Free Rad. Res., Vol. 29, pp. 359–365
Reprints available directly from the publisher
Photocopying permitted by license only

Oral Administration of (−)Catechin Protects against Ischemia-Reperfusion-induced Neuronal Death in the Gerbil

O. INANAMI[a,*], Y. WATANABE[b], B. SYUTO[b], M. NAKANO[c], M. TSUJI[a] and M. KUWABARA[a]

[a]*Department of Radiation Biology, Faculty of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan;*
[b]*Department of Veterinary Physiology, Faculty of Agriculture, Iwate University, Morioka 020-0066, Japan;*
[c]*Institute for Medical Science of Aging, Aichi Medical University, Nagakute 480-1103, Japan*

Accepted by Prof. N. Taniguchi

(Received 29 May 1998; In revised form 15 June 1998)

The effect of *ad libitum* oral-administration of (−)catechin solution on ischemia-reperfusion-induced cell death of hippocampal CA1 in the gerbil was histologically examined. When (−)catechin solution instead of drinking water was orally administered *ad libitum* for 2 weeks, dose-dependent protection against neuronal death following by transient ischemia and reperfusion was observed. To evaluate the involvement of reduction of reactive-oxygen-species (ROIs) by the antioxidant activity of (−)catechin in this protection, the superoxide scavenging activity of the brain in catechin-treated gerbils was measured by ESR and spin-trapping using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The superoxide scavenging activities of the brains obtained from catechin-treated gerbils were significantly higher than those of catechin-untreated animals. From these results, it was suggested that orally administered (−)catechin was absorbed, passed through the blood–brain barrier and that delayed neuronal death of hippocampal CA1 after ischemia-reperfusion was prevented due to its antioxidant activities.

Keywords: (−)Catechin, flavonoid, gerbil, hippocampus, ischemia-reperfusion, reactive-oxygen-species (ROIs)

INTRODUCTION

Flavonoid-related compounds contained in beverages, vegetables and fruits, etc., have been reported to have beneficial effects in various pathological conditions, including cancer,[1] hypertension[2] and allergy.[3] It is believed that these protective effects of flavonoids are due to their antioxidant properties. In fact, it was demonstrated that (−)epicatechin, (−)epicatechin gallate and quercetin serve as powerful antioxidants against lipid peroxidation when phospholipid bilayers are exposed to aqueous oxygen free radicals *in vitro*.[4–6] In the central nervous system (CNS), Yoneda *et al.*[7] showed that oral administration of a flavonoid-rich tea extract prevents iron-salt-induced lipid peroxide accumulation in the rat brain. Recently, we reported that the oral administration of rooibos

*Corresponding author. Tel.: +81-011-706-5237. Fax: +81-011-706-5236. E-mail: inanami@vetmed.hokudai.ac.jp.

tea (*Aspalathus linealis*) containing flavonoid derivatives suppresses age-related accumulation of neurotoxic lipid peroxides in rat brain.[8] These results suggested that flavonoids could pass through the blood–brain barrier and penetrate into the brain, and then protect against cell damage induced by oxidative stress.

Delayed neuronal death in hippocampal CA1 of the brain is known to be induced by transient ischemia and reperfusion,[9] and the induction of this cell death is associated with generation of reactive-oxygen-species (ROIs) just after ischemia.[10,11] It was demonstrated that the ROIs are abruptly produced by ischemia-activated hypoxanthine-xanthine oxidase (Hxn-XOD).[10] The ROIs from the respiratory chains in mitochondria are also known to be produced by NMDA receptors following ischemia reperfusion.[11] In this experiment, to evaluate the protective effect of flavonoids on CNS injury involving in ROIs, we examined whether the oral administration of (−)catechin inhibited this delayed neuronal death in gerbils. The superoxide scavenging activity of the brain of the catechin-treated gerbil was also examined by ESR and spin-trapping using 5,5-dimethyl-1-pyrroline-N-oxide (DMPO).

MATERIALS AND METHODS

Mongolian gerbils (*Meriones unguiculatus*) weighing 65–90 g were used. They were group-housed in a facility in accordance with the *Guide for the Care and Use of Laboratory Animals* of Iwate University with water and food *ad libitum* on a 12 : 12 h light : dark cycle. (−)Catechin (*Camellia sinensis* from green tea, Mitsui Norin Co. Japan) was dissolved in sterile water and administration was performed by oral *ad libitum* uptake of this (−)catechin solution instead of drinking water from 2 weeks prior to ischemia until 1 week after ischemia. Four groups, (1) sham operation without ischemia-reperfusion (control); (2) ischemia-reperfusion without administration of (−)catechin; (3) ischemia-reperfusion with oral administration of 0.1 mg/ml of (−)catechin; and (4) ischemia-reperfusion with oral administration of 1.0 mg/ml of (−)catechin, were used for the experiment. The uptake of groups receiving 0.1 mg/ml and 1.0 mg/ml (−)catechin were roughly estimated as 5 mg/kg/day and 50 mg/kg/day by consumption (about 4.0 ml/day/gerbil) of (−)catechin solution per day, respectively.

Surgery to induce transient focal brain ischemia and histological examination were performed under pentbarbital anesthesia (50 mg/kg, i.p.). Briefly, bilateral common carotid arteries were occluded with an aneurysm clip for 5 min under anesthesia, and then the skin lesion was sutured. After the gerbils recovered from anesthesia, the animals were allowed free access to water and food as in the normal condition. Seven days later, the animals were sacrificed by decapitation. The brains were removed and coronal sections 12 μm in thickness including the hippocampal region about 1.5 mm caudal to bregma were made with a cryostat microtome (Blight, UK) at −20°C and stained with cresyl violet. The cell damage in the CA1 region induced by ischemia-reperfusion was evaluated by counting neurons per 1 mm linear length of the stratum pyramidale of the CA1 region under a microscope (BH-2, Olympus, Tokyo, Japan).

Superoxide scavenging activity of the brain was assessed by ESR and spin-trapping techniques using DMPO as a spin-trapping reagent.[12] In this experiment, O_2^- was produced by the hypoxanthine-xanthine oxidase system in the presence of DMPO and brain homogenate, and O_2^- that could not be scavenged by brain-associated antioxidants was trapped by DMPO. The amount of DMPO–OOH produced by the reaction of DMPO with O_2^- was measured by ESR spectroscopy. Forebrains were obtained from gerbils treated with 1.0 mg/ml of (−)catechin solution instead of drinking water for 2 weeks as well for the histological experiment. Seven volumes of ice-cold PBS were added to brain

tissue (wet volume) and the brain was homogenized under ice-cold conditions using polytron homogenizer (Ultra-Turrax T25, Ika-Labortechnik, Co. Germany). The homogenate of the brain was diluted to 10-fold with PBS. DMPO was purified twice by activated charcoal and the concentration of DMPO was adjusted by absorbance at 234 nm ($\varepsilon = 7700\,M^{-1} \cdot cm^{-1}$).[13] The reaction mixture was prepared by mixing 100 µl of 1.0 mM hypoxanthine, 50 µl of 0.5 M DMPO and 10 µl of diluted brain homogenate. After 10 µl of XOD (0.2 unit/ml) was added to the reaction solution to start O_2^- production, the sample was immediately transferred to a flat cell ($0.2 \times 10 \times 40$ mm), fitted in the cavity of the ESR spectrometer (TE-200, JEOL Co. Japan). After incubation for 2 min at room temperature, the ESR spectrum was measured. The conditions for recording the ESR spectrum were as follows: time constant, 0.03 s; scan time, 5.0 mT/min; receiver gain, 5×10^2; microwave power, 20 mW; temperature, 23°C.

All statistical analyses were carried out using the Mann–Whitney U-test.

RESULTS AND DISCUSSION

Figure 1 presents photographs of coronal sections containing the hippocampal CA1 region obtained 7 days after the occlusion of bilateral carotid arteries for 5 min from various treatment groups and sham-operation (control). In the ischemia-reperfusion experiment without administration of (−)catechin, the pyramidal cells of the CA1 region in the saline treatment group

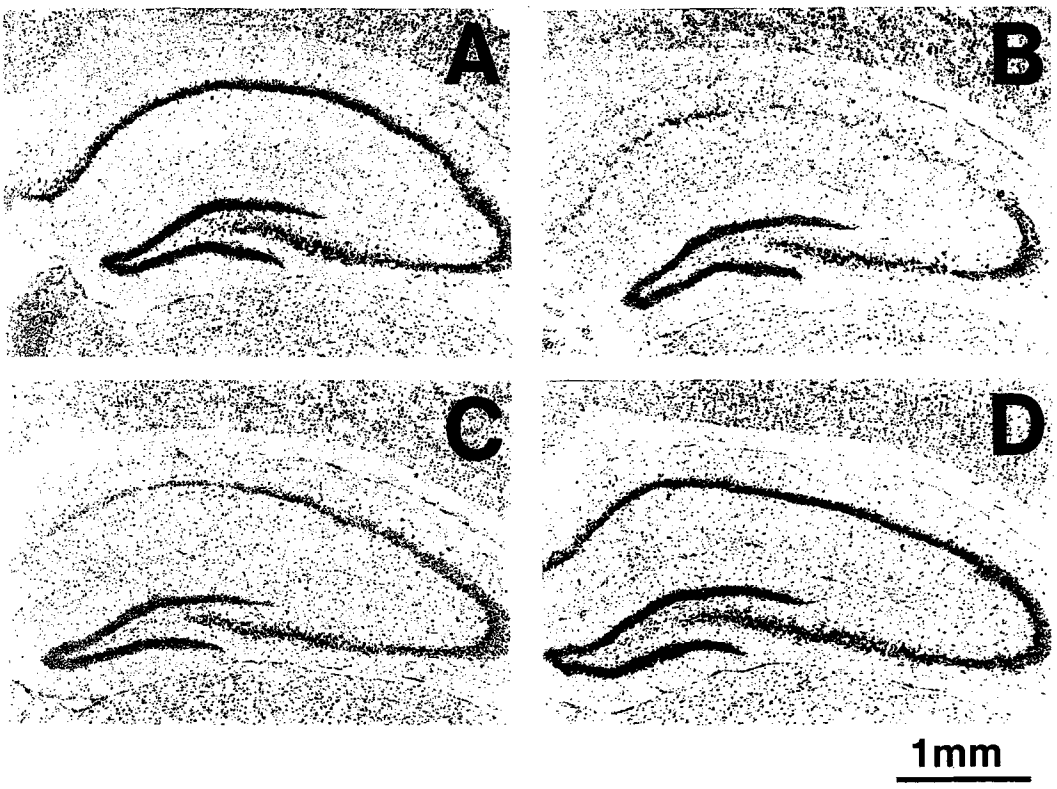

FIGURE 1 Photographs of coronal sections of the hippocampal CA1 region 7 days after 5 min ischemia of the forebrain in gerbils. (A) sham-operation (control); (B) ischemia-reperfusion only; (C) ischemia-reperfusion with *ad libitum* oral administration of 0.1 mg/ml of (−)catechin; (D) ischemia-reperfusion with *ad libitum* oral administration of 1.0 mg/ml of (−)catechin. Each section was stained with cresyl violet. Magnification: ×10. Bar: 1 mm.

completely disappeared after ischemia-reperfusion as shown in Figure 1B (16.98 ± 4.24% in Table I). In the groups with *ad libitum* oral administration of 0.1 mg/ml and 1.0 mg/ml of (−)catechin solution for 2 weeks prior to ischemia, 45.56 ± 12.26% and 96.19 ± 14.74% of cells in CA1 region, respectively, survived as shown in Table I. Thus, it was clearly demonstrated that the ischemia-reperfusion-induced cell death of the CA1 region was dose-dependently inhibited by oral administration of (−)catechin.

Since it was reported that the superoxide anion radical (O_2^-) is primarily produced by xanthine oxidase[10] or mitochondria[11] just after ischemia, we examined the superoxide scavenging activities of the brain of the catechin-treated gerbil. The activity of the antioxidant in the brain was evaluated by inhibition activity of brain homogenate against DMPO-OOH formation induced by the reaction of DMPO with O_2^- produced in the hypoxanthine-xanthine oxidase system. Figure 2A shows the ESR spectrum obtained after the reaction of DMPO and O_2^- in the absence of brain homogenate. This signal consisted of two components of 12 lines (A_N = 1.40 mT, $A_{H\beta}$ = 1.13 mT and $A_{H\gamma}$ = 0.14 mT) and 4 lines (A_N = 1.49 mT and $A_{H\beta}$ = 1.49 mT), respectively. These hyperfine coupling constants corresponded to those of DMPO-OOH and DMPO-OH, which were reported

TABLE I Protective effect of (−)catechin on delayed neuronal death of pyramidal cells in hippocampal CA1 of gerbils

	Surviving neuronal cells in ischemia-reperfusion
Sham operation (control)	100
Ischemia-reperfusion	16.98 ± 4.24*
Ischemia-reperfusion with (−)catechin (0.1 mg/ml)	45.56 ± 12.26*
Ischemia-reperfusion with (−)catechin (1.0 mg/ml)	96.19 ± 14.74^{NS}

(N) = 4 in each group. Surviving cells in a 1 mm linear length of the CA1 region were counted. Values are expressed as mean ± SEM in (%) of control.
*$p < 0.05$; significant difference from control.
NS; no significant difference from control.

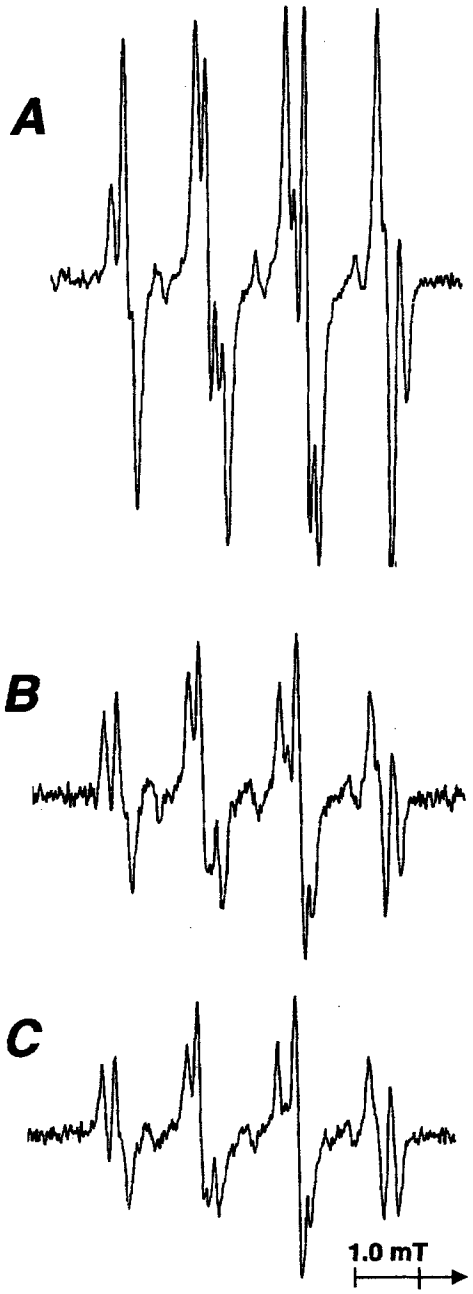

FIGURE 2 Superoxide scavenging activity of brain homogenate with and without catechin-treatment. (A) ESR spectrum obtained from the Hxn-XOD system in the presence of DMPO (control). (B) ESR spectrum obtained from the Hxn-XOD system in the presence of DMPO and brain homogenate of catechin-untreated gerbils. (C) ESR spectrum obtained from the Hxn-XOD system in the presence of DMPO and brain homogenate of catechin-treated gerbils. Experimental details are described in Materials and Methods.

previously.[15,16] When the brain homogenate obtained from catechin-untreated gerbils was added to this system, the intensity of ESR signals originating from DMPO-OOH (Figure 2B) was reduced to about half of the control signal level without homogenate. This result meant that half of the generated O_2^- was scavenged by SOD-like activity contained in brain homogenate. Furthermore, the addition of the brain homogenate from catechin-treated animals (*ad libitum* oral administration of 1.0 mg/ml of (−)catechin solution) to the reaction mixture significantly reduced the signal intensity of DMPO-OOH (Figure 2C) in comparison with that from catechin-untreated animals (Figure 2B). A summary of results obtained from 6 animals in each group is presented in Table II. The catechin-treatment decreased signal intensity originating from DMPO-OOH by about 27%. This meant that superoxide scavenging activity in the brain was increased about 27% by oral administration of (−)catechin. From these results, it was suggested that the (−)catechin passed through blood–brain barrier and that the delayed neuronal death was at least partly prevented by a reduction of O_2^- generation just after ischemia-reperfusion. As another explanation of this protective mechanism, it was also thought that endogenous antioxidants such as superoxide dismutase and glutathion peroxidase were induced by chronic administration of (−)catechin.

TABLE II Superoxide scavenging activities of forebrain in catechin-administrated gerbils

	Intensity of DMPO-OOH adduct
Hxn-XOD complete system (control)	100
+ Brain homogenate of catechin-untreated gerbils	52.2 ± 2.22*
+ Brain homogenate of catechin-treated gerbils	39.5 ± 2.09**

(N) = 6 in each group. Data are expressed as mean ± SEM in (%) of control.
*$p < 0.01$; significant difference from control.
**$p < 0.01$; significant difference from catechin-untreated group.

The mechanism of delayed neuronal death has not been definitely determined. However, in the early stage after ischemia, it is believed that burst release of N-methyl-D-aspartic acid (NMDA) occurs in the hippocampus after transient ischemia[15] and that the resulting stimulation of NMDA receptors in CA1 induces Ca^{2+} influx,[16] activation of Ca^{2+}-dependent protease,[17] phospholipase C,[18] protein kinase C[16] and Ca^{2+}-dependent superoxide production by the mitochondria.[19,20] The possibility of the production of O_2^- from Hxn-XOD system after ischemia was also reported independent of NMDA receptors.[10] At 2 days after ischemia, the oligometric laddering of DNA in the hippocampus as a biochemical marker of apoptosis was observed.[21] On the other hand, it was also reported that several flavonoids, including (−)catechin, have not only antioxidant activity[4–6] but also activity of inhibition of protein kinase C.[22] Therefore, in addition to reducing ROIs by the radical scavenging activity of (−)catechin, the inhibition of some signal transduction pathway to lead to cell death such as protein kinase C may participate in the protection of neuronal cells.

Recently, nitric oxide derived from inducible nitric oxide synthase (iNOS) was also observed from 2 to 3 days after injury in rat cerebral cortex.[23] Furthermore, it was also reported that iNOS is induced by NFκB as a transcription factor that is regulated by the intracellular redox-state[24] and antioxidants, i.e., pyrrolidine dithiocarbamate (PDTC)[25] and α-phenyl-N-*tert*-butylnitrone (PBN),[26] inhibit induction of iNOS by changing the intracellular redox-state. Recently, we demonstrated that the existence of PBN from 2 to 3 days after transient ischemia was essential for protection against ischemia-reperfusion-induced cell death (unpublished data). From this information, the prevention of cell death observed in this experiment may be explained by inhibition of the induction of iNOS, which produces neurotoxic nitric oxide.

In summary, our experiments showed that catechin treatment enhanced the antioxidant

activity in the brain and protected neuronal cells against death. We cannot describe the exact protection mechanism of the flavonoid but, considering the biological multi-functions of flavonoid-related compounds, it is inferred that not only reduction of ROIs but also inhibition of early and late events in the process of cell death contribute to protection of neuronal cells. Finally, from the point of view of development of safe food products and additives, to prevent oxidative-stress-induced cell injury in the CNS, it is noteworthy that oral uptake of a flavonoid was effective against ischemia-induced neuronal cell damage *in vivo*.

Acknowledgements

This work was supported, in part, by Grants-in-Aid for Basic Scientific Research from the Ministry of Education, Science, Sports and Culture, Japan (No. 09660311[O.I.], No. 08456146 [B.S.] and No. 09460133[M.K.] and No. 08308032[M.K.]). We are grateful to Prof. K. Taniguchi, Department of Veterinary Anatomy in Iwate University, for the use of his cryostat microtome.

References

[1] Y. Hara, S. Matsuzaki and K. Nakamura (1989) Anti-tumor activity of tea catechin. *Journal of Japanese Society of Nutrition Food Science*, **42**, 39–45.

[2] Y. Hara and F. Tonooka (1990) Hypotensive effect of tea catechins on blood pressure of rats. *Journal of Japanese Society of Nutrition Food Science*, **43**, 345–348.

[3] Y. Maeda, M. Yamamoto, T. Matui, K. Suguyama, N. Yokota, K. Nakagomi, H. Tanaka, T. Takahashi and T. Kobayashi (1989) Inhibitory effect of tea extracts on histamine release from mast cells. *Journal of Food Hygiene Society*, **30**, 295–299.

[4] T. Matuzaki and Y. Hara (1985) Antioxidative activity of tea leaf catechin. *Journal of Agricultural Chemical Society of Japan*, **59**, 129–134.

[5] J. Terao, M. Piskula and Q. Yao (1994) Protective effect of epicatechin, epicatechin gallate, and quercetin on lipid peroxidation in phospholipid bilayers. *Archives Biochemistry and Biophysics*, **308**, 278–284.

[6] N. Salah, N.J. Miller, G. Paganga, L. Tijburg, G.P. Bolwell and C. Rice-Evance (1995) Polyphenolitic flavonols as scavengers of aqueous phase radicals and as chain-breaking antioxidants. *Archives Biochemistry and Biophysics*, **322**, 339–346.

[7] T. Yoneda, M. Hiramatsu, N. Sakamoto, K. Togasaki, M. Komatsu and K. Yamaguchi (1995) Antioxidant effects of "β catechin". *Biochemistry and Molecular Biology International*, **35**, 995–1008.

[8] O. Inanami, T. Asanuma, N. Inukai, T. Jin, S. Shimokawa, N. Kasai, M. Nakano, F. Sato and M. Kuwabara (1995) The suppression of age-related accumulation of lipid peroxides in rat brain by administration of Rooibos tea (*aspalathus linearis*). *Neuroscience Letters*, **196**, 85–88.

[9] T. Kirino (1982) Delayed neuronal death in the gerbil hippocampus following ischemia. *Brain Research*, **239**, 57–69.

[10] J.W. Phillis, S. Sen and X. Cao (1994) Amflutizole, a xanthine oxidase inhibitor, inhibits free radical generation in the ischemic/reperfused rat cerebral cortex. *Neuroscience Letters*, **169**, 188–190.

[11] N. Lafon-Cazal, S. Pletrl, M. Culcasl and J. Bockaert (1993) NMDA-dependent superoxide production and neurotoxity. *Nature*, **364**, 535–537.

[12] M. Kuwabara, N. Inukai, O. Inanami, Y.-I. Miyake, N. Tsunoda, Y. Maki and F. Sato (1996) Lipid peroxide levels and superoxide-scavenging abilities of sera obtained from hotbred (Thoroughbred) horses. *Journal of Veterinary Medical Science*, **58**, 97–101.

[13] G.R. Rosen and E.J. Rauckman (1984) Spin-trapping of superoxide and hydroxyl radicals. In *Methods in Enzymology*, 105 (Ed. L. Packer), Academic Press, London, pp. 198–209.

[14] E.G. Janzen (1984) Spin-trapping. In *Methods in Enzymology*, 105 (Ed. L. Packer), Academic Press, London, pp. 188–198.

[15] H. Benveniste, J. Drejer, A. Shousboe and N.H. Diemer (1984) Elevation of the extracellular concentrations of glutamate and aspartate in the rat hippocampus during transient cerebral ischemia monitoring by intracerebral microdialysis. *Journal of Neurochemistry*, **43**, 1369–1374.

[16] A. Mitani, S. Takeyasu, H. Yanase, Y. Nakamura and K. Kataoka (1994) Changes in intracellular Ca^{2+} and energy levels during *in vitro* ischemia in the gerbil hippocampus slice. *Journal of Neurochemistry*, **62**, 626–634.

[17] N. Ogata, Y. Yonekawa, W. Taki, R. Kannagi, T. Murachi, T. Hamakubo and H. Kikuchi (1989) Degradation of neurofilament protein in cerebral ischemia. *Journal of Neurosurgery*, **70**, 103–107.

[18] T. Hattori, Y. Nishimura, N. Sakai, H. Yamada, Y. Kameyama, Y. Banno and Y. Nozawa (1987) Inhibitory effect of pentobarbital on phospholipase C activity in ischaemic rat brain. *Neurological Research*, **9**, 164–168.

[19] M. Lafon-Cazal, S. Pietri, M. Culcasi and J. Bockaert (1993) NMDA-dependent superoxide production and neurotoxicity. *Nature*, **364**, 535–537.

[20] L.L. Dugan, S.L. Sensi, L.M.T. Canzoniero, S.D. Handran, S.N. Rothman, T.-S. Lin, M.P. Goldberg and D.W. Choi (1995) Mitochondrial production of reactive oxygen species in cortical neurons following exposure to N-methyl-D-aspartate. *Journal of Neuroscience*, **15**, 6377–6388.

[21] J.P. MacManus, I.E. Hill, E. Preston, I. Rasquinha, T. Walker and A.M. Buchan (1995) Differences in DNA fragmentation following transient cerebral or decapitation ischemia in rats. *Journal of Cerebral Blood Flow Metabolism*, **15**, 728–737.

[22] G.M. Polya and L.Y. Foo (1994) Inhibition of eukaryote signal-regulated protein kinases by plant-derived catechin-related compounds. *Phytochemistry*, **35**, 1399–1405.

[23] K. Yamanaka, E. Kumara, K. Iwatsuki, T. Yoshimine, Y. Masana, T. Hayakawa, T. Shiga and H. Kosaka (1995) Increase in plasma nitric oxide and products following rat cortical injury. *Neuroscience Letters*, **194**, 124–126.

[24] D.J. Jamieson, S.L. Rivers and D.W. Stephen (1994) Analysis of Saccharomyces cerevisiae proteins induced by peroxide and superoxide stress. *Microbiology*, **140**, 3277–3283.

[25] Q.-W. Xie, R. Whisnant and C. Nathan (1993) Promoter of the mouse gene encoding calcium-independent nitric oxide synthase confers inducibility by interferon γ and bacterial lipopolysaccharide. *Journal of Experimental Medicine*, **177**, 1779–1784.

[26] T. Miyajima and Y. Kotake (1995) Spin-trapping agent, phenyl N-*tert*-butyl nitrone, inhibits induction of nitric oxide synthase in endotoxin-induced shock in mice. *Biochemical and Biophysical Research Communications*, **215**, 114–121.