原 著

Migration of neutrophils elicited by leukotriene B_4 is inhibited by fasudil, a Rho-kinase inhibitor, in the microvasculature of hamster cheek pouch

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Abstract

This study was designed to investigate possible effects of a Rho-kinase inhibitor, fasudil, on migration of neutrophils induced by leukotriene B_4. The neutrophil behavior was observed in the microvasculature of hamster cheek pouch using a trans-illumination microscope. Superfusion of lekotrience B_4 caused an increase in the number of neutrophils adhering the endothelium and migrating through the endothelium outside the venules. The migration induced by lekotrience B_4 was significantly attenuated in hamsters receiving intravenous infusion of 10 and 30mg/kg of fasudil prior to the leukotriene B_4 superfusion. These results suggest that inhibition of Rho-kinase by fasudil produces an inhibition of neutrophil migration and represents a new therapeutic strategy for neutrophil-mediated tissue damage.

Introduction

Fasudil is a Rho-kinase inhibitor that has shown clinical effectiveness in patients with subarachnoid hemorrhage[1] and that was launched for clinical use after subarachnoid hemorrhage in Japan. Rho-kinase contributes to the reorganization of the actin cytoskeleton and to the formation of stress fibers, and is thought to be one of the critical elements involved in a variety of cytoskeleton-dependent cell functions such as cell migration[2]. It has been shown that neutrophil chemotaxis as well as neutrophil and macrophage infiltration is inhibited by fasudil and its active metabolite, hydroxyfasudil[3~6]. In the present study, therefore, we investigated if the inhibition of Rho-kinase with fasudil would reduce neutrophil migration in vivo in a leukotriene-induced inflammatory model.

Materials and Methods

Ethical committee for animal experimentation of our institution approved this study. Thirty-one male Golden hamsters, weighing 110–160g at 10–16weeks old (Nihon SLC, Shizuoka, Japan) were anesthetized with urethane 1.2g/kg given intraperitoneally. A tracheal cannula was inserted to facilitate spontaneous respiration with a mixture of oxygen and room air. A cannula was placed in the femoral vein for drug infusion.

Preparation of hamster cheek pouch:

The hamster cheek pouch preparations were set up as previously described[7,8]. The cheek pouches were pulled out, cut longitudinally, and extended. The connective tissue was elaborately dissected away to expose the microvasculature of the mucous layer. The thin mucous membrane tissue was spread out in a plastic chamber.

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Microscopic observation and recording of leukocyte behavior:

The microvasculature of the hamster cheek pouch (unit: 50μm×100μm) was observed under a transillumination microscope (ECLIPSE, Nikon, Tokyo, Japan), using a W40 water immersion lens (×40, Nikon, Tokyo, Japan) and ×10 eyepieces (Nikon, Tokyo, Japan). Images of the microcirculation were projected onto a color television monitor screen (PVM-20M4J, SONY Co, Tokyo, Japan) via a color TV camera (DXC-5800, SONY Co, Tokyo, Japan) mounted at the top of the microscope. The behavior of the neutrophils in each experiment was recorded with a videotape recorder (SVO-5800, SONY Co, Tokyo, Japan).

Application of fasudil:

Fasudil (Asahi Kasei, Japan) at the dose of 3mg/kg (n=8), 10mg/kg (n=9), and 30mg/kg (n=7) in a total volume of 3ml/kg, or saline of 3ml/kg (n=7) as a vehicle were administrated via the femoral vein for 30 minutes.

Chemotactic agent:

Leukotriene B_4 (Paesel, GMBH, Frankfurt, Germany) was used as a chemoattractant[9]. A stock solution of leukotriene B_4 (30μm in absolute ethanol) was kept at −80℃ and diluted to 300nM with Tyrode's solution immediately before use. Ten minutes after initiating the administration of fasudil or saline, leukotriene B_4 (300nM) was applied to the microvasculature at the observation site with a 50μl micropipette.

Count of neutrophils:

Neutrophils could be individually visualized as bright white cells against the dark background of the blood stream, since they rolled slowly on the endothelial wall. Migration of neutrophils was determined when neutrophils were moving from the venular wall into the interstitial space (unit: 50μm×100μm).

Data analysis

All values are expressed in mean±SD. Data were statistically analyzed with ANOVA and Student's t-test was used for comparisons between the two groups, and paired t-test for comparisons before and after interventions in the same group. $P<0.05$ was considered as a statistically significant difference.

Results

Before the application of leukotriene B_4, several neutrophils were observed to move slowly along the vascular endothelium. The application of leukotriene B_4 caused a transient increase and a subsequent decrease in rolling of neutrophils. There were no significant differences in the alterations in rolling and adhesion of neutrophils among the four groups. Thickened wall was observed both in the vehicle and the fasudil 3mg groups, but not in the fasudil 10mg and fasudil 30mg groups (**Fig. 1**).

Migration of neutrophils started 20min after leukotriene B_4 application in all groups. **Fig. 2** demonstrates typical pictures in which migration of neutrophils occurred at 60min after initiating leukotriene B_4 in the vehicle group. In the vehicle group, 3.6± 1.3 and 5.2±1.8 counts/unit of neutrophils were migrated at 60 and 90min after leukotriene B_4, respectively. In the fasudil-10mg group, 1.3±0.5 and 1.4±0.2 counts/unit, and in the fasudil-30mg group, 1.1±0.5 and 1.6±0.7 counts/unit were migrated at 40 and 60min, respectively. The numbers of migrating neutrophils in the fasudil 10mg and the fasudil 30mg groups were significantly less than those in the vehicle and fasudil 3mg groups (**Fig. 3**).

Discussion

Leukotriene B_4 is a pro-inflammatory mediator synthesized in myeloid cells from arachidonic acid[10]. It induces recruitment and activation of neutrophils, monocytes and eosinophils by stimulating the production of a number of proinflammatory cytokines and mediators[9,10]. In our study, superfusion of leukotriene B_4 could also augment migration of neutrophils in the microvasculature of hamster cheek pouch. Pharmacological inhibition studies support a role for leukotriene B_4 in the pathogenesis of neutrophil mediated tissue damage, and treatments which reduce its production or block its effects may prove beneficial in neutrophil mediated inflammatory diseases[10~12]. Since

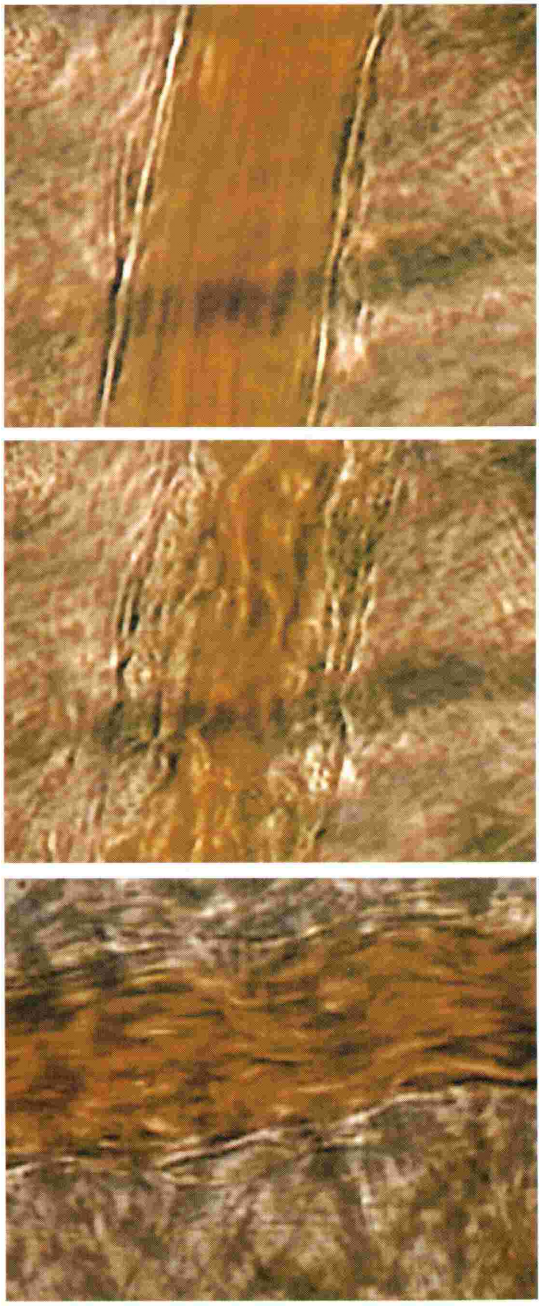

Figure 1

Photomicrograph of microcirculation in hamster cheek pouch. Intact venular wall was observed before superfusion of leukotriene B_4 (upper). Sixty min after superfusion of leukotriene B_4, the venular wall was thickened in a hamster receiving a vehicle (middle), whereas the venular wall was not thickened in a hamster treated with fasudil 30mg (lower). The thickened venular wall comprised of infiltrated neutrophils.

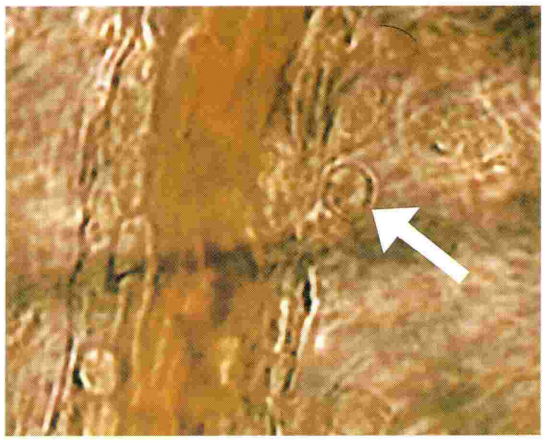

Figure 2

Photomicrograph of microcirculation in hamster cheek pouch. Migration of neutrophils was observed at 60 min after initiating leukotriene B_4 in a hamster receiving a vehicle. An arrow indicates a migrated neutrophil.

neutrophil migration elicited by superfusion of leukotriene B_4 was inhibited by pretreatment of fasudil in this study, it is suggested that fasudil can be beneficial in prevention of neutrophil mediated tissue damage.

Studies in animal models show fasudil to be promising in the treatment of stroke, angina, and renal fibrosis[5,13~15]. Fasudil and its active metabolite, hydroxyfasudil, inhibit Rho-kinase more effectively than they inhibit other protein kinases; e.g., protein kinase C, or myosin light chain kinase[16,17~19]. There is accumulating evidence that Rho is important regulators of endothelial barrier properties by influencing both the endothelial actin-based cytoskeleton and the integrity of interendothelial junctions[20]. The Rho family of small GTPases regulates many facets of cytoskeletal dynamics that underlie changes in cell shape and adhesion during migration[21,22]. It has also been shown that Rho-kinase is involved in controlling the development of polarity and migration of neutrophils[2]. All these together suggest that the inhibitory action of fasudil on neutrophil migration occurs through the inhibition of the Rho-kinase pathway. However, we could not exclude the possibility that fasudil causes the inhibitory effect through inhibition of other protein kinases because fasudil has a wide spectrum of action against

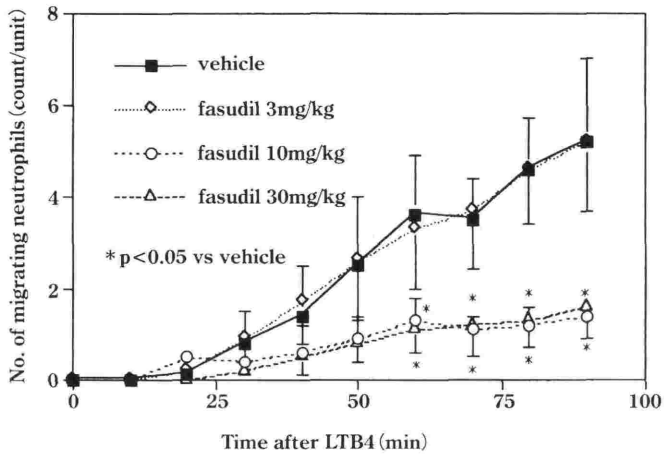

Figure 3

Changes in the numbers of migrating neutrophils after superfusion of leukotriene B_4. The abscissa indicates the time lapse after the application of leukotriene B_4. The ordinate indicates the numbers of migrating neutrophils in the detection window. Values are shown as mean±SD. Fasudil 10mg and 30mg significantly inhibited the migration of neutrophils elicited by leukotriene B_4 superfusion ($p<0.05$).

several kinds of protein kinase. Thus, the detailed mechanisms underlying the inhibition of neutrophil migration by fasudil have not been identified in this study.

In the present study, a significant inhibition in the migration of neutrophils induced by leukotriene B_4 was observed in hamsters receiving 10mg/kg and 30 mg/kg of fasudil. It has been reported that a significant inhibition of neutrophil chemotaxis induced by various chemoattractants, including N-formyl-methionyl-leucyl-phenylalanine, was observed at 3 to 30μM of fasudil[3]. In rats, the maximum plasma concentration of fasudil after intraperitoneal administration of fasudil at 10mg/kg was approximately 15μM[23], which is equivalent to the effective concentration for inhibition of neutrophil chemotaxis. Plasma concentrations of fasudil were not measured in this study, but intravenous infusion of fasudil 10mg/kg for 30min in hamsters could be suspected to reach a similar level as intraperitoneal administration of fasudil at the same dose.

It has been reported that fasudil 10mg/kg, which was administered daily for 2 weeks also attenuated interstitial fibrosis and macrophage infiltration in rat kidneys with unilateral ureteral obstruction[5]. It has also been reported that fasudil 10 and 30mg/kg significantly prevented the development of myocardial fibrosis in a chronic myocardial damage model in rats[24]. These previous reports are in agreement with our results in hamsters in terms of the doses of fasudil inhibiting neutrophil or macrophage infiltrations and the accompanied possible tissue damage.

In conclusion, this study demonstrated that fasudil, a Rho-kinase inhibitor, reduced neutrophil migration elicited by leukotriene B_4 in the microvasculature of hamster cheek pouch, representing a new therapeutic strategy for neutrophil-mediated tissue damage.

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References

1) Shibuya M, Suzuki Y, Sugita K, et al: Effect of AT877 on cerebral vasospasm after aneurysmal subarachnoid hemorrhage. Results of a prospective placebo-controlled double-blind trial. J Neurosurg 1992; 76: 571-7.
2) Niggli V: Rho-kinase in human neutrophils: a role in signaling for myosin light chain phosphorylation and cell migration. FEBS Lett 1999; 445: 69-72.
3) Satoh S, Kobayashi T, Hitomi A, et al: Inhibition of

neutrophil migration by a protein kinase inhibitor for the treatment of ischemic brain infarction. Jpn J Pharmacol 1999; 80: 41-8.
4) Ikegaki I, Hattori T, Yamaguchi T, et al: Involvement of Rho-kinase in vascular remodeling caused by long-term inhibition of nitric oxide synthesis in rats. Eur J Pharmacol 2001; 427: 69-75.
5) Satoh S, Yamaguchi T, Hitomi A, et al: Fasudil attenuates interstitial fibrosis in rat kidneys with unilateral ureteral obstruction. Eur J Pharmacol 2002; 455: 169-74.
6) Miyata K, Shimokawa H, Kandabashi T, et al: Rho-kinase is involved in macrophage-mediated formation of coronary vascular lesions in pigs in vivo. Arterioscler Thromb Vasc Biol 2000; 20: 2351-8.
7) Bjork J, Hedqvist P, Arfors KE: Increase in vascular permeability induced by leukotriene B4 and the role of polymorphonuclear leukocytes. Inflammation 1982; 6: 189-200.
8) Nagai K, Katori M: Possible changes in the leukocyte membrane as a mechanism of leukocyte adhesion to the venular walls induced by leukotriene B4 and fMLP in the microvasculature of the hamster cheek pouch. Int J Microcirc Clin Exp 1988; 7: 305-14.
9) Bjork J, Dahlen SE, Hedqvist P, et al: Leukotrienes B4 and C4 have distinct microcirculatory actions in vivo. Adv Prostaglandin Thromboxane Leukot Res 1983; 12: 1-6.
10) Crooks SW, Stockley RA: Leukotriene B4. Int J Biochem Cell Biol 1998; 30: 173-8.
11) Nakae H, Endo S, Inada K, et al: Nitrite/nitrate (NOX) and type II phospholipase A2, leukotriene B4, and platelet-activating factor levels in patients with septic shock. Res Commun Mol Pathol Pharmacol 1996; 92: 131-9.
12) Li EJ, Cook JA, Wise WC, et al: Effect of LTB4 receptor antagonists in endotoxic shock in the rat. Circ Shock. 1991; 34: 385-92.
13) Asano T, Ikegaki I, Satoh S, et al: Blockade of intracellular actions of calcium may protect against ischaemic damage to the gerbil brain. Br J Pharmacol 1991; 103: 1935-8.
14) Satoh S, Ikegaki I, Suzuki Y, et al: Neuroprotective properties of a protein kinase inhibitor against ischaemia-induced neuronal damage in rats and gerbils. Br J Pharmacol 1996; 118: 1592-6.
15) Utsunomiya T, Satoh S, Ikegaki I, et al: Antianginal effects of hydroxyfasudil, a Rho-kinase inhibitor, in a canine model of effort angina. Br J Pharmacol 2001; 134: 1724-30
16) Davies SP, Reddy H, Caivano M, et al: Specificity and mechanism of action of some commonly used protein kinase inhibitors. Biochem J 2000; 351: 95-105.
17) Shimokawa H, Seto M, Katsumata N, et al: Rho-kinase-mediated pathway induces enhanced myosin light chain phosphorylations in a swine model of coronary artery spasm. Cardiovasc Res 1999; 43: 1029-39.
18) Shimokawa H: Rho-kinase as a novel therapeutic target in treatment of cardiovascular diseases. J Cardiovasc Pharmacol 2002; 39: 319-27.
19) Nagumo H, Sasaki Y, Ono Y, et al: Rho kinase inhibitor HA-1077 prevents Rho-mediated myosin phosphatase inhibition in smooth muscle cells. Am J Physiol Cell Physiol 2000; 278: C57-65.
20) Nobes CD, Hall A: Rho GTPases control polarity, protrusion, and adhesion during cell movement. J Cell Biol 1999; 144: 1235-44.
21) Schwartz MA, Shattil SJ: Signaling networks linking integrins and rho family GTPases. Trends Biochem Sci 2000; 25: 388-91.
22) Dharmawardhane S, Bokoch GM: Rho GTPases and leukocyte cytoskeletal regulation. Curr Opin Hematol 1997; 4: 12-8.
23) Satoh S, Utsunomiya T, Tsurui K, et al: Pharmacological profile of hydroxy fasudil as a selective rho kinase inhibitor on ischemic brain damage. Life Sci 2001; 69: 1441-53.
24) Satoh S, Ikegaki I, Toshima Y, et al: Effects of Rho-kinase inhibitor on vasopressin-induced chronic myocardial damage in rats. Life Sci 2002; 72: 103-12.