

Figure 1. Schematic illustration (A) and block diagram (B) of a BBS. In the context of central baroreflex failure, the BBS automatically computes the frequency (STM) of a pulse train to stimulate sympathetic nerves through an epidural catheter placed at the level of lower thoracic spinal cord, while simultaneously sensing the change in AP. $H_{AP \rightarrow STM}$ denotes a transfer function for the controller functioning as an artificial vasomotor center. $H_{STM \rightarrow AP}$ is a transfer function showing the dynamic response of AP to STM. The overall transfer function of the BBS is given by $H_{AP \rightarrow STM} \times H_{STM \rightarrow AP}$. Therefore, the effect of an external disturbance (P_d) on AP is attenuated to $1/(1 + H_{AP \rightarrow STM} \times H_{STM \rightarrow AP})$.

cuts real-time operations that determine the frequency of electrical stimulation (STM) required to minimize the effect of an external disturbance (P_d) on AP and then commands an electrical stimulator to deliver a stimulus of the same frequency to the vasomotor sympathetic nerves via epidural-catheter electrodes placed at the lower thoracic level of the spinal cord. The lower thoracic level was selected as the site for the neural interface of the BBS because the abdominal splanchnic vascular bed is a major effector mechanism for the arterial baroreflex.²³⁻²⁵

According to a classic feedback-control theory, ie, feedback correction with proportional and integral gain factors,^{26,27} the following algorithm was used to program the controller for the calculation of STM in the frequency domain:

$$(1) \quad H_{AP \rightarrow STM} = K_p + \frac{K_i}{2\pi f j}$$

where $H_{AP \rightarrow STM}$ is a transfer function from AP to STM, K_p is the proportional correction factor, K_i is the integral correction factor, and j is the imaginary unit. The proportional factor determines the feedback amplification based on the absolute value of the instantaneous control error due to P_d , and the integral factor adjusts the feedback amplification based on the cumulative value of the instantaneous control error. Therefore, STM is computed as follows:

$$(2) \quad STM = -AP \cdot H_{AP \rightarrow STM}$$

and AP is also expressed as follows:

$$(3) \quad AP = STM \cdot H_{STM \rightarrow AP} + P_d$$

where $H_{STM \rightarrow AP}$ denotes the frequency response of AP to STM. From Equations 2 and 3, the effect of P_d on AP is estimated as follows:

$$(4) \quad AP = \frac{1}{1 + H_{AP \rightarrow STM} \cdot H_{STM \rightarrow AP}} P_d$$

Thus, if $H_{AP \rightarrow STM} \cdot H_{STM \rightarrow AP}$ is far larger than unity, the BBS can nullify the effect of P_d on AP.

Subjects and Experimental Protocols

A total of 33 patients (46 to 84 years old, 19 males) who underwent orthopedic operations were enrolled in the present study. Ten patients had hypertension, and 4 had diabetes mellitus. None of the subjects had frequent ectopic beats or atrial fibrillation. After induction anesthesia with propofol, an endotracheal tube was introduced orally. The patients were mechanically ventilated with 67% nitrous oxide and 1.5% to 2% end-tidal sevoflurane in oxygen during experimental protocols, while end-tidal carbon dioxide was maintained at 35 to 38 mm Hg. An arterial catheter was placed in the radial artery for AP measurement. To record central venous pressure (CVP), a central venous catheter was placed in the femoral vein, and the tip of the catheter was advanced into the inferior vena cava just above the diaphragmatic level. Furthermore, an epidural catheter was placed percutaneously, and the tip, which contained a pair of electrodes (Unique Medical, Tokyo; interelectrode distance 15 mm), was placed at the level of Th_{10-11} . Placement of the central venous catheter and the epidural catheter was verified by chest radiograph.²⁸

Before making an incision of affected areas, we performed 2 different protocols in separate groups of patients. In the first group of patients ($n=12$, 46 to 76 years old, 7 males) undergoing operations for cervical spondylosis and canal stenosis, the averaged $H_{STM \rightarrow AP}$ was estimated and the $H_{AP \rightarrow STM}$ was designed parametrically with Equation 1 to minimize the effect of P_d on AP. After we programmed the designed $H_{AP \rightarrow STM}$ into the computer, the efficacy of the BBS was tested against the rapid progressive hypotension induced by use of a thigh tourniquet²⁹⁻³¹ in the second group of patients ($n=21$, 64 to 84 years old, 12 males) undergoing operation for knee joint osteoarthritis. During each protocol, the muscle twitches induced by spinal cord stimulation were prevented by the intravenous administration of vecuronium bromide. Analgesia for the pain provoked by spinal cord stimulation and tourniquet inflation was provided by intravenous injection of fentanyl citrate. In a preliminary study, the validity of the analgesic preparation was confirmed for the experimental protocols, and the safety of spinal cord stimulation for 20 minutes was verified.

Estimation of Transfer Function From STM to AP

To characterize the dynamic nature of the AP response to STM, ie, $H_{STM \rightarrow AP}$, the lower thoracic sympathetic nerves were randomly stimulated for 15 minutes while we recorded AP. According to a white noise method for system identification, the STM was altered between 0 and 20 Hz every 4 seconds. The pulse width of electrical stimuli was fixed at 0.1 ms. The stimulation current was adjusted for each patient so as to produce a pressor response of ≈ 10 mm Hg at 20 Hz. This resulted in an average current of 15 ± 4 (mean \pm SD) mA. The electrical signals of STM and AP were digitized at 100 Hz. As described previously,²⁰⁻²² the transfer function from STM to AP, $H_{STM \rightarrow AP}$, was estimated with a fast Fourier transform algorithm. Finally, the average of $H_{STM \rightarrow AP}$ among 12 patients was calculated.

Design of Artificial Vasomotor Center

With substitution of the averaged $H_{STM \rightarrow AP}$ for Equation 4, the instantaneous AP response to P_d was simulated numerically, and a stepwise decline with an amplitude of 20 mm Hg was imposed on the BBS. While the feedback parameters of $H_{AP \rightarrow STM}$, ie, K_p and K_i , were altered, the effect of the parameters on the AP response was investigated. Finally, the parameters that enabled the BBS to quickly and stably minimize the effect of P_d on AP were determined.

Efficacy of BBS in a Clinical Model of Transient Hypotension

The performance of the BBS was evaluated in a clinical model of rapid transient hypotension ($n=21$). Rapid hypotension was evoked by the sudden deflation of a thigh tourniquet, which is widely used to achieve bloodless dissection during total knee arthroplasty.²⁹⁻³¹ Acute hypotension immediately after tourniquet release is a well-

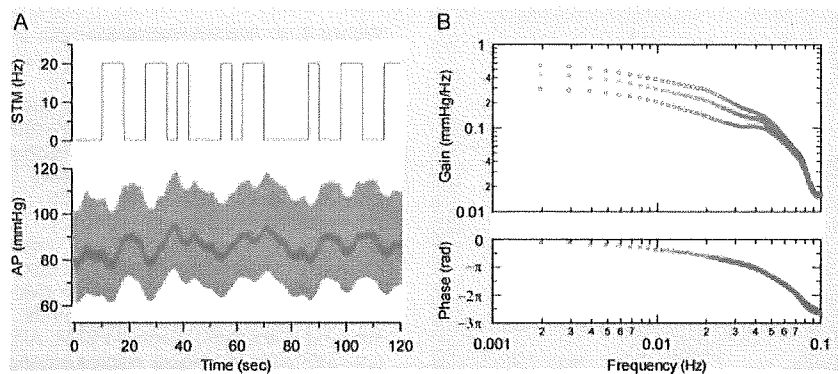


Figure 2. A, Representative example of time series data of the response of AP to random stimulation of the lower thoracic spinal cord. According to quasi-white noise, the STM was randomly altered between 0 and 20 Hz. The AP seems to slowly respond to STM with a delay. B, Transfer function of the AP response to the STM change. Data are expressed as mean \pm SD for 12 patients. rad indicates radians. See text for explanation.

known phenomenon that results from a rapid decrease in peripheral vascular resistance and an increase in venous pooling in the affected limb.²⁹ The degree of hypotension can be potentiated by the use of volatile anesthetic agents such as sevoflurane, which are central depressants of arterial baroreflex function.^{32,33} Therefore, tourniquet-related hypotension during sevoflurane anesthesia can be used as a model of orthostatic hypotension in central baroreflex failure.

Briefly, a tourniquet was applied to the upper femur and inflated at 300 mm Hg for 60 minutes and then quickly deflated for 10 minutes. The procedure was then repeated. The BBS was activated during 1 of the 2 trials of tourniquet-related hypotension, and the electrical signals of STM, CVP, and AP were digitized at 100 Hz.

Statistical Analysis

The hemodynamic responses to tourniquet release were measured for each subject while the BBS was being activated and inactivated. The effects of the BBS execution on the hemodynamic changes at 10, 50, and 100 seconds after tourniquet release were analyzed by paired *t* tests with Bonferroni adjustment. Differences were considered significant at overall $P < 0.05$.

Results

A representative example of original tracings of STM and AP during random stimulation of the spinal cord is shown in Figure 2A. Random on-off change in STM produced a delayed and slow change in AP. The relationship between STM and AP was quantitatively characterized by the frequency domain analysis (Figure 2B). The averaged transfer

function from STM to AP, $H_{STM \rightarrow AP}$, had low-pass characteristics with a corner frequency of 0.06 Hz. The gain factor was 0.43 ± 0.13 mm Hg \cdot Hz⁻¹ at the steady state (lowest frequency) and gradually decreased with input frequency. The phase spectrum showed that the input-output relationship was in phase and that the phase delay increased toward higher frequencies. The squared coherence, a measure of linear dependence between STM and AP, was > 0.9 in the frequency range of interest (data not shown).

The results of simulation for the design of the artificial vasomotor center, $H_{AP \rightarrow STM}$, are presented in Figure 3. The AP responses to the external disturbance P_d were simulated under 12 different combinations with feedback correction factors. Without feedback compensation, ie, when both feedback correction factors were zero, there was no attenuation of the effect of the external disturbance on AP. Therefore, AP fell by 20 mm Hg immediately after the imposition of P_d (Figure 3A, black line). By contrast, if either or both of the correction factors were too large, the underdamped oscillatory response of AP appeared, and the BBS became unstable. On the basis of these results, K_p was set at 1, and K_i was set at 0.1, so that the BBS could quickly and effectively attenuate the effect of the external disturbance (Figure 3B, red line).

A representative example of the results of the performance tests of the BBS is shown in Figure 4A. A sudden

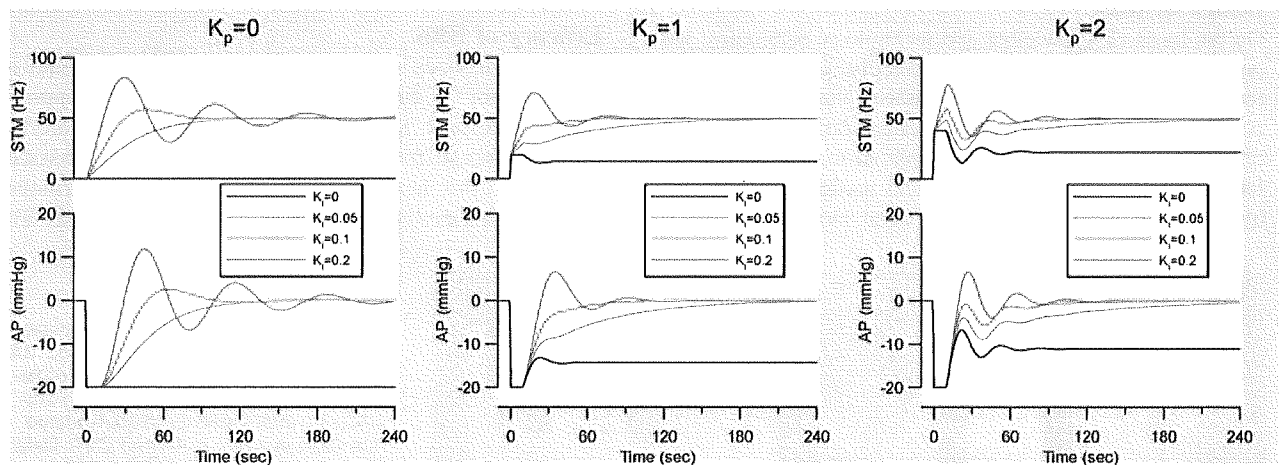


Figure 3. Numerical simulations of a feedback controller of the BBS. A stepwise pressure decline with an amplitude of 20 mm Hg is assumed to be imposed. Results are shown for 12 combinations of proportional (K_p) and integral (K_i) correction factors. See text for explanation.

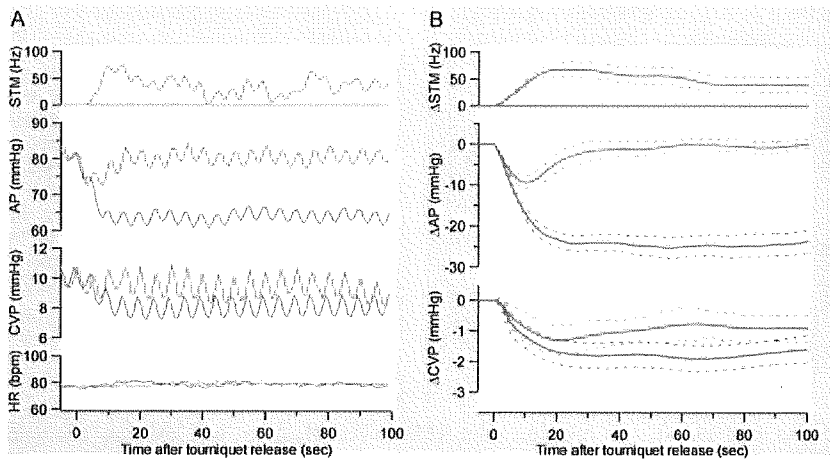


Figure 4. A, Representative example of original tracings of STM, AP, CVP, and heart rate (HR) during 2 episodes of rapid progressive hypotension induced by sudden deflation of a thigh tourniquet in a patient. When the BBS was inactive (blue line), AP decreased immediately after tourniquet release and did not return to baseline level. By contrast, when the BBS was activated (red line), the artificial vasomotor center automatically computed STM and drove an electrical stimulator to restore AP. B, Plots showing averaged changes in STM, AP, and CVP after tourniquet release among 21 patients. Data are expressed as mean (solid line)±SD (dotted line). See text for explanation.

deflation of the thigh tourniquet produced a rapid progressive fall in AP of ≈ 20 mm Hg within 10 seconds, while lowering CVP by 2 mm Hg. By contrast, when the BBS was activated, STM was computed automatically, and the spinal cord was stimulated appropriately to quickly and effectively attenuate the drop in AP and CVP. Figure 4B summarizes the results obtained from 21 patients, demonstrating effectiveness of the BBS performance in buffering the AP fall in response to the sudden release of the tourniquet. As demonstrated in Figure 5, tourniquet release resulted in an AP decrease of 17 ± 3 mm Hg at 10 seconds, 25 ± 2 mm Hg at 50 seconds, and 24 ± 3 mm Hg at 100 seconds. By contrast, during real-time execution of the BBS, the decrease in AP was 9 ± 2 mm Hg at 10 seconds, 1 ± 2 mm Hg at 50 seconds, and 0 ± 1 mm Hg at 100 seconds after the deflation. These data indicated that the BBS significantly attenuated the decrease in AP at these 3 time points and nullified the hypotensive effect of tourniquet release within 50 seconds. Similarly, the BBS significantly suppressed the decrease in CVP within 50 seconds after the release of the tourniquet.

Discussion

Design of BBS

On the basis of knowledge and technology of bionics, we previously developed an artificial feedback control system for automatic regulation of sympathetic vasomotor tone in animal models of central baroreflex failure.^{20–22} As a crucial first step to clinical application, we tested its feasibility and efficacy in a clinical model of orthostatic hypotension. A percutaneous epidural catheter approach

was established for the monitoring of spinal function during surgery and for pain management,²⁸ and the lower thoracic level was selected for spinal cord stimulation based on earlier reports that the abdominal splanchnic vascular bed is a major effector mechanism for arterial baroreflex in animals^{23,24} and humans.²⁵ Although the percutaneous epidural approach is less invasive than implantation surgery, spinal cord stimulation excites motor and sensory nerves^{12,22,28} in addition to sympathetic vasomotor efferents. Therefore, administration of sufficient doses of muscle relaxants and analgesics was required during experimental protocols. Under these conditions, the dynamic response of AP to STM was easily characterized by the white noise system identification method. Furthermore, the quantitatively estimated results of transfer function analysis (Figure 2B) enabled simulation of the effects of feedback correction factors²⁷ on performance of the BBS. As demonstrated in Figure 3, the simulation results suggested that the specific combination of feedback correction factors could optimize the performance of the BBS. On the basis of these results, the feedback correction factors were determined to allow the BBS to quickly stabilize AP against the external disturbances.

Efficacy of BBS

The present study utilized a tourniquet-related model of hypotension^{29–31} during general anesthesia^{32,33} to approximate orthostatic hypotension due to central baroreflex failure. Except for the change in peripheral vascular resistance, the hemodynamic changes after tourniquet deflation are similar to those achieved after upright tilt-

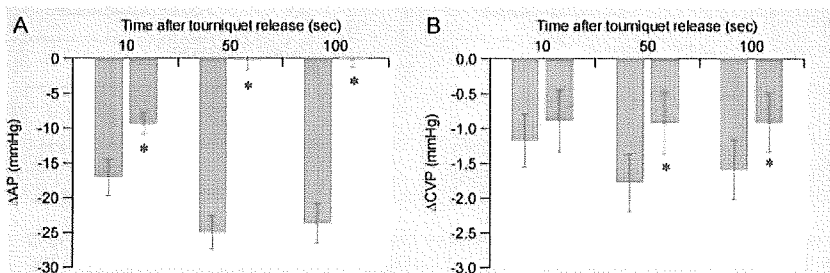


Figure 5. Bar graphs showing changes in AP (A) and CVP (B) at 10, 50, and 100 seconds after tourniquet release. Implementation of the BBS (red column) significantly attenuated tourniquet-related falls (blue column) in AP and CVP. Data are expressed as mean±SD for 21 patients. *Overall $P < 0.05$.

ing.^{29,31} For example, tourniquet release results in a rapid increase in venous pooling in the affected limb with a subsequent decrease in venous return and cardiac output. Under general anesthesia with volatile gases such as sevoflurane,^{32,33} arterial baroreflex function is inhibited, and the hemodynamic disturbance produced by the tourniquet inevitably results in abrupt hypotension. In rare instances, tourniquet deflation can also trigger fatal circulatory collapse.²⁹

Despite the fact that the BBS was implemented with fixed values of feedback correction factors for all patients, the BBS successfully stabilized AP against the hemodynamic challenge induced by sudden tourniquet release (Figure 4). These data indicate that the BBS may compensate for some individual differences in the dynamic response of AP to STM.

Finally, the CVP response to STM (Figure 4) in the present study suggests that the BBS attenuated a decrease in venous return. Previous studies have demonstrated that the baroreflex-mediated vasoconstriction in the splanchnic vascular bed is a major mechanism for recruitment of venous return during head-up tilting.^{23,25} Therefore, the BBS may functionally mimic the baroreflex control of venous return and control of AP.

Study Limitations

This study possessed several limitations. First, based on the previous results^{20–22} obtained from animal studies, the stimulation electrodes were placed in the epidural space at the level of the lower thoracic cord; however, further study to determine the optimal site of electrode placement would be of benefit. Second, it is unclear whether or not the feedback controller designed in the present study is universally applicable to other cases. Although preset parameters for feedback correction were used in the present study, other approaches based on a robust control theory could yield a better result. Finally, the epidural catheter method for sympathetic nerve stimulation is associated with significant pain and discomfort. Thus, practical use of the BBS requires an appropriate method for stimulating only efferent sympathetic nerves.

Clinical Implications

The present study confirmed the efficacy of the BBS in a clinical setting and suggests that the BBS has tremendous potential as a new therapeutic modality for treatment of severe orthostatic intolerance in patients with various syndromes of central baroreflex failure, including Shy-Drager syndrome, baroreceptor deafferentation, and traumatic spinal cord injuries.

Acknowledgments

This study was supported by a Health and Labor Sciences research grant (H14-NANO-002, H16-NANO-005, H15-KOKORO-019) from the Ministry of Health, Labor, and Welfare of Japan and by a grant-in-aid for scientific research (15300165) from the Ministry of Education, Science, Sports, and Culture of Japan.

Disclosures

None.

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CLINICAL PERSPECTIVE

Central baroreflex failure due to Shy-Drager syndrome, baroreceptor deafferentation, and traumatic spinal cord injuries results in severe orthostatic hypotension. However, most commonly used interventions, such as salt loading, cardiac pacing, and pharmacological approaches, can neither restore nor reproduce the functioning of a native vasomotor center. Here, we proposed a novel therapeutic strategy against central baroreflex failure and developed a bionic baroreflex system (BBS). The BBS consisted of a pressure sensor, computer, electrical stimulator, and epidural catheter with sympathetic nerve stimulation electrodes. While automatically calculating the frequency of a pulse train in response to a change in arterial pressure, the computer drove the stimulator at the appropriate frequency to stabilize arterial pressure against an external disturbance. According to a parametric negative-feedback control theory, we designed an algorithm of the computer functioning as an artificial vasomotor center. The efficacy of the BBS was tested in a clinical model of orthostatic hypotension during knee joint surgery. Without the implementation of the BBS, a sudden deflation of a thigh tourniquet resulted in rapid progressive hypotension. By contrast, during real-time execution of the BBS, arterial pressure was quickly restored to the baseline level before tourniquet release. These results suggest the technical feasibility of functional restoration of arterial baroreflex with the BBS.

Nitric Oxide Stimulates Vascular Endothelial Growth Factor Production in Cardiomyocytes Involved in Angiogenesis

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Abstract: Background: Hypoxia-inducible factor (HIF)-1 α regulates the transcription of lines of genes, including vascular endothelial growth factor (VEGF), a major gene responsible for angiogenesis. Several recent studies have demonstrated that a nonhypoxic pathway via nitric oxide (NO) is involved in the activation of HIF-1 α . However, there is no direct evidence demonstrating the release of angiogenic factors by cardiomyocytes through the nonhypoxic induction pathway of HIF-1 α in the heart. Therefore we assessed the effects of an NO donor, S-Nitroso-N-acetylpenicillamine (SNAP) on the induction of VEGF via HIF-1 α under normoxia, using primary cultured rat cardiomyocytes (PRCMs). Methods and Results: PRCMs treated with acetylcholine (ACh) or SNAP exhibited a significant production of NO. SNAP activated the induction of HIF-1 α protein ex-

pression in PRCMs during normoxia. Phosphatidylinositol 3-kinase (PI3K)-dependent Akt phosphorylation was induced by SNAP and was completely blocked by wortmannin, a PI3K inhibitor, and N^G-nitro-L-arginine methyl ester (L-NAME), a NO synthase inhibitor. The SNAP treatment also increased VEGF protein expression in PRCMs. Furthermore, conditioned medium derived from SNAP-treated cardiomyocytes phosphorylated the VEGF type-2 receptor (Flk-1) of human umbilical vein endothelial cells (a fourfold increase compared to the control group, $p < 0.001$, $n = 5$) and accelerated angiogenesis. Conclusion: Our results suggest that cardiomyocytes produce VEGF through a nonhypoxic HIF-1 α induction pathway activated by NO, resulting in angiogenesis.

Key words: vascular endothelial growth factor, angiogenesis, cardiomyocyte, Flk-1, nitric oxide.

The prognosis of patients with chronic heart failure remains poor because of progressive remodeling of the heart and lethal arrhythmia [1]. It has recently been reported that vagal nerve stimulation therapy markedly improved long-term survival in an animal model of chronic heart failure after myocardial infarction [2] and that acetylcholine (ACh) has a direct cardioprotective effect through the PI3K-Akt-hypoxia-inducible factor (HIF)-1 α pathway [3, 4]. Nitric oxide (NO) is supposed to be one of the signaling molecules induced by ACh; however, it remains to be clarified whether NO is involved in angiogenesis through the nonhypoxic induction pathway of HIF-1 α and vascular endothelial growth factor (VEGF), and is thereby related to the cardioprotective effects of ACh or vagal nerve stimulation.

VEGF is a key angiogenic factor and major target of HIF-1 α , which is produced by ischemic tissue and growing tumors [5–7]. Factors including VEGF secreted by noncardiomyocytes are known to possess significant paracrine effects on cardiomyocytes; however, the importance of such cardiomyocyte-derived factors as paracrine or autocrine effectors on angiogenesis in the heart remains

to be elucidated. The HIF-1 α protein level is usually regulated by the oxygen concentration. During hypoxia, HIF-1 α protein is stabilized by escaping from degradation through von Hippel-Lindau tumor-suppressor protein (VHL) [8, 9]. Furthermore, the PI3K-Akt signaling pathway, which is known for the antiapoptotic functions [10, 11], is demonstrated to be involved in HIF-1 α induction [12]. Recently it has been revealed that besides hypoxia, certain cytokines, growth factors, and NO increase the HIF-1 α protein level even under the normoxic conditions in some specific cells [13–15]. To our knowledge, however, the involvement of NO in this signaling pathway in cardiomyocytes under normoxic conditions remains to be elucidated. Moreover, it is also unclear whether NO is involved in angiogenesis in the heart, though NO is associated with many aspects of cellular biology involved in cell signaling, vasodilatory tone, and cell growth [16].

With this background, we speculated the nonhypoxic induction of HIF-1 α in the cardiomyocytes through NO-mediated pathway and that NO plays another role in producing an angiogenic factor through the pathway. To prove this hypothesis, we assessed the effect of a NO do-

nor, *S*-Nitroso-*N*-acetylpenicillamine (SNAP), on the nonhypoxic induction of HIF-1 α 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 μ M (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 μ M DAF-2 and 100 μ M L-arginine for 60 min, followed by 1 μ M 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 μ g/ml), and L-NAME (1 mM) on Akt, HIF-1 α , and VEGF by im-

munoblotting 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-1 α , 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-1 α to VEGF expression, HEK 293 cells were transfected with an expression vector for dominant-negative HIF-1 α (dn HIF-1 α) [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 μ M 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-1 α , VEGF, and Glut-1, as well as β -actin. The sense and antisense gene-specific primers were as follows:

HIF-1 α (sense), 5'-GGGAGAAAAGCAAGTCGTG-3',
 HIF-1 α (antisense), 5'-AGTCAGCAACGTGGAAGG-3';
 VEGF (sense), 5'-CCAGCACATAGGAGAGATGAGCTTC-3',
 VEGF (antisense), 5'-GGTGTGGTGGTGACATGGTTAATC-3';
 Glut-1 (sense), 5'-ACACCTCCCCACATACATG-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-1 α ; 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 μ l/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 μ g/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 \pm 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-1 α 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-1 α protein

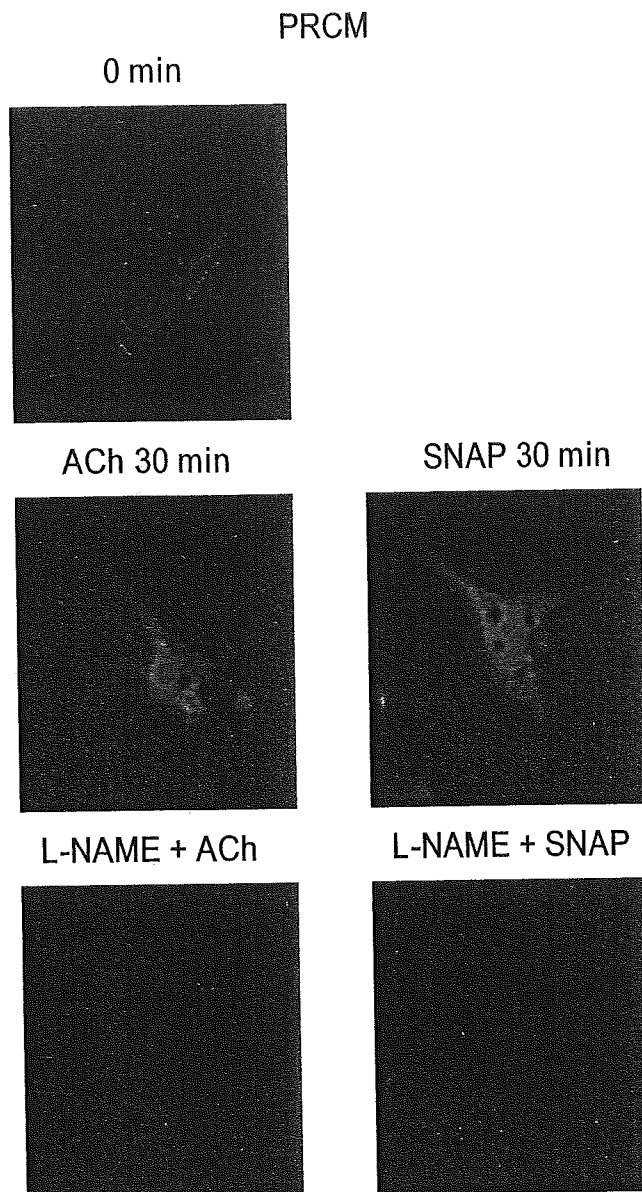


Fig. 1. Rat primary cardiomyocytes release NO in response to ACh or SNAP. PRCMs released NO after treatment with 1 mM ACh or 1 μ M SNAP, evaluated with DAF-2. NO release was observed within 30 min after ACh or SNAP treatment ($n = 3$). Pretreatment with 1 mM L-NAME for 60 min blocked NO production ($n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

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-1 α induction in the cardiomyocytes (Fig. 2a). Such an induction of HIF-1 α 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 μ g/ml), followed by stimulation with SNAP for 8 h. However, actinomycin D failed to inhibit the HIF-1 α induction by SNAP (Fig.

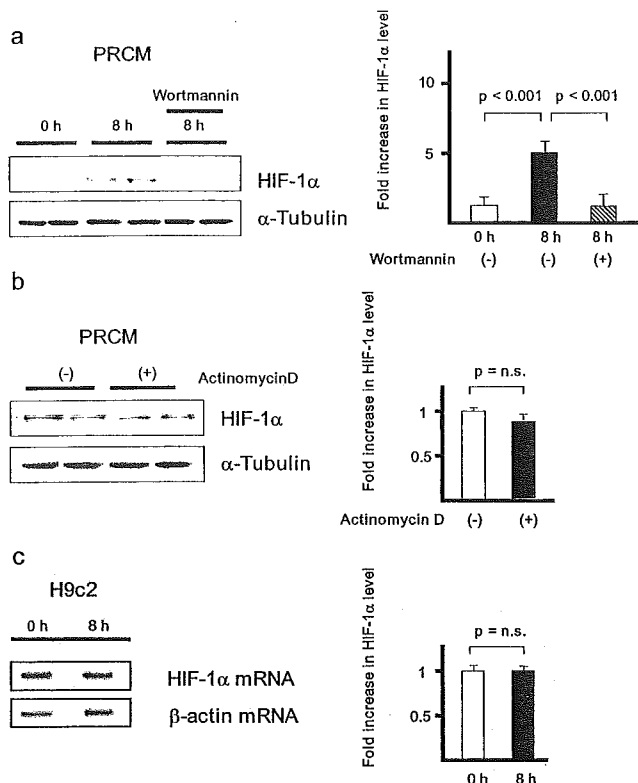


Fig. 2. The HIF-1 α protein expression level is increased by SNAP in cardiomyocytes in normoxia. Treating the PRCMs for 8 h with SNAP (1 μ M) already increased HIF-1 α protein expression in normoxia. Pretreatment of PRCMs with wortmannin (30 nM) for 30 min inhibited SNAP-induced HIF-1 α expression ($n = 5$) (a). However, treatment with actinomycin D (0.5 μ g/ml) for 15 min did not inhibit the upregulation of HIF-1 α protein expression by SNAP ($n = 5$) (b). In H9c2 cells, the HIF-1 α mRNA expression level was not increased by SNAP ($n = 5$) (c).

2b), and SNAP further did not increase the HIF-1 α mRNA level, evaluated by RT-PCR (Fig. 2c), thus suggesting that SNAP induces HIF-1 α 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-1 α 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-1 α in the cardiomyocytes under normoxic conditions.

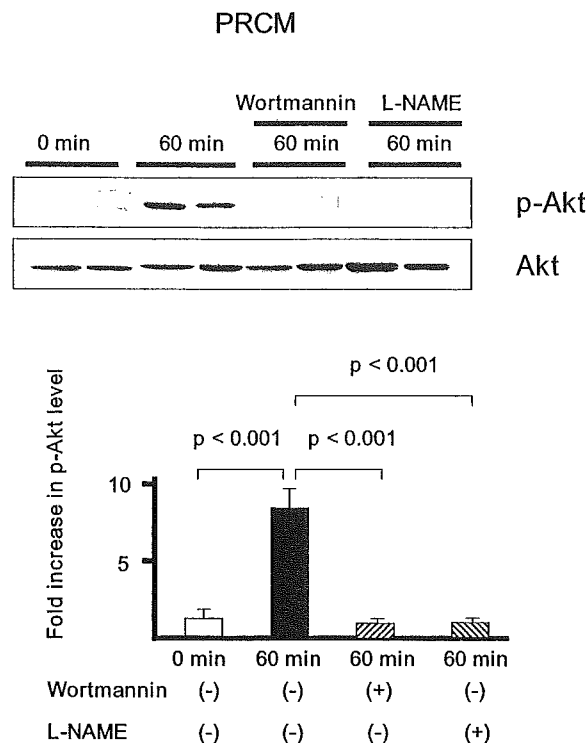


Fig. 3. Akt phosphorylation is increased by SNAP in cardiomyocytes under normoxia. Akt phosphorylation was increased by SNAP (1 μ M) in PRCMs with a rapid time course. However, pretreatment with wortmannin (30 nM) for 60 min or L-NAME (1 mM) for 60 min completely inhibited the Akt phosphorylation in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

Promotion of angiogenic signaling cascade by NO in cardiomyocytes under normoxia

To identify if SNAP-induced HIF-1 α 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-1 α -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-1 α induction pathway in the production of VEGF by the cardiomyocytes under normoxic conditions. Furthermore, to elucidate the contribution of HIF-1 α to VEGF protein expression, dn HIF-1 α was introduced into HEK293 cells, and it was demonstrated that dn HIF-1 α 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 HU-VECs. As expected, the conditioned medium-treated cells

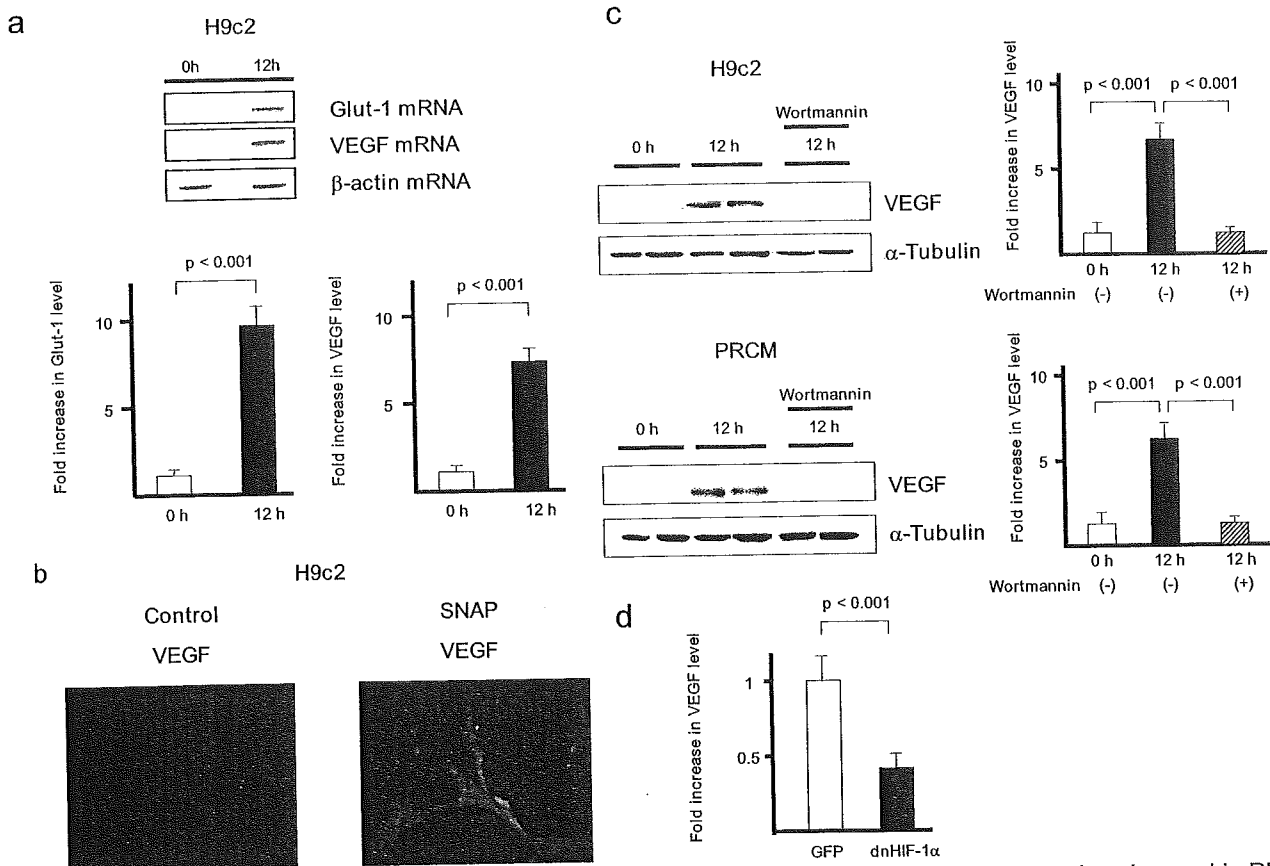


Fig. 4. SNAP increases Glut-1 and VEGF gene expression levels through HIF-1 α in cardiomyocytes under normoxia. In H9c2 cells, Glut-1 mRNA and VEGF mRNA were both increased by SNAP ($n = 5$) (a). VEGF immunoreactivity was increased by SNAP in H9c2 cells ($n = 5$) (b). The SNAP-induced

VEGF protein expression, which was also observed in PRCMs, was completely inhibited by 30 nM wortmannin ($n = 5$) (c). In contrast to control (GFP), VEGF induction by SNAP was blocked by dn HIF-1 α 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-1 α by ACh during normoxia [4]. In the present study, SNAP-treated cardiomyocytes revealed a similar pathway in the induction of HIF-1 α , suggesting that NO from cardiomyocytes activates an angiogenic signaling through HIF-1 α .

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

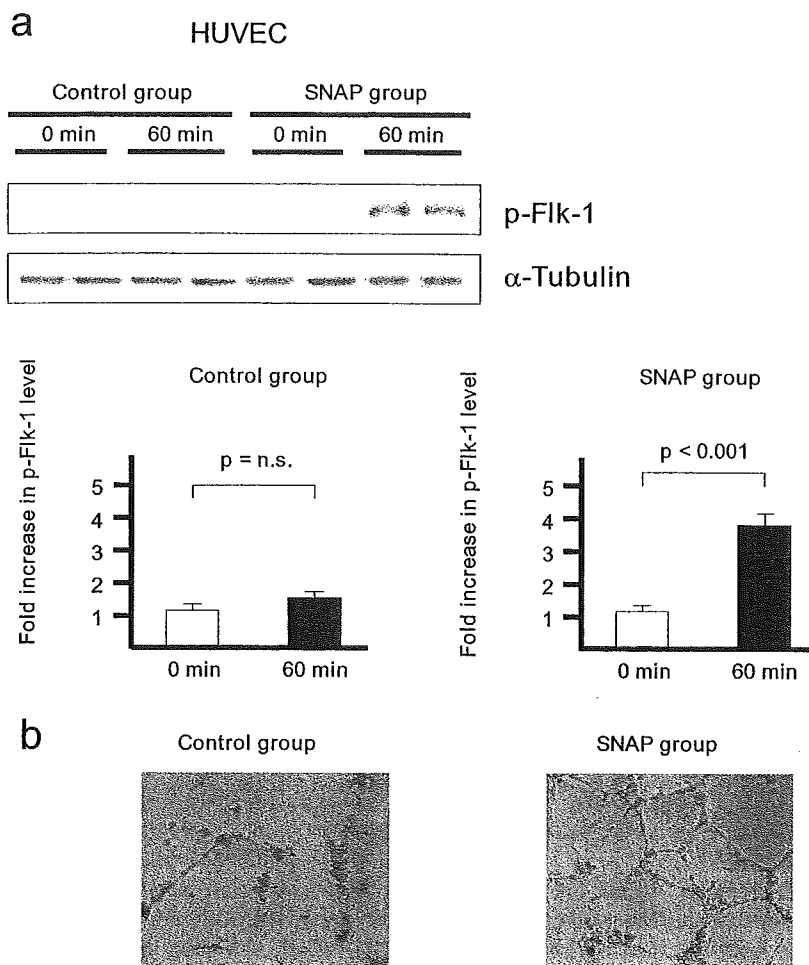


Fig. 5. VEGF derived from SNAP-treated cardiomyocytes induces angiogenesis. The SNAP-treated conditioned medium increased Flk-1 phosphorylation in HUVECs, compared with the nontreated conditioned medium ($n = 5$) (a). The SNAP-treated conditioned medium accelerated angiogenesis in HUVECs compared with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

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-1 α induction in cardiomyocytes.

Second, as shown in this study, NO increased the cardiac VEGF protein expression through HIF-1 α regulation, and dn HIF-1 α 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-1 α , 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-1 α -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 hypovascularity and depressed cardiac function. Our present study indicated that HIF-1 α induction through NO plays a main role in stimulating VEGF production by cardiomyocytes and accelerates angiogenesis.

In this study we focused on HIF-1 α as an upstream factor regulating cardiac VEGF expression. Unlike the hypoxic induction pathway of HIF-1 α , 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-1 α 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-1 α [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-1 α . 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-1 α was not decreased by actinomycin D; therefore this suggests that SNAP does not play a role in the transcriptional regulation of HIF-1 α , rather in the inhibition of protein degradation. Therefore in cardiomyocytes, such a mechanism might be involved in a NO-mediated Akt-HIF-1 α -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-1 α induction pathway, and furthermore, it contributes to angiogenesis through cardiac VEGF production, which phosphorylates Flk-1, a VEGF type-2 receptor.

This study was supported by a Health and Labor Sciences Research Grant (H15-PHYSI-001) for Advanced Medical Technology from the Ministry of Health, Labor, and Welfare of Japan.

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