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The Kinase Inhibitor Fasudil (HA-1077) Reduces Intimal Hyperplasia through Inhibiting Migration and Enhancing Cell Loss of Vascular Smooth Muscle Cells[1]

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Smooth muscle cell (SMC) migration plays an important role in restenosis after angioplasty. Myosin phosphorylation is necessary for cell migration. Fasudil is an inhibitor of protein kinases, including myosin light chain kinase and Rho associated kinase, thereby inhibiting myosin phosphorylation, and it has been clinically used to prevent vasospasm following subarachnoid hemorrage. Based on these findings, we examined the antimigrative action of fasudil. In SMC (SM-3), fasudil (1–100 μM) inhibited SMC migration in a dose-dependent manner ($p < 0.001$). Fasudil suppressed actin stress fiber formation dose dependently. In rabbit carotid artery, fasudil (10 mg/kg/day) markedly reduced intimal hyperplasia 14 days following balloon injury. Cell kinetic study showed that fasudil did not affect proliferation but enhanced cell loss in the media after injury. We concluded that fasudil reduced neointimal formation after balloon injury through both inhibiting migration and enhancing cell loss of medial SMC.

Accumulation of smooth muscle cells (SMCs) into the intima, which is due to both proliferation and migration, is a critical event in the formation of restenotic lesions after percutaneous transluminal coronary angioplasty (PTCA) (1–3). It has been suggested that myosin phosphorylation is necessary for cell migration as its driving force (4–6). Fasudil (HA-1077), an inhibitor of Ser-Thr protein kinases including myosin light chain (MLC20) kinase, inhibits myosin phosphorylation (7, 8) and has been already clinically used with great success to prevent delayed cerebral vasospasm following subarachnoid hemorrhage (9, 10). Therefore, fasudil is expected to inhibit SMC migration and accumulation into intima. We examined the usefulness of fasudil as a novel therapeutic drug to prevent coronary restenosis after PTCA.

MATERIAL AND METHODS

Cell culture. Human aortic SMCs (passage 5) were obtained from Clonetics Corp. (San Diego, CA) and cultured at 37°C in modified MCDB131 medium supplemented with 5% fetal calf serum (FCS) (11). The SM-3 cell line, a strain of rabbit aortic SMC (12), and primarily cultured SMC from rabbit aorta (third to fifth passage) were maintained in Minimum Essential Eagle Medium (MEM) containing 10% FCS.

Fluorescent staining of F-actin with FITC-labeled phalloidin. Fluorescent staining of F-actin was carried out according to the methods of Sasaki *et al.* (13) with minor modification. SMCs were plated on 8 mm square Lab-Tek chamber slides (Nunc Co, USA) and incubated for 2 h at 37°C in a CO_2 incubator with various concentration of fasudil. Fasudil was gifted from the Life Science Research Center, Asahi Chemical Industry Company, Japan. The cells were washed with PBS, fixed in a 4% paraformaldehyde-PBS solution, permeabilized with 0.5% Triton X-100 and stained with FITC-labeled phalloidin. Finally the slides were analyzed by a fluorescence microscope (Olympus).

Migration assay. Migration activity was measured by a modified Boyden's chamber method using microchemotactic chambers (Neuroprobe Inc. U.S.A.) and polycarbonate filters (Nucleopore Corp. U.S.A.) with a pore size of 5.0 mm, as described previously (14). Briefly, the filters were coated with 20 μg/ml fibronectin or 300 μg/ml collagen I and placed between the chambers. Cells were trypsinized and suspended at a concentration of 5.0×10^5 cells/ml in MEM supplement. The SMC suspension (50 μl) was placed in the upper chamber, and human recombinant 6 ng/ml PDGF-BB (Sigma, U.S.A.) was used as a chemotactic agent. The chamber was incubated at 37°C in 5% CO_2 in air for 3 h. The SMCs which had migrated to the lower side of the filter were fixed in methanol, stained with Diff-Quick staining solution, and counted under a microscope (×200) to quantify SMC migration. Migration activity was expressed as the mean number of cells that had migrated per high-power field.

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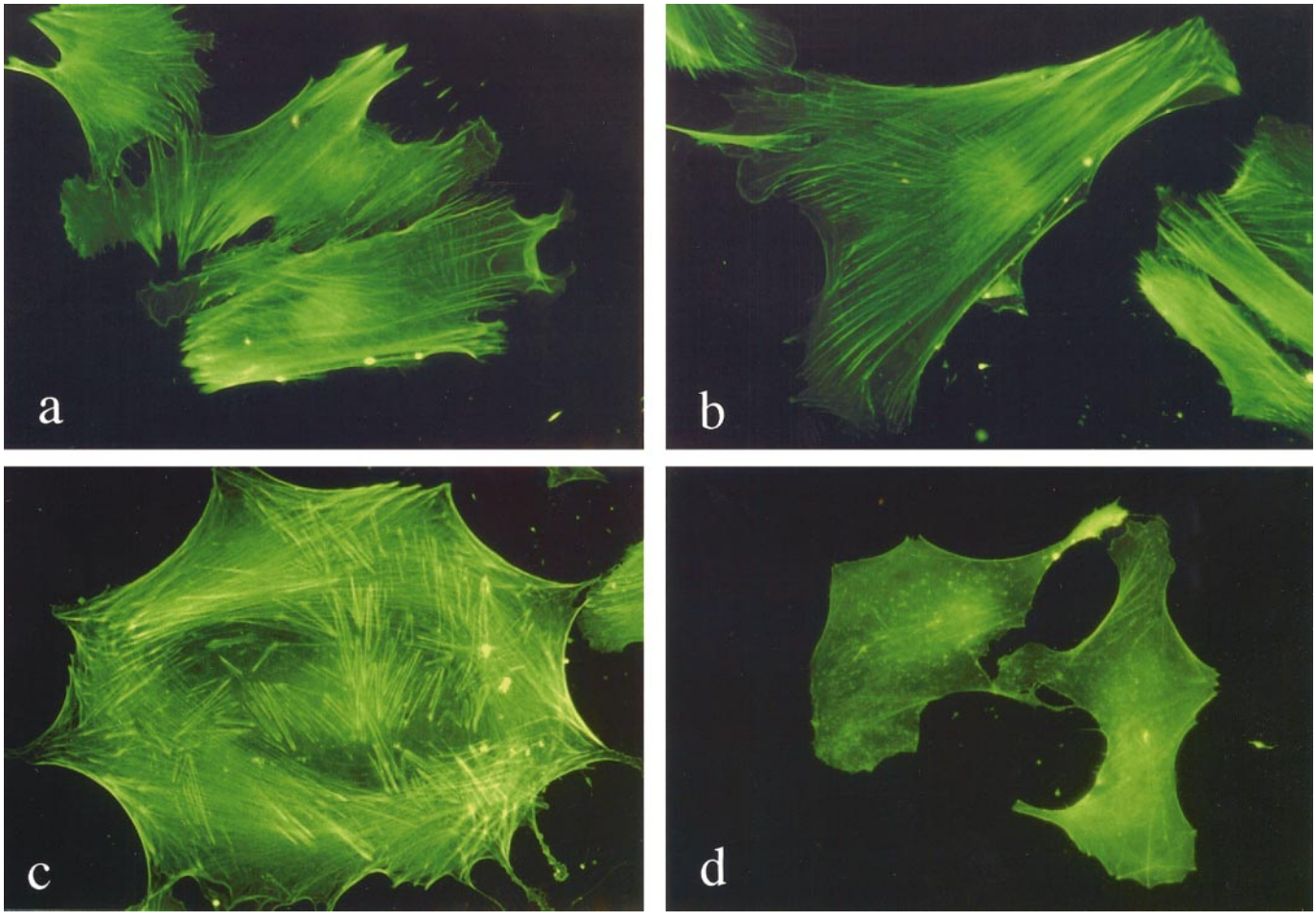

FIG. 1. Fasudil inhibits stress fibers formation in human aortic SMC. Human aortic SMCs, untreated (a) or treated with 1 μM (b), 10 μM (c), or 100 μM (d) of fasudil for 2 h, were fixed with 4% paraformaldehyde and stained with rhodamine-labeled pholloidine. Fasudil disorganized actin stress fibers in a dose-dependent manner. Original magnification: (a–d) $\times$80.

Animal study. All animal studies were performed with the approval of the Osaka Medical College Animal Care and Use Committee. Thirty-seven Japanese white rabbits (male, 2.5 to 3.0 kg) fed a normal diet were divided into two groups: fasudil treated group and control. Fasudil was administered orally, 10 mg/kg/day from 3 days before balloon injury until sacrifice. Each rabbit was anesthetized with sodium pentobarbital (25 mg/kg), a 4F sheath introducer (Medikit Co., Ltd., Tokyo, Japan) was inserted for arterial access via right femoral artery. A 2.75 mm-PTCA balloon catheter was advanced into the right carotid artery, inflated with 6 atm and denuded three times. Rabbits were sacrificed at 0 (n = 3), 7 (n = 12), and 14 (n = 10) days. Rabbits sacrificed at 0, 7 (n = 6 of 12), 14 days were perfusion-fixed by 4% paraformaldehyde-PBS solution and the rabbits sacrificed at 7 days were fixed in methanol-Carnoy's fixative (n = 6 of 12). In addition, for cell kinetic study as described below, twelve rabbits were sacrificed at 30 minutes (n = 3), and at 4 h (n = 3) in both control- and fasudil-treated group. The carotid arteries were removed, sectioned into eight 4-mm-blocks for paraffin embedding. Sequential 3-μm-thick cross sections were cut, stained with H&E and immunohistochemicaly. Morphometrical analyses were carried out using computer-assisted imaging analyzer. The intimal and medial areas of each cross section were measured, and the intimal/medial area ratio (I/M ratio) were calculated.

Cell kinetic study. Total cell number of the intima and media were estimated for each section by multiplying the intimal and medial cross-sectional areas, respectively, by the number of nuclei per square millimeter, which were calculated by counting nuclei and area in three representative high power fields ($\times$200). To identify proliferative cells, a monoclonal antibody against proliferative cell nuclear antigen (PCNA, DAKO A/S, Glostrup, Denmark), which is expressed in the late G1, S, and G2 phases of the cell cycle, was used. PCNA-positive cells in media per high-power field ($\times$100) were represented as a percentage of total cells. As the serum concentration of fasudil is not measurable directly, its metabolite, AT877, was measured by liquid chromatography mass spectrometry (LC-MS).

Statistical analysis. All values are expressed as mean $\pm$ SEM. Statistical analysis was carried out on a Macintosh computer (Apple Inc., Japan) using a commercially available statistical software (Statview). When two groups were compared, differences were assessed by the unpaired Student's t test.

RESULTS

Disorganization of actin stress fiber by fasudil. Cultured SMCs were incubated with various concentration of fasudil and analyzed by immunocytochemistry with F-actin staining. Human aortic SMCs grown in MEM-5% FCS medium showed a number of actin stress fibers and their associated focal adhesion (Fig. 1a). Fasudil induced

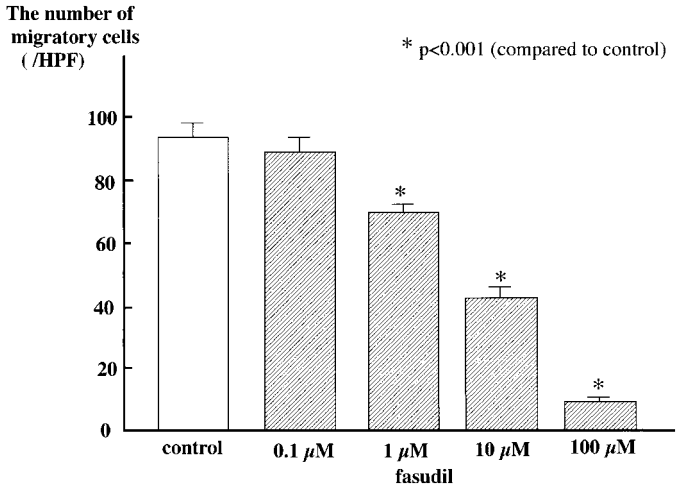

FIG. 2. Fasudil inhibits SMC migration. Migration assay using SM-3 cell with the Boyden's chamber method: open column, control; hatched column, fasudil-treated group; 0.1, 1, 10, and 100 μM, respectively. Fasudil inhibited SMC migration at doses greater than 1 μM ($p < 0.001$) in a dose-dependent manner. Human recombinant PDGF-BB was employed as a chemotactic agent.

disorganization of actin stress fiber, such as a decrease in number and length of the actin-containing microfilament, in a dose dependent manner (Fig. 1b–d). At 1 μM of fasudil treatment, it seemed that formation of stress fibers does not change significantly. At 10 μM, length of stress fibers were markedly shortened and cells became polygonal. At 100 μM, the filamentous actin structure was totally disrupted and an actin-containing circular structure was only seen in the peripheral regions. The same result was seen in cultured rabbit aortic SMCs (data not shown).

Fasudil inhibits SMC migration. Modified Boyden's chamber assay using the SM-3 cells was carried out with administration of fasudil at 0.1 to 100 μM (Fig. 2). Fasudil inhibited SMC migration in a dose dependent manner at more than 1 μM: 30% inhibition by 1 μM; 60% inhibition by 10 μM; 93% inhibition by 100 μM. Similar results were seen in both human aortic SMC and rabbit aortic SMC (data not shown).

Fasudil inhibits intimal hyperplasia after injury. In this rabbit model, a thick layer of neointima was developed at 14 days after balloon injury (Fig. 3a). Intimal formation was clearly suppressed by fasudil treatment (Fig. 3b). The morphometric analyses showed that I/M ratio was significantly decreased in the fasudil treated group compared to the control group (0.16 ± 0.03 vs. 0.64 ± 0.03, $p < 0.001$; Fig. 3c). At 14 days after injury, fasudil administration decreased intimal area but did not cause significant change in medial area (Table 1). The serum concentration of fasudil metabolite, AT-877, at sacrifice was 0.7 μM at two hours after last oral administration of fasudil.

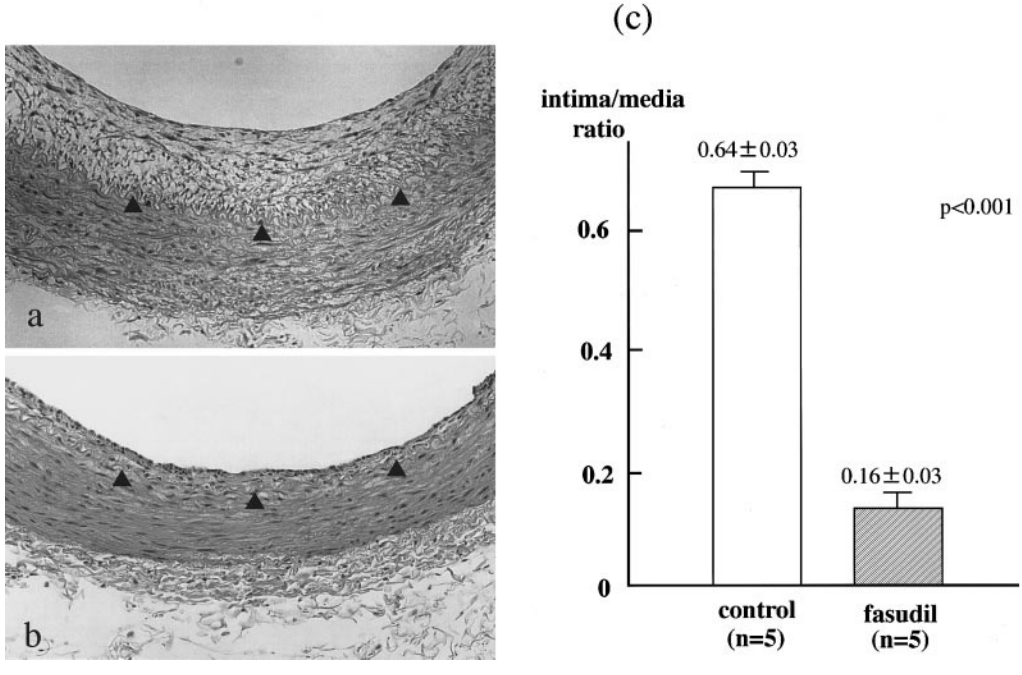

FIG. 3. Fasudil inhibits intimal hyperplasia after balloon injury. H&E-stained sections from the carotid artery at 14 days after balloon injury in control (a) and fasudil-treated rabbit (b). Orally administration of fasudil (10 mg/kg, once a day) was started 3 days before injury and administered until 14 days after injury; arrowhead indicates internal elastic lamina (IEL). Original magnification: (a, b) ×100. Morphometric analyses (c) showed that the intima/media ratio was significantly decreased by fasudil (0.64 ± 0.03; control vs 0.16 ± 0.03; fasudil, $p < 0.001$).

TABLE 1

Cell Kinetic Analysis of Vascular SMCs after Balloon Injury

	Area (mm^2)		Cell count	
	Control	Fasudil	Control	Fasudil
Media				
0	0.23 ± 0.01		1040 ± 52.6	
30 min	0.34 ± 0.02	0.34 ± 0.02	901 ± 66.4	883 ± 53.6
4 h	0.38 ± 0.02	0.32 ± 0.02	756 ± 75.8	748 ± 57.8
3 days	0.41 ± 0.03	0.45 ± 0.03	1010 ± 66.5	589 ± 70.6*
7 days	0.41 ± 0.03	0.48 ± 0.03	1560 ± 142	1460 ± 109
14 days	0.34 ± 0.02	0.43 ± 0.02	1340 ± 83.7	1850 ± 74.8*
Intima				
7 days	0.05 ± 0.01	0.03 ± 0.01	609 ± 104	392 ± 158
14 days	0.18 ± 0.01	0.08 ± 0.01*	1300 ± 145	735 ± 112*

* $p < 0.01$ vs. control.

Fasudil does not affect SMC proliferation in vivo. To elucidate the mechanism by which fasudil reduces intimal hyperplasia *in vivo*, a proliferation activity was analyzed. There was no significant difference in the frequency of medial PCNA positive cell 7 days after injury between the control and the fasudil-treated group (57.7 ± 2.36 vs. 49.2 ± 0.03% N.S. $p = 0.12$) (Fig. 4a–c), suggesting that fasudil did not affect SMC proliferation in this dosage. Occasionally we found that the cells in the inner half of the media were sparse without destruction of elastic fibers in the fasudil-treated group 7 days after injury (Fig. 4b).

Cell kinetic analyses after injury. To clarify the net effect of proliferation, migration and cell loss in this model, we counted the total cell number in both media and intima during time course (Table 1). In this model, the number of medial cell was decreased until 4 h after injury and increased to the level before injury at 3 days. In fasudil-treatment rabbit, the number of medial cells was still decreased until 3 days, suggesting that fasudil enhances and prolongs cell loss after injury. However, medial cell number was not different at 7 days between control and fasudil-treated animals, suggesting that the proliferation activity from 3 to 7 days after injury was not inhibited by fasudil. At 14 days after injury, the number of intimal cells was fewer in fasudil-treated animal than in control (735 ± 112 vs. 1300 ± 145, respectively), although the medial cell number was larger in fasudil-treated animal than in control (1850 ± 74.8 vs. 1340 ± 83.7, respectively). These data suggested that fasudil inhibit SMC migration from the media to the intima.

DISCUSSION

The present study showed that fasudil, a protein kinase inhibitor, reduced intimal hyperplasia after balloon injury in rabbit carotid artery. It is thought that myosin phosphorylation, focal adhesion complex, and actin stress fiber formation are necessary steps for cell migration (5). It has been suggested that MLCK and Rho-associated kinase are critical in these steps via phosphorylation of the ezrin/radixin/moesin (ERM) proteins (15) and addu-

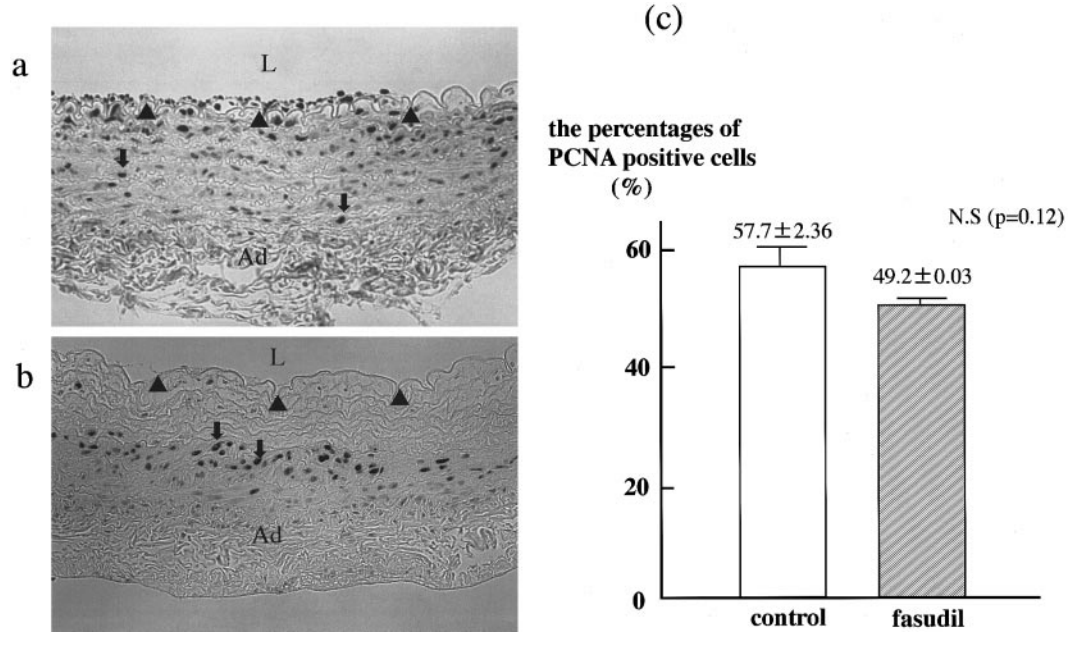

FIG. 4. Fasudil does not affect SMC proliferation in the media after injury *in vivo.* Immunohistochemistry with an antibody against PCNA 7 days after injury in control (a) or fasudil-treated group (b). Arrowheads indicate IEL and arrow indicates PCNA-positive cells. (L) lumen, (Ad) adventitia. PCNA-positive cells in the media were counted since the neointima was not found in the fasudil-treated group (C). SMCs disappeared from the inner half of the media. Original magnification: (a, b) ×200.

cin (16) as well as MLC (17–20). Recently, fasudil has been reported to inhibit Rho-kinase at Ki value of 0.33 μM (21) more efficiently than MLCK (Ki, 36 μM) (8). In this study, we demonstrated that fasudil disorganized actin stress fiber at least 10 μM and inhibited SMCs migration even at 1 μM. These results suggest that inhibition of SMC migration by fasudil is mainly due to inhibition of phosphorylation of Rho-kinase.

Our in vivo data showed that fasudil does not affect on medial SMC proliferation following injury (Fig. 4, Table 1). Sase *et al.* has reported that fasudil suppresses cultured SMC proliferation at doses greater than 30 μM (22). This concentration is much higher than that required for inhibition of SMC migration (Fig. 2). We cannot know the concentration of fasudil in the tissue, however, the serum concentration of fasudil metabolite at sacrifice was below 1 μM. It is suggested that the dosage which we used in vivo were sufficient to inhibit SMC migration but not its proliferation. In addition, there are not any study which reduce intimal hyperplasia through inhibiting SMC migration but not proliferation as far as we know.

Lastly we found a novel interesting phenomenon that fasudil enhanced cell loss in the media after balloon injury. Recent reports have suggested that apoptosis occurs during vascular remodeling both in human restenosis and in animal model following balloon injury (23–25). In animal study, apoptosis can be detected in the neointima or in the media within 4 h after injury (23, 26, 27). In the present study, cell kinetic study (Table 1) clearly showed that the cell loss in the media still occurred until 3 days after injury in fasudil-treated animal. Recently, Kondo *et al.* reported that cytoplasmic translocation of ERM protein was responsible for an early phase of apoptosis (28). It is possible that inhibition of ERM protein phosphorylation by fasudil might induce apoptosis. In conclusion, using fasudil, we can reduce intimal hyperplasia after balloon injury by inhibiting SMC migration and enhancing cell loss without altering proliferation activity, and they might be results from inhibition of Rho-K and MLCK by fasudil.

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REFERENCES

1. Hermans, W. R. M., Rensing, B. J., Strauss, B. H., and Serruys, P. W. (1991) *Am. Heart J.* **122,** 171–187.
2. Ueda, M., Becker, A. E., and Fujimoto, T. (1987) *Br. Heart. J.* **58,** 635–643.
3. Ip, J. H., Fuster, V., Badimon, L., Badimon, J., Taubman, M. B., and Chesebro, J. H. (1990) *J. Am. Coll. Cardiol.* **15,** 1667–1687.
4. Wilson, A. K., Gorgas, G., Claypool, W. D., and de Lanerolle, P. (1991) *J. Cell. Biol.* **114,** 277–283.
5. Stossel, T. P. (1993) *Nature* **260,** 1086–1094.
6. Adelstein, R. S. (1983) *J. Clin. Invest.* **72,** 1863–1866.
7. Sasaki, Y., and Sasaki, Y. (1990) *Biochem. Biophys. Res. Commun.* **171,** 1182–1187.
8. Seto, M., Sasaki, Y., Sasaki, Y., and Hidaka, H. (1991) *Eur. J. Pharmacol.* **195,** 267–272.
9. Satoh, S., Suzuki, Y., Harada, T., Ikegaki, I., Asano, T., Shibuya, M., Sugita, K., and Hidaka, H. (1992) *Eur. J. Pharmacol.* **220,** 243–248.
10. Shibuya, M., Suzuki, Y., Sugita, K., Saito, I., Sasaki, T., Takakura, K., Nagata, I., Kikuchi, H., Takemae, T., Hidaka, H., and Nakashima, M. (1992) *J. Neurosurg.* **76,** 571–577.
11. Morishita, R., Higaki, J., Hayashi, S., Yo, Y., Aoki, M., Nakamura, S., Moriguchi, A., Matsushita, H., Matsumoto, K., Nakamura, T., and Ogihara, T. (1997) *Diabetologia* **40**(9), 1053–1061.
12. Sasaki, Y., Uchida, T., and Sasaki, Y. (1989) *J. Biochem.* **106,** 1009–1018.
13. Sasaki, Y., Sasaki, Y., Kanno, K., and Hidaka, H. (1987) *Am. J. Physiol.* **253**(*Cell Physiol.* **22**), C71–C78.
14. Fukui, R., Shibata, N., Kohbayashi, E., Amakawa, M., Furutama, D., Hoshiga, M., Negoro, N., Nakakoji, T., Ii, M., Ishihara, T., and Ohsawa, N. (1997) *Atherosclerosis* **132**(1), 53–59.
15. Mackay, D. J. G., Esch, F., Furthmayr, H., and Hall, A. (1997) *J. Cell. Biol.* **138,** 927–938.
16. Kimura, K., Fukata, Y., Matsuoka, Y., Bennett, V., Matsuura, Y., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1998) *J. Biol. Chem.* **273,** 5542–5548.
17. Matsui, T., Amano, M., Yamamoto, T., Chihara, K., Nakafuku, M., Ito, M., Nakano, T., Okawa, K., Iwamatsu, A., and Kaibuchi, K. (1996) *EMBO. J.* **15,** 2208–2216.
18. Amano, M., Ito, M., Kimura, K., Fukata, Y., Chihara, K., Nakano, T., Matsuura, Y., and Kaibuchi, K. (1996) *J. Biol. Chem.* **271,** 20246–20249.
19. Kureishi, Y., Kabayashi, S., Amano, M., Kimura, K., Kanaide, H., Nakano, T., Kaibichi, K., and Ito, M. (1997) *J. Biol. Chem.* **272,** 12257–12260.
20. Fukata, Y., Kimuara, K., Oshiro, N., Saya, H., Matsuura, Y., and Kaibuchi, K. (1998) *J. Cell. Biol.* **141,** 409–418.
21. Uehata, M., Ishigaki, T., Satoh, H., Ono, T., Kawahara, T., Morishita, T., Tamakawa, H., Yamagami, K., Inui, J., Maekawa, M., and Narumiya, S. (1997) *Nature* **389,** 990–994.
22. Sase, K., Yui, Y., Hattori, R., Shirotani, M., Kawai, C., and Sasayama, S. (1992) *Jpn. Circ. J.* **56,** 1129–1233.
23. Han, D. K. M., Haudenschild, C. C., Hong, M. K., Tinkle, B. T., Leon, M. B., and Liau, G. (1995) *Am. J. Pathol.* **147,** 267–277.
24. Bennet, M. R., Evan, G. I., and Schwartz, S. M. (1995) *J. Clin. Invest.* **95,** 2266–2274.
25. Isner, J. M., Kearney, M., Bortman, S., and Passeri, J. (1995) *Circulation* **91,** 2703–2711.
26. Piallat, B. M. L., Gabbiani, F., Redard, M., Desmouliere, A., and Gabbiani, G. (1995) *Am. J. Pathol.* **146,** 1059–1064.
27. Perlman, H., Maillard, L., Krasinski, K., and Walsh, K. (1997) *Circulation* **95,** 981–987.
28. Kondo, T., Takeuchi, K., Doi, Y., Yonemura, S., Nagata, S., Tsukita, S., and Tsukita, S. (1997) *J. Cell. Biol.* **139,** 749–758.