A New Model of Cerebral Microthrombosis in Rats and the Neuroprotective Effect of a Rho-Kinase Inhibitor

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Background and Purpose—The aim of this study was to develop a new model of stroke based on endothelial damage and thrombotic occlusion in a perforating artery, leading to small cerebral infarcts and neurological deficits in rats. Moreover, the neuroprotective efficacy of fasudil, a rho-kinase inhibitor, was investigated in this model.

Methods—Fifty-six male Sprague-Dawley rats were used in the present study. Rats were anesthetized with sodium pentobarbital, and 100 mg of sodium laurate was injected into the left internal carotid artery on days 1 and 3. The thrombus induction and consequent of ischemic brain damage were examined by histopathological analyses and neurological deficit scoring in a posture reflex test. To investigate the neuroprotective effects of fasudil, 1 or 10 mg/kg was administered intraperitoneally 5 minutes after the first injection of sodium laurate and once daily thereafter on the following 2 days.

Results—One hour after the injection of sodium laurate, microscopic examination of phosphotungstic acid hematoxylin-stained sections (n=5) revealed that microthrombi containing fibrin strands obstructed the perforating arteries in the ipsilateral hemisphere. Under a transmission electron microscope (n=6), endothelial cells appeared exfoliated and the vascular lumen was obstructed by a thrombus composed of degranulated platelets, fibrin, leukocytes, and erythrocytes. No evidence of endothelial cell damage or thrombus could be found in the ipsilateral side of the pial artery (middle cerebral artery). Twenty-four hours after the second injection of sodium laurate (day 4), 13 of 15 rats (86.6%) showed mild to severe neurological deficits. Multiple small cerebral infarcts were observed in the hippocampus, cortex, and thalamus. Treatment with fasudil (1 and 10 mg/kg, n=15 each) resulted in a significant improvement in neurological deficits. Fasudil also significantly reduced the area of cerebral infarction.

Conclusions—We present a new model of stroke in rats, in which the perforating arteries are selectively occluded by microthrombi. This model is useful to investigate the pathophysiology and treatment of small cerebral infarction, which is caused by perforating arterial occlusive diseases such as lacunar infarcts. Fasudil may be beneficial in the treatment of acute ischemic stroke. (Stroke. 2000;31:2245-2250.)

Key Words: cerebral infarction ● cerebral thrombosis ● protein kinases ● rats

Lacunar infarcts are small, deep infarcts caused by occlusion of the perforating branches of the major cerebral arteries. The infarct is usually associated with one of several clinical lacunar syndromes, depending on the exact location of the lesion.1,2 The most common mechanism of arterial occlusion causing lacunar infarcts is thought to result from vasculopathy of the small perforating arteries. To our knowledge, there has been no previous animal model of cerebral infarction caused by selective perforating artery occlusion based on in situ small arterial injury. The development of a relevant animal model of lacunar infarcts is important for the study of their pathophysiology and for the evaluation of potential therapies.

Sodium laurate is known as a drug that induces tissue necrosis in peripheral regions when injected into an artery. It has been reported that intra-arterial injection of sodium laurate produced endothelial damage, which triggered platelet adhesion and aggregation to form occlusive thrombi.3 In this study, we aimed to develop a new model of stroke in rats as a result of in situ small perforating arterial damage with subsequent thrombotic occlusion, which was induced by internal carotid artery injection of sodium laurate. Lacunar patients usually display a series of neurological deficits after the onset of stroke, and neurological deficits are one of the most important parameters to indicate patient disability after stroke. Therefore, in addition to histopathological analyses, we assessed the neurological deficits in rats after internal carotid artery injection of sodium laurate. Furthermore, using this model, we examined the therapeutic potential of fasudil, a rho-kinase inhibitor, against the ischemic brain damage caused by perforating arterial occlusion.
Materials and Methods

Adult male Sprague-Dawley rats (SLC, Japan; n=56) weighing between 236 and 360 g were used in the present study.

Induction of Cerebral Microthrombosis

On day 1, rats were anesthetized with sodium pentobarbital (50 mg/kg IP) and immobilized in the supine position with spontaneous breathing. A longitudinal incision was made in the cervical skin. After exposure of the left common, external, and internal carotid arteries, the left external carotid, occipital, and pterygopalatine arteries were ligated with a thread. A polyethylene catheter was inserted into the external carotid artery, and the tip of the catheter was placed close to the carotid bifurcation. With use of a microvascular clip, the carotid artery was temporarily clamped during sodium laurate injection. Sodium laurate (Wako) was dissolved in saline, and 100 μg was injected into the internal carotid artery over 30 seconds. The catheter was removed after the injection, and the common carotid artery was ligated at a place slightly proximal to the injection site. Forty-eight hours after the first injection (day 3), rats received the second injection of sodium laurate. The rats were anesthetized with sodium pentobarbital (50 mg/kg IP). After exposure of the left common carotid artery, a polyethylene catheter was inserted into the common carotid artery. Then, 100 μg of sodium laurate was injected into the internal carotid artery. The catheter was removed after the injection, and the common carotid artery was ligated.

Neurological Examination

On day 2 and day 4, neurological deficits such as hemiplegia were evaluated in a posture test. In a postural reflex test, rats were tested for degree of abnormal posture when suspended by their tails 1 m above the floor. They were scored according to the following criteria: Rats extended both forelimbs straight, no observable deficit: 0 (normal). Rats attached the right forelimb to the breast and extended the left forelimb straight: 1 (mild). Rats twisted the upper half of the body, in addition to the behavior in score 1: 2 (severe).

Light Microscopic Analyses

One hour after the first injection of sodium laurate (day 1), rats (n=5) were anesthetized (sodium pentobarbital, 50 mg/kg IP), and the brain was perfused with heparinized saline followed by a 10% buffered formalin solution through the cardiac ventricle. The brains were dissected out and fixed in 10% buffered formalin solution until embedded in paraffin. Five coronal brain sections (5 μm) were prepared at 2-mm intervals. Slice 3 was selected to include the hippocampal area. To evaluate thrombus induction, the coronal brain sections were stained with phosphotungstic acid hematoxylin (PTAH).

At the end of the neurological examination (day 4), brains were dissected out and coronal brain sections were prepared as described above. The brain sections were stained with Luxol fast blue–hematoxylin and eosin for quantification of infarct area and histopathological evaluation.

Transmission Electron Microscopic Analyses

One hour after the injection of sodium laurate or saline (day 1), rats (n=6) were perfused transcardially under anesthesia (sodium pentobarbital 50 mg/kg IP) with heparinized saline followed by 2.5% glutaraldehyde in 0.1 mol/L phosphate buffer (pH 7.2). The brains of 3 of these 6 rats were removed from the cranial vault and cut into coronal sections. Selected areas of the brain, containing the cerebral cortex, hippocampus, and thalamus of the ipsilateral hemisphere, were sectioned into blocks. The left middle cerebral arteries of 3 other rats were removed from each brain and sectioned into coronal blocks. Each block was immersed in 2.5% glutaraldehyde for 2 hours at 4°C, and then fixed with 0.1 mol/L phosphate buffer (pH 7.2). These samples were postfixed in 1% osmium tetroxide in 0.1 mol/L phosphate buffer for 2 hours at 4°C, dehydrated through graded ethanol, and embedded in epoxy resin. Semithin sections were stained with toluidine blue, and ultrathin sections of the areas of interest, stained with uranyl acetate and lead citrate, were examined by transmission electron microscopy (H-7000, Hitachi).

Effect of Fasudil

To evaluate the neuroprotective effect of fasudil in rats injected with sodium laurate, 45 rats were randomly assigned to 3 groups: control, and those treated with 1 mg/kg and 10 mg/kg of fasudil. Fasudil (Asahi Chemical Industry) was dissolved in saline to prepare concentrations of 1 and 10 mg/mL. Fasudil or saline (1 mL/kg) was administrated intraperitoneally 5 minutes after the first injection of sodium laurate and once daily thereafter on the following 2 days.

Measurement of Infarct Area

Each coronal section stained with Luxol fast blue–hematoxylin and eosin was photographed. These photographic images were digitized and used to determine the area of infarct and the area of each ipsilateral hemisphere for each slice on a computerized image analysis system (Image 1.47, NTIS). Because the infarcts were multiple and not continuous, the size of the infarcted area was expressed as a percentage of the coronal section of the ipsilateral hemisphere to reduce errors associated with processing of tissue for histological analysis.

Statistical Analyses

Data are expressed as mean±SEM. Statistical analysis of the infarct size was performed by ANOVA, followed by Dunnett’s test. Neurological deficits were analyzed by nonparametric Dunnett’s-type multiple comparison test. A value of P<0.05 was considered significant.

Results

Neurological Deficits

Twenty-four hours after the first injection of sodium laurate (day 2), mild to severe neurological deficits were observed in 40% of rats. Twenty-four hours after the second injection of sodium laurate (day 4), 86.6% of rats showed mild to severe neurological deficits (Table).

Histopathology

One hour after the first injection of sodium laurate (day 1), microscopic examination of PTAH-stained sections revealed well-perfused vessels, as well as arteries obstructing thrombotic material in some brain regions. The vascular lumen was obstructed by thrombus containing fibrin strands, which were a deep blue color material stained with PTAH (Figure 1A). Fibrin deposition evaluated by PTAH staining was not detected in the contralateral hemisphere. Under a transmission electron microscope, endothelial damage and microthrombus was observed at perforating artery in the ipsilateral hemisphere. The endothelium was frequently exfoliated from the basal lamina and platelets adhered to the endothelium (Figure 1B). The vascular lumen was obstructed completely.
by thrombi containing degranulated platelets (Figure 1C). In some of the thrombi, there was an admixture of erythrocytes, leukocytes, and fibrin (Figure 1D). Endothelial damage or thrombi were not observed in sham-operated rats (Figure 1E). No evidence of endothelial cell damage or thrombus could be found in the ipsilateral side of the middle cerebral artery (Figure 1F).

Twenty-four hours after the second injection of sodium laurate (day 4), the coronal section of the brain stained with Luxol fast blue–hematoxylin and eosin showed multiple small infarcts in the form of poorly stained areas involving the hippocampus, cortex, and thalamus (Figures 2A through 2C).

Effect of Fasudil
Twenty-four hours after the second injection of sodium laurate (day 4), 13 of 15 rats in the control group (86.6%) showed mild to severe neurological deficits. Neurological deficits were observed in 8 of 15 rats (53.3%) and 3 of 15 rats (20.0%) in the groups treated with 1 mg/kg and 10 mg/kg of fasudil, respectively. Neurological deficits were significantly improved in the fasudil-treated rats (Figure 3). The infarct area in the rats treated with 10 mg/kg of fasudil was significantly reduced compared with that of control rats, with the effect being more prominent in slices 3, 4, and 5, in which largest infarcted areas were measured in the control rats (Figure 4).

Discussion
In the present study, we presented a new model of stroke in rats, which was produced by internal carotid artery injection of sodium laurate. Our new model has several advantages over previous stroke models: (1) endothelial damage and subsequent thrombotic occlusion are selectively induced in perforating arteries; (2) the thrombi contain fibrin; (3) the microthrombotic occlusion associated with small cerebral infarcts and neurological deficits can be induced; and (4) although distribution of the infarcts was unpredictable, the total size of infarcts was comparatively constant and neurological deficits were observed in most rats.

It was reported that intra-arterial injection of sodium laurate produced endothelial damage, which triggered platelet adhesion and aggregation to form thrombosis. The mechanism of stroke in this new model was likely thrombotic occlusion of perforating arteries due to in situ arterial wall injuries. The evidence for this is that (1) endothelial damage and obstructing thrombi were present in some perforating arteries 1 hour after the injection of sodium laurate, and (2) endothelial damage or thrombus formation were not observed in the large pial artery (MCA).

The effect of sodium laurate seemed to be dose dependent. In an earlier preliminary trial, 200 µg of sodium laurate was injected into the internal carotid artery, and all rats died soon after or within several hours after the injection (data not shown). The dose of 100 µg of sodium laurate used in the
The present study may be an appropriate dose that causes endothelial damage and thrombotic occlusion localizing in some perforating arteries but not in large pial arteries. It is well known that the blood flow velocity becomes gradually lower from large arteries to arterioles. It is speculated that the contact time between sodium laurate and perforating arterial walls is longer than that in large pial arteries and that this time difference contributes to the lesion site localizing in the perforating artery. In the present model, the thrombi were almost transient, because none were found in the ipsilateral hemisphere 24 hours after a single injection of 100 mg sodium laurate (data not shown). This finding suggests that the occluded perforating arteries had spontaneously recanalized within 24 hours of the sodium laurate injection. Therefore, injection of 100 µg of sodium laurate causes a state of ischemia-reperfusion in the cerebral microcirculation. Transcranial Doppler sonographic studies have demonstrated that spontaneous recanalization occurs several times after the onset of stroke in some proportion of patients.

Mild to severe neurological deficits were observed in 40% and 86.6% of rats 24 hours after the first or second injection of sodium laurate, respectively. The incidence and severity of neurological deficits developed with repetitive injection of 100 µg of sodium laurate. The second injection of sodium laurate may be an added insult to an artery already sensitized by the first injection or may be a means of increasing the total endothelial damage in the arteries. It has been reported that 20% to 50% of patients with lacunar infarction showed gradual progression of clinical deficits, sometimes for up to 72 hours. Therefore, 2 injections of sodium laurate can produce a situation that mimics the early neurological changes in acute lacunar patients.

The posture reflex test used in the present study was reported to be sensitive to damage in the cortex and striatum. It was recently reported that the infarction of deep structures of the brain, including internal capsule and hypothalamus, contribute to the manifestation of postural reflex abnormality. In the present model, the infarcts were multiple and distributed in both cortical and subcortical regions. It is difficult to demonstrate what area of brain damage specifically contributes to these behavioral deficits. However, the posterior region of the cerebrum may contribute to the manifestation of neurological deficits after sodium laurate—induced cerebral microthrombosis in day 4 rats. The bar graph shows proportion of the forelimb flexion score of 15 rats. The significance of difference was calculated by a nonparametric Dunnett’s-type multiple comparison.

Figure 2. Photomicrographs of the rat cerebrum 24 hours after the second injection of sodium laurate (day 4). A, Hippocampal region; B, cortical region; and C, thalamic region. The small infarcts observed were poorly stained with Luxol fast blue–hematoxylin and eosin (surrounded by arrowheads) (magnification ×10; bar=500 µm).

Figure 3. Protective effect of fasudil against neurological deficits after sodium laurate–induced cerebral microthrombosis in day 4 rats. The bar graph shows proportion of the forelimb flexion score of 15 rats. The significance of difference was calculated by a nonparametric Dunnett’s-type multiple comparison.

Figure 4. Effect of fasudil on cerebral infarction after sodium laurate–induced cerebral microthrombosis in rats. Coronal sections were prepared at 2-mm intervals, and slice 3 was selected to include the hippocampal area. Significant reductions of the infarct areas were observed in 10 mg/kg fasudil–treated rats compared with control rats. Control, ○; fasudil 1 mg/kg, ●; and fasudil 10 mg/kg, ■. Each data point represents the mean±SEM of 15 rats. *P<0.05 and **P<0.01 compared with the control group (Dunnett’s test).
these neurological abnormalities because the total size of the infarct area was largest in these sections (Figure 4).

Lacunar infarcts are caused by occlusion of small perforating arteries, and the possible causes of small-vessel occlusion include arterial wall disease, thrombus, or embolus. It has been reported\(^{10,11}\) that photochemical damage to the endothelium of the carotid artery of rats produces microemboli to the brain, resulting in small cerebral infarcts resembling lacunes in humans. In this model, the emboli were composed of platelets and did not contain fibrin. In addition, platelet accumulation occurs without endothelial damage within downstream microvascular beds or evidence for local platelet aggregation or adhesion.\(^{12,13}\) This model is useful for the study of the pathogenesis and treatment of small cerebral infarcts caused by platelet embolization, but not for those caused by microthrombosis or small arterial wall damage.

The results of the present study demonstrated that intraarterial administration of fasudil reduced the size of ischemic brain damage and improved neurological deficits in a microthrombosis model. Although we did not measure the physiological data after the fasudil treatment, previous studies\(^{14–16}\) have shown that fasudil does not affect changes in arterial blood gases, pH, or temperature. In the study for measurements of the changes in cerebral blood flow, we confirmed that fasudil did not alter physiological parameters in this microthrombosis model (data not shown).

Rho-kinase regulates the phosphorylation of myosin light chain\(^{17,18}\) and is implicated in physiological functions, such as smooth muscle contraction and cell migration.\(^{19–21}\) Fasudil potently inhibited rho-kinase (Ki \(= 0.33 \mu\) mol)\(^{22}\) and myosin light chain phosphorylations.\(^{23}\) Fasudil potently increased cerebral blood flow and inhibited inflammatory responses to the ischemic brain.\(^{14–16}\) These effects of fasudil may, at least in part, contribute to the mechanism of neuroprotective action in the present model. Further studies are required to define the mechanisms of fasudil in reducing ischemic brain damage in this microthrombosis model. Because rats were treated with fasudil only 5 minutes after the intravascular infusion of sodium laureate, it is possible that fasudil may affect the degree of primary insult of endothelial damage or microthrombus formation and therefore explain its effect on outcome. To rule out this possibility and evaluate potential therapy with fasudil, we are currently engaged in studies to examine delayed treatment (several hours after the onset of thrombosis) with fasudil against cerebral ischemia in this microthrombosis model.

In conclusion, we present here a new model of stroke in rats, in which the perforating arteries are selectively occluded by microthrombi. This model is useful to investigate the pathophysiology and treatment of small cerebral infarction, which is caused by perforating arterial occlusive diseases such as lacunar infarcts. Our present results suggest that fasudil is beneficial in the treatment of acute ischemic stroke.

References


Toshiro et al A New Model of Cerebral Microthrombosis 2249
The study by Toshima and colleagues describes the development of a new rat model of thrombotic occlusion in perforating arteries, leading to multiple small cerebral infarcts and neurological deficits. Following the intracarotid injection of sodium laurate, a drug that has previously been reported to produce endothelial damage and subsequent platelet activation, the authors describe microthrombi in perforating arteries in the ipsilateral hemisphere as early as 4 hours after treatment. At 24 hours after injection of sodium laurate, mild to severe neurological deficits and multiple small infarcts were reported. Additionally, the authors assessed the neuroprotective effects of a rho kinase inhibitor (fasudil) in this novel model. Treatment with fasudil 5 minutes after injection of sodium laurate improved neurological outcome and reduced histopathological damage 4 days after thrombus induction.

As the authors state, few experimental models are available that lead to endothelial damage with subsequent thrombotic occlusion of perforating cerebral arteries. Because lacunar infarcts are important clinically and are caused by occlusion of the perforating branches of the major cerebral arteries, this new model may potentially be used for evaluation of novel therapies to treat this disorder. The authors provide light and electron microscopic evidence for endothelial damage, with subsequent formation of fibrin-stabilized thrombi. Importantly, the microthrombotic occlusions lead to small cerebral infarcts and neurological deficits that can be quantitated.

Although the mechanism by which sodium laurate administration produces endothelial damage is unknown, the mechanism of stroke formation in this model appears to be the result of thrombotic occlusion of perforating arteries due to in situ arterial wall injury. Thus, this model may be ideal for the evaluation of new treatments that target vascular pathology and thrombotic processes. In this regard, it would be interesting to determine whether thrombolytic agents are neuroprotective in this stroke model.

The authors also provide data indicating that the intravenous administration of fasudil improves outcome. Although the authors do not know exactly how the drug works, previous data may provide clues. Fasudil is a rho-kinase inhibitor that has been implicated in smooth-muscle contraction. Thus, treatment with Fasudil may potentially inhibit rho-kinase and increase cerebral blood flow. Because treatment in the present study was started 5 minutes after injection of sodium laurate, there is a possibility that this treatment may actually affect the primary insult and thereby explain the present results. Thus, as stated by the authors, future studies are required to test more delayed treatment strategies to determine whether this therapy may have a potential benefit for treatment of acute ischemic stroke.

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