A Phenothiazine Derivative Reduces Rat Brain Damage After Global or Focal Ischemia

Melvin J. Yu, PhD; Jefferson R. McCowan, MS; E. Barry Smalstig, BS; David R. Bennett; Michael E. Roush, BS; and James A. Clemens, PhD

Background and Purpose: We previously reported that 2-(10H-phenothiazin-2-yl-oxy)-N,N-dimethylethylamine hydrochloride is a potent inhibitor of iron-dependent lipid peroxidation in vitro and can protect primary cultures of rat hippocampal neurons from hydrogen peroxide–induced toxicity. Because oxidants may play an important role in mediating posts ischemic tissue injury, we evaluated this agent in two rat models of transient cerebral ischemia.

Methods: In a model of global forebrain ischemia, 23 male Wistar rats were subjected to 10 minutes of four-vessel occlusion followed by 72 hours of reperfusion. The rats received three intraperitoneal injections of either vehicle (2% aqueous acacia) or test agent (40 mg/kg). In a model of focal stroke, 19 spontaneously hypertensive rats were subjected to 2 hours of tandem middle cerebral and ipsilateral common carotid artery occlusion followed by 24 hours of reperfusion. The rats received three intraperitoneal injections of either vehicle (2% aqueous acacia) or test agent (40 mg/kg).

Results: In the global model, the phenothiazine significantly protected the CA1 layer of the hippocampus, with a reduction in mean damage score from 2.1 ±0.3 for control rats to 1.0±0.4 for treated rats (p<0.05). In the transient focal stroke model, the compound reduced cortical infarct volume from 130.1±10.3 mm³ for control rats to 95.2±24.5 mm³ for treated rats (p<0.02).

Conclusions: Although the primary mechanism responsible for the protective effect is unclear at the present time, our study is consistent with the hypothesis that oxidant-mediated lipid peroxidation may be involved in the pathophysiology of posts ischemic brain injury. (Stroke 1992;23:1287-1291)

KEY WORDS • cerebral ischemia • phenothiazines • rats

The phenothiazines represent an important class of agents possessing a spectrum of clinically useful properties. Although primarily used in the management of psychiatric disorders, a number of phenothiazines have also been studied in settings of hepatic, renal, and myocardial ischemia. In particular, chlorpromazine, the oldest member of the phenothiazine class of drugs, exhibits a variety of properties, including stabilization of biomembranes; inhibition of calmodulin; and reduction of rat liver microsome and myocardial membrane phospholipid peroxidation. While a combination of activities may be important for attenuating ischemia-induced damage, we chose to focus on the latter because oxidants such as oxygen-derived free radicals and hydrogen peroxide are believed to be important mediators in the acute stage pathology of ischemia and reperfusion injury.

We recently reported on the chemistry, electrochemistry, and structure–activity relation for a series of phenothiazines as in vitro inhibitors of iron-dependent lipid peroxidation. Although chlorpromazine inhibits lipid peroxidation in vitro, it is weakly active and may suppress cardiac phospholipid peroxidation by inducing lipid structural changes rather than by acting as a chain-breaking antioxidant. However, based on earlier polarographic studies, we hypothesized that lowering the oxidation potential of this agent through appropriate structural modifications may yield a potent antioxidant that may function as a more effective inhibitor of nonenzymatic lipid peroxidation. Using cyclic voltammetry, we examined a series of N-10 unsubstituted phenothiazines and found that these compounds exhibited an oxidation potential lower than that for chlorpromazine.

Importantly, this lower redox potential was manifest functionally in that 2-(10H-phenothiazin-2-yl-oxy)-N,N-dimethylethylamine hydrochloride (minimum inhibitory concentration, 0.093 µM), the prototype from this investigation, was more potent than chlorpromazine (minimum inhibitory concentration, 10 µM) in blocking iron-dependent peroxidation of rabbit brain vesicular membrane lipids in vitro. Using a whole cell assay, we evaluated representative examples from that study as cytoprotective agents and found that these compounds exhibited an oxidation potential lower than that for chlorpromazine.

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Because oxidants such as hydrogen peroxide may be important causal factors in postischemic tissue injury, we investigated the activity of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride as an anti-ischemic agent by intraperitoneal administration in two rat models according to a pretreatment protocol, a four-vessel occlusion model of transient global forebrain ischemia and a transient tandem middle cerebral artery (MCA) and ipsilateral common carotid artery (CCA) occlusion model of focal stroke. In connection with our chemical efforts to identify salt forms with increased water solubility, we also prepared and subsequently evaluated the corresponding mesylate salt by continuous intravenous infusion using a rat model of permanent MCA occlusion.

Materials and Methods

We conducted the four-vessel occlusion study as previously described using male Wistar rats (Hilltop Laboratories, Scottsdale, Pa.) weighing 250-280 g, according to the method of Pulsinelli and Brierley. In the present study, however, the rats were subjected to a 10-minute period of four-vessel occlusion. Seventy-two hours after the ischemic period, the animals were killed; their brains were then perfused with 10% formalin, removed, sectioned through the hippocampus, mounted on glass slides, and stained with hematoxylin and eosin. Damage to the right and left hemispheres of the brain were scored in a blinded fashion on a scale of 0 to 3. A score of 0 indicated no cell loss, a score of 1 indicated approximately one third of the cells died, a score of 2 indicated approximately two thirds of the cells died, and a score of 3 indicated a >90% cell loss. Mean damage scores for each group were determined, and the level of significance was calculated using the Mann-Whitney U test. Vehicle (2% aqueous acacia, n = 11) or 40 mg/kg 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride (n = 12) was administered intraperitoneally 2 hours before occlusion, 4 hours after occlusion, and again 24 hours later. During the period of ischemia and for 30 minutes after ischemia, the rat's body temperature was measured with a rectal probe and regulated at 37°C by a Yellow Springs Instrument feedback system (Yellow Springs, Ohio) that turned on a heat lamp suspended above the rat when the temperature dropped below 37°C.

In the MCA occlusion and reperfusion studies, male spontaneously hypertensive rats (Taconic Farms, Germantown, N.Y.) weighing 280-350 g were subjected to focal cerebral ischemia according to the method of Brint and coworkers. Briefly, the rats were anesthetized with halothane, and the MCAAs and ipsilateral CCAAs were exposed. For studies 1 and 2, both arteries were clamped for a period of 2 hours and then reperfused for 24 hours. The brains were removed and sectioned histologically, and infarct volume was determined using image analysis. During the period of ischemia, the rat's body temperature was measured by means of a rectal probe and regulated at 37°C by a Yellow Springs Instrument feedback system that turned on a heating pad when the temperature dropped below 37°C. Vehicle (2% aqueous acacia, n = 10 in each study) or 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride (10 mg/kg, n = 8 in study 1 and 40 mg/kg, n = 9 in study 2) was administered intraperitoneally 2 hours before MCA occlusion, 2 hours after occlusion, and again 2 hours later. The level of significance between vehicle- and drug-treated groups was calculated using Student's t test.

In the permanent occlusion study, male spontaneously hypertensive rats were anesthetized with halothane, and the MCA was exposed and cauterized. After 24 hours the animals were killed. Because of limited aqueous solubility of the hydrochloride salt, the corresponding mesylate salt was subsequently prepared and used for this study. Either vehicle (0.312 M aqueous mannitol, n = 5) or 20 mg/kg 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine methanesulfonate (n = 5) was administered as a bolus intravenous injection 10 minutes before cauterization, followed by a continuous intravenous infusion (10 mg/kg per hour of the phenothiazine) for 24 hours through the jugular vein.

Results

We initially prepared 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride as our prototype in connection with our chemical and structure-activity relation investigation into the phenothiazines as lipid peroxidation inhibitors. Because of limited saline solubility, this compound was administered intraperitoneally as a 2% aqueous acacia suspension in the four-vessel occlusion study according to the experimental protocol outlined in Figure 1A. As summarized in Table 1, pretreatment significantly attenuated damage to the CA1 layer of the hippocampus after a 10-minute period of global ischemia with 72 hours of reperfusion (p < 0.05).

Two doses were examined in the spontaneously hypertensive rat MCA occlusion and reperfusion model. Pretreatment with 10 mg/kg i.p. 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride according to the experimental protocol outlined in Figure 1B did not result in a statistically significant reduction in cortical infarct size after 2 hours of tandem MCA and ipsilateral CCA occlusion with 24 hours of reperfusion (Table 2). Pretreatment with a higher dose (40 mg/kg), however, was associated with improved cerebral histology when administered according to an identical experimental protocol (p < 0.02).

In connection with our subsequent efforts to develop an intravenous formulation for this agent, we prepared the corresponding mesylate salt and evaluated it according to the experimental protocol outlined in Figure 1C. As summarized in Table 3, administration of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine methanesulfonate by an initial intravenous loading bolus injection followed by a continuous intravenous infusion was not effective in this model when infarct size was assessed 24 hours after cauterization of the MCA.

Discussion

A 10-minute period of severe global forebrain ischemia (four-vessel occlusion) followed by reperfusion in the rat results in selective damage to the CA1 layer of the hippocampus that progresses over several days. A 2-hour period of focal ischemia (tandem MCA and ipsilateral CCA occlusion) in the spontaneously hypertensive rat, on the other hand, results in rapid nonselective cell necrosis that yields a moderate-sized cortical infarct when measured 24 hours after initiation of reperfusion. Although the time course, pattern, and
extent of cell death differ dramatically in the four-vessel occlusion and MCA occlusion rat models of transient cerebral ischemia, the present study demonstrates that the phenothiazine 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride is efficacious in both settings.

Although the mechanisms underlying postischemic neuronal degeneration are likely to be multifactorial, oxidants have been proposed to be causative factors of cellular injury in animal models of global12,18 and focal19,20 cerebral ischemia. Lipid peroxidation inhibitors21–23 and free radical scavengers24,25 reportedly decrease ischemia-induced damage in the brain. The phenothiazine 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride exhibits a low oxidant potential measured electrochemically and can block nonenzymic lipid peroxidation in vitro as well as protect primary cultures of rat hippocampal neurons from hydrogen peroxide-induced toxicity. Thus, one potential mechanism by which this agent attenuates ischemic injury in vivo may relate to its ability to block lipid peroxidation in vitro.

However, while oxidants may play a key role in the pathogenesis of ischemia and reperfusion injury, other potentially important mechanisms including calcium overload, phospholipase activation,26 regional calcium–calmodulin binding,27 and increased neurotransmitter release (e.g., dopamine28,29 and excitatory amino acids30) may contribute significantly toward ischemia-induced injury.31 A large number of phenothiazines have been claimed in the patent literature as leukotriene biosynthesis inhibitors32 and as excitatory amino acid antagonists.33 In addition, certain phenothiazines such as chlorpromazine and trifluperazine have been studied as calmodulin inhibitors.34 Using rat hippocampal slices, Balestrino and Somjen35 reported that chlorpromazine protects against hypoxia-induced loss of synaptic transmission by delaying the occurrence of spreading depression and the resulting massive influx of calcium into the neuron. The phenothiazines chlorpromazine and trifluperazine were later examined by Zivin et al36 in two central nervous system rabbit models, a multiple cerebral embolism and a spinal cord ischemia model. In these two systems, ischemia-induced neurological func-

### Table 1. Effect of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine Hydrochloride on Hippocampal CA1 Damage in Rat Model of Transient Global Forebrain Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Damage score (hippocampus)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acacia vehicle</td>
<td>11</td>
<td>2.1±0.3</td>
</tr>
<tr>
<td>Treated</td>
<td>12</td>
<td>1.0±0.4*</td>
</tr>
</tbody>
</table>

n, Number of rats. Rats received three intraperitoneal injections of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride (40 mg/kg) suspended in 2% aqueous acacia. First injection occurred 2 hours before ischemic period and second and third occurred at 4 and 24 hours, respectively, after initiation of reperfusion. Body temperature was regulated at 37°C during and for 30 minutes after ischemia. Values are mean±SEM.

*p<0.05 by Mann-Whitney U test versus vehicle.

### Table 2. Effect of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine Hydrochloride on Cerebral Infarct Size in Rat Model of Transient Focal Ischemia

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Brain infarct volume (mm³)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Study 1 (10 mg/kg):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acacia vehicle</td>
<td>10</td>
<td>133.9±12.1</td>
</tr>
<tr>
<td>Treated</td>
<td>8</td>
<td>116.4±8.6</td>
</tr>
<tr>
<td>Study 2 (40 mg/kg):</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acacia vehicle</td>
<td>10</td>
<td>130.1±10.3</td>
</tr>
<tr>
<td>Treated</td>
<td>9</td>
<td>95.2±24.5*</td>
</tr>
</tbody>
</table>

n, Number of rats. Rats received three intraperitoneal injections of 2-(10H-phenothiazin-2-yloxy)-N,N-dimethylethanamine hydrochloride (10 mg/kg and 40 mg/kg for studies 1 and 2, respectively) suspended in 2% aqueous acacia. First injection occurred 2 hours before ischemic period and second and third occurred at 2 and 4 hours, respectively, after initiation of reperfusion. Values are mean±SEM.

*p<0.02 by Student's t test versus vehicle.
tion deficits were reduced by these agents, an effect attributed to inhibition of calcium-dependent kinases. Thus, multiple mechanisms in addition to in vitro lipid peroxidation inhibition and biomembrane stabilization may contribute to neuronal protection by chlorpromazine in vivo. However, the relative importance of these potential mechanisms as a basis for the salutary effects of 2-(10H-phenothiazin-2-yl)-N,N-dimethylethanamine hydrochloride in our global and focal models of transient cerebral ischemia remains to be established. Additional studies are needed to fully evaluate the properties of this agent and to help clarify its mechanism of action in vivo.

It is noteworthy that the corresponding mesylate salt was not protective in the permanent MCA occlusion model when administered by continuous intravenous infusion (10 mg/kg per hour) throughout the infarction experiment. A higher dose (20 mg/kg per hour) produced toxic effects. Although the basis for this observation is unclear, the mesylate salt at a maximal tolerated dose was not effective under the conditions of the model. Although potential differences in pathophysiology of the ischemic insult when not accompanied by reperfusion may account for this discrepancy, it is possible that other factors such as dose, vehicle, or route of administration may not have been optimal for this compound. Further experiments with more detailed monitoring of physiological variables may help address these issues and lead to a clearer understanding of the role that oxidants may play in mediating postischemic tissue injury.

In summary, the described phenothiazine derivative attenuated postischemic damage in two rat models of transient cerebral ischemia when administered by intraperitoneal injection according to a pretreatment protocol. The primary mechanism responsible for the cerebroprotective effect is unclear at the present time because multiple factors may influence the cascade of events leading to postischemic brain damage. However, the in vitro profile of 2-(10H-phenothiazin-2-yl)-N,N-dimethylethanamine hydrochloride supports the possibility that antioxidant mechanisms may underly its ability to ameliorate brain damage when administered before and after a transient ischemic insult. Our study is therefore consistent with the hypothesis that oxidant-mediated lipid peroxidation may be involved in the pathophysiology of postischemic brain injury.

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The generation of reactive products of univalent reduction of oxygen is an important mechanism for tissue injury in ischemia. Antioxidant therapy to minimize ischemic injury to the central nervous system has received accelerated attention in recent years. The first antioxidant drugs (superoxide dismutase and the 21-aminosteroid tirilazad mesylate) are already in clinical trials. Yu et al, in the study reported above, found that a phenothiazine derivative reduces brain damage from global or focal ischemia in rats. Studies such as this have important clinical significance for two reasons. The obvious one is the establishment of an experimental data base for selecting agents suitable for testing as therapeutic agents in humans. A second reason, equally important, is the identification of the mechanism by which ischemia injures neurons or blood vessels. Although the most popular mechanism thought to be involved in the damage induced by oxidants is lipid peroxidation, with the consequent dysfunction of cell membranes, it is important to remember that oxidants have multiple effects. They are capable of inactivating enzymes, damaging important tissue components such as DNA, and interacting with biologically active tissue products such as endothelium-derived relaxing factor. As Yu et al correctly point out, other important processes that take place during and after central nervous system ischemia may also contribute to cell injury and death. Some of these processes may interact with the oxidants in complex ways. By using a variety of agents and identifying precisely their mechanism of action, we may learn much about the process by which tissues deteriorate and die as a result of ischemia.

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