The protective effect of M40401, a superoxide dismutase mimetic, on post-ischemic brain damage in Mongolian gerbils

Vincenzo Mollace*1, Michelangelo Iannone2, Carolina Muscoli1,3, Ernesto Palma1, Teresa Granato2, Andrea Modesti4, Robert Nisticò5, Domenicantonio Rotiroti1 and Daniela Salvemini3

Address: 1Faculty of Pharmacy, University of Catanzaro "Magna Graecia", Roccelletta di Borgia, Catanzaro Italy, 2Institute of Neurological Science ISN – Section of Pharmacology, CNR, Roccelletta di Borgia, Catanzaro, Italy, 3Metaphore Pharmaceuticals, 1910 Innerbelt Business Center Dr, St Louis MO 63114, USA, 4Department of Experimental Medicine and Biochemical Science, University of Rome “Tor Vergata”, Rome 00161, Italy and 5Faculty of Pharmacy, University of Calabria – Arcavacata di Rende (CS), Italy

Email: Vincenzo Mollace* - mollace@libero.it; Michelangelo Iannone - iannone@unicz.it; Carolina Muscoli - cmuscoli@metaphore.com; Ernesto Palma - palma@unicz.it; Teresa Granato - granato@cs.cnr.it; Andrea Modesti - modesti@uniroma2.it; Robert Nisticò - gnistico@europarl.eu.int; Domenicantonio Rotiroti - rotiroti@unicz.it; Daniela Salvemini - cmuscoli@metaphore.com

* Corresponding author

Abstract

Background: Overproduction of free radical species has been shown to occur in brain tissues after ischemia-reperfusion injury. However, most of free radical scavengers known to antagonize oxidative damage (e.g. superoxide dismutase, catalase), are unable to protect against ischemia-reperfusion brain injury when given in vivo, an effect mainly due to their difficulty to gain access to brain tissues. Here we studied the effect of a low molecular weight superoxide dismutase mimetic (M40401) in brain damage subsequent to ischemia-reperfusion injury in Mongolian gerbils.

Results: In animals undergoing ischemia-reperfusion injury, neuropathological and ultrastructural changes were monitored for 1–7 days either in the presence or in the absence of M40401 after bilateral common carotid artery occlusion (BCCO). Administration of M40401 (1–40 mg/kg, given i.p. 1 h after BCCO) protected against post-ischemic, ultrastructural and neuropathological changes occurring within the hippocampal CA1 area. The protective effect of M40401 was associated with a significant reduction of the levels of malondialdehyde (MDA; a marker of lipid peroxidation) in ischemic brain tissues after ischemia-reperfusion.

Conclusion: Taken together, these results demonstrate that M40401 provides protective effects when given early after the induction of ischemia-reperfusion of brain tissues and suggest the possible use of such compounds in the treatment of neurological dysfunction subsequent to cerebral flow disturbances.
mechanisms involved in post-ischemic damage of nervous tissues have been widely explored over the last few years in order to develop selective and more suitable strategies for the treatment of ischemia/reperfusion-related neurological disorders [1–6]. In particular, mounting evidence suggests that a crucial role in triggering and maintaining the post-ischemic insult to brain tissues is represented by the oxidative stress which follows the reperfusion phase of stroke. Reperfusion of the ischemic brain, excessive release of excitatory amino acids, such as glutamate, and infiltration by neutrophils are major sources of reactive oxygen species (ROS) generation. These, in turn, amplify the profound imbalance found in the neurons and astroglial cells of the ischemic core and penumbra [7]. In particular, the reaction of superoxide anions with nitric oxide (NO), leads to the formation of peroxynitrite (ONOO-) [8], a powerful damaging oxidant and nitrating agent, which may induce many of the permanent ultra structural changes of ischemic brain tissue [9,10].

Despite the large amount of evidence showing the clear relationship between oxidative stress and post-ischemic brain damage, neuroprotection by free radical-scavenging molecules in vivo is not as straightforward as that observed when using in vitro models. In particular, superoxide dismutase (SOD) as well catalase, the catalytic scavengers for superoxide anions or hydrogen peroxide respectively, while producing a significant neuroprotective effect in vitro, were found unsatisfactory when used in experimental stroke models in vivo [11–14]. The reasons for such failures are not clear, but could be related to various factors including rapid clearance, lack of cellular penetration, short half-life and lack of blood brain barrier penetration [15–17]. On the other hand, these enzymes show modest protective effects when treatment is administered before ischemia, but little to no protection in a delayed-treatment protocol [14].

To overcome some of the limitations associated with the native SOD enzymes, low molecular weight synthetic enzymes possessing improved cellular and tissue penetration, with wide organ bio-distribution have been identified. One of these, EUK 134, a low molecular weight salen manganese complex was found to be protective when given in a rat model of focal cerebral ischemic without reperfusion [18]. Since EUK 134 possesses both SOD and catalase activity, these results suggest a role for both superoxide and hydrogen peroxide in brain damage evoked by ischemia without reperfusion.

Recently, a class of non peptidic low-molecular weight compounds has been suggested for assessing an improved therapeutic approach in diseases mediated by superoxide overproduction [19,20]. In particular, evidence exists suggesting that M40401 [21], an SOD mimetic compound able to gain access to brain tissues, produces protective effect against oxidative damage generated in the brain of paraquat-treated rats, when given peripherally [22]. These new SOD mimetics represent a breakthrough in chemical design in that they are stable in vivo, possess high activity, and are selective for superoxide with no activity toward H2O2, peroxynitrite, nitric oxide, or hypochlorite [23–25].

The present experiments have been carried out in order to study 1) the possible role of superoxide in electrocortical and ultra structural changes subsequent to brain damage associated with ischemia and reperfusion induced by unilateral occlusion of carotid artery (BCCO) in Mongolian gerbils, and 2) the protective effect of a SOD mimetic (M40401) which lacks catalase activity against ischemia-reperfusion brain damage.

**Results**

**Effects of ischemia-reperfusion in the hippocampus of Mongolian gerbils**

Induction of BCCO in Mongolian gerbils produced significant early neuropathological and ultrastructural changes, which were followed by significant neuronal loss within the CA1 area of gerbil’s hippocampus. In particular, histological and ultrastructural evaluation carried out at day 1 after induction of brain ischemia showed significant incidence, compared to sham operated animals (n = 10; Figure 1A), of eccentric pycnotic nuclei, intracellular oedema and vacuolar degeneration (n = 10; Figure 1B) found in CA1 and partially in CA2 areas of gerbil’s hippocampus. CA3, CA4 and Dentate gyrus (DG) showed a normal cytoarchitecture (n = 10; not shown). Furthermore, the examination of brain tissues by electron microscopy carried out 1 day after the ischemic insult showed, within CA1 area of the hippocampus of gerbils, the presence of vacuolated neurons with irregular shaped nuclei composed of euchromatin with margination of heterochromatin; in a few cells, large electron-light vacuoles occupied the entire cytoplasm and the cell shape was not maintained. Moreover, the mitochondria were dense and disorganized. In the same area, a few glial cells showed a suffering phenotype characterized by dispersion of the chromatin into the cytoplasm due to the absence of the nuclear membrane, nucleoli still prominent, dense cytoplasm with small vacuolization and endoplasmic reticulum preserved; the plasma membrane was not always visible and often interrupted and in the extracellular space myelinic fibers with alteration of the myelinic structure are visible (n = 10; Figure 2A,2B,2C). These features were not visible in sham animals where neurons and glial cells showed a normal cytoarchitecture.
Figure 1
Temporary (5 min) BCCO in Mongolian gerbils leads to damage in the hippocampal CA1 area, compared to sham-operated animals (A), characterized by neurons showing eccentric pycnotic nuclei, intracellular edema and vacuolar degeneration (B). M40401 (40 mg/Kg) given i.p. 1 h after BCCO protected against ischemia-reperfusion hippocampal early lesion (C)
Figure 2

(A-C): ischemia + 1 day recovery: prominent ultrastructural damages of CA1 area of the hippocampus in the site of carotid artery occlusion. In particular, are displayed vacuolated neurons with irregular nuclei and margination of the heterochromatin (pre-apoptotic neurons). Large electron-light vacuoles occupy the cytoplasm and the mitochondria are dense and disorganized. In many cells, vacuoles occupy the entire cytoplasm, cell shape is not maintained and the chromatin is dispersed into the cytoplasm (see A). (magnification × 3800). (D) M40401 (40 mg/kg) protected against damage. Indeed, the micrographs show the presence, in the hypothalamic CA1 area of normal neurons with large nuclei with euchromatin and prominent nucleoli. In addition, developed mitochondria, rough endoplasmic reticulum and normal cytoskeleton components are distinguished. In the extracellular space are found preserved myelinic fibers and blood vessels. (magnification × 3800)
The early post-ischemic lesion seen 1 day after BCCO was accompanied by loss of neurons within CA1 area when evaluated 7 days after induction of global ischemia. Indeed, in a group of 10 animals undergoing BCCO, histological examination of brain slices carried out at the day 7 after brain ischemia showed significant reduction in the number of neurons detected within CA1 area of gerbil’s hippocampus compared to sham operated animals (n = 10; Figure 3A and 3B).

**Effects of M40401**

Administration of M40401 (1–40 mg/Kg i.p. 1 h after BCCO; n = 10 for each dose), produced a significant and dose-dependent reduction of neuropathological and ultrastructural early changes within the hippocampus of gerbils undergoing ischemia-reperfusion (Figure 3B and 4D). Thus, the administration of M40401 (1–40 mg/Kg given i.p. after BCCO, n = 10 for each dose) in Mongolian gerbils reduced by 18 ± 4, 36 ± 2, 72 ± 4 and 100%, respectively, the number of cells showing ultrastructural changes in the hippocampus found 1 days after induction of BCCO. Sham operated Mongolian gerbils treated with 1–40 mg/kg i.p. of M40401 (n = 10 for each dose) did not show any significant histopathological and ultrastructural modification when compared to sham non-treated Mongolian gerbils (data not shown). Results showing lack of BCCO-related early damage in the hippocampal area after the highest dose of M40401 (40 mg/kg, i.p, n = 10), lead to protective effect against neuronal loss seen 7 days after BCCO within the CA1 area of the gerbil’s hippocampus (Figure 3A and 3B).

**Effect of M40401 in the post-ischemic elevation of MDA in brain tissues**

Ischemia-reperfusion was followed by significant elevation of the concentration of MDA in ischemic brain, mainly within the hippocampus. This effect was observed
that O$_2^-$ overproduced in a mitochondrial compartment, cerebral ischemia [32]. In addition, it has been suggested dant activity could be beneficial in the acute treatment of is reduced in stroke patients, and replacement of antioxi-

species (ROS) (mainly superoxide anion, hydroxyl radical and hydrogen peroxide) which, in turn, participate in the mechanisms leading to post-ischemic neuronal cell death and apoptosis of neuronal and non neuronal cells [26,27]. Importantly, accumulation of free fatty acids and adenine nucleotides has been described over the ischemic period after both global and focal ischemia in the brain. Thus, during reperfusion, metabolism of free fatty acids (via cyclooxygenase and lipoxygenase activation) and of adenine nucleotides (via xanthine oxidase activity) leads to ROS overproduction [8,10,28–31]. This is further accompanied by leukocyte infiltration, which generates both ROS and nitric oxide which, in turn, contribute to the generation of highly reactive and neurotoxic nitrogen free radical species, such as peroxynitrite [8,10].

The antioxidant status of the tissue affected by ischemia-reperfusion is of great importance for the primary endog-

loss of superoxide-overproduction has been shown to occur.

Discussion
It is known that ischemia-reperfusion of brain tissue is fol-

The antioxidant status of the tissue affected by ischemia-reperfusion is of great importance for the primary endogenous defense against the free radical-induced injury. In particular, evidence exists that the SOD activity in serum was in fact confirmed by the reduction of lipid peroxidation in injured brain (Figure 4B).

antioxidant activity could be beneficial in the acute treatment of cerebral ischemia [32]. In addition, it has been suggested that O$_2^-$ overproduced in a mitochondrial compartment, when uncoupled from antioxidant defenses, induces impairment of mitochondrial function and causes exacerbated of cerebral infarction after ischemia [33]. Moreover, it has also been shown that the endogenous antioxidant activity is differentially affected by the intensity of ischemic challenge and this suggests that the regional effects of ROS vary substantially following ischemia-reperfusion [34]. Thus, changes in the antioxidant status of brain tissues before, during and after ischemia-reperfusion greatly affects post-ischemic brain damage and has been widely studied in the last few years. In fact evidence has been acquired showing that extracel-

M40401 (1–40 mg/kg; Figure 4; n = 10 for each dose), dose-dependently attenuated the increase of MDA levels detected in the hippocampus 1 day after BCCO suggesting that, in this model, the SOD mimetic was able to reduce lipid peroxidation in injured brain (Figure 4B).

On the basis of this evidence, it is likely that an additional supply of antioxidant moieties over and above the natural SOD enzyme levels is crucial for a selective protection of brain tissue against ischemic injury [43,44]. In particular, the discovery that metalloporphyrin complexes of Mn and Fe that possess the capacity to destroy superoxide, perox-

In conclusion, our results indicate that low molecular weight synthetic SOD mimic which lack catalase activity may represent a novel and potentially useful approach in the treatment of many neurodegenerative disorders, such as ischemia/reperfusion brain injury, in which an abnormal release of free radicals has been shown to occur.

Methods
Bilateral Common Carotid Artery Occlusion and electro-

Adult male Mongolian gerbils (Meriones unguiculatus, 50–70 g; Charles River, Milan, Italy), housed in a temperature (20°C) and humidity (60%) – controlled colony room, were used for this study. The colony was maintained in a 12 h light/dark cycle with light on at 7:00 a.m. and both laboratory food and tap water were available ad libitum. Under chloral hydrate (400 mg/kg i.p.) anesthesia, 4 stainless steel wire electrodes were chronically implanted onto each fronto-parietal cortex under stereotaxic
guidance; the ground electrode was anchored to the nasal bone. Animals were allowed one week recovery before testing. Before experiments, the animals were placed individually in a perspex cage and allowed 30 min acclimatization to the new environment. Electro cortical (ECoG) recordings were made via an EEG machine (Mod. ERA-9; OTE Biomedica, Florence, Italy). A 30 min period of ECoG recording under basal conditions, as well as before induction of brain ischemia, was taken as basal value. Brain ischemia was induced by temporary bilateral occlusion of the common carotid artery (BCCO). Under anesthesia with ether, the common carotid arteries were dissected via a ventral neck incision and occluded with ligatures for 5 min (day 0). Then, the ligatures were removed, the skin incision sutured and animals were allowed to recover. A complete flattening of ECoG activity, was found for about 10 minutes after BCCO indicating the occurrence of brain ischemia. Animals that displayed no post-ischemic ECoG flattening were discarded from the study. Rectal temperature was monitored and kept between 36°C and 37°C during surgery by means of a heating pad. In addition, mean arterial blood pressure (MABP) and blood gases were evaluated immediately before, during, 5 minutes and 1, 2, 4 and 6 hours after BCCO either in untreated or M40401-treated gerbils.

**Neuropathological studies**

1, 3 or 7 days after BCCO, the gerbils were re-anesthetized with pentobarbital and transcardially perfusion-fixed with 4% buffered formaldehyde (pH 7.4) after a brief rinse with saline and heparin (0.1%) at room temperature. The brains were removed, kept in cold fixative for 2 hours, and stored in phosphate-buffered saline (PBS) overnight.

Histological stainings for nerve cells were performed to evaluate general brain morphology and tissue damage. Briefly, coronal slices of the dorsal hippocampus 400 µm thick were cut with a vibratome, dehydrated in graded alcohols and embedded in paraffin. Coronal sections 6 to 7 µm were stained with cresyl violet. The sections were coverslipped with permount and examined using a light microscope (Leica). Sham operated animals were used as a control group: these underwent similar handling but no BCCO was induced.

For Electron Microscopy studies, coronal sections (10 µm thick) collected every 50 microns were stained using Nissl method (cresol fast violet) and examined in a Leitz-orthophan photomicroscope. In particular, animals were perfusion-fixed with 0.01 M PBS (pH 7.4) followed by 3% paraformaldehyde and 0.1% glutaraldehyde in 0.15 M PB (pH 7.4) at room temperature. The brain was removed, blocks of the areas of interest were made and these were postfixed in osmium tetroxide 1.33% for 2 h at 4°C. After several washes in PBS, the tissue fragments were dehydrated in graded alcohol, transferred into toluene, and embedded in Epon 812 resin. The resin was allowed to polymerize in a dry oven at 60°C for 24 h. Thin sections were cut with a glass knife Reichert microtome, stained with toluidine blue and examined on Axioskop microscope (Zeiss). Ultra-thin sections were cut on a Reichert microtome using a diamond knife, stained with uranyl-acetate-lead-hydroxide and evaluated and photographed on a Philips electron microscope CM10 (Philips, End hoven, The Netherlands).

**Malondialdehyde Determinations**

Malondialdehyde (MDA; used as a biochemical marker for lipid peroxidation) was measured by a method previously described [46]. Levels of MDA were measured 1, 3 and 7 days after induction of brain ischemia in untreated or M40401-treated Mongolian gerbils. Briefly, hippocampal regions of perfused Mongolian gerbils were surgically identified, removed and then frozen in liquid nitrogen, and homogenized in potassium chloride (1.15%). Chloroform (2 ml) was then added to each homogenate and then spun for 30 min.

The organic layer of the sample was removed and dried under nitrogen gas and re-constituted with 100 µl of saline. MDA generation was evaluated by the assay of thiobarbituric acid (TBA)-reacting compounds. The addition of a solution of 20 µl of sodium dodecyl sulphate (SDS; 8.1%), 150 µl of 20% acetic acid solution (pH3.5), 150 µl of 0.8% TBA and 400 µl of distilled water, produced a chromogenic product which was extracted in n-butanol and pyridine. Then, the organic layer was removed and MDA levels read at 532 nm and expressed as nmol MDA/g wet tissue.

**Drug administration and experimental groups**

M40401 (Metaphore Pharm. Inc., St. Louis) was dissolved in 26 mM sodium bicarbonate buffer (pH = 8.1–8.3). M40401 (1–40 mg/kg) was given intraperitoneally (i.p.; 0.3 ml) 1 h after BCCO (day 0). ECoG changes and histopathological studies were carried out 1, 3 and 7 days after M40401 administration. The same drug administration protocol was performed in sham animals.

**Selectivity of M40401**

It has been shown that the pentaaza macrocylic ligand complexes of Mn(II) can not only be highly active catalysts for the dismutation of O$_2^-$, but that they are also highly selective [25]. In particular, evidence exists that this complex and others of this pentaaza macrocyclic ligand class, such as M40403 or M40401 do not react with hydrogen peroxide under the same conditions [25], nor do they react with other biologically relevant oxidants such as ONOO$^-$ or nitric oxide [25].
Materials
All the reagents used for this study were purchased from Sigma (Milan). M40401 was kindly provided by Dr. D.P. Riley (Metaphore Pharm. Inc, St. Louis, Mo, USA).

Statistics
Results are expressed as means ± s.e.m. for n experiments. The results were analysed independently by at least three observers and Student's unpaired t test was used to determine the significance between means. A P value of < 0.05 was taken as significant.

Author’s contributions
VM conceived the study and participated in the sequence alignment and drafted the manuscript. MI carried out the surgery and the immunohistochemistry and participated in the drafted of the manuscript. CM carried out the surgery and biochemical analysis and conceived the study. TG and AM carried out the electron microscopy studies. RN participated in the design of the study. DR and DS participated in the design of the study and its coordination.

Aknowledgements
This work was supported by Italian Ministry for Research and Development (MURST; COFIN 2000), CNR grant N. 99.00412.FF49 and by European Fund for Regional Development (P.O.P. 94–99). The authors thank Mrs. Simonelli Lucilla (University “La Sapienza”, Rome, Italy) for excellent technical support and Dr. Renia Botting for helpful suggestions in preparing the manuscript.

References
1. Nehls DG, Park CK and McCulloch J: Effect of the NMDA antagonist MK-801 on local cerebral blood flow in focal cerebral ischaemia in the rat / Cereb Blood Flow Metab 1989, 9:537-537.
2. Dinnagl U, Iadeoca C and Moskowitz MA: Pathobiology of ischaemic stroke: an integrated view Trends Neurosci 1999, 22:391-397.
3. Martin RL, Lloyd HG and Cowan AI: The early events of oxygen and glucose deprivation: setting the scene for neuronal death? Trends Neurosci 1994, 17:251-257.
4. Katsumura K, Kristian T and Siejo BK: Energy metabolism, ion homeostasis, and cell damage in the brain Biochem Soc Trans 1994, 22:591-996.
5. Furukawa K: The actin-severing protein gelsolin modulates calcium channel and NMDA receptor activities and vulnerability to excitotoxicity in hippocampal neurons / J Neurosci 1997, 17:8178-8186.
6. Chen ZL and Strickland S: Neuronal death in the hippocampus is promoted by plasmin-catalyzed degradation of laminin Cell 1997, 91:917-925.
7. Zhao P, Pahlmark K, Smith ML and Siejo BK: Delayed treatment with the spin trap alpha-phenyl-N-tert-butyl nitrone (PBN) reduces infarct size following transient middle cerebral arterial occlusion in rats Acta Physiol Scand 1994, 152:349-350.
8. Beckman JS, Beckman TW, Chen J, Marshall PA and Freeman BA: Apparent hydroxyl radical production by peroxynitrite: implications for endothelial injury from nitric oxide and superoxide Proc Natl Acad Sci 1990, 87:1620-1624.
9. Beckman JS and Koppenol WH: Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and the ugly Am J Physiol 1996, 271:C1424-C1437.
10. Iadeoca C: Bright and dark sides of nitric oxide in ischemic brain injury Trends Neurosci 1997, 20:132-139.
11. Cerchiaro EL, Hoel TM, Safar P and Sclarabassi RJ: Protective effects of combined superoxide dismutase and defereroxamine on reoxygenation of cerebral blood flow and function after cardiac arrest in dogs Stroke 1987, 18:869-878.
12. Forsman M, Fleischer JE, Milde JH, Steen PA and Michenfelder JD: Superoxide dismutase and catalase failed to improve neurologic outcome after complete cerebral ischaemia in the dog Acta Anaesthesiol Scand 1988, 32:152-155.
13. Cerchiaro EL, Sclarabassi RJ, Safar P and Hoel TM: Effects of combined superoxide dismutase and defereroxamine on recovery of brainstem auditory evoked potentials and EEG after asphyxial cardiac arrest in dogs Resuscitation 1990, 19:25-40.
14. Takuya M, Matsumoto M, Kitagawa K, Niinobe M, Ohtsuki T, Hata R, Ogawa S, Handa N, Mikoshiba K and Kamada T: Recombinant SOD can attenuate ischemic neuronal damage in gerbils Life Sci 1992, 51:253-259.
15. Freeman BA, Young SL and Crapo JD: Liposome-mediated augmentation of the native superoxide dismutase in endothelial cells prevents oxygen injury / J Biol Chem 1983, 258:12534-12542.
16. Turrens JF, Crapo JD and Freeman BA: Protection against oxygen toxicity by intravenous injection of liposome-entrapped catalase and superoxide dismutase J Clin Invest 1984, 73:87-93.
17. Beckman JS, Minor RL, White CW, Repine JE, Rosen GM and Freeman BA: Superoxide and catalase conjugated to polyethylene glycol increases endothelial enzyme activity and oxidant resistance / J Biol Chem 1988, 263:6884-6892.
18. Baker K, Markus CB, Huffman K, Kruk H, Malfroy B and Doctorow SR: Synthetic combined superoxide dismutase/ catalase mimetics are protective as a delayed treatment in a rat stroke model: a key role for reactive oxygen species in ischemic brain injury J Pharmacol Exp Ther 1998, 284:215-21.
19. Salvemini D, Riley DP, Lennon PJ, Wang ZQ, Currie MC, Macarthur H and Misko TP: Protective effects of a superoxide dismutase mimetic and peroxynitrite decomposition catalysts in endothelin-induced intestinal damage Br J Pharmacol 1999, 126:304-306.
20. Salvemini D, Riley DP and Cuzzocore S: SOD mimetics are coming of age Nature Reviews Drug Discovery 2002, 1:367-374.
21. Anton K, Rath N, Naik A, Slomczynska U, Schall OF and Riley DP: Computer-aided design (CAD) of Mn(II) complexes: superoxide dismutase mimetics with catalytic activity exceeding that of the enzyme J Am Chem Soc 2001, 123:1779-1789.
22. Mollace V, Iannone M, Muscoli C, Palm E, Granato T, Rispoli V, Nistico R, Rotrori D and Salvemini D: The role of oxidative stress in paraquat-induced neurotoxicity in rats: protection by non-peptidyl superoxide dismutase mimetic Neurosci Lett 2003, 335:163-6.
23. Salvemini D, Wang ZQ, Zweier JL, Samouilov A, Neumann WL and Weiss RH: Toward the rational design of superoxide dismutase mimics: Mechanistic studies for the elucidation of substrate effects on the catalytic activity of macrocyclic manganese (II) complexes J Am Chem Soc 1997, 119:6522-6528.
24. Salvemini D and Riley DP: M40403-Superoxide dismutase mimetic Drugs Fut 2000, 25:1027-1033.
25. Linnink MD, Zobrist RH and Hatfield MD: Evidence supporting a role for programmed cell death in focal cerebral ischaemia in rats Stroke 1993, 24:2002-2009.
26. Benfocico K, Kainic D, Alfibone C, Miotto P and Lipton SA: Apoptosis and necrosis: two distinct events induced, respectively, by mild and intense insults with N-methyl-D-aspartate or nitric oxide/superoxide in cortical cell cultures Proc Natl Acad Sci 1995, 92:7162-7166.
27. Porcellato G, DeMedio GE, Fini C, Floridi A, Goracci G, Horrocks LA, Lazarewicz JW, Palmieri CA, Strozsajder J and Trovarelli J: Phospholipid and its metabolism in ischaemia Proc Eur Soc Neurochem 1978, 1:491-498.
28. Van Wijlen DG, Park T, Rubino R and Berne RM: Increases in cerebrovascular fluid adenosine concentration during hypoxia, local potassium infusion, and ischemia J Cereb Blood Flow Metab 1986, 6:522-528.
29. Hallebeck JM, Dutka AJ, Tanishima T, Koochek M, Kumaroo K, Thompson CB, Obrenovitch TP and Contreras TJ: Polymorphismu-
clear leukocyte accumulation in the brain regions with low blood flow during the early postischemic period Stroke 1986, 17:246-253.

31. Kochanek PM, Dutka AJ and Hallenbeck JM: Indomethacin, prostacyclin and heparin improve postischemic cerebral blood flow without affecting early postischemic granulocyte accumulation Stroke 1987, 18:634-637.

32. Spranger M, Krempien S, Schwab S, Donneberg S and Hacke W: Superoxide dismutase activity in serum of patients with acute cerebral ischaemic injury. Correlation with clinical course and infarct size Stroke 1997, 28:2425-2428.

33. Murakami K, Kondo T, Kawase M, Li Y, Sato S, Chen SF and Chan PH: Mitochondrial susceptibility to oxidative stress exacerbates cerebral infarction that follows permanent focal cerebral ischemia in mutant mice with manganese superoxide dismutase deficiency Neurosci 1998, 18:205-213.

34. Toyoda T and Lee KS: Differential induction of superoxide dismutase in core and penumbra regions after transient focal ischaemia in the rat neocortex Neurosci Lett 1997, 235:29-32.

35. Sheng H, Brady TC, Pearselab RD, Crapo JD and Warner DS: Extracellular superoxide dismutase deficiency worsens outcome from focal cerebral ischemia in the mouse Neurosci Lett 1999, 267:13-16.

36. Kawase M, Murakami K, Fujimura M, Morita-Fujimura Y, Gasche Y, Kondo T, Scott RW and Chan PH: Exacerbation of ischemia in mutant mice with CuZn superoxide dismutase deficiency Stroke 1999, 30:1962-1968.

37. Kondo T, Reaume AG, Huang TT, Carlson E, Murakami K, Chen SF, Hoffman EK, Scott RW, Epstein CJ and Chan PH: Reduction of CuZn-superoxide dismutase activity exacerbates neuronal cell injury and edema formation after transient focal cerebral ischemia Neurosci 1997, 17:4180-4189.

38. Bidmon HJ, Kato K, Schleicher A, Witte OW and Zilles K: Transient increase of manganese-superoxide dismutase in remote brain areas after focal photothrombotic cortical lesion Stroke 1998, 29:203-210.

39. Keller JN, Kendy MS, Holtsberg FW, St Clair DK, Yen HC, Gernmeyer A, Steiner SM, Bruce-Keller AJ, Hutchins JB and Mattson MP: Mitochondrial manganese superoxide dismutase prevents neural apoptosis and reduces ischemic brain injury: suppression of peroxinitrate production, lipid peroxidation and mitochondrial dysfunction Neurosci 1998, 18:687-697.

40. Sheng H, Bart RD, Oury TD, Pearse RD, Crapo JD and Warner DS: Mice overexpressing extracellular superoxide dismutase have increased resistance to focal cerebral ischemia Neurosci 1999, 88:185-191.

41. Suegna G, Ceballos-Picot I, Chevalier E, Nicole A, Onteniente B and Sola B: Reduction of ischemic damage in NGF-transgenic mice: correlation with enhancement of antioxidant enzyme activities Neurobiol Dis 1999, 6:180-189.

42. Fujimura M, Morita-Fujimura Y, Narasimhan P, Copin JC, Kawase M and Chan PH: Copper-zinc superoxide dismutase prevents the early decrease of apurinic/apyrimidinic endonuclease and subsequent DNA fragmentation after transient focal cerebral ischemia in mice Stroke 1999, 30:2408-2415.

43. Wengenack TM, Curran GL and Poduslo JF: Post-ischemic, systemic administration of pyrimidine-modified superoxide dismutase reduces hippocampal CA1 neurodegeneration in rat global cerebral ischemia Brain Res 1997, 754:46-54.

44. Franci JW, Ren J, Warren L, Brown RHJr and Finklstein SP: Post-ischemic infusion of CuZn superoxide dismutase or SOD:TeT451 reduces cerebral infarction following focal ischemia/reperfusion in rats Exp Neurol 1997, 146:435-443.

45. Patel M and Day BJ: Metalloporphyrin class of therapeutic catalytic antioxidants Trends Pharmacol 1999, 20:359-364.

46. Ohkawa H, Oishi H and Yagi K: Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction Analytical Biochem 1979, 95:351-358.