Effect of Chronic Hypoxia on Inducible Nitric Oxide Synthase Expression in Rat Myocardial Tissue

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The purpose of our study was to evaluate the effect of chronic exposure to low cellular oxygen tension (90% N_2 and 10% O_2 for 14 days) in inducing apoptosis and activation of transcription and translation of inducible nitric oxide (NO) synthase (iNOS) in rat hearts tissue. Rats were divided into four groups: normoxic, hypoxic, rats maintained in normoxic condition for 7 days and subjected to hypoxic conditions for another 7 days, and rats maintained in hypoxic condition for 7 days and subjected to normoxic conditions for another 7 days. At the 7th and 14th days, five rats from each group were sacrificed. Immunohistochemical and Western blot analysis were performed on myocardial tissue to reveal the presence of iNOS. Expression of iNOS was determined by RT-PCR. Apoptosis was evaluated by terminal deoxynucleotidyl transferase-mediated deoxyuridine triphosphate nick-end labeling and by detection of internucleosomal DNA fragmentation by electrophoresis. Electrophoretic analysis of DNA showed oligonucleosomal fragmentation in the hypoxic groups, but no ladder was observed in the other groups. This data was confirmed through end labeling with streptavidin-biotin (biotin d-UTP). iNOS expression was evaluated through immunohistochemical techniques (Ab anti-iNOS) and Western blotting, and the results were quantified with a computerized imaging analysis. The expression of iNOS protein was greater in the hypoxic groups; in the normoxic groups, only a nonspecific background was detected. This data was supported with results obtained through RT-PCR, which showed the specific transcription of mRNA for iNOS in the same experimental conditions. In addition, the iNOS activity was also evaluated and was found to be more active in the hypoxic groups (0.1 ± 0.01 vs 0.02 ± 0.003). The present study shows that exposure to low oxygen tension is capable of inducing programmed cell death and activating iNOS. Exp Biol Med 228:935–942, 2003

Key words: nitric oxide; apoptosis; hypoxia; myocardial tissue; tunel

Nitric oxide (NO) is a paracrine substance that plays a role in a variety of pathophysiological events (1, 2). NO is produced from L-arginine on conversion into L-citrulline by the enzyme NO synthase (NOS) in a reaction that requires molecular oxygen (1). NOS is a family of enzymes with three major isoforms (3). The three isoforms were originally identified in brain as the neuronal NOS (nNOS), in macrophages as the inducible NOS (iNOS), and in endothelial cells as the endothelial NOS (eNOS) (4, 5). In the heart, the eNOS that is present in the endothelium of coronary vessels and myocardium normally accounts for most of the NO production (6). iNOS is induced by a wide variety of agents, including endotoxin, interleukin 1β, and tumor necrosis factor-α. NO may play an important role in hypoxic vasodilatation in coronary arteries (7).

Hypoxia enhances NO formation in cardiomyocytes (8), in the lung (9), and in pulmonary endothelial cells (10). Others reported an inhibition of NO production by hypoxia in pulmonary arteries (11), pulmonary endothelial cells (12), and aortic endothelial cells (13). Finally, there are also reports that hypoxia does not change NO formation in rat mesangial cells (14) and human endothelial cells (15). The heterogeneity of the results may be due to differences in experimental protocol, from animals and tissues examined, or from the duration of hypoxia.

Apoptosis is a normal physiological process during embryogenesis and tissue turnover. However, apoptosis also plays an important role in the pathophysiology of many diseases (16). Programmed cell death is characterized by cytoplasmic condensation and DNA fragmentation into oligomers (17). Several studies report that apoptosis may be

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induced by various stimuli, such as ischemia-reperfusion and mechanical stretch (18, 19).

In physiological conditions, low cellular oxygen tension is a feature of adaptation to high altitude and endurance exercises (20), whereas in pathophysiological conditions, lower tissue oxygen may be associated with ischemia and fibrosis. Mammalian cells respond to hypoxia in part by increasing the expression of genes that encode both tissue-specific and ubiquitous proteins (21, 22).

The study that follows was designed to test the possibility that chronic hypoxia may influence the expression of iNOS mRNA and may increase the iNOS protein content in rat hearts. Although hypoxia may increase the incidence of apoptosis in cultured cardiomyocytes, the influence of chronic oxygen tension changes on apoptosis in cardiac cells *in vivo* is less clear.

Materials and Methods

Animal Experiments. Male Wistar rats (250–300 g) that had free access to food and water were used for the experiments. Randomly, the rats were subdivided in four experimental groups: A) normoxic rats (n = 10) were kept in ambient air; five rats were sacrificed after 7 days (A7), and five rats were sacrificed after 14 days (A14); B) hypoxic rats (n = 10); five rats were sacrificed after 7 days (B7), and five rats were sacrificed after 14 days (B14). The hypoxic rats were maintained for 1 and 2 weeks in a chamber filled with O_2 10% and N_2 90%; C) (n = 10); five rats were maintained in normoxic condition for the first 7 days (C7) and five rats were maintained in hypoxic conditions for the second 7 days (C14); and D) (n = 10); five rats were maintained in hypoxic conditions for the first 7 days (D7) and five rats were maintained in normoxic conditions for the second 7 days (D14). In brief, rats were anesthetized with Nembutal (20 mg/kg) after injection of 1000 IU heparin into the femoral vein. The hearts were quickly excised, washed in phosphate-buffered saline (PBS), cut into four to five blocks, fixed in liquid nitrogen, and stored at –80°C (22). The animal studies were approved by the ethics committee of the University of Chieti.

Immunohistochemical Analysis. Detection of iNOS protein was performed in 5-μm-thick frozen tissue slices sectioned in a cryostat (Leica, Heidelberg, Germany). Slides were blocked in PBS with 5% normal goat serum, 0.1% bovine serum albumin, and 0.1% Tween 20 for 30 min at room temperature. They were incubated with anti-iNOS antibody of rabbit origin (Santa Cruz Biotechnologies, Santa Cruz, CA) diluted 1:100 in PBS and applied for 30 min at room temperature. Afterward, the slides were washed in PBS three times for 5 min each and were then incubated for 30 min with biotinylated secondary antibody. The sections were then washed in PBS three times for 5 min, incubated for 30 min with Avidin Biotin (AB), and washed again in PBS three times for 5 min. The sections were incubated with peroxidase substrate for 5 min, washed in tap water for 5 min, and dehydrated (Rabbit ABC Staining System; Santa Cruz Biotechnologies) (23).

Western Blot Analysis for iNOS. Frozen tissue was sliced very thinly and thawed in 2 ml of ice-cold of lysis buffer (RIPA) containing inhibitors per gram of tissue. Determination of iNOS protein in the tissue was performed by Western blotting of protein extracts. Fifty micrograms of protein determined by Lowery method from normoxic and hypoxic hearts was separated by electrophoresis in a 7.5% SDS-PAGE (Bio-Rad, Hercules, CA) and was transferred at 4°C to nitrocellulose membrane (Bio-Rad) in glycine-methanol buffer. Nitrocellulose was then blocked in Tris-buffered saline (TBS)-milk and was incubated overnight in primary the anti-iNOS (Santa Cruz Biotechnologies) antisera. The nitrocellulose was then washed in TBS, incubated with an alkaline phosphatase, conjugated with a secondary antibody for 2 hr, washed again, and developed in an alkaline buffer with nitroblue tetrazolium (NBT) as substrate (Alkaline Phosphatase Conjugate Substrate kit; Bio-Rad) (24). β-Actin was used as an internal standard.

RT-PCR. As previously described, total RNA was extracted using 1 ml of RNA-fast (Biotecx, Houston, TX). Reverse transcription was performed in a volume of 20 μl containing 1 μl of M-MLV reverse transcriptase, 4 μl of dNTP, 2 μl of random primers, and 1 μl of RNase inhibitor (Ambion, Austin, TX) for 60 min at 42°C. PCR amplification was performed in a Eppendorf Mastercycler 5330 thermocycler using 2.5 μl of cDNA, 2 mM $MgCl_2$, 2 U of Taq DNA polymerase (Ambion), and 1.25 μl of 18 S (ribosomal mRNA) as internal standard. The amplification was operated for 30 cycles at the following conditions: 94°C for 60 sec (denaturation), 55°C for 60 sec (annealing), and 72°C for 60 sec (extension). The following primer pairs were used: 5′-TCTGTGCCTTTGCTCATGAC-3′ (sense) and 5′-CATGGTGAACACGTTCTTGG-3′ (antisense) for rat iNOS (25).

Citrulline Synthesis (NO Activity). The measure of the conversion of L-arginine to L-citrulline is a standard assay method to quantify NOS activity (26). Briefly, 10 μl of radioactive arginine, L-[2,3,4,5–^{3}H]arginine monohydrochloride 1 μCi/μl (64 Ci/mM; Amersham, Arlington Heights, IL), 50 μl of NADPH 10 mM, and 50 μl of $CaCl_2$ 6 mM, (Calbiochem, La Jolla, CA) were added to each tissue homogenate sample and incubated for 30 min at room temperature. After incubation, the reactions were stopped with 400 μl of stop-buffer (50 mM HEPES, pH 5.5, and 5 mM EDTA) and samples were equilibrated with resin to bind unreacted arginine. After centrifugation, the radioactivity corresponding to L-(^{3}H)-citrulline was measured with liquid scintillation spectrometry. Calcium was omitted from these incubations to favor the determination of the calcium-independent iNOS isoform.

In Situ End Labeling. Terminal deoxynucleotidyl transferase [TdT]-mediated desoxyuridinetriphosphate [dUTP] nick end-labeling (TUNEL) is a technique to esti-

mate apoptosis in tissue sections. The method is based on the preferential binding of TdT to the 3′-hydroxyl ends of DNA (27). The ability of TUNEL assays to identify apoptotic nuclei is limited when the reagents cannot access the DNA due to interference from hypercondensation (pyknosis) or local proteins. Fresh tissue sections from five hearts per group were used. The slides were fixed with cold acetone and were then incubated with 20 μg/ml proteinase K. Endogenous peroxidase was inactivated by immersion in 2% hydrogen peroxide. Tissue sections were stained with TdT buffer (Boehringer Mannheim, Indianapolis, IN) and $CoCl_2$ (Sigma, St. Louis, MO) for 5 min at room temperature. Residues of biotinylated dUTP (Boehringer Mannheim) were catalytically added to the ends of DNA fragments with the enzyme TdT for 60 min at 37°C. After end labeling, the sections were incubated with a streptavidin-biotinylated horseradish peroxidase complex (Amersham) for 45 min at room temperature. They were stained with 50 m*M* 3-3′diaminobenzidine tetrahydrochloride (DAB) and 0.03% H_2O_2 (Sigma) for 5 min at room temperature and were counterstained with Mayers-hematoxylin (Bio Optica, Milan, Italy) to detect the biotin-labeled nuclei. Deionized water was substituted instead of TdT as a negative control. Apoptotic bodies were stained brown. The *in situ* hybridization photomicrographs were scored by two blinded observers. Ten slides for each group were stained for TUNEL and three slides and four fields were randomly selected from each were photographed. Later, two external blinded observers counted total nuclei and the TUNEL-positive nuclei. The percentage of apoptotic nuclei relative to total nuclei observed was computed. When the difference between the two observers was higher than 15%, the image was not included. In some cases, the slides were observed again and nuclei were morphologically characterized.

DNA Fragment Electrophoresis. Heart tissue was homogenated and resuspended in lysis buffer followed by incubation with 100 μg of proteinase K (Sigma) overnight at 37°C. DNA was extracted by phenol and chloroform and was followed by ethanol precipitation. The pellet was resuspended in Tris-EDTA (TE) buffer, treated with RNase for 1 hr at room temperature, extracted again by phenol and chloroform, and followed by ethanol precipitation. The DNA was resuspended in TE buffer. The concentration of DNA was normalized by spectrophotometric assay, and 1 μg was electrophoretically fractionated on a 2% agarose gel with ethidium bromide (28).

Image Processing and Analysis System. For the immunohistochemistry, areas were chosen at random and recorded. Each experimental frame was digitized into 512 × 512 pixels. The densitometric analysis of western blots was performed using a video camera (Sony, Tokyo, Japan) connected with a Quantimet 500 plus (Leica Cambridg, Cambridge, UK). The change in integrated optical density (IOD.) was determined using ISO transmission density standard CAT 152-3406 (Eastman Kodak, Rochester, NY) (29).

Statistical Analysis. The results were expressed as means ± SD. Statistical analysis was performed using analysis of variance (ANOVA). Probability of null hypothesis of <5% ($P < 0.05$) was considered statistically significant. Replicate samples were determined for all the quantitative data presented along with blinded reviews of the histochemical data.

Results

iNOS Immunohistochemistry of Myocardial Tissue. To assess the presence and the localization of iNOS in myocardial tissue, an immunohistochemical analysis was conducted. Slides from myocardial tissue were prepared and incubated with an anti-iNOS antibody (Fig. 1). In normoxic rats, myocardial iNOS immunoreactivity was low at both the 7th (A7) and 14th (A14) days compared with the same time intervals in the hypoxic rats (B7 and B14). Though the increase in immunoreactivity at hypoxic day 14 was visually more apparent, the error and small number of subjects precluded verifying a quantifiably progression be-

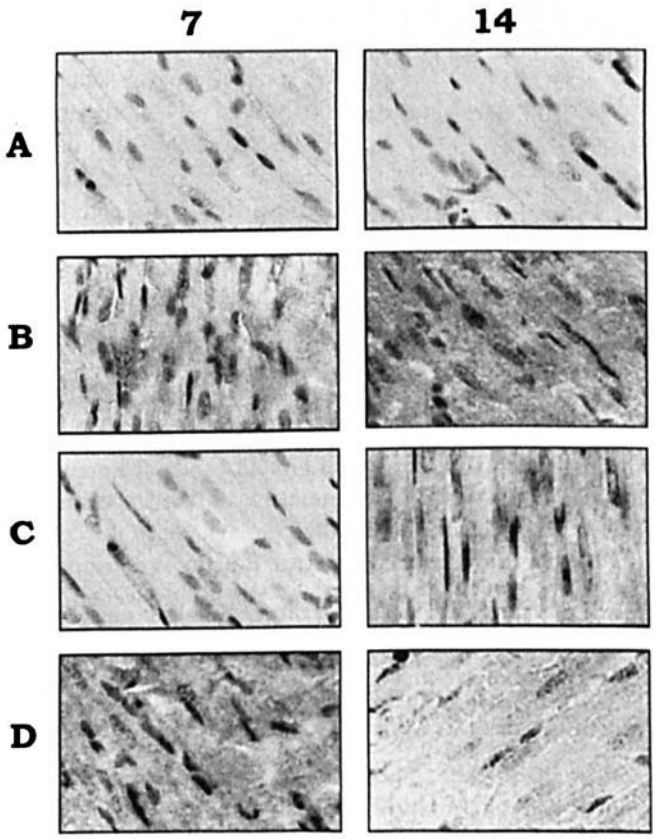

Figure 1. Examples of histological sections of rat hearts (magnification 40x) reacted with antisera specific for iNOS are pictured. Positive immunostaining is indicated by red-brown coloring. The individual panels represent myocardial sections from normoxic animals collected after 7 (A7) and 14 (A14) days, from hypoxic animals after 7 (B7) and 14 (B14) days, from normoxic animals after 7 (C7) days followed by hypoxia for 7 (C14) days, and from hypoxic animals after 7 (D7) days followed by normoxia for 7 (D14) days. The number of subject in each group was five. The quantitative densitometries of the staining intensity for these treatment groups are summarized in Tables I and II.

Table I. The Quantitative Densitometry of Samples Collected from Normoxic Animals after 7 (A7) and 14 (A14) Days and from Hypoxic Animals after 7 (B7) and 14 (B14) Days

iNOS	A7	A14	B7	B14
Immunohistochemistry	1.1 ± 0.5	1 ± 0.3	5.2 ± 2.8	8 ± 1.9
WB	0.15 ± 0.09	0.21 ± 0.16	0.6 ± 0.15	0.87 ± 0.09
RT-PCR	0.2 ± 0.09	0.3 ± 0.12	0.72 ± 0.2	0.95 ± 0.2
TUNEL + c(%)	0.02 ± 0.01	0.03 ± 0.01	0.7 ± 0.2	1.2 ± 0.4

Note. The abbreviations are WB for Western blot of the iNOS protein content, RT-PCR for iNOS mRNA amplified by reverse transcriptase-polymerase chain reaction, and %TUNEL + for the percentage of apoptotic cells estimated by TUNEL assay. There were five subjects in each experimental group.

tween Days 7 and 14. The IOD (Quantimet 500 plus; Leica) listed in Table I was lower in group A than that observed in group B (A7 versus B7, $P < 0.05$, A14 versus B14, $P < 0.01$). The effect of 7 days of hypoxia was confirmed in group C where the rats were maintained in normoxic condition for 7 days and were then subjected to hypoxia for other 7 days. In group C7 (1 ± 0.4 IOD), a low immunoreactivity was found, similar to group A7. The switch from normoxic to hypoxic conditions produced an increase in iNOS immunoreactivity 7 days later in group C14 (Table II, C7 versus D7, $P < 0.01$) similar to that observed after 7 days of hypoxia in group B7. When hypoxic animals were returned to normoxic conditions, the data were consistent with hypoxia-mediated induction of iNOS. The iNOS immunoreactivity appeared to decline between Days 7 and day 14 when one-half of the hypoxic animals were returned to normoxic conditions for 7 days (D7 versus D14, Table II). A significant sequential (D7 versus D14) decline was obscured by the large error in the D7 group, but within the crossover design, the D14 normoxic animals were different from their C14 hypoxic mates (D14 versus C14, $P < 0.05$).

iNOS Protein Levels. Western blots were prepared to confirm the immunohistochemical data and verify the changes in the tissue iNOS protein content. Figure 2 provides representative images of protein bands of immunoreactive iNOS at 130 kD from each of the experimental groups. Equal degrees of loading are evident in the consistent density of the internal standard, β-actin. A densitometer (Ultrascan XL; LKB/Pharmacia, Uppsala, Sweden) was used for normalization. Data are reported in Tables I and II as mean ± SD. The Western blots confirmed the immunohistochemistry and indicated that hypoxia increased iNOS protein in heart (A7 versus B7, $P < 0.01$, A14 versus B14, $P < 0.01$) and returning the animals to normoxic conditions restored tissue iNOS protein to control values (C14 versus D14, $P < 0.05$).

mRNA iNOS RT-PCR Analysis. To determine whether expression of mRNA encoding iNOS increased in response to hypoxia in heart tissue, RT-PCR was performed for all groups. As shown in Figure 3, a representative RT-PCR reveals iNOS mRNA in all groups. In this figure, the iNOS bands from the hypoxic groups (B7, B14, C14, and D7) are generally more intense than their respective normoxic partners (A7, A14, C7, and D14), which are very light. The 18S ribosomal standards are evident in each lane. The densitometry (Tables I and II) confirmed that hypoxia increased iNOS and mRNA (A7 versus B7, $P < 0.01$, A14 versus B14, $P < 0.01$) and that restoring animals to normoxic conditions returned iNOS and mRNA toward control values (C14 versus D14, $P < 0.005$).

TUNEL Reaction. The TUNEL reaction identifies apoptotic cells, and TUNEL-positive nuclei were evident in most sections (Fig. 4). There were fewer TUNEL-positive cells in the normoxic groups (A7 and A14, Table I) than in their hypoxic counterparts (B7 and B14). The distribution of TUNEL-positive nuclei was highly variable even within sample sections, but the percentage did increase when normoxic animals were made hypoxic in the crossover experiment (C7 versus C14, $P < 0.05$, Table II). The percentage of TUNEL-positive cells also declined when hypoxic animals were returned to normoxia (D7 versus D14, $P < 0.01$, Table II), although the value remained higher than normoxic controls after 7 days of normoxia (D14 versus A14, $P < 0.01$).

Electrophoretic Analysis for the Determination of DNA Fragments. Figure 5 illustrates representative separations of DNA fragments extracted from each of the

Table II. The Quantitative Densitometry of Samples Collected from Normoxic Animals after 7 (C7) Days Followed by Hypoxia for 7 (C14) days, and from Hypoxic Animals after 7 (D7) Followed by Normoxia for 7 (D14) Days

iNOS	C7	C14	D7	D14
Immunohistochemistry	1 ± 0.4	4.3 ± 0.4	5.4 ± 2.6	3.2 ± 0.8
WB	0.28 ± 0.1	0.57 ± 0.2	0.63 ± 0.2	0.29 ± 0.14
RT-PCR	0.2 ± 0.08	0.64 ± 0.25	0.58 ± 0.2	0.3 ± 0.1
TUNEL + c(%)	0.02 ± 0.01	0.67 ± 0.1	0.7 ± 0.04	0.4 ± 0.02

Note. WB, Western blot of the iNOS protein content; RT-PCR, iNOS amplified by RT-PCR; %TUNEL +, the percentage of apoptotic cells estimated by TUNEL assay. There were five subjects in each experimental group.

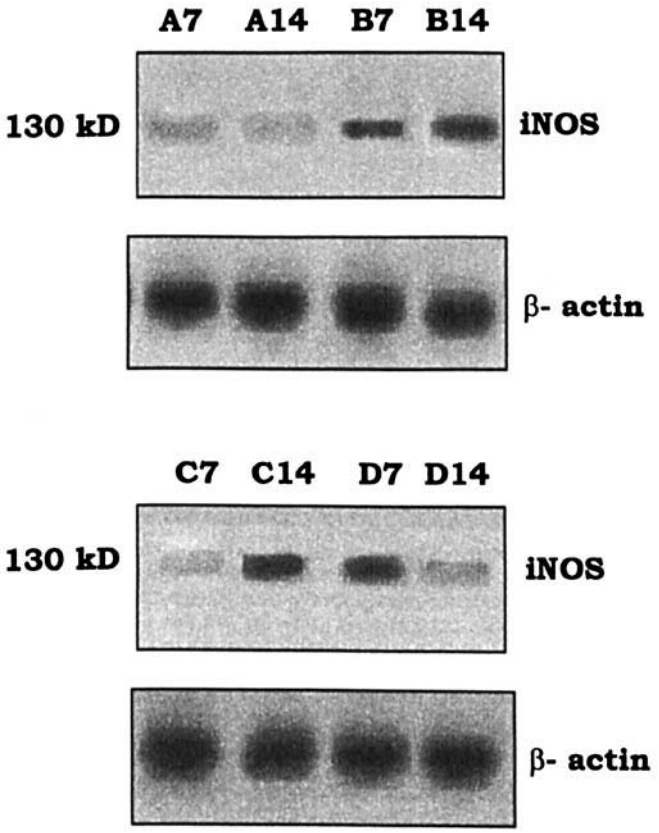

Figure 2. Sample Western blots are illustrated for myocardial iNOS protein extracted from each of the eight experimental groups described in the legend for Figure 1. Immunoreactivity for iNOS was identified at a position in the gel consistent with a mass of 130 kD. The gels were counterstained for β-actin to assure the application of equivalent tissue extracts. The quantitative densitometries for all of the western blots are summarized in Tables I and II.

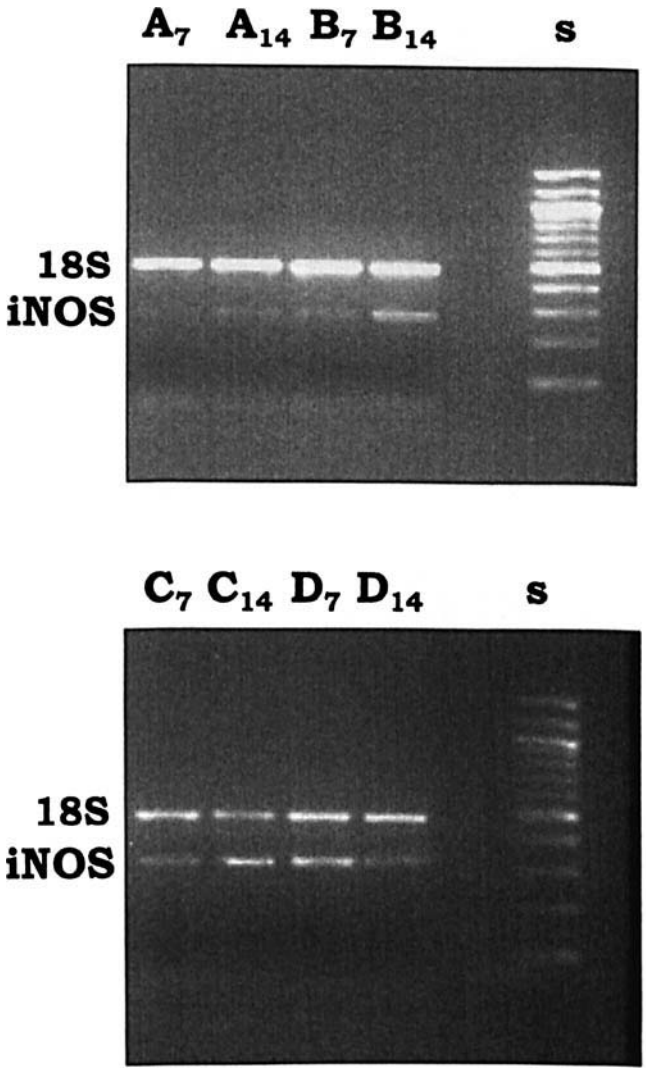

Figure 3. Sample electrophoretic gels illustrate the relative abundance of iNOS mRNA amplified by RT-PCR for each of the eight experimental groups described in the legend for Figure 1. Ribosomal mRNA (18S) and commercial molecular weight standards (S) were used as internal and external standards, respectively. The quantitative densitometries for this experiments are summarized for all treatment groups in Tables I and II.

experimental groups. DNA extracted from rat hearts subjected to hypoxic conditions (B7, B14, C14, and D7) contained fragmented DNA that produced a ladder consistent with internucleosomal DNA length of about 180 base pairs. Laddering was absent in extracts from normoxic rats (A7, A14, and C7). The persistence of laddering in group D14, which had been returned to normoxia, is unexplained but may reflect the persistence of an intermediate level of TUNEL-positive nuclei in that group.

iNOS Activity. Basal L-[^{3}H]citrulline production from L-[^{3}H]arginine was low but detectable in homogenates obtained from each of the normoxic groups (A7, A14, C7, and C14). NOS activity was elevated in samples obtained from each of the four hypoxic groups (B7, B14, C14, and D7). This increased enzyme activity was functionally consistent with the increased iNOS mRNA and immunoreactive iNOS protein observed above (Fig. 6).

Discussion

Hearts may be exposed to low oxygen tension in many circumstances, including pulmonary diseases, during cardiac arrest, and at high altitude. Chronic exposure to hypoxia and hyperoxia induces complex metabolic, functional, and structural modifications (30, 31). The chronic exposure to low cellular oxygen tension (hypoxia) was reportedly capable of inducing apoptosis in cultures of cardiomyocytes. Prior studies suggested that hypoxia influenced coronary eNOS activity in heart (32).

The current study was designed to specifically address possible alterations in iNOS in heart during chronic hypoxia. The basic findings indicate that immunoreactive iNOS was visually more evident in myocardial tissue sections from hypoxic animals than those from normoxic controls. The immunocytochemistry was confirmed by western blot.

The gene expression of iNOS also increased during hypoxic conditions. The increased iNOS during chronic hypoxia correlated with parallel increases in the apoptotic cells and DNA fragmentation. These observations are consistent with the hypothesis that hypoxia moderates iNOS

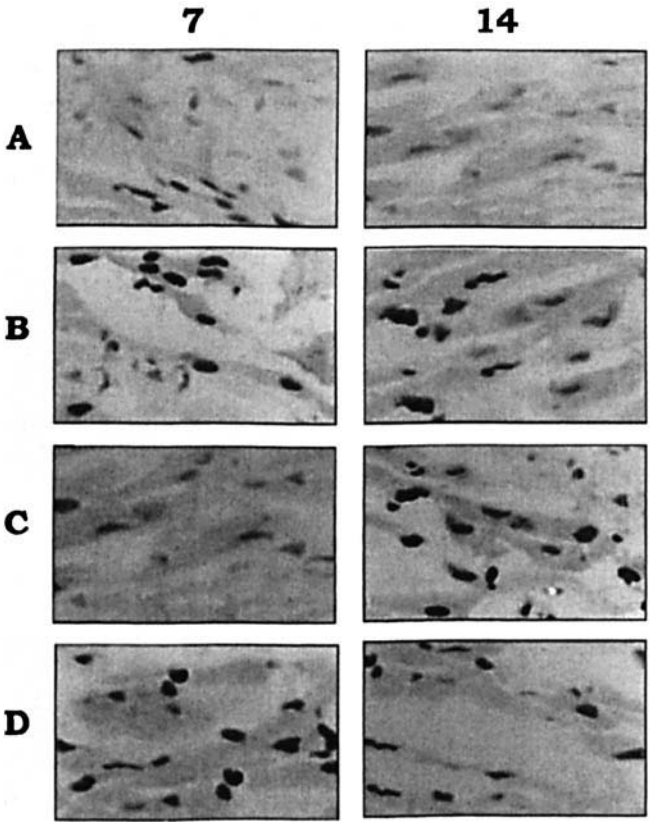

Figure 4. Examples of histological sections of rat hearts (magnification 40x) stained for TUNEL analysis are pictured from each of the experimental groups described in the legend for Figure 1. Apoptotic, TUNEL-positive cells are indicated by the dark nuclear coloration. The percentage of TUNEL-positive nuclei for all groups ranged from a minimum 0.6% to a maximum of 3.5% (mean 2.5 ± 1.07). The quantitative analyses of TUNEL-positive cells are summarized for all treatment groups in Tables I and II.

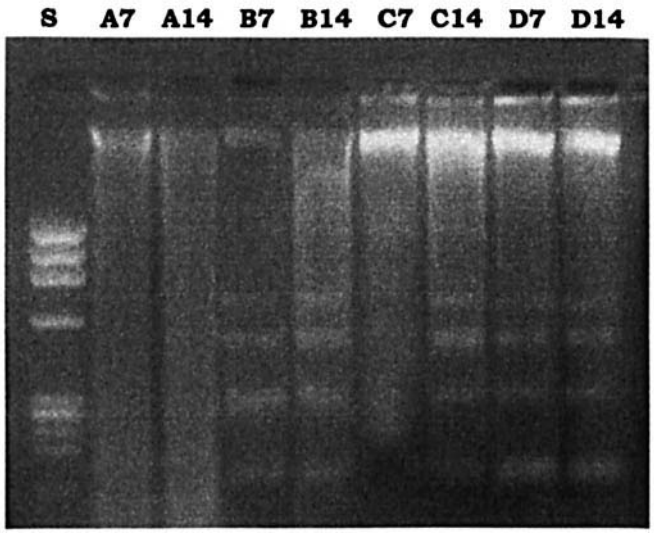

Figure 5. Sample agarose gel electrophoretic patterns are illustrated for DNA extracted from hearts from each of the experimental groups described in the legend for Figure 1. A standard 1-Kb ladder is provided for size comparisons in the far, left line (S). Five hearts from each of the experimental groups were analyzed in this manner, and the fragmentation pattern was consistently observed in groups B7, B14, C14, D7, and D14 as depicted in the representative gels.

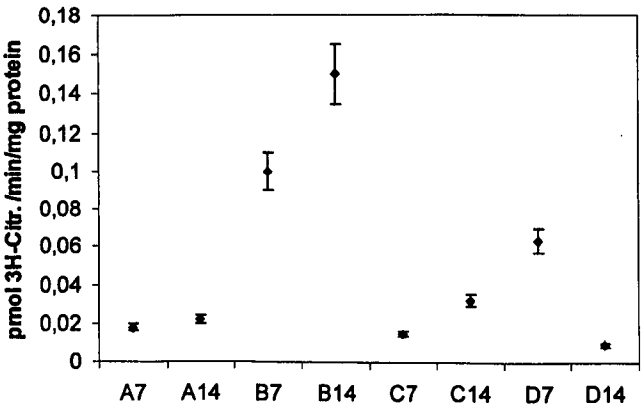

Figure 6. This graph illustrates NOS activity in tissue homogenates from each of the experimental groups described in the legend for Figure 1. The enzyme activity is expressed as radiolabeled citrulline (^{3}H-Cirt) produced.

expression in rat heart and that the resulting NO production contributes to programmed cell death.

The findings here show that the iNOS enzyme is more evident in hypoxic myocardial tissue compared with normoxic controls (Fig. 1) as confirmed by the generation of the iNOS protein in Western blots (Fig. 2). The gene expression of iNOS is also increased in hypoxic conditions (Fig. 3). The increase of iNOS in hypoxic myocardial tissue leads to an increase in the numbers of apoptotic cells (Fig. 4) and DNA fragmentation (Fig. 5). The iNOS gene could be regulated by hypoxia in rat hearts.

The regulation of NOS gene expression in response to low oxygen tension may be important in several physiological and pathological conditions in which available oxygen is decreased. In support of this concept, acute Simvastatin administration reduces ischemia-reperfusion injury and prevents coronary endothelial cell and cardiomyocyte damage by NO-dependent mechanisms (33).

Mechanisms by which hypoxia induces gene expression include transcriptional and posttranscriptional regulation (34). One mechanism by which hypoxia may increase iNOS expression is through the induction of transcription factors such as hypoxia-inducible factor (HIF-1). HIF-1 activates transcription of a number of genes in hypoxic conditions. Both HIF-1 mRNA and protein are induced by hypoxia and rapidly decay when the oxygen availability returns to normal conditions (35).

The majority of NO synthesized by constitutive and presumably eNOS activity produces beneficial effects on postischemic ventricular recovery and coronary flow (36). Endothelial NO serves as a second messenger in this respect and the rate of synthesis is comparatively low. In addition to facilitating the delivery of oxygen, studies have suggested that NO could also regulate oxygen demand (37) perhaps by reducing mitochondrial oxygen consumption independent of cyclic-GMP signaling (38). Therefore, moderate increases in NO during hypoxia might be helpful by matching both oxygen supply and demand. In contrast, NO synthesized by iNOS was associated with the myocytic and endo-

thelial injuries (39). Though eNOS is the predominant NO source in the heart, the capacity for NO production from increased iNOS after chronic hypoxia may substantially exceed production from the existing eNOS (40). Excessive or persistent production of NO may be responsible for the hypoxic tissue damage observed here and elsewhere (41, 42).

Cardiomyocyte apoptosis is an important pathogenic mechanism in myocardial ischemia/reperfusion injury. The effects of NO modulation by pharmacological species (such as statins) on myocardial apoptotic cell death was reported in several experimental studies (43). The precise mechanism of NO-mediated apoptosis is unknown. In this regard, the related cytoplasmic proteins Bcl-2 and Bax are proposed to prevent and induce apoptosis, respectively. Bax is believed to oppose the beneficial effect of Bcl-2 by forming heterodimers with Bcl-2. Therefore, the high Bax rather than Bcl-2 is proposed to accelerate cell death (44). NO may be involved in this process because NO stimulates the overexpression of Bax, and the subsequent overexpression of Bcl-2 suppresses NO-mediated apoptosis (45). A second connection between NO and apoptosis was provided by the observation that NO and NO donors induce Fas expression in multiple cell types (46). NO also induces the proinflammatory cytokine, tumor necrosis factor (TNF). TNF can activate pathways favoring apoptosis by binding to Fas, a member of the TNF receptor family (47, 48). The current observation correlating chronic hypoxia, increased expression of myocardial iNOS, and increased apoptotic activity are consistent with iNOS- and NO-mediated apoptosis in the heart. However, the precise mechanisms linking the hypoxic insult and apoptotic cell death have yet to be fully characterized.

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