

Hypoxic Regulation of Inducible Nitric Oxide Synthase via Hypoxia Inducible Factor-1 in Cardiac Myocytes

Frank Jung, Lisa A. Palmer, Nan Zhou, Roger A. Johns

Abstract—The relationship between hypoxia and regulation of nitric oxide synthase (NOS) in myocardial tissue is not well understood. We investigated the role of hypoxia inducible factor-1 (HIF-1) on expression of the inducible NOS (iNOS) in myocardial cells in vivo and in vitro. In situ hybridization in myocardial tissue from rats exposed to hypoxia for 3 weeks demonstrated increased iNOS mRNA expression. Northern analysis of RNA from hearts of those animals and from cells exposed to hypoxia for 12 hours in vitro demonstrated an increase of HIF-1 RNA expression. Electrophoretic mobility shift assays using oligonucleotides containing the iNOS HIF-1 DNA binding site and nuclear extracts from cardiac myocytes showed induction of specific DNA binding in cells subjected to hypoxia. Transient transfection of cardiac myocytes using the murine iNOS promoter resulted in a 3.43-fold increase in promoter activity under hypoxia compared with normoxia. Mutation or deletion of the HIF-1 site eliminated the hypoxic response. As cytokines have been shown to regulate iNOS expression in myocardial cells, cultured neonatal cardiac myocytes were stimulated with interleukin-1 β causing a dramatic induction of iNOS protein expression under normoxia, with further augmentation under hypoxia. Transient transfection of cells stimulated with interleukin-1 β showed an increased iNOS promoter activity under normoxic conditions compared with unstimulated cells, with a further increase in response to hypoxia, which was dependent on HIF-1. These results demonstrate that hypoxia causes an increase in iNOS expression in cardiac myocytes and that HIF-1 is essential for the hypoxic regulation of iNOS gene expression. (*Circ Res.* 2000;86:319-325.)

Key Words: inducible nitric oxide synthase ■ hypoxia inducible factor-1 ■ heart ■ hypoxia

Nitric oxide (NO) plays an important role as a regulator in the nervous, immune, and cardiovascular systems.¹ In cardiac tissue, basal production of NO is maintained by constitutive expression of endothelial NO synthase and contributes to the regulation of coronary circulation, heart rate, and myocardial contractility.² However, NO generated by the inducible form of NO synthase (iNOS) has been implicated in many pathophysiological states leading to myocardial dysfunction.³⁻⁹ It has also been reported that in ischemia/reperfusion injury, NO production can exhibit either a positive effect (attributed to a decrease in neutrophil and platelet adhesion or vasodilation) or a negative effect (related to the production of free radicals and inactivation of mitochondrial enzymes).¹⁰⁻¹³ Furthermore, there is recent evidence that iNOS plays a role as a mediator in the reduction of infarct size via late preconditioning.^{14,15}

The relationship between acute or chronic hypoxia and NO synthase regulation in cardiac myocytes is still not well established.¹⁶ Also, other factors, such as cytokines,^{1,3,17-20} may influence hypoxia-mediated iNOS regulation, although the exact mechanisms are not known.²¹⁻²⁵

It has been shown that low oxygen tension regulates a number of other genes.²⁶⁻²⁸ *cis*-acting sequences responsible for the induction of hypoxia-induced transcription of the erythropoietin gene have been identified. The *trans*-acting factor, hypoxia inducible factor-1 (HIF-1), binds to a conserved region in the enhancer located in the 3'-flanking region of the erythropoietin gene, which is required for hypoxic inducibility.^{29,30} This DNA binding protein is a heterodimer composed of 2 subunits, HIF-1 α and HIF-1 β .³¹⁻³³ Functionally important binding sites for HIF-1 (consensus 5'-RCGTG-3') have also been found in a number of other genes known to be regulated by hypoxia, such as vascular endothelial growth factor^{34,35}; the glycolytic enzymes aldolase A, enolase 1, and lactate dehydrogenase A; and phosphoglycerate kinase-1.³⁶⁻³⁹ A putative HIF-1 site in the murine iNOS gene was also shown to be required for hypoxia-induced transcription in a macrophage cell line and in pulmonary endothelial cells.^{22,23} We undertook the present study to examine what role HIF-1 and interleukin (IL)-1 β play on the effect of hypoxia-modulated iNOS expression in myocardium and isolated myocytes.

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Materials and Methods

Primary neonatal cardiac myocytes were isolated from 1- to 2-day-old Sprague Dawley rat pups, using a neonatal cardiac myocyte isolation procedure kit according to the manufacturer's instructions (NCMIS, Worthington Enzyme). Cardiac myocytes were then plated at a density of 2 to 3×10^6 cells per 60-mm culture dish and allowed to seed for 24 hours before transfection or cytokine stimulation. For studies involving hypoxic conditions, cells were purged with 95% N_2 -5% CO_2 for 20 minutes and then placed in a 1% to 2% O_2 -5% CO_2 -balanced N_2 incubator for 6 to 36 hours.

Cells were transfected using pGL-3 constructs containing the full-length iNOS promoter (pGLiNOS) or the iNOS promoter with a mutation of the HIF-1 binding site (pGL209) or the iNOS promoter with a deletion of the HIF-1 binding site (pGL220). After transfection, cells were placed into hypoxic (1% to 2% O_2) or nonhypoxic incubators for up to 36 hours before harvesting for luciferase assays. Cytokines (10 ng/mL) or vehicle (PBS/BSA) was added before hypoxic exposure. Promoter activity was measured in luciferase light units as fold increase over promoterless activity. β -Galactosidase and protein concentrations using a standard BSA curve were used to normalize for transfection efficiency and cell number.

Nuclear extracts were prepared from cardiac myocytes exposed to normoxia and hypoxia for 36 hours as previously described.³¹ Extract (3 μ g) was incubated with radiolabeled oligonucleotide probe (1.5 fmol) and loaded on a 4% nondenaturing polyacrylamide gel. Electrophoresis was carried out at 4°C. When used, competitor oligonucleotides were added at the beginning of the 5-minute preincubation period.

Procedures followed in the care and euthanization of the animals were approved by the Animal Research Committee of the University of Virginia. The protocol for the exposure of rats to hypoxia has been previously described.²⁵ Animals were euthanized after 3 weeks of exposure to normoxic or hypoxic conditions.

Crude protein from normoxic and hypoxic heart samples and cultured cardiac myocytes were prepared for Western blot analysis. Electrophoresis was carried out on a 7.5% SDS gel according to the method of Laemmli,⁴⁰ and blots were incubated with primary IgG iNOS antibody for 1 hour. Protein detection was carried out using enhanced chemiluminescence.

Total RNA was isolated from hearts of animals exposed to 3 weeks of hypoxia and normoxia and from normoxic and hypoxic myocytes. RNA was fractionated by glyoxyl-agarose gel electrophoresis and transferred to Hybond-N+ nylon membrane. cDNA probes for HIF-1 α were labeled with α -³²P-dCTP and hybridizations performed using 25 ng of end-labeled cDNA probe. The cDNA for HIF-1 α has been previously described.⁴¹ Blots were exposed to autoradiography at -70°C.

In situ hybridization was performed on serial sections of formalin-fixed, paraffin-embedded tissue from left ventricles of animals exposed to normoxia and hypoxia for 3 weeks. The conditions of target pretreatment, hybridization, and probe generation have been extensively characterized.^{42,43} Sense and antisense orientation probes specific for iNOS mRNA were used. The in situ hybridization data were analyzed using both brightfield morphology as well as darkfield optics to better visualize the full distribution of the silver grains generating the autoradiographic signal.

An expanded Materials and Methods section is available online at <http://www.circresaha.org>.

Results

Hypoxia Induces iNOS mRNA in the Myocardium of Hearts From Rats Exposed to Chronic Hypoxia

To examine the effect of chronic hypoxia on iNOS expression in myocardium, in situ hybridization was used to determine the presence and cellular location of iNOS mRNA expression

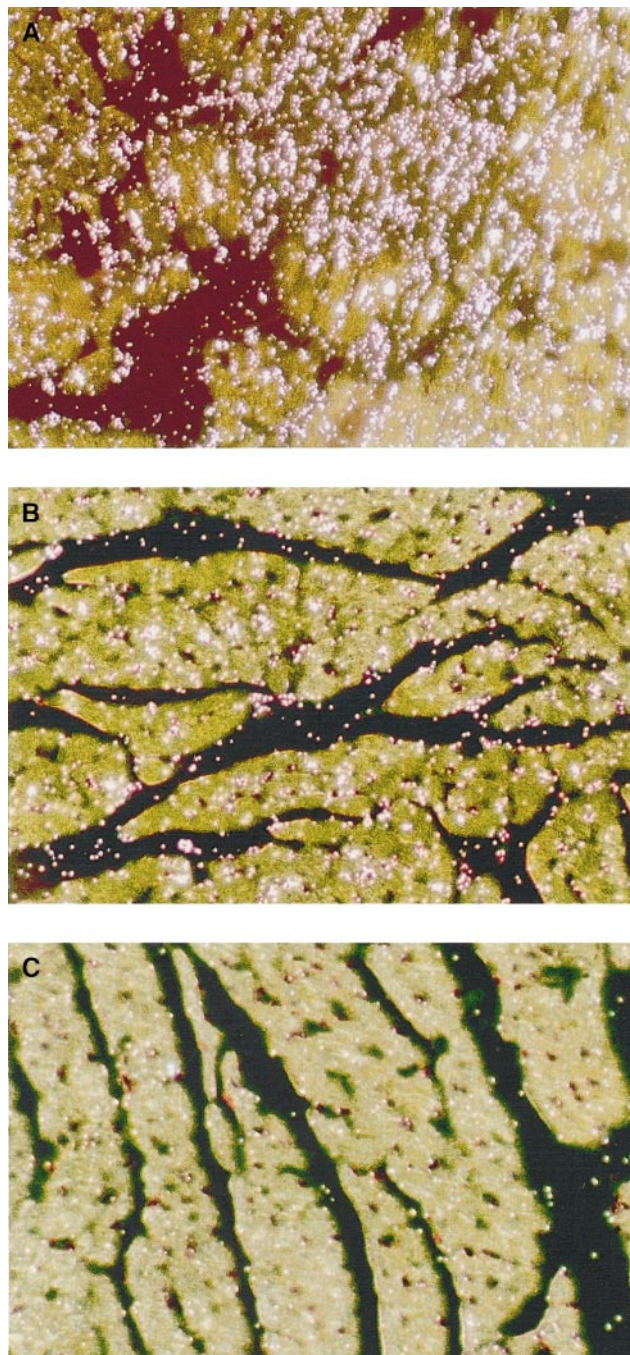


Figure 1. Hypoxia increases iNOS mRNA expression in myocardial cells in hearts of rats exposed to chronic hypoxia. In situ hybridization analysis was performed on hearts of rats exposed to 10% O_2 (hypoxia) (A) or room air (normoxia) (B) for 3 weeks. Tissues from left ventricles were probed with the antisense cRNA for iNOS to demonstrate iNOS RNA expression. Tissues from hypoxic hearts were also probed with the control sense cRNA for iNOS (C).

in hearts from rats subjected to 3 weeks of hypoxia. Compared with tissue sections from hearts of rats subjected to 3 weeks of normoxia (Figure 1A), iNOS mRNA expression was increased predominantly in the nuclei of myocardial cells from rat hearts exposed to chronic hypoxia (Figure 1B). These findings were observed independently of the origin of the tissue, in the right or left ventricle.

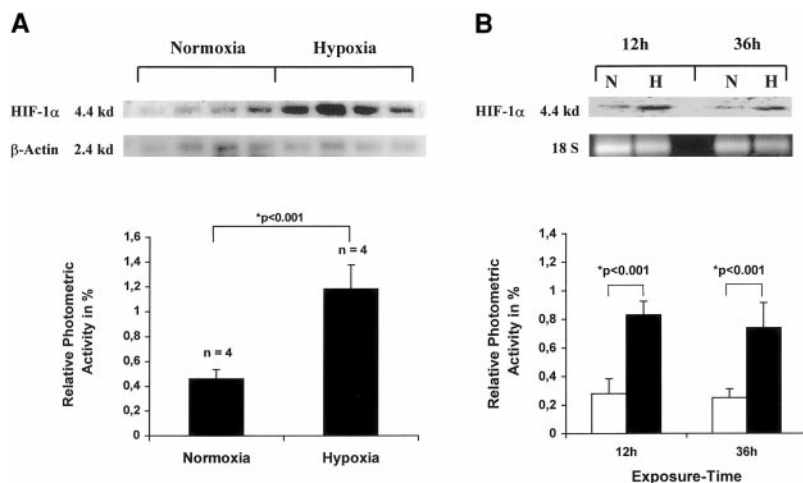


Figure 2. Analysis of HIF-1 α RNA expression in hypoxic rat hearts and hypoxic neonatal cardiac myocytes. Northern blot analysis was performed on total RNA from the hearts of rats exposed to room air (normoxia, n=4) or 10% O₂ (hypoxia, n=4) for 3 weeks (A) and from isolated neonatal cardiac myocytes exposed to 20% O₂ (normoxia) or 1% O₂ (hypoxia) up to 12 hours. Blots were hybridized to probes for HIF-1 α (B). Data are mean \pm SD of 3 experiments performed. Differences of photometric activity between normoxia and hypoxia were statistically significant using a 2-way variance analysis (* P <0.001).

Hypoxia Induces HIF-1 α mRNA Expression in Hearts From Rats Subjected to Chronic Hypoxia

It has not been shown whether HIF-1 is influenced by conditions of chronic hypoxia in cardiac cells in vivo or in vitro. To determine whether expression of RNA encoding HIF-1 increased in response to chronic hypoxia in myocardial cells and tissue, Northern blot analysis was performed using total RNA from hearts of rats subjected to normoxia and hypoxia for 3 weeks and from neonatal cardiac myocytes subjected to normoxia or hypoxia for up to 36 hours. In normoxic rat hearts and neonatal cardiac myocytes cultured under nonhypoxic conditions, low-level RNA expression of HIF-1 α was observed, whereas HIF-1 α RNA levels were increased in hearts from rats exposed to hypoxia (Figure 2A) and in isolated neonatal cardiac myocytes exposed to prolonged hypoxia (Figure 2B).

Hypoxia and IL-1 β Have an Additive Effect on the Induction of iNOS Protein Expression in Cardiac Myocytes

Cytokines, such as IL-1 β , are involved in the induction of iNOS expression not only in cardiac myocytes but also in other cell types.^{44,45} However, the effect of hypoxia on modulation of IL-1 β -induced iNOS expression is controversial. Therefore, we examined the effect of IL-1 β stimulation of cardiac myocytes on iNOS expression under normoxic and hypoxic conditions. In control cells stimulated with vehicle (PBS/BSA), no iNOS protein expression was observed under normoxia, whereas there was only weak induction under hypoxia. In contrast, stimulation of cells with IL-1 β caused a significant induction of iNOS protein expression under normoxia, which was further increased under hypoxic conditions (Figure 3).

Hypoxia Induces Nuclear Proteins That Bind to the HIF-1 Sequence in the iNOS Promoter

The HIF-1 binding site in the 5'-flanking region of the murine iNOS promoter has been shown to be involved in the regulation of iNOS gene expression in macrophages and endothelial cells.^{22,23} To determine whether hypoxic exposure of neonatal cardiac myocytes induces nuclear proteins that bind to the HIF-1 binding site, electrophoretic mobility shift

assays (EMSA) were performed using nuclear extracts prepared from cells cultured under normoxic or hypoxic conditions and a 30-bp wild-type (WT) oligonucleotide containing the consensus HIF-1 binding site (Figure 4A). Two constitutively expressed DNA binding activities were present in extracts from cardiac cells cultured under normoxic or hypoxic conditions. However, another DNA binding activity was specifically induced by hypoxia (Figure 4B, arrow). This DNA binding activity could be detected in cells as early as 6 hours after exposure to hypoxia. Competition experiments with excess unlabeled WT or mutated oligonucleotides (Figure 4A) demonstrated that binding of this hypoxia-induced factor was specific (Figure 4B).

Hypoxia Increases Transcriptional Activity of the iNOS Promoter in Cardiac Myocytes via HIF-1

To determine whether iNOS promoter activity was affected by hypoxia in cardiac myocytes, transient transfection experiments were performed using pGLiNOS (Figure 5A), which contains 1588 bp of the 5'-flanking promoter region of the murine iNOS gene linked to a luciferase reporter gene. iNOS promoter activity was increased 3.43-fold in cells subjected to hypoxia compared with cells exposed to normoxia (Figure 5B).

To determine whether the HIF-1 binding site is functionally required for hypoxic induction of the murine iNOS promoter in cardiac myocytes, transient transfection experi-

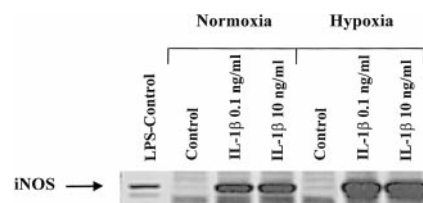


Figure 3. IL-1 β induces iNOS protein expression in cardiac myocytes, which is potentiated by hypoxia. Western blot analysis was performed using total protein from cardiac myocytes exposed to normoxia or hypoxia. The cytokine IL-1 β (0.1 or 10 ng/mL) or vehicle (PBS/BSA) was added before cells were subjected to normoxia or hypoxia. Cellular protein was prepared after a 36-hour exposure. Blots were hybridized with primary IgG iNOS antibody (Transduction Laboratories).

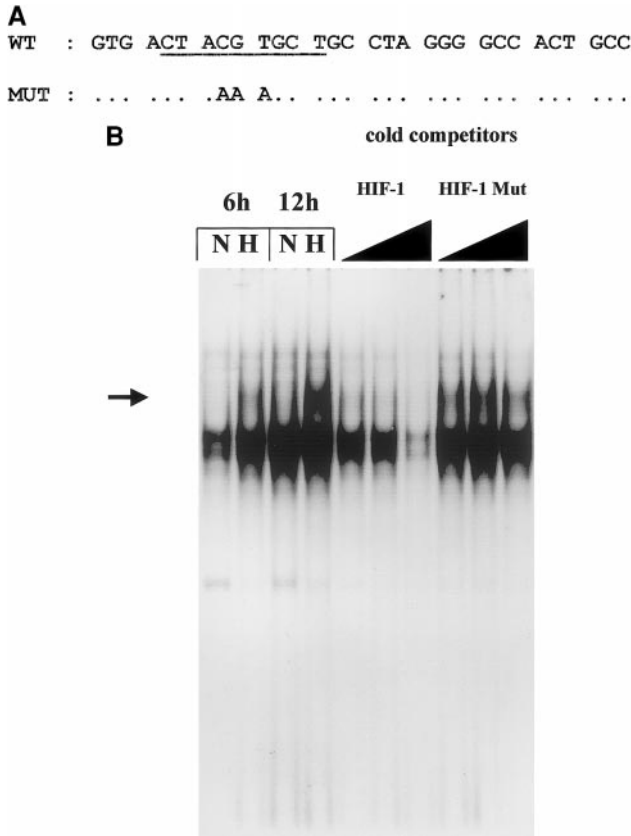


Figure 4. Hypoxia induces a factor that binds to iNOS gene promoter sequences. A, Sequences of the oligonucleotides used in the EMSA. The WT oligonucleotide contains the WT 9-bp sequence (underlined), which is identical to the HIF-1 binding site in the erythropoietin gene enhancer, in the context of the murine iNOS 5'-flanking region. MUT contains a 3-bp mutation previously shown to eliminate HIF-1 binding to the erythropoietin gene enhancer.⁴⁸ B, EMSAs were performed on nuclear extracts prepared from neonatal cardiac myocytes exposed to 20% O₂ (normoxia, N) or 1% O₂ (hypoxia, H) for 12 hours, using an oligonucleotide probe (WT) consisting of a WT 30-bp sequence from the iNOS promoter. Arrow denotes the location of the hypoxia-induced complex. Competition was performed using increasing molar excess (50×, 100×, and 200×) of unlabeled WT or mutated (Mut) oligonucleotides.

ments were performed using constructs with a deletion (pGL220) or a mutation (pGL209) of the HIF-1 binding site (Figure 5A). Both deletion and mutation of the HIF-1 binding site eliminated the increase in iNOS promoter activity seen in response to hypoxia when compared with cells exposed to normoxia (Figure 5B). This confirms that the HIF-1 binding site is required for transcriptional activation of the iNOS gene in cardiac myocytes under hypoxic conditions.

Hypoxic Augmentation of IL-1β–Induced iNOS Promoter Activity Is Dependent on HIF-1 Binding to the iNOS Promoter

To determine whether the inducibility of iNOS expression by IL-1β is also occurring at the transcriptional level and whether the increase of promoter activity under hypoxic conditions is dependent on interaction of IL-1β with HIF-1, we performed transient transfection experiments in cardiac myocytes with and without IL-1β stimulation

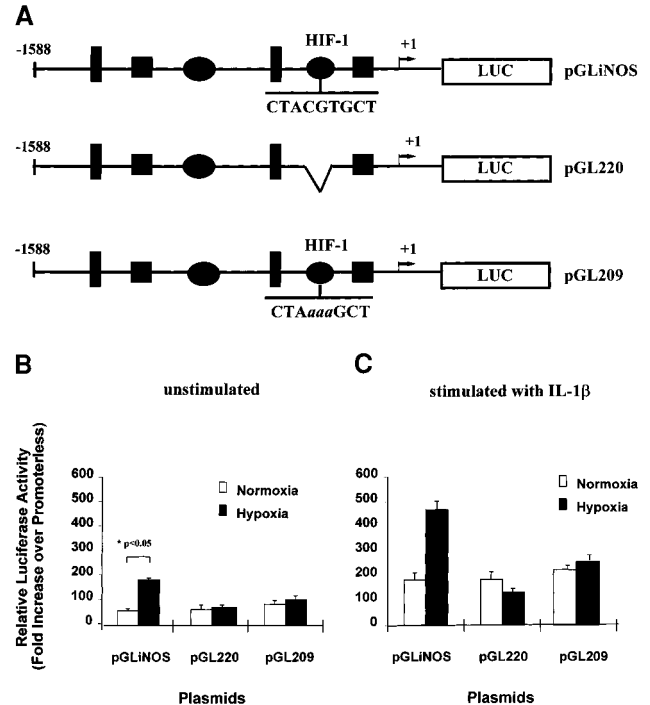


Figure 5. Hypoxia increases promoter activity of the iNOS gene via HIF-1. Augmentation by IL-1β of the iNOS promoter activity under hypoxic conditions is dependent on HIF-1 binding to the iNOS promoter. A, Schematic diagram illustrating the location of the HIF-1 binding site (−227 to −219 bp) in the 5'-flanking region of the murine iNOS gene (pGLiNOS), the HIF-1 binding site deletion construct (pGL220), and the construct containing the HIF-1 mutation (pGL209). Other *cis*-acting sequences are putative binding sites for activator protein-1 (rectangle), nuclear factor κB (square), and p53 (oval). LUC indicates luciferase. B and C, Neonatal cardiac myocytes were transiently transfected with the WT pGLiNOS, the HIF-1 deletion (pGL220), and HIF-1 mutation constructs (pGL209) or with backbone vector (pGLbasic) alone (not shown). After transfection, cells were cultured under nonhypoxic (open bars) or hypoxic (filled bars) conditions for 36 hours. Before exposure, cells were either incubated with vehicle (PBS/BSA) (B) or stimulated with IL-1β cytokine (10 ng/mL) (C). Cells were harvested and luciferase activity was determined. Promoter activity was expressed in luciferase light units as fold increase over promoterless activity (not shown). Protein concentrations using a standard BSA curve were used to normalize for cell number in the luciferase and β-galactosidase assay. Induction of iNOS promoter activity under hypoxia is shown relative to the activity of the construct under normoxic conditions. Data are mean±SD of triplicate samples. Results are shown for 3 representative experiments. Differences of luciferase activity between normoxia and hypoxia were statistically significant using a 2-way variance analysis (**P*<0.05).

(Figure 5B and 5C). Overall, stimulation with IL-1β caused a significant increase in iNOS promoter activity under normoxic conditions, which was further potentiated when cells were exposed to hypoxia. Using constructs containing a deletion (pGL220) or a mutation (pGL209) of the HIF-1 binding site (Figure 5A) still eliminated the increase in promoter activity seen in response to hypoxia in cells stimulated with IL-1β (Figure 5C). This confirms that hypoxic augmentation of IL-1β–induced iNOS promoter activity in cardiac myocytes is dependent on HIF-1 binding to the iNOS promoter.

Discussion

Our studies were designed to investigate the mechanism of hypoxia-induced regulation of iNOS in cardiac myocytes *in vitro* and *in vivo*. We have demonstrated that iNOS mRNA expression is increased in hearts from rats exposed to 3 weeks of hypoxia. iNOS mRNA expression was equally present in the right and left ventricles. There are now several other studies demonstrating increased iNOS expression under hypoxic conditions in cell types other than cardiac myocytes. Melillo et al²² found that interferon- γ -induced iNOS transcription is increased by hypoxia in a macrophage cell line. Additional evidence was provided earlier from our laboratory showing that iNOS gene expression was induced by hypoxia in pulmonary endothelial cells.²³ Kitakaze et al¹⁶ have shown that short-term exposure of cardiac myocytes to hypoxia resulted in increased NO production, although they did not specify whether this effect was mediated by the constitutive or the inducible form of NO synthase.

However, it is not entirely clear what mechanisms regulate iNOS gene expression under hypoxic conditions in cardiac myocytes. HIF-1 is one transcription factor known to play a role in oxygen regulation of several genes, such as erythropoietin, vascular endothelial growth factor, and recently the iNOS gene in pulmonary endothelial cells.^{23,29,34} In our study, we have uncovered for the first time a specific mechanism of hypoxia-induced iNOS gene regulation in cardiac myocytes that is mediated via the transcription factor HIF-1. HIF-1 DNA binding activity was only detected in nuclear extracts of cells grown under hypoxic conditions. Also, transient transfection experiments revealed that for the murine iNOS gene, HIF-1 is essential for the increased promoter activity in cardiac myocytes exposed to hypoxia, as mutation or deletion of the HIF-1 binding site abolished hypoxic induction of the iNOS promoter activity. It is known that both HIF-1 mRNA and protein are ubiquitously expressed in all organs of human and rodents⁴⁶ and that they are rapidly induced by hypoxia and rapidly decay upon return to nonhypoxic conditions.^{30–33,47} Our *in vivo* data demonstrated that induction of HIF-1 α RNA expression was observed in hearts of rats subjected to 3 weeks of hypoxia.

The role HIF-1 plays in the transcriptional regulation of gene expression in response to hypoxia may be both cell type specific and gene specific. For example, Semenza et al⁴⁸ have shown that in the human hepatoblastoma cell line Hep3B, which is transcriptional activation mediated by HIF-1, requires the binding of a second unidentified factor at site 2 of the erythropoietin gene enhancer. Comparison of sequences around the HIF-1 site present in the 5'-flanking region of the iNOS gene and the 3'-enhancer of the erythropoietin gene shows a region of similarity 10 bp downstream of the HIF-1 site. This 5-bp 5'-CACTG-3' sequence eliminated the ability of the erythropoietin enhancer to activate transcription in response to hypoxia. Thus, it is possible that the 5'-CACTG-3' sequence in the iNOS gene may also be involved in the hypoxia-induced increase of iNOS expression.

Compared with the murine sequence, there is 85% homology in the 5'-flanking region of the rat iNOS gene, which also contains an intact HIF-1 consensus site.⁴⁹ It is therefore likely that the rat iNOS gene is regulated in a similar manner under

hypoxic conditions. Conversely, in the human iNOS gene, there is no known HIF-1 binding site contained within the published sequence. It is possible that a HIF-1 site is present upstream of the known published sequence and may still be involved in the hypoxic regulation of the human iNOS gene. Alternatively, other factors may be responsible for the regulation of the human iNOS gene under hypoxic conditions. Putative binding sites of factors such as activator protein-1 and nuclear factor κ B, which have previously been implicated in the regulation of other genes by low oxygen tension, are also present in the human iNOS gene and may participate in its regulation by hypoxia.

It is not known whether additional factors are required for the activation of the iNOS gene via HIF-1 in cardiac myocytes under hypoxic conditions. For example, regulation of the lactate dehydrogenase A gene by hypoxia in the human cervical carcinoma cell line HeLa is augmented by forskolin and is dependent on the HIF-1 binding site and a cAMP response element.³⁹ In the murine macrophage line ANA-1, the effects of hypoxia on iNOS transcription, which requires the HIF-1 binding site, are augmented by interferon- γ treatment.²¹ Similarly, our transient transfection experiments with cardiac myocytes demonstrated that stimulation with IL-1 β further increased promoter activity not only under normoxic but also under hypoxic conditions. In contrast, mutation and deletion of the HIF-1 site still resulted in abolishment of the hypoxia-dependent response. Thus, our data suggest that IL-1 β is able to induce iNOS expression and that the increased iNOS gene expression under hypoxic conditions is still dependent on HIF-1.

Also on the translational level, stimulation of cells with IL-1 β resulted in a significant induction of iNOS protein expression in cells subjected to normoxia, which was further augmented under hypoxic conditions, which is in contrast to another study in which exposure of cardiac myocytes to prolonged hypoxia (48 hours) did not cause an increase of iNOS expression in cardiac myocytes. iNOS mRNA and protein expression as well as NO release in cardiac myocytes was only induced after stimulation of cells with IL-1 β . Interestingly, exposure of cells to prolonged hypoxia then led to a significant decrease in IL-1 β -mediated NO release and iNOS induction.²¹ This discrepancy with our study could be explained by differences in the stimulation protocol with IL-1 β . Although previous data from our laboratory suggested that the hypoxic response of iNOS expression was dependent on the concentration of the IL-1 β stimulus in cell types other than cardiac myocytes, we did not find a difference in hypoxia-induced iNOS expression in cardiac myocytes when they were stimulated with 2 different concentrations of IL-1 β (0.1 or 10 ng/mL). Also, the time of stimulation with IL-1 β may play a critical role in the response pattern of gene regulation in certain cell types. For example, Friedlander et al⁵⁰ demonstrated that IL-1 β exhibited pro- or antiapoptotic effects on hepatocytes, depending on the time of stimulation of cells exposed to hypoxia. This raises new, interesting thoughts regarding different roles of cytokines in specific cell types.

In summary, the mechanisms by which iNOS gene expression is increased in cardiac myocytes under pathophysiological

ical conditions in which oxygen availability is compromised are not known. For the first time, we have shown that increased expression of HIF-1 results in the transcriptional activation of iNOS gene expression under hypoxic conditions in myocardial cells, thus demonstrating a specific mechanism of hypoxia-induced iNOS expression in this cell type. Our studies have also demonstrated that IL-1 β induced iNOS gene and protein expression in cardiac myocytes, which is further augmented under hypoxic conditions via the transcription factor HIF-1. The functional role of NO production under conditions of low oxygen tension in cardiac myocytes is not established. One could speculate that the increase of NO production and iNOS gene expression in cardiac myocytes observed under different pathophysiological conditions such as myocardial ischemia/reperfusion or myocardial infarction, stunning, or hibernation, may potentially contribute to the impairment in myocardial contraction. However, there is increasing evidence that increased NO production and expression of iNOS may have a protective role as a mediator in late cardiac preconditioning.^{14,15} Therefore, identification and elucidation of regulatory pathways, such as the HIF-1 pathway, on the regulation of iNOS gene expression may have important therapeutic consequences and remains a subject for further investigation.

Acknowledgments

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Hypoxic Regulation of Inducible Nitric Oxide Synthase via Hypoxia Inducible Factor-1 in Cardiac Myocytes

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Correction

In an article by Jung et al (*Circ Res.* 2000;86:319–325), “Hypoxic Regulation of Inducible Nitric Oxide Synthase via Hypoxia Inducible Factor-1 in Cardiac Myocytes,” the labels associated with the radiographs in Figures 2A and 2B are incorrect. In Figure 2A, the radiograph labels should read “HIF-1 α 4.4 kb” and “ β -Actin 2.4 kb.” In Figure 2B, the radiograph label should read “HIF-1 α 4.4 kb.”

Materials and Methods

Constructs: pCAT-Constructs containing the full length iNOS promoter (pCATiNOS) or the iNOS promoter with a mutation of the HIF-1 binding site (pCAT209) or the iNOS promoter with a deletion of the HIF-1 binding site (pCAT220) were obtained from Dr. G. Melillo (22). Subcloning of the promoter fragments into pGL3-luciferase reporter plasmids was carried out as follows: pCATiNOS plasmids were digested with HIND III and subsequently blunted with Klenow. The promoter fragments were isolated through partial digestion with Xba I and cloned into a Nhe-I and a blunted KpnI site of a pGL3-basic vector. The new plasmids, pGLiNOS (containing the full length iNOS promoter) , pGL209 (containing the iNOS promoter with a mutation of the HIF-1 binding site) and pGL220 (containing the iNOS promoter with a deletion of the HIF-1 binding site), were then sequenced to check the presence of the insert and their correct orientation.

The cDNA for HIF-1 α has been previously described (40).

Cell culture: Primary neonatal cardiac myocytes were isolated from 1-2 day old Sprague Dawley rat pups, using a neonatal cardiac myocyte isolation procedure kit according to the manufacturer's instructions (NCMIS, Worthington Enzyme, NY). Briefly, rat pups were anesthetized and sacrificed. The beating heart was extracted, washed in a Hanks salt solution and minced with a razor blade. Trypsin was added and the tissue incubated for up to eight hours at 4⁰C. Cells were then digested with collagenase for 45 min on a rotating shaker at 37⁰C. Careful trituration was performed using a wide mouth pipette to dissociate cells which were centrifuged and subsequently preplated for 2 hours in a 37⁰C incubator to separate cardiac myocytes from fibroblasts. This resulted in a 95% pure cardiac myocyte population. Cardiac myocytes were then plated at a density of 2 - 3 x 10⁶ cells per 60 mm culture dish and

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allowed to seed for 24 hours prior to transfection or cytokine stimulation in minimum essential medium (MEM, GIBCO BRL Grand Island, NY), containing L-glutamine, 10% fetal bovine serum, 100 U/ml Penicillin, 100 µg/ml Streptomycin and 0.1 mmol/L BrDU to suppress fibroblast growth. Cells were maintained in a humidified 37⁰C, 5% CO₂ incubator. Cytokines (0.1 ng/ml or 10 ng/ml) or vehicle (Phosphate Buffered Saline/Bovine Serum Albumin - PBS/BSA) were added before placing cells into hypoxic conditions. For studies involving hypoxic conditions, cells were placed in a modular incubator and purged with 95% N₂, 5% CO₂, for 20 min. The modular incubator was then placed in a 1 - 2% O₂, 5% CO₂, balanced N₂ incubator for 6h to 36 hours. pO₂, pCO₂ and pH of the medium were measured in a blood gas analyzer (Corning model 178). Normoxia values were as follows: pH 7.31 ± 0.1, pO₂ 119 ± 2.3, pCO₂ 44.3 ± 4.8; hypoxia values: pH 7.27 ± 0.13, pO₂ 21.6 ± 2.9, pCO₂ 33.1 ± 1.4.

Transient transfections: Neonatal cardiac myocytes were transfected using Lipofectin Reagent (GIBCO BRL, Grand Island, NY), according to the manufacturer's instructions. Briefly, after the isolation procedure, cardiac myocytes were allowed to seed at a density of 2 - 3 x 10⁶ onto 60 mm culture dishes for 24 hours in the presence of 0.1 mmol/L BrDU, to suppress any growth of residual fibroblasts. Cells were washed three times with non-serum containing culture medium to remove any cellular debris and transfection was carried out using 20 µl Lipofectin Reagent, 5 µg pGLiNOS, pGL209, pGL220 plasmid DNA or backbone vector (pGL3basic), and 0.5 µg pSVβgal in Opti-serumfree Medium (Gibco BRL, Grand Island, NY). The medium was changed after 6 hours to serum containing medium. After another 6 hours of recovery-time the cells were placed into hypoxic (1 - 2% O₂) or non-hypoxic incubators for up to 36 hours before harvesting for luciferase assays. Cytokines (10

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ng/ml) or vehicle (PBS/BSA) were added prior to hypoxic exposure. All transfections were performed at least three times in triplicate.

Luciferase Assay: Luciferase assays were performed according to the instructions of the manufacturer (Promega Corporation, Madison, WI). Briefly, cells were washed three times with PBS and lysed in luciferase lysis buffer for 15 min. Lysates were prepared with one freeze/thaw cycle. For the luciferase assay, 20 μ l of the lysate were used with 100 μ l of luciferase substrate. Promoter activity was measured in luciferase light units as fold increase over promoterless. β gal, and protein concentrations using a standard BSA curve were used to normalize for transfection efficiency and cell number.

Nuclear Extracts Preparation: Nuclear extracts were prepared from cardiac myocytes exposed to normoxia and hypoxia for 36 hours as previously described (31). Briefly, cells were washed twice in cold phosphate buffered saline (PBS) and once in 4 packed cell volumes of buffer A (10 mmol/L Tris HCL (pH 7.4), 1.5 mmol/L MgCL₂, 10 mmol/L KCL, 2 mmol/L DTT, 0.4 mmol/L PMSF, 1 mmol/L Na₃VO₄, 2 g/L leupeptin, 2 g/L pepstatin, 2 g/L aprotinin). Cell pellets were resuspended in 4 packed cell volumes of buffer A, incubated on ice for 10 min and homogenized with 50 strokes in a dounce homogenizer. The nuclei were pelleted by centrifugation and resuspended in 3 packed nuclear volumes of buffer C (0.42 mol/L KCL, 20 mmol/L Tris HCL (pH 7.4), 1.5 mmol/L MgCL₂, 20% glycerol, 2 mmol/L DTT, 0.4 mmol/L PMSF, 1 mmol/L Na₃VO₄, 2 g/L leupeptin, 2 g/L pepstatin, 2 g/L aprotinin). The resulting mixture was mixed on a rotator for 30 min, centrifuged to remove nuclear debris and subsequently dialyzed with one change of buffer D (20 mmol/L Tris HCL (pH 7.5), 0.1 mol/L KCL, 0.2 mmol/L EDTA, 20% glycerol) for 4 hours at 4⁰C. The extracts were aliquoted and stored at -80⁰C.

Electromobility Shift Assay: Nuclear extracts (3 μg) prepared from neonatal cardiac myocytes were preincubated in binding buffer (10 mmol/L Tris, 50 mmol/L KCL, 50 mmol/L NaCL, 1 mmol/L MgCl_2 , 1 mmol/L EDTA, 5 mmol/L DTT and 5% glycerol) for 5 min at 4°C . Radiolabeled oligonucleotide probe (1.5 fmol) was added and incubated for 15 min. The mixture was loaded on a 4% nondenaturing polyacrylamide gel and electrophoresis was carried out in 0.3X TBE at 4°C (1X TBE = 89 mmol/L Tris Borate, 20 mmol/L EDTA [pH 8.0]). The gel was dried and autoradiography performed. When used, competitor oligonucleotides were added at the beginning of the 5 min preincubation period. The mixture was incubated on ice for 20 min before loading onto the gel.

In vivo animal protocol: Procedures followed in the care and sacrifice of the animals were approved by the Animal Research Committee of the University of Virginia. The protocol for the exposure of rats to hypoxia has been previously described (25). Briefly, male Sprague-Dawley rats (250-300 g) were placed in a Plexiglas chamber maintained at a 10% O_2 -atmosphere (hypoxic group) or in a chamber open to room air (normoxic group) for 3 weeks, with a 12/12 hour light/dark cycle. Hypoxia was maintained using a Pro:ox model 350 unit (Reming Bioinstruments, Reffield, NY), which controlled fractional concentration of O_2 in inspired gas by solenoid controlled infusion of N_2 (Roberts Oxygen, Rockville, MD) balanced against an inward leak of air through holes in the chamber. The hypoxic rats were exposed to room air for 10-15 min daily while their cages were changed. CO_2 , water vapor, and ammonia were removed by pumping the atmosphere of the hypoxia chamber through Bara Lyme (Barium hydroxide lime, USP; Chemetron Medical Division, Allied Healthcare Products, St Louis, MO), Drierite (anhydrous calcium sulfate; Fisher Scientific, Atlanta, GA), and activated carbon (Fisher Scientific, Atlanta, GA).

Isolation of Protein and Western Blot Analysis: Crude protein from normoxic and hypoxic heart samples and cultured cardiac myocytes were prepared in homogenization buffer (50 mmol/L Tris (pH 7.4), 0.1 mmol/L EDTA, 0.1 mmol/L EGTA, 1 mmol/L PMSF, 2 mmol/L Leupeptin, 1 mmol/L Pepstatin A and 0.1% β -Mercaptoethanol). Western blot electrophoresis was then carried out on a 7.5% SDS gel according to the method of Laemmli et al. (41) using 120 μ g of protein from crude rat hearts and 30 μ g of protein from cultured cardiac myocytes. Protein was transferred to nitrocellulose using an electrophoresis transfer chamber. Blots were incubated with primary IgG iNOS antibody for 1 hour (Transduction Laboratories, Lexington, KY). Blots were then washed 6x and incubated with peroxidase labeled anti-iNOS antibody. Protein detection was carried out using enhanced chemiluminescence (ECL-Amersham International, Buckinghamshire, England).

Isolation of RNA and Northern Analysis: Total RNA was purified from hypoxic and normoxic cardiac myocytes using the Qiagen RNA isolation kit according to the manufacturer's instructions (Qiagen Inc., Valencia, CA). Briefly, cells were scraped off the culture dish in RNA lysis buffer and subsequently homogenized using a Qias shredder column. Total RNA was then isolated over a "Rneasy" spin column and eluted in diethylpyrocarbonate (DEPC) water. Total RNA from hearts of animals exposed to 3 weeks hypoxia and normoxia was isolated as previously described (24). 10 μ g total RNA from cultured cardiac myocytes and 50 μ g of total RNA from rat heart samples were fractionated by glyoxyl-agarose gel electrophoresis and transferred to Hybond-N+ nylon membrane (Amersham Life Science, Arlington, IL). cDNA probes for HIF-1 α were labeled with (α^{32}) dCTP using the RTS-RadPrime DNA labeling system (Gibco BRL, Grand Island, NY). Hybridizations were performed in 5x Denhardt's and 40% Formamide solution at 42 $^{\circ}$ C using 25 ng of end-labeled cDNA probe. Blots were exposed to autoradiography at 70 $^{\circ}$ C after high

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stringency washes in 2X SSC, 0.1% SDS at room temperature and 0.1X SSC, 0.1% SDS at 65°C.

In-Situ Hybridization: In-situ hybridization was performed on serial sections of formalin fixed paraffin embedded heart mounted on 2-aminopropyltriethoxysilane coated slides. The conditions of target pretreatment, hybridization and probe generation have been extensively characterized (42,43). Sense and antisense orientation probes specific for iNOS mRNA were generated from a Bluescript vector containing the iNOS promoter-fragment (pBSiNOSprom). For the antisense riboprobe, pBSiNOSprom was digested with Eco RI and transcribed using T7 RNA polymerase, for the sense riboprobe pBSiNOSprom was digested with Xho I and transcribed using T3 RNA polymerase. The riboprobes were labeled to a specific activity of 1.1×10^8 dpm/ μ g using tritiated UTP and CTP, and were applied to the sections at a fully saturating concentration of 0.2 μ g/ml/kb. Following stringent washing at 60°C in 0.1X SSC (1X SSC = 0.15 mol/L NaCl, 0.0015 mol/L Na-citrate, pH 7.0) the sections were autoradiographed for 4 weeks, photographically developed, and counterstained with hematoxylin and eosin prior to microscopic observation. The above conditions were established in a series of preliminary experiments. In addition, RNA preservation of the specimens was assessed using a 1.8 kb probe directed against a ubiquitously expressed actin mRNA. The in situ hybridization data were analyzed using both brightfield morphology as well as darkfield optics to better visualize the full distribution of the silver grains generating the autoradiographic signal.