IGF-1 Overexpression Inhibits the Development of Diabetic Cardiomyopathy and Angiotensin II–Mediated Oxidative Stress

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Stimulation of the local renin-angiotensin system and apoptosis characterize the diabetic heart. Because IGF-1 reduces angiotensin (Ang) II and apoptosis, we tested whether streptozotocin-induced diabetic cardiomyopathy was attenuated in IGF-1 transgenic mice (TGM). Diabetes progressively depressed ventricular performance in wild-type mice (WTM) but had no hemodynamic effect on TGM. Myocyte apoptosis measured at 7 and 30 days after the onset of diabetes was twofold higher in WTM than in TGM. Myocyte necrosis was apparent only at 30 days and was more severe in WTM. Diabetic nontransgenic mice lost 24% of their ventricular myocytes and showed a 28% myocyte hypertrophy; both phenomena were prevented by IGF-1. In diabetic WTM, p53 was increased in myocytes, and this activation of p53 was characterized by upregulation of Bax, angiotensinogen, Ang type 1 (AT1) receptors, and Ang II. IGF-1 overexpression decreased these biochemical responses. In vivo accumulation of the reactive O2 product nitrotyrosine and the in vitro formation of H2O2-˙OH in myocytes were higher in diabetic WTM than TGM. Apoptosis in vitro was detected in myocytes exhibiting high H2O2-˙OH fluorescence, and apoptosis in vivo was linked to the presence of nitrotyrosine. H2O2-˙OH generation and myocyte apoptosis in vitro were inhibited by the AT1 blocker losartan and the O2 scavenger Tiron. In conclusion, IGF-1 interferes with the development of diabetic myopathy by attenuating p53 function and Ang II production and thus AT1 activation. This latter event might be responsible for the decrease in oxidative stress and myocyte death by IGF-1. Diabetes 50:1414–1424, 2001

Diabetes alters the structure and function of the human heart, but the mechanisms involved are unknown (1). Type 1 insulin-dependent diabetes is characterized experimentally by cardiac myopathy, in which cell death by apoptosis predominates (2). Hyperglycemia activates the local renin-angiotensin system (RAS), resulting in the formation of angiotensin (Ang) II and stimulation of the endogenous cell death pathway (2). These observations raise the possibility that IGF-1 may protect the myocardium from the consequences of diabetes. This growth factor interferes with the myocyte RAS and the synthesis and secretion of Ang II (3). IGF-1 enhances the expression of MDM2, which in turn forms MDM2-p53 complexes inhibiting p53 DNA binding (3). Downregulation of p53 function decreases transcription of angiotensinogen (Aogen), which is the limiting factor in the synthesis of Ang II in myocytes (4). Therefore, IGF-1 may exert a therapeutic effect on ventricular dysfunction (5) and diabetic cardiomyopathy by attenuating the cellular RAS. This hypothesis is supported by the observation that ACE inhibition reduces cardiovascular events, improving the morbidity and mortality of diabetic patients (6,7).

Diabetes is associated with an exponential increase in oxidative damage (8). In various cell systems, a direct link has been found between Ang II and reactive O2 (9–11). However, it is unknown whether the generation of reactive oxygen species (ROS) constitutes the intermediate event in the transmission of death signals to myocytes by Ang II. Alternatively, cooperation between Ang II and ROS may be required for cell death to occur in the diabetic heart. In the absence of ROS-induced DNA damage, Ang II may not be able to execute the death process. We hypothesized that IGF-1 overexpression may protect the myocardium from diabetes by depressing the synthesis of Ang II and thus the formation of ROS and cellular damage. To address these issues, diabetes was induced in mice homozygous for the IGF-1 transgene (12). In vivo determinations were performed in control and diabetic animals to identify the effects of IGF-1 on p53 function, p53-dependent genes, activation of the local RAS, accumulation of oxidative stress products, myocyte death, and cardiac hemodynamics. In vitro studies analyzed both the impact of diabetes on ROS formation and the efficacy of Ang type 1 (AT1) blockade and reactive O2 scavenger on oxidative challenge and myocyte death. Transgenic mice (TGM) with targeted

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Ang, angiotensin; Aogen, angiotensinogen; AT1, Ang receptor type 1; CM-H2DCFDA, 5-(6)-chloromethyl-2,7-dichlorodihydrofluorescein diacetate; LVEDP, left ventricular end-diastolic pressure; LVSP, left ventricular systolic pressure; OD, optical density; PI, propidium iodide; RAS, renin-Ang system; ROS, reactive oxygen species; STZ, streptozotocin; TdT, terminal deoxynucleotidyl transferase; TGM, transgenic mice; WTM, wild-type mice.
expression of IGFI-1 in myocytes were preferred over non-transgenic animals with chronic administration of growth factor, because our objective was to characterize the impact of IGFI-1 on the development of diabetic cardiomyopathy. Systemic injection of IGFI-1 would have influenced other organs, complicating the interpretation of the results in the heart.

RESEARCH DESIGN AND METHODS

**Diabetes.** A total of 31 male wild-type mice (WTM) and 31 homozygous IGFI-1 TGM at 5 months of age were injected into the tail vein with streptozotocin (STZ) (200 mg/kg body wt) dissolved in a citrate-saline solution (pH 4.5) (2). Control mice, 23 in each group, were injected with the diluent and fed a restricted diet to match the decrease in body weight with diabetes. This was done to avoid the influence of caloric loss (caused by glycosuria) on body weight. Mice were killed at 7 and 30 days. Blood glucose was measured at the time of killing. Serum levels of IGFI-1 were obtained at 7 days by Nichols Advantage-chemiluminescence immunoassay. All protocols used were in accordance with institutional guidelines.

**Cardiac function.** Just before death, animals were anesthetized with tribromoethanol (1.2%, 0.2 ml i.p.). The carotid artery was cannulated with a microtip pressure transducer (SPR-671, Millar Instruments) connected to an electrostatic chart recorder. The transducer was advanced into the left ventricle for the evaluation of ventricular pressures and the rate of pressure rise (+dP/dt) and decay (–dP/dt). Rectal temperature was maintained at 36–38°C (2–4).

**Tissue fixation and sampling.** The heart was arrested in diastole with 0.15 ml calcium chloride (100 mmol/l), and the myocardium was perfused through the aorta with 10% formalin (13,14). The heart was excised, cardiac weights were recorded, and three slices of the left ventricle were embedded in paraffin; 48 animals were studied (6 in each group) at 7 and 30 days after STZ or diet injection. Sections were stained with hematoxylin-eosin, and 60 fields in each heart were examined at ×400 with a reticle containing 42 points to yield the volume percentage of myocytes and interstitium. Ventricular volume was multiplied by the volume fraction of myocytes to compute the aggregate volume of myocytes in the ventricle (13–15).

**Myocyte death.** This terminal analysis was performed in 48 animals. Myocyte apoptosis was determined by deoxynucleotidyl transferase (TdT) and hairpin probe with single-base 3’ overhangs, and myocyte necrosis was determined by hairpin probe with blunt ends. Apoptosis-necrosis was evaluated by TdT and hairpin probe with blunt ends (16). These techniques have been described previously (2,13–18). Myocyte cytoplasm and nuclei were stained by a-sarcomeric actin antibody and propidium iodide (PI), respectively (2,13–18).

Sections from the base, mid-region, and apical portion of each left ventricle were examined by confocal microscopy, and the numbers of myocyte nuclei that were labeled by TdT, hairpin probes, and by TdT and hairpin probe with blunt ends were recorded by analyzing a minimum of 13.5 mm² to a maximum of 44.5 mm² of tissue. The total number of myocyte nuclei sampled for TdT at 7 days of diabetes was 78,393, 51,643, 72,255, and 69,486 in control and diabetic nontransgenic mice and control and diabetic TGM, respectively. Corresponding values for hairpin probe with single-base 3’ overhangs were 77,827, 74,764, 101,469, and 81,027. Values with hairpin probe with blunt ends were 81,252, 77,464, 111,660, and 105,912. In a comparable manner, the total number of myocyte nuclei sampled for TdT at 30 days of diabetes was 81,613, 80,849, 77,796, and 81,282 in control and diabetic nontransgenic mice and control and diabetic TGM, respectively. Corresponding values for hairpin probe with single base 3’ overhangs were 82,904, 86,658, 90,559, and 77,796. Values with hairpin probe with blunt ends were 92,117, 80,849, 83,687, and 81,282. Values for TdT and hairpin probe with blunt ends are not indicated because this simultaneous association was never found. The density of myocyte nuclei was obtained by counting (per unit area of tissue) the number of nuclei in a-sarcomeric actin-positive cells. The number of apoptotic and necrotic myocytes per 10² cells was then calculated.

**Nitrotyrosine labeling.** This analysis was performed in 24 animals (6 in each group) at 30 days of diabetes. Sections were incubated overnight with rabbit anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) diluted 1:40 in phosphate-buffered saline (19). Fluorescein isothiocyanate–labeled goat anti-rabbit IgG was used as a secondary antibody. Simultaneous presence of cell nuclei was evaluated by a secondary antibody labeled with tetramethyl rhodamine isothiocyanate. Sections treated with 10% peroxidinir was used as a positive control. The percentage of myocytes containing nitrotyrosine was obtained by confocal microscopy; 300 myocyte profiles were examined in each animal.

**Myocyte size and number.** Myocyte cell volume was obtained by three-dimensional section reconstruction of enzymatically dissociated cells. In each left ventricle, 150–200 myocytes were measured. The total number of ventricular myocytes was computed by dividing the aggregate volume of myocytes by the average cell volume. This methodology has been described previously (12,19).

**Myocyte isolation.** This was performed in 44 mice, 6 in each group, at 7 days after diabetes and in 5 at 30 days after diabetes. Left ventricular myocytes were enzymatically dissociated following a protocol well established in our laboratory (12–14). Myocyte yields were 2.1 ± 0.4 × 10⁶ in control WTM, 1.4 ± 0.5 × 10⁶ in diabetic WTM, 2.9 ± 0.8 × 10⁶ in control TGM, and 2.6 ± 0.7 × 10⁶ in diabetic TGM. Contamination from nonmyocytes ranged from 1 to 3% in all cases.

**Band shift.** Oligonucleotides corresponding to the p53 binding site in the Bax, Agen, and AT1 promoters were used in mobility shift assays (3,14,17). As a negative control, nuclear extracts were exposed to a p53 antibody (Fab 240, Santa Cruz Biotechnology) before AT1, and Bax probes were used as competitors, and unlabeled mutated Agen, AT1, and Bax were used as noncompetitors (14,17).

**Western blot.** A total of 50 μg of myocyte proteins were separated by 12% SDS-PAGE. Proteins were transferred to nitrocellulose and exposed to anti-human p53 (Fab 240, Santa Cruz Biotechnology), anti-human Bax (P19, Santa Cruz Biotechnology), anti-Agen (Swant), anti-human AT1 (306; Santa Cruz Biotechnology) antibodies, and anti-human AT2 (C-18; Santa Cruz Biotechnology) antibodies (3,14,17).

**Ang II labeling.** This was performed in 24 animals (6 in each group) at 7 days after diabetes. Sections were incubated with Ang II antisense (2,3). Specificity was determined by preabsorption of antibody with antigen and by staining with Ang II–immunoreactive rabbit serum. Fluorescein isothiocyanate–labeled myocytes and Ang II sites per millimeter squared of myocytes were determined (2,3). A total of 200 myocyte profiles were analyzed in each experimental condition.}

**Reactive O2.** This analysis included five separate myocyte isolations in each group of mice at 7 days of diabetes, using a total of 20 animals. Isolated myocytes were attached to laminin-coated petri dishes, and cells were loaded with 5-(6-chloromethyl-2,7-dichlorofluorescein diacetate (CM-H₂DCFDA) (Molecular Probes, Eugene, OR) for 30 min. Fluorescence intensity from individual cells was measured using an excitation wavelength of 485 nm and an emission wavelength of 530 nm (20,21); 100–140 cells were sampled at random in each preparation using an Olympus IX70 inverted microscope equipped with a digital cooled CCD-IEEE camera (Optronics, Goleta, CA) and ImagePro image analysis software (Medis Image Cybernetics, Silver Spring, MD). To avoid changes in fluorescence intensity, only a single measurement per field was collected. Fluorescence was calibrated with InSpeck microspheres (Molecular Probes). Calibration curves were generated and cell brightness was measured. Probe fluorescence was established and this background was subtracted from all determinations. Preliminary experiments were also performed to assess the uptake of CM-H₂DCFDA with time. Maximum fluorescence was reached between 20 and 30 min. This time interval was used. The volume of each analyzed myocyte was measured by confocal microscopy (9,10), and fluorescence intensity per cell was divided by its volume to correct for differences in cell size. After the completion of the measurements of ROS, cells were fixed and stained for apoptosis.

**Ang II, ROS and apoptosis.** The effects of 30 min of exposure to Ang II (10⁻⁶ mol/l) on ROS formation were detected as described above. Freshly isolated myocytes were cultured in laminin-coated petri dishes and serum-free medium for 1 h before the addition of Ang II. Identical cultures were exposed to losartan (10⁻⁷ mol/l) and to losartan plus Ang II, and ROS generation was measured. Five separate myocyte isolations were used in each condition. For the evaluation of apoptosis, myocytes were cultured in the presence of Ang II for 24 h. Additionally, the effect of losartan or the superoxide anion scavenger Tiron (0.1 mmol/l) on Ang II–mediated apoptosis was determined. In these experiments, losartan or Tiron were added to the culture 30 min before Ang II. Again, six myocyte isolations were used in each protocol.

**Statistics.** All tissue samples were coded, and the code was broken at the end of the study. Results are presented as the mean ± SD. Statistical significance between two measurements was determined by the two-tailed unpaired Student’s t test, and among groups it was determined by the Bonferroni’s method (22). Probability values of P < 0.05 were considered significant.

**RESULTS**

**Ventricular function.** Blood glucose in WTM increased from 11.4 ± 2.1 to 27.5 ± 3.3 and 36.5 ± 3.6 mmol/l at 7 and 30 days after STZ administration, respectively. Comparably changes in blood glucose were detected in TGM: for control, diabetic TGM at 7 days, and diabetic TGM at 30 days.
The 1.7-fold difference between the two groups of diabetic WTM and TGM had higher heart weight–body weight ratios (4.3 \pm 0.5 and 4.7 \pm 0.6). Control and diabetic TGM had higher heart weight–body weight ratios (P < 0.01 to P < 0.001) than corresponding WTM (12).

It should be noted that the serum level of IGF-1 was <3 ng/ml in nondiabetic and diabetic WTM. Conversely, IGF-1 was 5.7 \pm 1.2 ng/ml in nondiabetic and diabetic TGM, respectively. It cannot be excluded, therefore, that the higher circulating level of IGF-1 in TGM may have had an additional effect on the response of the heart to diabetes in this group.

**Myocyte loss.** Cell death values at 7 and 30 days were similar in control WTM and control TGM and thus were combined. Diabetes was associated with an increase in myocyte apoptosis, which was higher at 7 days than it was at 30 days in both WTM and TGM (Fig. 2A and B). However, at either interval, cell death was twofold greater in diabetic WTM than in TGM. The extent of apoptosis did not differ when measured by TdT or hairpin probe with single-base 3' overhangs. Myocyte necrosis, evaluated by a hairpin probe with blunt ends, was not increased in diabetic WTM and TGM at 7 days. Conversely, myocyte necrosis with diabetes at 30 days increased 2.9-fold and 1.8-fold in diabetic WTM and TGM, respectively (Fig. 2C).

The 1.7-fold difference between the two groups of diabetic mice was significant. Cells dying by both apoptosis and necrosis were never found. In comparison with control WTM, a 24% reduction in the total number of left ventricular myocytes was found in diabetic WTM at 30 days (Fig. 2D). Cell loss in these animals was accompanied by a 28% myocyte hypertrophy (Fig. 2E), which resulted in preservation of ventricular mass (Fig. 2F). The 24% loss of cells in the ventricle reflected an absolute dropout of 1.1 \times 10^8 myocytes. In contrast, diabetes did not change the number, size, and aggregate myocyte volume in TGM (Fig. 2D-F).

**p53 DNA binding and myocyte RAS.** p53 Function and expression of the p53-dependent and regulated genes (Bax, Aogen, and AT_2) and p53-independent genes (renin and AT_1 receptor) were measured in myocytes isolated from control and diabetic WTM and TGM (Fig. 3A). These determinations were restricted to 7 days because apoptosis with diabetes was greater at this interval. p53 Binding to its cognate DNA sequence on the promoter of Bax (5'-AGCTTGTCTCACAAGTGAACAGCTGGCGGTGGGCTATATTGA-3'), Aogen (5'-AGCTCCTCGTTACAGATCAAGGTAGCCCTGGGAATAGATCCATCTTC-3'), and AT_2 (5'-GCTGAGCTTGGATCTGGAGGCGACACTGGG-3') was increased in myocytes of diabetic WTM (Fig. 3B–D). The optical density (OD) of the p53-shifted band for each of these three genes was significantly higher than in diabetic TGM (OD data not shown). These differences were coupled with larger quantities of p53 (Fig. 3E) and Bax (Fig. 3F) proteins in WTM myocytes with diabetes. Similarly, Aogen (Fig. 3G), renin (Fig. 3I), and AT_1 (Fig. 3H) increased more in diabetic WTM than in diabetic TGM. AT_2 protein did not change in diabetic mice (Fig. 3J).

The quantitative analysis of Ang II localization in the myocardium (Fig. 4A–D) showed labeling in 51 \pm 7% (n = 6) and 31 \pm 6% (n = 6, P < 0.001) of the myocytes in nondiabetic WTM and TGM, respectively. Diabetes increased the fraction of Ang II–positive myocytes to 77 \pm 9% (n = 6, P < 0.001) in WTM and to 50 \pm 8% (n = 6, P < 0.001) in TGM. The number of Ang II sites per millimeter squared of myocytes was twofold (P < 0.001) higher in nondiabetic WTM (14,000 \pm 3,500 per mm^2) than in TGM (7,000 \pm 2,100). With diabetes, these values increased 2.3-fold (P < 0.001) in WTM (32,100 \pm 8,300) and 1.7-fold (P < 0.001) in TGM (12,000 \pm 3,400). Thus, Ang II sites in
myocytes were 2.7-fold ($P < 0.001$) more numerous in diabetic WTM than in TGM.

**Nitrotyrosine localization.** Nitrotyrosine is formed by the interaction of peroxynitrite with cytoplasmic proteins (23). This modified amino acid is a product of oxidative stress that can be detected in the myocardium using a nitrotyrosine-specific antibody (Fig. 5A and B). Its association with myocyte apoptosis can be identified as well (Fig. 5C and D). The analysis of nitrotyrosine localization in myocytes was performed at 30 days of diabetes to allow its accumulation with time. The percentage of myocytes expressing nitrotyrosine was 28 ± 6% ($n = 6$; $P < 0.001$) in nondiabetic WTM and TGM, respectively. Diabetes increased the fraction of nitrotyrosine-positive myocytes to 71 ± 6% ($n = 6$, $P < 0.001$) in WTM; this parameter did not change in diabetic TGM, which had a value of 16 ± 4% ($n = 6$, NS). The presence of nitrotyrosine does not permit the examination of all nuclei of positive cells; nuclei are often not included in the section plane (Fig. 5A and B). Moreover, nitrotyrosine labeling exceeds by several orders of magnitude the extent of cell death. A total of 63 apoptotic myocytes were examined in diabetic WTM to detect the potential implication of nitrotyrosine in apoptosis; in all cases, apoptosis was accompanied by nitrotyrosine labeling (Fig. 5C and D). To confirm the role of oxidative stress in cell death, 38, 26, and 18 apoptotic myocytes were analyzed in diabetic TGM and nondiabetic WTM and TGM, respectively; nitrotyrosine was present in every cell undergoing apoptosis.

**ROS formation.** The increase in nitrotyrosine in myocytes with diabetes suggested that a causative link existed between oxidative stress and this disease. Additionally, IGF-1 attenuated the effects of oxidative stress on nitrotyrosine accumulation in the diabetic heart. To establish whether a relationship was present between diabetes and Ang II formation and between ROS production and cell death, in vitro studies were performed after 7 days of diabetes. This interval was selected because Ang II labeling of myocytes was obtained at this time and apoptosis in vivo was higher than at 30 days (see above). Myocytes were isolated from nondiabetic and diabetic WTM, and TGM and ROS generation was measured using a fluorescent probe detecting H$_2$O$_2$ and 'OH in living cells (Fig. 6A and B). In each cell, fluorescence intensity ($f$) was normalized by the corresponding myocyte cell volume ($V$). ROS production was 44% ($P < 0.01$) higher in myocytes from nondiabetic WTM ($n = 5, 1.34 ± 0.11 f/V$) than in cells from nondiabetic TGM ($n = 5, 0.93 ± 0.08$). Diabetes increased the formation of H$_2$O$_2$-'OH in myocytes of WTM ($n = 5, 2.40 ± 0.29 f/V$) and TGM ($n = 5, 1.23 ± 0.12$) 1.8-fold ($P < 0.001$) and 1.3-fold ($P < 0.05$), respectively. Using 0.2-f/V increments, the frequency distribution of fluorescence intensity of myocytes was plotted (Fig. 6C–F). In comparison with WTM, IGF-1 overexpression in nondiabetic and diabetic hearts was characterized by a shift to the left of the reactive O$_2$ signal. In nondiabetic WTM and TGM and diabetic WTM and TGM, respectively, a total of 563, 585, 601, and 643 myocytes were examined and 1, 0, 7, and 2 apoptotic cells were found. All dying myocytes had high levels of H$_2$O$_2$-'OH; $f/V$ values ranged from 3 to 5. The frequency of apoptotic myocytes found in vitro were higher than in vivo. This is a consistent phe-

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**FIG. 2.** Effects of diabetes on myocyte apoptosis evaluated with TdT (A) and Hairpin probe (B), and the effects of diabetes on necrosis (C), myocyte number (D), myocyte cell volume (E), and aggregate myocyte mass in the left ventricle (F). See legend to Fig. 1 for symbols. *$P < 0.05$ vs. C; **$P < 0.05$ vs. D 7 days; and †$P < 0.05$ vs. nontransgenic mice. A–C: C 7–30 days, $n = 12$; D 7 days, $n = 6$; D 30 days, $n = 6$. D–F: $n = 5$. **
FIG. 3

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nomenon associated with cell isolation and culture conditions (4,17).

To identify whether the cellular release of Ang II was responsible for the induction of H$_2$O$_2$-OH at baseline and with diabetes or whether oxidative stress was independent from AT$_1$ receptor activation, myocytes were exposed for 30 min, respectively, to the AT$_1$ antagonist losartan (10$^{-7}$ mol/l) or the intracellular reactive O$_2$ scavenger Tiron (0.1 mmol/l). H$_2$O$_2$-OH fluorescence was reduced by losartan (Fig. 6G–J) in myocytes obtained from diabetic WTM (from 2.40 ± 0.29 to 1.53 ± 0.13 fV; 582 cells, two apoptosis) and TGM (0.91 ± 0.14; 610, one). Values in losartan-treated nondiabetic WTM and TGM were, respectively, 1.26 ± 0.18 fV (541 cells, one apoptosis) and 0.85 ± 0.09 fV (572 cells, no apoptosis). Similarly, Tiron-attenuated oxidative stress mimicked the effects of losartan at a more distal level: WTM = 1.12 ± 0.17 fV (551 cells, no apoptosis), TGM = 0.78 ± 0.11 (539, none), diabetic WTM = 1.29 ± 0.21 (680, one), and diabetic TGM = 0.91 ± 0.14 (564, none).

**Ang II, ROS formation and apoptosis.** Myocytes from control WTM were exposed to Ang II, and the H$_2$O$_2$-OH signal was measured (Fig. 7A). Fluorescence intensity per cell nearly doubled with Ang II. In contrast, pretreatment of cells with losartan prevented the effects of Ang II on ROS production. Myocyte apoptosis markedly increased 24 h after the addition of Ang II. Importantly, Tiron or losartan inhibited the apoptotic signal transmitted by Ang II (Fig. 7B). Thus, Ang II activated apoptosis via the AT$_1$ receptor by enhancing oxidative stress.

**DISCUSSION**

**IGF-1 overexpression protects from diabetic cardiomyopathy.** Whether diabetes per se—in the absence of coronary artery disease and hypertension—leads to cardiac myopathy in humans has been a controversial question (1). Animal models of diabetes have not resolved this issue. Biochemical, mechanical, structural, and electrophysiological alterations have been identified in combination with modest abnormalities in the diastolic properties of the heart (2,24,25). However, indexes of severe ventricular dysfunction and failure have not been observed (1,2).

Results presented here document for the first time that in a mouse model of type 1 insulin-dependent diabetes, cardiac performance is impaired soon after the onset of the disease and deteriorates chronically. Myocyte loss and hypertrophy of the remaining cells characterize the diabetic decompensated heart, mimicking cardiac myopathies in humans and animals (20).

Clinical and experimental studies aiming at the identification of a therapeutic role for exogenously administrated IGF-1 or growth hormone in pathologic states of the heart have not provided a consistent answer. Positive observations (5,27) have been contrasted by negative results (28, 29). IGF-1 overexpression in TGM has previously been shown to inhibit both myocyte apoptosis in the surviving myocardium after infarction (13) and myocyte necrosis after nonocclusive coronary artery constriction (14). Interference with cell death improved cardiac anatomy and decreased diastolic wall stress in both situations. However, these beneficial effects were not accompanied by a corresponding amelioration in ventricular hemodynamics, possibly due to the presence of large infarcts and restriction in coronary perfusion, respectively. Therefore, the therapeutic impact of IGF-1 on the diseased heart remains unclear. The current findings demonstrate unequivocally that IGF-1 overexpression affected the level of activation of myocyte death with diabetes and preserved ventricular performance. Myocyte death has been questioned as an etiological factor capable of inducing functional alterations. Cell death has been claimed to be an epiphenomenon that has little influence on the onset and evolution of cardiac failure (30). Current data in diabetic nontransgenic mice and TGM challenge this contention.

**IGF-1 overexpression inhibits Ang II synthesis and ROS formation.** Recent observations in diabetic patients (7) and in rats after STZ administration (2) have demonstrated that the systemic and local RAS are activated with diabetes, exerting a detrimental effect on the course of the disease. Formation of Ang II in the myocardium and stimulation of AT$_1$ receptors cause myocyte apoptosis and cardiac remodeling (2). The in vivo results obtained here are consistent with the concept that upregulation of p53 leads to enhanced expression of the p53-regulated gene (Aogen) responsible for the increased levels of Ang II in myocytes with diabetes. Aogen is the limiting factor in the formation of Ang II in cardiac muscle cells (4,17,31), and inactivation of p53 inhibits generation of the octapeptide (4). IGF-1 attenuates p53 transcriptional activity and thereby downregulates Aogen and the synthesis of Ang II. This negative modulation of RAS by IGF-1 is mediated by MDM2 and the generation of MDM2-p53 inactive complexes (3,32). Ang II leads to oxidative stress in several cell systems through NADH/NADPH oxidase (9). This enzyme is the major source of superoxide; p22phox is critical for the transfer of electrons from NADH or NADPH to O$_2$ and the production of reactive O$_2$ (33).

Ang II increases the formation of ROS in neonatal myocytes (34), endothelial cells (11), and smooth muscle cells (9) in vitro by activating AT$_1$ receptors (11). However, a link between ROS and apoptosis has not been established. It is technically impossible to measure the generation of ROS in cardiac myocytes in vivo. Therefore, the localization of nitrotyrosine in myocytes was evaluated. Superoxide anion interacts with nitric oxide, forming peroxynitrite (ONOO$^-$) (23). ONOO$^-$ induces oxidative damage to proteins, leading to the production of a modi-

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**FIG. 3.** Left ventricular myocytes from a diabetic nontransgenic mouse at 7 days (A); α-sarcromere actin staining of the cytoplasm (red fluorescence and PI labeling of nuclei (yellow fluorescence), ×300. B–D: Gel mobility assays of p53 binding to its consensus sequence in the promoter of bax (B), Aogen (C), and AT$_1$ (D). Bax, Ao, and AT$_1$; probes in the absence of nuclear extracts. Co, competition with an excess of unlabeled self-oligonucleotide; Ab, competition with monoclonal p53 antibody; Mut, preincubation with unlabeled mutated form of oligonucleotides; SV-T2, nuclear extracts from SV-T2 cells; C, control; D, diabetic; W, nontransgenics; T, transgenics. OD values of the shifted bands are not listed. E–J: Western blots of p53 (E), Bax (F), Aogen (G), AT$_1$ (H), renin (I), and AT$_1$ (J). ODs for p53: WC = 1.9 ± 0.31, WD = 6.7 ± 1.5 (P < 0.001); TC = 0.56 ± 0.16, TD = 2.1 ± 0.48 (P < 0.05). ODs for Bax: WC = 1.4 ± 0.26, WD = 5.7 ± 0.81 (P < 0.001); TC = 0.50 ± 0.15, TD = 0.38 ± 0.52 (P < 0.05). ODs for Aogen: WC = 6.2 ± 1.3, WD = 19 ± 4 (P < 0.001); TC = 1.3 ± 0.44, TD = 2.4 ± 0.84 (P < 0.01). ODs for AT$_1$: WC = 3.7 ± 1.0, WD = 13 ± 3 (P < 0.001); TC = 0.59 ± 0.29, TD = 1.8 ± 0.54 (P < 0.01). ODs for renin: WC = 0.88 ± 0.29, WD = 2.1 ± 0.64 (P < 0.001); TC = 0.71 ± 0.19, TD = 1.1 ± 0.28 (NS). ODs for AT$_1$: WC = 0.93 ± 0.33, WD = 0.91 ± 0.24 (NS); TC = 0.85 ± 0.26, TD = 0.82 ± 0.27 (NS), n = 6 in all cases.
FIG. 4. Myocardium of nondiabetic (A and B) and diabetic (C and D) WTM. Green fluorescence (A and C) and yellow fluorescence (B and D) reflect Ang II labeling (arrows) and laminin staining of the interstitium. Red fluorescence shows α-sarcomeric actin staining of the cytoplasm (B and D), and blue fluorescence shows PI labeling of nuclei (B and D). Magnification: ×1,200.
FIG. 5. Nitrotyrosine localization in the myocardium of a diabetic WTM at 30 days (A and B), shown by blue fluorescence (A) and pink fluorescence (B). α-Sarcomeric actin staining of the cytoplasm is shown by red fluorescence (B) and PI-labeling of nuclei (green-yellow fluorescence, A and B). C and D from the same heart show an apoptotic nucleus (green-yellow fluorescence, arrow) detected by hairpin probe with single-base 3’ overhangs. Stainings for nitrotyrosine and myocyte cytoplasm are the same as those in A and B. Magnification: ×1,200.
fied amino acid (nitrotyrosine). The increased frequency of nitrotyrosine-positive myocytes in diabetic nontransgenic mice pointed to an oxidative challenge in vivo. This cellular response was prevented in diabetic IGF-1 TGM, correlating with the lower level of Ang II in the myocardium. However, these in vivo results did not prove whether Ang II was the trigger for the induction of ROS or whether IGF-1 reduced oxidative stress by interfering only with the synthesis of Ang II. IGF-1 could have affected the activity of NADH/NADPH oxidase, limiting superoxide formation (23). Importantly, AT1 blockade in myocytes from diabetic WTM and TGM decreased the H2O2 signals. Although the levels of H2O2 varied in the presence and absence of IGF-1 overexpression, inhibition of Ang II binding markedly depressed the generation of ROS in either myocyte population. Similar results were obtained with the reactive O2 scavenger Tiron. Thus, Ang II was the mediator of reactive O2, and IGF-1 attenuated oxidative stress by reducing the local synthesis of Ang II in the myocardium with diabetes.

IGF-1 overexpression attenuates myocyte death with diabetes. Myocyte apoptosis and necrosis are both involved in the development of diabetic cardiomyopathy. Necrosis temporally follows apoptosis, contributing to the chronic loss of ventricular myocytes with diabetes. The strict association between nitrotyrosine and apoptosis suggests that oxidative damage is causally implicated in the activation of this form of cell death. Although a similar link was not investigated for myocyte necrosis, different levels of reactive O2 trigger apoptosis or necrosis; high quantities induce necrosis and low amounts promote apoptosis (35). IGF-1 attenuated necrosis and apoptosis but did not prevent cell death completely. However, myocyte apoptosis and necrosis in TGM did not affect the aggregate number of ventricular myocytes 1 month after STZ administration. This apparent inconsistency may be explained by myocyte regeneration, which could have occurred with IGF-1 overexpression (12).

In conclusion, the positive correlation between the extent of oxidative challenge and myocyte apoptosis in vitro...
and between nitrotyrosine localization and myocyte apoptosis persist over long time periods remains an important unanswered question.

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