Molecular Mechanism of Cardiac Adaptation to Hypoxic Stresses: A Role of Vascular Endothelial Growth Factor (VEGF) in Increased Adhesion of Cardiac Myocytes to the Extracellular Matrix

低酸素ストレスに対する心筋細胞適応の分子機序
― 心筋細胞の細胞外基質との接着増強における VEGFの役割 ―

高　橋　直　之

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Contents

Abstract	*page*	1
Introduction		2
Materials and Methods		6
Results		12
Discussion		28
Acknowledgments		34
References		35

Figures

1.	Stress and mitogen activate MAPK signaling pathways.	*page*	3
2.	Hypoxia induces increase in VEGF content in the culture medium.		12
3.	KDR/Flk-1 and Flt-1 are expressed in cardiac myocytes.		13
4.	KDR/Flk-1 is tyrosine phosphorylated in response to VEGF.		14
5.	VEGF mediates hypoxia-induced tyrosine phosphorylation of $p125^{FAK}$ and paxillin.		16
6.	VEGF stimulates protein tyrosine kinase activity of $p125^{FAK}$.		17
7.	Effects of tyrosine kinase inhibitors, genistein and tyrphostin, on VEGF-induced tyrosine phosphorylation of $p125^{FAK}$.		18
8.	VEGF induces association of $p125^{FAK}$ with $p60^{c\text{-}src}$, GRB2, or Shc.		19
9.	VEGF activates SAPK/JNKs in cardiac myocytes.		20
10.	VEGF activates p38MAPK in cardiac myocytes.		20
11.	VEGF phosphorylates ATF-2 (Thr71) in cardiac myocytes.		21
12.	VEGF alters the subcellular localization of $p125^{FAK}$ in cardiac myocytes and fibroblasts.		22
13.	Effect of VEGF on subcellular distribution of $p125^{FAK}$.		24
14.	Effects of VEGF on adhesion of cardiac myocytes to the ECM.		25
15.	Effects of p160ROCK-specific inhibitor Y-27632 on the phosphorylation of $p125^{FAK}$, SAPK/JNKs, and p38MAPK.		26
16.	Effects of p160ROCK-specific inhibitor Y-27632 on adhesion of cardiac myocytes to the ECM.		27
17.	Scheme of VEGF-induced signaling cascades.		31

Abstract

Vascular endothelial growth factor (VEGF) has been proposed to be one of the most potential candidate factors in ischemia-induced collateral vessel formation. Evidence has accumulated that VEGF is expressed in the heart tissue, and its expression is markedly increased in response to hypoxia. Recently we found that VEGF activated mitogen-activated protein kinase (MAPK) cascade in cultured rat cardiac myocytes. To elucidate how VEGF affects adhesive interaction of cardiac myocytes with the extracellular matrix (ECM), one of important cell functions, I investigated molecular mechanism of activation of focal adhesion related proteins in cultured rat cardiac myocytes. I also analyzed the effects of VEGF on stress-inducible MAPKs, stress-activated protein kinase (SAPK) (also called Jun N-terminal kinase [JNK]), and p38MAPK cascades. Furthermore, I examined the role of Rho/p160ROCK in these VEGF-induced signaling cascades, using a p160ROCK-selective inhibitor Y-27632.

I found that cardiac myocytes not only rapidly released VEGF in response to hypoxia but also expressed the 2 VEGF receptors, KDR/Flk-1 and Flt-1, and that KDR/Flk-1 was significantly tyrosine phosphorylated on VEGF stimulation. VEGF mediated hypoxia-induced activation of $p125^{FAK}$ and induced subcellular translocation of $p125^{FAK}$ from perinuclear sites to focal adhesions. Activation of $p125^{FAK}$ was accompanied by its association with adapter proteins GRB2, Shc, and non-receptor type tyrosine kinase $p60^{src}$. VEGF also activated SAPK/JNK and p38MAPK cascades. VEGF-induced activation of SAPK/JNK, p38MAPK and $p125^{FAK}$ was almost completely inhibited by p160ROCK-specific inhibitor Y-27632. Furthermore, I confirmed that VEGF significantly strengthened adhesion of cardiac myocytes to ECM, which was abolished by Y-27632, using an electric cell-substrate impedance sensor. These results indicate that Rho/p160ROCK plays a critical role in the VEGF-induced cardiac responses, especially in adhesion of cardiac myocytes to ECM, which may facilitate various self-protective actions. These data strongly suggest a pivotal role of VEGF in cardiac adaptation to hypoxic stress.

Introduction

Vascular endothelial growth factor (VEGF), also known as vascular permeability factor, is an important regulator of endothelial cell proliferation, migration, and permeability, and is secreted from tumor cells and cells exposed to hypoxia (1-4). VEGF has been proposed to be one of the most potential candidate factors in ischemia-induced collateral vessel formation as well as tumor neovascularization (5). VEGF has been reported to be induced by hypoxia in several cell types *in vitro* (4), including cardiac myocytes (6, 7). Levy *et al* demonstrated that cardiac myocytes synthesize and secrete VEGF in response to hypoxia *in vitro* (6). We previously reported that serum level of VEGF was markedly elevated in patients with acute myocardial infarction and it rapidly returned to basal level after reperfusion therapy (8). This indicates that most of the tissue cells, including cardiac myocytes, are exposed to high levels of VEGF in conditions such as acute myocardial infarction, as well as that VEGF is one of the most sensitive indicators of hypoxia. Recently, we found that VEGF activated mitogen-activated protein kinases (MAPKs), S6 kinase ($p90^{rsk}$), and Raf-1 in cultured rat cardiac myocytes (9). This indicates that cardiac myocytes themselves are also target cells for VEGF.

Adhesive interactions between cells and the extracellular matrix (ECM) are known to be mediated by the integrin family (10). The cell-ECM interaction plays a fundamental role in regulating cellular behaviors such as migration, proliferation, and differentiation, especially for cardiac myocytes to perform continuously repetitive contractions and to adapt to external stresses including hypoxia (10). Recent studies suggest that integrins transduce extracellular signals across the plasma membrane (10, 11). The identification of focal adhesion kinase ($p125^{FAK}$) provided the first evidence for the activation of an intracellular signaling molecule by integrins (12, 13). $p125^{FAK}$ is a non-receptor protein tyrosine kinase that is widely expressed in different cell types and phosphorylated on tyrosine residues accompanied with formation of focal adhesions. $p125^{FAK}$ has also been proved to be involved in signal transduction from cell surface receptors for neuropeptides and growth factors (14-18). Recently, Abedi and Zachary (19) have reported that VEGF induces tyrosine phosphorylation of $p125^{FAK}$ and paxillin, another focal adhesion protein, in endothelial cells, as well as increases immunofluorescent staining of them in focal adhesions, which suggests that adhesion between endothelial cells and ECM may be modulated by VEGF stimulation. However, it is not known whether VEGF modulates cell-substrate adhesion of cardiac myocytes.

The MAP kinases ERK1, ERK2, p38MAPK, and stress-activated protein kinase (SAPK) (also called Jun N-terminal kinase [JNK]) appear as central elements of 3 homologous pathways used by mammalian cells to transduce the messages generated by stress agents or

growth factors (20) (see Fig. 1). SAPK/JNKs and p38 MAPKs are recognized as stress-sensitive kinases (21-23). These kinases are supposed to play critical roles in genetic responses of many components of the cardiovascular system to disease processes, including ischemia, reperfusion injury, atherosclerosis, and heart failure (24). The activation cascades for SAPK/JNK and p38MAPK are less understood than those for ERK are. It is supposed that the sequential stimulation of the Ras-like monomeric GTP-binding protein, Rac or Cds42,

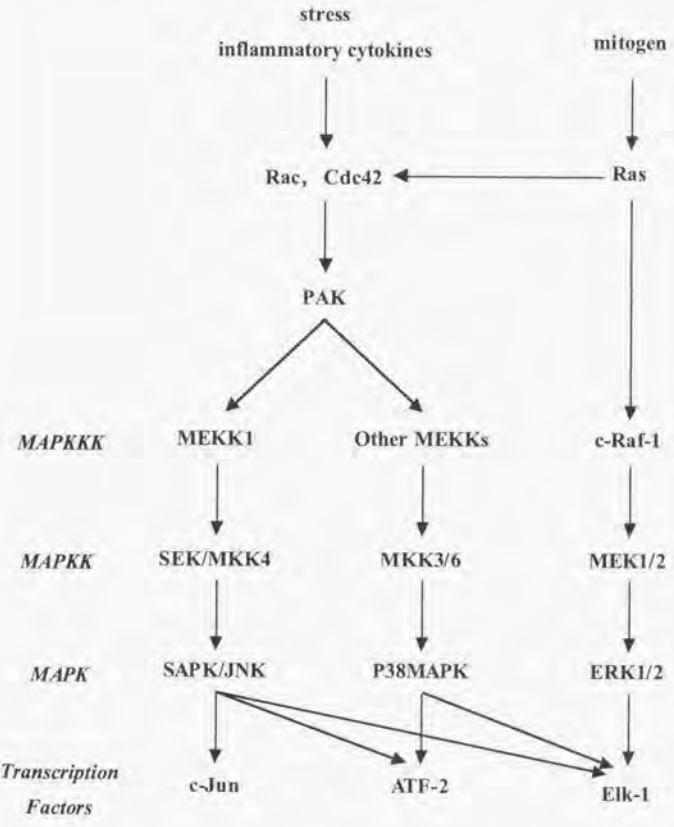

Fig. 1. Stress and mitogen activate MAPK signaling pathways.

perhaps by Ras itself, leads to the activation of MEKK1, a MAPKKK that then activates the MAPKK, MEK4, also called SEK, culminating in the activation of SAPK/JNKs (25-27). Activated SAPK/JNKs phosphorylate c-Jun on specific N-terminal serine residues (Ser63 and Ser73), major phosphorylation sites required for its transcriptional activity (28). Activation of SAPK/JNKs also leads to activation of other transcription factors, Elk-1 (29, 30) and ATF-2 (23, 31, 32). Recently, MKK3 (33) and MKK6 (34) were cloned, either of which specifically activates p38MAPK. Further upstream in the p38MAPK cascades are supposed to be Rac and Cdc42 as are in the SAPK/JNK cascades (35, 36). Activated p38MAPK phosphorylates and activates transcription factors, including CHOP, CREB, ATF-1 and ATF-2, and thereby may regulate the expression of a number of genes influencing growth and differentiation (37-39). Regarding that ischemia/reperfusion injury induces SAPK/JNK and p38MAPK in heart (40) and that the cardiac myocytes are exposed to high level of VEGF in such a condition (8), it is important to know how VEGF modulates SAPK/JNK and p38MAPK cascades in cardiac myocytes.

The Rho-related small GTP-binding proteins play a key role in regulating the assembly and organization of the actin cytoskeleton in response to extracellular growth factors (41, 42). Cardiac myocytes has been reported to express RhoA (43), which is an essential component of signaling pathways linking extracellular factors to the formation of stress fibers and focal adhesions (41). Several lines of evidence suggested that Rho stimulated tyrosine phosphorylation of $p125^{FAK}$ and paxillin (44, 45). Several direct targets of RhoA have been identified recently, including protein kinase N (46, 47), rhophilin (48), rhotekin (49), p140mDia (48), and a family of Rho-associated kinases, p160ROCK (ROCK-I) (50) and ROKα/Rho-kinase/ROCK-II (51, 52), although their functions remain almost unknown. Among them, Rho-associated kinase isozymes, p160ROCK and ROKα/Rho-kinase/ROCK-II, are known to mediate RhoA-induced formation of focal adhesions and actin stress fibers (53-55). In cardiac myocytes, it has been reported that Rho and Rho kinase are required for Gαq and α1-adrenergic receptor-mediated hypertrophic gene expression (56, 57), and supposed that Rho kinase mediates Rho-induced hypertrophic response (58). SAPK/JNKs and p38MAPK are also required for the Gαq and α1-adrenergic receptor mediated hypertrophic gene expression in cardiac myocytes (56, 59). These facts raises the possibility that the activity of p38MAPK or SAPK/JNK might be controlled by Rho or Rho-associated kinases in cardiac myocytes.

In this study, I examined whether hypoxia caused cardiac myocytes to release VEGF rapidly. To know the roles of VEGF in hypoxia-induced signaling cascades, I investigated the effects of VEGF on adhesion between cardiac myocytes and ECM, especially focusing on the

activation of adhesion related proteins, including $p125^{FAK}$, comparing with those of hypoxia. To confirm that VEGF really modulates cell adhesion, I evaluated the state of cell adhesion to ECM using an electrical impedance sensor. I also investigated the effects of VEGF on stress-sensitive kinases, p38MAPK and SAPK/JNK cascades. To know whether Rho/p160ROCK are involved in the VEGF-induced signaling cascades, I analyzed the effects of p160ROCK-selective inhibitor Y-27632 (60) on the VEGF-induced activation of $p125^{FAK}$, SAPK/JNK, and p38MAPK. Here I show that cardiac myocytes rapidly release VEGF in response to hypoxia and express the 2 VEGF receptors, KDR/Flk-1 and Flt-1, with KDR/Flk-1 significantly tyrosine phosphorylated upon VEGF stimulation. VEGF mediated hypoxia-induced activation of $p125^{FAK}$ and induced subcellular translocation of $p125^{FAK}$ from perinuclear sites to focal adhesions. Activation of $p125^{FAK}$ was accompanied by its association with adapter proteins GRB2, Shc, and non-receptor type tyrosine kinase $p60^{c-src}$. VEGF also activated SAPK/JNK and p38MAPK cascades. Furthermore, VEGF definitely accelerated the adhesion of cardiac myocytes to ECM. p160ROCK-inhibitor Y-27632 significantly reduced basal phosphorylation levels of $p125^{FAK}$, p38MAPK, and SAPK/JNK, and almost completely abolished their VEGF-induced activation. Furthermore, Y-27632 markedly impaired cell-substrate adhesion of cardiac myocytes. These findings suggest p160ROCK plays a pivotal role in VEGF-induced signaling pathway in cardiac myocytes.

Materials and Methods

Cell culture Primary culture of ventricular cardiac myocytes was prepared from neonatal rats as previously described (61). Briefly, heart ventricles were aseptically removed from neonatal Wistar rats, minced in calcium-free phosphate-buffered saline (PBS), and digested with 0.025% trypsin-EDTA in PBS. The isolated cells were washed in Dulbecco's modified Eagle's medium (DMEM; Gibco Laboratories, Grand Island, NY) containing 10% fetal bovine serum and preplated onto plastic dishes for 1 hr to selectively remove fibroblasts. Nonadherent cells (enriched in cardiac myocytes) were collected and replated onto gelatin-coated culture dishes. They were cultured for 2 days in DMEM supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 34 μmol/L streptomycin until they were confluent. They were starved for 24 hr before stimulation with VEGF or hypoxia.

The percentage of myocytes was estimated to be around 90%, as judged by immunoperoxidase staining with a mouse anti-cardiac myosin heavy chain monoclonal antibody (mAb) derived from the CMA-19 clone, followed by counterstaining with hematoxylin. Preparation of mouse anti-cardiac myosin heavy chain mAb (CMA19) was previously described (62). Contamination by endothelial cells was estimated to be less than 1%, as judged by immunoperoxidase staining with mouse anti-factor VIII related antigen mAb (Z002; Zymed Laboratories, San Francisco, Inc. CA, USA) (data not shown). Therefore, I concluded that the rest of the contaminating non-muscle cells (about 10% of total cells) mostly consisted of fibroblasts.

Hypoxia Medium was made hypoxic by an agent which absorbs O_2 down to less than 0.1% and provides 21% CO_2 (AnaeroPack; Mitsubishi Gas Chemical Co., Inc., Tokyo, Japan) in an anaerobic jar (AnaeroPack Series; Mitsubishi Gas Chemical Co., Inc.). Cultured cardiac myocytes were subjected to hypoxia by replacing their medium with the hypoxic medium. To keep hypoxic condition, all the procedures were performed in a glove bag filled with 95% N_2 and 5% CO_2.

Measurement of VEGF released from cardiac myocytes The concentrations of VEGF in conditioned medium of cardiac myocytes subjected to normoxia or hypoxia, were measured using a quantitative sandwich enzyme immunoassay kit (*Quantikine™ M* mouse VEGF Immunoassay; R & D Systems, Inc., Minneapolis, USA), which also recognized rat VEGF (according to the manufacturer).

Analyses of phosphotyrosine content of KDR/Flk-1, p125^{FAK}, and paxillin Cardiac myocytes were treated with hypoxia or 22.8 pmol/L (1.0 ng/ml) recombinant human VEGF (rhVEGF; Upstate Biotechnology Incorporated, NY, USA) for various periods as

indicated. To investigate the effects of neutralization of VEGF, the cells were preincubated with anti-human VEGF neutralizing mAb (MAB293; R & D Systems, USA) at 10 μg/ml for 1 hr before stimulation. They were frozen in liquid nitrogen and lysed on ice with NP-40 buffer (25 mmol/L Tris/HCl pH 7.6, 25 mmol/L NaCl, 1 mmol/L Na_3VO_4, 10 mmol/L sodium pyrophosphate, 10 nmol/L okadaic acid, 0.5 mmol/L EGTA, 1 mmol/L phenylmethylsulflonyl fluoride [PMSF], and 1% Nonidet P-40). The cell lysates were centrifuged, and the supernatants containing detergent soluble proteins were collected. Proteins were immunoprecipitated at 4 °C over night with mouse anti-phosphotyrosine mAb (PY20; Transduction Laboratories, KY, USA) and protein G-Sepharose (Pharmacia LKB). The immunoprecipitates were subjected to SDS-polyacrylamide gel electrophoresis (PAGE) and then transferred onto polyvinylidene difluoride (PVDF) transfer membranes (NEN Research Products, MA, USA). PVDF membranes were blocked with 1 % bovine serum albumin in PBS and then incubated overnight at 4 °C with rabbit polyclonal anti-KDR/Flk-1 antibody (C20; Santa Cruz Biotechnology, Inc., CA, USA), rabbit polyclonal anti-$p125^{FAK}$ antibody (a gift from Dr. Hisataka Sabe, Department of Molecular Biology, Osaka BioScience Institute, Osaka, Japan), or mouse anti-paxillin mAb (349; Transduction Laboratories, KY, USA). After incubation with alkaliphosphatase-conjugated anti-rabbit or anti-mouse immunoglobulin G (IgG) antibody (both antibodies from Vector Laboratories, Inc., Burlingame, CA, USA), the blots were developed with a chemiluminescence detection kit (New England Biolabs, Inc., Beverly, MA, USA). Tyrosine phosphorylation of $p125^{FAK}$ was confirmed by immunoprecipitation using anti-$p125^{FAK}$ polyclonal antibody followed by Western blotting with anti-phosphotyrosine mAb (4G10; Upstate Biotechnology Incorporated, NY, USA) or anti-$p125^{FAK}$ polyclonal antibody. The increase in tyrosine phosphorylation of KDR/Flk-1 was quantified by scanning densitometry. For analysis of expression of VEGF receptors, total cell lysates in Laemmli's sample buffer were also electrophoresed and immunoblotted using rabbit polyclonal anti-KDR/Flk-1 antibody or rabbit polyclonal anti-Flt-1 antibody (C-17; Santa Cruz Biotechnology, Inc.).

Kinase assay of $p125^{FAK}$ Cardiac myocytes were treated with rhVEGF (22.8 pmol/L) for various periods as indicated, and then they were frozen in liquid nitrogen. Immune complex tyrosine kinase assays of $p125^{FAK}$ were performed with a non-RI solid phase enzyme-linked immunosorbent assay (ELISA) kit using the exogenous substrate, poly(Glu-Tyr) (12, 63, 64) (Universal Tyrosine Kinase Assay Kit, Takara Shuzo, Co. Ltd., Kyoto, Japan) according to the manufacturer's instructions. Briefly, the cell lysates were centrifuged and the supernatants were immunoprecipitated with rabbit polyclonal anti-$p125^{FAK}$ antibody. The immunoprecipitates were incubated with ATP in the microtiter plate onto which poly(Glu-Tyr)

had been precoated. The amount of phosphorylated poly(Glu-Tyr) was measured by ELISA using a horseradish peroxidase-linked anti-phosphotyrosine (PY20) antibody.

Analyses of the effects of tyrosine kinase inhibitors on tyrosine phosphorylation of p125^{FAK} Cells were preincubated for 30 min with or without either of the tyrosine kinase inhibitors, genistein (Gibco BRL , Inc. Grand Island, NY) at 37 μmol/L, or tyrphostin (Gibco BRL) at 50 μmol/L, and then they were stimulated with 22.8 pmol/L rhVEGF. The cell lysates were immunoprecipitated with mouse anti-phosphotyrosine mAb (PY20) and subjected to Western analysis using rabbit polyclonal anti-p125^{FAK} antibody. The increase in tyrosine phosphorylation at the 125 kDa band was quantified by scanning densitometry.

Analyses of association of p125^{FAK} with p60^{src}, GRB2 or Shc After stimulation with 22.8 pmol/L rhVEGF for various periods as indicated, the cell lysates were immunoprecipitated with mouse anti-v-Src mAb (LA074; Quality Biotech, Camden, NJ, USA), rabbit polyclonal anti-GRB2 antibody (Santa Cruz Biotechnology, Inc., CA, USA), or mouse anti-Shc mAb (PG-797; Santa Cruz Biotechnology, Inc.) along with protein G-Sepharose and subjected to Western analysis using rabbit polyclonal anti-p125^{FAK} antibody.

Kinase assay of p38MAPK and SAPK/JNK Cardiac myocytes were treated with rhVEGF (22.8 pmol/L) for various periods as indicated, then they were frozen in liquid nitrogen and lysed on ice. p38 MAP kinase activity was assayed with an assay kit using ATF-2 fusion protein beads as substrate (p38 MAP Kinase Assay Kit, New England Biolabs, Inc.), according to the manufacturer's instructions. Briefly, the cell lysates were centrifuged and the supernatants were immunoprecipitated with anti-p38MAPK antibody. The immunoprecipitates were incubated with ATF-2 fusion protein in the presence of ATP. Phosphorylation of ATF-2 at Thr71, a major phosphorylation site required for its transcriptional activity, was measured by Western blotting using an anti-phospho-specific-ATF-2 (Thr71) antibody. SAPK/JNK kinase activity was assayed with an assay kit using N-terminal c-Jun (1-89) fusion protein beads which contained a high affinity binding site for SAPK/JNK as its substrate (SAPK/JNK Assay Kit, New England Biolabs, Inc.), according to the manufacturer's instructions. Briefly, SAPK/JNK was pulled down using the c-Jun fusion protein beads. Kinase reaction was performed using the same protein as its substrate in the presence of ATP. Phosphorylation of c-Jun fusion protein at Ser63, a major phosphorylation site required for its transcriptional activity, was measured by Western blotting with an anti-phospho-specific-c-Jun (Ser63) antibody.

Analysis of phosphorylation of p38MAPK and SAPK/JNK Cardiac myocytes were exposed to rhVEGF (22.8 pmol/L) for various periods as indicated. Then they were

frozen in liquid nitrogen and lysed on ice with NP-40 buffer. The cell lysates were centrifuged and the supernatants were suspended in Laemmli's sample buffer. Aliquots of the samples were subjected to Western analysis using a rabbit polyclonal anti-phospho-specific p38MAPK (Tyr182) antibody (New England Biolabs, Inc.) or a rabbit polyclonal anti-phospho-specific SAPK (Thr183/Tyr185) antibody (New England Biolabs, Inc.). To confirm that almost equal amounts of SAPK/JNK protein or p38MAPK protein were electrophoresed in each reaction, the membranes were reprobed with phosphorylation-state independent rabbit polyclonal anti-SAPK/JNK antibody (New England Biolabs, Inc.) or anti-p38MAPK antibody (New England Biolabs, Inc.), respectively.

Analysis of phosphorylation of ATF-2 Cardiac myocytes were exposed to rhVEGF (22.8 pmol/L) for various periods as indicated. Then they were frozen in liquid nitrogen and lysed on ice with NP-40 buffer, and the cell lysates were centrifuged. The pellets, which mainly consisted of nuclei, were suspended in Laemmli's sample buffer. Aliquots of the samples were subjected to Western analyses using a rabbit polyclonal anti-phospho-specific ATF-2 (Thr71: a major phosphorylation site required for its transcriptional activity) antibody or a rabbit polyclonal phosphorylation-state independent anti-ATF-2 antibody (New England Biolabs, Inc.), respectively.

Subcellular fractionation After stimulation with 22.8 pmol/L rhVEGF for various periods as indicated, cytosolic and membrane fractions were prepared from cardiac myocytes according to modified methods previously described (65, 66). Briefly, cells were washed twice with PBS and then harvested in a buffer containing 20 mmol/L Hepes and 250 mmol/L sucrose (pH 7.4) followed by centrifuging at 1000×g for 3 min. The supernatant was discarded, and the pellet was suspended in hypotonic Tris Buffer containing 10 mmol/L Tris (pH 7.5), 1 mmol/L $MgCl_2$, 100 μmol/L leupeptin, 1 mmol/L PMSF, and 1 mmol/L Na_3VO_4. The pellet was homogenized by 80 strokes of Dounce homogenizer. The homogenate was centrifuged at 1000×g for 15 min to remove nuclei and debris. The supernatant was centrifuged at 48,000×g for 30 min, resulting in a pellet, which was resuspended in hypotonic Tris buffer containing 1% Nonidet P-40 and stored as a membrane fraction, and the supernatant, which was recentrifuged at 246,000×g for 90 min to remove the microsome-rich fraction. The resulting supernatant was stored as a cytosolic fraction. All of the above steps were carried out at 4 °C unless otherwise indicated. Protein estimation was carried out using the BCA protein assay reagent (Pierce, IL, USA). The fractions were suspended in Laemmli's sample buffer and incubated at 37 °C for 30 min, and then their aliquots were subjected to SDS-PAGE followed by immunoblotting with polyclonal anti-$p125^{FAK}$ antibody or rabbit polyclonal anti-rat Na, K-ATPase α1 fusion protein (Upstate Biotechnology, Inc.).

Immunocytochemistry To investigate whether subcellular distribution of $p125^{FAK}$ in cardiac myocytes and fibroblasts is altered in response to VEGF, I performed double staining for $p125^{FAK}$ and cardiac myosin heavy chain. rhVEGF-treated (60 min) or untreated cardiac myocytes were fixed in acetone for 5 min at room temperature. The cells were incubated first with rabbit polyclonal anti-$p125^{FAK}$ antibody for 1 hr at 37 °C, then incubated sequentially with biotinylated anti-rabbit IgG (Vector Laboratories, Inc.) for 1 hr at 37 °C and fluorescein isothiocyanate (FITC)-conjugated avidin D (Vector Laboratories, Inc.) for 30 min at 37 °C. The cells were then incubated sequentially with mouse anti-cardiac myosin heavy chain mAb (CMA-19) for 1 hr at 37 °C and tetramethylrhodamine isothiocyanate (TRITC)-conjugated anti-mouse IgG1 antibody (Chemicon International, Inc.) for 30 min at 37 °C. To investigate the relationship between the immunofluorescent staining of $p125^{FAK}$ and focal adhesions in rhVEGF-treated (60 min) cells, I performed double staining for $p125^{FAK}$ and vinculin. The cells were first stained with rabbit polyclonal anti-$p125^{FAK}$ antibody, and then were sequentially with biotinylated anti-rabbit IgG and FITC-conjugated avidin D as described above. They were then incubated with mouse anti-vinculin mAb (V-4505; Sigma, USA) for 1 hr at 37 °C and TRITC-conjugated anti-mouse IgG1 antibody for 30 min at 37 °C. The sections were examined and photographed under a fluorescence microscope (Microphoto-FX, Nikon, Tokyo, Japan).

Analyses of the effects of p160ROCK-specific inhibitor Y-27632 on phosphorylation of $p125^{FAK}$, p38MAPK and SAPK/JNK Cells were preincubated for 60 min with or without p160ROCK-specific inhibitor Y-27632 at 10 μmol/L, and then they were stimulated with 22.8 pmol/L rhVEGF. Y-27632 was supplied by Yoshitomi Pharmaceutical Industries (Saitama, Japan). VEGF-induced phosphorylation of $p125^{FAK}$, p38MAPK or SAPK/JNKs was analyzed as above.

Cell-substrate adhesion assay Cell-substrate adhesion was measured using the electrical cell-substrate impedance sensing system (Applied BioPhysics, Inc., NY, USA) previously reported by Keese and Giaever (67-69). In this system, the cells were cultured on a small gold electrode (area ~ 10^{-4} cm^2) deposited on the bottom of tissue culture vessels. A small alternating current signal (1μA) at a frequency of 4000 Hz was passed between the small electrode and a larger counter electrode (area ~ 10^{-1} cm^2) placed at a distant. The voltage between the small and large electrodes was monitored. As cell membranes have very high impedance, the attachment of cells to the small electrode blocks the current, forcing it to flow under the cells, causing an increase in the impedance. Primary cultures of ventricular cardiac myocytes were prepared as above. Cardiac myocytes were seeded on gelatin-precoated electrodes and cultured for 2 days in DMEM supplemented with 10% fetal bovine serum, 50

U/ml penicillin, and 34 µmol/L streptomycin. They were starved for 24 hr, and then stimulated with rhVEGF (34.2 pmol/L). To examine a role of p160ROCK in cell-substrate adhesion, cells were preincubated for 60 min with 10 µmol/L Y-27632 prior to addition of rhVEGF. The resistance, which reflects the extent of cell-substrate adhesion, was monitored as described previously (70).

Statistics Statistical comparisons of control group with treated groups were carried out using the unpaired *t*-test with *P* values corrected by the Bonferroni method. Values of $P < 0.05$ were considered significant.

Results

Hypoxia causes cardiac myocytes to release VEGF rapidly. To examine whether hypoxia stimulates cardiac myocytes to release VEGF rapidly, concentrations of VEGF in conditioned medium of cardiac myocytes subjected to normoxia or hypoxia for 5 min were measured. The concentration of VEGF was significantly increased in hypoxic condition as compared with in normoxic condition, which indicates that cardiac myocytes rapidly secrete VEGF molecules into culture medium in response to hypoxia (Fig. 2).

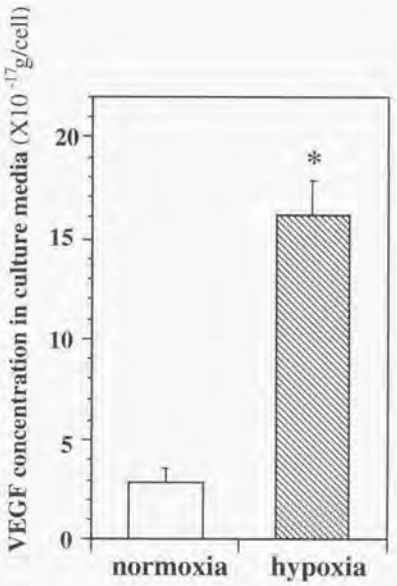

Fig. 2. Hypoxia induces increase in VEGF content in the culture medium.

Conditioned media from cells under normoxia or hypoxia for 5 min were collected. Concentration of VEGF was measured by quantitative sandwich enzyme immunoassay. * $P<0.001$ vs. normoxia (unpaired t-test). Results shown are mean ± SD.

Cardiac myocytes express KDR/Flk-1 and Flt-1. It has been shown that VEGF binds to 2 structurally related tyrosine kinase receptors, Flt-1 (71, 72) and KDR/Flk-1 (73, 74). I examined whether cardiac myocytes expressed these receptors by Western analysis. As shown in Fig. 3, KDR/Flk-1 and Flt-1 were expressed in the cultured cardiac myocytes. To examine tyrosine phosphorylation of these VEGF receptors, the cell lysates from rhVEGF-treated and untreated myocytes were immunoprecipitated with anti-phosphotyrosine mAb (PY20) followed by Western blotting using anti-KDR/Flk-1 or anti-Flt-1 antibodies. KDR/Flk-1 was significantly tyrosine phosphorylated on rhVEGF stimulation, with phosphorylation reaching a maximum level at 5 to 10 min with rhVEGF (Fig. 4A), whereas Flt-1 was not (data not shown). The increase in tyrosine phosphorylation of KDR/Flk-1 was quantified by scanning densitometry of the autoradiogram and expressed as ratio to the initial phosphorylation before addition of rhVEGF. As shown in Fig. 4B, tyrosine phosphorylation of KDR/Flk-1 was significantly increased at 5 to 10 min after rhVEGF treatment as compared with the initial value.

Fig. 3. KDR/Flk-1 and Flt-1 are expressed in cardiac myocytes.

To investigate whether cardiac myocytes express VEGF receptors, KDR/Flk-1 and Flt-1, total cell lysates in Laemmli's sample buffer were electrophoresed and immunoblotted using anti-KDR/Flk-1 antibody or anti-Flt-1 antibody. Mr indicates molecular weight.

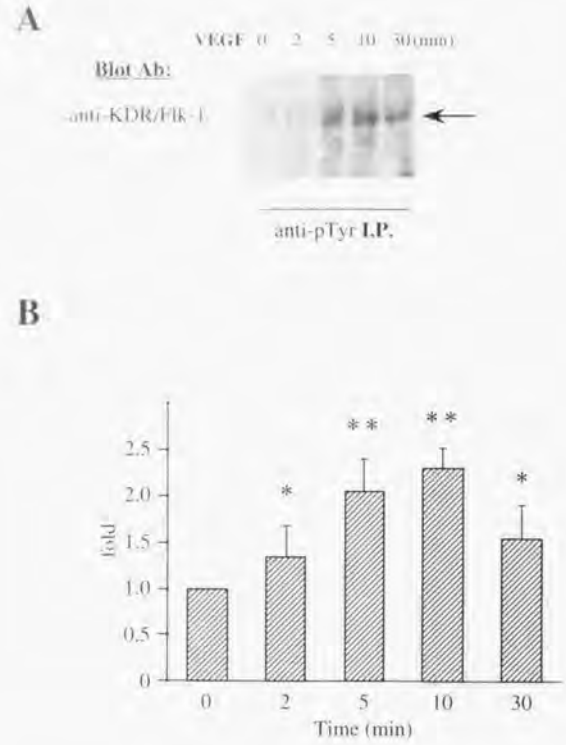

Fig. 4. KDR/Flk-1 is tyrosine phosphorylated in response to VEGF.

Cultured rat cardiac myocytes were exposed to rhVEGF (22.8 pmol/L) for the indicated periods. The cell lysates were centrifuged, and the supernatants were immunoprecipitated with an anti-phosphotyrosine mAb (PY20) and then immunoblotted with anti-KDR/Flk-1 antibody (A). In addition, the increase in tyrosine phosphorylation of KDR/Flk-1 was quantified by scanning densitometry of the autoradiograms (B). The initial phosphorylation of KDR/Flk-1 before addition of rhVEGF at 0 min is defined as 1.0. Fold induction values represent average of 5 independent experiments. An *arrow* indicates position of KDR/Flk-1; Ab, antibody; anti-pTyr I.P., anti-phosphotyrosine immunoprecipitation. $*P < 0.05$ and $**P < 0.01$ vs. initial value. Results shown are mean ± SD.

VEGF mediates hypoxia-induced tyrosine phosphorylation of p125^{FAK} and paxillin. To investigate the effects of hypoxia on the adhesion of cardiac myocytes to the ECM, I examined whether hypoxia induced phosphorylation of a focal adhesion-associated tyrosine kinase, p125^{FAK}. The cell lysates from untreated or hypoxia-treated cardiac myocytes were immunoprecipitated with anti-phosphotyrosine mAb (PY20), then immunoblotted with anti-p125^{FAK} polyclonal antibody. p125^{FAK} was significantly tyrosine-phosphorylated in response to hypoxia within 5 min, and that hypoxia-induced phosphorylation of p125^{FAK} at 5 min was markedly suppressed by pretreatment with the neutralizing anti-VEGF mAb (Fig. 5A). Then, I examined whether exogenous VEGF could stimulate tyrosine phosphorylation of p125^{FAK}. The cell lysates from untreated or rhVEGF-treated myocytes were immunoprecipitated with anti-phosphotyrosine mAb (PY20) followed by Western blotting with anti-p125^{FAK} polyclonal antibody. Conversely, immunoprecipitates with anti-p125^{FAK} polyclonal antibody were also analyzed by Western blotting with anti-phosphotyrosine mAb (4G10) or anti-p125^{FAK} polyclonal antibody. As shown in Fig. 5B (*top panel*) and 5C, VEGF significantly increased tyrosine phosphorylation of p125^{FAK}, which peaked at 5 to 10 min after addition of rhVEGF. I confirmed that almost equal amounts of p125^{FAK} protein were electrophoresed in each reaction (Fig. 5C *bottom panel*). These results suggest that hypoxia-induced activation of p125^{FAK} is primarily mediated by VEGF. I also investigated whether or not VEGF caused tyrosine phosphorylation of paxillin, another focal adhesion-associated protein that interacted with several proteins, including p125^{FAK}, members of src family of tyrosine kinases, transforming protein v-crk, and cytoskeletal protein vinculin. Immunoprecipitates with anti-phosphotyrosine mAb (PY20) from the stimulated and non-stimulated cardiac myocytes were analyzed by Western blotting with anti-paxillin mAb. As shown in Fig. 5B (*bottom panel*), VEGF also caused a significant increase in tyrosine phosphorylation of paxillin. The tyrosine phosphorylation of paxillin reached a maximum level at 5 to 10 min after addition of rhVEGF and decreased subsequently.

VEGF stimulates protein tyrosine kinase activity of p125^{FAK}. To examine whether the catalytic activity of p125^{FAK} is stimulated by VEGF, kinase activity of p125^{FAK} immunoprecipitates from untreated or rhVEGF-treated cardiac myocytes was assayed using poly(Glu-Tyr) as substrate. As shown in Fig. 6, VEGF induced activation of p125^{FAK} as early as 2 min, peaking at 5 min, which paralleled its tyrosine phosphorylation.

Tyrosine kinase inhibitors partially inhibit VEGF-induced activation of p125^{FAK}. Next, to examine whether VEGF-induced tyrosine-phosphorylation of p125^{FAK} was dependent on tyrosine kinase activity, the cardiac myocytes were pretreated with or without either of the tyrosine kinase inhibitors genistein (37 μmol/L) or tyrphostin (50 μmol/L) before addition of

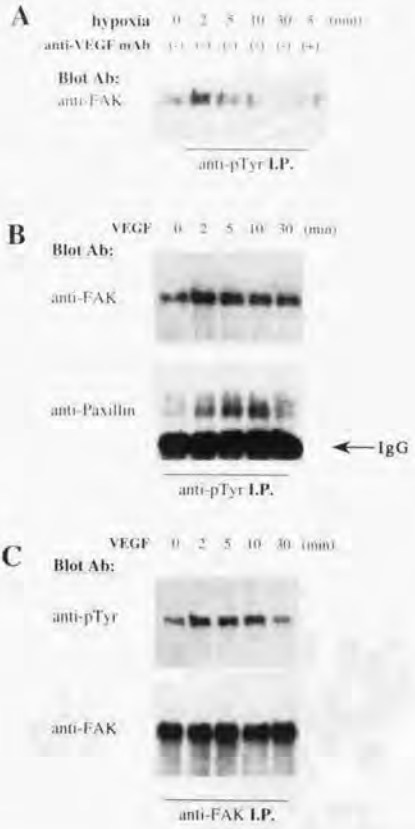

Fig. 5. VEGF mediates hypoxia-induced tyrosine phosphorylation of p125^{FAK} and paxillin.

A. Cultured cardiac myocytes were exposed to hypoxia for the indicated periods in the absence or presence of neutralizing anti-VEGF mAb. In the anti-VEGF mAb-treated group, the cells were preincubated with anti-VEGF mAb for 1 h before VEGF stimulation. The cell lysates were immunoprecipitated with anti-phosphotyrosine mAb (PY20), then immunoblotted with anti-p125^{FAK} antibody. B. Cells were treated for the indicated periods with 22.8 pmol/L rhVEGF and lysed with NP-40 buffer. The cell lysates were centrifuged, and the supernatants were immunoprecipitated with an anti-phosphotyrosine mAb (PY20), then immunoblotted with anti-p125^{FAK} antibody (*top panel*), or anti-paxillin mAb (*bottom panel*). C. Tyrosine phosphorylation of p125^{FAK} was also analyzed by immunoprecipitation using anti-p125^{FAK} polyclonal antibody followed by Western blotting with anti-phosphotyrosine mAb (4G10) (*top panel*). To ascertain equal loading of p125^{FAK} protein, immunoprecipitates with anti-p125^{FAK} antibody were analyzed by anti-p125^{FAK} Western blotting (*bottom panel*). Results shown are representative of 3 independent experiments.

rhVEGF. One of the representative results among 3 independent experiments is shown in Fig. 7. Both genistein and tyrphostin partially inhibited VEGF-induced increase in tyrosine phosphorylation of $p125^{FAK}$ at 5 min.

VEGF induces association of $p125^{FAK}$ with Shc, GRB2, and $p60^{c\text{-}src}$. To determine whether c-Src plays a role in VEGF-induced tyrosine phosphorylation of $p125^{FAK}$, immunoprecipitates with anti-v-Src mAb were subjected to SDS-PAGE followed by immunoblotting with anti-$p125^{FAK}$ antibody. As shown in Fig. 8*a*, VEGF caused a significant increase in the association of $p60^{c\text{-}src}$ with $p125^{FAK}$, which peeked at 5 to 10 min after addition of rhVEGF. To determine whether the VEGF-activated $p125^{FAK}$ signaling complexes contained other known Src homology (SH) 2-containing proteins, such as the SH2/SH3 adapter proteins GRB2 and Shc, I examined the VEGF-induced association of $p125^{FAK}$ with these adapter proteins. Immunoprecipitates with either anti-GRB2 polyclonal antibody or anti-Shc mAb

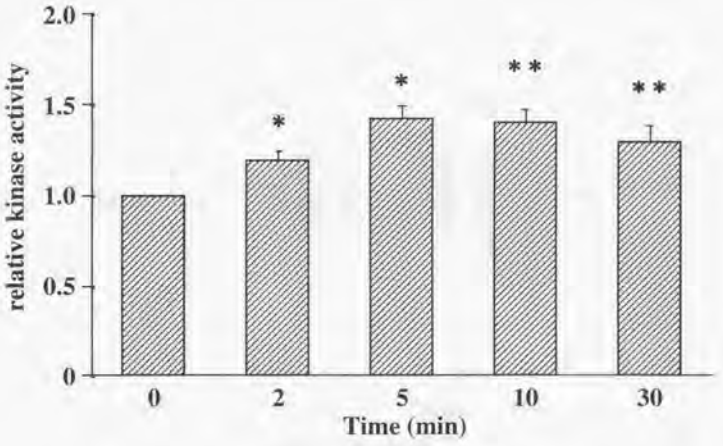

Fig. 6. VEGF stimulates protein tyrosine kinase activity of $p125^{FAK}$.

Protein tyrosine kinase activity of $p125^{FAK}$ immunoprecipitates from untreated or 22.8 pmol/L rhVEGF-treated cardiac myocytes were assayed with a non-radioactive isotope solid phase enzyme-liked immunosorbent assay (ELISA) kit, using poly(Glu-Tyr) as substrate. The immunoprecipitates were incubated with ATP in the microtiter plate onto which poly(Glu-Tyr) had been pre-coated. The amount of phosphorylated poly(Glu-Tyr) was measured by ELISA using a horseradish peroxidase-linked anti-phosphotyrosine (PY20) antibody. $*P<0.001$, $**P<0.01$ vs. control. Results shown are mean ± SD.

from rhVEGF-stimulated cardiac myocytes were subjected to SDS-PAGE followed by immunoblotting with anti-p125^{FAK} polyclonal antibody. As shown in Fig. 8*b* and 8*c*, VEGF caused significant increase in the association of Shc and GRB2 with p125^{FAK}. The association peeked at 5 to 10 min after addition of rhVEGF.

VEGF activates p38MAPK and SAPK/JNKs in cardiac myocytes. SAPK/JNKs (21, 26) and p38MAPK (22, 75, 76) are protein serine/threonine kinases related to ERK1/2, and are weakly activated by growth factors in general. Instead, they are markedly activated in response to cellular stresses such as inflammatory cytokines, UV irradiation, ischemia, reperfusion, heat shock, endotoxin, and genotoxic stress. I investigated whether VEGF activated SAPK/JNK or p38MAPK. Fig. 9A shows typical temporal change in SAPK/JNK activity represented by phosphorylation of substrate c-Jun fusion protein at Ser63,

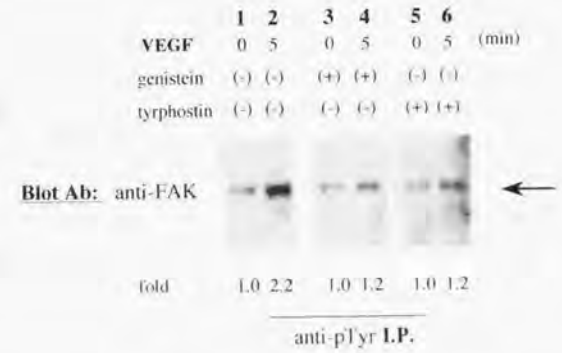

Fig. 7. Effects of tyrosine kinase inhibitors, genistein and tyrphostin, on VEGF-induced tyrosine phosphorylation of p125^{FAK}.

Serum-starved cardiac myocytes were pretreated for 30 min with or without 37 μmol/L genistein or 50 μmol/L tyrphostin and were then challenged with 22.8 pmol/L rhVEGF for the indicated periods. The cell lysates were centrifuged, and the supernatants were immunoprecipitated with anti-phosphotyrosine antibody (PY20), and further analyzed by anti-p125^{FAK} Western blotting. An *arrow* indicates position of p125^{FAK}. The increase in tyrosine phosphorylation of p125^{FAK} was quantified by scanning densitometry of the autoradiogram (the corresponding figures underneath the panel). Initial phosphorylation of p125^{FAK} before addition of rhVEGF at 0 min is defined as 1.0. Fold induction values represent average of 3 independent experiments.

which peaked at 5 to 10 min after addition of rhVEGF. In addition, marked phosphorylation of p46 SAPK/JNK and moderate phosphorylation of p54 SAPK/JNK at Thr183/Thy185 were induced by rhVEGF stimulation (Fig. 9B *top panel*). Those phosphorylation was led to a maximum level at 5 to 10 min, concomitantly with the activation. I confirmed that almost equal amounts of p46 and p54 SAPK/JNK protein were electrophoresed in each reaction by reprobing with a phosphorylation-state independent anti-SAPK antibody (Fig. 9B *bottom panel*). Fig. 10A shows typical temporal change in p38 MAP kinase activity represented by phosphorylation of substrate ATF-2 fusion protein at Thr71. The kinase activity was led to a maximum level at 5 to 10 min after addition of rhVEGF. I also investigated VEGF-induced phosphorylation of p38MAPK at Tyr182, which indicated activation of this kinase. VEGF caused a significant increase in the phosphorylation of p38MAPK at Tyr182, which peaked at 5 to 10 min after addition of rhVEGF, concomitantly with the activation (Fig. 10B *top panel*). I confirmed that almost equal amounts of p38MAPK protein were electrophoresed in each reaction by reprobing with a phosphorylation-state independent anti-p38MAPK antibody (Fig. 10B *bottom panel*).

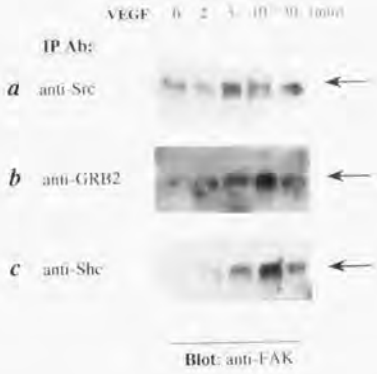

Fig. 8. VEGF induces association of p125^{FAK} with p60$^{c\text{-}src}$, GRB2, or Shc.

Quiescent cardiac myocytes were treated with 22.8 pmol/L rhVEGF for the indicated periods. Cell lysates were centrifuged, and the supernatants were immunoprecipitated with anti-v-Src mAb (LA074) (*panel a*), polyclonal anti-GRB2 antibody (*panel b*), or anti-Shc mAb (PG-797) (*panel c*). Immunoprecipitates were analyzed by Western blotting with anti-p125^{FAK} polyclonal antibody. *Arrows* indicate positions of p125^{FAK}. Results shown are representative of 3 independent experiments.

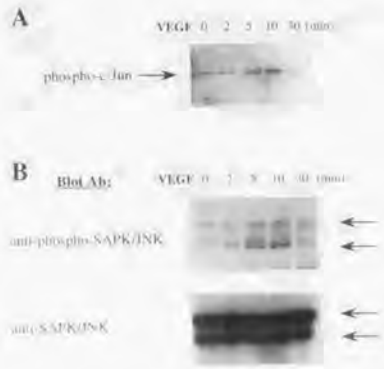

Fig. 9. VEGF activates SAPK/JNKs in cardiac myocytes.

Serum-starved cardiac myocytes were treated with rhVEGF (22.8 pmol/L) for the indicated periods. SAPK/JNK kinase activity was assayed with an assay kit using N-terminal c-Jun (1-89) fusion protein beads as substrate, according to the manufacturer's instructions (A). In addition, VEGF-induced phosphorylation of SAPK/JNKs was analyzed. The cell lysates were centrifuged, and the supernatants were subjected to Western analysis using an anti-phospho-specific SAPK (Thr183/Tyr185) (B *top panel*) antibody or a phosphorylation-state independent anti-SAPK antibody (B *bottom panel*). *Arrows* indicate positions of SAPK/JNKs.

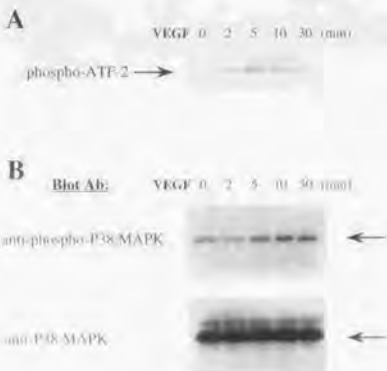

Fig. 10. VEGF activates p38MAPK in cardiac myocytes.

Serum-starved cardiac myocytes were treated with rhVEGF (22.8 pmol/L) for the indicated periods. p38 MAP kinase activity was assayed with an assay kit using ATF-2 fusion protein as substrate according to the manufacturer's instructions (A). In addition, VEGF-induced phosphorylation of p38MAPK was analyzed. The cell lysates were centrifuged, and the supernatants were subjected to Western analyses using an anti-phospho-specific p38MAPK (Tyr182) antibody (B *top panel*) or a phosphorylation-state independent anti-p38MAPK antibody (B *bottom panel*). *Arrows* indicate positions of p38MAPK.

VEGF activates ATF-2 in cardiac myocytes. It is known that the transcriptional activity of ATF-2 is stimulated by activation of SAPK/JNKs and p38MAPK in response to a variety of stresses, including genotoxic agents, cytokines, and UV irradiation (32, 77, 78). Therefore, I examined whether ATF-2 is phosphorylated by VEGF. As shown in Fig. 11A, VEGF significantly phosphorylated Thr71 of ATF-2, indicating activation of this transcription factor. The phosphorylation was led to a maximum level at 5 to 10 min after rhVEGF stimulation. I confirmed that almost equal amounts of ATF-2 protein were electrophoresed in each reaction by reprobing with a phosphorylation-state independent anti-ATF-2 antibody (Fig. 11B).

VEGF induces subcellular translocation of p125^{FAK} in cardiac myocytes and fibroblasts. It has been shown that cell adhesion to ECM, such as fibronectin, through integrins causes increased tyrosine phosphorylation of p125^{FAK} as well as accumulation of p125^{FAK} in focal adhesions (11, 79-81). I examined the effects of VEGF on subcellular localization of p125^{FAK} in cardiac myocytes and nonmuscle cells (which mostly consisted of fibroblasts). To distinguish cardiac myocytes from nonmuscle cells, I performed double

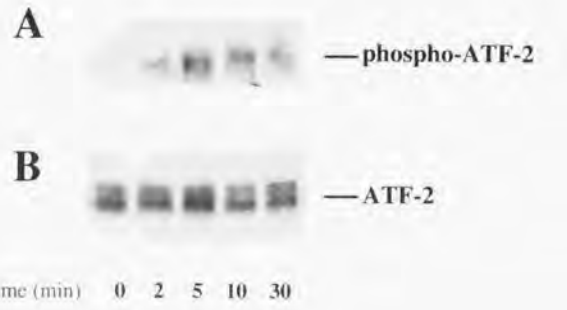

Fig. 11. VEGF phosphorylates ATF-2 (Thr71) in cardiac myocytes.

Serum-starved cardiac myocytes were exposed to rhVEGF (22.8 pmol/L) for the indicated periods. Cell lysates were centrifuged, and the pellets were subjected to Western analyses using an anti-phospho-specific ATF-2 (Thr71) antibody (A) or a phosphorylation-state independent anti-ATF-2 antibody (B).

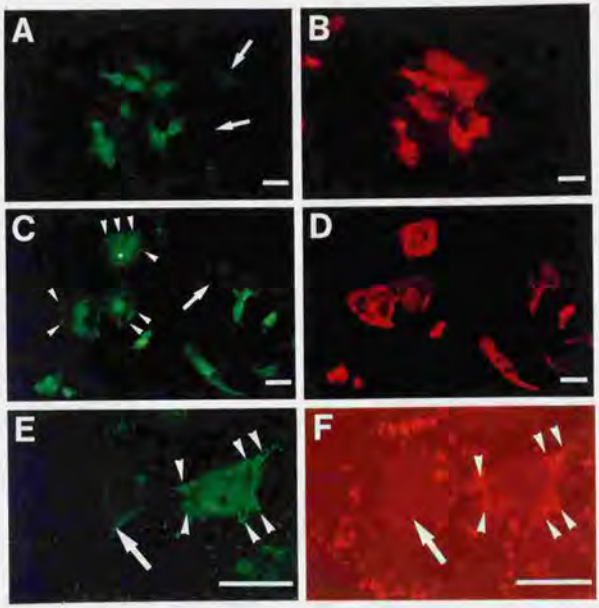

Fig. 12. VEGF alters the subcellular localization of p125^{FAK} in cardiac myocytes and fibroblasts.

Quiescent cardiac myocytes were given no treatment (A and B) or stimulated with 22.8 pmol/L rhVEGF for 60 min (C, D, E, and F) and fixed in acetone. To distinguish cardiac myocytes from contaminating fibroblasts, I performed double-staining for cardiac myosin heavy chain (B and D) and p125^{FAK} (A and C) by immunofluorescence. For the rhVEGF-treated (60 min) cells, I also performed double-staining for p125^{FAK} (E) and vinculin (F) by immunofluorescence. *Arrowheads* indicate localization of p125^{FAK} (C and E) and that of vinculin (F) in cardiac myocytes. *Arrows* indicate localization of p125^{FAK} (A, C, and E) and that of vinculin (F) in fibroblasts. Bars=10 μm.

staining for cardiac myosin heavy chain and $p125^{FAK}$ by immunofluorescence. Fig. 12B and 12D show that myofibrils of cardiac myocytes were strongly stained, making it easy to distinguish cardiac myocytes from fibroblasts. As shown in Fig. 12A, $p125^{FAK}$ predominantly localized in the perinuclear region in non-stimulated cardiac myocytes. Only weak fluorescent dots of $p125^{FAK}$ were seen in some of non-stimulated fibroblasts (Fig. 12A, *arrows*). Fluorescent dots of $p125^{FAK}$, which mostly localized in the central regions in a higher density, appeared to scatter to the peripheral cytoplasm in cardiac myocytes stimulated with rhVEGF for 60 min (Fig. 12C, *arrowheads*). In fibroblasts stimulated with rhVEGF for 60 min, fluorescent dots of $p125^{FAK}$ were observed to be concentrated in the patchy arrowhead-like structures at the peripheral cytoplasm reminiscent of focal adhesions (Fig. 12C, *arrows*). To compare the localization of $p125^{FAK}$ staining to focal adhesions in rhVEGF-treated cells, I performed double staining for $p125^{FAK}$ (Fig. 12E) and vinculin (Fig. 12F) by immunofluorescence. Fluorescent dots of $p125^{FAK}$ showed localization similar to that of vinculin in cardiac myocytes (Fig. 12E and 12F, *arrowheads*), which suggests that $p125^{FAK}$ was localized to focal adhesions in response to rhVEGF, although the rest of $p125^{FAK}$ remained in cytoplasm. In fibroblasts, the patchy arrowhead-like shaped fluorescent dots of $p125^{FAK}$ were clearly observed with higher-resolution (Fig. 12E, *arrow*). Incubation of cardiac myocytes with control non-immune rabbit serum yielded no significant staining (data not shown).

Next, to confirm that VEGF induces subcellular translocation of $p125^{FAK}$, I analyzed $p125^{FAK}$ content in subcellular fractions by Western analysis with or without rhVEGF stimulation. As shown in Fig. 13, the amount of $p125^{FAK}$ in the membrane fraction was increased in response to rhVEGF (Fig. 13*b top panel*), whereas that in the cytosolic fraction remained almost unchanged (Fig. 13*a*). I confirmed that almost equal amounts of the membrane fraction were electrophoresed in each reaction by Western analysis using anti- Na, K ATPase α1 subunit antibody (Fig. 13*b*, *bottom panel*).

VEGF strengthens adhesion of cardiac myocytes to the ECM. To assess the effects of VEGF on the extent of cell-substrate adhesion, I monitored changes in cell-substrate resistance after addition of rhVEGF, using an electric cell-substrate impedance sensor. Typical results are shown in Fig. 14A. The extent of cell-substrate adhesion is expressed as "normalized" resistance, which was defined as a ratio of the resistance to the initial value before addition of rhVEGF. VEGF induced a significant increase in normalized resistance, indicating strengthening of the cell-substrate adhesion at 3 hr after addition of rhVEGF (Fig. 14B).

p160ROCK-inhibitor Y-27632 inhibits VEGF-induced activation of $p125^{FAK}$, p38MAPK, and SAPK/JNK. Rho and its direct target p160ROCK have been

reported to stimulate tyrosine phosphorylation of $p125^{FAK}$ and paxillin (44, 45, 53-55). I investigated whether p160ROCK is involved in the VEGF-induced signaling cascades, using p160ROCK-specific inhibitor Y-27632 (60). As shown Fig. 15A, pretreatment with 10 μmol/L Y-27632 diminished the basal phosphorylation of $p125^{FAK}$ and almost abolished VEGF-induced phosphorylation of $p125^{FAK}$. I also investigated the effects of Y-27632 on VEGF-induced activation of SAPK/JNKs and p38MAPK. Pretreatment with Y-27632 at 10 μmol/L diminished the basal phosphorylation of p38MAPK (Fig. 15B) as well as SAPK/JNKs (Fig. 15C) and markedly diminished VEGF-induced phosphorylation of both kinases.

p160ROCK-inhibitor Y-27632 diminishes adhesion of cardiac myocytes to the ECM. To determine whether p160ROCK really modulates adhesion of cardiac myocytes to the ECM, I analyzed the effects of p160ROCK-inhibitor Y-27632 on their cell-substrate adhesion, using an electric cell-substrate impedance sensor. Typical results are shown in Fig. 16. Preincubation with 10 μmol/L Y-27632 substantially diminished the normalized resistance, which reflects the extent of cell-substrate adhesion. There was no significant difference in the normalized resistance values between rhVEGF-treated and untreated groups.

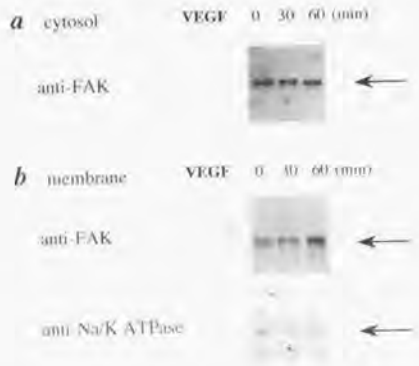

Fig. 13. Effect of VEGF on subcellular distribution of $p125^{FAK}$.

After stimulation with 22.8 pmol/L rhVEGF for the indicated periods, cytosolic and membrane fractions were prepared from cardiac myocytes as described in "Materials and Methods", and subjected to Western analysis using anti-$p125^{FAK}$ antibody (*a* and *b top panel*). To confirm that almost equal amounts of membrane fractions were electrophoresed in each reaction, the same amounts of membrane fractions were subjected to Western analysis using anti-Na, K ATPase α1 subunit (isoform specific) antibody (*b bottom panel*).

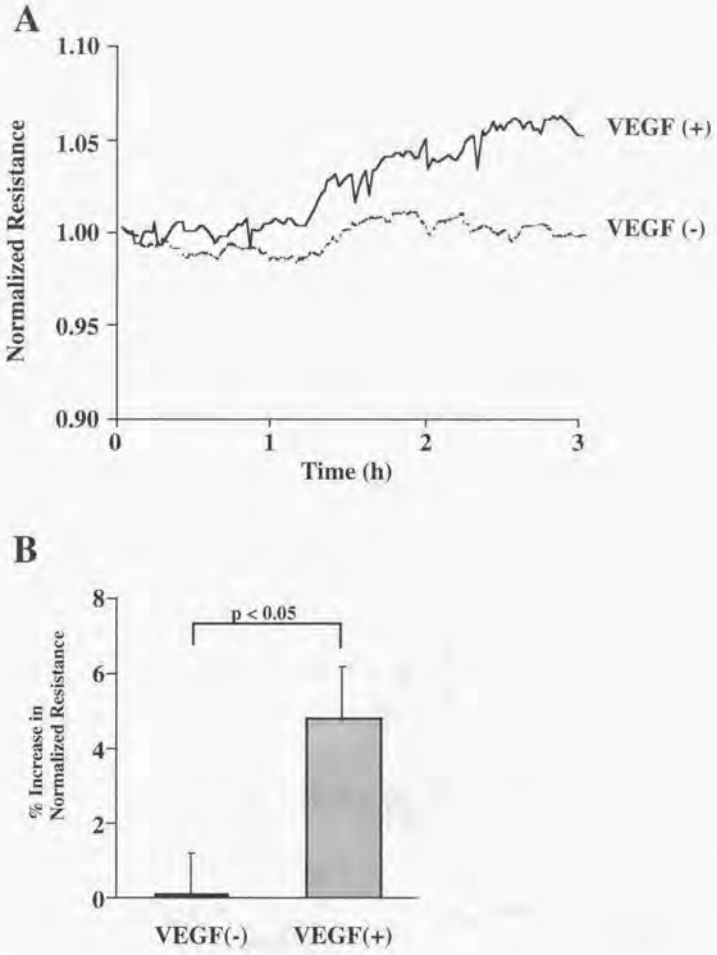

Fig. 14. Effects of VEGF on adhesion of cardiac myocytes to the ECM.

Cardiac myocytes were cultured on the surface of gelatin-coated wells manufactured especially for measurement of resistance. After starvation for 24 hr, they were stimulated with rhVEGF (34.2 pmol/L). A. Typical tracing representative of 4 replicate experiments. In all of these experiments, initial resistance was within the range of 5000 to 7000 ohms. To simplify the comparison, ordinate represents normalized resistance defined as a fraction of the initial resistance before addition of rhVEGF. B. Comparison of percentage increase in normalized resistance at 3 hr after addition of rhVEGF, VEGF(+), with control, VEGF(-). Results shown are mean ± SD from 4 independent experiments.

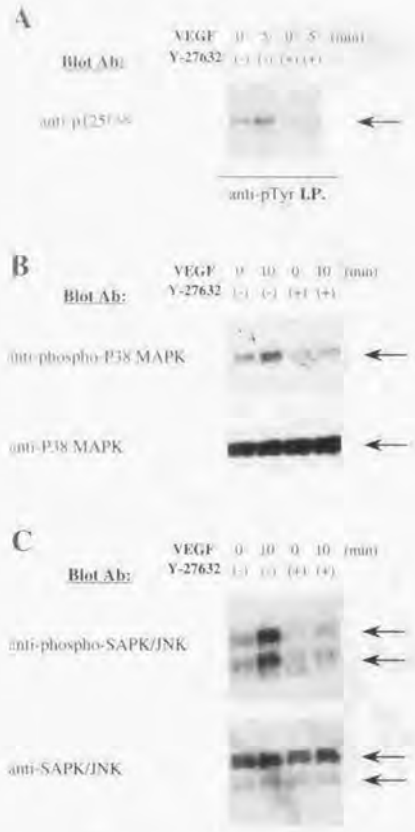

Fig. 15. Effects of p160ROCK-specific inhibitor Y-27632 on the phosphorylation of $p125^{FAK}$, SAPK/JNKs, and p38MAPK.

Serum starved cardiac myocytes were preincubated for 60 min with or without p160ROCK-specific inhibitor Y-27632 at 10 μmol/L, then they were stimulated with 22.8 pmol/L rhVEGF for the indicated periods. Tyrosine phosphorylation of $p125^{FAK}$ was analyzed by immunoprecipitation using anti-phosphotyrosine mAb (PY20) followed by Western blotting with anti-$p125^{FAK}$ antibody (A). VEGF-induced phosphorylation of p38MAPK was analyzed using an anti-phospho-specific p38MAPK (Tyr182) antibody (B *top panel*) or a phosphorylation-state independent anti-p38MAPK antibody (B *bottom panel*). Phosphorylation of SAPK/JNKs was analyzed using an anti-phospho-specific SAPK (Thr183/Tyr185) antibody (C *top panel*) or a phosphorylation-state independent anti-SAPK antibody (C *bottom panel*). *Arrows* indicate positions of $p125^{FAK}$ (A), p38MAPK (B), or SAPK/JNKs (C).

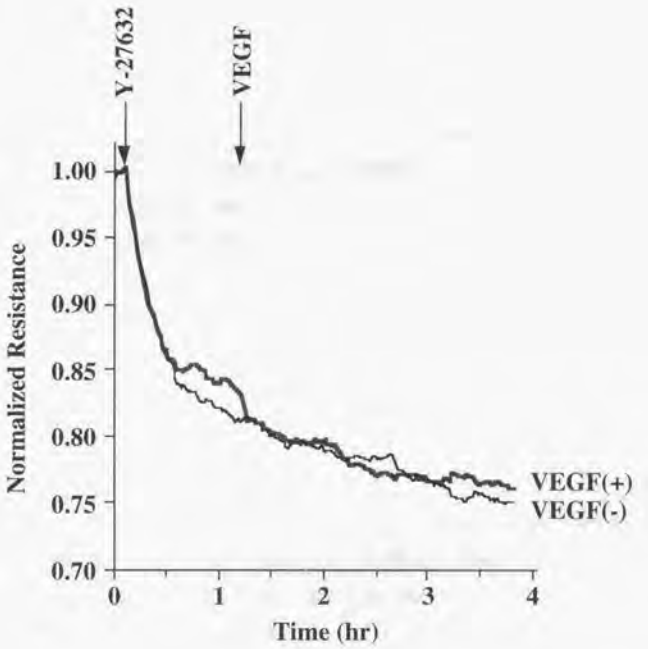

Fig. 16. Effects of p160ROCK-specific inhibitor Y-27632 on adhesion of cardiac myocytes to the ECM.

Cardiac myocytes were cultured on the surface of gelatin-coated wells manufactured especially for measurement of resistance. After starvation for 24 hr, they were preincubated with 10 μmol/L Y-27632 for 60 min, and then stimulated with rhVEGF (34.2 pmol/L). Typical tracing representative of 4 replicate experiments was shown. Ordinate represents normalized resistance expressed as a fraction of the initial resistance before addition of rhVEGF.

Discussion

Evidence has accumulated that VEGF is expressed in the heart tissue, and its expression is markedly increased in response to hypoxia (6, 7, 82). I demonstrated for the first time that cardiac myocytes rapidly secreted VEGF in response to hypoxia.

The biological effects of VEGF are mediated by specific cell surface receptors. VEGF is known to bind with high affinity to 2 structurally related tyrosine kinase receptors, Flt-1 (71, 72) and KDR/Flk-1 (73, 74). Studies on signal transduction from Flt-1 and KDR showed that these 2 receptor tyrosine kinases have different signal transduction properties (83, 84). It was reported that KDR-expressing cells showed striking changes in cell morphology, actin reorganization and membrane ruffling, chemotaxis and mitogenicity on VEGF stimulation, whereas Flt-1-expressing cells lacked such responses in a cultured human umbilical vein endothelial cell population (83). I confirmed by Western blot analysis that KDR/Flk-1 as well as Flt-1 was expressed in the cultured cardiac myocytes and that KDR/Flk-1 was significantly tyrosine phosphorylated on VEGF stimulation, whereas Flt-1 was not. It has been reported that KDR is autophosphorylated much more efficiently than Flt-1 in response to VEGF, on the basis of a comparison between Flt-1-transfected porcine aortic endothelial cells and KDR-transfected ones (83). By analogy, it is possible that detectable tyrosine phosphorylation was not induced for Flt-1 protein in response to VEGF in cardiac myocytes at least at the concentration used in this study, although KDR/Flk-1 protein was efficiently tyrosine phosphorylated. It might also in part reflect the difference in the sensitivity between the anti-KDR/Flk-1 antibody and the anti-Flt-1 antibody used in the present study. These results suggest that the VEGF-induced signaling pathway may be more dependent on KDR/Flk-1 than Flt-1 or that it requires formation of heterodimeric complexes between KDR and Flt-1. To clarify which of the 2 receptor tyrosine kinases (or both) mediates VEGF-induced activation in cardiac myocytes, further investigation is needed.

$p125^{FAK}$ is a widely expressed nonreceptor protein-tyrosine kinase that localizes to focal adhesion structures. $p125^{FAK}$ is thought to be one of the key elements in the signal transduction pathway underlying changes in cell behavior induced by diverse stimuli, including integrin engagement, oncogenic transformation, several neuropeptides, and growth factors (12, 14-18). It was shown that tyrosine-phosphorylated $pp125^{FAK}$ directly interacts with $pp60^{c\text{-}src}$ and $pp59^{fyn}$ as one of their major substrates (64, 85). Indeed, tyrosine phosphorylation of $p125^{FAK}$ by Src-family kinases has been shown to be directly correlated with increased protein tyrosine kinase activity, which is an important step in the formation of an active signaling complex (12). Paxillin is a cytoskeletal protein involved in actin-membrane attachment at sites

of cell adhesion to the ECM, which has also been demonstrated to be one of the major substrates of $pp60^{c\text{-}src}$ in Rous sarcoma virus-transformed cells as $p125^{FAK}$ (86). It becomes tyrosine-phosphorylated concomitantly with $p125^{FAK}$ in response to multiple stimuli, including integrin-mediated cell adhesion, several neuropeptides and growth factors (87-89).

In the present study, I have shown that hypoxia induces activation of $p125^{FAK}$, which is inhibited by neutralization of VEGF. In addition, exogenous VEGF induced activation of $p125^{FAK}$ with similar time course as hypoxia, which suggests that VEGF mediates hypoxia-induced activation of $p125^{FAK}$. Although the time courses of the change in tyrosine phosphorylation and the activation of the kinase activity in response to VEGF stimulation were similar, the extent of $p125^{FAK}$ activation seemed to be smaller than that expected from the increase in its tyrosine phosphorylation. The kinase activity assayed with exogenous substrate poly(Glu-Tyr) reflected only the ability to phosphorylate downstream substrates, including other kinases. On the other hand, tyrosine phosphorylation of $p125^{FAK}$ results not only from autophosphorylation but also from phosphorylation by other upstream protein kinases, including Src family kinases (64, 85). The contribution from the latter might be relatively large compared with that from the former. This may be the reason for the discrepancy between the extent of its activation measured with the exogenous substrate and tyrosine phosphorylation.

I also showed that VEGF stimulated tyrosine phosphorylation of paxillin, and that VEGF-induced tyrosine phosphorylation of $p125^{FAK}$ was inhibited at least partially by tyrosine kinase inhibitors, genistein and tyrphostin. Moreover, VEGF caused increased association of $p125^{FAK}$ with $pp60^{c\text{-}src}$ concomitantly with increased tyrosine phosphorylation of $p125^{FAK}$. Recently, Abedi and Zachary (19) have reported that VEGF induces tyrosine phosphorylation of $p125^{FAK}$ and paxillin in endothelial cells, suggesting that they are components in a VEGF-stimulated signaling pathway. My results indicate that tyrosine phosphorylation of $p125^{FAK}$ and paxillin is also a part of the signal transduction by VEGF in cardiac myocytes as in endothelial cells, resulting in organization of the cytoskeleton. Tyrosine kinases, especially $p60^{c\text{-}src}$ might take some part in VEGF-induced tyrosine phosphorylation and activation of $p125^{FAK}$ in cardiac myocytes.

Transduction of various mitogenic signals from the cell membrane to the nucleus involves the adapter proteins Shc and GRB2, which mediate activation of the Ras/MAPK pathway (90-92). Shc is an immediate substrate of receptor tyrosine kinase and serves to physically link activated receptors to downstream signaling components (93, 94). GRB2 is a ubiquitously expressed 24 kDa mammalian protein, which directly binds autophosphorylated tyrosine kinase receptors as well as phosphorylated Shc proteins and $p125^{FAK}$ through its SH2 domain (90, 91,

95, 96). VEGF was reported to induce association of KDR with Shc and GRB2, leading to activation of Raf-1/MEK/MAPK pathway in porcine aortic endothelial cells overexpressing KDR (97). It has also been demonstrated that adhesion of fibroblasts to fibronectin promotes SH2-domain-mediated association of GRB2 and $p60^{c\text{-}src}$ with $p125^{FAK}$ *in vivo*, resulting in activation of MAPK (95). In cardiac myocytes, the tyrosine kinase-Shc-GRB2-Sos pathway was shown to be involved in signal transduction from Gq protein-coupled angiotensin II receptor to activation of $p21^{ras}$ (98). In the present study, I demonstrated VEGF induced association of $p125^{FAK}$ with GRB2 and Shc, occurring concomitantly with increased tyrosine phosphorylation of $p125^{FAK}$. This strongly suggests that VEGF induces formation of signaling complex consisted of $p125^{FAK}$, GRB2, Shc, $p60^{c\text{-}src}$, and paxillin in cardiac myocytes, which links activated VEGF receptors (VEGF-Rs) to downstream signaling components (see Fig. 17).

Furthermore, the immunocytochemical study demonstrated that VEGF stimulation significantly altered the subcellular localization of $p125^{FAK}$ from the perinuclear region to the peripheral cytoplasm and increased accumulation of $p125^{FAK}$ in the patchy arrowhead-like structures at the peripheral cytoplasm (ie, focal adhesions) in cardiac myocytes as well as in fibroblasts. In addition, I confirmed quantitatively by Western analysis that the amount of $p125^{FAK}$ in the membrane-rich fraction significantly increased in response to VEGF. These results indicate that $p125^{FAK}$ translocates to focal adhesions in response to VEGF. Taken together, VEGF causes activation as well as subcellular translocation of $p125^{FAK}$ to focal adhesions, where it works. Activation and accumulation of $p125^{FAK}$ in focal adhesions strongly suggests that adhesive interaction between cardiac myocytes and ECM may be strengthened in response to VEGF. Using an electric cell-substrate impedance sensor, I confirmed that VEGF really strengthened adhesion between cardiac myocytes and ECM.

Recently, several direct target molecules of Rho were isolated (46-52). Among them, p160ROCK and ROKα/Rho-kinase/ROCK-II has been suggested to mediate the effects of Rho on the formation of focal adhesions and stress fibers through regulation of actomyosin system (53-55). I demonstrated that p160ROCK-specific inhibitor Y-27632 diminished the basal phosphorylation of $p125^{FAK}$ and almost abolished its phosphorylation in response to VEGF. Consistently, Y-27632 substantially diminished the cell-substrate adhesion and abolished its increase in response to VEGF. The extent of Y-27632-induced decrease in cell-substrate adhesion was much larger than that of VEGF-induced increase. The reason may be that almost full attachment to the ECM was already achieved in basal condition and there was left little room to be strengthened. These results suggest that Rho/p160ROCK play a critically important role in VEGF-induced signaling cascades leading to strengthening cell-substrate

adhesion.

p38MAPK and SAPK/JNKs participate in cellular responses to mitogenic stimuli, environmental stresses and apoptotic agents (24). VEGF was reported to induce activation of both p38MAPK (99) and SAPK/JNK (100, 101) in endothelial cells. In a human bone marrow endothelial cell line, VEGF was shown to phosphorylate and activate RAFTK (related adhesion focal tyrosine kinase), the second member of the focal adhesion kinase family, and

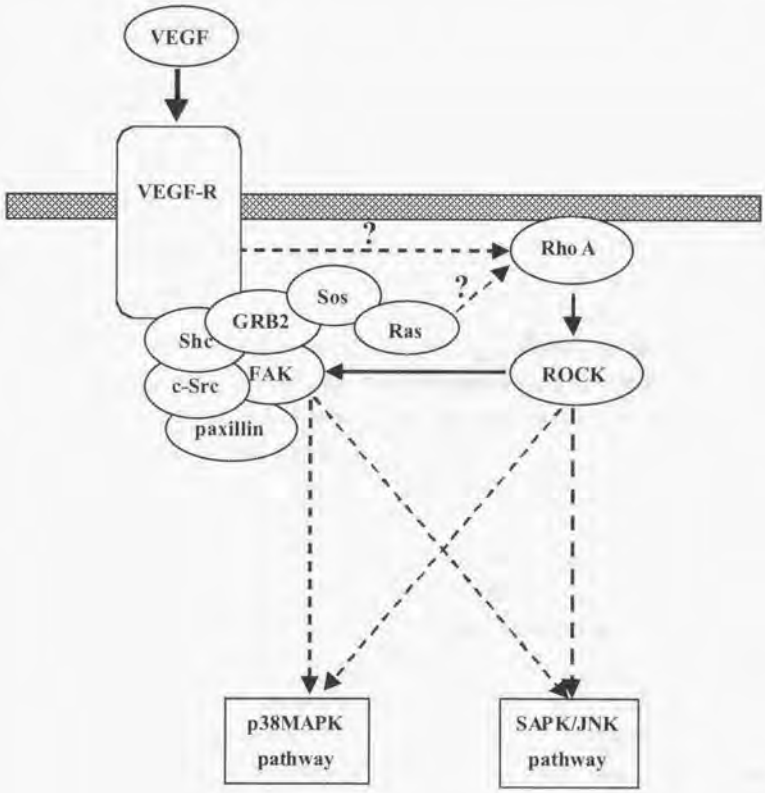

Fig. 17. Scheme of VEGF-induced signaling cascades

Dotted arrows indicate tentative connections, and *question marks* indicate purely hypothetical connections. See text for details.

SAPK/JNK (102). It has also been reported that a number of growth factors including VEGF, basic fibroblast growth factor, and VEGF-related protein (VRP), the ligand for the FLT-4 receptor, induce phosphorylation and activation of RAFTK, leading to activation of SAPK/JNK in Kaposi's sarcoma cells (103). In myocardial cells, p38MAPK has been demonstrated to play an important role in α1-adrenergic receptor-mediated hypertrophic growth (104).

In this study, I demonstrated that VEGF activated both SAPK/JNKs and p38MAPK in cultured rat cardiac myocytes, resulting in activation of ATF-2, the downstream transcription factor, while SAPK/JNKs were activated more markedly than p38MAPK. It was reported that VEGF-induced activation of p38MAPK in endothelial cells led to activation of MAP kinase activated protein kinase-2/3, followed by phosphorylation of the F-actin polymerization modulator, heat shock protein 27, resulting in formation of stress fibers, recruitment of vinculin to focal adhesions, and increased cell migration (99). VEGF-activated p38MAPK might play similar roles in actin reorganization in cardiac myocytes as in endothelial cells.

The activation cascades for SAPK/JNK and p38MAPK are still not defined (40, 102, 103). It has been known that constitutively activated Rac and Cdc42 but not RhoA regulate the activity of SAPK/JNK and p38MAPK in some cell lines (36, 105). In a human kidney epithelial cell line, however, Cdc42 and Rho, but not Rac and Ras can induce activation of SAPK/JNKs (106). In cultured vascular endothelial cells, shear stress was shown to induce translocation of Cdc42 and Rho from cytosol to membrane, leading to activation of SAPK/JNK, followed by cell alignment and stress fiber formation, which were inhibited by dominant-negative mutants of Rho and p160ROCK, but not by the dominant-negative mutant of Cdc42 (107). In endothelial cells, ICAM-1 cross-linking caused activation of Rho, followed by activation of SAPK/JNK and tyrosine phosphorylation of $p125^{FAK}$ and paxillin (108). These discrepancies among different cell species might be attributed to the diversity of target molecules for small GTP-binding proteins and their tissue specific distribution. In cardiac myocytes, it was reported that Rho and Rho kinase as well as SAPK/JNK and p38MAPK were required for Gαq and α1-adrenergic receptor-mediated hypertrophic gene expression (56-59), suggesting possible link among them. In this study, I demonstrated that p160ROCK-specific inhibitor Y-27632 diminished the basal phosphorylation of SAPK/JNK as well as that of p38MAPK and almost abolished VEGF-induced phosphorylation of both kinases. These results suggest that Rho/p160ROCK may be upstream in the VEGF-induced SAPK/JNK and p38MAPK cascades in cardiac myocytes. Fig. 17 shows possible signaling cascades which were suggested by this study. It was reported that integrin-mediated signal transduction leading to activation of SAPK/JNK was $p125^{FAK}$-depedent in endothelial cells (109-111). By analogy, it is possible that VEGF-mediated activation of $p125^{FAK}$ in cardiac myocytes may facilitate signal transduction

to the cytoskeleton as well as to the p38MAPK or SAPK/JNK pathway. Further investigations is needed to determine whether signal transduction pathways from p160ROCK to p38MAPK or SAPK/JNK in cardiac myocytes are $p125^{FAK}$-dependent or not. How RhoA receives signals from VEGF receptors is still not determined. Ras may lie upstream of RhoA, or some other proteins such as phospholipase γ, protein kinase C, or nitric oxide synthase, which have been proved to play important roles in VEGF-induced signaling cascades in endothelial cells, may mediate signal transduction downstream to RhoA.

It has been noted that there is a reciprocal relationship between intercellular and cell-matrix adhesion. For instance, focal contacts are quite abundant in sparsely plated cells, while cell-cell adherens junctions are predominant in dense cultures. It was also reported that integrity and phosphotyrosine content of cell-cell adherens junctions were reciprocally related to the number and size of focal contacts in an epithelial cell line (112). Regarding that cardiac myocytes have close intercellular contacts *in vivo*, the *in vivo* roles of molecular components of cell-ECM adhesion may be different from those *in vitro*, although cell-ECM adhesion is also integral *in vivo* for the connective tissue to provide for mechanical coupling of myocytes and function in force distribution. Indeed, immunofluorescent study revealed that $p125^{FAK}$ was predominantly localized in intercalated disks *in vivo* heart and there were no distinct focal adhesions as seen in cultured cells (data not shown). In addition, its staining was not significantly different between in normoxic and hypoxic hearts (data not shown). To investigate further the *in vivo* roles of $p125^{FAK}$ in myocardial ischemia, further study using adult cardiac myocytes in long-term culture, which have been known to express intercalated disks, or $p125^{FAK}$-overexpressing or knockout mice, is needed.

In conclusion, cardiac myocytes rapidly secrete VEGF in response to hypoxia and they themselves are one of the target cells for VEGF. VEGF activates several signaling cascades, including focal adhesion related kinases as well as stress-sensitive kinases, and strengthened the adhesion of cardiac myocytes to ECM. p160ROCK plays a critical role in these cardiac responses to VEGF. These data strongly suggest a pivotal role of VEGF in cardiac adaptation to hypoxic stress.

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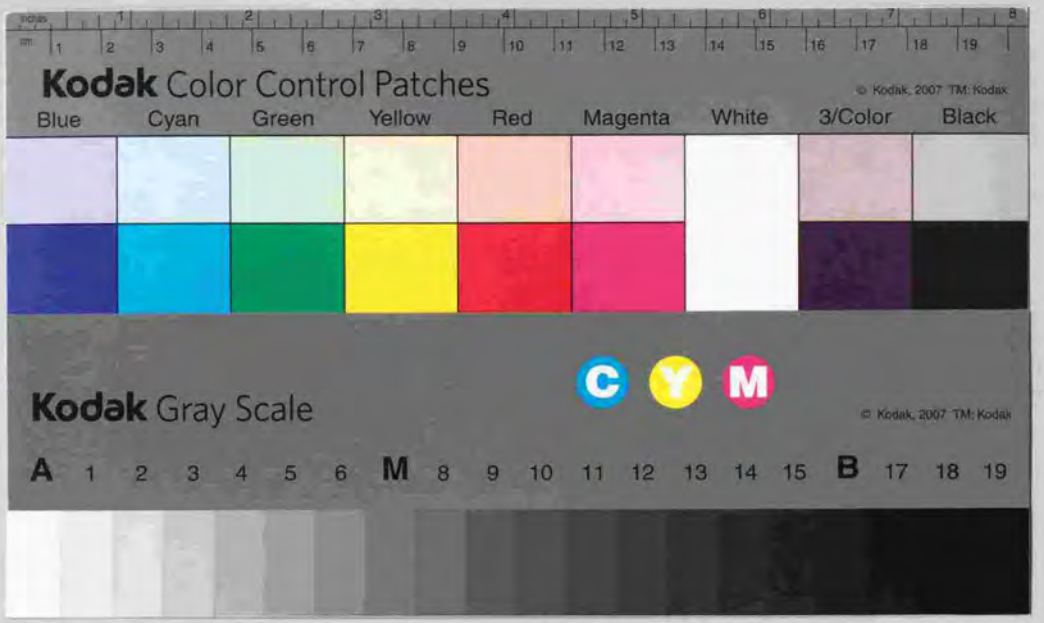
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