

An Alternative Treatment in Warm Cerebral Ischemia-Reperfusion Injury

Inhibition of Glutamate Neurotransmission

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Abstract: Glutamate neurotoxicity has been implicated in the pathophysiology of cerebral ischemia-reperfusion injury. Therefore, we investigated the effect of glutamate receptor antagonist riluzole in a rabbit model of warm cerebral ischemia-reperfusion. A total of 16 New Zealand rabbits were randomly assigned to one of three groups: Riluzole group (n = 6), which received riluzole (8 mg/kg), control group (n = 5), which received only vehicle before ischemic period, and sham group (n = 5), which had the same operation but did not undergo clamping. To induce warm cerebral ischemia the left carotid artery was occluded for 15 minutes and then reperfusion was allowed. The rabbits were killed after 4 hours of reperfusion. The brain slices were harvested for immunohistochemical examination of platelet endothelial cell adhesion molecule (PECAM) expression and blood samples were taken for measurement of serum superoxide dismutase (SOD), catalase, myeloperoxidase (MPO) and malondialdehyde (MDA) levels. The results indicated that riluzole treatment reduced the relative levels of malondialdehyde and myeloperoxidase and increased SOD levels ($P < 0.001$). No statistically important difference was determined between riluzole and sham group with respect to these results. We did not find any significant difference in catalase levels among three groups. Immunohistochemical examination showed significant decrease of PECAM expression in riluzole treated animals ($P < 0.05$). Our findings suggested that riluzole may protect brain in a setting of severe ischemia-reperfusion injury and therefore, be considered for clinical usage.

Key Words: cerebral ischemia-reperfusion, riluzole, oxidative stress, endothelial inflammatory response

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Ischemia reperfusion (I/R) injury is associated with the production of cytokines and increased expression of adhesion molecules by hypoxic parenchymal and endothelial cells. These agents recruit circulating polymorphonuclear leukocytes to reperfused tissue and the various molecular families (selectins, integrins) mediate various of neutrophil extravasation in endothelial-neutrophil interaction.¹

Much evidence indicates that the cell death after cerebral ischemia is mediated by the massive release of excitatory aminoacids, overproduction of reactive oxygen species and lipid peroxidation.²

During cerebral ischemia there is excess release into the synaptic cleft of the neurotransmitters that activate the various calcium influx processes and the activation of enzymes by elevated cytosolic calcium results, in addition to structural damage, in the liberation of fatty acids from membranes, over production of reactive oxygen species and lipid peroxidation. Observations that these events contribute to neuronal cell death indicate that blocking oxidative stress and excitatory aminoacid neurotransmission are targets for reducing the degree of neurologic deficits observed following cerebral injury.

Recent studies suggested that the release of excitatory aminoacids into the extracellular space of the central nervous system by ischemic cells might play a key role in the neuronal cell death.³ Glutamate may destroy neuronal cells through its action on N-methyl-D-aspartate (NMDA) and non-NMDA receptors by inducing massive sodium and calcium ion influxes into the cell, resulting in neuronal death (4-B4). NMDA and non-NMDA receptor antagonists have demonstrated neuroprotective properties both in vitro and in vivo.^{4,5}

Riluzole (2-amino-6-trifluoromethoxybenzothiazole) is a neuroprotective drug that blocks glutamatergic neurotransmission in the central nervous system and has demonstrated anti ischemic properties.⁶ Experimental studies suggested that it has a complex mechanism of action, including a direct, but non-competitive, blockade of ionotropic excitatory aminoacid (EAAs) receptors, inhibition of presynaptic glutamate release and inactivation of voltage dependent calcium and sodium channels.⁶ Although the effect of riluzole treatment was examined using measures of oxidative stress, no data are available whether it has a role in a later phase of endothelial inflammation.^{7,8,9,10}

Therefore, the aim of this study was to determine whether riluzole was really capable of reducing oxidative stress and demonstrate whether it has beneficial effects on endothelial inflammatory response in warm global cerebral I/R injury.

MATERIALS AND METHODS

Surgical procedure

A total of 16 New Zealand rabbits were used in the experiment. All animals were randomly assigned to one of three groups: Riluzole group (n = 6), control group (n = 5) and sham group (n = 5).

Prior to surgery, animals were anaesthetized with ketamine (50 mg/kg)-diazepam (2 mg/kg) I.M. and a 0.9% NaCl solution was started to infuse at a similar dose (15–20 mL for the whole procedure) via the ear vein. A catheter was inserted into the right femoral artery for mean arterial blood pressure (MAP), arterial blood gas monitoring and blood sampling for biochemical analysis.

Throughout the experiment, body temperature was maintained at 37°C using a heating pad and arterial pH, arterial oxygen pressure (paO₂), arterial carbon dioxide pressure (pCO₂) was maintained at physiologic levels. Arterial blood gases were analyzed at the following times: baseline, before ischemia was induced; ischemia, at the end of ischemia; reperfusion, 2 hours after the start of reperfusion.

After the blood samples were taken for baseline analysis of biochemical parameters, a midline neck incision was made, left carotid artery was exposed and separated from vagosympathetic trunks. Ischemia was induced using a microvascular clamp across the left carotid artery and was maintained for 15 minutes. Then reperfusion was allowed by releasing the clamp. After 4 hours, according to the study of Engelhard et al, blood samples were obtained again for biochemical analysis and the rabbits were killed by lethal thiopental (200 mg/kg) injection.² The brain was removed and sliced into coronal sections for immunohistochemical examination.

The animals in sham group underwent the same surgical procedure, but no cerebral ischemia was induced and the control group animals received only vehicle before carotid artery occlusion.

Drug Treatment

Riluzole (RBI, R-116, Sigma, USA) was dissolved in a small volume of HCL (0.1 N), diluted in saline at a concentration of 2.5 mg/ml, and pH adjusted to 7.4 with NaOH. The solution was administered to animals by I.V. injection at a dose of 8 mg/kg., 15 minutes prior to the occlusion of left carotid artery. The dose was based on those used in previous studies.^{11,12}

Immunohistochemical Examination

Immunohistochemistry for PECAM-1 was performed using a combination streptavidin-biotin-peroxidase method and microwave antigen retrieval on formalin-fixed paraffin-embedded tissues. After deparaffinization, endogenous peroxidase activity was blocked with H₂O₂. To retrieve antigen, sections were boiled with 0.01 mol/L Citrate buffer in a microwave pressure cooker for 10 minutes and cooled at

room temperature. Non-specific binding was blocked with normal horse serum for 15 minutes at room temperature. A monoclonal mouse Anti-Human endothelial cell, CD31 antibody, clone JC/70A (DAKO, Denmark) was used as a marker of endothelial cells at a 1:30 dilution. The sections were then stained using the avidin-biotin complex (ABC) immunoperoxidase technique employing commercially available reagent (ABC kit, Labvision, Fremont, USA). The sections were counterstained with Mayer's haematoxylin and mounted with paramount Human tonsil tissue served as positive control. The intensity of immunostaining was scored as follows: (0), no staining; (1+), weak staining; (2+), moderate staining; and (3+), strong staining. Only cytoplasmic staining was considered positive.

Biochemical Assay

Measurement of SOD

Pyrogallol auto-oxidizes rapidly in aqueous solution to produce a yellow color that can be read at 420 nm. This process is dependent on the presence of superoxide anions. The enzyme superoxide dismutase (SOD) inhibits the auto-oxidation of pyrogallo by catalyzing the breakdown of superoxide. The inhibition of pyrogallol oxidation by SOD is monitored at 420 nm, and the amount of enzyme producing 50% inhibition is defined as one unit of enzyme activity.^{13,14}

Measurement of catalase activity

The determination of CAT activity was measured according to the Beutler method.¹⁵

Principle: Catalase catalyzes the breakdown of H₂O₂ to H₂O and O₂. The rate of decomposition of H₂O₂ by catalase is measured spectrometrically at 230 nm, because H₂O₂ absorbs light at this wavelength. Ethanol is added to stabilize the hemolysate by breaking down "complex II" of catalase and H₂O₂. After the addition of 50 µL tris buffer, 900 µL of H₂O₂, 30 µL of H₂O to the cuvettes system is incubated at 37°C for ten minutes the hemolysate is added and in the following ten minutes the decrease of optical density is measured against blank at 412 nm.¹⁵

Measurement of Myeloperoxidase (MPO)

The level of sera MPO activity is directly related to the fact that it reduces o-dianisidine. Therefore, the reduction in o-dianisidine was measured by spectrophotometry at the wavelength of 410 nm for estimation of MPO levels.¹⁶

Measurement of Malondialdehyde (MDA)

The MDA level was measured as an index of lipid peroxidation, by the thiobarbituric acid reaction according to the methods of Yagi.^{17,18} The principle of this method depends on assessment of the pink color produced by the interaction of the barbituric acid with MDA elaborated as a result of lipid peroxidation. The color reactant 1,1,3,3-tetraethoxypropane was used as the primary standard.

Statistical Analysis

Statistical analysis of the physiological parameters and data from each biochemical measure were performed using repeated measurement analysis of variance with two factors.

One way ANOVA (Analysis of Variance) was used for comparing three groups by means of PECAM. The data were expressed as the mean score ± SD and a *P* value of less than 0.05 was considered as statistically significant.

RESULTS

Table 1 shows the physiologic variables throughout the experiment. There were no differences in MAP, PaO₂ and PaCO₂ between control group and animals treated with riluzole. In sham operated animals physiologic variables did not change over time (Table 1).

The results of biochemical analysis revealed a significant effect of drug treatment on lipid peroxidation levels. The relative levels of malondialdehyde and myeloperoxidase increased in control group animals when compared with sham and riluzole treated animals (*P* < 0.05), whereas no statistically important difference was determined between riluzole and sham groups (Table 2).

SOD levels of control group animals were dramatically lower than the other two groups (*P* < 0.05), whereas no statistically important difference was detected between riluzole and sham groups. The activity of other scavenger enzyme, catalase, did not change either in control or riluzole and sham groups (Table 2).

The unique immunohistochemical examination in this study was performed for determination of endothelial inflammation. PECAM expression of brain tissue, which was detected for this purpose, diminished significantly in riluzole treated animals (*P* < 0.05) (Figs. 1, 2).

DISCUSSION

The results of the present study provide the evidence that glutamate release following ischemic events is a critical step in the appearance of oxidative stress and inflammatory response. Our results also demonstrate that detrimental effects of cerebral ischemia-reperfusion (I/R) injury can be reduced by drugs that inhibit glutamate release. The dose of riluzole used in this study (8 mg/kg) is the dose that has been reported to be neuroprotective in several studies.^{6,19} It is likely that, with less glutamate available, glutamate mediated lipid peroxidation is

TABLE 1. Physiological Variables

	Baseline	Ischemia	Reperfusion
MAP (mm.Hg)			
Control	120 ± 10	34 ± 2*	125 ± 23
Riluzole	130 ± 15	32 ± 2*	128 ± 20
Sham	125 ± 10	120 ± 8	127 ± 13
PaO ₂ (mm.Hg)			
Control	134 ± 20	140 ± 12	132 ± 30
Riluzole	120 ± 33	130 ± 22	120 ± 26
Sham	122 ± 30	110 ± 30	114 ± 14
PaCO ₂ (mm.Hg)			
Control	42 ± 4	38 ± 5	40 ± 6
Riluzole	40 ± 2	40 ± 6	42 ± 4
Sham	40 ± 2	41 ± 3	42 ± 2

*Statistically low (*P* < 0.05) when compared to baseline values.

TABLE 2. Levels of Lipid Peroxidation Products (MPO, MDA) and Scavenger Enzymes (SOD, Catalase) in Blood Samples

	Baseline	After Reperfusion
MPO (U/L)		
Control	1.20 ± 0.08	2.45 ± 0.03*
Riluzole	1.28 ± 0.01	1.13 ± 0.22
Sham	1.25 ± 0.07	1.35 ± 0.07
MDA (nmol/ml)		
Control	1.22 ± 0.09	7.02 ± 2.20*
Riluzole	1.23 ± 0.01	1.40 ± 0.17
Sham	1.30 ± 0.01	1.30 ± 0.01
SOD (U/L)		
Control	1591.00 ± 74.59	787.25 ± 65.45†
Riluzole	1232.50 ± 96.94	1298.17 ± 76.31
Sham	1635.00 ± 7.07	1624.00 ± 5.65
Catalase (IU/L)		
Control	588.48 ± 153.31	275.05 ± 83.02
Riluzole	355.27 ± 201.72	332.85 ± 199.34
Sham	232.90 ± 10.53	227.77 ± 10.22

*Statistically high (*P* < 0.05) when compared with riluzole and sham group.

†Statistically low (*P* < 0.05) when compared with riluzole and sham group.

reduced, which consequently would preserve the activity of glutamate transport proteins in cell membranes.

Infiltration of neutrophils to the infarct areas following I/R plays a crucial role in the development of cerebral injury.^{20,21} This begins with enhanced firm adhesion of peripheral neutrophils to the endothelium and subsequent transmigration of neutrophils to injured sites where they release large quantity of ROS and proteolytic enzymes that modulate I/R injury.^{21,22,23}

Accumulation of ROS, particularly superoxide anion (O⁻²) and hydrogen peroxide (H₂O₂) causes damage to membrane phospholipids, proteins and DNA.^{24,25} Under normal circumstances, these oxidant species can be effectively scavenged by antioxidant enzymes, namely superoxide dismutase (SOD), catalase and glutathione peroxidase. However, the sudden

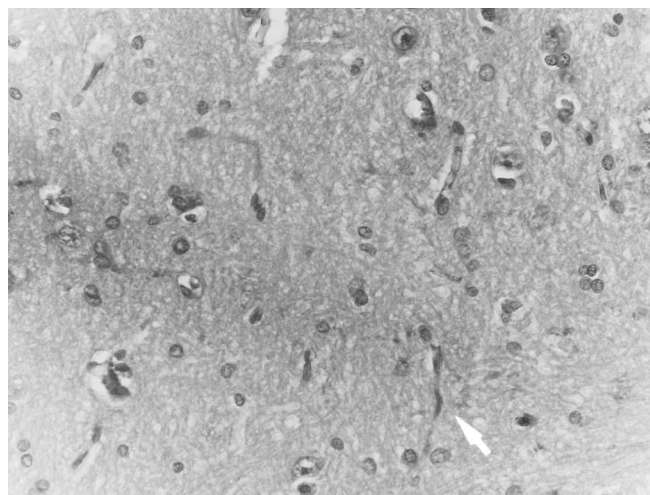


FIGURE 1. Strong immunoreactivity for PECAM in brain tissues of control group animals (HEX400).

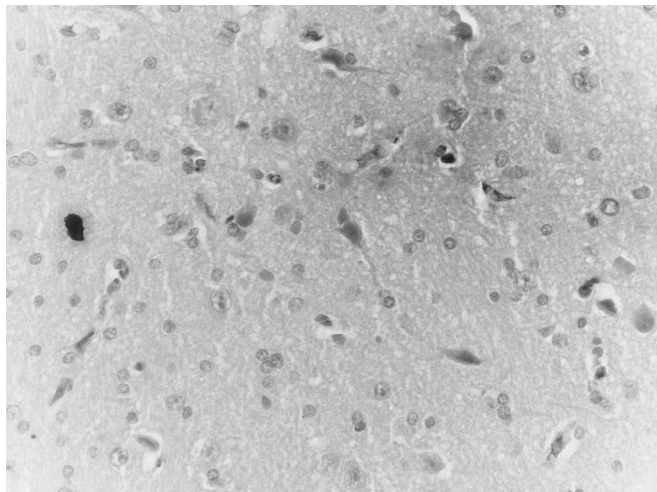


FIGURE 2. Weak immunoreactivity for PECAM in brain tissues of riluzole treated animals (HEX400) PECAM: (The score of the staining intensity).

burst of ROS during I/R can overwhelm the antioxidant defense, resulting in lipid peroxidation and hence cerebral injury.^{26,27}

One of the mechanisms involved in the pathogenesis of I/R injury is glutamate excitotoxicity. Rapid removal of glutamate from the extracellular space is required to prevent the neurotoxic effects of glutamate receptor overstimulation. This is accomplished by a family of high affinity Na⁺ dependent glutamate transporters denoted excitatory aminoacid transporter EAAT1-EAAT5. Reactive oxygen species have been found to disrupt the function of glutamate transporters and eventually a loss of glutamate uptake.^{28,29}

Results obtained from our study showed that, riluzole treatment applied before cerebral I/R increased SOD and catalase activities with decrease in serum MDA, a marker of lipid peroxidation and MPO, an index of neutrophil infiltration. This finding represents that, glutamate-mediated lipid peroxidation was reduced by riluzole and elevated SOD activity resulted in less oxidative stress.

Different mechanisms may involve in reduction of oxidative stress by SOD. First, reduced superoxide levels in the cells could reduce ROS reactions with the sulfhydryl groups of glutamate transporter proteins. By scavenging superoxide, SOD could also prevent superoxide-induced release of metal ions normally sequestered in proteins and thereby prevent the formation of highly reactive hydroxyl radicals from Fenton reaction. Furthermore, SOD may act to prevent the oxidative chain reactions in which superoxide serves as initiator or propagator. Additionally, SOD may preserve the function of glutamate transporters by preventing ROS activation of protein kinase C, because protein kinase C is reported to phosphorylate and down-regulate EAAT1 function.²⁸

Several lines of evidence indicate that G proteins may be involved in neuroprotective effects.³⁰ Diminishing ROS production through impediment of G-protein mediated calcium influx accounts for the inhibition of neutrophil firm adhesion.^{31,32} Therefore, inhibiting neutrophil adhesion, calcium mobilization

and ROS production can reduce neutrophil infiltration or recruitment to cerebral I/R injured tissues. It seems that riluzole, being an inactivator of voltage dependent calcium and sodium channels and stimulator of a G protein dependent signal transduction pathway, has beneficial effects on cerebral I/R injury.

PECAM-1 (platelet endothelial cell adhesion molecule) or CD31 is constitutively expressed on platelets, most circulating cells and endothelial cells and is thought to participate in the interactions that affect the leukocyte transmigration and microvascular permeability. Antibodies of this molecule have demonstrated to inhibit adhesion of leukocytes to the endothelial surface, binding of platelets to the injured endothelium and blocking the passage of leukocytes between endothelial cells.³³

In this study, inhibition of glutamate release by riluzole treatment was found to prevent partially the detrimental effects of PECAM-1. And the results also indicate that MDA, MPO, SOD and catalase levels are in correlation with the reduced PECAM-1 expression. This confirms that a single dose riluzole given before clamping may result in significant cerebral protection, even in a setting of severe ischemia.

In conclusion, we can say that; inhibition of glutamatergic neurotransmission, with the absence of adverse effects, may have potentially beneficial effects on cerebral I/R.

REFERENCES

1. Catron RS, Kumar V, Collins T. Cellular Pathology I: Cell injury and Cell death in Robbins: Pathologic Basis of Disease. W.B Saunders, Philadelphia, USA, 1999, p.12.
2. Engelhard K, Werner C, Eberspacher E, et al. The effect of the α_2 -agonist dexmedetomidine and the N-Methyl-Aspartate antagonist S(+)-Ketamine on the expression of apoptosis-regulating proteins after incomplete cerebral ischemia and reperfusion in rats. *Anesth Analg.* 2003;96:524–531.
3. Lang-Lazdunski L, Heurteaux C, Dupont H, et al. Prevention of ischemic spinal cord injury: Comparative effects of magnesium sulphate and riluzole. *J Vasc Surg.* 2000;32:179–189.
4. Rokkas CK, Cronin CS, Lobner DC, et al. Dextorphan inhibits the release of excitatory aminoacids during spinal cord ischemia. *Ann Thorac Surg.* 1994;58:312–320.
5. Bowes MP, Swanson S, Zivin JA, et al. The AMPA antagonist LY293558 improves functional neurological outcome following reversible spinal cord ischemia in rabbits. *J Cereb Blood Flow Metab.* 1996;16:967–972.
6. Doble A. The pharmacology and mechanism of action of riluzole. *Neurology.* 1996;47:233–241.
7. Azbill RD, Mu X, Bruce-Keller AJ, et al. Impaired mitochondrial function, oxidative stress and altered antioxidant enzyme activities following traumatic spinal cord injury. *Brain Res.* 1997;765:283–290.
8. Cheramy A, Barbetio L, Godeheu G, et al. Riluzole inhibits the release of glutamate in the caudate nucleus of the cat in vivo. *Neurosci Lett.* 1992;147:209–212.
9. Martin D, Thompson MA, Nadler JV. The neuroprotective agent riluzole inhibits release of glutamate and aspartate from slices of hippocampal area CA1. *Eur J Pharmacol.* 1993;250:473–476.
10. Wokke J. Riluzole. *Lancet.* 1996;348:795–799.
11. Springer JE, Azbill RD, Kennedy SF, et al. Rapid calpain 1 activation and cytoskeletal protein degradation following traumatic spinal cord injury: attenuation with riluzole pretreatment. *J Neurochem.* 1997;69:1592–1600.
12. Wahl F, Renou E, Stutzmann JM. Riluzole reduces brain lesions and improves neurological function in rats after a traumatic brain injury. *Brain Res.* 1997;756:247–253.
13. Marklund S, Marklund G. involvement of the superoxide anion radical in the auto-oxidation of pyrogallol and a convenient assay for superoxide dismutase. *Eur J Biochem.* 1974;47:469–474.
14. Roth EF Jr, Gilbert HS. the pyrogallol assay for superoxide dismutase: absence absence of a glutathione artifact. *Anal Biochem.* 1984;137:50–53.

15. Beutler E. "Glutathion." In: Beutler E (ed). *Red cell metabolism: A manual of biochemical methods*. Grune and Stratton: New York; 1975. pp 105–107.
16. Golowich SP, Kaplan SD. *Methods in enzymology*. Vol.II. New York; Academic Press. 1955, p. 769.
17. Yagi K. Lipid peroxides and related radicals in clinical medicine. In: D. Armstrong, ed. *Free radicals in diagnostic medicine*. New York: Plenum; 1994, p. 1–15.
18. Bishop ML, Janet LP. *Free radicals in clinical chemistry*. 3rd ed. Philadelphia, New York: JB Lippincot; 1996, p. 765–77.
19. Lang-Lazdunski L, Heurteaux CN, Vaillant N, et al. Riluzole prevents ischemic spinal cord injury caused by aortic crossclamping. *J Thorac Cardiovasc Surg*. 1999;117:881–889.
20. Liou K-T, Shen Y-C, Chen C-F, et al. Honokiol protects rat brain from focal cerebral ischemia-reperfusion injury by inhibiting neutrophil infiltration and reactive oxygen species production. *Brain Res*. 2003; 992:159–166.
21. Nagy Z, Bori S, Bori S. Regulatory mechanisms in focal cerebral ischemia. New possibilities in neuroprotective therapy. *Ideggyogy sz. Szle*. 2002;55:73–85.
22. Pabst MJ. Priming of neutrophils. In: PG Hellewell and TJ Williams, Eds. *Immunopharmacology of neutrophils*, Academic Press, London. 1994, p. 195–221.
23. Pabst MJ. Priming of neutrophils. In: PG Hellewell and TJ Williams, Eds. *Immunopharmacology of neutrophils*, Academic Press, London. 1994, p. 245–257.
24. Siu FK, Lo SC, Leung MC. Electroacupuncture reduces the extent of lipid peroxidation by increasing superoxide dismutase and glutathion peroxidase activities in ischemic reperfused rat brains. *Neurosci Lett*. 2004;2: 158–162.
25. Michels C, Raes M, Tossaint O, et al. Importance of Se-Glutathione peroxidase, catalase and Cu/Zn-SOD for cell survival against oxidative stress. *Free Radic Biol Med*. 1994;17:235–248.
26. Weisbrot-Lefkowitz M, Reuhl K, Perry B, et al. Overexpression of human glutathione peroxidase protects transgenic mice against focal cerebral ischemia-reperfusion damage. *Brain Res Mol Brain Res*. 1998;53:333–338.
27. Fujimura M, Morita-Fujimura Y, Copin J, et al. Reduction of copper, zinc-superoxide-dismutase in knockout mice does not affect edema or infarction volumes and the early release of mitochondrial cytochrome c after permanent focal cerebral ischemia. *Brain Res*. 2001;889:208–213.
28. Chen Y, Ying W, Simma V, et al. Over expression of Cu, Zn speroxide dismutase attenuates oxidative inhibition of astrocyte glutamate uptake. *J Neurochem* 2000, 75: 3; p. 939–945.
29. Agostinho P, Duarte CB, Oliveira CR. Impairment of excitatory aminoacid transporter activity by oxidative stress conditions in retinal cells: effect of antioxidants. *FASEB J*. 1997;11:154–163.
30. Doble A, Hubert JB, Blanchard JC. Pertussis toxin pretreatment abolishes the inhibitory effect of riluzole and carbachol on aspartat release from cultured cerebellar granule cells. *Neurosci Lett*. 1992;140:251–254.
31. Shen YC, Chen CF, Wang SY, et al. Impediment to calcium influx and reactive oxygen production accounts for the inhibition of neutrophil Mac-1 up-regulation and adhesion by tetrandrine. *Mol Pharmacol*. 1999; 55:186–193.
32. Shen YC, Chen CF, Sung YJ. Tetrandrine ameliorates ischemia-reperfusion injury of rat myocardium through inhibition of neutrophil priming and activation. *Br J Pharmacol*. 1999;128:1593–1601.
33. Liao F. Soluble domain of platelet-endothelial cell adhesion molecule (PECAM) is sufficient to block transendothelial migration in vitro and in vivo. *J Exp Med*. 1997;185:1349–1354.