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Neuroprotection by LY341122, a novel inhibitor of lipid peroxidation, against focal ischemic brain damage in rats

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Abstract

LY341122 (2-(3,5-di-*t*-butyl-4-hydroxyphenyl)-4-(2-(4-methylethylaminomethyl-phenyloxy)ethyl)oxazole) is a potent inhibitor of lipid peroxidation which has been shown to protect against global ischemia and traumatic brain injury in rats. The purpose of this study was to examine the effect of LY341122 on ischemic injury in a highly reproducible model of focal cerebral ischemia in rats. Male Sprague–Dawley rats were anesthetized with halothane and subjected to 120 min of temporary middle cerebral artery occlusion by retrograde insertion of an intraluminal nylon suture coated with poly-L-lysine. The drug (LY341122, $n = 19$) or vehicle (phosphate-buffered saline (PBS), $n = 10$) was administered i.v. (as a 5 or 10 mg/kg bolus followed by a 5 or 10 mg/kg/h infusion for 20 h, respectively, starting 1 or 2 h after the onset of middle cerebral artery occlusion). Neurological status was evaluated during middle cerebral artery occlusion (60 min) and daily for 3 days thereafter. Three days after ischemia, brains were perfusion-fixed and infarct volumes and brain edema were determined. LY341122 significantly improved the neurological score compared to vehicle at 24, 48 and 72 h after middle cerebral artery occlusion. Treatment with LY341122 significantly reduced total infarct volume in all treated groups compared to vehicle rats. Cortical infarct volume was significantly reduced by LY341122 treatment in the 10 mg/kg (1 h) and LY341122 10 mg/kg (2 h) groups compared to vehicle rats (14.7 ± 9.5 vs. 106.8 ± 20.9 mm^3, and 36.9 ± 20.1 vs. 106.8 ± 20.9 mm^3, respectively (mean ± S.E.M.)). Striatal infarct volume was also significantly reduced by treatment with LY341122 in the 10 mg/kg (1 h) group compared to vehicle (23.7 ± 3.4 vs. 68.2 ± 6.7 mm^3). These results demonstrate the neuroprotective efficacy of LY341122 in focal cerebral ischemia.

Keywords: LY341122; Cerebral ischemia, focal; Neuroprotection; Stroke; Oxygen radical; (Rat)

1. Introduction

Cerebral ischemia, in particular the period following reperfusion, is accompanied by the enhanced formation of oxygen free radicals in brain tissue. Excessive production of reactive oxygen species such as superoxide anion, hydroxyl radical, and hydrogen peroxide is a known cause of oxidative stress which may result in cell injury. These oxygen intermediates react with and cause damage to lipid membranes, proteins, nucleic acids, and extracellular matrix glucosaminoglycans by abstracting electrons and propagating free radicals (Traystman et al., 1991). Reactive oxygen species are detoxified by an arsenal of endogenous antioxidants, including superoxide dismutase, catalase, glutathione peroxidase, and thioredoxin (Hall, 1994; Ginsberg, 1995).

LY341122 (2-(3,5-di-*t*-butyl-4-hydroxyphenyl)-4-(2-(4-methylethylaminomethyl-phenyloxy)ethyl)oxazole) (Fig. 1) is a potent inhibitor of lipid peroxidation, which has been shown to protect against global ischemia (Clemens et al., 1999) and traumatic brain injury in rats (Wada et al., 1999). Recent studies have shown that LY341122 is able to reduce neuronal damage in primary cultures of hippocampal neurons exposed to reactive oxygen species (Clemens et al., 1999). The only known characteristic of this compound is its ability to inhibit lipid peroxidation;

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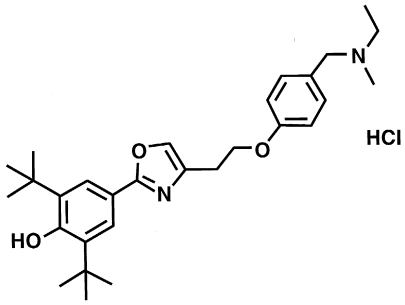

Fig. 1. Chemical structure of LY341122.

this is probably due to its tertiary butylphenol moiety, which is known to be antioxidant.

The purpose of this study was to evaluate the effect of LY341122 on neurological deficit, infarct size and the extent of edema following transient middle cerebral artery occlusion in rats.

2. Materials and methods

2.1. Animal preparation

Adult male Sprague–Dawley rats (270–330 g, Charles River Laboratories, Wilmington, MA) were fasted overnight but were allowed free access to water. Animal protocols for these studies were approved by the University of Miami Animal Care and Use Committee. Following atropine sulfate (0.5 mg/kg, i.p.), anesthesia was induced with 3.5% halothane in a mixture of 70% nitrous oxide and 30% oxygen. Rats were orally intubated, immobilized with pancuronium bromide (0.6 mg/kg, i.v.), and mechanically ventilated. Temperature probes were inserted into the rectum and the left temporalis muscle, and separate heating lamps were used to maintain rectal and cranial temperatures at 37.0°C to 37.5°C. Polyethylene catheters were

Table 1
Physiological variables. Values are mean ± S.E.M. (MCAo, middle cerebral artery occlusion; MABP, mean arterial blood pressure)

	Vehicle ($n = 10$)	LY341122			
		5 mg/kg		10 mg/kg	
		1 h ($n = 5$)	2 h ($n = 4$)	1 h ($n = 5$)	2 h ($n = 5$)
Before MCAo (15 min)					
Cranial temperature (°C)	37.4 ± 0.08	37.2 ± 0.04	37.3 ± 0.11	37.4 ± 0.12	37.4 ± 0.11
Rectal temperature (°C)	37.2 ± 0.08	37.1 ± 0.10	37.1 ± 0.08	37.3 ± 0.06	37.2 ± 0.11
pH	7.42 ± 0.01	7.44 ± 0.01	7.44 ± 0.01	7.42 ± 0.02	7.42 ± 0.01
pO_2 (mm Hg)	118 ± 12	124 ± 11	106 ± 10	119 ± 6	126 ± 10
pCO_2 (mm Hg)	39.2 ± 0.7	38.0 ± 0.5	38.5 ± 0.5	38.5 ± 0.8	39.9 ± 0.3
MABP (mm Hg)	109 ± 4	105 ± 6	116 ± 10	107 ± 6	122 ± 7
Plasma glucose (mg/dl)	125 ± 7	128 ± 11	133 ± 14	120 ± 5	132 ± 9
During MCAo (15 min)					
Cranial temperature (°C)	37.4 ± 0.10	37.2 ± 0.06	37.4 ± 0.11	37.3 ± 0.09	37.3 ± 0.12
Rectal temperature (°C)	37.3 ± 0.08	37.2 ± 0.04	37.2 ± 0.07	37.3 ± 0.09	37.1 ± 0.14
pH	7.41 ± 0.02	7.42 ± 0.01	7.43 ± 0.01	7.41 ± 0.01	7.41 ± 0.01
pO_2 (mm Hg)	136 ± 11	118 ± 6	101 ± 9	116 ± 7	116 ± 9
pCO_2 (mm Hg)	40.0 ± 1.9	39.4 ± 0.8	39.7 ± 0.6	40.0 ± 0.3	39.7 ± 0.4
MABP (mm Hg)	111 ± 4	109 ± 7	120 ± 5	107 ± 4	109 ± 11
Plasma glucose (mg/dl)	122 ± 5	133 ± 8	142 ± 12	122 ± 6	131 ± 10
15 min after recirculation					
Cranial temperature (°C)	37.3 ± 0.08	36.9 ± 0.07	37.1 ± 0.08	37.1 ± 0.10	37.2 ± 0.13
Rectal temperature (°C)	37.3 ± 0.06	37.2 ± 0.12	37.1 ± 0.10	37.2 ± 0.10	37.1 ± 0.04
MABP (mm Hg)	92 ± 4	105 ± 5	105 ± 6	107 ± 4	102 ± 7
24 h after MCAo					
Rectal temperature (°C)	37.3 ± 0.16	37.0 ± 0.12	37.2 ± 0.57	36.9 ± 0.25	36.9 ± 0.24
MABP (mm Hg)	98 ± 7	100 ± 4	105 ± 9	103 ± 4	93 ± 7
48 h after MCAo					
Rectal temperature (°C)	37.4 ± 0.24	37.2 ± 0.57	37.7 ± 0.13	37.4 ± 0.14	37.2 ± 0.31
MABP (mm Hg)	100 ± 0	98 ± 3	98 ± 3	98 ± 3	100 ± 6
72 h after MCAo					
Rectal temperature (°C)	37.3 ± 0.14	37.1 ± 0.33	37.4 ± 0.16	37.0 ± 0.43	37.5 ± 0.18
MABP (mm Hg)	100 ± 0	98 ± 3	98 ± 3	98 ± 3	96 ± 2

introduced into the femoral artery and vein for blood pressure recording, blood sampling and for drug infusion.

2.1.1. Drug-infusion system

The femoral vein cannula was tunneled subcutaneously so as to exit from the dorsal neck and was connected to a swivel device (Stoelting, Wood Dale, IL) attached to the roof of the spacious cage. This device allowed bidirectional rotation. PE-50 tubing extending from the other end of the device was joined to a disposable syringe attached to an infusion pump (KDS-100, KD Scientific, Boston, MA). This tethering system allowed the rats' free movement without interfering with the patency of the cannula during drug administration.

2.2. Middle cerebral artery occlusion

Middle cerebral artery occlusion was induced as described by Zea et al. (1989) and modified by us (Belayev et al., 1996). Under an operating microscope, the right common carotid artery was exposed through a midline neck incision and was carefully dissected free from surrounding nerves and fascia from its bifurcation to the base of the skull. The occipital artery branches of the external carotid artery were then isolated, and these branches were dissected and coagulated. The internal carotid artery was isolated and carefully separated from the adjacent vagus nerve, and the pterygopalatine artery was ligated. Next, a 4-cm length of 3–0 monofilament nylon suture was inserted via the proximal external carotid artery into the internal carotid artery and thence into the circle of Willis, effectively occluding the middle cerebral artery. In producing middle cerebral artery occlusion, we made use of poly-L-lysine-coated suture as previously described (Belayev et al., 1996). The suture was inserted 19 to 20 mm from the bifurcation of the common carotid artery, according to the animal's body weight. After the intraluminal suture was placed, the neck incision was closed with a silk suture. Animals were then allowed to awaken from anesthesia and were returned to their cages. Rats that did not demonstrate a left upper extremity paresis during this recovery period were excluded from further study (see Behavioral testing, below). After 2 h of middle cerebral artery occlusion, rats were reanesthetized with the same anesthetic combination. Temperature probes were reinserted, and the intraluminal suture was carefully removed. The neck incision was closed with silk sutures, and the animals were allowed to survive for 3 days with free access to food and water.

2.3. Treatment groups

The drug (LY341122, 5 or 10 mg/kg), or vehicle (phosphate-buffered saline, PBS), were administered i.v. at 1 or 2 h after the onset on middle cerebral artery occlusion. Animals were divided into the six following treatment groups:

1. LY341122, 5 mg/kg (1 h) group, ($n = 7$): 5 mg/kg bolus (at a constant rate over 15 min) plus 5 mg/kg/h infusion for 20 h starting 1 h after onset of middle cerebral artery occlusion.
2. LY341122, 5 mg/kg (2 h) group, ($n = 4$): 5 mg/kg bolus (at a constant rate over 15 min) plus 5 mg/kg/h infusion for 20 h starting at the time of reperfusion (i.e., after 2 h middle cerebral artery occlusion).
3. LY341122, 10 mg/kg (1 h) group, ($n = 5$): 10 mg/kg bolus (at a constant rate over 15 min) plus 10 mg/kg/h infusion for 20 h starting 1 h after onset middle cerebral artery occlusion.
4. LY341122, 10 mg/kg (2 h) group, ($n = 7$): 10 mg/kg bolus (at a constant rate over 15 min) plus 10 mg/kg/h infusion for 20 h starting at the time of reperfusion.
5. Vehicle (1 h) group, ($n = 4$): PBS vehicle bolus plus 0.4 ml/kg/h infusion for 20 h starting 1 h after onset of middle cerebral artery occlusion.

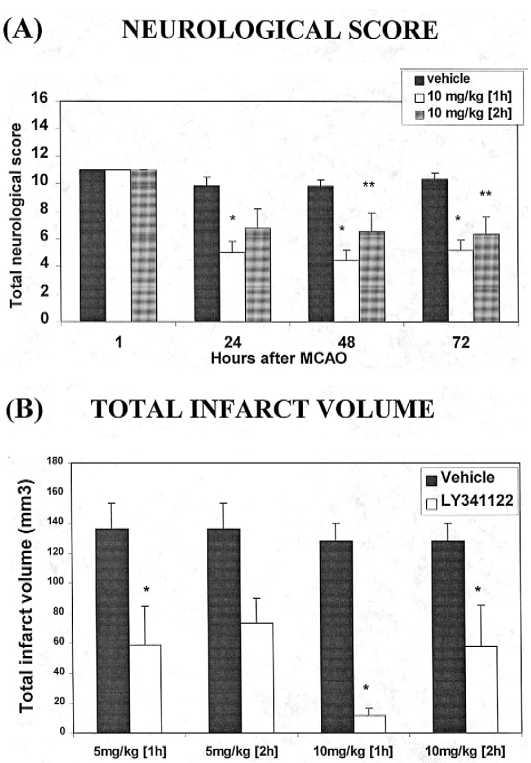

Fig. 2. Panel A: Total neurological score (normal score = 0; maximal score = 12) at various times after middle cerebral artery occlusion in LY341122- and vehicle-treated rats. $^{*}P < 0.05$, LY341122 10 mg/kg (1 h) vs. vehicle; $^{**}P < 0.05$, LY341122 10 mg/kg (2 h) vs. vehicle (Student's *t*-tests). Panel B: Total corrected infarct volume in LY341122 and vehicle groups. $^{*}P < 0.05$, treated vs. vehicle groups (one-way ANOVA followed by Dunnett's test). Numbers of animals: $n = 10$ in combined vehicle-treated groups; $n = 5$ in LY341122 5 mg/kg (1 h) group; $n = 4$ in LY341122 5 mg/kg (2 h) group; $n = 5$ each in LY341122 10 mg/kg (1 h) and LY341122 10 mg/kg (2 h) groups.

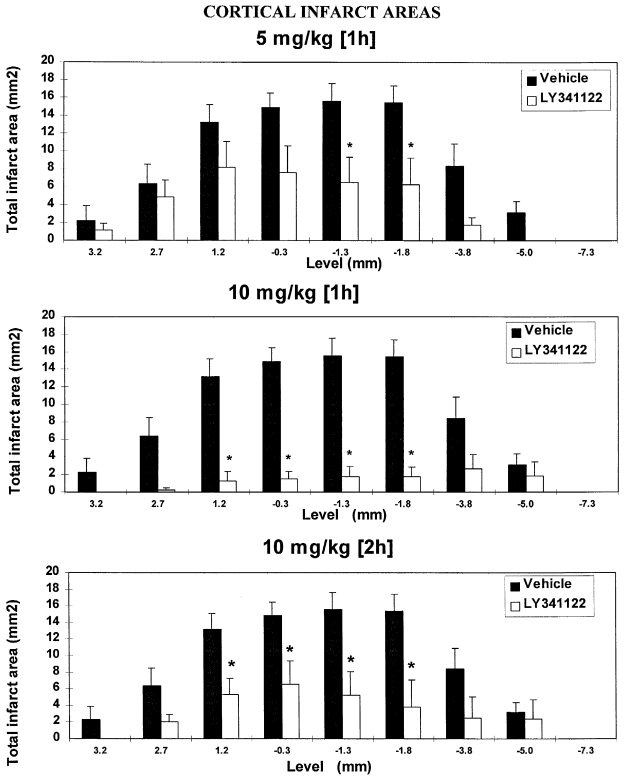

Fig. 3. Bar graphs showing rostrocaudal distribution of areas of *cortical infarction* at nine coronal levels in LY341122- (5 mg/kg (1 h), 10 mg/kg (1 h), and 10 mg/kg (2 h)) and the combined vehicle-treated groups. Data are presented as means ± S.E.M. $^{*}P < 0.05$, treated vs. vehicle (repeated-measures ANOVA followed by Dunnett's test).

6. Vehicle (2 h) group, ($n = 6$): PBS vehicle bolus plus 0.9 ml/kg/h infusion for 20 h starting 1 h after onset of middle cerebral artery occlusion.

2.4. Behavioral testing

Behavioral tests were performed in all rats before middle cerebral artery occlusion, during occlusion (at 50–60 min), and 24, 48 and 72 h after reperfusion. The battery consisted of two tests that have been used previously to evaluate various aspects of neurologic function: the postural reflex test, to examine upper-body posture, and the forelimb placing test, to examine sensorimotor integration in forelimb placing responses to visual, tactile and proprioceptive stimuli.

2.4.1. Postural reflex test

This test, designed to examine upper-body posture, is regarded as being sensitive to both cortical and striatal lesions (Bederson et al., 1986). Rats were suspended by the tail 1 m above the floor. Intact rats extended both forelimbs toward the floor; animals displaying this behavior were assigned a score of 0. A score of 1 was given if the rat flexed one or both forelimbs. The animal was then given the lateral push test, which involved placing the rat on a sheet of plastic-coated paper and applying lateral pressure behind the shoulders in the left and right directions. If the rat was unable to resist the force equally in both directions, it received a score of 2.

2.4.2. Elicited forelimb placing

Forelimb placing reactions to visual, tactile, and proprioceptive stimuli were measured for each forelimb to examine sensorimotor integration (De Ryck et al., 1989). For visual placing, the animal was cupped in the experimenter's hands with the forelimb hanging free and was slowly tilted and lowered from each side toward a tabletop. Intact rats reached for the table with both forelimbs extended. By moving the animal laterally toward the table edge, sideways visual placing could be assessed as well. Tactile placing was judged by lightly contacting the dorsal and then the lateral surface of the rat's forepaw to the table edge while slightly elevating its head to obscure its view of the table. Intact rats immediately placed the paw on the tabletop, while impaired rats either were slow to place or did not place at all. Proprioceptive placing was also assessed by pressing the rat's paw against the table edge to stimulate limb muscles. For each of these tests, a score of 0 was given for normal, immediate placing. A score of 1

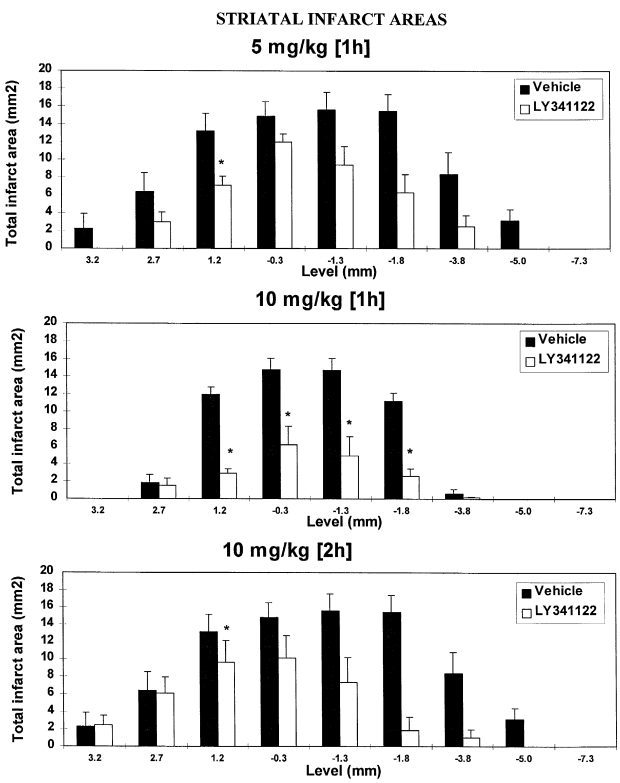

Fig. 4. Bar graphs showing rostrocaudal distribution of areas of *striatal infarction* at nine coronal levels in LY341122- (5 mg/kg (1 h), 10 mg/kg (1 h), and 10 mg/kg (2 h)) and the combined vehicle-treated groups. Data are presented as means ± S.E.M. $^{*}P < 0.05$, treated vs. vehicle (repeated-measures ANOVA followed by Dunnett's test).

was given if the placing was delayed or incomplete. A score of 2 indicated absent placing.

2.4.3. Total neurological score

Total neurological score was calculated as the sum of scores on forward visual placing (range, 0–2), lateral visual placing (0–2), dorsal (0–2) and lateral (0–2) tactile placing, proprioceptive placing (0–2) and postural reflex (0–2). Thus, the maximal possible score was 12, as previously described (Belayev et al., 1996; Zhao et al., 1997).

Rats with convulsions or sustained disturbances of consciousness were excluded from the study; most of these cases prove to have subarachnoid hemorrhage secondary to suture-induced rupture of the internal carotid artery. In the present study, three animals developed subarachnoid hemorrhage, as evidenced by persistent unconsciousness, before assignment to treatment groups. They were thus excluded from the study. Autopsy confirmed subarachnoid hemorrhage in all cases.

2.5. Histological assessment of infarction and edema volume

Animals were allowed to survive for 3 days. Brains were then perfusion-fixed with a mixture of 40% formaldehyde, glacial acetic acid, and methanol (FAM, 1:1:8 by volume), and brain blocks were embedded in paraffin. Ten-micron thick sections were cut in the coronal plane and stained with hematoxylin and eosin. To quantitate infarct volume and depict infarct frequency distribution, histological sections were digitized at nine standardized coronal levels by means of a digital camera (Xillix Technologies, Vancouver, Canada) interfaced to an MCID image-analysis system (Imaging Research, St. Catherines,

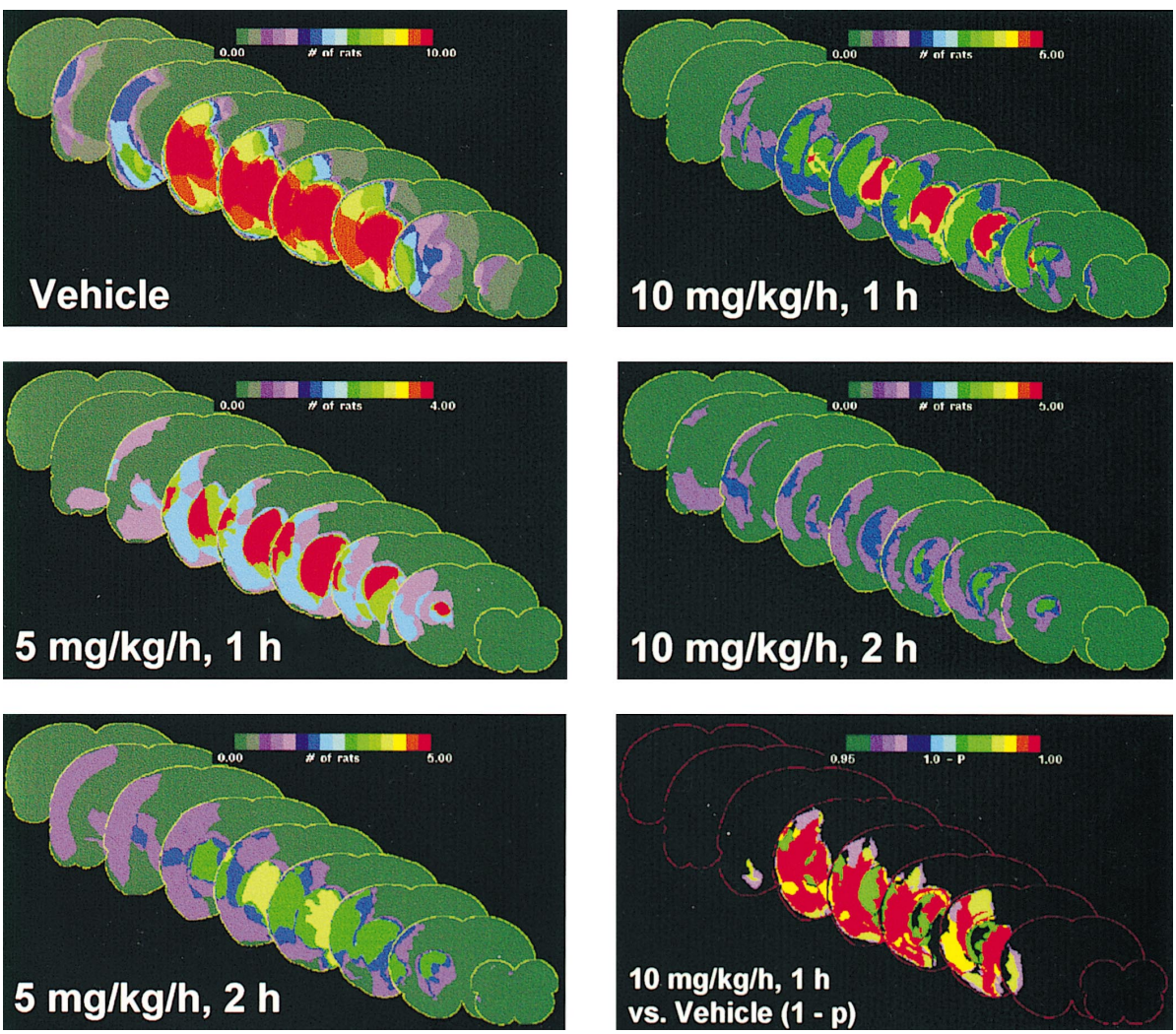

Fig. 5. Pixel-based frequency maps of histological infarction at nine stereotactic levels in rat brains treated with vehicle and LY341122. The color bars depict numbers of animals with infarction at each pixel location. (The lower right panel presents the results of a Fisher exact test comparing the LY341122 10 mg/kg (1 h) and vehicle groups; the color bar depicts $(1-p)$, thresholded from 0.95 to 1.00 (i.e., $P < 0.05$). The topographic distribution of LY341122-associated cortical and subcortical neuroprotection is evident.)

Ontario, Canada), from which data were exported to a DEC-Alpha workstation (Digital Equipment, Maynard, MA) for processing. An investigator blinded to the experimental groups then outlined the zones of infarction as well as the left and right hemisphere contours for each section. The volume of infarction was calculated as the product of cross-sectional area for all sections and the distance between sections. To compensate for brain swelling in the ischemic hemisphere, total infarct volume in each rat was corrected by first computing the volume of the left and right hemispheres, and applying the following formula: corrected infarct volume = left hemisphere volume − (right hemisphere volume − measured infarct volume) (Swanson et al., 1990). The degree of associated brain edema was determined as the difference in brain volume between the two hemispheres. To generate frequency maps of histological infarction, digitized histology data-sets of individual animals were dichotomized on a pixel-by-pixel basis and were mapped at corresponding coronal levels into a common atlas template as previously described (Zhao et al., 1996, 1997).

2.6. Statistical analysis

Physiological variables, neurological score, and infarct volumes were compared among groups by analysis of variance (ANOVA) with post-hoc Bonferroni's tests to correct for multiple comparisons. Infarct areas and brain edema at various coronal levels were analyzed by repeated-measures ANOVA with post-hoc Bonferroni's and Dunnett's tests. Infarct frequency maps in vehicle- and LY341122-treated rats were compared on a pixel-by-pixel basis by the Fisher's exact test (Siegel, 1956). $P < 0.05$ was regarded as significant. Values are presented as mean values ± S.E.M.

3. Results

3.1. General physiological variables

Rectal and cranial (temporalis muscle) temperatures, arterial blood pressure, plasma glucose and blood gases showed no significant differences among groups (Table 1).

3.2. Neurological assessment

Contralateral forelimb placing deficits were clearly present at 50–60 min after the onset of middle cerebral artery occlusion in all rats. LY341122 (5 mg/kg (2 h)) significantly improved the neurological score compared to vehicle at 48 and 72 h after middle cerebral artery occlusion

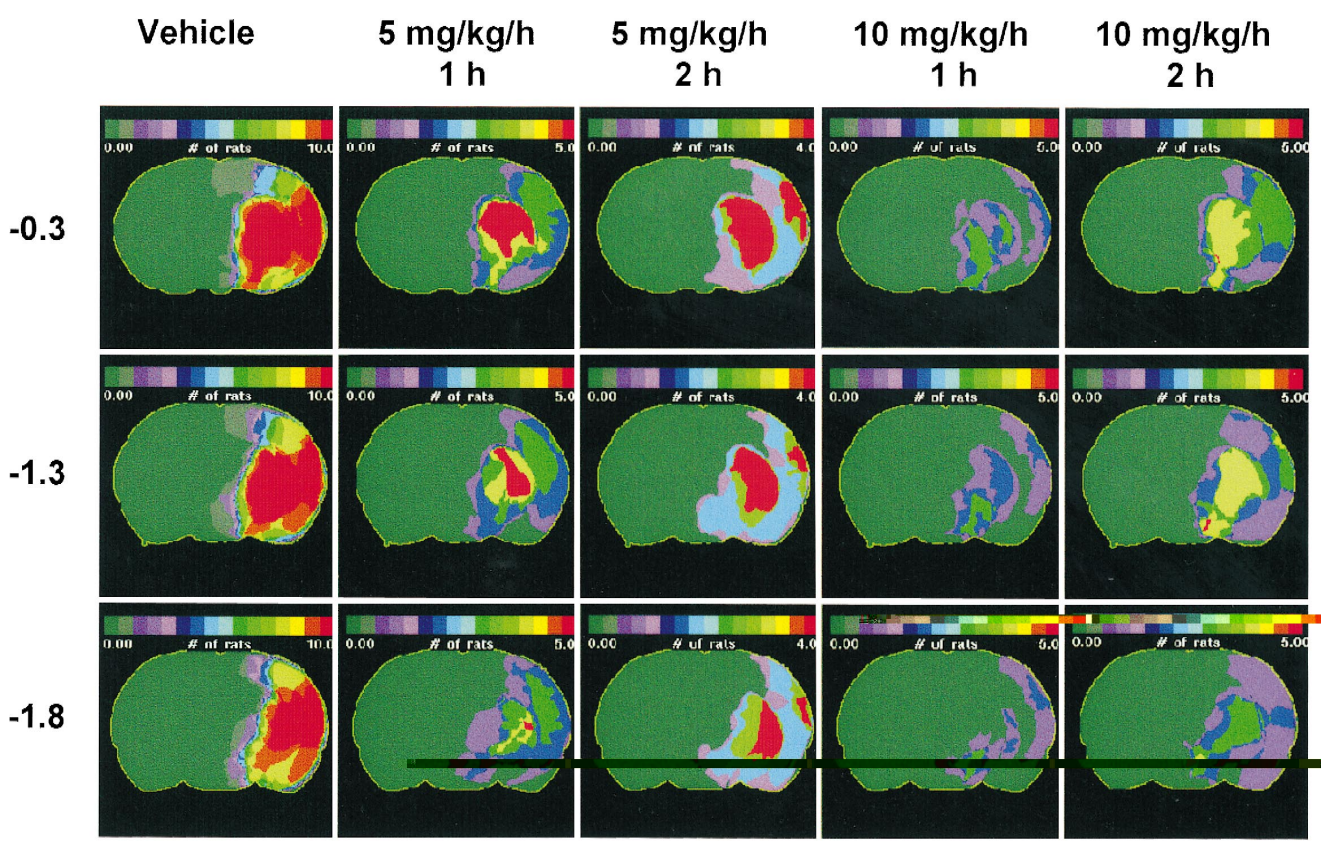

Fig. 6. Pixel-based frequency maps of histological infarction in vehicle- and LY341122-treated groups at coronal levels of bregma −0.3, −1.3 and −1.8 mm. Color bars represent numbers of animals having infarction at each pixel location.

(8.0 ± 0.6 vs. 9.8 ± 0.5, $P < 0.05$; and 7.8 ± 0.8 vs. 10.3 ± 0.5, $P < 0.03$, respectively). Higher doses of LY341122 (10 mg/kg (1 h) and 10 mg/kg (2 h)) resulted in more marked improvements of neurological score compared to vehicle which were significant at 24, 48 and 72 h after middle cerebral artery occlusion (Fig. 2A). Rats treated with LY341122 (5 mg/kg (1 h)) tended to have an improved neurological score at 24 h, but this did not reach statistical significance.

3.3. Histopathology

Treatment with LY341122 significantly reduced total (cortical + subcortical) corrected infarct volume in all treated groups compared to vehicle-treated rats (Fig. 2B). When LY341122 treatment was begun at 1 h at the higher dose (10 mg/kg (1 h)), a profound degree of neuroprotection was attained, amounting to 91% reduction in mean infarct volume. In the other LY341122 treatment groups, more moderate, statistically significant degrees of neuroprotection were also achieved (46–56% mean reductions in total infarct volume).

When considered separately, cortical infarct volume was significantly reduced by treatment with LY341122 in the 10 mg/kg (1 h) and 10 mg/kg (2 h) groups compared to vehicle rats (14.7 ± 9.5 vs. 97.8 ± 19 mm^3, $P < 0.005$; and 36.9 ± 20.1 vs. 106.8 ± 20.9 mm^3, $P < 0.04$, respectively). Cortical infarct areas were significantly smaller in drug-treated rats than in the vehicle groups at multiple coronal levels (Fig. 3). *Striatal* infarct volume was significantly reduced by treatment with LY341122 only in the 10 mg/kg (1 h) group compared to the vehicle group (23.7 ± 3.4 vs. 68.2 ± 6.7 mm^3, $P < 0.0004$), and striatal infarct areas were correspondingly smaller in treated rats of that group (Fig. 4). Mean hemispheral brain edema measured volumetrically ranged from 6.4% to 11.2% and did not differ statistically between LY341122- and vehicle-treated groups.

Figs. 5 and 6 present pixel-based frequency maps depicting the topography of cerebral infarction in rats treated

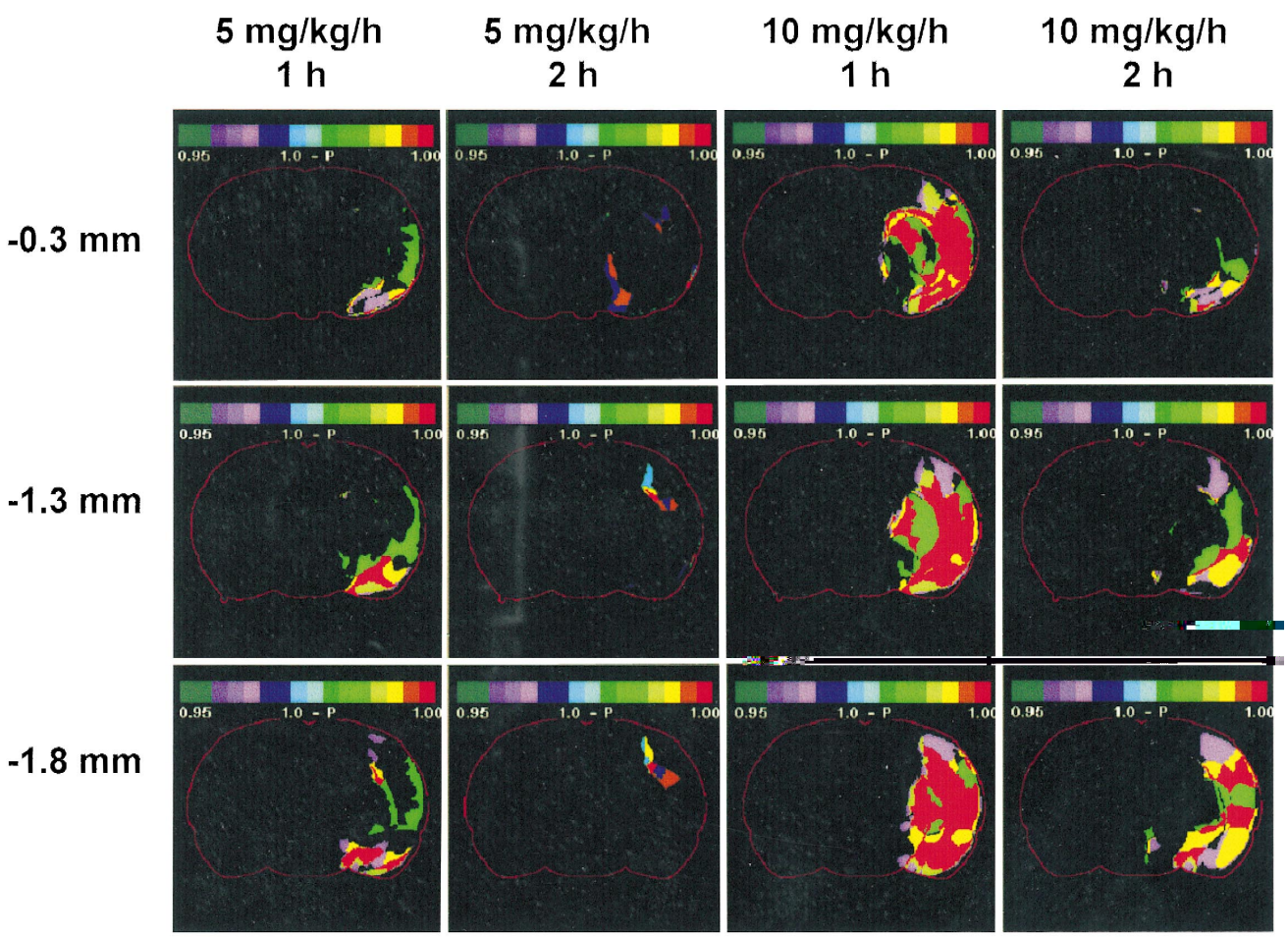

Fig. 7. The results of Fisher exact tests comparing histological frequency maps in each of the four LY341122-treatment groups vs. vehicle-treated animals, performed on a pixel-by-pixel basis. (Coronal levels are the same as shown in Fig. 6.) The color bars depict $(1 - p)$, where p represents the level of statistical significance, and are thresholded at 0.95 to 1.00 (i.e., $P < 0.05$). The relative neuroprotective efficacy of the four LY341122 dosing regimens is readily evident.

with LY341122 and vehicle. Fig. 7 shows the results of Fisher exact tests comparing infarction in vehicle- vs. drug-treated groups. LY341122 treatment, particularly in the 10 mg/kg (1 h) group, was associated with marked reductions in the probability of infarction throughout the neocortex.

All LY341122-treated animals developed transient hematuria and bloody ocular discharges, which disappeared after 24 h. (Previous studies (J.A. Clemens, unpublished) suggest that this is due to a minor degree of hemolysis when the compound is dissolved in PBS, attributable to the mild detergent property of the compound coupled with the low pH of the injection vehicle.) Four animals died in our study: two of seven rats in the LY341122 5 mg/kg (1 h) group (one on the second and one on the third day), and two of seven rats in the LY341122 10 mg/kg (2 h) groups (both on the second day). Autopsy revealed large ipsilateral hemispheric infarcts and extensive brain edema. No animal died in the control group.

4. Discussion

The present results show that the lipid-peroxidation inhibitor LY341122, when administered to rats with reversible middle cerebral artery occlusion, confers substantial neuroprotection that is evident both on neurobehavioral examination and by quantitative histopathology. These data provide useful information regarding the dose–response characteristics of this agent as well as the therapeutic window within which it is effective. Our study was designed specifically to assess the *postischemic* neuroprotective efficacy of LY341122, and our results establish that the therapeutic window for this agent extends to at least 2 h after the onset of reversible middle cerebral artery occlusion. Maximal histopathological protection (amounting to greater than 90% reduction in infarct size) was attained with the 10 mg/kg dose initiated at 1 h after the onset of ischemia, while substantial though lesser neuroprotection (46–56% infarct-size reduction) was achieved by doses of either 5 mg/kg (initiated at 1 h), 5 mg/kg (initiated at 2 h), or 10 mg/kg (initiated at 2 h). The protective effect of LY341122 in this study could not be explained by differences in systemic or brain temperatures, arterial pressure, or arterial blood gases because these variables were carefully controlled and did not differ among groups.

The doses of LY341122 used in this study were chosen on the basis of previous experience with this agent. In a rat model of global forebrain ischemia by four-vessel occlusion, Clemens et al. (1999) reported that immediate postischemic treatment with 10 and 5 mg/kg/h doses of LY341122 for 20 h following a 20-min insult reduced hippocampal CA1 neuronal damage by 85% and 33–46%, respectively; significant neuroprotection was also obtained when treatment was started 2 h after reperfusion.

In a study of moderate fluid-percussion brain injury in rats, early post-treatment with LY341122 (10 mg/kg bolus and 5 mg/kg/h infusion × 20 h) led to a significant reduction in overall contusion volume and also reduced the frequency of damaged cortical neurons (Wada et al., 1999). In others studies, when LY341122 was administered in vivo for 20 h and brain homogenates were then subjected to in vitro oxidation, the agent reduced the formation of thiobarbituric acid reactive substances by 79% (Clemens et al., 1999).

There is extensive experimental support for the early occurrence and pathophysiological relevance of oxygen radical formation and cell-membrane lipid peroxidation in the injured nervous system (Hall, 1994). The radical-initiated peroxidation of neuronal, glial, and vascular cell membranes and myelin is promoted catalytically by free iron released from hemoglobin, transferrin, and ferritin under conditions of lowered tissue pH or oxygen-radical attack. If untreated, lipid peroxidation is a propagating process that impairs phospholipid-dependent enzymes, disrupts ionic gradients, and promotes membrane lysis (Hall, 1998; Watson, 1998). These considerations have prompted the development and application of a variety of antioxidant approaches to attenuate neuronal injury.

Superoxide radicals generated during and after ischemia appear to play a crucial role in development of neuronal damage (Liu et al., 1994; Chan, 1996). Several reports have disclosed that superoxide dismutase, an enzymatic scavenger of superoxide radicals, effectively diminishes ischemic neuronal damage both in global and focal cerebral ischemia animal models. Copper/zinc superoxide dismutase (CuZn-superoxide dismutase) and manganese superoxide dismutase (Mn-superoxide dismutase) are two major forms of mammalian superoxide dismutase existing in the cytosol and mitochondria, respectively (Fridovich, 1975). Cerebral ischemia has been extensively studied in transgenic mice overexpressing human CuZn-superoxide dismutase (Chan, 1996). Transgenic mice overexpressing CuZn-superoxide dismutase showed no differences in infarct areas from their nontransgenic littermates under conditions of permanent middle cerebral artery occlusion (Chan et al., 1993) but exhibited a one-quarter reduction of infarct volume after temporary middle cerebral artery occlusion followed by reperfusion (Yang et al., 1994). Taken together, these studies suggest that antioxidant therapy is likely to be more beneficial when reperfusion is permitted, possibly owing to the more effective delivery of the therapeutic agent to the reperfused brain, or to the more extensive production of oxygen radicals during the reperfusion phase. Microdialysis studies have suggested that hydroxyl radicals are generated in the cortical penumbral region throughout the period of middle cerebral artery occlusion and early reperfusion periods, and that reduced cerebral blood flow levels during reperfusion favor this process

(Morimoto et al., 1996). The protection afforded by free radical scavengers appears most evident in penumbral areas of cortex (Martz et al., 1990).

A great variety of compounds to inhibit free radical production or lipid peroxidation have been evaluated as potential therapeutic agents to attenuate reperfusion injury following cerebral ischemia (Hall, 1998). These include polyethylene glycol conjugated-superoxide dismutase and catalase (Liu et al., 1989), dimethylthiourea (Martz et al., 1990), PBN (*N-tert*-butyl-α-phenylnitrone) (Cao and Phillis, 1994), α-tocopherol (Hall, 1994), 21-aminosteroids (Hall, 1998), and pyrrolopyrimidines (Hall et al., 1997; Soehle et al., 1998). In previous studies, free radical scavengers reduced infarct size only moderately (typically by 25% to 35%) after focal ischemia (Hall, 1998). Greater successes have been achieved with the spin-trap agent PBN (Folbergrova et al., 1995). Nitrone-based spin-traps penetrate the brain readily at high concentrations and react with transient free radicals to form stable adducts (Sakamoto et al., 1991). In transient focal ischemia in rats, 50% protection has been reported with PBN treatment (Folbergrova et al., 1995).

Protective drugs that are able to cross the blood–brain barrier may have an advantage in ameliorating ischemic damage (Moore and Traystman, 1994; Belayev et al., 1998). These agents may be more efficacious because they are lipophilic and thus localize to neuronal membranes, where they can more effectively inhibit lipid peroxidation (Traystman et al., 1991). LY341122 is highly lipophilic drug; 1 h after its intravenous administration, brain levels are about five-fold greater than serum levels, and brain levels remain elevated at 24 h, when serum levels are undetectable (Clemens et al., unpublished data).

As vascular endothelial cells are known to be targets of free radical injury and may also be a source of free radical production (Hall, 1998), the beneficial effect of free radical scavengers in cerebral ischemia may be mediated in part via protection of the cerebral microvasculature. Thus, the administration of endothelial-targeted antioxidants might be beneficial in treating cerebral ischemia (Schmid-Elsaesser et al., 1997). Antioxidant protection of injured cerebrovascular endothelium might help to preserve perfusion through collateral channels so as to maintain blood flow above the threshold for tissue damage.

Middle cerebral artery occlusion in rodents may produce varying volumes of infarction, depending upon the rat strain employed, the method of middle cerebral artery occlusion, and the anatomic location of the occlusion site (Oliff et al., 1995). Intraluminal occlusion of the middle cerebral artery occlusion has become increasingly popular as a focal-ischemia model owing to its relative simplicity and minimally invasive nature (Zea et al., 1989; Laing et al., 1993). Different groups, however, have reported varying degrees of reproducibility with this approach [e.g., (Laing et al., 1993; Belayev et al., 1996). One source of infarct-size variability appears to be related to the extent of insertion of the suture, its size, and other characteristics of the suture itself (Kuge et al., 1995). In the present study, and in recently published observations (Belayev et al., 1996; Zhao et al., 1997), we have employed a poly-L-lysine-coated suture and have found that this technique leads to reliable and highly consistent results (coefficient of variation of infarct volume, 9%).

Observation of neurological deficits is generally important not only in clinical cases of stroke but also in experimental cerebral ischemia models. Various neurological and behavioral examinations have been employed to evaluate functional impairment of rats after cerebral ischemia. Focal ischemia induced a neurological deficit characterized by sensorimotor dysfunction, which has been noted as well by previous workers (Bederson et al., 1986; De Ryck et al., 1989; Markgraf et al., 1994). It is important that the design of preclinical drug trials be relevant to clinical stroke in humans, in which functional outcome rather than infarct volume is the typical study end-point. For these reasons, complementing histological analyses with behavioral testing in the preclinical evaluation of potential stroke therapies is desirable. The behavioral tasks in this study were chosen to address specific functional deficits associated with the neural areas damaged by middle cerebral artery occlusion (i.e., striatum and dorsolateral neocortex) (Belayev et al., 1996; Zhao et al., 1997). LY341122 significantly improved the neurological score compared to vehicle at 24–72 h after middle cerebral artery occlusion. These data agree with the findings of other workers who have noted significant neurological improvement after antioxidant-treatment (pyrrolopyrimidine and 21-aminosteroid) of focal cerebral ischemia in rats (Schmid-Elsaesser et al., 1997).

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