Early Expression of Myocardial HIF-1α in Response to Mechanical Stresses

Regulation by Stretch-Activated Channels and the Phosphatidylinositol 3-Kinase Signaling Pathway

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Abstract—Vascular endothelial growth factor (VEGF) expression is upregulated by hypoxia-inducible factor-1 (HIF-1) in ischemic tissues and growing tumors. Normally, HIF-1 activity depends on the amount of HIF-1α subunit, which is tightly regulated by the oxygen tension. In the myocardium, VEGF expression has been shown to be induced under nonhypoxic conditions by mechanical stresses. However, the cellular mechanism of stress-mediated VEGF induction remains unclear. Therefore, we examined the possible involvement of HIF-1 in stress-mediated VEGF induction in rat hearts. In this study, we increased the left ventricular wall tension using 3 different methods, namely by inducing regional ischemia, by expanding an intraventricular balloon, and by producing hemodynamic overload using an aortocaval shunt. In all cases, HIF-1α accumulated in the nuclei of cardiac myocytes in the early phase, and this was followed by VEGF induction. Phosphatidylinositol 3-kinase (PI3K)–dependent Akt phosphorylation was found to be activated by mechanical stress and completely blocked by wortmannin (a PI3K inhibitor). Moreover, the stress-mediated induction of HIF-1α and VEGF was suppressed by gadolinium (a stretch-activated channel inhibitor), wortmannin, and rapamycin (a FRAP inhibitor). Our results suggest that HIF-1α plays an important role in the induction of VEGF in nonischemic and mechanically stressed myocardium, and that this is regulated by stretch-activated channels and the PI3K/Akt/FRAP pathway. Moreover, this signaling pathway, which induces HIF-1α, seems to play an important role in the adaptation of the myocardium to stresses. The full text of this article is available at http://www.circresaha.org.

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Key Words: hypoxia-inducible factor-1α ■ vascular endothelial growth factor ■ phosphatidylinositol 3-kinase ■ heart ■ mechanical stress

Vascular endothelial growth factor (VEGF) is a key angiogenic factor, which is produced by ischemic tissues and growing tumors.1 The transcriptional upregulation of VEGF has been shown to play a major role in the hypoxic induction of VEGF, which is mediated by the binding of hypoxia-inducible factor-1 (HIF-1) to the hypoxia-response element in the 5′-flank region.2 HIF-1 is composed of HIF-1α and HIF-1β, both of which belong to the PAS family of basic helix-loop-helix (bHLH) transcription factors.3 Normally, HIF-1 activity depends on the amount of HIF-1α protein, which is markedly increased by hypoxia, whereas HIF-1β protein is constitutively present regardless of oxygen tension.4 Under normoxic conditions, HIF-1α protein is not detected because it is targeted for proteasomal degradation by the oxygen-dependent degradation domain (ODDD).5 During this process, the von Hippel-Lindau tumor suppressor protein (pVHL) plays an important role as part of the multiprotein ubiquitin E3 ligase complex.6,7 pVHL directly interacts with the subdomains within the ODDD and mediates the ubiquitylation of HIF-1α, which then becomes the target of proteasomal proteolysis.7,8 However, under hypoxic conditions, HIF-1α escapes from the degradation process and is transported to the nucleus, in which it is codimerized with HIF-1β to form HIF-1, which in turn, transactivates the VEGF gene.

Recently, it has been demonstrated that the hydroxylation of 2 proline residues located in the C-terminal9,10 and N-terminal11 ends of ODDD regulates pVHL binding to HIF-1α and determines the stability of HIF-1α. Comparatively, few studies on HIF-1α have been reported in cardiac myocytes.12,13 Recently, HIF-1α protein was observed to be induced in the ischemic human myocardium. In biopsy specimens obtained from ischemic or infarcted myo-
cardium, HIF-1α and VEGF proteins were detected by immunohistochemical staining, whereas they were undetectable in specimens of the nonischemic myocardium. This suggests that the early induction of HIF-1α mediates the transcription of the VEGF gene in the ischemic myocardium, which is one of the first adaptations of human myocardium to ischemia.

In the myocardial infarction model of rat heart, VEGF expression was found to be induced in areas of the left ventricle remote from the infarct area. Because these areas are neither ischemic nor hypoxic, stimuli other than ischemia seem to be responsible for this VEGF induction. One of possible stimuli is the diastolic wall stress caused by an increased left ventricular end-diastolic pressure typically observed in hearts with extensive left ventricular infarctions.

Recently, Li et al. demonstrated that VEGF gene expression was induced by mechanically stretching the left ventricle in an isolated rat heart model. They also suggested that VEGF induction by stretching is mediated in part by TGF-β and PKC. However, the role of HIF-1 in VEGF induction in the nonischemic or stretched myocardium has not been investigated, even though HIF-1 is a major transcription factor known to regulate VEGF gene expression. In this study, we examined the possibility that HIF-1α is induced in the nonischemic myocardium remote from the ischemic area and in stretched myocardium, and that this induction is followed by VEGF expression.

Materials and Methods

Regional Ischemia of Rat Heart

The investigation conformed to the Guide for the Care and Use of Laboratory Animals protocol, published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). Specific pathogen-free adult male Sprague-Dawley (250 to 300 g; Daehan Biolink Co Ltd, Korea) rats were anesthetized with pentobarbital sodium (50 mg/kg), intubated, and mechanically ventilated with a rodent ventilator with room air. Anterolateral thoracotomy was performed and the heart was rapidly exteriorized. A 6-0 silk suture was snared around the proximal left anterior descending (LAD) coronary artery and tightly ligated to occlude the vessel. The heart was then placed back and the chest was closed in 2 layers with 4-0 silk sutures. The animal was then allowed to recover. Sham-operated rats were treated in the same manner but the coronary artery was not ligated. Ventricular tissues were prepared from 2 different areas: the ischemic area in the center of the territory of the left anterior descending coronary artery, and the nonischemic area in the posterior part of the left ventricle far from the ischemic area. The tissues were then washed with cold saline and processed as outlined later. Periischemic tissue around the ischemic zone was removed and not used in this study. The central venous pressure (CVP) and the left ventricular end-diastolic pressure (LVEDP) were monitored using a suprapubic catheters inserted into the superior vena cava and the left ventricular lumen, as described previously.

Ex Vivo Stretch and In Vivo Hemodynamic Overload Models

Rats were anesthetized by the intravenous administration of 30 mg/kg of pentobarbital sodium. Hearts were excised, immediately connected to an aortic cannulae, and then perfused at a constant pressure in the nonrecirculating Langendorff mode with Krebs-Henseleit buffer containing (in mmol/L) 118 NaCl, 4.7 KCl, 1.25 CaCl2, 1.2 MgSO4, 10 glucose, 25 NaHCO3, and 1.2 KH2PO4. The buffer solution was saturated with a 95% O2/5% CO2 mixture at 37°C, and the perfusion pressure was maintained at 80 cm of H2O. The left ventricular pressure was monitored using a plastic catheter with a small balloon tip, which was inserted into the left ventricle through the mitral valve. To stretch the left ventricle of the rat heart ex vivo, the balloon was swollen until the end diastolic pressure reached 5 mm Hg. After the cardiac function had stabilized, the left ventricle was subjected to stretch for 1 or 2 hours by expanding the inserted balloon to raise the end diastolic pressure to 35 mm Hg.

To induce mechanical stress in the rat myocardium in vivo, aortocaval shunt (ACS) surgery was performed as described previously. Rats were anesthetized with pentobarbital sodium (50 mg/kg, IP), and the inferior vena cava and the abdominal aorta were exposed through a midline abdominal incision under sterile conditions. The AC shunt was produced using an 18-gauge needle at a point 5 mm caudal to the left renal vein. The patency of the shunt was verified visually based on the swelling of the vena cava and the mixing of arterial blood with venous blood. Sham operations were performed in control rats in an identical manner, except for needle insertion. After the abdominal incision was closed, the animals were allowed to recover. Various inhibitors were administered to the rats through a tail vein 30 minutes before ACS. Gadolinium chloride (GdCl3) was purchased from Sigma; wortmannin and rapamycin from Alexis Biochemical, and chelerythrine and diltiazem from RBI.

Immunoprecipitation and Immunoblot Analysis for HIF-1α

The expression of HIF-1α protein was determined in the ventricular myocardium using immunoprecipitation and immunoblotting methods. Myocardial tissues excised from each heart region were homogenized in 5 volumes of a lysis buffer (20 mmol/L Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/L KCl, 2 mmol/L EDTA, 0.5 mmol/L DTT, 0.4 mmol/L PMSF, and 2% NP-40) containing a protease inhibitor cocktail (Sigma) in a Potter-Elvehjem homogenizer. The homogenate was centrifuged at 12,000 g for 10 minutes 4°C, and the resulting supernatant was analyzed for HIF-1α. Protein concentrations were determined using the bicinchoninic acid method (Pierce, Rockford, IL) with bovine serum albumin as a standard. The lysates (150 μg protein) were incubated with 5 μL of a purified rabbit anti-HIF-1α antibody developed previously for 12 hours, followed by incubation with 10 μL of protein A-Sepharose beads (Amersham Pharmacia Biotech) for 3 hours. After washing, the immunocomplexes were eluted by boiling for 3 minutes in 20 μL of the SDS sample buffer containing 10 mmol/L DTT. The elute was electrophoresed on 7% SDS-polyacrylamide gels, and then the proteins were transferred to Immobilon-P membranes (Millipore), which were then incubated with 5% skim milk in Tris-buffered saline for 2 hours to block nonspecific binding. The membranes were incubated overnight at 4°C with a rat anti-HIF-1α antibody (1:5000), as described previously, and then incubated with a goat anti-rat IgG antiserum conjugated with horseradish peroxidase (1:5000) for 2 hours. Immunoreactive protein bands were visualized using the enhanced chemiluminescence plus detection system (ECLplus, Amersham-Pharmacia).

Immunoblot Analyses for VEGF and Akt

Myocardial tissue excised from each heart region was homogenized in ice-cold buffer (in mmol/L: 50 Tris, pH 7.4, 1 EDTA, 0.5 DTT, 0.4 PMSF, and 1 sodium vanadate) containing a protease inhibitor cocktail (Sigma) in a Potter-Elvehjem homogenizer. Homogenates were centrifuged at 10,000 g for 15 minutes at 4°C, and the supernatants were submitted for immunoblot analysis. Twenty micrograms of each protein sample was electrophoresed on 12% SDS-polyacrylamide gels under reducing conditions. Gels were run in sets consisting of control and experimental samples from the same region. Proteins on the gels were then transferred to Immobilon-P membranes (Millipore) and incubated with 5% skim milk in Tris-buffered saline for 1 hour, to block nonspecific binding. The membranes were then incubated overnight at 4°C with a mouse monoclonal antibody against amino acids 1 to 140 of human VEGF (Santa Cruz Biotechnology, 1:500), anti-Akt (Cell Signaling Technology, 1:100), or anti-phospho-Akt (Cell Signaling Technology, 1:100) and then further incubated with horseradish peroxidase-
conjugated secondary antibodies (1:5000) for 2 hours. Immunoreactive protein bands were visualized using the enhanced chemiluminescence plus detection system.

**Semiquantitative Reverse Transcriptase–Polymerase Chain Reaction Assay**

Total RNA was extracted with Trizol reagent (Gibco/BRL) according to manufacturer’s instructions. RNA (1 μg) was added to a 50-μL reverse transcriptase–polymerase chain reaction (RT-PCR; PCR-Access, Promega). The reaction master mix was prepared according to the manufacturer’s instructions to give final concentrations of 1× AMV/TII reaction buffer, 0.2 mmol/L dNTP, 5 μCi (α-32P)CTP, 1.5 mmol/L MgSO4, 0.1 U/μL AMV reverse transcriptase, 0.1 U/μL TII DNA polymerase, and 250 mmol/L primers. To quantify VEGF and ANP mRNAs, we used specific primers in a reaction involving 1 cycle of reverse transcription at 48°C for 45 minutes and 20 cycles of denaturation at 94°C for 30 seconds, annealing at 53°C for 30 seconds, elongation at 68°C for 1 minute, and a final extension at 68°C for 5 minutes. The resulting PCR fragments (5 μL) were electrophoresed on 4% polyacrylamide gels at 100 V in a 0.3% Tris/Borate/EDTA buffer (15 mmol/L Tris, 30 mmol/L Borate, 0.06 mmol/L EDTA, pH 7.5) at 4°C, and the dried gels were autoradiographed. The nucleotide sequences of the primers used were 5′-CTGACCTTCCCTGCTCTTG-3′ and 5′-CTTTCTTGTCGCTGACATTCA-3′ for VEGF; 5′-GACATGGGCTTCCTTTCCA-3′ and 5′-TTATCTTCAGTACCGGAAGCT-3′ for ANP; 5′-CACGACACCGAGTTACAGAAGCT-3′ and 5′-TCAGTTAACTTTGACCACTGTC-3′ for HIF-1α; 5′-GTGGGGTGATGGTCCAGAA-3′ and 5′-TGATCTCTGTACCGCATG-3′ for β-actin.

**Immunohistochemistry**

Hearts were frozen in OCT compound, and 8-μm sections were used for immunostaining. Endogenous peroxidase activity was blocked by treating the sections with 0.3% H2O2 in PBS for 30 minutes. After several washes with PBS, the sections were incubated with 5% normal goat serum in PBS for 1 hour to block nonspecific binding and then incubated overnight at 4°C with the purified rabbit anti-HIF-1α antibody (1:100).19 Subsequently, they were incubated with a biotinylated anti-rabbit IgG antibody (1:200, Vector Laboratories) for 2 hours and incubated with an avidin-biotin peroxidase complex (Vectastain ABC Kit, Vector Laboratories) for 1 hour. Finally, the sections were developed in diaminobenzidine and hydrogen peroxide solution. Rabbit preimmune serum was used in place of HIF-1α antibody as the negative staining control.

**Results**

**VEGF Is Induced in the Nonischemic Myocardium as well as in the Ischemic Myocardium**

Myocardial tissues were obtained from ischemic and nonischemic regions in the left ventricles of regionally ischemic rat hearts. The VEGF protein level increased in both the ischemic and nonischemic region. Temporal changes in VEGF protein levels were observed in these tissues. In the ischemic region, an initial increase in the VEGF protein level relative to that of the sham-operated rats was observed 4 hours after regional ischemia, and a further increase 12 and 24 hours after ischemia (Figure 1A). Thereafter, it slightly decreased 48 hours after ischemia and remained at a higher level for at least 72 hours after ischemia (data not shown). Similarly, in the nonischemic region, the VEGF protein level was also elevated 12 hours after ischemia and reached a peak at both the 24- and 48-hour time period (Figure 1B). The VEGF protein level subsequently declined 72 hours after ischemia (data not shown). The membrane used for immunoblotting was washed with TTBS and stained with Coomassie Brilliant Blue R-250 to verify the quantity and quality of the protein samples. To examine whether the VEGF expression was upregulated at the transcriptional level, the mRNA level of VEGF was determined and found to be induced in both regions and this was comparable to VEGF protein induction. In the ischemic region, the increase in the mRNA level was observed 12 hours after ischemia and continued to increase at both the 24- and 48-hour time period (Figure 1C). In the nonischemic region, the mRNA expression was also elevated 12 hours after ischemia and reached its peak at the 24- and 48-hour time period (Figure 1D). To examine whether the induction of HIF-1α mRNA precedes the VEGF gene expression in these conditions, the temporal changes in the HIF-1α mRNA levels were observed. However, the expression of HIF-1α mRNA was not induced both in the ischemic and nonischemic myocardium at all (Figures 1C and 1D). The tissue level of β-actin mRNA, which was measured as an internal standard, was not altered significantly.

**HIF-1α Accumulates in the Nuclei of Myocytes in the Nonischemic Myocardium**

Because HIF-1α is an important transcriptional factor that regulates VEGF expression, the expression level of HIF-1α protein was determined using immunoprecipitation and immunoblotting methods, which were performed using 2 kinds of anti–HIF-1α antibodies raised from rabbit and rat. The immunoblot assays revealed that the HIF-1α antibodies used in this study recognized an inductive protein of about 120 kDa in ischemic myocardial tissues. The immunopositive protein might be rat HIF-1α because it is recognized by 2 different anti–HIF-1α antibodies and induced by ischemia, and its molecular mass is well matched with that of human HIF-1α. Notably, the expression of HIF-1α protein was enhanced in the nonischemic myocardium as well as in the ischemic myocardium (Figure 1E). HIF-1α accumulated both in the ischemic and nonischemic myocardium 4 hours after ischemia. HIF-1α was maximally induced 24 hours after ischemia and subsequently declined. Immunohistochemical analysis also revealed that HIF-1α accumulated in the nuclei of myocytes in the ischemic region 4 hours after regional ischemia compared with sham-operated rats. Consistent with our immunoblot observation, intense immunostaining for HIF-1α was also observed in the nuclei of myocytes in the nonischemic region 4 and 24 hours after regional ischemia (Figure 1F). In Figure 1G, the HIF-1α protein level determined immunohistochemically is represented as the numbers of cells containing immunopositive nuclei. This was conducted on 3 different areas of each slide for 3 consecutive slides, and the mean values were calculated. The number of nuclei stained normally with hematoxylin showed no statistical difference. HIF-1α positive cells were observed in the nonischemic ventricles as well as in the ischemic ventricles of RI rats. HIF-1α accumulated in the nuclei of myocytes both in the ischemic and nonischemic region to an extent measurably greater than that in the sham-operated rats as early as 2 hours after regional ischemia. The number of HIF-1α positive cells continued to increase in both regions in a time-dependent manner. In the nonischemic myocardium, the nuclear accumulation of HIF-1α reached a peak at the
24-hour time point and subsequently declined (Figure 1G). However, the nuclear localization of HIF-1α in the ischemic region 24 hours after ischemia could not be counted because the morphology of myocytes was too shrunk to identify the nuclei.

Mechanical Stresses Induce the Nuclear Accumulation of HIF-1α and VEGF Gene Expression

Contractile dysfunction of the ischemic myocardium places the left ventricle, including the nonischemic myocardium, under diastolic wall stress. To examine whether mechanical stress affects HIF-1α–mediated VEGF induction, a soft balloon was placed in the left ventricle of an isolated heart and inflated to 35 mm Hg for 1 or 2 hours. One part of the left ventricle was then homogenized to measure the VEGF mRNA level, and the other part was quickly frozen for HIF-1α detection by immunohistochemistry. The VEGF mRNA level was found to increase markedly after only 1 hour of stretching and further induced after 2 hours (Figure 2A). Concomitantly, strong immunoreactive signals of HIF-1α protein were detected in the nuclei of myocytes (Figure 2B). To rule out the possibility that the myocardium was under hypoxic stress due to disturbance of the microcirculation caused by increased intramural pressure, the lactate content was measured by enzymatic assay (Lactate Reagent, Sigma Chemical). No significant difference in the lactate levels was found in the nonstretched and stretched hearts (data not shown). Moreover, after deflating the balloon, pressure parameters, such as systolic and diastolic pressures and heart rate, returned to the control values before stretching. These results suggest that mechanical stretch per se without hypoxic stress can induce HIF-1α protein and VEGF gene expression in isolated hearts.

To determine the extent of diastolic wall stress on the left ventricle, CVP and LVEDP were measured 12 hours after inducing regional ischemia (RI) and fitting the ACS. CVP and LVEDP were significantly elevated in RI models, which confirms that diastolic wall stress occurs in the ischemic heart, as has previously been reported.17 Increased stress
parameters in the ACS rats indicates that ACS surgery adequately induces wall stress in the rat heart (Figure 3A). However, no change in myocardial lactate level after ACS was found (data not shown). In addition, atrial natriuretic peptide (ANP) mRNA was measured as another index for myocardial stretch because it is a cardiac hormone that is known to be induced in response to myocardial stretch.21 As shown in Figure 3B, myocardial ANP mRNA levels were substantially increased 12 hours after RI and ACS surgery compared with that of the sham-operated rats.

To investigate whether mechanical stress enhances VEGF gene expression through HIF-1α in vivo, VEGF mRNA was measured in the heart tissues of ACS rats. Figure 4A shows that the VEGF protein level gradually increased, achieving a peak at the 24-hour time period and that it remained elevated for at least 48 hours. VEGF mRNA levels were also increased in parallel with its protein expression in the hearts of ACS rats (Figure 4A). Immunoblot analysis revealed that HIF-1α protein were induced in the left ventricles 8 hours after ACS and further increased 12 hours after ACS (Figure 4A). Immunohistochemical analysis also demonstrated early expression of myocardial HIF-1α in ACS rats. HIF-1α in the nuclei started to appear as early as 1 hour after ACS. The number of HIF-1α positive cells continuously increased until 24-hour time point and subsequently declined 24 hours later (Figure 4B). However, the HIF-1α mRNA level did not increase in the left ventricles of ACS rats (Figure 4A). These results suggest that HIF-1α accumulates in the nuclei of myocytes in early responses to mechanical stresses at the posttranscriptional step and is followed by enhanced expression of the VEGF gene.

Mechanical Stress Response Is Mediated Through the SAC/PI3K/Akt/FRAP Pathway

We next tried to identify the cellular mechanism responsible for the expression of HIF-1α by mechanical stress. Because oxygen tension in the nonischemic zone of the RI heart and the stretched heart might be near normoxic, we thought that some nonhypoxic mechanism might be involved in mechanical stress-mediated HIF-1α induction. In prostate cancer cells, HIF-1α and VEGF overexpression under normoxic conditions are mediated via the PI3K/Akt/FRAP pathway.22 Because this nonhypoxic HIF-1α induction pathway has been identified in only cancer cells, it has been considered to be related with angiogenesis or the hypoxic adaptation of tumors. However, it is also possible that this pathway features in cardiac myocytes in the case of nonhypoxic, mechanical stress-mediated HIF-1α induction. In terms of myocardial stretch, stretch-activated ion channels (SACs) are also possible candidates to cause HIF-1α induction because SACs are thought to be related with the upstream pathway of stretch-mediated cellular responses.23 The TGF-β/PKC pathway could also be a possible candidate mechanism responsible for the stretch induction of HIF-1α because this pathway has been reported to be partly involved in stretch-mediated VEGF induction in the myocardium.16,24 Therefore, we set out to determine which of the above pathways is involved in mechanical stress-mediated HIF-1α and VEGF induction in the myocardium. Accordingly, HIF-1α levels were measured...
in the hearts of ACS rats and ACS rats pretreated with various inhibitors, namely, chelerythrine (a PKC inhibitor), wortmannin (a PI3K inhibitor), rapamycin (a FRAP inhibitor), or gadolinium (Gd³⁺, a blocker of stretch-activated ion channels), which were administered intravenously 30 minutes before ACS surgery. As shown in Figure 5A, stress-mediated HIF-1α induction was significantly inhibited by wortmannin, rapamycin, and Gd³⁺, but not by chelerythrine. Concomitantly, both VEGF protein and its mRNA induction by ACS were markedly reduced by wortmannin, rapamycin, and Gd³⁺ (Figure 5B). Although Gd³⁺ is widely used to inhibit SACs, it is also known to block L-type calcium channel. To exclude the involvement of L-type calcium channel in the stress responses, we treated ACS rats with diltiazem, a specific L-type calcium channel blocker, but it neither inhibited HIF-1α nor VEGF induction at the doses used (Figures 5A and 5B). To determine whether the myocardial stretch was accompanied by the PI3K-dependent activation of Akt, we measured the levels of phosphorylated Akt in the hearts of ACS rats. The extent of Akt phosphorylation greatly increased 1 hour after ACS and this slightly decreased over the next hour, whereas the total amount of Akt protein was unchanged (Figure 5C). Because Akt phosphorylation was almost completely abolished by wortmannin, this confirmed that the Akt was activated mainly by PI3K in the myocardium of the ACS rat. Because the time course of the PI3K/Akt signaling might be helpful in the elucidation of the mechanism of HIF-1α induction, we examined the Akt phosphorylation at an earlier phase. Figure 5D shows that Akt was phosphorylated in the myocardium as early as 10 minutes after ACS compared with the 60 minutes of the sham control. The phosphorylated Akt increased time-dependently, peaking at the 30-minute time point. The phosphorylation of Akt was maintained for 2 hours after ACS at a higher level than that of the sham control (Figure 5C). Taken together, these results suggest that mechanical stress-mediated HIF-1α and VEGF induction is highly dependent on SACs and the PI3K/Akt/FRAP pathway.

**Discussion**

In this study, we found that HIF-1α accumulated in the nuclei of cardiac myocytes in the nonischemic myocardium remote from the ischemic area as early as 2 hours after regional ischemia, and that this was followed by the induction of the VEGF gene. Likewise, HIF-1α and VEGF were induced by mechanical stress in an ex vivo–heart model and in an in vivo–heart model of ACS. PI3K-dependent Akt phosphorylation was activated during the early phase of ACS and was blocked by wortmannin. Mechanical stress-mediated induction of HIF-1α and VEGF was suppressed by Gd³⁺, wortmannin, and rapamycin, but not by chelerythrine. These results suggest that HIF-1α plays an important role in the induction of VEGF in the nonischemic and mechanically stressed myocardium, and that this is regulated by SACs and the PI3K/Akt/FRAP pathway. Our hypothesis for mechanical stress-mediated induction of HIF-1α in rat heart is summarized in Figure 6.

Because HIF-1α induction was first described for conditions of decreased oxygen pressure, most mechanistic studies on HIF-1α regulation have concerned hypoxic conditions. However, recent evidence suggests that HIF-1α can be induced under normoxic conditions by growth factors, hormones, or nitric oxide as well, although information on the signal transduction pathways involved is limited. Zhong et al. demonstrated a role of the PI3K/Akt/FRAP pathway in HIF-1α induction in response to serum and epidermal growth factor treatment. Likewise, it has been demonstrated that nitric oxide and tumor necrosis factor-α induce HIF-1α via the PI3K/Akt pathway. Therefore, the PI3K signaling is believed to be a typical example of nonhypoxic HIF-1α induction in cancer cells. In this study, we found that PI3K-dependent HIF-1α induction occurred in the nonhypoxic myocardium under mechanical stress. To our knowledge, this is the first report suggesting the possible involvement of this pathway in the angiogenic process in the myocardium undertaken mechanical wall stress.

In this study, we did not examine the role of TGF-β in VEGF gene induction in the nonischemic or the stretched myocardium, and on the basis of several previous reports, TGF-β could be another important factor responsible for VEGF induction. Li et al. showed that the inflation of an
intraventricular balloon increased VEGF mRNA levels in isolated hearts and that this effect of stretch was suppressed by treatment with a TGF-β/H9252 antiserum or a PKC inhibitor. Zheng et al24 also demonstrated the involvement of TGF-β/H9252 in the stretch-mediated induction of VEGF in cardiac myocytes, which were stretched using a Flexercell Unit. Moreover, in a recent work27 that analyzed the 5′-flank region of the VEGF gene, it was suggested that TGF-β/H9252 positively regulates VEGF expression through Smad proteins, which are key mediators of TGF-β/H9252 signal transmission from the plasma membrane to the nucleus.28 Because the Smad-binding site is located very near the HIF-1α-binding site in the VEGF promoter, Smad is physically associated with HIF-1 and synergistically upregulates VEGF gene expression with HIF-1. Therefore, both the TGF-β/Smad pathway and our SAC/P13K/Akt/FRAP/HIF-1α pathway are likely to be essential for the induction of VEGF in stretched myocardium. Thus, VEGF expression might be inhibited by a variety of inhibitors capable of blocking any step of these pathways. On the other hand, PKC may not be essential for VEGF induction in hearts stretched in vivo because chelerythrine, a PKC inhibitor, failed to block VEGF induction. The mechanism of VEGF gene induction in the in vivo stretch model seems to differ from that in the ex vivo stretch model, as has been previously reported.16

In the majority of experiments that have demonstrated the hypoxic or the nonhypoxic induction of HIF-1α, it has been reported that HIF-1α expression is regulated predominantly at the posttranscriptional stage. However, several reports concerned with HIF-1α induction in cardiac cells have suggested that HIF-1α protein is regulated at the mRNA level in the myocardium. Notably, HIF-1α mRNA increased in biopsy specimens obtained from ischemic human hearts14 and in rat hearts with an impaired energy metabolism.29 In cultured HL-1 cardiomyocytes, HIF-1α mRNA was induced by 50% after 24-hour hypoxia, but not after 12-hour hypoxia.12 According to these reports, the induction of HIF-1α mRNA is likely to be responsible for the sustained increase in HIF-1α observed several days after ischemia or heart failure.
In the present study, we observed that the induction of HIF-1α protein occurred without a concomitant change in its mRNA level during the early responses to ischemia and mechanical stress, which is an induction pattern commonly observed in other types of cells. Summarizing, HIF-1α seems to be regulated by 2 different mechanisms according to circumstances.

How does PI3K signaling induce HIF-1α protein in mechanically stressed hearts? Normally, HIF-1α induction by hypoxia or transition metals is due to increased protein stability, as described previously in the Introduction. However, Laughner et al. have recently demonstrated that the HIF-1α induction caused by stimulating the PI3K/Akt/FRAP pathway, is because of increased protein synthesis and is not associated with an increased HIF-1α mRNA level or with increased HIF-1α protein stability. Moreover, they have found that the 5′-untranslated region of HIF-1α mRNA appears to be targeted by the PI3K signaling and that this regulates HIF-1α translation. Although we did not examine the rate of HIF-1α protein synthesis in this study, we speculate that HIF-1α protein in the myocardium is induced by mechanical stresses, probably via the same mechanism. However, we cannot exclude the possibility that the mechanism of PI3K-dependent HIF-1α induction differs in the myocardium because of different experimental conditions used. Our experiment was mainly performed in vivo whereas they used cancer cell-lines in vitro. Moreover, PI3K-dependent HIF-1α induction was observed only in serum-free conditions, whereas HIF-1 induction in the stressed myocardium occurred under in vivo conditions, which are always influenced by the presence of serum.

How cells sense mechanical stimuli, and how this translates into gene expression, is not understood. Because mechanical stimuli alone can direct activate the transcription factors involved in the early response to mechanical stresses, i.e., heat-shock factor and the immediate early genes, it is believed that cells possess a putative mechanosensor and mechanotransducer. SACs have been proposed as candidate mechanotransducers, as such channels have been found to produce cation influx nonselectively in response to mechanical stretch. Because Gd³⁺ can effectively inhibit SACs, it has been used to examine the involvement of SACs in the response to mechanical stresses. Recently, Chang et al. demonstrated the activation of HSF by SACs in rat hearts using Gd³⁺. In this study, we found that Gd³⁺ blocked the HIF-1α and VEGF expression induced by hemodynamic overload, whereas diltiazem had no effect. Our results further support the possible role of SAC as a mechanotransducer. However, how SAC are linked with the PI3K signaling remains to be determined.

In conclusion, this study demonstrates that HIF-1α and VEGF are induced in the nonhypoxic myocardium remote from the ischemic area and in hearts subjected to mechanical stresses ex vivo and in vivo. The results obtained with various inhibitors suggest that HIF-1α expression is upregulated through SACs and the PI3K/Akt/FRAP pathway. Moreover, because HIF-1α has been found to be induced in 3 separate mechanically stressed heart models, we suggest that HIF-1α plays an important role in the adaptation of the myocardium to mechanical stresses.

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