Inhibition of Neutrophil Migration by a Protein Kinase Inhibitor for the Treatment of Ischemic Brain Infarction

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ABSTRACT—This study investigated the therapeutic potential of agents that inhibited neutrophil infiltration in cerebral ischemic infarction. The migration of neutrophils elicited by N-formyl-methionyl-leucyl-phenylalanine, tumor necrosis factor, C5a or platelet-activating factor was potently inhibited by fasudil, an inhibitor of protein kinases including rhokinase, protein kinase C and myosin light chain kinase, and hydroxy fasudil, a metabolite of fasudil, in vitro. In a microembolism model in rats, myeloperoxidase-quantified neutrophil accumulation in the ischemic brain was observed 24 hr after embolization. Intravenous administration of fasudil prevented the accumulation of neutrophils. In rats given fasudil, myeloperoxidase activity in the ipsilateral hemisphere (0.04±0.01 unit/g wet tissue) was significantly lower than that in ischemic rats (0.11±0.02 unit/g wet tissue). Fasudil also significantly reduced the size of the infarct area and improved neurological functions. These results suggest that neutrophil infiltration into the ischemic brain is involved in the pathogenesis of ischemic injury and that inhibiting neutrophil infiltration may provide an effective therapeutic intervention to reduce ischemic injury.

Keywords: Cerebral ischemia, Neutrophil, Migration, Fasudil, Protein kinase inhibitor

In patients in the early stage of stroke, leukocytes, especially neutrophils, accumulate in regions with depressed circulation, and neutrophils remain numerous for some days but with some decrease (1, 2). The poor neurological outcome and large infarction volumes in patients with severe neutrophil accumulation sheds light on the importance of neutrophil involvement in brain tissue damage as a result of ischemia (2). In experimental studies, it has also been demonstrated that neutrophils play an important role in the development of ischemic brain damage, and depletion of circulating neutrophils or inhibition of neutrophil adhesion or infiltration is thought to ameliorate cerebral ischemic injury (3–7).

Activated neutrophils may aggravate ischemic tissue damage in the brain by adhesion to the endothelium, infiltration or releasing various mediators such as O²⁻ and proteases. The migration of neutrophils is mediated by numerous agents, such as N-formyl-methionyl-leucyl-phenylalanine (fMLP), C5a, tumor necrosis factor (TNF) or platelet-activating factor (PAF) (8–12). However, the subsequent signal transduction pathway mediating neutrophil movement has remained obscure.

Fasudil is a newly developed antivasospastic drug (13–16) that inhibits myosin light chain kinase (MLCK) (17), protein kinase C (PKC) (18) and rho kinase (19). Fasudil also decreases ischemic damage in the brain (20, 21). Initial studies suggested that the effect could be due to increased cerebral blood flow (14, 22, 23). More recently, other mechanisms have been found. Maiese et al. (24) reported that fasudil had neuroprotective properties, as observed in cultures of neuronal cells. Arai et al. (25) found that fasudil inhibited the production of superoxide anions in human neutrophils. We examined the neuroprotective properties of fasudil (3 and 10 mg/kg, i.p.) in a rat model of microembolization stroke as a preliminary examination (26). Fasudil dose-dependently protected the brain from ischemic infarction. In the present study, the experiment was designed to examine the effect of fasudil at a high dose (10 mg/kg, i.v.). We previously demonstrated that intra-arterial and intravenous administration of fasudil reversed vasospasm with similar potency (16). To determine the effects of fasudil in the
clinical setting of stroke therapy, fasudil was administered intravenously.

This study was designed to investigate the role of protein kinase pathways in neutrophil migration. We also examined the therapeutic potential of agents that inhibited neutrophil infiltration in cerebral ischemic infarction by using fasudil or ozagrel, a thromboxane A₂ (TXA₂) synthetase inhibitor used in the treatment of acute ischemic stroke.

MATERIALS AND METHODS

Cell preparation and chemotaxis

Neutrophils were obtained from peripheral blood of healthy volunteers. The yield of the cell preparation was >90% neutrophils, as judged from the morphology on Giemsa staining. Chemotaxis of neutrophils was measured using a 96-well Boyden chamber in which a 3-μm-pore-sized filter separates the upper and lower chambers. Before placing in the upper compartment, neutrophil suspensions (5 × 10⁵/ml) were preincubated for 30 min with fasudil, hydroxyfasudil, a metabolite of fasudil, or ozagrel. Neutrophils (10² per well) were then placed in the upper well and exposed to 10⁻⁶ M fMLP, 10⁻⁷ M C₅a, 10⁻⁷ M PAF or 10 ng/ml TNF placed in the lower well across a membrane filter. Incubation was carried out for 30 min (fMLP, C₅a, PAF) or 60 min (TNF) at 37°C. The number of neutrophils responding to the chemotactic stimulus and appearing on the opposite side of the filter was determined after Diff-Quik (International Reagents Corp., Kobe) staining. Migrated neutrophils were quantified by measuring specific light absorbance using a densitometer (600 nm).

Measurement of myosin light chain (MLC) phosphorylation

Neutrophils (5 × 10⁶ cells) were incubated with fasudil or hydroxyfasudil at 37°C for 30 min in Hanks' balanced solution and then treated with 10⁻⁶ M fMLP for 30 min at 37°C. The reaction was terminated with trichloroacetic acid (TCA). The neutrophils were then washed 3 times with acetone containing 10 nM dithiothreitol (DTT) to remove TCA, dried and exposed to 50 μl of glycercol-polyacrylamide gel electrophoresis (PAGE) sample buffer that contained 20 nM Tris (hydroxymethyl) aminoethane base–22 mM glycine (pH 8.6), 10 nM DTT, 8 M urea and 0.1% bromophenol blue.

The extent of MLC phosphorylation in the neutrophils was measured by separation of non- and mono-phosphorylated forms by glycercol-PAGE followed by electrophoretic transfer of the proteins to a nitrocellulose membrane; the relative amount of each form was quantified by an immunoblot procedure. Production and characterization of polyclonal anti-MLC antibody was described by Seto et al. (27). The blots were developed using ECL Western-blotting reagents (Amersham, Bucks, UK). Densitometry of immunoblots and quantification of the absorbance peaks were carried out using a Model GS-700 Imaging Densitometer (Bio Rad, Hercules, CA, USA) equipped with a recording integrator. The extent of MLC phosphorylation was expressed as the percentage MLC in the monophosphorylated form.

Microembolization study

Rats, weighing 235 to 450 g, were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and placed in the supine position with spontaneous respiration. The left common, external and internal carotid arteries were exposed through a ventral midline incision. The left external carotid, occipital and pterygopalatine arteries were ligated, and a catheter was inserted via the left external carotid artery into the common carotid artery. Cerebral microembolism was produced by injecting 4,000 polymer microspheres (o.d.: 50.0±1.0 μm; Duke Scientific Corp., Palo Alto, CA, USA) into the internal carotid artery. A catheter was inserted into the tail vein to administer fasudil or ozagrel. An intravenous infusion of fasudil (10 mg/kg per 60 min), ozagrel (10 mg/kg per 60 min) or vehicle (saline, 5 ml/kg per 60 min) was begun 5 min after the injection of microspheres and subsequently maintained for 60 min.

Twenty-four hours after embolization, the behavior of rats was scored on the basis of the severity of the following symptoms: truncal curvature, circling behavior and rolling fit, all considered to be typical for stroke. The score consisted of 3 (severe), 2 (moderate), 1 (slight) and 0 (normal) for each symptom.

Neutrophil infiltration within the brain was quantified using a myeloperoxidase (MPO) activity assay. Measurements were performed on normal rats (n=6), ischemic rats (n=9) and rats given fasudil (n=9) 24 hr after embolization. For MPO analysis in ischemic brain tissue, rats were anesthetized with pentobarbital sodium (50 mg/kg, i.p.) and perfused transcardially with 100 ml heparinized saline solution before brain removal to flush all blood components from the vasculature. Both cerebral hemispheres were stored at −80°C until MPO analysis. Brain samples were thawed and wet weight in grams was rapidly measured. Each sample was homogenized (1:20, wt./vol.) in 5 nM potassium phosphate buffer (pH 6.0, 4°C) using a Teflon homogenizer and centrifuged at 30,000×g for 30 min at 4°C. The supernatant was discarded, and the pellet was washed again as described above. The pellet was extracted by suspending the material in 0.5% hexadecyltrimethylammonium bromide in 50 nM potassium phosphate buffer (1:10), pH 6.0 at
25°C. The specimens were frozen using dry ice and subjected to three freeze-thaw cycles, after which sonication (10 sec) was repeated between cycles. After the last sonication, the samples were then centrifuged at 12,000 x g for 15 min at 4°C, and MPO activity in the supernatant was assayed. The supernatant (0.1 ml) was mixed with 2.8 ml of 50 mM potassium phosphate buffer (pH 6.0) containing 0.167 mg/ml o-dianisidine dihydrochloride and with 0.1 ml of 0.05% hydrogen peroxide. The change in absorbance at 460 nm was measured using a spectrophotometer.

To examine the related histology, the rats were anesthetized (pentobarbital, 50 mg/kg, i.p.) and the brains perfused with a 10% buffered formalin solution through the cardiac ventricle. The study was carried out on 28 rats, divided into 9 control, 9 ozagrel-treated and 10 fasudil-treated groups. 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 and stained with Luxol fast blue-hematoxylin and eosin. Slice 3 was prepared to include the hippocampal area. Infarct areas were quantified using a computerized image analysis system (NIH Image 1.47; NTIS, Springfield, VA, USA), and were expressed as a percentage of the coronal section of the half hemisphere.

**Statistical analyses**

Values are expressed as means±S.E.M. The significance of difference was calculated by Student's t-test, Student's t-test for paired samples, Dunnett's test or nonparametric Dunnett-type multiple comparisons. P values of 0.05 or less were considered to indicate significant differences.

**Drugs**

The drugs used were fasudil and hydroxy fasudil (Asahi

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**Fig. 1.** Dose-response effect of fasudil (○), hydroxy fasudil (●) or ozagrel (△) on neutrophil chemotaxis induced by fMLP, 10⁻⁶ M (A); TNF, 10 ng/ml (B); C5a, 10⁻⁷ M (C); or PAF, 10⁻⁷ M (D). Each data point represents the mean±S.E.M. of three to nine experiments. The chemotactic activity by fMLP, TNF, C5a or PAF-stimulated neutrophils (control) was defined as 100%. The asterisks indicate a significant difference from the control (**P < 0.01, *P < 0.05; Student's t-test for paired samples).
Chemical Industry, Tokyo), ozagrel (Ono Pharmaceutical Co., Ltd., Osaka) and pentobarbital sodium (Pittman-Moore, Indianapolis, IN, USA).

RESULTS

Neutrophil chemotaxis

When neutrophil chemotaxis was elicited with the chemoattractant fMLP, TNF, C5a or PAF, a marked migration response was observed. Figure 1 shows the effects of fasudil or hydroxy fasudil on the migration elicited by the optimum concentration of fMLP, TNF, C5a or PAF. The migration so elicited was dose-dependently inhibited by fasudil or hydroxy fasudil. The lowest concentration of fasudil or hydroxy fasudil, at which significant inhibition of the chemotaxis induced various chemoattractants, was 3–30 or 1–3 \( \mu M \), respectively. Hydroxy fasudil was about 3–30 times more potent in inhibiting neutrophil infiltration than was fasudil. Ozagrel (10 and 100 \( \mu M \)) did not significantly inhibit neutrophil infiltration.

Measurement of MLC phosphorylation

The level of MLC phosphorylation was measured after adding \( 10^{-6} M \) fMLP in the presence of fasudil or hydroxy fasudil (Fig. 2). In neutrophils stimulated with fMLP, the extent of MLC phosphorylation (40.7±1.7% before stimulation, \( n=10 \)) increased to a value of 52.4±2.7% (\( n=10, P<0.01 \) vs before stimulation). Fasudil or hydroxy fasudil (1–100 \( \mu M \)) dose-dependently inhibited MLC phosphorylation in neutrophils. The lowest concentration of fasudil or hydroxy fasudil at which significant inhibition of the MLC phosphorylation induced by fMLP occurred was 1 \( \mu M \).

Microembolization study

Few neutrophils were observed in the brain tissue of normal rats. Twenty-four hours after embolization, a large number of neutrophils was observed in the ipsilateral hemisphere of ischemic rats, and MPO activity

![Graph showing MPO activity before and after fasudil treatment](image)

**Fig. 3.** Effect of fasudil (10 mg/kg) on MPO activity 24 hr after embolization. The saline treated-control is shown as an open column and fasudil as a hatched column. Each column represents the mean±S.E.M. of the number of experiments shown in parentheses. Statistical significance was assessed by Student's \( t \)-test.

![Graph showing MLC phosphorylation](image)

**Fig. 2.** Effects of fasudil or hydroxy fasudil on MLC phosphorylation in neutrophils. Each column represents the mean±S.E.M. of five experiments. The asterisks indicate a significance from the fMLP control (\( **P<0.01, ^*P<0.05 \): Student's \( t \)-test for paired samples).

![Graph showing infarction area](image)

**Fig. 4.** Effect of fasudil or ozagrel on the areas of ischemic damage on 5 coronal slices. Coronal slices were prepared at 2-mm intervals, and slice 3 was prepared to include the hippocampal area. A significant reduction of infarct size was induced by fasudil (○, \( n=10 \)) or ozagrel (●, \( n=9 \)) compared with the control (△, \( n=9 \)). Each data point represents the mean±S.E.M. The asterisks indicate a significant difference from the control (\( **P<0.01, ^*P<0.05 \): Dunnnett's test).
Table 1. Protective effect of fasudil or ozagrel against the impairment of neurological function

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Score 0</th>
<th>Score 1</th>
<th>Score 2</th>
<th>Score 3</th>
<th>Total</th>
<th>Significant difference (vs saline-treated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline-treated/ischemic</td>
<td></td>
<td>0 (0.0)</td>
<td>1 (10.0)</td>
<td>2 (20.0)</td>
<td>10 (100.0)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fasudil-treated/ischemic</td>
<td>10 mg/kg</td>
<td>6 (60.0)</td>
<td>0 (0.0)</td>
<td>3 (30.0)</td>
<td>1 (10.0)</td>
<td>10 (100.0)</td>
<td>P &lt; 0.05</td>
</tr>
<tr>
<td>Ozagrel-treated/ischemic</td>
<td>10 mg/kg</td>
<td>0 (0.0)</td>
<td>2 (22.2)</td>
<td>5 (55.6)</td>
<td>2 (22.2)</td>
<td>9 (100.0)</td>
<td>NS</td>
</tr>
</tbody>
</table>

Values are numbers of rats and percentage (in parentheses) of the total within each group. The significance of difference was calculated by the nonparametric Dunnett’s test. NS indicates no significant difference.

(0.11 ± 0.02 unit/g wet tissue, P < 0.01 vs normal) was significantly greater than that in normal rats (0.00 ± 0.00 unit/g wet tissue) (Fig. 3). In rats given fasudil, MPO activity in the ipsilateral hemisphere (0.04 ± 0.01 unit/g wet tissue) was lower than that in ischemic rats. In the contralateral hemisphere, MPO activity was slightly, but not significantly, increased (0.02 ± 0.01 unit/g wet tissue).

The infarct area averaged 44.1 ± 3.3% of the cortical section of the half hemisphere (slice No. 4) in the ischemic rats (Fig. 4), a significant difference compared with findings in the mean area of 17.1 ± 2.9% (P < 0.01) and 32.8 ± 2.3% (P < 0.05) observed in rats treated with fasudil or ozagrel, respectively. All animals in the ischemic group showed typical symptoms of stroke such as truncal curvature, circling behavior and rolling fits (Table 1). Neurological function was significantly improved in the fasudil-treated animals. Ozagrel (10 mg/kg) did not significantly improve the ischemia-induced neurological function.

DISCUSSION

The present results showed that fasudil or hydroxy fasudil inhibited neutrophil chemotaxis to fMLP, C5a, TNF or PAF, in vitro. The lowest concentration of hydroxy fasudil, at which a significant inhibition of the chemotaxis induced by various neutrophil activators occurred, was 1 - 3 μM; and hydroxy fasudil was about equally potent in inhibiting the migration of neutrophils by fMLP, C5a, TNF or PAF. Fasudil also inhibited various chemoattractant-induced migrations of neutrophils, at concentrations of 3 - 30 μM. Fasudil or hydroxy fasudil induced similar inhibitory effects in neutrophils migrated using fMLP, C5a, TNF or PAF, suggesting that the inhibitory action of fasudil or hydroxy fasudil may occur through an intracellular common pathway of the migration of neutrophils rather than through a specific receptor or membrane channel.

In a clinical trial, hydroxy fasudil was formed rapidly following administration of fasudil (28); and at 45 min after the initiation of infusion, the concentration of hydroxy fasudil present in plasma was approximately 80% of the parent drug. Hydroxy fasudil was eliminated more slowly than fasudil (data not shown). Hydroxy fasudil, as well as fasudil, may be effective in inhibiting the migration of neutrophils and in preventing brain injury associated with ischemic stroke.

Proteins such as actin and myosin are not only responsible for the contraction of smooth muscles but also play an important role in the movement and shape changes of non-muscle cells, such as leukocytes and platelets (29). The actomyosin cytoskeleton was found to be involved in leukocyte chemotaxis, and alterations in the cytoskeleton may dramatically affect cell motility (30). Signaling pathways appear to play a part in controlling basic cellular functions, such as cell migration, phagocytosis, and secretion. The role of protein kinase or phosphatase in neutrophil functions has been discussed (31, 32). Activation of neutrophils by fMLP was accompanied by an increase in MLC phosphorylation. Fasudil or hydroxy fasudil dose dependently inhibited MLC phosphorylation, with a similar potency, a finding inconsistent with observations that fasudil was approximately 30 times less potent in inhibiting neutrophil infiltration than was hydroxy fasudil. While the occurrence of neutrophil infiltration may be related to the MLC phosphorylation pathway, the related mechanisms may involve other pathways.

PKC may also play a critical role in the signal transduction of the neutrophil function. Dang et al. (33) demonstrated that fMLP stimulates rapid and sustained translocation of PKC to the membrane fraction in neutrophils. Since such translocation is an indication of PKC activation, activation of PKC is considered to be one of the critical processes by which fMLP stimulates neutrophil functions. Fasudil and hydroxy fasudil (34) potently inhibits PKC and may produce a suppressive effect on neutrophil infiltration by inhibiting PKC activation. The detailed mechanisms underlying the infiltration of neutrophils have not been identified. Fasudil, an inhibitor
of protein kinases including rho kinase, PKC and MLCK, inhibited the neutrophil migration in vitro; thus, protein kinases may be involved in neutrophil migration. However, we could not exclude the possibility that fasudil or hydroxy fasudil inhibits other protein kinases because these compounds have a wide spectrum of action against several kinds of protein kinases (34). More evidence is required to clearly established the mechanism by which fasudil or hydroxy fasudil inhibits neutrophil migration.

In the present study, neutrophil accumulation in cerebral ischemic infarct was measured by determining MPO activity as a marker for quantification of infiltrated neutrophils (35), and it was observed in a model of microembolism. Neutrophil infiltration was not evident in the brain of normal rats (MPO activity: 0.00±0.00 unit/g wet tissue). In the ischemic group, neutrophil infiltration was observed in the ipsilateral hemisphere of ischemic brains (MPO activity: 0.11±0.02 unit/g wet tissue), and all rats showed typical symptoms of stroke such as truncal curvature, circling behavior and rolling fits. In the ipsilateral brain hemisphere of ischemic rats, few neutrophils were observed in the normal brain area, while many neutrophils were observed in the infarction region by light microscopy. Based on these results, neutrophil infiltration into the ischemic area of the brain may be correlated with the pathogenesis of ischemic injury and neurological impairment. Fujinuma et al. (1) reported that accumulation of leukocytes was greater in the region of more severe ischemia and the central zone of the ischemia in acute embolic stroke in humans.

Ischemic brain injury evokes not only endogenous brain parenchymal cell damage but also an inflammatory response, which includes adhesion, infiltration and accumulation of neutrophils. The primary mechanism of neutrophil-mediated damage occurs when neutrophils are within the microvasculature. Neutrophils promote ischemic cell damage by reducing cerebral blood flow, damaging the endothelium by free radicals or proteases and increasing permeability of the blood-brain barrier. Leukocyte infiltration into ischemic tissue potentiates ischemic cell damage, all of which may lead to a vicious cycle of ischemia and inflammatory response. We previously reported findings of an increase in the water content of the ipsilateral hemisphere after embolization and the occurrence of cerebral edema in this model (26). If ischemic cell damage is exacerbated by a delayed process of leukocyte presence in the affected tissue, it seems reasonable to assume that postischemic interference with processes of inflammation may reduce the extent of ischemic injury. Our finding was that intravenous administration of fasudil inhibited the ischemic-induced neutrophil infiltration as well as impairing neurological function and changing morphology. Fasudil has no effect on the number of peripheral neutrophils and platelets (data not shown), and this suggests that neuroprotective properties of fasudil are due not to depletion of circulating neutrophils, but to inhibition of neutrophil infiltration. Arai et al. (25) reported that fasudil inhibited the production of superoxide anions in neutrophils, and it has been reported that fasudil administered intraperitoneally significantly protected against edema in a microembolism model in rats (26). These results suggest that fasudil was effective in protecting against ischemic injury by limiting inflammatory responses, at least in part.

The cerebral protective effects of hypothermia have been reported (36). Fasudil had no significant effect on the magnitude of body temperature (data not shown). This suggests that the neuroprotective effect of fasudil is due not to hypothermia, but it may be required to determine whether fasudil affects brain temperature. The method of inducing a microembolism model was essentially the same as in previous reports (37). In this model, regional cerebral blood flow (rCBF) in the embolized hemisphere was decreased (30–50% of the control value). Previously, it was reported that fasudil increased rCBF in animal models of cerebral ischemia (21, 23). To define the mechanisms of fasudil in preventing brain injury, it may be useful to examine the effects of fasudil on rCBF in the model of microembolism stroke.

A slight but significant reduction in ischemic brain damage was found in the ozagrel-treated group. Ozagrel did not significantly inhibit the neutrophil chemotaxis induced by various chemoattractants. Neuroprotective effects of ozagrel may involve mechanisms other than the inhibition of the neutrophil chemotaxis. Hiraku et al. (38) reported that ozagrel exhibited a suppressive effect on the vascular contraction and the generation of the thrombosis and cerebral infarction through an inhibition of TXA2 production. These effects through TXA2 synthetase inhibition may be associated with the neuroprotective effect of ozagrel.

Our present findings suggest that neutrophil infiltration into the ischemic brain is involved in the pathogenesis of ischemic injury and that inhibiting neutrophil infiltration may provide an effective therapeutic intervention to reduce ischemic injury.

REFERENCES

Neutrophil Migration and Protein Kinases


