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Nitric oxide induces oxidative stress and apoptosis in neuronal cells

Taotao Wei [a], Chang Chen [a], Jingwu Hou [a], Wenjuan Xin [a,*], Akitane Mori [b]

[a] *Department of Molecular and Cellular Biophysics, Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Chaoyang District, Beijing 100101, PR China*

[b] *Department of Neuroscience, Institute of Molecular and Cellular Medicine, Okayama University Medical School, Okayama 700, Japan*

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Abstract

Within the central nervous system and under normal conditions, nitric oxide (NO) is an important physiological signaling molecule. When produced in large excess, NO also displays neurotoxicity. In our previous report, we have demonstrated that the exposure of neuronal cells to NO donors induced apoptotic cell death, while pretreatment with free radical scavengers L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1) or superoxide dismutase attenuated apoptosis effectively, suggesting that reactive oxygen species (ROS) may be involved in the cascade of events leading to apoptosis. In the present investigation, we directly studied the kinetic generation of ROS in NO-treated neuronal cells by flow cytometry using 2′,7′-dichloro-fluorescein diacetate and dihydrorhodamine 123 as redox-sensitive fluorescence probes. The results indicated that exposure of cerebellar granule cells to the NO donor *S*-nitroso-*N*-acetylpenicillamine (SNAP) induced oxidative stress, which was characterized by the accumulation of cytosolic and mitochondrial ROS, the increase in the extracellular hydrogen peroxide level, and the formation of lipid peroxidation products. SNAP treatment also induced apoptotic cell death as confirmed by the formation of cytosolic mono- and oligonucleosomes. Pretreating cells with the novel antioxidant EPC-K1 effectively prevented oxidative stress induced by SNAP, and attenuated cells from apoptosis.

Keywords: Nitric oxide; Reactive oxygen species; Oxidative stress; Cerebellar granule cell; Apoptosis; EPC-K1

1. Introduction

In respiring cells, a small amount of the consumed oxygen is reduced in a specific way, yielding a variety of highly reactive oxygen species (ROS) such as superoxide anion, hydrogen peroxide and hydroxyl radical [1]. ROS are capable of causing oxidative damage to biomacromolecules, leading to lipid peroxidation, the oxidation of amino acid residues (especially cysteine residues), the formation of protein–protein cross-links and DNA oxidative damage. Under normal physiological conditions, the endogenous ROS are generally produced as a consequence of electron transfer reactions in mitochondria, peroxisomes and cytosol, and can be scavenged by the cellular defending systems including both nonenzymatic (L-ascorbic acid, α-tocopherol, glutathione, etc.) and enzymatic antioxidants (superoxide dismutase (SOD), catalase, glutathione peroxidase, etc.). But under certain pathological conditions, the dynamic balance between the generation and elimination of ROS may be broken and thus the cellular

* Corresponding author. Fax: +86-10-64888566; E-mail: xinwj@sun5.ibp.ac.cn

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ROS levels increase significantly. High levels of ROS may cause the oxidative damage of various cellular components, and finally result in cell death [2].

Nitric oxide (NO) is a poisonous, unstable free radical gas that has been known for years to be a constituent of air pollutant and is involved in the depletion of the ozone layer. However, recent studies revealed that NO can be generated endogenously in several types of cells [3] and plays diverse biological roles including host defense, vascular regulation and neuronal communication [4]. In the mammalian central nervous system, NO exerts a number of important functions including neurotransmission, synaptic plasticity and memory [5,6]. On the other hand, when produced in large excess or produced with ROS concurrently, NO also displays neurotoxicity and can induce apoptotic cell death in different types of neuronal cells. It has been proposed that NO-mediated neuronal injury is involved in several neuronal disorders such as Parkinson's disease [7,8], but the underlying mechanisms are still not clearly known. In this regard, studying the mechanism of NO neurotoxicity is very significant. In our previous work we have found that pretreating cells with antioxidants such as SOD or L-ascorbic acid 2-[3,4-dihydro-2,5,7,8-tetramethyl-2-(4,8,12-trimethyltridecyl)-2*H*-1-benzopyran-6-yl-hydrogen phosphate] potassium salt (EPC-K1; a novel derivative of α-tocopherol and L-ascorbic acid) effectively attenuated NO-induced apoptosis [9], which was in accordance with other reports [10,11], while down-regulation of SOD expression potentiated NO-induced neuronal apoptosis [12]. These findings suggested that ROS may be, at least in part, involved in NO-induced apoptosis. However, there is no report on the direct measurement of endogenous ROS generated in NO-treated cells, and it is still unclear by which pathways NO generates ROS and induces oxidative stress in neuronal cells. In this paper, we directly studied the kinetic formation of endogenous ROS in cerebellar granule cells treated with *S*-nitroso-*N*-acetylpenicillamine (SNAP; a NO donor) for the first time, and the scavenging effects of EPC-K1 on endogenous ROS were examined. The results may help us to understand the mechanisms of NO toxicity more clearly, which will lead to potential therapeutic methods for diseases associated with the excessive generation of NO.

2. Materials and methods

2.1. Materials

Wistar rats were purchased from the Experimental Animal Center of Peking University Medical School (Beijing, China). Cell culture plastic ware was purchased from Corning Costar (Acton, MA, USA). Phenol red-free Dulbecco's modified Eagle's medium (DMEM), cell culture supplements, fetal bovine serum and trypsin (1:250) were products of Gibco BRL (Grand Island, NY, USA). Poly-L-lysine, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid) (HEPES), SNAP, horseradish peroxidase (HRP) and 2,2′-azinobis(3-ethylbenzthiazoline-6-sulfonic acid) diammonium salt (ABTS) were purchased from Sigma (St. Louis, MO, USA). Dihydrorhodamine 123 (DHR123) and 2′,7′-dichloro-fluorescein diacetate (DCFH-DA) were purchased from Molecular Probes (Eugene, OR, USA). EPC-K1 was a generous gift from Senju Pharmaceutical Co. (Osaka, Japan). Other reagents made in China were of analytical grade.

2.2. Cell culture and NO exposure

Primary cultures of rat cerebellar granule cells were prepared following procedures described previously [13]. Briefly, cerebella were dissected out from 7-day-old Wistar rats. After the removal of meninges and blood vessels, cerebella were rinsed with HBSS, minced and dissociated by mild trypsinization. Cells were plated on six-well multidishes (2×10^6 cells/ml, 2 ml/well) or 24-well multidishes (2×10^6 cells/ml, 0.5 ml/well) previously coated with poly-L-lysine. Culture medium consisted of phenol red-free 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.

Forty-eight h after plating, cells were treated with NO by the addition of freshly prepared SNAP stock solution (final concentration 500 μM) and cells were cultured for an indicated time. In some experiments, the antioxidant EPC-K1 (final concentration 25 μM) was added to cells 15 min before the addition of SNAP. The effects of decomposed SNAP on cells

were examined by treating cells with SNAP stock solution that had been incubated at 37°C for 24 h.

2.3. Detection of apoptosis

Apoptosis induced by NO was detected quantitatively by determining the nucleosomal fragmentation of DNA using the Boehringer Mannheim Cell Death Detection ELISA^{PLUS} Kit, which is based on the quantitative sandwich-enzyme-immunoassay principle using mouse monoclonal antibodies directed against DNA and histones [14]. The enrichment factor, which represented the accumulation of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasm, was used as an index of apoptosis.

2.4. Determination of cytosolic ROS

The levels of intracellular ROS were determined by flow cytometry using DCFH-DA as a ROS-sensitive fluorescence probe [15,16]. DCFH-DA is a non-fluorescent compound that can permeate cells freely. When inside cells, it is hydrolyzed to DCFH and is trapped inside cells. Upon oxidation by the cytosolic ROS, it is converted to the fluorescent compound 2′,7′-dichlorofluorescein (DCF), which can be detected by flow cytometry. The oxidation of DCFH-DA is relatively specific for the detection of intracellular ROS.

Cells were treated with 500 μM SNAP for an indicated time, detached with 0.05% trypsin–20 μg/ml DNase I, washed twice with phenol red-free DMEM containing 0.2% bovine serum albumin, and loaded with 1 μM DCFH-DA at 37°C for 45 min. After being washed twice, 10 000 cells were analyzed by a Becton Dickinson FACS420 flow cytometer with excitation set at 488 nm and emission at 525 nm.

2.5. Determination of mitochondrial ROS

DHR123 was used as a fluorescence probe to measure the ROS produced by the mitochondria, namely superoxide, hydrogen peroxide and peroxynitrite [17,18]. Briefly, after exposure to 500 μM SNAP for an indicated time, cells were harvested as described above and loaded with 1 μM DHR123 at 37°C for 25 min. After being washed twice, 10 000 cells were analyzed with a Becton Dickinson FACS420 flow cytometer with excitation set at 488 nm and emission at 525 nm.

2.6. Determination of extracellular hydrogen peroxide

The level of extracellular hydrogen peroxide was determined by the HRP/ABTS method [19], which is based on the HRP-catalyzed oxidation of ABTS. Cells cultured in phenol red-free DMEM were exposed to 500 μM SNAP for 24 h. Then 0.4 ml of cell culture supernatant was mixed with 0.4 ml of reaction mixture (5 mM ABTS, 0.2 U HRP, 0.2 M acetate buffer, pH 4.0). After incubation at 37°C for 20 min, the absorption at 420 nm was measured immediately. Hydrogen peroxide was used as an external standard.

2.7. Determination of thiobarbituric acid reactive substances (TBARS)

TBARS were used as an index of lipid peroxidation [20]. Briefly, 1.2×10^7 cells exposed to SNAP for an indicated time were washed twice with phosphate-buffered saline, mixed with 0.4 ml of 2.8% trichloroacetic acid and 0.6 ml of 0.67% thiobarbituric acid, and heated at 95°C for 1 h. After being cooled, *n*-butanol was added followed by vigorous shaking. The absorption of the organic layer was determined at 532 nm.

2.8. Statistical analysis

Each experiment was performed at least three times and the results are presented as mean ± S.D. The data were analyzed by one-way analysis of variance (ANOVA). A level of $P < 0.05$ was considered significant.

3. Results

3.1. NO-induced apoptosis

After incubation with 500 μM of SNAP which generates NO upon decomposition, cerebellar granule cells underwent apoptosis gradually, which was characterized biochemically by the internucleosomal

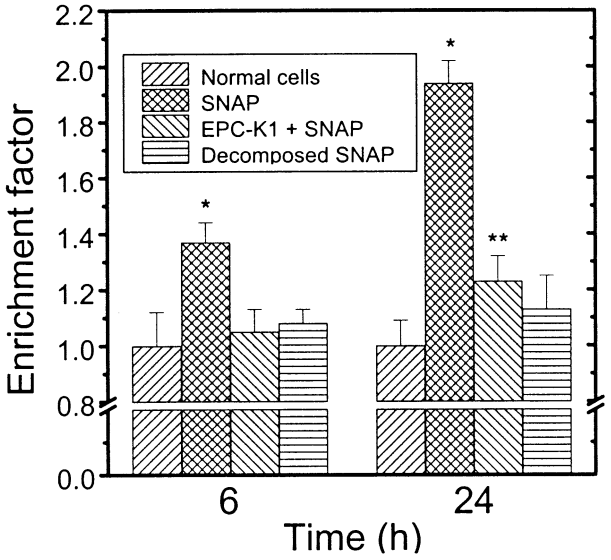

Fig. 1. Quantitative determination of DNA nucleosomal fragmentation of cerebellar granule cells exposed to NO donor SNAP. The cerebellar granule cells were pretreated with 25 μmol/l EPC-K1 for 15 min and then exposed to 500 μmol/l SNAP for indicated time. The DNA fragments were quantified by ELISA. An enrichment factor was employed as an index of DNA fragmentation. Data are mean ± S.D. of three samples. $*P < 0.05$ in comparison with normal cells; $**P < 0.05$ in comparison with cells exposed to peroxynitrite.

fragmentation of DNA measured by enzyme-linked immunosorbent assay (ELISA). The enrichment factor, which represented the accumulation of histone-associated DNA fragments (mono- and oligonucleosomes) in the cytoplasm, was used as an index of DNA fragmentation. Fig. 1 showed that in cerebellar granule cells treated with 500 μM SNAP for 24 h, the enrichment factor was 1.94 ± 0.017, which was significantly higher than that of normal cells (1.00 ± 0.009). The enrichment factor of EPC-K1-pretreated cells was 1.23 ± 0.09, suggesting that pretreatment with the antioxidant EPC-K1 (25 μM) could effectively prevent NO-induced DNA fragmentation. Decomposed SNAP showed no significant effect on cells, suggesting that SNAP induced apoptosis by releasing NO.

3.2. Formation of intracellular and mitochondrial ROS

ROS generated endogenously inside cells could be detected by flow cytometry using oxidant-sensitive fluorescence probe DCFH-DA or DHR123. NO itself may also oxidize DCFH-DA and DHR123 directly. To exclude the interference of NO, the NO donor SNAP added to cells was washed out before loading with the probes. Under our experimental conditions the formation of the fluorescent species

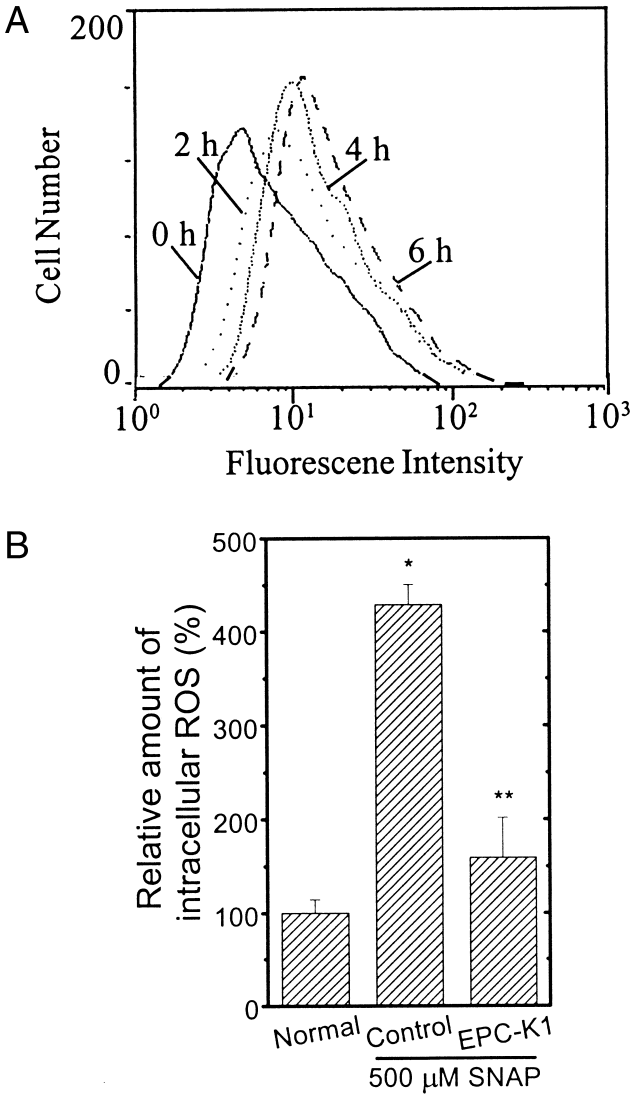

Fig. 2. (A) Time-course of NO-induced cytosolic ROS formation in cerebellar granule cells. Cells were exposed to 500 μmol/l SNAP for 0, 2, 4 and 6 h, respectively, and then loaded with 1 μmol/l DCFH-DA for 45 min and analyzed by flow cytometry. (B) Effects of EPC-K1 on NO-induced cytosolic ROS formation in cerebellar granule cells. Cells were pretreated with 25 μmol/l EPC-K1 for 15 min and then exposed to 500 μmol/l SNAP for 6 h, loaded with 1 μmol/l DCFH-DA for 45 min and analyzed by flow cytometry. Data are mean ± S.D. of three samples. $*P < 0.01$ in comparison with normal cells; $**P < 0.01$ in comparison with cells exposed to NO donor SNAP.

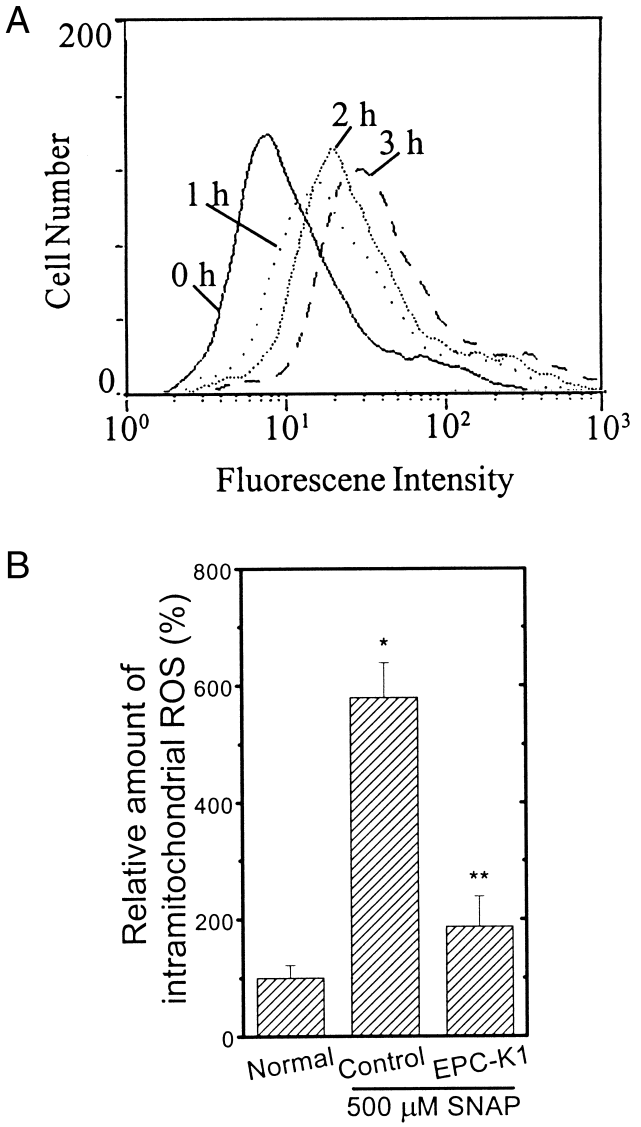

Fig. 3. (A) Time-course of NO-induced mitochondrial ROS formation in cerebellar granule cells. Cells were exposed to 500 μmol/l SNAP for 0, 1, 2 and 3 h, respectively, and then loaded with 1 μmol/l DHR123 for 25 min and analyzed by flow cytometry. (B) Effects of EPC-K1 on NO-induced mitochondrial ROS formation in cerebellar granule cells. Cells were pretreated with 25 μmol/l EPC-K1 for 15 min and then exposed to 500 μmol/l SNAP for 3 h, loaded with 1 μmol/l DHR123 for 25 min and analyzed by flow cytometry. Data are mean ± S.D. of three samples. $*P < 0.01$ in comparison with normal cells; $**P < 0.01$ in comparison with cells exposed to NO donor SNAP.

DCF and Rh123 was due to the endogenous ROS generated within the cerebellar granule cells.

Fig. 2A showed the kinetic formation of intracellular ROS using DCFH-DA as the fluorescence probe. The exposure of cells to SNAP caused a time-dependent increase in the cytosolic ROS level as measured by flow cytometry. The cytosolic ROS reached the maximum level 6 h after the addition of SNAP, which was 429% of the normal cells. Then the cytosolic ROS level decreased gradually. In cells pretreated with EPC-K1, the formation of cytosolic ROS was suppressed significantly as shown in Fig. 2B.

Fig. 3A showed the kinetic formation of mitochondrial ROS measured by flow cytometry using DHR123 as the fluorescence probe. Similarly, the exposure of cells to SNAP caused a time-dependent increase in the mitochondrial ROS level. The mitochondrial ROS reached the maximum level 3 h after the addition of SNAP, which is 579% of the normal cells. Pretreatment with EPC-K1 also inhibited the formation of mitochondrial ROS effectively as shown in Fig. 3B.

3.3. Accumulation of extracellular hydrogen peroxide

Upon exposure to NO donor SNAP, the extracellular hydrogen peroxide level increased time-dependently as shown in Fig. 4. The extracellular hydrogen

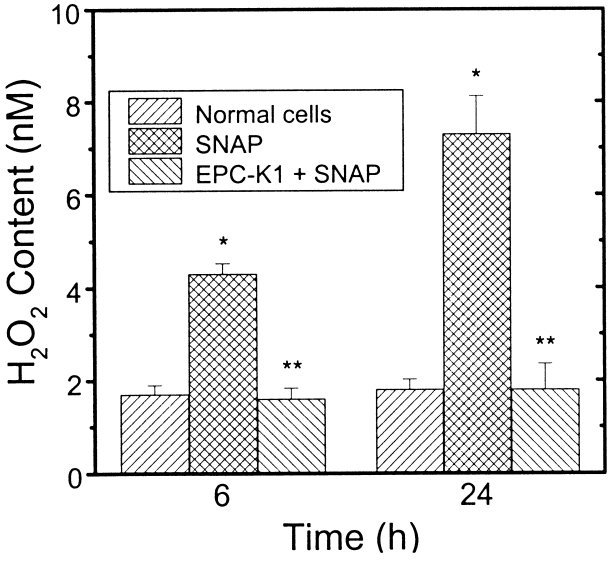

Fig. 4. Extracellular hydrogen peroxide accumulation in NO-treated cerebellar granule cells. Cells were exposed to 500 μmol/l SNAP for indicated time and the extracellular hydrogen peroxide was determined. Data are mean ± S.D. of six samples. $*P < 0.01$ in comparison with normal cells; $**P < 0.01$ in comparison with cells exposed to NO donor SNAP.

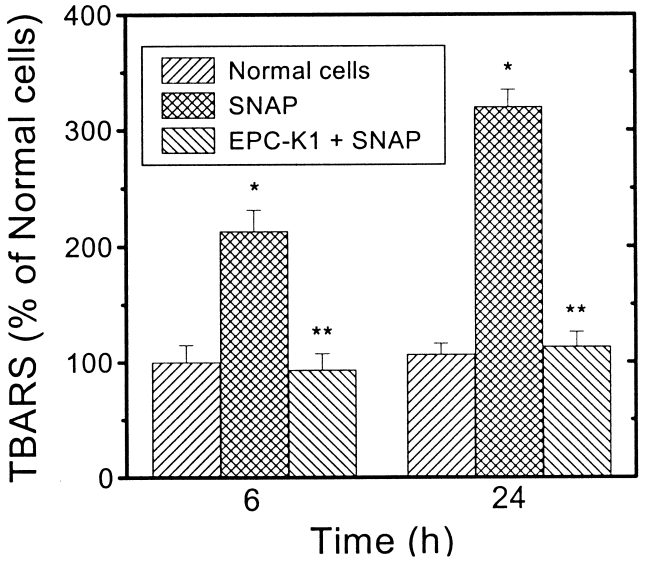

Fig. 5. Lipid peroxidation in NO-treated cerebellar granule cells. Cells were exposed to 500 μmol/l SNAP for indicated time and the TBARS was determined by TBA assay. Data are mean ± S.D. of three samples. $*P < 0.01$ in comparison with normal cells; $**P < 0.01$ in comparison with cells exposed to NO donor SNAP.

peroxide level was 7.3 ± 0.82 nM 24 h after the addition of SNAP, which was much higher than that of normal cells (1.8 ± 0.22 nM). Pretreatment with EPC-K1 effectively prevented the accumulation of extracellular hydrogen peroxide.

3.4. Lipid peroxidation in cells

Treatment with SNAP induced lipid peroxidation in cerebellar granule cells as measured by the TBA assay. In cells exposed to 500 μM SNAP for 24 h, the TBARS level in cells was 320 ± 14.7% of normal cells as shown in Fig. 5. Pretreatment with EPC-K1 inhibited the lipid peroxidation induced by SNAP significantly.

4. Discussion

Under certain pathological conditions such as Parkinsonism [8] and ischemic brain injury [21], a high concentration of NO is synthesized by neurons or activated glial cells. A high concentration of NO could induce apoptotic cell death in neuronal cells, and thus cause neuronal injury. The mechanisms of NO-induced apoptosis are not clearly known yet. Many pathways, such as the interaction with excitatory amino acid receptors [22], the depletion of cellular NAD^+ [23], and the activation of caspases [24] are involved in the cascade of events leading to NO-induced apoptosis. Considering that antioxidants could effectively prevent NO-induced neuronal apoptosis, it has been proposed that the induction of oxidative stress may also be involved in NO-induced apoptosis [9,12]. However, this hypothesis still lacks experimental evidence. In the present investigation, we measured the ROS content of rat cerebellar granule cells treated with NO, and found that SNAP-induced neuronal apoptosis was accompanied by the accumulation of intracellular ROS. The results strongly support the hypothesis that oxidative stress is one of the important pathways leading to neuronal apoptosis induced by NO.

Accumulated evidence indicates that mitochondria are the major location for the generation of endogenous ROS under certain pathological conditions [25,26]. In the present study, we studied the generation of mitochondrial ROS by flow cytometry using a specific redox-sensitive fluorescence probe, DHR123. Upon exposure of cells to SNAP, the mitochondrial ROS levels increased to the maximum within 3 h as measured by flow cytometry, suggesting that SNAP caused the formation of ROS in mitochondria. By a similar method using DCFH-DA as the fluorescence probe, we found that the intracellular ROS levels increased to the maximum 6 h after treatment with SNAP. Generally, the production of ROS by mitochondria is a consequence of the blockade of the electron transfer chain. It has been well documented that NO can inhibit the activity of several enzymes of the mitochondrial respiratory chain including complex I, complex II–III, and complex IV in neuronal cells [27]. The inhibition of mitochondrial respiration by NO may increase the electron leakage, and cause the formation of endogenous ROS (mainly superoxide anion), which was observed in submitochondrial particles [28]. Superoxide anion is a relatively stable oxygen free radical. After being protonized, it can diffuse across the biological membranes, including the mitochondrial and cytoplasmic membranes, and then convert to hydrogen peroxide inside the cells or in the extracellular circumstances by both enzymatic reaction (SOD-catalyzed dismuta-

tion) and non-enzymatic reaction (Harber–Weiss reaction). It has been reported that NO can potentiate the cytotoxicity of hydrogen peroxide [29]. Furthermore, NO reacts with superoxide at a high rate ($k \geq 6.7 \times 10^9$ M^{-1} s^{-1}) [30] to form peroxynitrite, which is far more reactive and damaging than its precursors. These downstream products of superoxide, including hydrogen peroxide and peroxynitrite, are potent oxidants which induced oxidative injury of cells and resulted in apoptosis. Pretreating cells with the novel antioxidant EPC-K1 effectively inhibited the formation of endogenous ROS, and prevented cells from apoptosis.

It has been proposed that NO is a potential pathogen of several neuronal diseases. In this paper, we directly prove that the formation of endogenous ROS is an important pathway leading to NO-induced neurotoxicity. Accordingly, some effective antioxidants that can scavenge the endogenous ROS, could protect neuronal cells from apoptosis effectively and might be used as potential drugs for NO-associated neuronal diseases. EPC-K1 is such an effective antioxidant. Our previous reports showed that EPC-K1 is a potent scavenger on both hydrophilic and hydrophobic free radicals, and can increase the antioxidant capacity of brain in rats [31]. In cells pretreated with EPC-K1, the oxidative stress and apoptosis induced by NO is effectively attenuated. The results of the present investigation suggest that EPC-K1 might be used as a potential drug for diseases associated with the excessive production of NO.

References

[1] B. Halliwell, J.M.C. Gutteridge, Role of free radicals and catalytic metal ions in human disease: an overview, Methods Enzymol. 186 (1990) 1–85.
[2] M.D. Jacobson, Reactive oxygen species and programmed cell death, Trends Biochem. Sci. 21 (1996) 83–86.
[3] B. Mayer, B. Hemmens, Biosynthesis and action of nitric oxide in mammalian cells, Trends Biochem. Sci. 22 (1997) 477–481.
[4] H.H.H.W. Schmidt, U. Walter, NO at work, Cell 78 (1994) 919–925.
[5] C. Hölscher, Nitric oxide, the enigmatic neuronal messenger: its role in synaptic plasticity, Trends Neurosci. 20 (1997) 298–303.
[6] R.D. Hawkins, NO honey, I don't remember, Neuron 16 (1996) 465–467.
[7] S.J.R. Heales, J.P. Bolaños, V.C. Stewart, P.S. Brookes, J.M. Land, J.B. Clark, Nitric oxide, mitochondria and neurological disease, Biochim. Biophys. Acta 1410 (1999) 215–228.
[8] E.C. Hirsch, S. Hunot, Nitric oxide, glial cells and neuronal degeneration in parkinsonism, Trends Pharmacol. Sci. 21 (2000) 163–165.
[9] T.T. Wei, C. Chen, J.W. Hou, B.L. Zhao, W.J. Xin, A. Mori, The antioxidant EPC-K1 attenuates NO-induced mitochondrial dysfunction, lipid peroxidation and apoptosis in cerebellar granule cells, Toxicology 134 (1999) 117–126.
[10] S.A. Lipton, Y.B. Choi, Z.H. Pan, S.Z. Lei, H.S.V. Chen, N.J. Sucher, J. Loscalzo, D.J. Singel, J.S. Stamler, A redox-based mechanism for the neuroprotective and neurodestructive effects of nitric oxide and related nitroso-compounds, Nature 364 (1993) 626–632.
[11] E.M. Grasbon-Frodl, P. Brundin, Mesencephalic neuron death induced by congerners of nitrogen monoxide is prevented by the lazaroid U-83836E, Exp. Brain Res. 113 (1997) 138–143.
[12] C.M. Troy, D. Derossi, A. Prochiantz, L.A. Greene, M.L. Shelanski, Downregulation of Cu/Zn superoxide dismutase leads to cell death via the nitric oxide–peroxynitrite pathway, J. Neurosci. 16 (1996) 253–261.
[13] Y.C. Ni, B.L. Zhao, J.W. Hou, W.J. Xin, Preventive effect of Ginkgo biloba extract on apoptosis in rat cerebellar neuronal cells induced by hydroxyl radicals, Neurosci. Lett. 214 (1996) 115–118.
[14] M. Sandoval, X.J. Zhang, X.P. Liu, E.E. Mannick, D.A. Clark, M.J.S. Miller, Peroxynitrite-induced apoptosis in T84 and RAW 264.7 cells: attenuation by L-ascorbic acid, Free Radic. Biol. Med. 22 (1997) 489–495.
[15] Y.H. Li, P. Maher, D. Schubert, A role for 12-lipoxygenase in nerve cell death caused by glutathione depletion, Neuron 19 (1997) 453–463.
[16] H.D. Teixeira, R.I. Schumacher, R. Meneghini, Lower intracellular hydrogen peroxide levels in cells overexpressing CuZn-superoxide dismutase, Proc. Natl. Acad. Sci. USA 95 (1998) 7872–7875.
[17] A.C. Rego, M.S. Santos, C.R. Oliveira, Influence of the antioxidants vitamin E and idebenone on retinal cell injury mediated by chemical ischemia, hypoglycemia, or oxidative stress, Free Radic. Biol. Med. 26 (1999) 1405–1417.
[18] R.J. Mark, J.N. Keller, I. Kruman, M.P. Mattson, Basic FGF attenuates amyloid β-peptide-induced oxidative stress, mitochondrial dysfunction, and impairment of Na^+, K^+-ATPase activity in hippocampal reurons, Brain Res. 756 (1997) 205–214.
[19] Y. Higuchi, S. Matsukawa, Active oxygen-mediated chromosomal 1–2 Mbp giant DNA fragmentation into internucleosomal DNA fragmentation in apoptosis of glioma cells by glutamate, Free Radic. Biol. Med. 24 (1998) 418–426.
[20] M.G. Salgo, W.A. Pryor, Trolox inhibits peroxynitrite-medi-

ated oxidative stress and apoptosis in rat thymocytes, Arch. Biochem. Biophys. 333 (1996) 482–488.
[21] C. Iadecola, Bright and dark sides of nitric oxide in ischemic brain injury, Trends Neurosci. 20 (1997) 132–139.
[22] E. Bonfoco, M. Leist, B. Zhivotovsky, S. Orrenius, S.A. Lipton, P. Nicotera, Cytoskeletal breakdown and apoptosis elicited by NO donors in cerebellar granule cells require NMDA receptor activation, J. Neurochem. 67 (1996) 2484–2493.
[23] U.K. Meßmer, B. Brüne, Nitric oxide (NO) in apoptotic versus necrotic RAW 264.7 macrophage cell death: the role of NO-donor exposure, NAD^+ content, and p53 accumulation, Arch. Biochem. Biophys. 327 (1996) 1–10.
[24] L. Boscá, S. Hortelano, Mechanisms of nitric oxide-dependent apoptosis: involvement of mitochondrial mediators, Cell. Signal. 11 (1999) 239–244.
[25] L.L. Dugan, S.L. Sensi, L.M.T. Canzoniero, S.D. Handran, S.M. Rothman, T.S. Lin, M.P. Goldberg, D.W. Choi, Mitochondrial production of reactive oxygen species in cortical neurons following exposure to *N*-methyl-D-aspartate, J. Neurosci. 15 (1995) 6377–6388.
[26] G. Du, A. Mouithys-Mickalad, F.E. Sluse, Generation of superoxide anion by mitochondria and impairment of their functions during anoxia and reoxygenation in vitro, Free Radic. Biol. Med. 25 (1998) 1066–1074.
[27] J.P. Bolaños, A. Almeida, V. Stewart, S. Peuchen, J.M. Land, J.B. Clark, S.J.R. Heales, Nitric oxide-mediated mitochondrial damage in the brain: mechanisms and implications for neurodegenerative diseases, J. Neurochem. 68 (1997) 2227–2240.
[28] J.J. Poderoso, M.C. Carreras, C. Lisdero, N. Riobo, F. Schopfer, A. Boveris, Nitric oxide inhibits electron transfer and increases superoxide radical production in rat heart mitochondria and submitochondrial particals, Arch. Biochem. Biophys. 328 (1996) 85–92.
[29] I. Ioannidis, H. DeGroot, Cytotoxicity of nitric oxide in FU5 hepatoma cells: evidence for co-operative action with hydrogen peroxide, Biochem. J. 296 (1993) 341–345.
[30] R.E. Huie, S. Padmaja, Reaction of $^{\bullet}NO$ with $^{\bullet}O_2^-$, Free Radic. Res. Commun. 18 (1993) 195–199.
[31] T.T. Wei, C. Chen, F.M. Li, B.L. Zhao, J.W. Hou, W.J. Xin, A. Mori, Antioxidant properties of EPC-K1: a study on mechanisms, Biophys. Chem. 77 (1999) 153–160.