The Antioxidant LY231617 Reduces Global Ischemic Neuronal Injury in Rats

J.A. Clemens, PhD; R.D. Saunders, PhD; P.P. Ho, PhD; L.A. Phebus, PhD; and J.A. Panetta, PhD

Background and Purpose: In the rat four-vessel occlusion model with 30 minutes of ischemia most agents have failed to be of benefit when given after ischemia. Because posts ischemia administration is more clinically relevant, we evaluated the antioxidant LY231617 (2,6-bis(1,1-dimethylethyl)-4-[[1-(1-ethyl)amino]methyl]phenol hydrochloride) when administered after 30 minutes of four-vessel occlusion.

Methods: Male Wistar rats were subjected to 30 minutes of four-vessel occlusion. LY231617 was either given orally 30 minutes before ischemia or intravenously beginning at 30 minutes after the onset of ischemia. Hippocampal CA1 layer and striatal damage were rated on a scale of 0–3 (0, no damage; 3, >90% cell loss). We also evaluated the ability of LY231617 to prevent iron-dependent lipid peroxidation and to prevent hydrogen peroxide–induced neuronal death of hippocampal neurons in primary culture by exposing cultures to a 50-µM concentration of hydrogen peroxide for 15 minutes in the presence of LY231617.

Results: Oral administration of LY231617 reduced both striatal and hippocampal CA1 damage by >75% \( (p < 0.0001) \). In two separate experiments in which LY231617 was given intravenously beginning 30 minutes after occlusion, hippocampal and striatal damage were reduced by approximately 50% \( (p < 0.03) \) in the first experiment and by approximately 41% \( (p < 0.002) \) in the second experiment. Addition of 5 \( \mu M \) of LY231617 to primary hippocampal neuronal cultures antagonized the lethal effect of hydrogen peroxide \( (p < 0.05) \). Iron-dependent lipid peroxidation was also inhibited in a dose-related fashion.

Conclusions: The significant reduction of ischemia-induced or hydrogen peroxide–induced neuronal damage and inhibition of lipid peroxidation by LY231617 observed in this study suggest that reactive oxygen intermediates play an important role in the events leading to neuronal death after global ischemia/reperfusion. (Stroke 1993;24:716–723)

Key Words • cerebral ischemia • free radicals • neuronal death • rats

Damage resulting from the generation of oxygen-derived free radicals has been proposed as a mechanism that contributes to ischemia-induced cell death.\(^1,6\) Evidence that supports this hypothesis is derived from studies showing that compounds possessing free radical scavenging and/or lipid peroxidation–inhibiting activities reduced brain damage after ischemia.\(^2,7–12\) Although several compounds with radical scavenging properties have been shown to be effective in ameliorating ischemia-induced brain damage, actual proof of the presence of free radicals during ischemia and reperfusion in the brain has been somewhat difficult to obtain. Recently, through the use of electron paramagnetic resonance techniques and spin trap reagents, direct measurement of free radicals resulting from ischemia/reperfusion has been reported.\(^13\) The presence of free radicals during ischemia/reperfusion implicates them as factors that are, at least in part, responsible for the resulting neuronal cell death because free radicals are among the most toxic chemical species known.

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Our laboratory recently reported that LY178002, a compound that inhibits both iron-dependent lipid peroxidation and key enzymes of the arachidonic acid cascade, was effective in reducing damage in the hippocampal CA1 layer and in corpus striatum in animals subjected to 30 minutes of four-vessel occlusion.\(^4,11\) The low aqueous solubility of LY178002 prompted us to search for related chemical structures that would be equally or more efficacious and suitable for intravenous administration. LY231617 (2,6-bis(1,1-dimethylethyl)-4-[[1-(1-ethyl)amino]methyl]phenol hydrochloride) is such a compound. Similar to LY178002, the aromatic portion of the LY231617 molecule (Figure 1) contains the backbone structure of butylated hydroxytoluene, a known antioxidant. Thus, because of the similarity of this compound to known antioxidants we decided to examine its ability to inhibit lipid peroxidation and to evaluate it in a model of oxidant-induced neuronal injury in vitro and then in the four-vessel occlusion model of global ischemia in vivo. A study was also performed to determine if LY231617 is able to penetrate the blood–brain barrier.

Materials and Methods

Peroxidation of rabbit brain vesicular membrane lipids was assayed as previously described.\(^13\) Frozen ma-
ture stripped rabbit brain (Pell-Freeze Biologicals, Rogers, Alaska) was thawed, minced, homogenized in 25 mM tris(hydroxymethyl)aminomethane HCl containing 0.15 M potassium chloride, pH 7.5 (10% wt/vol), and centrifuged at low speed (1,000g for 15 minutes). The test agent (dissolved in 5 uL dimethyl sulfoxide) was added to the supernatant fraction (0.5 mL), which was then incubated with 220 mM adenosine diphosphate and 2 mM ferric chloride at 37°C for 1 hour. The control supernatant was not incubated. The reaction was terminated by adding 0.4 mL of thiobarbituric acid–reactive substance reagent (0.02% trichloroacetic acid and 0.8% thiobarbituric acid) and boiled for 15 minutes. The sample was acidified with 2.3N hydrochloric acid (0.5 mL), extracted with 1-butanol (1 mL), and centrifuged at 3,000g for 5 minutes. The absorbency of the butanol phase was determined at 532 nm in a Spectronic-20 spectrophotometer (Westbury, N.Y.), and the amount of malondialdehyde present was determined by linear regression analysis of a standard curve.

For the in vitro evaluation of the ability of LY231617 to inhibit oxidative damage, rat hippocampal neurons were used. Primary cultures of hippocampal neurons were prepared by modifications of the methods described by Banker and Cowan

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and Novelli et al

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using rat fetuses of 18–19-day gestation. The fetuses were removed, and the hippocampi were dissected under a dissecting microscope. Hippocampi were placed in solution I containing (mM): NaCl 124, KCl 5.37, Na2HPO4 1, glucose 14.5, N-2-hydroxyethylpiperezine-N'-2-ethanesulfonic acid (HEPES) 25, MgSO4 1.2, bovine serum albumin (3 mg/mL), and penicillin/streptomycin (5,000 units/L) at pH 7.4. The tissue was disrupted by trypsin (0.75% stock trypsin in 29 mL solution I) treatment for 15 minutes at 37°C. The tissue was centrifuged at 1,400 rpm for 3 minutes, the trypsin solution removed, and a DNase/soybean trypsin inhibitor solution (1 mL DNase [400 uM/mL]+1 mL soybean trypsin inhibitor [2.5 mL]+14 mL solution I) added. The suspension was vortexed and incubated at 37°C for 15 minutes. The suspension was gently triturated with a Pasteur pipette and then passed through a cell strainer (70-μm nylon mesh; Falcon). The cell suspension was centrifuged at 1,400 rpm for 5 minutes, the supernatant removed, and then 10 mL of plating media (Dulbecco’s modified Eagle’s medium with low glucose, 10% fetal calf serum, 4.0 g/L glucose, 1.5 g/L KCl, and 5,000 units/L penicillin/streptomycin, pH 7.4) was added. Cells were plated in 35-mM six-well cluster dishes (approximately 15,000 cells/cm²), which had been precoated with poly-d-lysine. Hydrogen peroxide toxicity studies were performed on mature (10–14 days in vitro) cultures as follows. The conditioned media was removed, and the cultures were rinsed with a HEPES-buffered saline (HBS) solution (136 mM NaCl, 5.4 mM KCl, 0.62 mM MgSO4, 1.0 mM CaCl2, 1.1 mM KH2PO4, 1.1 mM Na2HPO4, 25 mM HEPES, and 5,000 units/L penicillin/streptomycin, pH 7.4). The cultures were exposed to various concentrations of hydrogen peroxide in HBS for 15 minutes at 37°C. A dose–response curve was determined for hydrogen peroxide–induced lethality. For neuroprotection evaluation, LY231617 was added concurrently with hydrogen peroxide during the 15-minute exposure. After 15 minutes the test solutions were removed, the cultures were rinsed three times with HBS, and the conditioned media was returned to the cultures. Viability was assessed the next day with the vital stains fluorescein diacetate and propidium iodide (Jones and Senff

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In the global ischemia studies we used male Wistar rats from Hilltop Laboratories, Scottsdale, Pa., weighing 250–280 g. The rats were subjected to 30 minutes of forebrain ischemia by the method of Pulsinelli and Brierley.

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After the period of ischemia, the animals were allowed free access to food and water. Seventy-two hours after four-vessel occlusion, the rats were killed, and the brains were removed, frozen, and sectioned through the striatum and hippocampus. The sections were mounted on slides and stained with hematoxylin and eosin. Damage to the right and left hemispheres of the brain was scored on a scale of 0–3 (0, no cell loss; 1, approximately one third of cells had died; 2, approximately two thirds of cells had died; and 3, >90% cell loss). The individual who performed the four-vessel occlusion surgery as well as the individuals who performed the drug treatment were blinded to the identity of the treatment groups. The brains were also scored in a blinded fashion. The average damage score for each group was computed, and the level of significance was calculated using the Mann-Whitney U test.

Three separate experiments were performed. In the first experiment, LY231617 was evaluated by the oral route of administration. In this experiment the compound was given orally in acacia suspension at a dose of 50 mg/kg p.o. 30 minutes before occlusion and again 4 hours after the onset of reperfusion and the following morning. In the second experiment LY231617 was dissolved in a 10% dimethylsulfoxide–90% physiological saline vehicle and evaluated by the intravenous route of administration. In this experiment the compound was given as a 20 mg/kg i.v. bolus, starting at the time of reperfusion, which was 30 minutes after the onset of ischemia. After injection of the intravenous bolus, the rat was connected to an infusion pump and received 5 mg/kg per hour for 24 hours through a jugular cannula. In the third experiment compound LY231617 was administered at a dose of 10 mg/kg over a 5–7-minute period immediately on reperfusion, which was 30 minutes after the onset of ischemia. After the administration of the bolus, the compound was infused at the rate of 5 mg/kg per hour i.v. for 24 hours.

In an experiment to determine if LY231617 can pass the blood–brain barrier, rats were treated orally with 50 mg/kg of LY231617 and killed by decapitation 1 hour later. Blood was collected, and brains were removed. Brains were frozen on dry ice and subsequently main-

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**Figure 1. Diagram of structure of LY231617.**
The LY231617 concentrated from the 15-minute hydrogen peroxide-treated brains was centrifuged at 12,000g. The supernatant, or serum diluted with 0.1 M, pH 9 phosphate buffer, was applied to cyano solid-phase extraction columns, which were subsequently washed with water. The LY231617 concentrated on these columns was eluted by the mobile phase onto the analytical column. The mobile phase consisted of pH 6, 0.05 M phosphate buffer containing 50% acetonitrile. A 4.6 mm×15 cm silica-based cyano analytical column was used for the separation. The UV detector was set to a wavelength of 215 nm and a sensitivity of 0.01 absorbance units (full scale). Standards were made up in serum or brain homogenates from nontreated rats and subjected to the same extraction procedures as samples from treated rats.

Results

The ability of LY231617 to antagonize hydrogen peroxide–induced neuronal injury is shown in Figure 2. Hydrogen peroxide–induced lethality and the protective effect of LY231617 in vitro are shown in Figure 2. Exposure to 50 μM hydrogen peroxide for 15 minutes produced no acute morphological changes, but 24 hours later there was widespread neuronal death as assayed by the fluorescent vital stains (Figure 2). This apparent delayed neuronal cell death parallels what is observed in models of transient forebrain ischemia. Five μM LY231617 added during the 15-minute hydrogen peroxide exposure increased neuron viability from 20% (untreated) to 70% (Figure 2). The decrease in efficacy of LY231617 at higher doses may be due to limited toxicity of the compound caused by its hydrophobic nature. Representative photomicrographs of control, hydrogen peroxide–treated, and hydrogen peroxide+LY231617–treated neuronal preparations are shown in Figures 3A, 3B, and 3C, respectively. The effect of LY231617 alone on hippocampal cultures was evaluated, and no toxicity was observed until concentrations >50 μM were added (data not shown).

The effect of LY231617 on iron–dependent lipid peroxidation is illustrated in Figure 4. Addition of LY231617 to brain homogenates resulted in a dose-related inhibition of lipid peroxidation.

Seventy-two hours after 30 minutes of global forebrain ischemia the hippocampus and striatum both show massive neuronal damage. The hippocampal CA1 pyramidal cells are generally totally obliterated, and the majority of the neurons in the corpus striatum die; however, on occasion the most medial part of the striatum, which borders on the ventricle, may survive. Figures 5 and 6 show examples of the extent of striatal and hippocampal damage, respectively, and also illustrate the protective effect of LY231617. In experiment 1, oral administration of 50 mg/kg of LY231617 given three times significantly protected both the hippocampus (p<0.0001) and striatum (p<0.0001) from 30 minutes of four-vessel occlusion (Table 1). In experiments 2 and 3, LY231617 significantly protected the hippocampus and the striatum when given 30 minutes after the onset of the ischemia at the time of reperfusion. Table 2 shows that intravenous LY231617 treatment resulted in a 56% reduction in hippocampal CA1 damage (p<0.03) and a 49% reduction in striatal damage (p<0.03) in one experiment and a 48% reduction in hippocampal CA1 damage (p<0.001) and a 41% reduction in striatal damage (p<0.002) in a second experiment using a lower bolus dose. The neuroprotective effect of LY231617 appeared to be mediated through an action on the brain rather than in the periphery. One hour after administration of LY231617, the brain levels were 28-fold higher than serum levels (Figure 7).

In studies in which mean arterial blood pressure (MABP) was recorded, LY231617 produced a sustained 10–15% decline in MABP when infused intravenously at 10 mg/kg per hour. Constant infusion of 5.0 mg/kg i.v. had no significant effect on MABP.

Discussion

The four-vessel occlusion model used in this study produces an incomplete global cerebral ischemia with reperfusion. The 30-minute period of ischemia used results in profound and highly reproducible neuronal damage in both the CA1 layer of the hippocampus and the corpus striatum. The administration of LY231617 in this model significantly attenuated the ischemia-induced damage. Brain levels of LY231617 were 28-fold higher than serum levels at 1 hour after administration. The high brain levels of LY231617 demonstrate that LY231617 is crossing the blood–brain barrier and accumulating in the brain. These high brain levels of LY231617 in the presence of low serum levels strongly indicate that LY231617 is exerting its neuroprotective effects directly at the level of the central nervous system. The precise mechanism of the protective effect of LY231617 is not entirely clear. However, because of
the chemical structure of the compound and the ability of LY231617 to significantly reduce oxidative damage resulting from hydrogen peroxide exposure and inhibition of iron-dependent lipid peroxidation, we believe that one potential mechanism of action of this compound is its ability to antagonize oxidative damage. During ischemia and subsequent reperfusion, the substrates for oxidative free radical formation are present. For example, our laboratory and others have reported massive release of dopamine into the extracellular fluid compartment of the corpus striatum during ischemia, and when the nigrostriatal dopaminergic tract is destroyed by 6-hydroxydopamine, ischemic damage to the striatum is reduced. On reperfusion of the ischemic striatum, dopamine is likely oxidized by molecular oxygen, which could result in the production of two superoxide anion radicals for each molecule of catecholamine. Because superoxide dismutase does not require adenosine triphosphate for its activity, it is likely that superoxide was converted into hydrogen peroxide. It is not unreasonable to propose or suggest that much of the neuronal damage seen in ischemia/reperfusion experiments could result from toxic oxygen species possibly produced from hydrogen peroxide. Substantially elevated levels of hydrogen peroxide have been measured in brain after ischemia/reperfusion. In another study using direct measurement of hydrogen peroxide from striatal dialysates during reperfusion after 30 minutes of four-vessel occlusion, large increases in hydrogen peroxide concentration (approximately 150 μM) were observed (P. Hyslop and L. Phebus, unpublished observations). In addition, reports of increased lipid peroxidation in vulnerable brain regions after transient forebrain ischemia in rats and the demonstration of free radicals during the early reperfusion phase by electron spin resonance spectroscopy support the present view. The idea of free radical involvement in ischemia-induced neuronal damage is further supported by recent reports showing that agents believed to block free radical mechanisms, or scavenge hydrogen peroxide, reduced neuronal damage in ischemia models. These agents include dimethylthiourea, superoxide dismutase (liposome entrapped), U74006F, certain di-
Damage in the Four-Vessel Occlusion Model of Global Ischemia

<table>
<thead>
<tr>
<th>Treatment</th>
<th>No. of rats</th>
<th>Damage score</th>
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<tbody>
<tr>
<td></td>
<td></td>
<td>Hippocampus</td>
</tr>
<tr>
<td>2% Acacia</td>
<td>12</td>
<td>2.95±0.03</td>
</tr>
<tr>
<td>LY231617*</td>
<td>11</td>
<td>0.66±0.29†</td>
</tr>
</tbody>
</table>

Values are mean±SEM. *LY231617 was administered at a dose of 50 mg/kg orally 30 minutes before occlusion and again 4 hours after onset of reperfusion and the following morning. †p<0.0001 by Mann-Whitney U test.

Butylphenols, α-tocopherol, and a variety of other compounds. Many other proposed sources of ischemia-induced damage may have a free radical–mediated component. For example, glutamate neurotoxicity is widely believed to be a source of neuronal damage in response to ischemia. Murphy et al.24,25 reported that antioxidants could block glutamate excitotoxicity in a neuroblastoma cell line. Other investigators found that kainate neurotoxicity could be blocked by free radical scavengers.26 Interestingly, a recent report suggested that nitric oxide mediates glutamate neurotoxicity in primary cortical cultures27 and inhibitors of nitric oxide synthetase selectively prevented glutamate neurotoxicity. Nitric oxide contains an unpaired electron, is paramagnetic, and can react with the superoxide anion radical to form peroxynitrite anion. Peroxynitrite anion can undergo further decomposition when protonated to yield highly toxic oxygen species such as highly reactive hydroxyl free radicals.28 Thus, in addition to the contribution to hydroxyl free radical formation from hydrogen peroxide by the iron-catalyzed Haber-Weiss reaction, another source of hydroxyl radicals may originate from the superoxide–nitric oxide pathway. Other sources of cell damage from ischemia/reperfusion injury may have a free radical–mediated component. For example, activation of phospholipase A₂ with the subsequent production of thromboxanes and platelet activating factor has been proposed to have a role in ischemia-induced damage.29–35 In addition to enhancing the formation of thromboxanes and platelet activating factor, phospholipase A₂ may attack the integrity of cell membranes. This action of phospholipase A₂ can be an additional source of cellular damage during ischemia. Oxidant-mediated activation of phospholipase A₂ in endothelial cells has been documented.32 Exposure of cells to hydrogen peroxide induces activation of phospholipase A₂.30 In addition to activation of phospholipases, these oxygen-derived radicals are capable of attacking proteins and nucleic acids, thereby leading to tissue injury and cell death. Potential sources of oxygen radicals during reperfusion after ischemia and their effects on cerebral vessels and brain have been recently reviewed by Kontos.36

Although LY231617 is a highly effective agent in protecting against brain damage from global ischemia, it is not totally free of other activities. The major apparent side effect of LY231617 is a reduction in MABP. Intravenous infusion of LY231617 at a dose of 10 mg/kg per hour resulted in a sustained 10–15% decrease in MABP. Infusion of 5.0 mg/kg per hour had no significant effect on MABP; however, this dose was effective in reducing damage in the four-vessel occlusion model. The dose-limiting toxicity of this compound, therefore, appears to be its hypotensive effect.

In conclusion, several factors may participate in the cascade of events that ultimately lead to cell death after ischemia and reperfusion. There are reasons to believe that reactive oxygen species can play an important role in many of these processes and may actually have an initiating role. Other potential pharmacological activities that may have a role in the anti-ischemic activity of LY231617 are currently being investigated. However, the dramatic reduction in brain damage seen in the current study in a global ischemia/reperfusion model and the reduction in hydrogen peroxide–induced neuronal cell death in vitro as well as inhibition of lipid peroxidation demonstrate that LY231617 is a highly effective anti-ischemia agent and could potentially exert its effect through antagonism of an oxidative-mediated mechanism of cellular destruction.

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Editorial Comment

Using a newly synthesized antioxidant (LY231617) that has a backbone structure of butylated hydroxytoluene, Clemens and colleagues report in this study that both hydrogen peroxide–induced cortical neuronal death in vitro and global cerebral ischemia and reperfusion–induced neuronal damage in the hippocampus and striatum are significantly reduced. Several important observations are made in this study. First, the antioxidative amelioration of hydrogen peroxide–induced neuronal damage in vitro appears to be dose...
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