Contributory role of VEGF overexpression in endothelin-1-induced cardiomyocyte hypertrophy

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ENDOTHELIN (ET)-1 is a potent vasoconstrictor polypeptide containing 21 amino acids that was originally isolated from the culture supernatant of porcine aortic endothelial cells (38). However, we now know that many different cells, including cardiac myocytes, can produce this polypeptide (13). Moreover, besides its vasoconstrictive action, ET-1 has been shown to initiate a wide variety of biological actions, such as mitogenesis of a number of cells, including cardiac myocytes (30). ET-1 can also stimulate the production of adrenomedullin in vascular smooth muscle cells (29) and atrial natriuretic peptide in cardiomyocytes (10). Thus the regulation of cell growth by ET-1 may be partly due to possible involvement of released cytokines or growth factors under physiological or pathological conditions. In the heart, endogenous ET-1, which is locally generated and secreted by cardiomyocytes, may contribute to cardiac hypertrophy via an autocrine/paracrine fashion (15). Although the actions of ET-1 are mediated through its inter-action with two classes of cell surface receptors, ET type A (ET\(_A\)) receptors are associated with vasoconstriction and cell growth, whereas ET type B (ET\(_B\)) receptors are linked to endothelial cell-mediated vasodilation and release of other neurohormones, such as aldosterone (18).

Vascular endothelial growth factor (VEGF) is an endothelial cell-specific mitogen in vitro and an angiogenic inducer in vivo models (8). The VEGF effects are mediated mainly through two VEGF receptor tyrosine kinases, \(\text{fms-like tyrosine kinase 1 (Flt-1)}\) and \(\text{fetal liver kinase 1 [Flk-1/kinase domain region (KDR)]}\) (8). The tissue distribution of these VEGF receptors includes vascular smooth muscle cells (14), osteoblasts (6), cardiomyocytes (31), myofibroblasts (5), neurons (4), and various tumor cells (36). In balloon-injured rat carotid artery, local delivery of VEGF has been demonstrated to accelerate reendothelialization and thereby attenuate intimal hyperplasia (1). Treatment with VEGF has also been shown to exacerbate neointimal thickening after balloon denudation vascular injury in dogs (16). Meanwhile, experimental evidence for involvement of ET-1 in neointimal formation after angioplasty has been provided (7). Importantly, ET-1 has been documented to enhance VEGF mRNA expression via activation of ET\(_A\) receptors in rat vascular smooth muscle cells (17). From these reports, we assume that VEGF may serve as a key regulator of ET-1-induced cell proliferation in cardiovascular disorders.

Our recent work (13, 27) showed that ET-1 is a potent inducer of the development of cardiomyocyte hypertrophy. It was of interest therefore to investigate whether ET-1-induced cardiomyocyte hypertrophy is associated with a change in expression of VEGF in neonatal rat cardiomyocytes. We found that treatment of cardiomyocytes with ET-1 for 24 h caused VEGF overexpression in a concentration-dependent fashion. It is now established that hypoxia-inducible factor (HIF)-1 is a key mediator of hypoxic responses (26). Activation of HIF-1 regulates the VEGF gene by its binding to a hypoxia-responsive element in the 5′-flanking region of the VEGF gene (9). Thus HIF-1 is a strong inducer of VEGF mRNA expression. In ovarian carcinoma cells, ET-1 stimulates the secretion of VEGF through HIF-1α protein increase (28). We thus examined the effect of ET-1 treatment on expression of the HIF family in cardiomyocytes. We also investigated possible involvement of ET\(_A\) receptors in ET-1-induced overexpression of VEGF in cardiomyocytes using an ET\(_A\)-selective receptor blocker, TA-0201.

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Finally, we tested whether the specific inhibition of VEGF bioactivity with the use of its neutralizing peptides can prevent ET-1-induced cardiomyocyte hypertrophy.

**MATERIALS AND METHODS**

*Myocyte isolation and culture.* Ventricular cardiac myocytes were isolated from 2- to 3-day-old Sprague-Dawley rats, as described previously (13), and were incubated on fibronectin-coated dishes in DMEM-Ham’s F-12 medium supplemented with 0.1% fatty acid-free BSA (Sigma, St. Louis, MO). Cells were cultured for 3 days after differential adhesion and then used for further experiments. Myocytes were exposed to only vehicles for test drugs (control, in 95% air-5% CO2). At day 4 of culture, cardiomyocytes were divided into groups; 24 h after drug treatment, cardiomyocytes were harvested for analysis. TA-0201 (10^-6 M), an ETA selective antagonist, was given 30 min before ET-1 administration. VEGF-neutralizing peptides and anti-rat VEGF-specific goat IgG (5 and 10 µg/ml; R&D Systems, Minneapolis, MN) were applied 60 min before ET-1 administration. Animal experiments were carried out in a humane manner after we received approval from the Institutional Animal Experiment Committee of the University of Tsukuba and were in accordance with the Regulation for Animal Experiments in our university and Fundamental Guidelines for Proper Conduct of Animal Experiment and Related Activities in Academic Research Institutions under the jurisdiction of Ministry of Education, Culture, Sports, Science and Technology.

*Cardiac myocyte surface area.* To determine changes in cell size, the peripheries of cell images captured by a charge-coupled device camera (Olympus, Tokyo, Japan) were traced and analyzed with NIH Image software (National Institutes of Health, Bethesda, MD). These values were then doubled to account for the portion of the cell surface in contact with the dish. All cells from randomly selected fields in two or three dishes were examined for each experimental group. A total of 60 cells in each experimental group were examined.

*Protein synthesis.* The rate of protein synthesis in cardiomyocytes treated was investigated, as described previously (27). Briefly, protein synthesis in cultured neonatal rat ventricular myocytes was assessed by measuring [14C]leucine incorporation into acid-insoluble cellular material. The cells were plated in 24-well dishes at a density of 10^5 cells/well. After treatment with ET-1 or vehicle for 24 h, 0.1 µCi/ml [14C]leucine was added and cells were incubated for 24 h. The cells were washed twice with cold PBS, and 5% TCA was added for 10 min. The cells were then incubated with 0.25% trypsin at 37°C for 30 min, and cell residues were solubilized in 0.5 N NaOH for 10 min. Aliquots were counted with a scintillation counter (Beckman LS-6500 scintillation counter; Beckman Coulter, Fullerton, CA).

*Western blot analysis.* Cardiomyocytes were plated at a field density of 2 x 10^6 cells/cm² on 60-mm culture dishes with 2 ml of culture medium. Cardiomyocytes of different groups were lysed on ice with buffer (10 mM Tris·HCl, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.1% sodium deoxycholate, and 0.1% SDS). The protein concentration of supernatant was determined with the bicinchoninic acid protein assay (Pierce, Rockford, IL). Samples were run on SDS-PAGE, using 7.5–10% gel, and electrotransferred to polyvinylidene difluoride (PVDF) filter membrane. To reduce nonspecific binding, the PVDF membrane was blocked for 2 h at room temperature with 5% nonfat milk in PBS containing 0.1% Tween 20 (TPBS). Thereafter, the PVDF membrane was incubated overnight at 4°C with primary antibodies in TPBS. After washing three times with TPBS, we incubated the PVDF membrane with horseradish peroxidase-conjugated anti-rabbit (Amer sham Biosciences UK, Little Chalfont, Buckinghamshire, UK), anti-mouse (Amer sham Biosciences), or anti-goat antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), diluted at 1:2,000–10,000 in TPBS at room temperature for 60 min. The blots were visualized with an enhanced chemiluminescence detection system (Amer sham Biosciences), exposed to X-ray film, and analyzed by free NIH Image software produced by Wayne Rashand (National Institutes of Health). Negligible loading or transfer variation was observed between samples.

The following antibodies, which are commercially available, were used: rabbit anti-human VEGF polyclonal antibody (Immuno-Biological Laboratories, Fujioka, Japan), rabbit anti-human Flt-1 polyclonal antibody (Santa Cruz Biotechnology), rabbit anti-human Flt-1 polyclonal antibody (Santa Cruz Biotechnology), goat polyclonal phospho-Flk-1 (Tyr951) antibody (Santa Cruz Biotechnology), mouse monoclonal HIF-1a antibody (Novus, Littleton, CO), goat polyclonal HIF-2a (EPAS-1) antibody (Santa Cruz Biotechnology), mouse monoclonal HIF-1ß antibody (Novus), and mouse monoclonal β-actin antibody (Abcam, Cambridge, UK).

Enzyme immunoassay of VEGF, VEGF receptors, and ET-1. Levels of VEGF, Flt-1, KDR, and ET-1 in cardiomyocytes were determined with ELISA kits [R&D Systems (VEGF, Flt-1 and KDR) and ImmunoBiological Laboratories (ET-1)], according to the manufacturers’ instructions.

*Statistical analysis.* Statistical assessment of the data was made by one-way ANOVA with multiple comparisons by Fisher’s protected least significant difference t-test. Nonparametric data were analyzed by the Mann-Whitney U-test or Wilcoxon’s signed rank test. P < 0.05 was considered statistically significant.

**RESULTS**

Effects of ET-1 treatment on morphology and VEGF, its two principal receptors (Flt-1 and KDR), and HIF family expression in cardiomyocytes. When cardiomyocytes were treated with ET-1 at different concentrations for 24 h, hypertrophy was observed in a concentration-dependent fashion, as assessed by the measurement of cell surface area, which showed a 2.3-fold increase at its maximum level (Fig. 1A). Furthermore, [14C]leucine uptake by cardiomyocytes was increased after ET-1 treatment (Fig. 1B). It was dependent on the concentrations of ET-1 treated and peaked at a 1.9-fold increase.

![Fig. 1. Effects of endothelin-1 (ET-1) treatment on cell surface area (A) and [14C]leucine uptake (B) in rat ventricular cardiomyocytes. Cardiomyocytes were treated with ET-1 at different concentrations for 24 h. Cell surface area and [14C]leucine uptake obtained from control cardiomyocytes are normalized as 1.0. Data are means ± SE; n = 100 (A) and n = 6 (B). *P < 0.01 and **P < 0.0001, compared with control (Con).](http://ajpheart.physiology.org/)
VEGF protein expression was upregulated in ET-1-treated cardiomyocytes by increasing the treatment concentrations of ET-1 (Fig. 2A). The protein expression level of Flt-1, which is a vascular, permeable receptor of VEGF, was also increased in ET-1-treated cardiomyocytes in a manner dependent on the treatment concentrations of ET-1 (Fig. 2B). Moreover, the protein expression level of another VEGF receptor (KDR), whose activation shows an angiogenic effect, was similarly increased in ET-1-treated cardiomyocytes (Fig. 2C).

VEGF, Flt-1, and KDR protein levels in cardiomyocytes were also determined by ELISA. These data confirmed the immunoblotting data. Thus ELISA analysis showed that treatment with 10 nM ET-1 increased VEGF levels from 161 pg/mg (n = 9) to 261 pg/mg (n = 7), Flt-1 levels from 205 pg/mg (n = 2) to 417 pg/mg (n = 3), and KDR levels from 11 pg/mg (n = 2) to 20 pg/mg (n = 2).

Although HIF-1α showed no significant change in its protein expression in ET-1-treated cardiomyocytes (Fig. 3A), HIF-1β...
and HIF-2α were upregulated with increased concentrations of ET-1 treatment to cardiomyocytes (Fig. 3, B and C).

**Effect of TA-0201 on the morphological and protein expression changes in ET-1-treated cardiomyocytes.** As depicted in Fig. 4A, cardiomyocyte hypertrophy was evident after 10−8 M ET-1 treatment for 24 h. This ET-1-induced cardiomyocyte hypertrophy was strongly prevented by the ETA-receptor-selective antagonist TA-0201 (10−6 M), which was given 30 min before ET-1 treatment. When the cell surface area was calculated, ET-1 treatment produced a 2.3-fold increase in cardiomyocyte surface area, an effect that was completely blocked by TA-0201 (Fig. 4B). Treatment with 10−6 M TA-0201 reduced the increase in [14C]leucine uptake in ET-1-treated cardiomyocytes nearly to the control level (Fig. 4C). It should be noted that there was no change in cell surface area and [14C]leucine uptake, even though TA-0201 was given to control myocytes (Fig. 4, Band C).

The protein expression levels of VEGF, Flt-1, and KDR, which were increased by treatment of cardiomyocytes with 10−8 M ET-1, were reduced nearly to the respective control level when 10−6 M TA-0201 was given in advance (Fig. 5). TA-0201 by itself did not affect protein expression of VEGF, Flt-1, and KDR in control cardiomyocytes.

**Effect of VEGF-neutralizing peptide treatment on morphological changes in ET-1-treated cardiomyocytes.** When VEGF-neutralizing peptides (5–10 μg/ml) were given 60 min before 10−8 M ET-1 treatment, the ET-1-induced increases in cell surface area and [14C]leucine uptake in cardiomyocytes were significantly and dose-dependently inhibited (Fig. 6, A and B). However, these inhibitory effects of VEGF-neutralizing peptides were not so pronounced. VEGF-neutralizing peptides at doses used in this study had no effect on cell surface area and [14C]leucine uptake in control cardiomyocytes. We also tested the effect of recombinant VEGF on cell surface area of cardiomyocyte. Treatment with recombinant VEGF increased cardiomyocyte surface area in a dose-dependent manner (Fig. 6C).

**Effects of TA-0201 and VEGF-neutralizing peptides on phosphorylation of KDR in ET-1-treated cardiomyocytes.** We examined phosphorylation of KDR as evidence for VEGF system activation (8). The phosphorylated KDR level was upregulated in ET-1-treated cardiomyocytes. This upregulation was significantly arrested by treatment with either TA-0201 or VEGF-neutralizing peptides (Fig. 7).

**DISCUSSION**

We demonstrate for the first time that ET-1 concentration-dependently increased the expression levels of VEGF and its two receptors, Flt-1 and KDR, in neonatal rat cardiomyocytes, which may be associated with upregulation of HIF-2α. ET₆
receptors appeared to play a dominant role in the ET-1-induced upregulation of the VEGF system in cardiomyocytes because treatment with the selective ET_A-receptor antagonist TA-0201 blocked this upregulation. Finally, ET-1-induced cardiomyocyte hypertrophy, which was also mediated through ET_A receptors, was slightly but significantly inhibited by VEGF-neutralizing peptides, suggesting that the upregulated VEGF system may contribute, at least in part, to the development of cardiomyocyte hypertrophy by ET-1.

In the present study, cardiomyocyte hypertrophy was observed when treated with ET-1 at concentrations over $10^{-10}$ M. This is compatible with our recent data (27).
have represented many lines of published evidence that ET-1 production is markedly increased in the hypertrophied heart and the failing heart (19, 22-24, 37, 39). We have demonstrated that treatment with ETA-receptor antagonists can significantly block the development of cardiac hypertrophy and phenotypic transition in the failing heart of monocrotaline-induced pulmonary hypertensive rats (19) and cardiomyopathic hamsters (37).

In this study, we show that ET\(_A\) receptor blockade with TA-0201 put back the protein expression levels of the VEGF system and the phosphorylation level of KDR in ET-1-treated cardiomyocytes. In the present study, the VEGF system, including Flt-1, was upregulated in ET-1-treated cardiomyocytes. Furthermore, we found that TA-0201 suppressed not only overexpression of the VEGF system but also cardiomyocyte hypertrophy induced by ET-1. In light of experimental evidence that cardiomyocyte-specific deletion of the VEGF gene results in ventricular thinning, dilatation, and contractile dysfunction in the heart (12), we interpret this observation to assume that the upregulated VEGF system may be closely associated with the development of hypertrophy in ET-1-treated cardiomyocytes.

We found that the expression levels of HIF-1\(\beta\) and HIF-2\(\alpha\) proteins were increased with the increase in the concentration of ET-1 given to cardiomyocytes. However, no significant change in HIF-1\(\alpha\) protein expression was observed when cardiomyocytes were treated with ET-1. HIF-1\(\alpha\) and HIF-1\(\beta\) are the subunits composing HIF-1, which is a transcription factor under hypoxic conditions (33). Whereas HIF-1\(\beta\) is constitutively expressed, HIF-1\(\alpha\) is a specific factor responsible for hypoxic responses. Under hypoxic conditions, HIF-1\(\alpha\) protein is stabilized without being degraded through oxygen-dependent proteolysis and initiates a multistep pathway of activation, including dimerization with its partner HIF-1\(\beta\) (34).

HIF-2\(\alpha\) is also subject to oxygen-dependent proteosomal destruction and binds as a heterodimer with HIF-\(\beta\) subunit to the hypoxia-response element in the promoter of target genes (35). Gene-inactivation studies have revealed a role of HIF-2\(\alpha\) in cardiovascular development and angiogenesis in the embryo (20, 32). Despite the report that ET-1 induces VEGF by increasing HIF-1\(\alpha\) accumulation in ovarian carcinoma cells (28), whether ET-1 increases HIF-2\(\alpha\) accumulation and activates the HIF-2 transcription complex is unknown. Thus, to the best of our knowledge, this is the first report demonstrating that treatment of cardiomyocytes with ET-1 resulted in an increase in HIF-2\(\alpha\) expression, which may be implicated as one of the potential mechanisms for ET-1-induced VEGF production. Although ET-1 is a well-known target gene of HIF-1\(\alpha\) (25), HIF-binding sites within hypoxia-response elements of the ET-1 gene, which reveal a sequence distinct from the tentative HIF-1 consensus sequence, have been suggested to represent a consensus recognition sequence specific for HIF-2 (3). Interestingly, pulmonary ET-1 levels have been documented to be markedly upregulated during hypoxia in wild-type but not in HIF-2\(\alpha\)-deficient mice (2). Together, there may be a positive feedback loop between ET-1 and HIF-2\(\alpha\).

A potential role of VEGF in ET-1-induced cardiomyocyte hypertrophy was tested with VEGF-neutralizing peptides and human recombinant VEGF. We showed that human recombinant VEGF was capable of inducing cardiomyocyte hypertrophy in a dose-dependent manner. When given at doses of 5 and 10 \(\mu\)g/ml, VEGF-neutralizing peptides did not affect cell surface area and \(^{14}\)C]leucine uptake by cardiomyocytes in the absence of ET-1. However, the peptides showed a significant dose-dependent inhibition of these variables that were increased by ET-1. This suggests VEGF-neutralizing peptides were able to inhibit ET-1-induced cardiomyocyte hypertrophy. Nevertheless, the inhibitory effect of VEGF-neutralizing peptides was not so pronounced. It should be noted that the doses of VEGF neutralizing peptides employed in this study can cause a complete inhibition of VEGF-associated angiogenesis in rat corneas (21), human choroidal endothelial cells (11), and quail cultured embryonic hearts (40). We thus suggest an accessory role of VEGF in the development of cardiomyocyte hypertrophy induced by ET-1.

It has to be considered that this in vitro study was performed with cardiomyocytes without endothelial cells and fibroblasts. VEGF may be produced by numerous different cell types, and its expression could be induced by a variety of bioactive substances, including ANG II. We are thus aware that this in vitro study does not simply predict the regulatory mechanism of VEGF in cardiac hypertrophy in vivo and that further work is necessary to confirm the broader applicability of this observation.

In conclusion, treatment of neonatal rat cardiomyocytes with ET-1 resulted in concentration-dependent hypertrophy through activation of ET\(_A\) receptors. ET-1 treatment also caused an ETA-receptor-mediated increase in expression of VEGF and its two principal receptors, Flt-1 and KDR. This upregulation of the VEGF signaling system may be associated with the increased production of the transcription factor HIF-2\(\alpha\) protein. Our present finding that VEGF-neutralizing peptides showed a slight but significant inhibition of ET-1-induced increases in cell surface area and \(^{14}\)C]leucine uptake by cardiomyocytes suggests that the upregulated VEGF signaling system appears
to contribute, at least in part, to ET-1-induced cardiomyocyte hypertrophy.

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