

Expression of Angiogenic Factors During Acute Coronary Syndromes in Human Type 2 Diabetes

Raffaele Marfella,^{1,2} Katherine Esposito,^{1,2} Francesco Nappo,¹ Mario Siniscalchi,¹ Ferdinando Carlo Sasso,¹ Michele Portoghese,³ Maria Pia Di Marino,⁴ Alfonso Baldi,⁴ Salvatore Cuzzocrea,⁵ Clara Di Filippo,^{2,6} Guglielmo Barboso,⁶ Feliciano Baldi,⁴ Francesco Rossi,^{2,6} Michele D'Amico,^{2,6} and Dario Giugliano^{1,2}

Inadequate angiogenic response to ischemia in diabetic myocardium could result in poor collateral formation. Because hypoxia-inducible factor (HIF)-1 α is a transcriptional activator of vascular endothelial growth factor (VEGF) and is critical for initiating angiogenic responses to hypoxia, we investigated the expression of HIF-1 α and VEGF in specimens of human heart tissue to elucidate the molecular responses to myocardial ischemia in diabetic patients during unstable angina. Moreover, accumulation of a marker of protein nitration nitrotyrosine, as well as the superoxide anion (O₂⁻) levels and inducible nitric oxide synthase (iNOS), were evaluated. Ventricular biopsy specimens from 15 type 2 diabetic and 14 nondiabetic patients presenting with unstable angina (ischemic group) and from 20 patients (11 type 2 diabetic and 9 nondiabetic patients) who underwent coronary bypass surgery without angina within the preceding 10 days (control group) were collected during coronary bypass surgery. Nondiabetic patients had higher HIF-1 α and VEGF expressions compared with diabetic patients ($P < 0.001$). As compared with nondiabetic specimens, diabetic specimens showed higher levels of both iNOS mRNA and protein levels ($P < 0.001$) associated with the highest tissue levels of nitrotyrosine and O₂⁻ ($P < 0.001$). Diabetes is associated with increased myocardial tissue levels of iNOS, O₂⁻, and nitrotyrosine and reduced expression of myocardial angiogenesis factors during ischemia. *Diabetes* 53:2383–2391, 2004

D diabetic patients whose tests sustain a nonfatal myocardial infarction (MI) experience a more complicated course, including more frequent postinfarction angina, infarction extension, and congestive heart failure (1). The basis for these differences

in outcome remains unclear. The survival of myocardial tissue subjected to ischemia can be increased by the ability to promote growth of new blood vessels into ischemic areas, thus limiting regions of impairment and ultimately preserving myocardial function (2). Although the impairment of collateral vessel development is well established in diabetic patients (3), the factors responsible for this alteration are not well known.

Hypoxia, subsequent to ischemia, is a potent regulator of a variety of biologic processes, including angiogenesis, vascular contractility, and erythropoiesis (4). When a coronary artery is partially or totally occluded, metabolic and contractile changes are initiated in the heart within seconds (5). Some of these early changes facilitate cellular preservation and functional survival of the heart. If the myocardium remains deprived of blood, changes of progressively greater severity eventually culminate in cell death, tissue necrosis, and myofibrillar remodeling (6). Hypoxia-inducible factor (HIF)-1 α is a transcriptional factor that is expressed in response to a decrease in the partial pressure of cellular oxygen and activates the transcription of gene whose protein products mediate adaptive responses to hypoxia/ischemia. HIF-1 α is an 826-amino acid protein that functions as a *trans*-acting transcriptional activator of vascular endothelial growth factor (VEGF) and inducible nitric oxide (NO) synthase (iNOS) (7). Peak of expression of HIF-1 α and VEGF, as well as the NO production from iNOS, may contribute to limitation of ischemic injury by promoting angiogenesis and vascular remodeling in the human heart during ischemia (8,9). Although an impairment of HIF-1 α and VEGF expressions as well as an increment of iNOS expression have been evidenced in diabetic animals after ischemia-reperfusion injury (10–12), there are no data that indicate these alterations in human diabetic hearts.

This study was undertaken to examine whether the angiogenic process during unstable angina is influenced by diabetes. To address these issues, HIF-1 α and VEGF expressions were evaluated in myocardial biopsies obtained from patients with and without type 2 diabetes who were admitted to emergency wards for unstable angina and had to undergo coronary bypass surgery. Additionally, iNOS expression, the localization of nitrotyrosine, and the production of superoxide anion (O₂⁻) in myocytes were measured quantitatively. Nitrotyrosine was determined because this modified amino acid is a product of reactive O₂ (13).

From the ¹Department of Geriatrics and Metabolic Diseases, Section of Pathology, Second University of Naples, Naples, Italy; ²Centro di Eccellenza Cardiovascolare, Section of Pathology, Second University of Naples, Naples, Italy; the ³Cardiovascular Surgery Unit, Hospital Sassari, Sassari, Italy; the ⁴Department of Biochemistry, Section of Pathology, Second University of Naples, Naples, Italy; the ⁵Department of Pharmacology, University of Messina, Messina, Italy; and the ⁶Department of Experimental Medicine, Section of Pathology, Second University of Naples, Naples, Italy.

Address correspondence and reprint requests to Dott. Raffaele Marfella, Via Emilio Scaglione 141, 80145 Napoli, Italy. E-mail: raffaele.marfella@unina2.it. Received for publication 13 March 2004 and accepted in revised form 26 May 2004.

HIF, hypoxia-inducible factor; iNOS, inducible nitric oxide synthase; MI, myocardial infarction; VEGF, vascular endothelial growth factor.

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RESEARCH DESIGN AND METHODS

The study group comprised 15 type 2 diabetic and 14 nondiabetic patients presenting with unstable angina without previous MI and who were to undergo coronary bypass surgery (ischemic group). The assessment of type of unstable angina was based on Braunwald's classification (14)—they had evidence of myocardial ischemia at rest within 48 h, defined as angina or heart failure without Q waves on the electrocardiogram and without an increase in serum levels of troponin I (class III/B). Patients were admitted into the emergency wards from January 2001 to January 2004. The criteria for enrollment included the need for coronary bypass surgery in unstable angina (15). Coronary angiography and left ventricular cineangiography were performed by standard techniques. Twenty patients (11 diabetic and 9 nondiabetic patients) who underwent coronary bypass surgery but had not had angina or heart failure within the preceding 10 days were included in our study as the control group. Patients with interfering noncardiac diseases, such as inflammatory disorders, malignancy, or infection, as well as three-vessel disease, were not eligible for the study. The study protocol was approved by the institutional ethics committee for human subjects. Written informed consent was obtained from all patients.

Measurements of blood pressure and routine analyses were obtained on admission before patients started full medical therapy, including β -blockers and/or calcium antagonists, low-dosage aspirin, and continuous intravenous infusion of nitrates, and before coronary angiography and left ventricular cineangiography. Venous blood for troponin I levels was collected in EDTA-coated tubes immediately after patients arrived at the emergency department. Troponin I was measured with an Opus Magnum device (Behring Diagnostics). A discriminator value of 2.0 $\mu\text{g/l}$ was used for troponin I, as recommended by the manufacturer.

Biopsy of myocardium. After the induction of anesthesia and median sternotomy, the heart of each patient was examined, and 3-mm partial-thickness biopsy specimens were taken from the left ventricle. All biopsies were performed before coronary artery bypass, during ventilation with a fraction of inspired oxygen of 40% and peripheral oxygen saturations of $>95\%$. In patients with unstable angina (ischemic group), specimens were taken from the area of presumed ischemia (ischemic area) and from an area of the ventricle free of coronary disease (nonischemic area) that could have caused ischemia. In this way, each patient with ischemia served as his or her own control. In patients without evidence of ischemia (control group), a single ventricular biopsy specimen was obtained. Biopsy sites were closed with polypropylene sutures.

Analysis of specimens. Half of each biopsy specimen was fixed in formalin, sectioned to a thickness of 5 μm , mounted on slides, and stained with hematoxylin and eosin. The mounted specimens were then examined for evidence of ischemia or kept for immunohistochemistry. A portion of the other half of the specimen was frozen in liquid nitrogen for the following Western blot analysis, and the remaining portion was prepared for RT-PCR.

Measurement of RNA. Each sample was analyzed for the presence of transcripts encoding iNOS, human VEGF, and HIF-1 α as already published (10). Total RNA was extracted using RNazol reagent (Biotex Laboratories) according to the manufacturer's protocol. Levels of HIF-1 α , VEGF, and iNOS were measured by RT-PCR amplification by using the following primer sequences: human VEGF₁₆₅ 5'-3' = ATGAACTTTCTGTCTTGGGTG and 3'-5' = TCACCGCTCGGCTTGTCACA; iNOS sense 5'-GCCTCGCTCG GAAAGA-3' and the antisense 5'-TCCATGCAGACAAGCTT-3'; human HIF-1 α 5'-3' = CTGCTTGGTGCTGATTTGTGA and 3'-5' = TCCTGTACTGTCCTGT GGTGA.

Appropriate regions of the hypoxanthine-phosphorybosyl transferase cDNA were amplified as control. Amplifications were carried out for 32 cycles, using the following conditions: 94°C for 1 min, 57°C for 1 min, and 72°C for 1 min. Each RT-PCR experiment was repeated at least three times. Amplification products were electrophoresed on 2% agarose gel in 1 \times Tris acetic acid EDTA. Semiquantitative analysis of mRNA levels was carried out using the software associated with the Gel Doc 1000 (Biorad, Hercules, CA).

Western blot analysis. HIF-1 α , VEGF, and iNOS were determined by immunoblot analyses. Heart samples were lysed in a buffer containing 1% Nonidet P-40 for 30 min at 4°C. The lysates were then centrifuged for 10 min at 10,000g at 4°C. After centrifugation, 20 μg of each sample were loaded, electrophoresed in polyacrylamide gel, and electroblotted onto a nitrocellulose membrane. Primary antibodies to detect HIF-1 α , VEGF, and iNOS were obtained from Santa Cruz Biotechnology (Santa Cruz, CA) and were utilized according to the manufacturer's instructions. Immunoreactive signals were detected with a horseradish peroxidase-conjugated secondary antibody (Santa Cruz) and reacted with an enhanced chemiluminescence system (Amersham Pharmacia, Uppsala, Sweden). Each Western blot was repeated at least three times.

Immunohistochemical staining. Portions of the biopsy specimens were fixed in 10% formalin and prepared as 5- μm -thick tissue sections on slides. The paraffin was then removed with a xylene substitute (Hemo-De; Fisher Scientific), and the sections were rehydrated with ethanol gradient washes. Tissue sections were quenched sequentially in 3% hydrogen peroxide in aqueous solution and blocked with PBS/6% nonfat dry milk (Biorad) for 1 h at room temperature.

The sections of affected tissue and normal tissue from patients with ischemia and the sections from patients without ischemia were incubated with rabbit polyclonal anti-iNOS (cat. no. Sc-650; Santa Cruz) or mouse monoclonal anti-VEGF (cat. no. Sc-7269; Santa Cruz,) at a dilution of 1:100 and visualized by the streptavidin-biotin system (Dako) using diaminobenzidine (DAB) as the final chromogen. Tyrosine nitration, an index of the nitrosylation of proteins by peroxynitrite and/or reactive oxygen species (ROS), was determined by immunohistochemistry as previously described (13). Sections were incubated overnight with anti-nitrotyrosine rabbit polyclonal antibody (1:500 in PBS, vol/vol). Sections were washed with PBS and incubated with secondary antibody. Specific labeling was detected with a biotin-conjugated goat anti-rabbit IgG and avidin-biotin peroxidase complex (DBA, Milan, Italy). The sections were then scored for intensity of immunostaining (0 = absent, 1 = faint, 2 = moderate, and 3 = intense) for each antibody, and the average value was calculated for each section.

Measurement of O_2^- . Production of O_2^- was measured as the superoxide dismutase-inhibitable reduction of cytochrome c, as previously described (16).

Statistical analysis. The statistical analysis was performed with one-way ANOVA, followed by Duncan's multiple range test. $P < 5\%$ was considered to be statistically significant. All statistical analysis was made on IBM computers with the SOLO software package (BMDP, statistical software). The strength of the association of plasma HbA_{1c} with HIF-1 α , VEGF, and iNOS expression in ischemic specimens was assessed by linear regression analysis. All data are presented as means \pm SD.

RESULTS

Preoperative characteristics of the patients and classification of ventricular specimens. The characteristics and cardiac measurements of the patients before coronary bypass surgery and heart biopsy are shown in Table 1. The unstable angina therapy was similar in both groups (data not shown). There were no significant differences in the indication for coronary artery bypass grafting (CABG) among the groups (Table 1). As expected, on admission patients with diabetes had significantly higher plasma glucose levels compared with nondiabetic patients ($P < 0.001$). The glycosylated fraction of the major component of adult hemoglobin (HbA_{1c}) was elevated in diabetic patients; this elevation ranged from 7.1 to 10.9%, with a median of 8.2%. Among the diabetic subjects, eight were being treated with insulin therapy, eight with sulfonylureas, and six with metformin. The duration of diabetes ranged from 2 to 14 years, averaging 7 years. All ventricular biopsy specimens were examined by light microscopy for evidence of ischemia by a cardiac pathologist who was unaware of the patients' identity. Fifteen specimens had evidence of acute ischemia that had occurred within 48 h before biopsy (the ischemic group), and 12 specimens had no evidence of ischemia (the control group). For each patient in ischemic group, the second ventricular biopsy specimen, taken from an area remote from ischemic area, was found on microscopical examination to be normal.

Molecular analysis of ventricular specimens

HIF-1 α . Figure 1 shows the results of the analysis of HIF-1 α and HIF-1 α mRNA levels from ventricular specimens from the groups of patients. Nondiabetic patients of the ischemic group had detectable steady-state levels of HIF-1 α and HIF-1 α mRNA in specimens from the ischemic area and did not have detectable levels of both HIF-1 α expressions in the nonischemic area: HIF-1 α expression

TABLE 1
Clinical characteristics of the study patients

	Ischemic group		Control group	
	Nondiabetic patients	Diabetic patients	Nondiabetic patients	Diabetic patients
<i>n</i>	14	15	9	11
Age (years)	59 ± 3	58 ± 5	57 ± 6	56 ± 6
Male/female	8/6	9/6	4/5	7/4
BMI (kg/m ²)	25 ± 0.4	28 ± 0.6	25 ± 0.3	27 ± 0.4
Systolic blood pressure (mmHg)	118 ± 7	120 ± 11	119 ± 9	122 ± 10
Diastolic blood pressure (mmHg)	81 ± 7	81 ± 6	83 ± 9	82 ± 8
Heart rate (bpm)	70 ± 2	76 ± 3	71 ± 10	75 ± 12
Blood glucose (mmol/l)	6 ± 0.9	12.6 ± 1.3*	6.1 ± 0.8	10.4 ± 1.6†
Total cholesterol (mmol/l)	6.69 ± 0.04	6.82 ± 0.05	6.70 ± 0.03	6.79 ± 0.06
HDL cholesterol (mmol/l)	1.28 ± 0.04	1.24 ± 0.03	1.29 ± 0.05	1.25 ± 0.04
Triglycerides (mmol/l)	2.24 ± 0.16	2.61 ± 0.18	2.22 ± 0.17	2.53 ± 0.20
Smokers	6 (43)	6 (40)	2 (22)	3 (27)
Serum creatinine (μmol/l)	78.5 ± 2.7	79.2 ± 2.6	78.6 ± 2.3	77.5 ± 2.2
Previous disorder				
Angina pectoris	4 (28)	4 (26)	2 (22)	2 (18)
Hypertension	4 (28)	5 (33)	3 (33)	4 (36)
Active therapy				
β-Blocker	3 (21)	3 (20)	2 (22)	3 (27)
Diuretic	1 (7)	1 (6)	1 (11)	2 (18)
ACE inhibitors	1 (7)	4 (26)	2 (22)	4 (36)
Ejection fraction (%)	39.8 ± 7.6	33.1 ± 6.6	44.8 ± 6.8	43.5 ± 5.8
Troponin I (μg/l)	0.9 ± 0.6	1.1 ± 0.8	0.8 ± 0.5	0.9 ± 0.6
Vessel disease				
Left main coronary artery stenosis	9 (64)	10 (67)	3 (33)	4 (36)
Stenosis of LAD and LCA	5 (36)	5 (33)	6 (67)	7 (64)

Data are means ± SD or *n* (%). **P* < 0.001 vs. nondiabetic ischemic group; †*P* < 0.001 vs. nondiabetic control group. LAD, left anterior descending artery; LCA, left circumflex artery.

reached a value of 757.3 ± 36% and HIF-1α mRNA reached a value of 875.8 ± 99% in specimens of the ischemic area compared with those of the nonischemic area (*P* < 0.001). In diabetic patients, HIF-1α mRNA transcripts were slightly detected in specimens from the ischemic area: HIF-1α expression reached a value of 163.7 ± 51% and HIF-1α mRNA reached a value of 185.6 ± 69% in specimens of the ischemic area compared with those of the nonischemic area (*P* < 0.001). These values are only 21.6 and 21.2% of the incremental expressions seen in nondiabetic patients in ischemic specimens (*P* < 0.001). Both diabetic and nondiabetic patients without acute ischemia (control groups) had no detectable expressions of HIF-1α in their single specimens. Notably, HIF-1α expression in ischemic specimens was strongly dependent on glycemic control (Fig. 1C), as also reflected by the statistically significant inverse correlation (*R* = -0.533, *P* < 0.001) between plasma HbA_{1c} and HIF-1α concentrations.

VEGF. Figure 2 shows the results of the analysis of VEGF and VEGF mRNA levels from ventricular specimens from the groups of patients. Nondiabetic patients in the ischemic group had detectable steady-state levels of VEGF and VEGF mRNA in specimens from the ischemic region and did not have detectable levels of VEGF mRNA in the nonischemic area (VEGF 765.9 ± 94% and VEGF mRNA 818.7 ± 101% vs. the nonischemic region). In diabetic patients, VEGF mRNA transcripts were slightly detected in specimens from the ischemic area of the ventricle (VEGF 148.4 ± 43% and VEGF mRNA 176.7 ± 55% vs. the nonischemic area). These values are only 19.4 and 21.6% of the incremental VEGF expressions seen in nondiabetic

ischemic areas (*P* < 0.001). Both diabetic and nondiabetic control groups had no detectable expressions of VEGF in their single specimens. Strong immunostaining for VEGF was seen in biopsy from the ischemic area of nondiabetic patients. VEGF protein in these specimens was found in cytoplasm of endothelial cells lining small vessels and in cardiomyocytes (Fig. 2). In contrast, VEGF protein was almost undetectable in the ischemic area of diabetic patients, where it was confined to the myocardial vasculature (Fig. 2). Moreover, VEGF expression was inversely correlated with plasma HbA_{1c} in ischemic specimens (*R* = -0.483, *P* < 0.001).

iNOS. Figure 3 shows the results of the iNOS and iNOS mRNA levels from ventricular specimens from the groups of patients. In the ischemic group, iNOS and iNOS mRNA expressions were present in specimens from the nonischemic areas in both groups, although at a higher level in the diabetic group (*P* < 0.001). Nondiabetic patients with acute ischemia had levels of iNOS and iNOS mRNA in the specimens from the ischemic area significantly higher compared with levels of iNOS and iNOS mRNA in the nonischemic area (*P* < 0.001). Diabetic patients with acute ischemia had significantly higher levels of both iNOS and iNOS mRNA in specimens from the ischemic area compared with the levels of iNOS expressions in the nonischemic area: iNOS expression reached a value of 466 ± 76% and iNOS mRNA reached a value of 568 ± 79% in specimens of the ischemic area compared with those of the nonischemic area (*P* < 0.001). These values are significantly higher compared with the increments of iNOS expressions seen in the ischemic area of nondiabetic

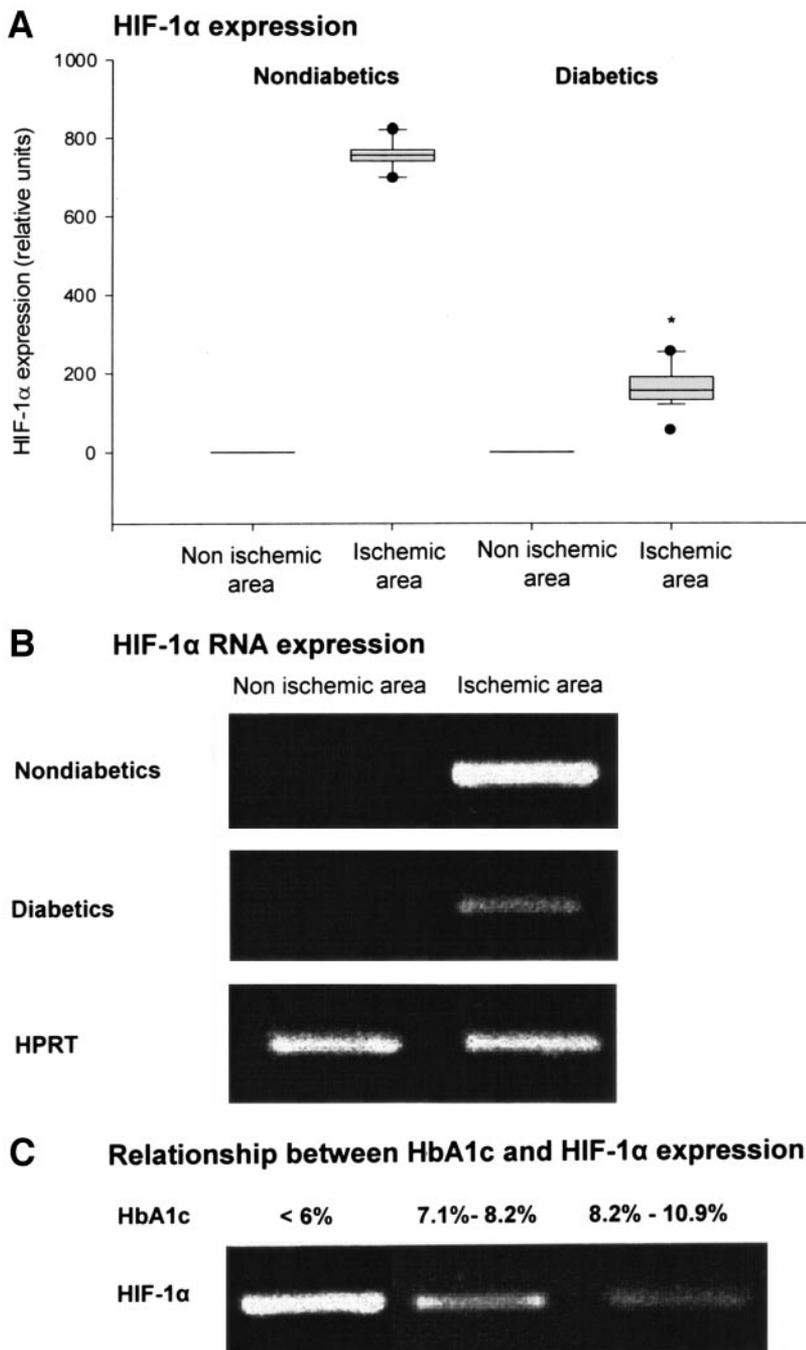


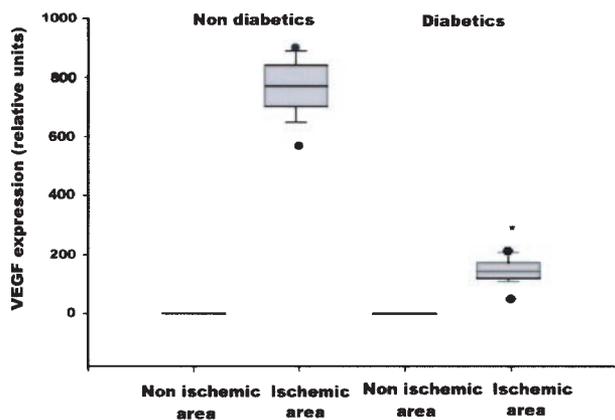
FIG. 1. *A*: Western blot analysis of HIF-1 α content in heart specimens from diabetic and nondiabetic patients (boxplot—a plot type that displays the 10th, 25th, 50th, 75th, and 90th percentiles as lines on a bar centered around the mean and the 5th and 95th percentiles as error bars; the mean line and data points beyond the 5th and 95th percentiles can also be displayed). *B*: Representative PCR analysis of HIF-1 α content in heart specimens from diabetic and nondiabetic patients. *C*: Relationship between HIF-1 α expression and HbA_{1c}. * $P < 0.001$ vs. nondiabetic patients.

patients (iNOS $269.7 \pm 76\%$ and iNOS mRNA $333.7 \pm 74\%$ vs. the nondiabetic ischemic area, $P < 0.001$). Nondiabetic patients of the control group had slightly detectable expressions of iNOS and iNOS mRNA in their single specimens that were significantly lower than iNOS expressions seen in the diabetic control group ($P < 0.001$). Immunohistochemical staining with antibody to iNOS of sectioned biopsy specimens from diabetic patients with ischemia revealed iNOS protein in areas of ischemia. Specifically, immunoreactivity was seen in the cytoplasm of cardiomyocytes and endothelial cells lining the small vessels (Fig. 3). iNOS protein from nondiabetic patients compared with diabetic patients showed a significantly lower immunostaining in both ischemic and nonischemic areas and in specimens from patients without ischemia (Fig. 3). Nota-

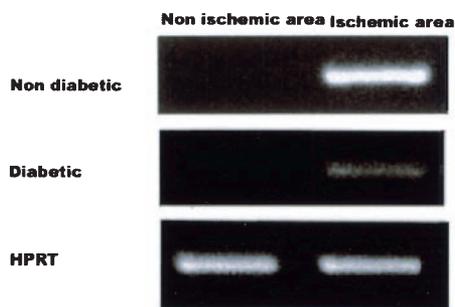
bly, iNOS expression in ischemic specimens was strongly dependent on glycemic control, as also reflected by the statistically significant correlation ($R = 0.677$, $P < 0.001$) between plasma HbA_{1c} and iNOS concentration

Nitrotyrosine. When immunostaining for the nitrotyrosine antigen was compared, differences were found between tissues from diabetic and nondiabetic ischemic specimens. Significantly intense nitrotyrosine immunostaining was present in diabetic tissue compared with nondiabetic tissues (score 3.4 ± 0.43 vs. 1.2 ± 0.31 , $P < 0.001$) (Fig. 4). In the nonischemic areas, significantly intense nitrotyrosine immunostaining was present in diabetic compared with nondiabetic patients (1.8 ± 0.12 vs. 0.28 ± 0.05 , $P < 0.001$). Nitrotyrosine immunostaining from specimens of the diabetic control group was signifi-

A



B



C

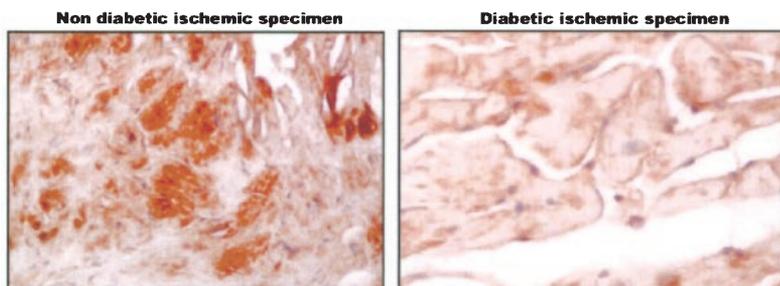


FIG. 2. A: Western blot analysis of VEGF content in heart specimens from diabetic and nondiabetic patients. B: PCR analysis of VEGF content in heart specimens from diabetic and nondiabetic patients. C: Representative immunohistochemical analysis of VEGF from ischemic ventricular biopsy specimens. * $P < 0.001$ vs. nondiabetic patients.

cantly higher than those of the nondiabetic control group ($P < 0.001$).

O_2^- . When O_2^- production was compared, differences were found between tissues from diabetic and nondiabetic ischemic specimens. Significantly higher levels of O_2^- were present in diabetic tissue compared with nondiabetic tissue ($P < 0.001$) (Fig. 5). In nonischemic areas, significantly higher levels of O_2^- were present in diabetic tissue compared with nondiabetic tissue ($P < 0.001$). O_2^- levels from specimens of the diabetic control group were significantly higher than those of the nondiabetic control group ($P < 0.001$).

DISCUSSION

To the best of our knowledge, there have been no studies investigating the association among diabetes, angiogenic factors, and oxidative stress in human heart tissue affected by ischemic insult. The main findings of our study demonstrate an association between diabetes, reduced expression of HIF-1 α and VEGF, and increment of iNOS, O_2^- , and

nitrotyrosine levels in heart specimens of patients with unstable angina. Diabetes amplifies oxidative reaction and worsens the angiogenic process. In nondiabetic patients, we detected increased steady-state levels of HIF-1 α and VEGF after unstable angina. This accumulation of both mRNA and protein was limited to the region of affected myocardium. No HIF-1 α and VEGF transcripts or proteins were detectable in nonischemic specimens. In diabetic patients, the picture is quite different because both HIF-1 α and VEGF levels were significantly lower than those in nondiabetic heart specimens. Thus, the roles of HIF-1 α and VEGF in beginning angiogenic process after myocardial ischemia seem to be reduced in diabetes.

Development of collateral vessels is triggered by the pressure gradient between the coronary bed of arteries caused by an obstruction and myocardial ischemia (17). Diabetes has been found to be an inhibiting factor on coronary collateral vessels development in a large patient population (3) and in a postmortem study (18). However, a lack of collateral vessels in diabetic patients, despite the

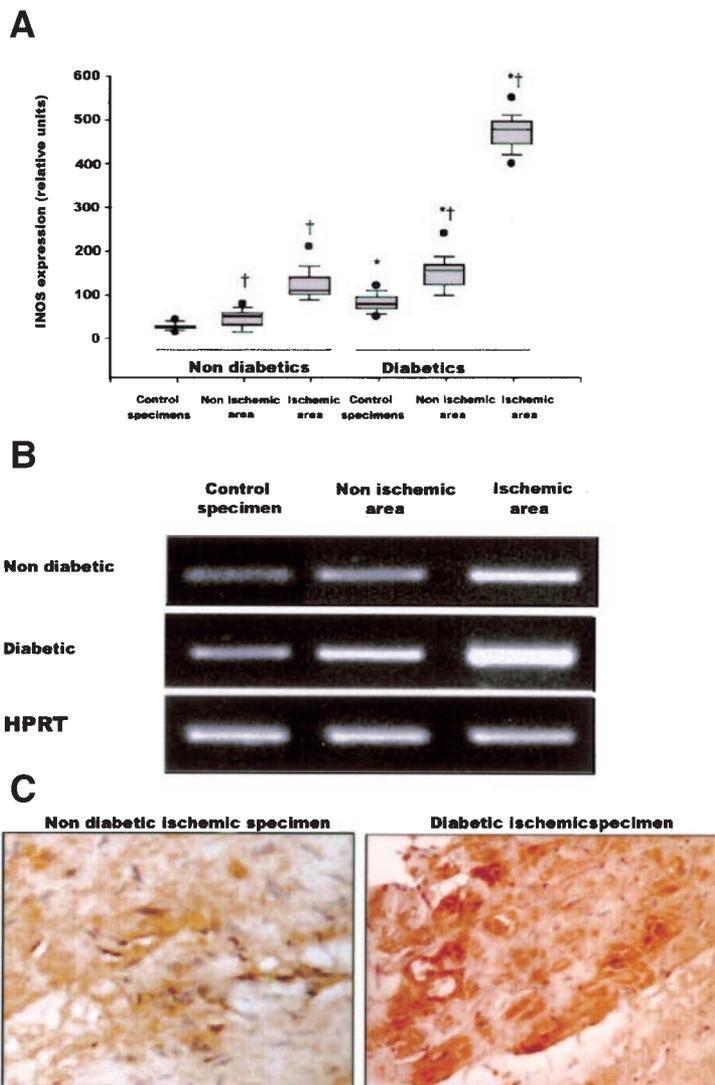


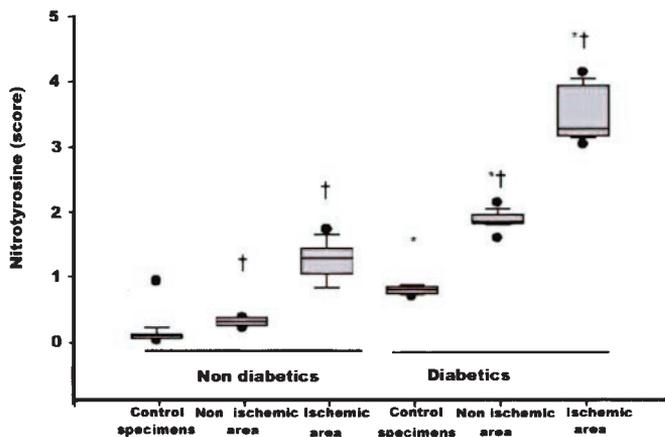
FIG. 3. *A:* Western blot analysis of iNOS content in heart specimens from diabetic and nondiabetic patients. *B:* PCR analysis of iNOS content in heart specimens from diabetic and nondiabetic patients. *C:* Representative immunohistochemical analysis of iNOS from ischemic ventricular biopsy specimens. * $P < 0.001$ vs. nondiabetic patients; † $P < 0.001$ vs. control specimens.

presence of coronary obstruction and evidence of myocardial ischemia, suggests that additional factors may contribute to collateral development. It is now widely accepted that myocardial ischemia somehow triggers collateral growth (19). A biochemical signal produced by ischemic myocardium may trigger the events leading to DNA synthesis and to mitosis in collateral vessels (20). Over the past decade, numerous angiogenic factors have been purified, and their amino acid sequences have been determined with subsequent gene cloning (21). Semenza (7) has shown in both in vitro and in vivo models of ischemia that one of the first genes upregulated by hypoxia is the gene encoding HIF-1 α . The expression of the gene for HIF-1 α is exquisitely sensitive to the onset of cellular hypoxic conditions, making it one of the earliest effectors of the response to ischemia (7). Thus, the decrease in the partial pressure of cellular oxygen induced by ischemia is a potent stimulator of HIF-1 α expression and of HIF-1 α -mediated VEGF gene transcription. VEGF has an important role in stimulating the growth of new capillaries in several organ systems and thus is a good candidate for the role of stimulating neovascularization to limit damage from ischemia in the heart. Although the mechanism of enhanced VEGF expression remains to be determined, it is

worth noting that the gene for VEGF has an HIF-1 α regulatory consensus sequence (a hypoxia-responsive element) in its promoter region (22). These observations, together with the previous finding that HIF-1 α is responsible for the increase in VEGF in cultured hypoxic myocytes (23), suggest that the increase in myocardial HIF-1 α protein that we detected in nondiabetic ischemic tissue is necessary for the enhanced expression of myocardial VEGF in states of acute ischemia. In contrast, the reduction of HIF-1 α gene and protein by diabetes may lead to impairment of VEGF-mediated angiogenesis during acute ischemia. Several investigations have shown increased VEGF-mediated angiogenesis in microvascular complications associated with diabetes (24), but the decrease in cardiac mRNA expression of VEGF and its protein in ischemic heart tissue is also consistent with pathological reports that collateral vascular formation after myocardial ischemia is blunted in diabetic patients (3). The paradoxical changes in the expression of VEGF and its receptors suggest that local regulatory factors differ between myocardium and microvessels.

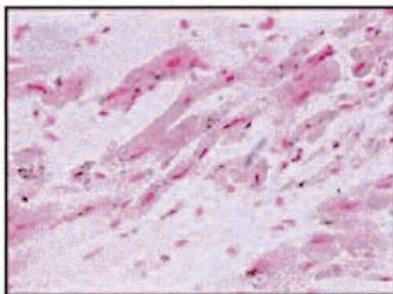
The recent demonstrations that reduced NO availability (25) and iNOS overexpression (26) may inhibit HIF-1 α activity reveal a negative feedback loop in the HIF-1 α -

A



B

Non diabetic ischemic specimen



Diabetic ischemic specimen

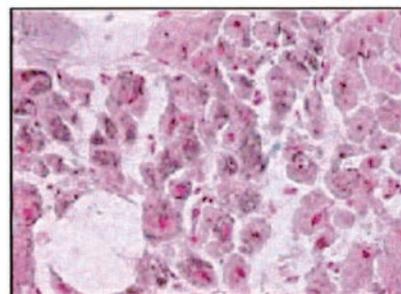


FIG. 4. A: Nitrotyrosine score in specimens from patients with and without diabetes. B: Representative nitrotyrosine immunostaining from ischemic ventricular biopsy specimens. * $P < 0.001$ vs. nondiabetic patients; † $P < 0.001$ vs. control specimens.

iNOS cascade and suggest that the reduction of the angiogenic factors may be linked to a greater oxidative stress evoked by diabetes, acting to either reduce NO availability (27) or stimulate iNOS expression (16). An effect of diabetes to enhance iNOS expression has recently been reported (12). iNOS is a calcium-independent enzyme and produces high levels of NO. Although increased NO production from iNOS may enhance early defensive response against reperfusion injury (9), high levels of NO generated by iNOS expression, through formation of per-

oxynitrite, may inhibit HIF-1 α and VEGF expressions (26). According to these observations, our findings demonstrate a presence of overexpression of iNOS gene and protein and nitrotyrosine formation in ischemic diabetic myocardial tissue that was not present in nondiabetic heart specimens. Although these findings do establish a cause-and-effect relationship between these cellular events, they could suggest an involvement of the NO-peroxynitrite pathway in the impaired angiogenesis during diabetes. Recently it has been demonstrated that in isolated rat

Non Diabetic Patients

Diabetic Patients

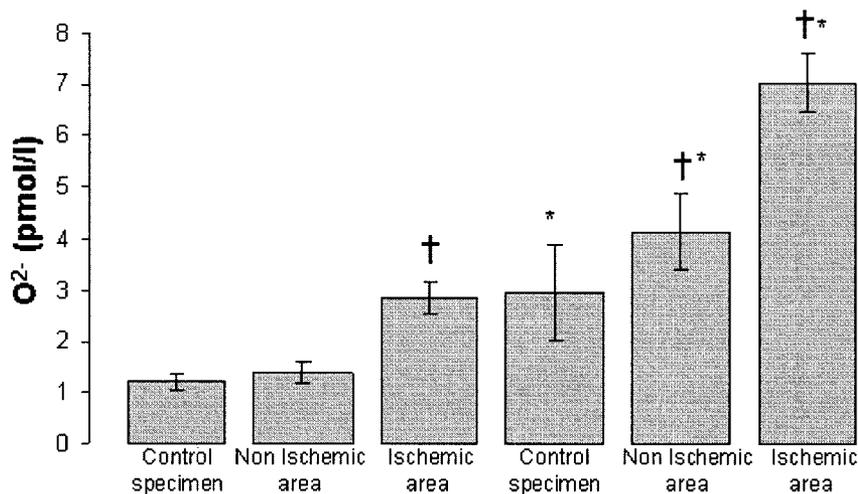


FIG. 5. O₂⁻ levels in heart specimens from patients with and without diabetes. * $P < 0.001$ vs. nondiabetic patients; † $P < 0.001$ vs. control specimens.

hearts acute exposure to high glucose increases iNOS gene expression, paralleled by a simultaneous increase of both NO and O_2^- production (16). The interaction of O_2^- with NO is very rapid and leads to inactivation of NO and production of the potent oxidant peroxynitrite (28). Because nitrotyrosine is considered a good marker of peroxynitrite formation (29), detection of high levels of nitrotyrosine in diabetic hearts, but not in nondiabetic hearts, is strongly suggestive for the increased generation of peroxynitrite. On the other hand, we cannot exclude that the increase of the nitrotyrosine could be evoked by other nitrating pathways as well as by an increase of the inflammatory cells. However, the large production of O_2^- , as evidenced in diabetic heart specimens, and the simultaneous rise of both NO and O_2^- production evoked by diabetes may be implicated in the impaired HIF-1 α and VEGF expressions during diabetes through peroxynitrite formation. Moreover, increased oxidative stress may reduce HIF-1 α expression, as suggested by the observation that the pretreatment of cerebral cells with N-acetyl-L-cysteine, an antioxidant, nullified the inhibitory effect of iNOS on HIF-1 activity (30). Following this line of thought, it might be speculated that the detrimental effect of diabetes in the angiogenic process might stem from its ability to increase iNOS gene expression in order to enhance the production of nitrotyrosine- and peroxynitrite-reducing NO availability during ischemia. NO is thought to mediate angiogenesis via multiple mechanisms (31). Using a rat hindlimb ischemia model, Namba et al. (32) established the importance of NO by showing that capillary formation was impaired by NO synthase inhibitors and that NO upregulated VEGF expression. Moreover, NO donors have been reported to stimulate VEGF synthesis in a variety of cultured cells (33–36). Similarly, NO has been shown to be an important downstream mediator of HIF-1 α stabilization (36) and other angiogenic factors, including basic fibroblast growth factor (bFGF), transforming growth factor (TGF)- β , and angiopoietin-1. NO stimulates endothelial proliferation and migration functions as an endothelial survival factor and may augment angiogenesis through vasodilation-induced increase in local blood flow (31). In addition, impaired NO production decreases the angiogenic response. In fact, Matsunaga et al. (37) demonstrated that the antiangiogenic effects of angiotensin, an endogenous angiogenesis inhibitor, are augmented in conditions of impaired NO production. In the study by Verma et al. (38), the inflammatory marker C-reactive protein (CRP) was shown to inhibit endothelial NO synthase expression and NO production, as well as basal and VEGF-induced angiogenesis.

The present study introduces an additional aspect of how diabetes might contribute to cardiovascular death in myocardial ischemia patients: diabetes results in increased tissue levels of myocardial iNOS and O_2^- and higher nitrotyrosine levels, leading to impaired myocardial angiogenesis factors. Whether this plays a role in the lesser ability to develop collateral blood vessels in diabetic patients with coronary artery disease awaits further elucidation.

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