nor, *S*-Nitroso-*N*-acetylpenicillamine (SNAP), on the nonhypoxic induction of HIF-1α and the VEGF production in cardiomyocytes, using the primary cultured rat cardiomyocytes (PRCMs).

MATERIALS AND METHODS

Reagents. Reagents including the NO donor, *S*-nitroso-*N*-acetylpenicillamine (SNAP), acetylcholine (ACh), a phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin, a specific nitric oxide synthase inhibitor, N^G-nitro-L-arginine methyl ester (L-NAME), and a transcriptional inhibitor, actinomycin D, were purchased from Sigma (Sigma Chemical Co., St. Louis, Missouri, USA).

Cell culture. This study followed the guidelines of the Council for Animal Care and was approved by an ethical committee of the Laboratory Animal Center, Kochi Medical School, Nankoku, Japan. According to the guideline, the Wistar rats used in this study were sacrificed. Primary cultured rat cardiomyocytes (PRCMs) were isolated from the hearts of 2-day-old neonatal rats and incubated on a gelatin-coated dish in DMEM/Ham F12 medium including 10% horse serum and ITS supplement according to our previous studies [17]. H9c2 cells have been frequently used to study the signal transductions and channels [18, 19]. H9c2 cells have been established as cell lines derived from the rat ventricular myocytes and thus far are widely used for many biological, biochemical, and electrophysiological studies because they have characteristics similar to PRCMs. Therefore they have often been utilized instead of PRCMs in studies where tons of rat cardiomyocytes are indispensable to perform experiments. To prepare many neonatal PRCMs for RNA isolation followed by RT-PCR, we used H9c2 cells, which, along with HEK 293, derived from human embryonic kidney cells, were incubated in DMEM supplemented with 10% FBS with antibiotics. To examine the effect of SNAP, cardiomyocytes in the serum-deficient medium were treated with either 1 μM (PRCMs, HEK 293 cells) or 1 mM (H9c2 cells) of SNAP.

Determination of NO from cardiomyocytes. To determine whether ACh and SNAP release NO in cardiomyocytes, we used an NO-sensitive fluorescent dye, diaminofluorescein-2 (DAF-2) (Daiichi Pure Chemicals Co. Ltd., Tokyo, Japan) [20]. PRCMs were treated with 10 μM DAF-2 and 100 μM L-arginine for 60 min, followed by 1 μM SNAP or 1 mM ACh. To examine the effect of L-NAME on NO production, the PRCMs were first pretreated with 1 mM L-NAME for 60 min, followed by the addition of DAF-2 and L-arginine. After incubation at 37°C, the cells were washed with PBS and observed under a fluorescence microscopy.

Western blotting analysis. To investigate the signal transduction pathway from SNAP to VEGF, we evaluated the effect of wortmannin (30 nM), actinomycin D (0.5 μg/ml), and L-NAME (1 mM) on Akt, HIF-1α, and VEGF by immunoblotting assay [21, 22]. Cardiomyocytes were pretreated with one of these agents prior to the addition of SNAP. After the incubation with SNAP, the cells were lysed and the total proteins isolated. The samples were then fractionated by 10% SDS-PAGE and transferred onto a PVDF membrane. Immunoblotting was performed with the primary antibodies against HIF-1α, VEGF (Santa Cruz Biotechnology, Santa Cruz, California, USA), Akt, phospho-Akt (Cell Signaling Technology, Beverly, Massachusetts, USA), or tubulin-α (Lab Vision, Fremont, California, USA), and was then reacted with an appropriate HRP-conjugated secondary antibody. The signal was detected with an enhanced chemiluminescence system (ECL Plus, Amersham, Piscataway, New Jersey, USA). Each experiment was performed in a duplicated fashion and repeated five times ($n = 5$), and representative data were shown.

Transfection. To investigate the direct contribution of HIF-1α to VEGF expression, HEK 293 cells were transfected with an expression vector for dominant-negative HIF-1α (dn HIF-1α) [23], using Effectene (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. HEK293 cells are derived from human embryonic kidney cells. It is known that the transient transfection of PRCMs with a conventional method is difficult and that the efficacy is extremely low. Compared with PRCMs, HEK293 cells have been extensively used for the transient transfection of an interested gene because of the extremely high efficiency of transfection and the higher protein expression level. Therefore we used HEK293 cells. Thirty-six hours after transfection, the HEK 293 cells were pretreated with 1 μM SNAP for 12 h, followed by an evaluation of the VEGF protein level. As a control, the cells were transfected with a vector for green fluorescent protein (GFP).

Reverse transcription-PCR (RT-PCR). RNA isolation and RT-PCR were performed as described earlier [17]. The synthesized cDNA was amplified with gene-specific primers for HIF-1α, VEGF, and Glut-1, as well as β-actin. The sense and antisense gene-specific primers were as follows:

HIF-1α (sense), 5'-GGGAGAAAAGCAAGTCGTG-3',
HIF-1α (antisense), 5'-AGTCAGCAACGTGGAAGG-3';
VEGF (sense), 5'-CCAGCACATAGGAGAGATGAGCTTC-3',
VEGF (antisense), 5'-GGTGTGGTGGTGACATGGTTAATC-3';
Glut-1 (sense), 5'-ACACCTCCCCCACATACATG-3',
Glut-1 (antisense), 5'-TGGAGTTTGGCTATAACACC-3';
β-actin (sense), 5'-GAAGATCCTGACCGAGCGTG-3',
β-actin (antisense), 5'-CGTACTCCTGCTTGCTGATCC-3'.

The optimal annealing temperature and the number of cycles for each template is as follows: 54°C, 30 cycles for HIF-1α; 62°C, 34 cycles for VEGF; 62°C, 36 cycles for Glut-1; and 60°C, 32 cycles for β-actin. PCR was performed in the range that gave a linear correlation between the amount of cDNA and the yield of PCR products. The

ratio of the RT-PCR product for each gene to that of β-actin was quantified and compared.

Immunohistochemistry. After SNAP treatment, H9c2 cells were fixed with 4% paraformaldehyde for 10 min and treated with 1% Triton X-100 for another 10 min. To block nonspecific antibody binding, the cells were incubated with 5% skim milk and successively incubated with a VEGF antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA) in 1% skim milk at 4°C overnight and an FITC-labeled secondary antibody (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) at 4°C overnight, then examined with an immunofluorescence microscope.

Human umbilical vein endothelial cells (HUVECs) culture. To understand if NO induces the cardiomyocytes to produce a factor responsible for angiogenesis, we examined the effect of conditioned medium derived from H9c2 cells treated with SNAP on HUVECs. The HUVECs were cultured in EGM-2 culture medium supplemented with angiogenic and growth factors (Cambrex Bio Science Walkersville, Inc., Walkersville, Maryland, USA). The H9c2 cells were treated with SNAP for 2 h and then incubated in the serum-free fresh medium. After 10 hours, the supernatant was collected and added to the HUVECs by replacing EGM-2 medium. The samples were collected before and after 60 min of stimulation with conditioned medium to evaluate the phosphorylation of VEGF receptor (Flk-1), using anti–pFlk-1 antibody (Santa Cruz Biotechnology, Santa Cruz, California, USA).

To further investigate the angiogenic effect of the conditioned medium derived from cardiomyocytes, the HUVECs were cultured on Matrigel (Becton Dickinson Labware, Bedford, Maryland, USA). The 96-well plates were coated with the diluted Matrigel (50 μl/well), incubated at 37°C for 1 h, then washed with serum-free DMEM. The HUVECs (1×10^4 cells) were seeded onto each well and cultured at 37°C for 10 h in DMEM, supplemented with 20% FBS, 25 μg/ml endothelial cell growth supplement (ECGS), 10 U/ml heparin, and conditioned medium derived from SNAP-treated or nontreated H9c2 cells.

Statistical analysis. Data are presented as mean ± SE. The differences were assessed by ANOVA followed by Fisher's PLSD for multiple comparisons. The results were considered statistically significant at $p < 0.05$.

RESULTS

A nonhypoxic induction of HIF-1α by NO through PI3K-Akt pathway

ACh or SNAP treatment rapidly increased the NO release in PRCMs within 30 min (Fig. 1); the release was continued and peaked at 8 h. In contrast, the cells pretreated with a nitric oxide synthase inhibitor L-NAME (1 mM) failed to show the NO signal (Fig. 1). The HIF-1α protein

PRCM

0 min
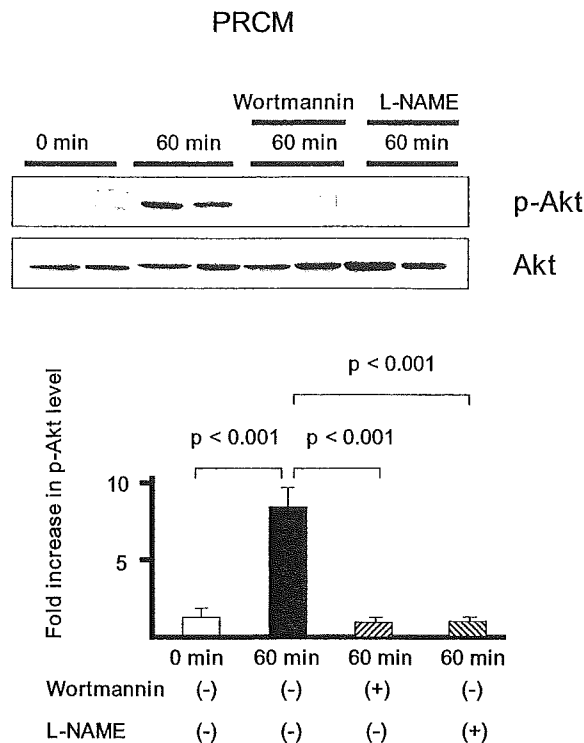

ACh 30 min SNAP 30 min

L-NAME + ACh L-NAME + SNAP
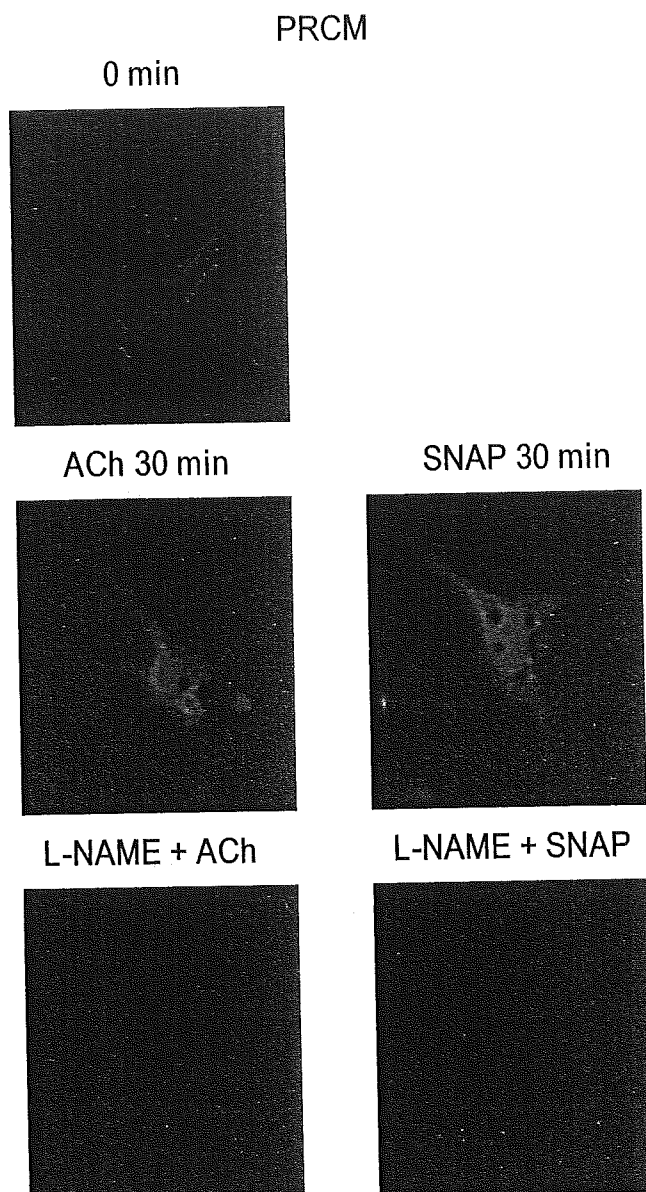
Fig. 1. Rat primary cardiomyocytes release NO in response to ACh or SNAP. PRCMs released NO after treatment with 1 mM ACh or 1 μM SNAP, evaluated with DAF-2. NO release was observed within 30 min after ACh or SNAP treatment (n = 3). Pretreatment with 1 mM L-NAME for 60 min blocked NO production (n = 3).

expression was gradually increased within 8 h since the SNAP treatment (a fivefold increase compared to the baseline (0 h), $p < 0.001$, $n = 5$) in PRCMs under normoxic conditions, thus confirming the occurrence of a nonhypoxic pathway for the HIF-1α induction in the cardiomyocytes (Fig. 2a). Such an induction of HIF-1α was also observed in H9c2 cells (data not shown). To understand if this induction is regulated at the transcriptional level, we pretreated cardiomyocytes with a commonly used transcriptional inhibitor, actinomycin D (0.5 μg/ml), followed by stimulation with SNAP for 8 h. However, actinomycin D failed to inhibit the HIF-1α induction by SNAP (Fig.
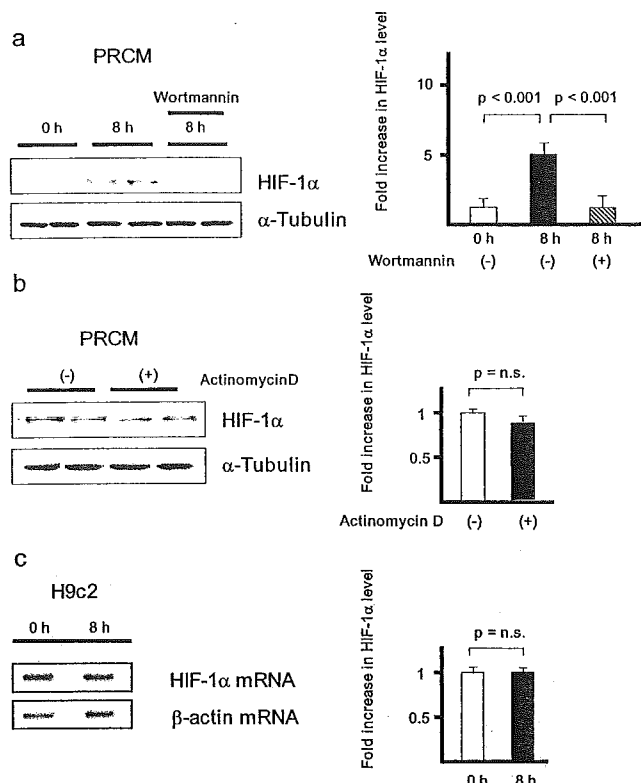
Fig. 2. The HIF-1α protein expression level is increased by SNAP in cardiomyocytes in normoxia. Treating the PRCMs for 8 h with SNAP (1 μM) already increased HIF-1α protein expression in normoxia. Pretreatment of PRCMs with wortmannin (30 nM) for 30 min inhibited SNAP-induced HIF-1α expression (n = 5) (**a**). However, treatment with actinomycin D (0.5 μg/ml) for 15 min did not inhibit the upregulation of HIF-1α protein expression by SNAP (n = 5) (**b**). In H9c2 cells, the HIF-1α mRNA expression level was not increased by SNAP (n = 5) (**c**).


Fig. 3. Akt phosphorylation is increased by SNAP in cardiomyocytes under normoxia. Akt phosphorylation was increased by SNAP (1 μM) in PRCMs with a rapid time course. However, pretreatment with wortmannin (30 nM) for 60 min or L-NAME (1 mM) for 60 min completely inhibited the Akt phosphorylation in cardiomyocytes (n = 5).

Promotion of angiogenic signaling cascade by NO in cardiomyocytes under normoxia

2b), and SNAP further did not increase the HIF-1α mRNA level, evaluated by RT-PCR (Fig. 2c), thus suggesting that SNAP induces HIF-1α posttranslationally in normoxic conditions. Western blotting analysis further revealed an increased Akt phosphorylation with SNAP treatment for 60 min compared to the baseline (0 min) (an eightfold increase from the baseline, $p < 0.001$, n = 5) in PRCMs (Fig. 3). Pretreating the cells with PI3K inhibitor wortmannin (30 nM) or nitric oxide synthase inhibitor L-NAME (1 mM) prevented the SNAP-induced Akt phosphorylation (Fig. 3), thus demonstrating an important role for PI3K and NO in the Akt signaling pathway. Even though wortmannin (30 nM) was able to inhibit the SNAP-induced Akt or HIF-1α induction, it failed to block the NO release by the SNAP-treated cardiomyocytes (data not shown), thus confirming that NO remains upstream to the PI3K-Akt pathway. Moreover, these results also suggest the NO-dependent induction of HIF-1α in the cardiomyocytes under normoxic conditions.

To identify if SNAP-induced HIF-1α actually affects transcriptional activation of the target genes, the gene expression levels of the Glut-1 and VEGF were evaluated by the use of RT-PCR. The treatment of H9c2 cells with SNAP for 12 h under normoxic conditions increased the gene expressions of Glut-1 and VEGF, major HIF-1α–regulated genes (Fig. 4a). The protein expression level of VEGF was also increased following SNAP treatment, as demonstrated by the immunohistochemical and Western blotting analysis (Fig. 4 b and c). Consistent with the earlier findings, wortmannin was also able to inhibit the SNAP-induced VEGF expression in H9c2 cells and PRCMs (Fig. 4c), thus suggesting the PI3K-Akt mediated HIF-1α induction pathway in the production of VEGF by the cardiomyocytes under normoxic conditions. Furthermore, to elucidate the contribution of HIF-1α to VEGF protein expression, dn HIF-1α was introduced into HEK293 cells, and it was demonstrated that dn HIF-1α partially inhibits the VEGF induction by SNAP (Fig. 4d).

VEGF production in cardiomyocytes was further confirmed by an addition of conditioned medium derived from SNAP-treated or nontreated H9c2 cells to the HUVECs. As expected, the conditioned medium-treated cells
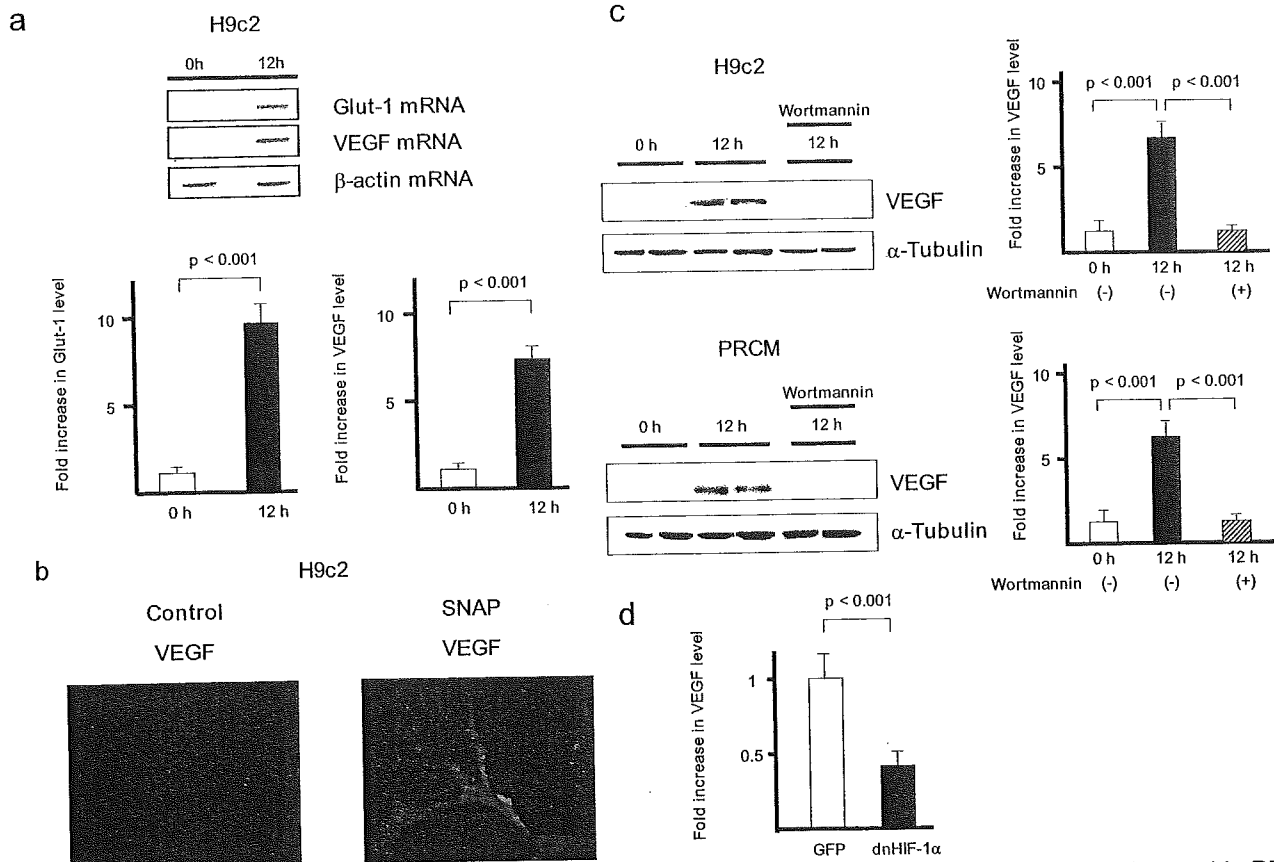
Fig. 4. SNAP increases Glut-1 and VEGF gene expression levels through HIF-1α in cardiomyocytes under normoxia. In H9c2 cells, Glut-1 mRNA and VEGF mRNA were both increased by SNAP ($n = 5$) (**a**). VEGF immunoreactivity was increased by SNAP in H9c2 cells ($n = 5$) (**b**). The SNAP-induced VEGF protein expression, which was also observed in PRC-Ms, was completely inhibited by 30 nM wortmannin ($n = 5$) (**c**). In contrast to control (GFP), VEGF induction by SNAP was blocked by dn HIF-1α in HEK293 cells ($n = 5$) (**d**).

(SNAP group) revealed increased Flk-1 phosphorylation (a fourfold increase compared to the control group, $p < 0.001$, $n = 5$), a VEGF type-2 receptor responsible for angiogenesis, in HUVECs (Fig. 5a). Furthermore, HUVECs were cultured on Matrigel in the presence of conditioned medium. Compared with the control group, the SNAP group activated more angiogenesis. It is suggested that SNAP exerts an acceleration of angiogenesis partially via cardiomyocyte-derived angiogenic factors, including VEGF (Fig. 5b).

DISCUSSION

It is well known that NO plays a critical role in modulating the vascular tone. According to the vascular effect, the depressed functional capacity of NO production would result in vasoconstriction and poor collateral circulation. Therefore NO or a NO donor has been used for coronary vasodilatation and decreasing blood pressure in systemic or pulmonary hypertension. However, the other effect of NO or a NO donor on cardiomyocytes remains to be fully investigated. It is known that NO is synthesized through eNOS in endothelial cells, and it is speculated that it has a significant paracrine effect on cardiomyocytes; however, it is unclear whether cardiomyocyte-derived NO possesses the direct action on cardiomyocytes to produce angiogenic factors.

Our previous study demonstrated the involvement of PI3K-Akt pathway in inducing the expression of HIF-1α by ACh during normoxia [4]. In the present study, SNAP-treated cardiomyocytes revealed a similar pathway in the induction of HIF-1α, suggesting that NO from cardiomyocytes activates an angiogenic signaling through HIF-1α.

As shown in the present study using DAF-2, a NO-sensitive dye, NO was detected in cardiomyocytes in response to SNAP as well as ACh, suggesting that cardiomyocytes release NO. The NO release by SNAP appeared in a rapid time course 30 min after the treatment, and it was not detected in PRCMs pretreated with L-NAME. Other studies have also reported the inhibitory effects of L-NAME on SNAP without the exact mechanisms being identified [24–27]; however, the speculated mechanism could be that the L-NAME pretreatment for 60 min of PRCMs might inhibit NO synthase, thereby reducing the basal NO production. Even if SNAP was thereafter added for 30 min to enhance NO release, the NO level in

a HUVEC

Control group		SNAP group	
0 min	60 min	0 min	60 min

p-Flk-1

α-Tubulin
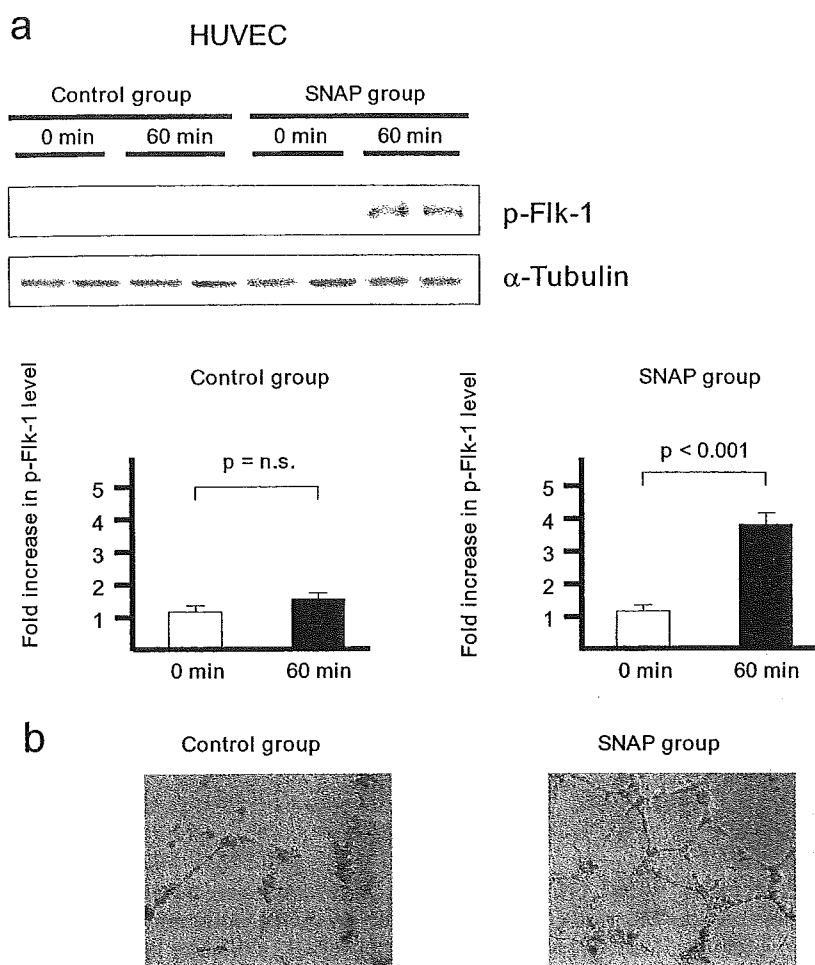
Fig. 5. VEGF derived from SNAP-treated cardiomyocytes induces angiogenesis. The SNAP-treated conditioned medium increased Flk-1 phosphorylation in HUVECs, compared with the nontreated conditioned medium ($n = 5$) (a). The SNAP-treated conditioned medium accelerated angiogenesis in HUVECs compared with the nontreated conditioned medium (b).

Control group — p = n.s. — 0 min / 60 min

SNAP group — $p < 0.001$ — 0 min / 60 min

b

Control group   SNAP group

the treated cardiomyocyte might be too low, compared with the nontreated cell, to be detected by DAF-2. Therefore, these results suggest that cardiomyocyte-derived NO as a paracrine or autocrine effector plays a critical role in the HIF-1α induction in cardiomyocytes.

Second, as shown in this study, NO increased the cardiac VEGF protein expression through HIF-1α regulation, and dn HIF-1α decreased the VEGF expression by SNAP. VEGF itself has been reported to be involved in cell survival through the tyrosine kinase receptors, including VEGF type-2 receptor (Flk-1), activating Akt via a PI3K-dependent pathway [28], leading to eNOS upregulation. Furthermore, as suggested in our study, the cardiomyocyte-derived VEGF plays a crucial role in accelerating angiogenesis by endothelial cells in a paracrine fashion because VEGF produced by cardiomyocytes phosphorylated Flk-1 in HUVECs. These results suggest that cardiomyocytes can not only be a target for a NO donor to activate a nonhypoxic pathway of HIF-1α, but can also play a role in producing angiogenic factors in the heart. Taken together, the beneficial effects of NO might in part be a result of the cell signaling through PI3K-Akt, and also in part a result of the angiogenic signaling through HIF-1α–VEGF.

In the recent study by Giordano et al. [29], a cardiomyocyte-specific knockout of VEGF caused impaired cardiac development with hypovascularity in the heart, suggesting that cardiomyocyte-induced VEGF production is essential for cardiac development; however, their study did not reveal the precise cellular mechanism by which cardiac VEGF deletion leads to hypovasculature and depressed cardiac function. Our present study indicated that HIF-1α induction through NO plays a main role in stimulating VEGF production by cardiomyocytes and accelerates angiogenesis.

In this study we focused on HIF-1α as an upstream factor regulating cardiac VEGF expression. Unlike the hypoxic induction pathway of HIF-1α, there is no direct evidence for a nonhypoxic induction pathway of cardiomyocytes through NO involved in angiogenesis. Consequently, this study revealed another pathway of cardiac HIF-1α induction. PI3K-Akt signal has many aspects in cell survival, including an antiapoptotic activity, such as an inhibition of Bad-binding to Bcl-2 through Akt phosphorylation, an inhibition of proapoptotic caspases, including caspase 9 and Fas, and an inhibition of the activity of proapoptotic glycogen synthetase kinase-3 [30, 31]. In previous studies, which used other cell lines, the PI3K-

Akt pathway has been demonstrated to be involved in the NO-dependent stabilization of HIF-1α [14, 32–34]. As demonstrated in this study, in the presence of actinomycin D, the dose of which is adequate to inhibit transcriptional activity, SNAP posttranslationally regulated HIF-1α. Actinomycin D was used to identify which mechanisms are responsible for the increased protein expression, i.e., de novo synthesis or posttranslational modification. The protein level of HIF-1α was not decreased by actinomycin D; therefore this suggests that SNAP does not play a role in the transcriptional regulation of HIF-1α, rather in the inhibition of protein degradation. Therefore in cardiomyocytes, such a mechanism might be involved in a NO-mediated Akt-HIF-1α-VEGF signaling pathway, leading to cell protection.

In conclusion, it is suggested that NO has beneficial effects on cardiomyocytes by the activation of the nonhypoxic HIF-1α induction pathway, and furthermore, it contributes to angiogenesis through cardiac VEGF production, which phosphorylates Flk-1, a VEGF type-2 receptor.

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