Effects of Tirilazad Mesylate on Vasospasm and Phospholipid Hydroperoxides in a Primate Model of Subarachnoid Hemorrhage

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Background and Purpose—Tirilazad mesylate has been used in the attempt to prevent cerebral vasospasm after subarachnoid hemorrhage (SAH), although the actual targets of this agent in vivo have thus far been controversial. Chemiluminescence/high-performance liquid chromatography provided a new method for direct measurements of phosphatidylcholine hydroperoxide (PCOOH) and phosphatidylethanolamine hydroperoxide (PEOOH) in vivo and showed that phosphatidylcholine is the lipid class most susceptible to lipid peroxidation. In the present study we measured those levels in a primate model of SAH for determination of the effects of tirilazad on vasospasm.

Methods—Fourteen Macaca monkeys of both sexes were randomly assigned into 2 groups: a tirilazad group receiving a dosage of 0.3 mg/kg and a placebo group receiving only the vehicle in which tirilazad was delivered. After the induction of experimental SAH around the right middle cerebral artery on day 0, tirilazad or vehicle was administered intravenously every 8 hours for 6 days. On day 7, the animals were killed after angiography and regional cerebral blood flow measurements were performed. The levels of PCOOH and PEOOH were measured in the clots, bilateral parietal cortices, right frontal cortex contact with clots, cerebellar hemispheres, bilateral middle cerebral arteries, and basilar arteries.

Results—In the placebo group, a significant vasospasm occurred in the cerebral arteries on both sides, but most prominently on the right side. The degree of vasospasm in the cerebral arteries was significantly attenuated in the tirilazad group (P<0.005). There were no significant differences in regional cerebral blood flow, PCOOH, and PEOOH levels in the clots, cerebral cortices, and cerebellar hemispheres between the 2 groups. In contrast, the levels of PCOOH in the cerebral arteries were significantly higher in the placebo group than in the tirilazad group (P<0.025). It was remarkable that the tirilazad treatments eliminated PCOOH in any vascular territory after SAH.

Conclusions—PCOOH in the artery wall may be an important indicator for vasospasm, and the inhibition of PCOOH may explain the efficacy of tirilazad on vasospasm. (Stroke. 1999;30:450-456.)

Key Words: cerebral ischemia, transient ■ lipid peroxidation ■ phospholipids ■ subarachnoid hemorrhage ■ monkeys

Tirilazad mesylate1–7 and its derivative8 have been shown to attenuate vasospasm in experimental models of subarachnoid hemorrhage (SAH). A recent cooperative study also suggested that tirilazad might have a neuroprotective effect in patients with SAH.9–11 However, its precise mechanism of action in improving vasospasm has yet to be fully understood. Only one study has demonstrated that tirilazad reduces malondialdehyde (MDA), a byproduct of lipid peroxidation, in subarachnoid clots.2

Lipid peroxidation proceeds by a free radical chain mechanism and gives lipid hydroperoxides as primary products.12 Since both phosphatidylcholine (PC) and phosphatidylethanolamine (PE) are important lipids in the cellular membrane and are major structural constituents of tissue lipids, the determination of PC hydroperoxide (PCOOH) and PE hydroperoxide (PEOOH) seems important for assessment of the degree of membrane lipid damage. Miyazawa et al13 showed that PC is the class most susceptible to lipid peroxidation in vivo. Thus, a chemiluminescence/high-performance liquid chromatography (CL-HPLC) method has been developed for the specific assay of PCOOH and PEOOH in biological tissues.14,15 In the present study we measured PCOOH and PEOOH levels in the clots, cerebral arteries, cerebral cortices, and cerebellar hemispheres obtained from a primate model of SAH and determined the mechanism of action of tirilazad on vasospasm.
Materials and Methods

All protocols were evaluated and approved by the Animal Ethics Review Committee of the Mie University School of Medicine. The animals were cared for in accordance with the Guidelines for Animal Experiments in the Mie University School of Medicine.

Study Protocol

Fourteen monkeys (Macaca fuscata and M fascicularis) of either sex weighing between 3.4 and 14.0 kg were divided into 2 groups of 7 animals. Tirilazad was provided by the Upjohn Company (Kalamazoo, Mich). The tirilazad group received a dose of 0.3 mg/kg in a citric acid vehicle, and the placebo group received only the vehicle. On day 0, monkeys in both groups underwent cerebral angiography to determine the baseline vessel caliber before SAH induction. Intravenous administration of tirilazad or placebo commenced 20 hours after SAH induction and was repeated every 8 hours for 6 days. On day 7, cerebral angiography and regional cerebral blood flow (rCBF) measurements were undertaken to evaluate the degree of vasospasm. The animals were killed by high-dose pentobarbital (50 mg/kg) followed by exsanguination. The brain was rapidly removed, and samples were immediately frozen in liquid nitrogen. The PCOOH and PEOOH levels were measured by CL-HPLC as described below.

For the angiography and SAH induction on day 0, the animals were anesthetized with an intramuscular injection of ketamine hydrochloride (6 to 10 mg/kg) and atropine sulfate (0.02 mg/kg). The animals were intubated, and a peripheral venous line was placed. An intravenous injection of sodium pentobarbital (20 mg/kg per hour) maintained the anesthesia, and pancuronium bromide (0.05 mg/kg per hour) maintained the paralysis. Gentamycin sulfate (2 mg/kg) was administered intramuscularly. A 2:1 mixture of N2O:O2 on a ventilator (SN-480 to 3, Shimano Co) was used to maintain the end-tidal CO2 (ETCO2) level at ∼40 mmHg during continuous ETCO2 monitoring (POET 601, Criticare System, Inc). Body temperature was monitored by a rectal thermometer (TF-DN, Terumo) and maintained at 37°C with a heating pad. With the use of a sterile technique, the axillary artery was dissected and catheterized with a 4F polyethylene catheter so that the tip was at the right innominate artery. The catheter was connected with a 3-way stopcock to a pressure transducer (MK12030 US, Baxter). An arterial phase anteroposterior cerebral angiogram was obtained by manually injecting a contrast medium (10 mL of Omnipaque 300, Daichi Setyaku Co). On day 7, angiography was repeated before rCBF measurement.

Induction of SAH

After the baseline cerebral angiography on day 0, the ETCO2 level was adjusted to ∼40 mmHg by controlled ventilation. The animal’s head was placed in a 3-point pin-fixation system, and a right frontotemporal craniectomy was performed with a sterile technique. Under an operating microscope, the dura mater was opened, and the temporal lobe was posteriorly retracted. The sylvian fissure was entered by sharp dissection, and the arachnoid membrane over the sphenoid segment of the middle cerebral artery (MCA), the precommunicating segment of the anterior cerebral artery (ACA), and the internal carotid artery (ICA) was opened. Five milliliters of autologous arterial blood clot was placed around the exposed cerebral arteries. The dura was closed in a watertight fashion, and the temporal muscle and skin were sutured in layers. After the operation, paralysis was reversed by an intravenous administration of neostigmine methylsulfate (0.07 mg/kg) and atropine sulfate (0.02 mg/kg). The animals were extubated after they recovered the gag reflex. The animals were observed for the development of operative complications and/or delayed neurological deficits.

Measurements of rCBF

On day 7, rCBF was measured by a hydrogen clearance technique. Under the same anesthesia as angiography, burr holes were made over both parietal bones. The head of the animal was fixed in a head frame, and platinum needle electrodes, 0.3 mm in diameter (UHE-100, Unique Medical Co), were placed 20 mm lateral to the midline, 10 mm posterior to the coronal suture, and in the gray matter 2 mm below the brain surface. A reference Ag-AgCl electrode (UHE-001, Unique Medical) was placed under the scalp. Hydrogen gas was administered via the endotracheal tube for 2 minutes at a concentration of 10%. The clearance curve for the hydrogen concentration in the brain tissue was recorded (UR 2P, Unique Medical), and the rCBF value was calculated by the initial-slope method (PHG-201, DDU-100, Unique Medical).

Drug Administration

After an intramuscular administration of 3 to 5 mg/kg ketamine hydrochloride, the animals received intravenous tirilazad (0.3 mg/kg) or placebo (0.02 mol/L citric acid) 3 times daily (every 8 hours) through an indwelling catheter inserted in the posterior calf region. To maintain patency, a heparin-lock solution was injected into the indwelling venous catheter after each drug infusion. The dose level of tirilazad was selected from previous experiments.

Euthanasia of Animals

On day 7 after SAH, the animals were killed with sodium pentobarbital (50 mg/kg IV) followed by transcardiac perfusion with 1.0 L of cold saline under 100 mm Hg pressure to wash out the circulating blood. The brains and any remaining subarachnoid clots were rapidly removed, and the clots were immediately frozen in liquid nitrogen. The bilateral parietal cortices, right frontal cortex contact with clots, cerebellar hemispheres, bilateral sphenoidal segments of MCAs, and basilar arteries were excised and immediately frozen in liquid nitrogen. The samples were stored in the liquid nitrogen until measurements of PCOOH and PEOOH could be made.

Radiological Assessment

During the angiography, exposure factors were kept constant, and a radiopaque control standard was used for correction to constant magnification. Subtraction films of the anteroposterior projection were made. An experienced person who was unaware of the treatment groups measured the diameter of the intracranial cerebral arteries 9 times with a calibrated optical micrometer (Scale Lupe No. 1983, PEAK) and determined a mean value. The arteries were measured bilaterally at the following points: sphenoidal segment of the MCA, precommunicating segment of the ACA, intradural ICA (C1), cavernous portion of the ICA (C2), and the basilar artery.

Determination of PCOOH and PEOOH Levels

The PCOOH and PEOOH contents were determined by CL-HPLC according to the method described by Miyazawa et al. All procedures were performed under ice cooling. The clots were weighed, homogenized, washed 4 times by centrifugation at 3000g for 30 minutes in 5 mol/L phosphate-buffered saline containing 0.1 mol/L ethylenediaminetetraacetate, pH 8.0, and replaced with ghosts to avoid the effects of iron during the following procedures. The total lipid was extracted with a mixture of chloroform and methanol (2:1, vol/vol; containing 0.002% butylated hydroxytoluene as an antioxidant) from the brain, ghosts, and cerebral arteries. The CL-HPLC system and experimental conditions for measuring PCOOH and PEOOH in total lipid prepared from samples were as follows: the HPLC column was Finepak SIL NH2-5 (5 μm, 250×4.6 mm; Japan Spectroscopic Co), the mobile phase was a mixture of 2-propanol-methanol-water in the brain (27:9:4, vol/vol) and clots (26:9:5, vol/vol) or hexane-2-propanol-methanol-water in the cerebral arteries (7:22:5:3, vol/vol), and the flow rate was 1.0 mL/min. After being passed through a JASCO 880-UV detector set at 210 nm to monitor the lipid extraction rate (detection of double bond), the column eluant was mixed with a luminescent reagent. The luminescent reagent was prepared to give a mixture of 6 μg cytochrome c (horse heart, type IV; Sigma Chemical Co) and 1.5 μguminol (3-aminophthaloyl hydrazine; Wako Pure Chemical Co) in the brain and clots, or 30 μg cytochrome c and 1.5 μguminol in the cerebral arteries in 1 mL of 30 mmol/L borate buffer (pH 10.0). The flow rate of the chemiluminescence reagent was 1.0 mL/min in the brain, 1.6 mL/min in the clots, and 3.2 mL/min in the cerebral arteries.
arteries (JASCO 880-PU pump). The chemiluminescence generated by the reaction of the hydroperoxide with the luminescent reagent at the post column was measured with a CLD-110 chemiluminescence detector (Tohoku Electronic Industries Co). Calibrations of PCOOH and PEOOH were done with an authentic standard PCOOH according to the method of Miyazawa et al. The residual extracted lipid was used for the quantitative determination of phospholipids with the use of a phospholipid test (Wako Co) in the brain or the method for determining phospholipid phosphorus described by Bartlett in the clots and cerebral arteries. Both PCOOH and PEOOH levels are expressed as moles per 10^5 mol phospholipids in the brains (n=7) and clots (n=7) and picomoles per gram tissue in the cerebral arteries (n=6 tirilazad and n=4 placebo groups) because of the failure of the quantitative determination of phospholipids in the cerebral arteries due to contamination.

Statistical Analysis
All data are expressed as mean±SD. Comparisons within groups were made by the paired t test, and intergroup comparisons at days 0 and day 7 were determined with the unpaired t test. The level of significance for all comparisons was P<0.05.

Results
Neurological Deficits and Physiological Parameters
The monkeys developed no delayed neurological deficits. When the mean values for body weight, volume of clots, mean arterial blood pressure, heart rate, and ETCO2 measured at baseline and on day 7 were compared, there was no significant difference between placebo and tirilazad groups (Table 1). No drug-induced complications were observed in either group.

Angiographic Vasospasm
In the placebo group on day 7, significant vasospasm occurred in the right MCA (P<0.001), ACA (P<0.005), C1 (P<0.025), C2 (P<0.005), and left MCA (P<0.01) and ACA (P<0.025) compared with the baseline values (Table 2 and Figure 1). In the tirilazad group, slight but significant vasospasm occurred in the right MCA (P<0.05), right C2 (P<0.05), left MCA (P<0.025), and left C2 (P<0.05) compared with the baseline values. Notwithstanding, the degree of vasospasm was significantly attenuated in the right MCA and ACA in the tirilazad group in comparison with the placebo group (P<0.005) (Table 2 and Figure 1).

rCBF
There was no significant difference in rCBF between the 2 groups (data not shown).

PCOOH and PEOOH Levels in Clots
In the tirilazad group, the total PCOOH and PEOOH levels in the subarachnoid clots were lower than in the placebo group (Figure 2, left panel). However, this difference was not significant. The PCOOH and PEOOH levels, expressed as moles per 10^5 mol phospholipids, were also not different between the 2 groups (Figure 2, right panel).

PCOOH and PEOOH Levels in Brain
There were no significant differences in the brain PCOOH and PEOOH levels between the 2 groups (Figure 3). The levels in the right frontal cortex in contact with subarachnoid clots were 5 times higher than in the other regions; however, this difference did not reach significance because of the small number of samples (Figure 3).

PCOOH and PEOOH Levels in Cerebral Arteries
The PCOOH and PEOOH levels were also measured in the cerebral arteries in the tirilazad (n=6) and placebo (n=4) groups (Figure 4). All samples in each group had to be mixed for the measurements because each artery sample was too small to measure both PCOOH and PEOOH levels. In the placebo group, a significant amount of PCOOH was detected in the right MCA, left MCA, and basilar arteries (Figure 4). Indeed, PCOOH was not detected in the cerebral arteries in the tirilazad group (Figure 4). There was a trend for higher PCOOH levels to be accompanied by more severe vasospasm (Figure 4). A significant difference was noted in PCOOH

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<th>TABLE 1. Physiological Parameters Before and After SAH</th>
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<td>Body Weight, kg</td>
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<td>Heart Rate, bpm</td>
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Values are means±SD. ND indicates not determined.

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<th>TABLE 2. Changes in Vessel Diameter</th>
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<td>Tirilazad</td>
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Values are mean±SD, expressed in millimeters. R indicates right; L, left; and BA, basilar artery.

*P<0.05, †P<0.025, ‡P<0.01, §P<0.005, ¶P<0.001, significant difference from baseline value on day 0.

¶P<0.005, significant difference from values of placebo-treated group.
levels between the placebo and tirilazad groups ($P<0.025$). PEOOH was not detected in the cerebral arteries in either group (data not shown).

**Discussion**

The major findings of the present study were that tirilazad was beneficial for the prevention of vasospasm and that the mechanism of action of this agent might be the inhibition of lipid peroxidation, in terms of PCOOH, in cerebral arteries. Moreover, the degree of vasospasm was correlated with the levels of PCOOH. This is the first study to directly demonstrate lipid peroxidation in cerebral arteries by using CL-HPLC.

Lipid hydrolysis with subsequent eicosanoid production is an early pathochemical event in SAH. However, lipid hydrolysis with the release of arachidonate may be closely tied to peroxidation-induced changes in membrane calcium permeability. In addition, a synergistic interaction between calcium and lipid peroxidation during cell damage has been demonstrated. Elevated levels of vasoconstrictor prostanoids occur in lumbar cerebrospinal fluid after SAH.

Moreover, there was a close correlation between the increase in the amount of lipid peroxides and the decrease in the activity of superoxide dismutase in the cerebrospinal fluid. It is likely that the increased level of lipid peroxides in the cerebrospinal fluid may cause lipid peroxidation in the arterial wall, resulting in prolonged vasospasm. However, we are unaware of any previous demonstration of the effects of tirilazad on lipid peroxidation in the spastic arteries.

The previous study demonstrated that 0.3-mg/kg tirilazad treatments significantly attenuated MDA contents in the clots. However, the present study did not demonstrate significant differences in clot PCOOH levels between tirilazad and placebo groups. Possible explanations for this discrepancy are as follows: (1) although PCOOH is a lipid hydroperoxide produced in the initial stage of lipid peroxidation, MDA is an end product and may reflect the productions of other lipid hydroperoxides; (2) the peak productions of PCOOH and MDA may take different time courses after SAH; (3) differences in the methods for sample preparation and/or in the measurements may explain the discrepancy; and (4) there are trends in that the clot PCOOH levels were lower...
in the tirilazad group than in the placebo group. One would expect that if there were more samples included in this study, the statistics would reach significance.

A decrease in rCBF on the clot side in the same model was demonstrated.\textsuperscript{26} However, the rCBF was not affected by either SAH or tirilazad treatments in the present study. One possible explanation for this may be the difference in blood pressure between the 2 studies: blood pressure in the present study was \(\approx 38\)% higher than in the previous study. Relatively high blood pressure might improve rCBF in the SAH model.\textsuperscript{27} Since lipid peroxidation may play a significant role in neuronal death due to cerebral ischemia,\textsuperscript{28} restoration of rCBF may explain the evidence that neither PCOOH nor PEOOH levels in the parietal cortex changed after SAH.

Our results are consistent with the observation that PC is the lipid class most susceptible to lipid hydroperoxidation in vivo.\textsuperscript{13} PC is a major phospholipid class in the cell membranes of cerebral arteries, and the enhanced turnover of PC might have been correlated with cerebral vasospasm.\textsuperscript{29} Possible explanations for the relationship between PC hydroperoxidation and vasospasm are as follows: (1) cytotoxic products may be generated during oxyhemoglobin-induced peroxidation of PC, leading to cellular membrane perturbation\textsuperscript{10}; and (2) lysophosphatidylcholine derived from membrane PC inhibits nitric oxide– and endothelium-dependent hyperpolarization factor–mediated vascular relaxation.\textsuperscript{31,32}

Since the lack of relaxation for nitric oxide and endothelium-dependent hyperpolarization factor may be important initiation factors for the occurrence of vasospasm,\textsuperscript{33–37} lysophosphatidylcholine may play a role in the pathogenesis of vasospasm. In addition, lysophosphatidylcholine stimulates protein kinase C.\textsuperscript{38} The activation of protein kinase C has been implicated in the maintenance of sustaining contraction after SAH.\textsuperscript{29,39–42} Diacylglycerol, an endogenous activator of protein kinase C, is also liberated through PC turnover.\textsuperscript{39} The activation of diacylglycerol-liberating enzyme, phospholipase C, in cerebrospinal fluid after SAH has been reported.\textsuperscript{43}

The vascular endothelium is able to produce significant amounts of superoxide radical\textsuperscript{44,45} and hydrogen peroxide.\textsuperscript{46} The vascular endothelium and perivascular cots together were the sites of the free radical generation, which is further supported by evidence of the ability of tirilazad to decrease in PCOOH. For this reason, tirilazad localizes in the brain endothelial cells after intravenous injections.\textsuperscript{47}

In the clinical trials for the treatment of SAH patients, tirilazad has been both effective\textsuperscript{9,10} and ineffective\textsuperscript{41} for improvement of overall outcome. The major differences between experimental and clinical studies are as follows: (1) Dosage regimen is different: 0.3 mg/kg every 8 hours for 6 days in the present study and 2 and 6 mg/kg per day every 6 hours for up to 10 days in the clinical studies.\textsuperscript{9–11} (2) Mean time to treatment is different: 20 hours after SAH in the present study and >30 hours after SAH in the clinical studies.\textsuperscript{9–11} (3) The volume and cause of SAH are essentially different; although human SAH is caused by rupture of aneurysms, primate SAH is induced by clot placement without vascular injury. (4) Clinical profiles are different between primate model and human cases; no monkey developed neurological deficits, but >30% of SAH patients developed symptomatic vasospasm.\textsuperscript{11} (5) Sex-related difference in effectiveness was reported in clinical trials\textsuperscript{10} (tirilazad was more effective in men than in women); however, tirilazad was very effective in female monkeys.\textsuperscript{1} Thus, the metabolism of tirilazad may be different between experimental animals and human cases. Finally, recent studies of acute spinal cord injury have demonstrated that tirilazad treatment started within 8 hours of injury improved motor recovery.\textsuperscript{48} If the tirilazad treatment were commenced earlier, ie, within 8 hours after SAH, a better functional outcome would be expected in the patients.

In conclusion, we found that 0.3 mg/kg tirilazad every 8 hours for 6 days after SAH was effective for the inhibition of lipid peroxidation in the cerebral artery and was beneficial for the prevention of vasospasm.

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References


artery diameter but no reduction in cerebral blood flow. The authors note the curious appearance of vasospasm in the arteries on the left side of the circle of Willis that are remote from the clot, which is placed on the right side. In our experience with blood clot placement in monkeys, the left-sided arteries have never become narrowed. The cerebrospinal fluid remains clear and there is no diffuse subarachnoid bleeding in this model, since clotted blood is placed in the subarachnoid space. The explanation for the left-sided vasospasm observed by Suzuki et al is not evident. In any case, tirilazad treatment significantly attenuated vasospasm, which is, as discussed above, consistent with previous animal studies and inconsistent with human trials.

The main finding of this study is that a measure of lipid peroxidation (PCOOH) was significantly lower in the arteries from animals treated with tirilazad than from those treated with drug vehicle. There was no reduction in the level of PCOOH in the brains or blood clots in animals treated with tirilazad. There was a trend for higher levels of PCOOH to be associated with more severe vasospasm. The authors believe that tirilazad works by inhibiting lipid peroxidation in cerebral arteries. What is the clinical importance of these findings? Is the arterial wall level of PCOOH an end point to use to determine whether tirilazad is being administered in quantities adequate to prevent vasospasm? It is certainly not a practical one, and in addition, I have some reservations about the measurements that were made and about the conclusions that were drawn. I suggest that because of this and the conflicting results compared with other experiments, further work needs to be done to confirm the hypothesis that 21-aminosteroids prevent vasospasm by inhibiting lipid peroxidation in the arterial wall. The measurements were conducted on a small number of pooled arterial samples and are essentially repetitive measurements on a sample size of one. There was no significant correlation between PCOOH levels and vasospasm, although a “trend” was evident. The normal basilar artery contained PCOOH but did not develop vasospasm. Previous experiments by one of the authors demonstrated that tirilazad did decrease lipid peroxidation in the subarachnoid blood clots, a finding not replicated here. Furthermore, other investigators have not been able to demonstrate reductions in lipid peroxidation in subarachnoid blood clots or the cerebral arteries of vasospastic dogs despite the prevention of vasospasm. The lack of correlation between the ability of 21-aminosteroids to inhibit lipid peroxidation and prevent vasospasm is consistent with studies in vitro that have shown no correlation between the ability of tirilazad to decrease lipid peroxidation and prevent cell injury as measured by cell permeability. These findings led to the hypothesis that 21-aminosteroids work through a nonspecific membrane-stabilizing action. The authors are encouraged to continue their studies. Perhaps more effective treatments for vasospasm will arise from a better understanding of the mechanism of action of 21-aminosteroids.

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