Fasudil-induced hypoxia-inducible factor-1α degradation disrupts a hypoxia-driven vascular endothelial growth factor autocrine mechanism in endothelial cells

Keiko Takata,¹ Ken-ichiro Morishige,² Toshifumi Takahashi,¹ Kae Hashimoto,² Seiji Tsutsumi,³ Limei Yin,³ Tsuyoshi Ohta,¹ Jun Kawagoe,¹ Kazuhiro Takahashi,¹ and Hirohisa Kurachi¹

¹Department of Obstetrics and Gynecology, Yamagata University, School of Medicine, Yamagata, Japan and ²Department of Obstetrics and Gynecology, Osaka University Graduate School of Medicine, Osaka, Japan

Abstract

Hypoxic response of endothelial cells (EC) is an important component of tumor angiogenesis. Especially, hypoxia-inducible factor-1 (HIF-1)–dependent EC-specific mechanism is an essential component of tumor angiogenesis. Recently, the Rho/Rho-associated kinase (ROCK) signaling has been shown to play a key role in HIF-1α induction in renal cell carcinoma and trophoblast. The present study was designed to investigate whether low oxygen conditions might modulate HIF-1α expression through the Rho/ROCK signaling in human umbilical vascular ECs (HUVEC). Pull-down assay showed that hypoxia stimulated RhoA activity. Under hypoxic conditions, HUVECs transfected with small interfering RNA of RhoA and ROCK2 exhibited decreased levels of HIF-1α protein compared with nontargeted small interfering RNA transfectants, whereas HIF-1α mRNA levels were not altered. One of ROCK inhibitors, fasudil, inhibited hypoxia-induced HIF-1α expression without altering HIF-1α mRNA expression. Furthermore, proteasome inhibitor prevented the effect of fasudil on HIF-1α expression, and polyubiquitination was enhanced by fasudil. These results suggested that hypoxia-induced HIF-1α expression is through preventing HIF-1α degradation by activating the Rho/ROCK signaling in ECs. Furthermore, fasudil induced both vascular endothelial growth factor (VEGF) and VEGF receptor-2 expression through the Rho/ROCK/HIF-1α signaling in HUVECs. Thus, augmented VEGF/VEGF receptor-2 autocrine mechanism stimulated HUVEC migration under hypoxic conditions. In summary, the Rho/ROCK/HIF-1α signaling is an essential mechanism for hypoxia-driven, VEGF-mediated autocrine loop in ECs. Therefore, fasudil might have the antimigratory effect against ECs in tumor angiogenesis. [Mol Cancer Ther 2008;7(6):1551–61]

Introduction

Growth and metastasis of solid tumors depend on the formation of new blood vessels which originate from the preexisting vascular system. These blood vessels grow into the tumor and thus provide the necessary nutrients and growth factors for tumor progression. At the same time, the newly formed blood vessels allow tumor cells to disseminate and form metastases in distant organs (1). Tumor-induced angiogenesis is mainly sustained by the production and secretion of angiogenic factors originating from tumor and stroma cells. The most prominent angiogenic factor is the vascular endothelial growth factor (VEGF), which is secreted by the tumor cells themselves and infiltrating immune cells, such as monocytes (2). VEGF binds to its receptors on the preexisting endotheium, stimulating endothelial cell (EC) proliferation and migration into the tumor, resulting in vascular sprouting. These sprouts ultimately form new blood vessels within the tumor. VEGF also plays a role in vasculogenesis by recruiting endothelial progenitor cells from the bone marrow (2).

Because VEGF plays such an important role in tumor progression and metastasis, it is an attractive target in the treatment of cancer. In combination with conventional chemotherapy, antiangiogenic strategies have a synergistic effect. Many antiangiogenic strategies have been undertaken to date, including anti-VEGF antibodies, such as bevacizumab (Avastin, Genentech, Inc.). Several studies have shown that combining anti-VEGF treatment with chemotherapy (3) or radiation therapy (4, 5) results in greater antitumor effects than either treatment alone. Recently, Willett and colleagues (6) have shown that VEGF blockade using bevacizumab decreases tumor perfusion, vascular volume, microvascular density, interstitial fluid pressure, and the number of viable, circulating endothelial and progenitor cells in colorectal cancer patients, providing direct evidence for antivascular effects after VEGF blockade. Several additional phase III studies are currently ongoing to fully assess the benefit of bevacizumab (7) and other anti-VEGF therapies, such as small molecule kinase inhibitors (8, 9) in patients with advanced cancer.
Within tumors, the availability of oxygen and nutrients is limited by competition among actively proliferating cells, and diffusion of metabolites is inhibited by high interstitial pressure (10). In response to intratumoral hypoxia, angiogenesis-stimulating factors produced by tumor cells induce the formation of a new blood supply from the preexisting vasculature, which is critical for tumor cells to survive and proliferate in a hostile microenvironment (11, 12). Hypoxia-inducible factor-1 (HIF-1) was identified and purified as a nuclear factor that was induced in hypoxic cells and bound to the cis-acting hypoxia response element located in the 3'-flanking region of the human EPO gene, which encodes erythropoietin (13). HIF-1 is a heterodimeric transcription factor composed of a HIF-1α subunit and a HIF-1β subunit (13). The level of HIF-1α is dependent on the cellular oxygen concentration (14), whereas the level of HIF-1β is not (15). HIF-1α is maintained at a low level in normoxic cells by degradation of the protein through the ubiquitin-proteasome pathway (16). In the presence of normal levels of oxygen, the binding of von Hippel-Lindau protein to the conserved oxygen degradation domain of HIF-1α results in iron-dependent hydroxylation, whereas this hydroxylation is inhibited under hypoxic conditions. This mechanism accounts for HIF-1α stabilization in hypoxic cells, allowing HIF-1α to translocate into the nucleus and to dimerize with HIF-1β (17, 18). The expression of over 40 genes, including VEGF, is activated at the transcriptional level by HIF-1 as determined by the most stringent criteria (19). In addition to controlling the production of angiogenic factors in hypoxic or ischemic tissue, HIF-1α controls angiogenesis at the transcriptional level by binding to the hypoxia response element located in the 3'-flanking region of the HIF-1α gene (17, 18).

One of the cellular events that follow low oxygen tension is ATP depletion, which results in the disruption of the actin cytoskeleton in diverse types of cells (21–23). Regulation of actin stress fibers is mediated mainly by the Rho GTPase family (24), which also participates in cell adhesion, migration, invasion, and gene transcription (25). A few studies have investigated the role of Rho proteins in the process of hypoxia-induced ATP depletion (26). Recent reports showed that the Rho protein is up-regulated in hypoxia and involved in HIF-1α induction in renal cell carcinoma (27) and trophoblast cells (28). On the other hand, Mizukami et al. reported an alternative mechanism for the hypoxic induction of VEGF in colon cancer that does not depend on HIF-1α but instead requires the activation of phosphatidylinositol 3-kinase/Rho/Rho-associated kinase (ROCK) and c-Myc (29). Then, we characterized Rho GTPase expression and activity under hypoxic conditions and investigated its potential roles in hypoxic responses in ECs.

In this report, we showed, for the first time, that RhoA is significantly activated and affects HIF-1α protein expression under hypoxic conditions in ECs. In addition, incubation of cells with fasudil, which is a specific inhibitor of ROCK (30) prevented HIF-1α protein expression under hypoxic conditions. Our findings showed that the Rho/ROCK pathway is necessary for HIF-1α accumulation in ECs and permit a better understanding of tumor angiogenesis during hypoxia.

Materials and Methods

Reagents
Fasudil, Y-27632, DMSO, and Hoechst 33258 were purchased from Sigma. VEGF receptor-2 (VEGFR-2) inhibitor SU1498 and proteasome inhibitor MG132 were purchased from Calbiochem. Chemotaxicell was obtained from Kurabo Industries Ltd. Rhotekin-RBD protein glutathione S-transferase beads were from Cytoskeleton. HIF-1α monoclonal antibody for Western blotting, HIF-1α polyclonal antibody for immunoprecipitation and immunohistochemistry, anti-RhoA monoclonal antibody, ROCK1 monoclonal antibody, ROCK2 polyclonal antibody, normal mouse IgG, and horseradish peroxidase–conjugated anti-mouse and anti-rabbit IgG were all purchased from Santa Cruz Biotechnology. VEGFR-2 polyclonal antibody, myosin light chain 2 (MLC-2) antibody, phosphorylated MLC-2 (Ser19) antibody, and anti-ubiquitin mouse monoclonal antibody were from Cell Signaling Technology. Polyvinylidene difluoride membranes (Hybond-P) and enhanced chemiluminescence Western blotting detection reagents were obtained from Amersham. Anti-human VEGF antibody was purchased from R&D Systems. Protein G-sepharose 4 fast flow was obtained from GE Healthcare Bio-Science AB. HIF-2α polyclonal antibody was purchased from Novus Biologicals. FITC-conjugated goat anti-rabbit antibody was from Jackson ImmunoResearch Laboratories, Inc.

Cells and Culture Conditions
Human umbilical vascular ECs (HUVEC) were isolated by trypsin digestion of umbilical veins from undamaged sections of fresh cords. The umbilical vein was cannulated, washed with PBS, and perfused with trypsin for 20 min at room temperature. After perfusion, the detached cells were collected, then the vein was washed with PBS, and the wash-off was pooled with the perfusate. After washing, cells were resuspended in HuMedia-EG2 medium (Kurabo Industries) and then plated on plastic culture dishes. HuMedia-EG2 medium consists of the base medium (HuMedia-EB2) supplemented with 2% fetal bovine serum, 10 ng/mL hEGF, 5 ng/mL hFGF-B, 1 μg/mL hydrocortisone, 50 μg/mL gentamicin, 50 ng/mL amphotericin B, and 10 μg/mL heparin. Subcultures were obtained by trypsinization and were used for experiments at passages 3 to 5. Cultures were maintained at 37°C in a humidified atmosphere containing 5% CO2. Before experiments, cells were cultured under serum-free conditions in M199 medium for 18 to 24 h. A hypoxic condition (1% O2) was attained by incubation of the cells in a controlled oxygen incubator. The gas was maintained at the above oxygen concentration by a compact gas oxygen controller Te-Her system O2−CO2 incubator (Hirasawa Works) with a residual gas mixture composed of 94% to 95% N2−5% CO2.
Analysis of Migration

The cell migration assay was done in a modified Boyden chamber using previously described techniques (31). Porous filters (8-μm pores) were coated on the underside by a passive adsorption of type I collagen (BD Biosciences). Starved cells were preincubated under normoxia for 24 h or under hypoxia with or without 10 μmol/L fasudil for 24 h, then washed twice with PBS, harvested, resuspended in serum-free M199 medium, and plated into the upper chamber (5 × 10^4/well). Lower chamber contained only serum-free M199 medium. Some cells preincubated under normoxia or hypoxia for 24 h were treated with 5 μg/mL of normal mouse IgG, anti-VEGF antibody, DMSO, or 10 μmol/L SU1498 for 30 min under hypoxia, and then cells were plated into the upper chamber (5 × 10^4/well). Upper and lower chambers contained the above reagents. Cells were allowed to migrate for 4 h under normoxia or hypoxic conditions. Nonmigrating cells were removed from the upper chamber with cotton swab, and migrated cells adherent to underside of the filter were fixed, stained with Hoechst 33258. Filter was mounted onto microscope slides, and stained cells were counted using the Image J Imaging System Software version 1.3 (NIH) in three fields per filter.

Analysis of Tube Formation

The surface of 96-well plates was coated with 30 μL of growth factor-reduced Matrigel matrix (BD Biosciences), which was allowed to polymerize at 37°C for 1 h. Starved cells were preincubated under normoxia or hypoxia with or without 10 μmol/L fasudil for 24 h, and then cells were washed twice with PBS and harvested with serum-free M199 medium and then plated into the Matrigel-coated wells (2 × 10^4/well) in triplicate. Moreover, some cells preincubated under normoxia or hypoxia for 24 h were treated with 5 μg/mL of normal mouse IgG, anti-VEGF antibody, DMSO, or 10 μmol/L SU1498 for 30 min under hypoxia, and the cells were seeded to the Matrigel-coated wells (2 × 10^4/well) in triplicate. After incubation for 5 h, the wells were photographed and four random fields per condition were recorded at 50× magnification. The total tube number of every field was counted.

Western Blot Analysis

Cells were washed twice with PBS and lysed in ice-cold lysis buffer containing 20 mmol/L Tris- HCl (pH 7.5), 150 mmol/L NaCl, 1 mmol/L Na₂EDTA, 1 mmol/L EGTA, 1% Triton, 2.5 mmol/L sodium P Pi, 1 mmol/L β-glycerophosphate, 1 mmol/L Na₃VO₄, 1 μg/mL leupeptin, and 1 mmol/L phenylmethylsulfonyl fluoride. Equal amounts of samples were resolved by SDS-PAGE and transferred to Hybond-P. The transferred samples were incubated with the antibody as indicated in the text and then incubated with the corresponding secondary horseradish peroxidase–conjugated IgG, and the immunoblotted proteins were visualized with enhanced chemiluminescence reagents.

Rho Pull-Down Assay

The Rho pull-down assay was done as described previously (32). Briefly, starved cells were incubated under normoxia for 30 min or under hypoxia with or without 10 μmol/L fasudil for 30 min. After treatment, cells were washed twice with PBS and lysed in ice-cold lysis buffer. Cell lysates were clarified by centrifugation, and equal volumes of lysates were incubated with Rho-tekin RBDagarose beads (200 μg) at 4°C overnight. The beads were washed four or five times with lysis buffer. Bound Rho proteins were detected by Western blotting using a monoclonal antibody against RhoA. Western blotting of the total amount of RhoA in cell lysates was done for the comparison of Rho activity (level of GTP-bound Rho) in the same samples.

VEGF ELISA

Starved cells were incubated under normoxia for 24 h or under hypoxia with or without 10 μmol/L fasudil for 24 h. After treatment, the supernatant was collected and 27× concentrated by centrifugation for 25 min at 4,000 × g at 4°C using Amicon ultra-15 centrifugal filter devices (Millipore). The levels of VEGF protein was measured by ELISA kit (R&D Systems) specific for the 165–amino acid form of human VEGF according to the manufacturer's protocol.

RNA Isolation and Reverse Transcription– PCR

Total RNA was isolated from cells by using the ISOGEN (Nippon Gene Co., Ltd.) according to the manufacturer's directions. Total RNA (0.5 μg) was reverse-transcribed using a first-strand cDNA synthesis kit, following the manufacturer's instructions (SuperScript First-Strand Synthesis System for reverse transcription–PCR, Invitrogen Corp.). An aliquot (1 μg) of reverse transcription product was used for PCR amplification in total volume of 50 μL. The PCR primer set used for HIF-1α, VEGF, VEGFR-2, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA amplification were as follows: HIF-1α sense 5'-GTC GGA CAG CCT CACCAA ACA GAC GAG C-3', antisense 5'-GTT AAC TTG ATC CAA AGC TCT GAG-3'; VEGF sense 5'-CCA TGA ACT TTC TGC TGT CTT-3', antisense 5'-ATC GCA TCA GGG GCA CAC AG-3'; VEGFR-2 sense 5'-GTT AAT AAC TGT AC-3', antisense 5'-GTT ATC AGC TCC ACC TGT ACC CTC-3'; GAPDH sense 5'-ACC ACA GTCT CAT GCC ATC AC-3', antisense 5'-TCC ACC CTG TTT AGA TG-3'. The thermal cycle profile used for HIF-1α, VEGF-2, and GAPDH was 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. The profile for VEGF was 29 cycle of denaturation at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 30 s. PCR fragments were analyzed by electrophoresis on 1.0% to 1.5% agarose gels and stained with ethidium bromide.

Real-Time Reverse Transcription– PCR

Quantitative PCR was done using the AB 7300 Real-Time PCR System (Applied Biosystems) under the following conditions: 10 min at 95°C, 40 cycles of 30 s at 95°C, 1 min at 60°C, and 1 min at 72°C. All TaqMan Probes VEGF (HS00175392), VEGFR-2 (HS00176676), HIF-1α (HS00153153), and GAPDH (HS99999905) were ordered from Gene Expression Assays (Applied Biosystems). Gene expression was quantitated using the comparative C<sub>T</sub> method of relative quantification using 7300 System SDS
software (Applied Biosystems). GAPDH transcript was tested as an endogenous reference to calculate the relative expression levels of target genes according to manufacturer’s instructions.

**Small Interfering RNA Analysis**

HUVECs were transfected with indicated concentrations of RhoA small interfering RNA (siRNA; siGENOME SMARTpool L-003860-00-0010; Dharmacon, Inc.), ROCK1 siRNA (siGENOME SMARTpool M-003536-01-0005; Dharmacon, Inc.), ROCK2 siRNA (siGENOME SMARTpool L-004610-00-0020; Dharmacon, Inc.), or nontargeted siRNA (siCONTROL Nontargeted siRNA 1 D-001210-01-20; Dharmacon, Inc.) using DharmaFECT. After 24 h, the transfection medium was removed and replaced with serum-free medium. Inhibition of RhoA, ROCK1, and ROCK2 protein expression were verified by Western blot analysis.

**HIF-1α Immunofluorescence Study**

Cells were fixed with methanol at -20°C for 10 min. The nonspecific binding sites were blocked with 3% bovine serum albumin in PBS for 1 h. The cells were incubated with a mouse monoclonal anti–HIF-1α antibody diluted 1:20 in 3% bovine serum albumin in PBS overnight at 4°C. After washing, cells were incubated with FITC-conjugated goat anti-rabbit antibody (1:100) for 1 h at room temperature.

**Figure 1.** The functional role of Rho/ROCK and VEGF autocrine signaling in EC migration and tube formation under hypoxic conditions. Migration of HUVEC was analyzed in Boyden chambers (A and C) and tube formation was analyzed by tube formation assay (B and D). A, starved cells were preincubated under normoxia for 24 h or under hypoxia with or without 10 μmol/L fasudil for 24 h, then plated into the upper chamber (5×10^4/well), and allowed to migrate for 4 h under normoxia or hypoxia. Upper and lower chamber contained only serum-free M199 medium. Representative photos of migrating cells were taken after Hoechst staining: treated with normoxia (a), hypoxia (b), and hypoxia and fasudil (c). Migrated cells were counted by Image J software in three random fields per filter. B, after preincubation under normoxia or hypoxia with or without 10 μmol/L fasudil for 24 h, cells were harvested with serum-free M199 medium and then plated into the Matrigel-coated wells in triplicate. After 5-h incubation, the wells were photographed and four random fields per condition were recorded at 50× magnification; treated with normoxia (a), hypoxia (b), and hypoxia and fasudil (c). The total tube number of every field was counted. C, the cells preincubated under normoxia or hypoxia for 24 h were treated with 5 μg/mL of normal mouse IgG, anti-VEGF antibody, DMSO, or 10 μmol/L SU1498 for 30 min under hypoxia and plated into the upper chamber (5×10^4/well). Upper and lower chamber contained the above reagents. Cells were allowed to migrate for 4 h under normoxic or hypoxic conditions. Migrated cells were counted by Image J software in three random fields per filter. D, after preincubation under normoxia or hypoxia for 24 h, cells treated with 5 μg/mL of normal mouse IgG, anti-VEGF antibody, DMSO, or 10 μmol/L SU1498 for 30 min under hypoxia. Cells were then seeded onto the Matrigel-coated wells in triplicate (2×10^4/well). After 5-h incubation, the wells were photographed and four random fields per condition were recorded at 50× magnification and total tube number of every field was counted. Data were expressed as the ratio of cell number (A and C) and tube number (B and D) per field of treated cells to that of the normoxic group. Columns, mean; bars, SE (n = 4). Experiments were repeated thrice with consistent results. Significant differences were indicated by asterisks (*, P < 0.05; **, P < 0.01; ***, P < 0.001).
After a further washing, cells were stained with propidium iodide (1 μg/mL) for 15 min at room temperature. Subsequently, the slides were mounted and analyzed by fluorescence microscopy (Olympus).

Immunoprecipitation

Whole-cell lysates were prepared with lysis buffer. After centrifugation (15,000 × g for 30 min at 4°C), supernatants were transferred to fresh tubes and total protein amounts of each sample were equally adjusted. Supernatants were rotated with 60 μL of protein G-sepharose beads at 4°C for 2 h for the process of preclean. After centrifugation (5,000 × g for 3 min at 4°C), supernatants were transferred to fresh tubes, then mixed with 2 μg of anti–HIF-1α antibody, and incubated at 4°C overnight. Thereafter, 40 μL of protein G-sepharose beads were added, and the mixture was incubated at 4°C overnight. Beads were washed four times with the lysis buffer and resolved by SDS-PAGE and transferred to Hybond-P. Then polyubiquitination was assessed by immunoblot analysis with anti-ubiquitin antibody.

Statistical Analysis

Differences between means were assessed by one-way analysis of variation (ANOVA) using SPSS software (SPSS, Inc.). Data are expressed as the mean ± SE. Significant differences are defined as P < 0.05.

Results

The Effect of Rho/ROCK and VEGF/VEGFR-2 Signaling Pathways on Hypoxia-Induced HUVEC Migration and Tube Formation

Migration of ECs toward solid tumors that are secreting VEGF or other angiogenic factors is a critical aspect of tumor angiogenesis (2). To determine the functional role of Rho/ROCK and VEGF autocrine signaling in EC migration and angiogenesis under hypoxic conditions, we used random motility assay through a Boyden chamber and tube formation assay. To evaluate the role of the Rho/ROCK and VEGF/VEGFR-2 signaling in hypoxia-induced EC migration, HUVEC were treated with ROCK inhibitor fasudil, anti-VEGF neutralizing antibody, and VEGFR-2 specific inhibitor SU1498. The cell migration was significantly increased up to 2.5-fold under hypoxic conditions compared with normoxia and fasudil inhibited hypoxia-induced cell migration (Fig. 1A). Similarly, the tube formation in vitro was significantly increased under hypoxic conditions compared with normoxia, and fasudil inhibited the hypoxia-induced increase in the tube formation (Fig. 1B). Besides, the hypoxia-induced cell migration and tube formation were suppressed by both anti-VEGF neutralizing antibody (5 μg/mL) and SU1498 (10 μmol/L; Fig. 1C and D). These data suggest that both ROCK and
VEGF/VEGFR-2 signaling are important for the hypoxia-induced EC migration and tube formation.

**Fasudil Inhibited the Hypoxia-Induced VEGF/VEGFR-2 Autocrine Loop Augmentation**

VEGF acts on ECs as chemotactic and mitogenic factors, via EC-specific receptors: VEGFR-1 (Flt-1), VEGFR-2 (Flk-1/KDR), and VEGFR-3 (Flt-4; ref. 33). And it has been reported that VEGF secretion was increased by HIF-1α under hypoxic conditions (33). To determine whether fasudil inhibits the hypoxia-induced expression of VEGF autocrine loop, we examined VEGF and VEGFR expression in HUVECs. Hypoxia significantly increased the expression of VEGF mRNA and the hypoxia-induced VEGF mRNA expression was inhibited by 10 μmol/L fasudil (Fig. 2A). The secretion of VEGF was significantly increased while HUVECs were cultured under hypoxic conditions for 24 h, whereas it returned to the normoxic level in fasudil-treated cells under hypoxic conditions (Fig. 2B). These results suggest that the hypoxia-induced increase of VEGF secretion is mediated through the Rho/ROCK pathway.

VEGFR-2 is a major mediator of mitogenic, angiogenic, and permeability-enhancing effects of VEGF (34, 35). The expression of VEGFR-2 is up-regulated by hypoxia, and this phenomenon is mediated by posttranscriptional regulation (36). To determine whether hypoxia affects VEGFR-2 mRNA and protein expression in our experimental system and to investigate the effect of fasudil on VEGFR-2 expression, we analyzed by reverse transcription–PCR and Western blot analysis. Our data showed that VEGFR-2 expression, we analyzed by reverse transcription–PCR and Western blot analysis. Our data showed that VEGFR-2 expression, was inhibited by 10 μmol/L fasudil (Fig. 2C). In contrast, HUVECs cultured under hypoxic conditions (Fig. 2D). These results suggest that the Rho/ROCK pathway might be involved in modulating the degradation of HIF-1α protein in hypoxic ECs. Taken together, the Rho/ROCK signaling is important for VEGF/VEGFR-2 autocrine loop augmentation in ECs under hypoxic conditions.

**Fasudil Inhibited Hypoxia-Induced HIF-1α Expression**

Hypoxia-induced VEGF secretion was regulated by HIF-1α (19). Furthermore hypoxia-induced VEGF/VEGFR-2 protein expression was regulated by VEGF induced by HIF-1α (20). So we next investigated the effects of fasudil on HIF-1α mRNA and protein expression by reverse transcription–PCR and Western blot analysis. Our data showed that hypoxia and fasudil had no effect on the HIF-1α mRNA expression (Fig. 3A). In contrast, the amount of HIF-1α protein increased in HUVECs under hypoxic conditions compared with that under normoxia. Furthermore, fasudil inhibited hypoxia-induced expression of HIF-1α protein (Fig. 3B). Inhibition of hypoxia-induced expression of HIF-1α protein was also observed by a specific ROCK inhibitor Y-27632 (Fig. 3C). These results suggest that the Rho/ROCK pathway might be involved in inhibiting the degradation of HIF-1α protein in hypoxic ECs. Therefore, the Rho/ROCK pathway might activate VEGF, VEGFR-2 autocrine loop through inhibiting HIF-1α degradation.

**Rho Signaling Was Activated Under Hypoxic Conditions in ECs**

RhoA has been reported to be activated under hypoxic conditions in renal cancer cells and trophoblast (27, 28). To investigate whether RhoA is activated under hypoxic conditions in ECs, we measured the intracellular level of the GTP-bound Rho, which is the active form, using the

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**Figure 3.** The effect of fasudil and Y-27632 on the expression of HIF-1α mRNA and protein. Expression of HIF-1α mRNA in HUVEC was examined by real-time PCR. Total RNA was isolated from cells cultured under normoxia (lane 1), or under hypoxia with or without 10 μmol/L fasudil (lanes 2 and 3) for 3 h. A, the mRNA levels of HIF-1α were quantified by real-time PCR, with gene expression normalized to GAPDH. The PCR product of the normoxic group was set at 1. B, expression of HIF-1α protein in HUVEC was examined by immunoblot analysis. Starved cells were pretreated with or without 10 μmol/L fasudil under normoxia (lanes 1 and 2) or hypoxia (lanes 3 and 4) for 4 h. C, expression of HIF-1α protein in HUVEC was examined by immunoblot analysis. Starved cells were pretreated with or without 10 μmol/L Y-27632 under normoxia (lanes 1 and 2) or hypoxia (lanes 3 and 4) for 4 h. Whole cells were lysed, and Western blot analysis was done using anti-mouse HIF-1α antibody (top). Blots were stripped and reprobed for α-tubulin as a loading control (bottom). Western blot results were analyzed by a scanner and Scion Image software and shown in the bar graph. The densitometry of the control band (normoxia without fasudil) was set at 1. Columns, mean from three separate experiments; bars, SE. Significant differences were indicated by asterisks: ***, P < 0.01.
pull-down assay. Although the level of total Rho showed no difference, the level of the active form of Rho was remarkably elevated under hypoxic conditions (Fig. 4A).

**Fasudil Inhibited Hypoxia-Induced MLC Phosphorylation**

It is well known that Rho/ROCK signaling promotes acto-myosin contractility by phosphorylation and inactivation of MLC phosphatase and also by direct phosphorylation of MLC (37). To confirm that hypoxia activates the Rho/ROCK signaling pathway and the activation can be blocked by fasudil, we investigated the phosphorylation status at Ser19 of MLC (one of ROCK substrates). In the presence of fasudil, hypoxia-induced MLC phosphorylation was significantly inhibited (Fig. 4B). These results showed that hypoxia-induced activation of the Rho/ROCK pathway is blocked by fasudil.
The Rho/ROCK Signaling Was Necessary for HIF-1α Protein Expression in ECs

To directly assess the role of the Rho/ROCK signaling pathway in hypoxia-induced HIF-1α expression, we tested whether HIF-1α protein expression was inhibited by introducing siRNA for RhoA, ROCK1, and ROCK2 into HUVECs (Fig. 4C). HIF-1α protein expression was investigated in the siRNA transfected (Fig. 4D). In comparison with the nontargeted siRNA transfected HUVECs, the HIF-1α expression was not changed in the ROCK1 siRNA transfected HUVECs. On the other hand, in the RhoA and ROCK2 siRNA transfected HUVECs, the HIF-1α expressions were significantly decreased (Fig. 4D). Thus, it was suggested that Rho/ROCK signaling pathway was essential for hypoxia-induced HIF-1α expression.

Fasudil Inhibited the Nuclear Translocation of HIF-1α Protein

Under hypoxic conditions, HIF-1α protein accumulates and translocates to the nucleus, where it forms an active complex with HIF-1β and activates transcription of target genes. We next studied the intracellular localization of HIF-1α and determined whether fasudil affects the nuclear translocation of hypoxia-induced HIF-1α protein. As shown by immunofluorescence analysis, HIF-1α immunoreactivity predominantly localized in the cytoplasm under normoxic conditions. However, the exposure of the ECs to MG132 or hypoxia resulted in a very striking nuclear accumulation of HIF-1α with hardly any detectable immunoreactivity remaining in the cytoplasm. Fasudil treatment under hypoxic conditions reduced nuclear translocation of HIF-1α protein, similar to the treatment with Y-27632 (Fig. 5). By these results, it is suggested that Rho/ROCK signaling pathway is involved in hypoxia-induced HIF-1α nuclear translocation.

The Effect of MG132 on Inhibitory Action of Fasudil to Hypoxia-Induced HIF-1α Protein Expression

Under normoxic conditions, HIF-1 subunits are unstable, being rapidly targeted to the ubiquitin-proteasome.

**Figure 5.** Fasudil prevented the nuclear accumulation of HIF-1α under hypoxic condition. Intracellular localization of HIF-1α was examined by immunofluorescence. After pretreatment with 10 μmol/L MG132, 10 μmol/L fasudil, or 10 μmol/L Y-27632 for 30 min, HUVECs were incubated under normoxia or hypoxia for 4 h. The ECs were prepared for indirect immunofluorescence analysis as described in Materials and Methods. Representative photos of HUVECs were taken at 400× magnification. Left, HIF-1α staining using FITC-conjugated secondary antibody; middle, propidium iodide (PI) staining of the nuclei; right, merged HIF-1α and propidium iodide staining.
pathway. Degradation is mediated by a ubiquitin-protein isopeptide ligase (E3) complex, in which the von Hippel-Lindau protein binds to a specific hydroxylated proline residue (Pro-564) within the oxygen-dependent degradation domain of HIF-1α (17–19). A major action of hypoxia is to suppress prolyl hydroxylation and degradation of HIF-1α by the proteasome. As shown in Fig. 3, fasudil might block the inhibition of HIF-1α degradation, which was caused by hypoxia. To investigate the mechanism of this effect, proteasome inhibitor MG132 was used in analyzing HIF-1α protein expression (Fig. 6A). A large accumulation of HIF-1α protein was observed even under normoxia in the presence of MG132, similar to hypoxic condition, and the accumulation was prevented in the presence of fasudil under hypoxic conditions. Whereas, in the presence of fasudil together with MG132 under hypoxic conditions, HIF-1α protein accumulated again. These data indicated that the Rho/ROCK pathway might be involved in the prevention of HIF-1α degradation under hypoxic conditions.

**Fasudil Stimulated the Polyubiquitination of HIF-1α Under Hypoxic Conditions**

Because HIF-1 protein is degraded mainly through the ubiquitin-proteasome pathway and ubiquitinated HIF-1α is immediately degraded by proteasome, we examined the status of polyubiquitination of HIF-1α using proteasome inhibitor MG132. The polyubiquitinated HIF-1α was accumulated under the prevention of proteasome degradation by MG132 (Fig. 6B, lane 2). Hypoxia inhibited the accumulation of HIF-1α polyubiquitination in the presence of MG132 (compare lane 4 with lane 2). These profiles are consistent with previous reports (38). Interestingly, fasudil reversed the suppressed polyubiquitination of HIF-1α even under the hypoxic conditions (compare lane 6 with lane 4). These data indicated that a ROCK inhibitor fasudil promotes the HIF-1α polyubiquitination under hypoxic conditions.

**Discussion**

The results of the present study showed that hypoxic conditions induce activation of Rho and the Rho/ROCK pathway is involved in hypoxia-induced HIF-1α protein expression in ECs. We did not investigate the mechanisms of hypoxia-induced Rho activation in this study. In renal cell carcinoma, Turcotte et al. showed that hypoxia-induced Rho activation might be mediated by the disruption of actin organization, which was induced by ATP depletion under hypoxia (27). We need to examine whether this mechanism is also available in ECs.

The Rho/ROCK pathway might play a major role in hypoxia-induced HIF-1α expression because they are prevented by knockdown of RhoA and ROCK2, or treatment with fasudil. These observations are in agreement with recent studies reporting the relation between Rho and HIF-1α induction in other cell types (27, 28). These two reports using renal cell carcinoma (27) and trophoblast cell (28) described that both HIF-1α mRNA and protein expression increased under hypoxic conditions and inhibited by C3 exotoxin (Rho inhibitor) and dominant-negative Rho. Then, they concluded that the Rho/ROCK pathway was involved in HIF-1α mRNA induction under hypoxic conditions. In contrast, in this study using ECs, HIF-1α mRNA expression was not altered under hypoxic conditions, although HIF-1α protein expression was increased. Besides hypoxia-induced HIF-1α protein expression was inhibited using fasudil or knockdown of RhoA and ROCK2. Moreover, we showed that fasudil inhibited hypoxia-induced HIF-1α protein accumulation via increased ubiquitination, followed by proteasome-mediated degradation. These data suggest that the Rho/ROCK pathway is involved in inhibition of HIF-1α protein degradation under hypoxic conditions, rather
than stimulates HIF-1α mRNA transcription. The difference between previous reports and our study could be explained by the variation of cell types. Further study should be investigated to determine the target molecule, which the Rho/ROCK pathway regulates in the modulation of HIF-1α ubiquitination.

RhoA and ROCK2 knockdown suppressed the HIF-1α induction by hypoxia suggesting that the Rho/ROCK signaling is an upstream regulator of HIF-1α accumulation in ECs. However, in colon cancer cells, Mizukami et al. clarified a role for the activation of c-Myc by Rho/ROCK signaling as an alternative HIF-1α-independent mechanism for the induction of VEGF in hypoxia (29). HIF-1α-independent mechanism also might be available in hypoxia-induced VEGF autocrine loop in ECs.

It has been known that VEGFR-2 is not a target gene of HIF-1. Besides, it has been reported that VEGFR-2 mRNA expression is not altered under hypoxia, although VEGFR-2 protein expression increases under hypoxia (36, 39, 40). In the present study, VEGFR-2 protein expression increased under hypoxia and fasudil inhibited the increase without any changes in mRNA level. It suggests that the Rho/ROCK pathway might be involved in hypoxia-induced increase in VEGFR-2 protein level at a posttranscriptional level. Thus far, the posttranscriptional mechanism to increase the protein expression through Rho/ROCK signal modification has not been clarified. Recent study revealed that ROCK inhibition increases the expression of F-box protein Skp2, a substrate-binding subunit of SCF ubiquitin ligase complex (41), whose substrates are p27, a cyclin-dependent kinase inhibitor, and c-Myc. We need a further study to identify the target molecule that participates in ROCK-dependent HIF1α ubiquitination.

Fasudil has been reported to show neuroprotective properties and has a role in cardiovascular protection (37, 42–44) and an approved drug in Japan since 1995 for the clinical treatment of cerebral vasospasm (37, 42). On the other hand, our study showed that fasudil inhibited hypoxia-induced HIF-1α expression in ECs and disrupted VEGF/VEGFR-2 autocrine loop, followed by the prevention of hypoxia-induced migration. Furthermore, we have shown that fasudil inhibited VEGF-induced EC migration by suppressing the post–VEGFR-2 signaling in vivo and in vitro (45). Therefore, fasudil might have a potential to be useful as an antiangiogenic drug, because ECs in malignant tumor are under hypoxic and VEGF-rich conditions (2).

In summary, this study may provide clues for future directions in clarifying the molecular mechanisms of endothelial HIF-1α regulation during tumor angiogenesis. Additional studies will be required to understand the consequences of Rho activation and HIF-1α expression in ECs and their roles in the tumor angiogenesis.

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Keiko Takata, Ken-ichirou Morishige, Toshifumi Takahashi, et al.


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