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LABORATORY INVESTIGATION

Antiangiogenic Properties of Fasudil, a Potent Rho-kinase Inhibitor

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Abstract

Purpose: Vascular endothelial growth factor (VEGF) plays a pivotal role in pathological angiogenesis. In this study, we addressed the therapeutic potential of fasudil, a potent Rho-kinase inhibitor, for VEGF-elicited angiogenesis and also for the intracellular signalings induced by VEGF.

Methods: In vitro, the inhibitory effects of fasudil on the VEGF-dependent VEGF receptor 2 (VEFGR2 or KDR), extracellular signal-related kinase (ERK) 1/2, Akt and myosin light chain (MLC) phosphorylation, as well as on the migration and proliferation of bovine retinal microvascular endothelial cells (BRECs) were analyzed with Western blotting, [^{3}H]-thymidine uptake, and modified Boyden chamber assay. VEGF-elicited in vivo angiogenesis was analyzed with a mouse corneal micropocket assay coembedded with or without fasudil.

Results: VEGF caused enhanced MLC phosphorylation of BRECs, which was almost completely attenuated by 10 μM fasudil. VEGF-dependent phosphorylation of ERK1/2 and Akt were also partially but significantly attenuated by treatment with fasudil without affecting VEGFR2 (KDR) phosphorylation. Moreover, both VEGF-induced [^{3}H]-thymidine uptake and the migration of BRECs were significantly inhibited in the presence of fasudil. Finally, VEGF-elicited angiogenesis in the corneal micropocket assay was potently attenuated by coembedding with fasudil ($P < 0.01$).

Conclusions: These findings indicate that fasudil might have a therapeutic potential for ocular angiogenic diseases. The antiangiogenic effect of fasudil appears to be mediated through the blockade not only of Rho-kinase signaling but also of ERK and Akt signaling. **Jpn J Ophthalmol** 2008;52:16–23 © Japanese Ophthalmological Society 2008

Key Words: angiogenesis, myosin light chain phosphorylation, retina, Rho-kinase, vascular endothelial growth factor

Introduction

The small GTPase RhoA and its downstream effector, Rho-kinase, play a central role in diverse cellular functions, including smooth muscle contraction, cytoskeletal rearrangement, cell migration, cell proliferation and gene expression.[1] As pharmacological inhibitors of Rho-kinase, fasudil[2] and Y-27632[3] have inhibitory Rho-kinase activity in a competitive manner with ATP. Accumulating evidence from animal and clinical studies indicates that Rho-kinase inhibitors have broad pharmacological properties for treating various diseases, including cardiovascular and renal diseases.[1]

In the pathogenesis of proliferative diabetic retinopathy, neovascularization is regulated by various growth factors and cytokines, especially by hypoxia-inducible vascular endothelial growth factor (VEGF), which regulates endothelial cell proliferation, migration, and permeability, and by the adhesive contacts of endothelial cells with the extracellular matrix.[4,5]

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During the last several years, a number of therapeutic agents, such as bevacizumab (Avastin, Genentech, South San Francisco, CA, USA), a monoclonal antibody that binds to human VEGF with high affinity, and pegaptanib sodium (Macugen, Eyetech Pharmaceuticals, New York, NY, USA; and Pfizer, New York, NY, USA), an anti-VEGF aptamer that specifically blocks the 165 isoform of VEGF, have been investigated for their efficacy in the pharmacological treatment of intraocular neovascularization.[6,7] Moreover, many products, such as alpha-defensins, VEGF165b (an endogenous C-terminal splice variant of VEGF), Ephrin A1, and vasohibin, have been reported to suppress retinal angiogenesis.[8–11]

VEGF is essential for various angiogenic processes. The binding of VEGF to its cognate receptors induces dimerization and subsequent phosphorylation of the receptors, leading to activation of several intracellular signaling molecules such as phosphatidylinositol 3-kinase/Akt, and mitogen-activated protein kinases (MAPKs).[12]

Rho-kinase is reported to be implicated in endothelial cell migration, which is an essential step for angiogenesis.[13,14] The initial event in cell migration is polarization and extension of protrusions in the direction of migration. Rho-kinase controls the formation of these prominences (lamellipodia and filopodia) by regulating the cytoskeleton and cell adhesion.[15] In endothelial cells Rho-kinase regulates stress fiber formation and cell–cell junctions in response to VEGF,[13,16] which might be associated with the phosphorylation of the myosin light chain (MLC), a target protein of Rho-kinase.[17] Moreover, recent in vivo studies have demonstrated that the Rho/Rho-kinase pathway plays a critical role in angiogenesis.[18,19]

In the clinic, fasudil, a potent Rho-kinase inhibitor, is effective for the treatment of a wide range of cardiovascular diseases, including cerebral and coronary vasospasm, angina, hypertension, pulmonary hypertension, and heart failure, with reasonable safety.[20–23] Rho-kinase is thus an important therapeutic target in cardiovascular medicine today. In the present study, we first demonstrated that fasudil could inhibit VEGF-elicited bovine retinal endothelial cell (BREC) migration and proliferation, and corneal neovascularization, possibly by suppressing both extracellular signal-related kinase (ERK) 1/2 and Akt phosphorylation involved in cell survival and migration[24,25] in addition to Rho-kinase activity.

Materials and Methods

Materials

Recombinant human $VEGF_{165}$ and recombinant mouse $VEGF_{164}$ were purchased from R & D Systems (Minneapolis, MN, USA). Goat polyclonal antibodies against MLC and phosphorylated MLC (pMLC) were obtained from Santa Cruz Biotech (Santa Cruz, CA, USA). A specific Rho-kinase inhibitor, Y-27632, was obtained from Calbiochem (San Diego, CA, USA). Fasudil, a potent Rho-kinase inhibitor, was generously provided by Asahi Kasei, Tokyo, Japan. Anti-phospho-p44/42 MAPK (Thr^{202}/Tyr^{204}) E10 mouse monoclonal antibody and phospho-Akt (Thr^{308}) and anti-total Akt rabbit polyclonal antibodies were purchased from Cell Signaling Technology (Beverly, MA, USA). Rabbit polyclonal antibody against Flk-1/KDR (VEGF receptor 2; VEGFR2) agarose conjugate (C-1158; SC-504AC), Flk-1/KDR (A-3; SC-6251) and ERK1 (K-23; SC-94) were obtained from Santa Cruz Biotech. Mouse monoclonal anti-phosphotyrosine (clone PY20) antibody was obtained from MP Biomedicals (Aurora, OH, USA).

Cell Culture

Isolated BRECs from eyes of freshly killed cattle were primarily cultured on a fibronectin-coated dish. Thereafter, typical cobblestone cells were selected and cultured on a type 1 collagen-coated dish up to passage 9 in Humedia-EB2 supplemented with Humedia-EG [2% fetal bovine serum (FBS), human epidermal growth factor (10 ng/ml), hydrocortisone (10 μg/ml), heparin (10 μg/ml), gentamicin (50 μg/ml), and amphotericin-B (50 ng/ml)] (Kurabo, Osaka, Japan) and 5% heat-inactivated horse serum (Sigma Chemical, St. Louis, MO, USA) at 37°C in a humidified 5% CO_2 atmosphere.

Western Blot Analysis

BRECs were incubated up to a 90% confluent state and starved in Dulbecco's modified Eagle medium (DMEM, Gibco, Grand Island, NY, USA) supplemented with 3% heat-inactivated bovine serum (BS, Gibco) for 24 h. After incubation in the presence or absence of fasudil (10 μM) for 30 min, cells were stimulated with human VEGF (25 ng/ml) and mechanically scraped for collection. Equal amounts of total cell lysates or immunoprecipitated KDR were separated on sodium dodecyl sulfate-polyacrylamide gels and transferred to nitrocellulose membranes. The blots were blocked with skimmed milk and incubated overnight at 4°C with primary antibodies (1 : 1000). After being washed three times for 10 min each time with t-TBS (20 mM Tris, pH 7.5; 500 mM NaCl; and 0.1% Tween-20), the membranes were incubated with horseradish peroxidase-labeled antibodies (Bio-Rad, Richmond, CA, USA), 1 : 4000 for 30 min at room temperature. Visualization was performed with an enhanced chemiluminescence (Amersham, Arlington Heights, IL, USA) detection system according to the manufacturer's protocol.

Migration Assay

BREC migration was measured by using modified Boyden chambers with 8-μm-pore filters (Kurabo), as previously described.[26] Briefly, BRECs were suspended at a density of 3×10^5 cells/ml with 1% BS DMEM with or without

fasudil (10 μM), and 300 μl of the suspensions were seeded on the fibronectin-coated upper chambers. Then, the upper chambers were inserted into the lower wells of 24-well plates filled with 700 μl of 1% BS DMEM, if needed, containing human VEGF (25 ng/ml). After 4 h of culture, the BRECs located on the top surfaces of the transwells were scraped off with cotton swabs, fixed in 70% ethanol, and stained with Giemsa's solution for 1 h. After the meshes were punched out, BRECs on the bottom surfaces of the meshes were counted at predefined positions under a microscope.

[^{3}H]-Thymidine Uptake

BRECs were seeded into 24-well plates at a density of 1.0×10^4 cells/well. The media were replaced by DMEM with 3% FBS the next day. After 24 h, the cells were stimulated with human VEGF (25 ng/ml) for 18 h with or without fasudil (10 μM). [^{3}H]-thymidine was then added (0.25 μCi/well) for an additional 6 h, after which the cells were washed, fixed, and lysed. Incorporated [^{3}H]-thymidine was determined by scintillation counting, as previously described.[26]

Immunoprecipitation

Immunoprecipitation was performed as described below; 500 μl of whole cell lysate was added with 500 μl of 1% NP-40 lysis buffer (2% TritonX-100, 300 mM NaCl, 20 mM Tris pH 7.4, 1% Nonidet P-40, 2 mM EDTA, 2 mM EGTA, 0.4 mM sodium orthovanadate, 0.4 mM phenylmethylsulfonyl fluoride), and 5 μg of rabbit polyclonal anti-Flk-1/KDR antibody agarose conjugate, and then incubated at 4°C overnight with mixing. Pellets, collected by centrifugation at 4000 *g*, were washed three times with 0.5% NP-40 lysis buffer and resuspended in 60 μl of 4× electrophoresis sample buffer. After boiling for 5 min, samples were subjected to Western blotting.

Corneal Micropocket Assay in Mice

All animal experiments were approved by the Committee on the Ethics of Animal Experiments, Kyushu University Graduate School of Medical Sciences, Japan. Male BALB/c mice (6–10 weeks old) were purchased from Seac Yoshitomi (Fukuoka, Japan) and anesthetized with pentobarbital sodium (60 mg/kg intraperitoneally). Hydron pellets (0.3 μl) (IFN Sciences, New Brunswick, NJ, USA) containing 200 ng mouse VEGF and with or without fasudil (750 ng/pellet) were prepared and implanted into the corneas. After 6 days, images of the corneal vessels were recorded by using Viewfinder 3.0 (Pixera), with standardized illumination and contrast, and were saved onto disks. Quantitative analysis of neovascularization in the mouse corneas was performed with the NIH image software package.[27]

Statistical Analysis

The experimental data are expressed as means ± SD. Statistical significance was assumed when results showed $P < 0.05$, using the Student *t* test in a normally distributed population.

Results

Phosphorylation State of MLC in the Presence of VEGF

To examine the effect of VEGF on MLC phosphorylation in BRECs, we assessed the time course of MLC phosphorylation in VEGF-stimulated BRECs with Western blotting. As shown in Fig. 1A, VEGF transiently dephosphorylated MLC within 5 min (48.2 ± 19.7%; $^{**}P < 0.01$) and then sequentially increased the phosphorylated state of MLC (201 ± 6.63% at 60 min; $^{**}P < 0.01$).

Inhibitory Potential of Fasudil on MLC Phosphorylation in Endothelial Cells

The phosphorylation state of MLC is increased when endothelial cells start stretching to remodel the vascular network.[28] To examine whether fasudil could inhibit the activation of Rho-kinase in BRECs, we examined the phosphorylation state of MLC with Western blotting. We first examined dose-dependent inhibitory effects of fasudil on MLC phosphorylation under 10% serum conditions. The phosphorylation state of MLC was almost completely inhibited within 30 min by fasudil treatment at a concentration of 10 μM (2.91 ± 1.00% versus 0 μM, $^{*}P < 0.01$). Moreover, pretreatment with fasudil for 30 min strongly suppressed the phosphorylation state of MLC induced by VEGF (4.66 ± 2.53% versus VEGF alone; $^{**}P < 0.01$) (Fig. 1B, C).

Fasudil Attenuated VEGF-Dependent Migration of BRECs

We investigated the effect of fasudil on endothelial cell migration by using a modified Boyden chamber assay. VEGF induced BREC migration markedly, and the effect was significantly attenuated by fasudil. The number of cells in the VEGF-stimulated group was markedly increased compared with the vehicle-treated group (vehicle alone, 95.2 ± 18.1 cells/field; VEGF alone, 326 ± 56.0 cells/field). However, VEGF-dependent BREC migration was significantly inhibited in the presence of 10 μM fasudil (VEGF alone, 326 ± 56.0 cells/field; VEGF with fasudil, 79.0 ± 15.8 cells/field). (Fig. 2A, B) We also confirmed the inhibitory effect of fasudil on VEGF-induced BREC migration in a scratch wound assay, and the results were consistent with those of the Boyden chamber assay (data not shown).

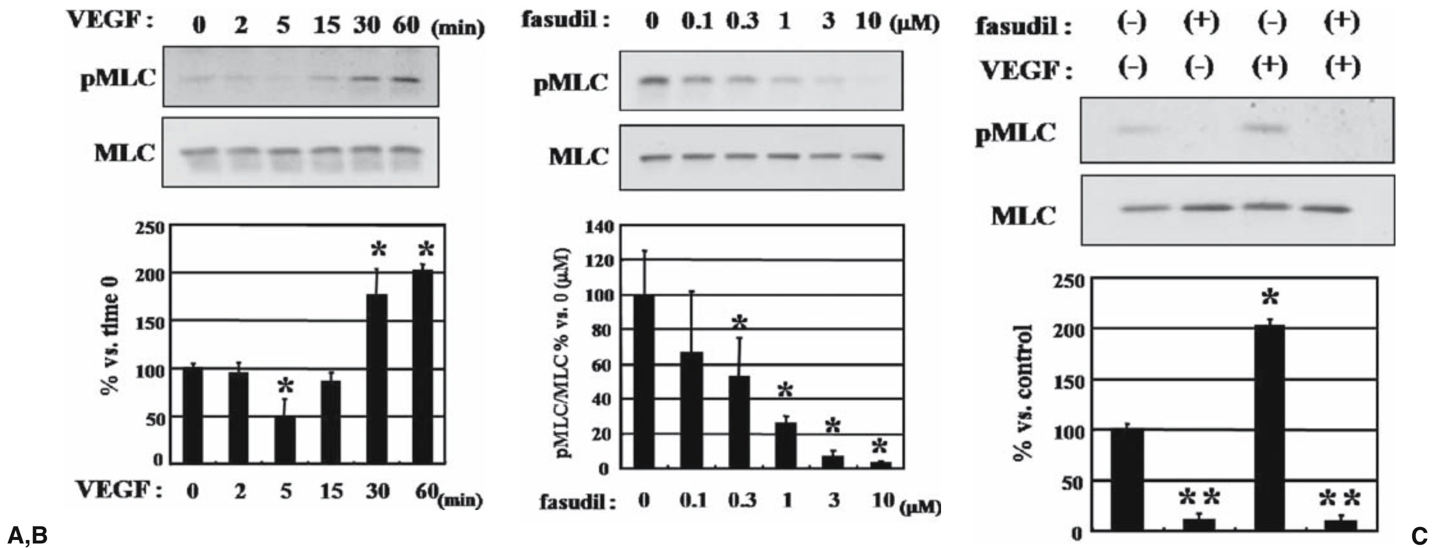

Figure 1A–C. Vascular endothelial growth factor (*VEGF*)-dependent phosphorylation of the myosin light chain (*MLC*) and the inhibitory effect of fasudil. **A** Bovine retinal endothelial cells (BRECs) were incubated in 3% bovine serum (BS)/Dulbecco's modified Eagle medium (DMEM) for 24h and then stimulated with human VEGF (25ng/ml) for the indicated time. Representative immunoblots for MLC phosphorylation (*pMLC*) and total MLC are shown. Values are means ± SD from three independent experiments. $*P < 0.01$ versus time 0. **B** BRECs incubated in 3% BS DMEM for 24h were further incubated with (0.1–10μM) or without fasudil for 30min. Typical immunoblots for pMLC and total MLC are shown. Values are means ± SD from three independent experiments. $*P < 0.01$ versus 0μM. **C** BRECs were incubated in 3% BS DMEM for 24h and then treated with vehicle or 10μM fasudil, and stimulated with human VEGF (25ng/ml) for 60min. Values are means ± SD from three independent experiments. $*P < 0.01$; $**P < 0.01$ versus each control.

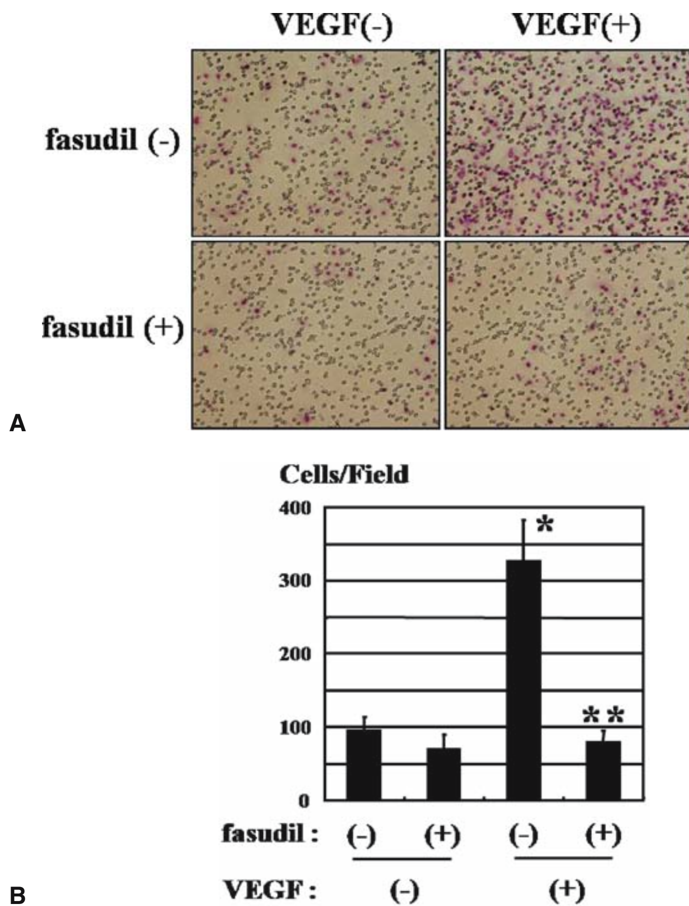

Figure 2A,B. VEGF-dependent migration of BRECs. **A** BREC migration was measured by using modified Boyden chambers with 8-μm-pore filters. After 4h of culture with or without human VEGF (25ng/ml), BRECs on the bottom surfaces of the mesh were photographed. **B** Transmigrated BRECs on the bottom surfaces of the mesh were counted at predefined positions under a microscope and quantified. Values are means ± SD from six cultures. $*P < 0.01$ versus vehicle alone, $**P < 0.01$ versus VEGF alone.

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BREC Thymidine Uptake Induced by VEGF Was Inhibited by Fasudil

It has been unclear whether Rho-kinase inhibition suppresses VEGF-induced endothelial cell proliferation. To examine whether treatment by fasudil affected de novo DNA synthesis in BRECs, we examined the effect of fasudil on thymidine uptake, an index of proliferation. We confirmed that thymidine uptake was promoted by VEGF (135.0 ± 14.3% versus control) and that fasudil inhibited thymidine uptake in a dose-dependent manner (data not shown). In BRECs treated with 10 μM fasudil, mean thymidine uptake induced by VEGF was significantly reduced (74.07 ± 6.34% versus VEGF alone) (Fig. 3).

Fasudil Had No Inhibitory Effect on VEGF Receptor-2 (KDR) Phosphorylation

VEGF signaling in endothelial cells is mainly mediated via KDR.[12] To investigate the selectivity of the inhibitory effect of fasudil on Rho-kinase activation, the phosphorylation state of KDR was assessed by Western blot analysis. We observed that KDR was phosphorylated by VEGF and that the phosphorylation state of KDR was not significantly affected by fasudil (Fig. 4A).

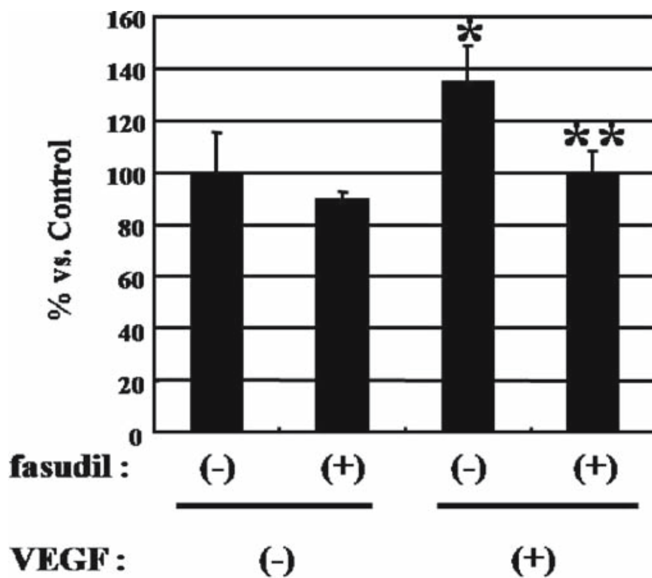

Figure 3. Inhibitory effect of fasudil on VEGF-induced thymidine uptake by BRECs. BRECs were seeded on a type 1 collagen-coated 24-well plate. The media were replaced by DMEM with 3% fetal bovine serum the next day. After 24 h, the cells were stimulated with human VEGF (25 ng/ml) for 18 h with or without fasudil (10 μM). [^{3}H]-thymidine was then added (0.25 μCi/well) for an additional 6 h, after which the cells were washed, fixed, and lysed. Incorporated [^{3}H]-thymidine was determined by scintillation counting. **P* < 0.01 versus vehicle alone; ***P* < 0.01 versus VEGF alone.

Fasudil Inhibited VEGF-Induced Phosphorylation of ERK1/2 and Akt

To investigate the inhibitory mechanisms of VEGF-induced angiogenesis by fasudil, we examined the effect of fasudil on the signal transduction by VEGF in BRECs. VEGF is known to activate ERK1/2 and Akt in endothelial cells during angiogenesis.[12,29] By Western blotting, we investigated which signaling pathway fasudil affected in VEGF-induced angiogenesis. VEGF induced the phosphorylation of ERK1/2, and the phosphorylation was partly inhibited by fasudil (Fig. 4B). We also examined whether fasudil inhibited VEGF-induced Akt phosphorylation. Fasudil significantly inhibited VEGF-induced phosphorylation of Akt in BRECs (Fig. 4C).

Fasudil Inhibited VEGF-elicited Angiogenesis in a Corneal Pocket Assay

The mouse cornea is a useful organ for estimation of angiogenesis because of its avascular character.[27] To examine whether fasudil could inhibit VEGF-induced angiogenesis in vivo, we implanted VEGF-embedded pellets into mouse corneas. VEGF-induced corneal neovascularization was almost completely inhibited by coembedding mouse VEGF and fasudil in the pocket (11.1% versus VEGF alone) (Fig. 5A, B). These results showed fasudil to be a potent inhibitor of VEGF-dependent angiogenesis in vivo.

Discussion

Accumulating evidence, obtained using the specific Rho-kinase inhibitor Y-27632, demonstrates that the Rho/Rho-kinase pathway plays a crucial role in cell migration.[13,14] However, few studies have examined the participation of the Rho-kinase pathway in ocular angiogenesis, and this is the first report indicating the inhibitory potency of fasudil on ocular angiogenesis in retinal microvascular endothelial cells.

Fasudil markedly attenuated VEGF-induced BREC migration in the Boyden chamber assay, and this inhibitory effect was in parallel with the phosphorylation state of MLC. Additionally, we confirmed the inhibitory effect of fasudil on BREC migration with a scratch wound assay, and the result was similar to that of the Boyden chamber assay (data not shown). Consistent with previous data concerning Y-27632 (10 μM), in the scratch wound motility assay, fasudil (10 μM) inhibited VEGF-dependent BREC migration almost completely, but not basal migration, without affecting cell viability (data not shown).

Endothelial cell proliferation is also an essential process for angiogenesis. In this study, fasudil significantly inhibited VEGF-induced BREC [^{3}H]-thymidine incorporation and ERK1/2 phosphorylation, whose activity indicates the proliferative activities of endothelial cells in angiogenic processes.[29]

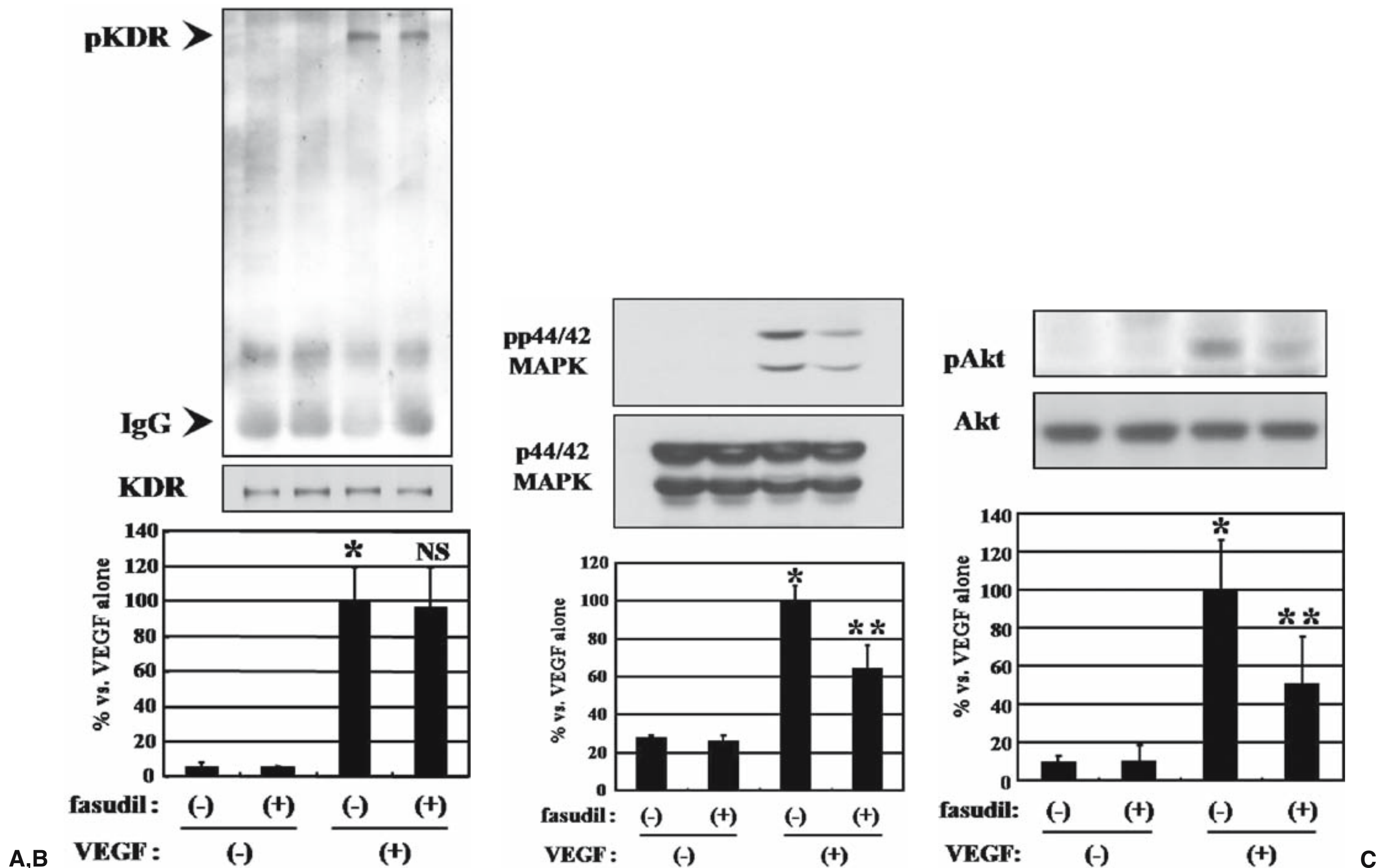

Figure 4A–C. Effect of fasudil on VEGF-dependent VEGF receptor 2 (*KDR*), extracellular signal-related kinase (ERK) 1/2 [*p44/42* mitogen-activated protein kinase (*MAPK*)] and Akt phosphorylation. **A** After pretreatment with fasudil (10 µM), BRECs stimulated with human VEGF (25 ng/ml) for 5 min were lysed, and immunoprecipitated KDR were loaded on a 6% sodium dodecyl sulfate polyacrylamide gel; an anti-phosphor-tyrosine antibody (PY-20) was used for detection of phosphorylated KDR (*pKDR*). Values are means ± SD from three independent experiments. *$P < 0.01$ versus control; *NS*, not significant versus VEGF alone. **B, C** After pretreatment with fasudil (10 µM) or vehicle for 30 min, BRECs stimulated with human VEGF (25 ng/ml) for 5 min were lysed and subjected to Western blotting. The phosphorylated state of phosphorylated ERK1/2 (*pp44/42 MAPK*) or Akt was detected by immunoblot analysis (*top*). Both membranes were reblotted with anti-ERK1/2 or Akt antibody (*bottom*). Values are means ± SD from three independent experiments. *$P < 0.01$ versus control; **$P < 0.01$ versus VEGF alone.

Mavria et al.[30] reported that ERK–MAPK promotes endothelial cell survival and sprouting by downregulation of Rho-kinase signaling. They demonstrated that the blockade of angiogenesis by inhibition of ERK1/2 signaling was overcome by the Rho-kinase inhibitor Y-27632. Their results suggest that the Rho-kinase inhibitor might allow endothelial cells to sprout, leading to neovascularization. Therefore, there might be alternative possibilities concerning the effect of fasudil on angiogenic processes. In fact, fasudil strongly attenuated VEGF-induced corneal neovascularization. Our study using BRECs demonstrated that lysophosphatidic acid (LPA), an agonist of the Rho-kinase pathway, affected neither ERK1/2 nor Akt phosphorylation (data not shown). On the other hand, phosphomolybdic acid (PMA), which caused ERK1/2 phosphorylation in BRECs, did not affect the phosphorylation state of MLC, which is one of the downstream mediators of the Rho-kinase pathway (data not shown). Nevertheless, fasudil significantly suppressed not only Rho-kinase but also ERK1/2 signaling pathways. The interplay between Rho-kinase and ERK1/2 signaling thus seems to be independent in BRECs. Moreover, we verified that PMA induced Akt phosphorylation in a time-dependent manner in BRECs (data not shown), a finding that is consistent with previous reports that VEGF induces protein kinase C-dependent Akt phosphorylation in human umbilical vein endothelial cells.[31] On the other hand, LPA had no apparent effect on the phosphorylation state of Akt (data not shown). Our study provides evidence that the antiangiogenic effect of fasudil in VEGF-induced angiogenesis may be mediated independently by the blockade of Rho-kinase, ERK1/2, and Akt signaling.

At a concentration of 10 µM, fasudil is known to be relatively selective for Rho-kinase and to have minimal effects on other signaling pathways, such as MLC kinase and protein kinase C.[32] Furthermore, Y-27632, another Rho-

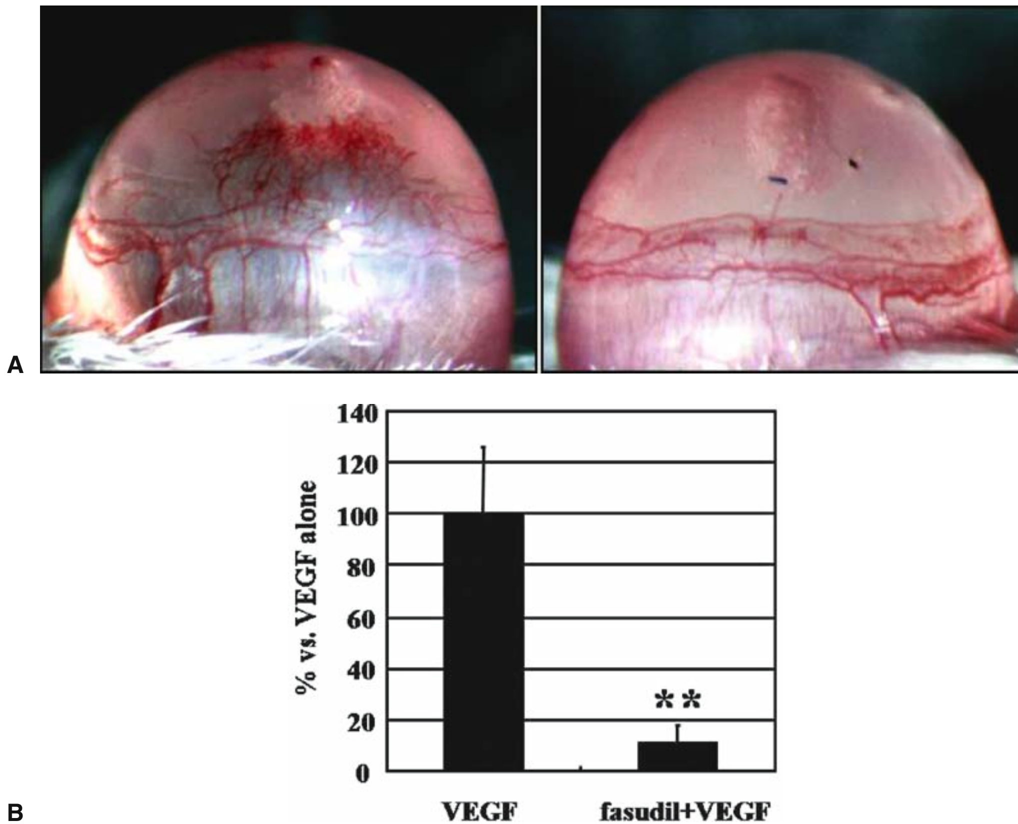

Figure 5A,B. In vivo antiangiogenic effect of fasudil. **A** 0.3 μl Hydron pellets containing 200 ng mouse VEGF with or without fasudil (750 ng/pellet) were prepared and implanted into the corneas of BALB/c mice. After 6 days, images of the corneal vessels were recorded with standardized illumination and contrast. Two representative *photographs* are shown. **B** Two quantitative analyses of corneal neovascularization on day 6. Coimplantation of fasudil inhibited VEGF-induced angiogenesis ($n = 12$ each). $^{**}P < 0.01$ versus VEGF alone-implanted mice.

kinase inhibitor, had similar effects on the inhibition of ERK1/2 signaling, but not on Akt phosphorylation (data not shown), suggesting that fasudil has a broader and more suppressive effect than Y27632 on the migration or survival of retinal endothelial cells, whereas Wolfrum et al.[33] observed that 10 μM fasudil slightly increased Akt phosphorylation in human microvascular endothelial cells. The differences in additional effects in each type of endothelial cells will require further examination.

Finally, we examined whether fasudil could suppress VEGF-induced in vivo neovascularization in a corneal pocket assay. Fasudil strongly attenuated VEGF-induced angiogenesis with no apparent adverse effects such as corneal edema or inflammation. However, the effective concentration of fasudil on angiogenesis in vivo was not estimated, since the concentration of fasudil in the tissue surrounding the pellet could not be measured precisely in this study. Further investigation in vivo is thus needed to determine the clinically efficient and safe use of fasudil.

Our previous reports also demonstrated that Rho-kinase inhibition by fasudil might have therapeutic potential for the prevention of proliferative vitreoretinal diseases.[34,35] However, pharmacological treatment sometimes leads to undesirable side effects in vivo. Further in vivo examination, therefore, is necessary to evaluate the therapeutic potential and safety of fasudil for clinical use in ocular diseases.

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