure 3).

When the cell membrane integrity is damaged, cytoplasmic enzyme LDH is released into the culture medium. Exposure of cells to SIN-1 also caused LDH

leakage. Pre-treating cells with 50 μM of EPC-K1 prevented cells from peroxynitrite-induced lysis effectively (Figure 4).

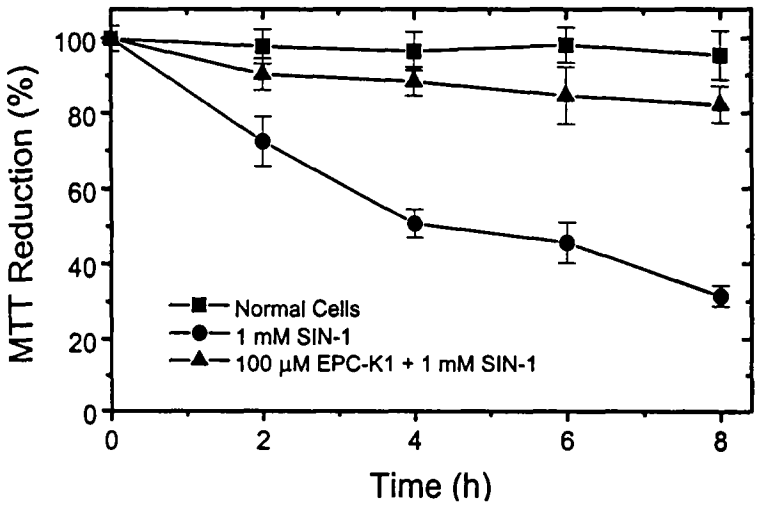

Figure 3. Time-course of cell death in cerebellar granule cells induced by SIN-1. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, and the cell viability was assessed by MTT assay. Data are means ± SD of 8 experiments.

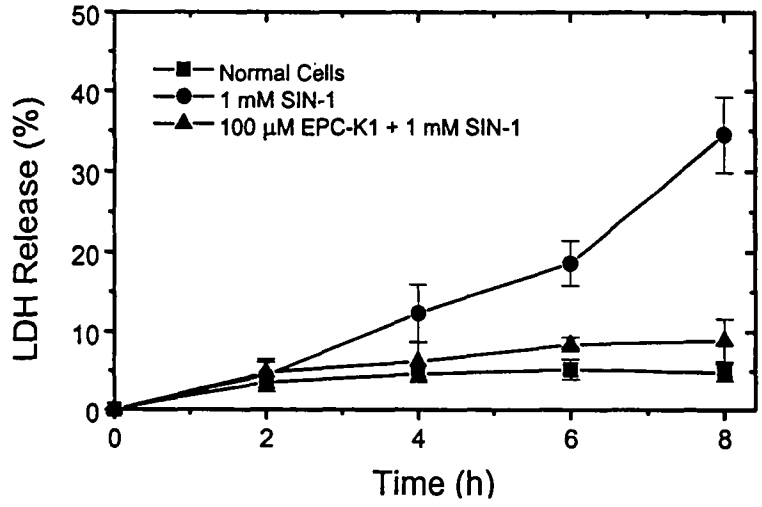

Figure 4. Loss of cell membrane integrity in cerebellar granule cells induced by SIN-1. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, and the cell membrane integrity was assessed by LDH efflux assay. Data are means ± SD of 3 experiments.

Lipid Peroxidation

Exposure to peroxynitrite induced lipid peroxidation in cerebellar granule cells time-dependently as indicated by the marked increase in TBARS levels. In cells pretreated with EPC-K1, the formation of TBARS was markedly suppressed (Figure 5).

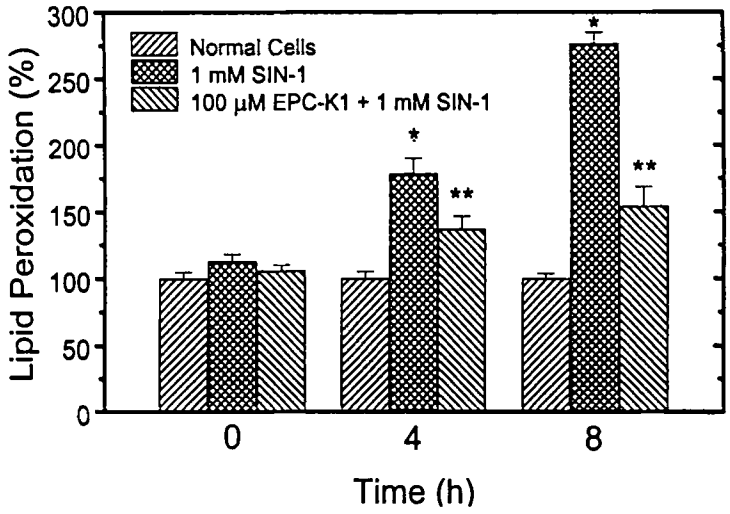

Figure 5. Lipid peroxidation in cerebellar granule cells. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, and the lipid peroxidation level was assessed by TBA assay. Data are means ± SD of 3 experiments. *: $P < 0.01$ in comparison with normal cells; **: $P < 0.01$ in comparison with cells treated with 1 mM SIN-1.

Decrease of Cellular GSH Level

Peroxynitrite treatment caused decrease in the cellular GSH level, which was prevented by pretreatment with EPC-K1 (Figure 6).

Alteration of Cell Membrane Biophysical Characteristics

The typical ESR spectra of cell membrane spin labeled with 5-doxyl, 16-doxyl and 3-mal were shown in Figure 7. The nitroxide groups of 5-doxyl are located in the shallow layers of membrane lipids. The order parameter (S) calculated from the ESR spectra reflects the degree of order of the lipid chain around the nitroxide radicals. The high S values correspond to high anisotropic motion and low membrane fluidity; accordingly, the low S values correspond to low anisotropic and high membrane fluidity. The nitroxide groups of 16-doxyl are located in the deep layers of membrane lipids. The correlation rotational time (τ_c) reflects the time during which molecules are rotated from one conformation to another. Membrane fluidity and τ_c are inversely

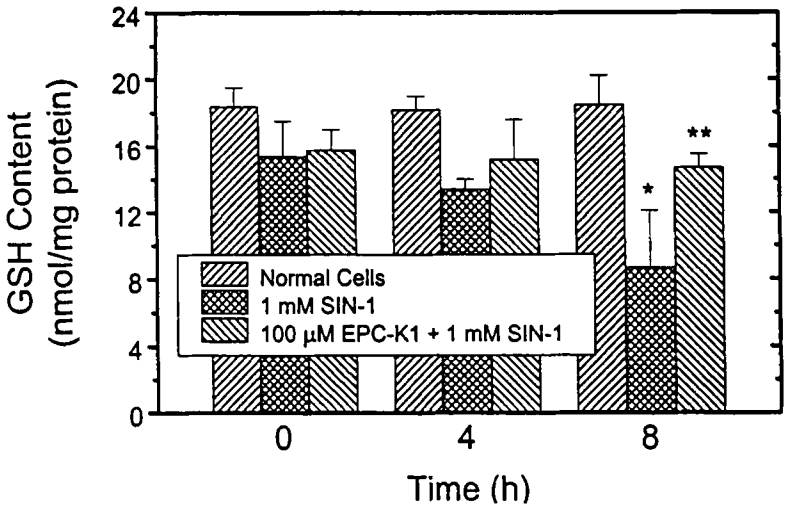

Figure 6. Decrease in cellular GSH level in cerebellar granule cells. Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for indicated time, and the cellular GSH level was assessed by fluorescent method. Data are means ± SD of 3 experiments. *: $P < 0.05$ in comparison with normal cells; **: $P < 0.05$ in comparison with cells treated with 1 mM SIN-1.

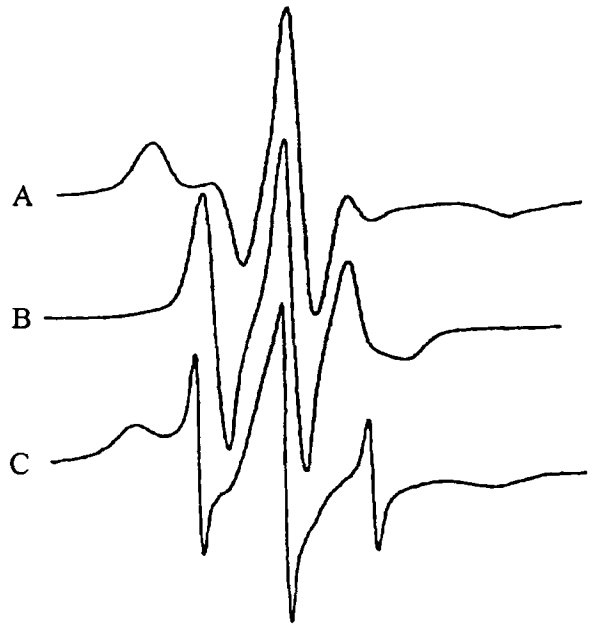

Figure 7. ESR spectra of cell membrane spin labeled with 5-doxyl (A), 16-doxyl (B), and 3-mal (C). Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for 8 h and then spin labeled with 5-doxyl, 16-doxyl, and 3-mal. The ESR spectra were recorded by a Varian E-109 spectrometer at room temperature (298 K).

correlated. There are two types of sulfhydryl groups binding sites on cell membrane proteins. One of them is on the surface of the membrane protein. When 3-mal spin labels are bound to surface, the nitroxide groups of 3-mal can move freely. Hence, the ESR spectra are weakly immobilized. The other type of binding site is in the deep layers of the protein tertiary structure. When maleimide spin labels are bound to the internal sites, the movement of the nitroxide is limited in space. Hence strongly immobilized spectra are produced.

After treatment with peroxynitrite, the order parameter and the rotational correlation time of cells increased markedly, suggesting the decrease in the fluidity of both the surface layer and the deep layer of the cell membrane (Table 1). The ratio of strongly immobilized component to the weakly immobilized component also increased markedly, suggesting the alteration of the membrane protein conformation. Pre-treatment with EPC-K1 showed significant protective effects against oxidative stress-induced alteration of cell membrane biophysical characteristics.

Table 1
SIN-1-induced cell membrane biophysical characteristics alteration in cerebellar granule cells.[a]

	Normal cells	1 mM SIN-1	100 μM EPC-K1 + 1 mM SIN-1
S	0.724 ± 0.011	0.754 ± 0.006*	0.718 ± 0.006**
τ_c (10^{-10} s)	9.27 ± 0.09	9.89 ± 0.15*	9.31 ± 0.12**
s/w	0.193 ± 0.009	0.239 ± 0.016*	0.208 ± 0.008**

[a]Cells were treated with 1 mM SIN-1 for 1 h, cultured in fresh medium for 8 h and then spin labeled with 5-doxyl, 16-doxyl, and 3-mal. The cell membrane biophysical characteristics order parameter (S), rotational correlation time (τ_c), and the ratio of strongly immobilized component to the weakly immobilized component (s/w) were calculated form the ESR spectra. Data were mean ± SD of 4 experiments. *: $P < 0.05$ in comparison with normal cells; **: $P < 0.05$ in comparison with cells treated with 1 mM SIN-1 for 8 h.

DISCUSSION

The CNS is especially prone to oxidative damage for several reasons. First, the brain has a very high oxygen consumption ratio, which may lead to the formation of endogenous reactive oxygen species; second, neuronal cell membrane is rich in polyunsaturated fatty acid side chains; third, the antioxidant defense systems in the CNS are relatively poor. Peroxynitrite-mediated oxidative injury might be the common pathway of several neuronal diseases.

Incubation with SIN-1, which generates peroxynitrite, caused the decrease in the cellular GSH level. GSH is the predominant low molecular thiol in mammalian cells and plays a major role in cellular defenses against oxidative attack. GSH not only scavenges peroxynitrite and hydroxyl radicals directly, but also reduces peroxides catalyzed by glutathione peroxidase (GPx). It was reported that GSH was of great importance in maintaining mitochondrial respiratory chain complex activity [17]. Thus, loss of GSH may cause the dysfunction of mitochondrial enzymes and result in cell death finally.

Results of TBA assay showed that peroxynitrite treatment induced lipid peroxidation in cerebellar granule cells as confirmed by the formation of TBARS. The intermediates formed during lipid peroxidation chain reactions may cause protein cross-link and thus damage the normal structure of the neuronal cell membrane, which was confirmed by the results of ESR spin labeling. The oxidative injury of neuronal cell membrane may trigger a series of signals such as Ca^{2+} influx [18]. Ca^{2+} overload causes more serious damage to the neuronal cells, and results in cell death finally [19]. Treatment with peroxynitrite also causes the release of endogenous aspartate in cerebellar granule cells [20], which may induce excitotoxicity in neurons.

EPC-K1 effectively protected neuronal cells form oxidative damage mediated by peroxynitrite. EPC-K1 is a kind of diester derivative of vitamin C and vitamin E. Our previous work indicated that EPC-K1 could scavenge both hydrophilic and hydrophobic radicals at high rates, and inhibit lipid peroxidation effectively [21]. Results of present investigation showed that EPC-K1 could scavenge peroxynitrite at high dose (2 mM). However, low concentration of EPC-K1 (100 μM) effectively protect neuronal cells from oxidative damage mediated by peroxynitrite. The results suggested that EPC-K1 protect neuronal cells form peroxynitrite-mediated damage not by scavenging peroxynitrite directly, but by inhibiting secondary reactions triggered by peroxynitrite such as lipid peroxidation, oxidation of amino acid residues, and cross-linkage of proteins.

In conclusion, exposure of cerebellar granule cells to peroxynitrite triggered oxidative damage, and pretreatment with EPC-K1 could effectively prevent cells from damage. The effective protection of EPC-K1 on peroxynitrite-induced neuronal damage made it possible to be used as a potential therapeutic agent on neuronal diseases associated with the peroxynitrite.

Acknowledgement

This work was supported in part by a grant from the National Natural Science Foundation of China.

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