Early blood pressure-independent cardiac changes in diabetic rats

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Abstract

Cardiac remodeling is a key event in both diabetic and hypertensive heart diseases. In the present study, we investigated early myocardial changes in an animal model, the male Sabra rat model (SBH/y) of salt-induced hypertension-rendered diabetic with streptozotocin. Control non-diabetic (C), diabetic (D), and D or C rats made hypertensive by salt loading (DS or CS) were studied after 6 weeks. M-mode echocardiography revealed that left ventricular internal dimension during diastole and systole were significantly increased in D and DS, but not in C or CS. Concurrently, we found in D and DS an increase in cardiac β -myosin heavy chain, atrial natriuretic peptide, skeletal α -actin mRNA, type III collagen, and transforming growth factor- β .

Myocardial angiotensin-converting enzyme (ACE) mRNA levels were increased while ACE2 mRNA levels were decreased in both D and DS groups. Cardiac angiotensin-1 (AT1) receptor protein levels were unchanged but the levels of phosphorylated (p) ERK and Jun-NH₂-protein kinase (JNK) were increased in D and DS. In conclusion, we detected early cardiac changes in diabetic rats that were unrelated to hypertension. The increase in ACE, the decrease in ACE2, and the increase in cardiac pERK and pJNK suggest an increase in free angiotensin II and AT1R signaling in the diabetic myocardium as a possible mechanism contributing to cardiac remodeling in diabetes.

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Introduction

Heart disease, particularly coronary artery disease, is a major cause of morbidity and mortality among patients with diabetes mellitus (DM). Another important cardiovascular problem in diabetic patients, as suggested by the Framingham study, is the development of heart failure which is due to an ill-defined cardiomyopathy, even in the absence of coronary artery disease (Galderisi *et al.* 1991). Preclinical evidence for the occurrence of diabetic heart disease has been demonstrated in the form of both diastolic and systolic dysfunction and of structural abnormalities (Marwick 2006). In asymptomatic diabetic patients, diastolic dysfunction appears to be, in fact, very common (Zabalgaitia *et al.* 2001). Since hypertension is commonly associated with diabetes, however, and since hypertension *per se* has also been identified as an important cause of heart failure, it is difficult to identify the relative importance in the development of heart failure with diabetes and hypertension as single contributing factors.

An early phase in the development of heart failure is cardiac remodeling. Cardiac remodeling is thought to be an important aspect of disease progression in heart failure, regardless of cause (Cohn *et al.* 2000). It is manifested clinically by changes in cardiac size, shape, and function in response to cardiac injury or increased load. The role of the renin-angiotensin system in cardiac remodeling is exemplified by the fact that angiotensin-converting enzyme (ACE) inhibitors, which improve survival in patients with heart

failure, can slow down and in some cases even reverse certain parameters of cardiac remodeling (Greenberg *et al.* 1995).

Angiotensin II (AngII) effects through its specific receptor (mostly AT1R) may depend on its local production. Free AngII levels are determined by the rate of its synthesis, regulated mostly by ACE, and degradation. The newly discovered ACE2 enzyme plays a role in this process (Burrell *et al.* 2004).

Cardiac remodeling in DM and hypertension has been less extensively investigated in comparison with post-myocardial infarction models. The aim of the present study was to investigate cardiac remodeling at the functional and gene expression level in an experimental model of DM. We studied cardiac gene expression and the activation of 'fetal' cardiac genes, which encode proteins involved in contraction, calcium handling, and metabolism (Marks 2003). Since hypertension is more commonly associated with diabetes in the clinical setting, we also tested the hypothesis that hypertension *per se* is an important contributor to the development of cardiac remodeling in DM.

Materials and Methods

Animals

The study was performed in the salt-sensitive hypertensive male Sabra rat model. This model consists of two strains: the Sabra hypertension-prone (SBH/y) rats that remain normotensive when fed regular diet but become hypertensive when salt

loaded, and the Sabra hypertension-resistant (SBN/y) rats that remain normotensive not only when fed regular diet but also when salt loaded. For the purposes of the present study, only animals from the SBH/y strain were used. The animals were procured from the Israeli Rat Genome Center in Ashkelon, Israel (www.irgc.co.il).

Animals were housed in compliance with institutional regulations and in accordance with 'Principles of Laboratory Animal Care' (NIH publication no. 85-23, revised 1985) and the guidelines of the American Society of Physiology for the care of laboratory animals. Unless stated otherwise, animals were provided tap water and standard rat chow containing 0.65% NaCl (Koffolk, Tel-Aviv, Israel) *ad libitum*. Climate-controlled conditions and regular-timed diurnal cycles were maintained. The local institutional review committee approved the study protocol.

Study groups

Animals were divided into four groups: Group 1 (C), non-diabetic SBH/y that were provided regular chow and thus normotensive; Group 2 (D), SBH/y-rendered diabetic rats that were provided regular chow and thus normotensive; Group 3 (CS), non-diabetic SBH/y that were salt loaded and thus hypertensive; and Group 4 (DS), diabetic SBH/y that were salt loaded and thus hypertensive.

Study protocol

Animals from the four study groups were studied over 6 weeks, during which D and DS groups were rendered diabetic, and CS and DS groups were made hypertensive. The animals ($n=7$ per group) were killed by the end of the 6-week study period. Cardiac function was evaluated by echocardiography on the last week of the experiment. Blood was collected from the bifurcation of the aorta, and serum was separated and frozen at -20°C for later measurements. The heart apex was separated and fixed in a 4% paraformaldehyde solution for histomorphological assessment (by hematoxylin/eosin, Masson, and periodic acid Schiff (PAS) stainings). The remaining heart was immediately frozen in liquid nitrogen and then at -70°C for later determination of mRNA and protein levels.

Induction of diabetes

DM was induced in 8-week-old male rats by i.p. injection of streptozotocin (60 mg/kg body weight (BW) in 100 mM citrate buffer at a pH of 4.5). In the control group, an equal volume of buffer was injected. Only animals with serum glucose levels above 18 mmol/l and glucosuria without ketonuria were included in the study.

Diabetic animals were provided with human insulin glargine s.c. injections daily of 1 unit/day (Lantus, Aventis Pharma, 100 U/ml). Urine was checked for the presence of glucose twice a week with chemstrips (Ketostix, Bayer-Ames,

UK). Whole blood glucose was measured from the tip of the tail with a glucometer when the urine glucose test was positive (Elite, Bayer Diagnostics). Food intake (energy supply) was assessed after 4 weeks. Food intake was determined by first cleaning the cages and changing the bedding. Animals were housed individually in cages and given 50 g/day of their standard (normal or high salt) rodent chow. The water bottle for each cage was kept full. Food placed into the feeder at the beginning and any left over at the end of each 24-h period was weighed. Any visible food in the cage was scavenged and included in the measurements.

Induction of hypertension

Hypertension was induced in the SBH/y immediately after the induction of DM by streptozotocin (STZ) by feeding the animals with chow containing 8% NaCl for 6 weeks. This type of diet invariably leads to the development of hypertension in SBH/y.

Systolic blood pressure (BP) was measured before salt loading and at weekly intervals thereafter for a total of 6 weeks. BP measurements were performed at ambient temperature ($26\text{--}28^{\circ}\text{C}$) in awake animals by the tail-cuff method using the IITC-31 computerized BP device (IITC Life Science, Woodland Hills, CA, USA), as described previously (Yagil & Yagil 1998).

Echocardiographic evaluation

Rats in all groups were anesthetized with tribromoethanol 200 mg/kg i.p. 2 days prior to killing. This type of anesthesia was previously shown not to depress cardiac function in rats (Schaefer *et al.* 2005). The rat chest was shaved and a layer of acoustic coupling gel was applied to the thorax. Two-D and M-mode echocardiography was performed by a commercially available 12 MHz linear array transducer system (Hewlett-Packard, Andover, MA, USA). The animals were placed on a supine position, care was taken that the transducer had adequate contact with the amount of gel while avoiding excessive pressure on the chest. M-mode recordings were obtained of the left ventricle (LV) at the level of the mitral valve in the parasternal view, using two-dimensional echocardiographic guidance in the short-axis view. A pulse-wave Doppler was used to examine mitral diastolic inflow from the apical four chamber view. For each measurement, data from three consecutive cardiac cycles were averaged. All measurements were made from digital images captured at the time of the study by the use of analysis software installed in the echocardiographic machine. The M-mode and Doppler images were obtained at a speed of 150 mm/s. LV internal dimension during diastole (LVIDD) and during systole (LVIDS) were measured. The percentage of left ventricular shortening (fractional shortening) was calculated. Based on the curve of mitral diastolic flow, peak flow velocity (E) of early filling wave (E wave), peak flow velocity (A) of atrial

filling wave (*A* wave), *E/A* ratio, and acceleration and deceleration time of *E* wave were determined.

Extraction of RNA

Total RNA was isolated from frozen heart tissues by the Tri-reagent method (Molecular Research Center, Cincinnati, OH, USA) according to the manufacturer's recommendation. The integrity of the RNA was assessed by visual inspection of the ethidium bromide-stained 28S and 18S RNA bands after electrophoresis through 1.25%/2.2 M formaldehyde gels. The quantity and quality of the total RNA extracted were evaluated at 260 and 280 nm u.v. wavelengths, and the optical density 260/280 nm was maintained between 1.7 and 2.1.

RT-PCR

We quantified heart β -myosin heavy chain (β -MHC), atrial natriuretic peptide (ANP), skeletal α -actin, type III collagen, transforming growth factor- β (TGF- β), ACE, and ACE2 mRNA by RT-PCR method. RNA was converted to cDNA by adding 13 μ l reverse transcriptase reaction mixture, containing 1 μ l moloney murine leukemia virus reverse transcriptase (MMLV-RT; 200 U/ μ l, Sigma), 0.5 μ l dithiothreitol (0.1 M, Sigma), 0.5 μ l RNase inhibitor (40 U/ μ l, Sigma), 1 μ l oligo-d(T) 12–18 primer (0.5 μ g/ μ l, Life Technologies, BRL), and 1 μ l dNTP (2.5 nmol/ μ l each nucleotide, Sigma) to each sample of RNA. The reaction tube was incubated for 1 h at 37 °C. The volume of each sample was then adjusted to 60 μ l and the enzyme was inactivated by incubation for 10 min at 65 °C. The resulting cDNAs were then amplified by PCR using specific primers, as shown in Table 1. To 45 μ l PCR mixture containing 32.75 μ l H₂O, 5 μ l reverse transcription product, 2.5 μ l 5'-primer (20 μ M), 2.5 μ l 3'-primer (20 μ M), 2 μ l dNTP (2.5 nmol/ μ l of each nucleotide, Sigma), 5 μ l of 10 $\times$ reaction buffer, and 0.25 μ l Taq DNA polymerase (Sigma) were added. A negative control consisting of the reaction mixture without the cDNA was included in each run. Thermal cycling was initiated with incubation at 95 °C for 5 min for activation of Taq DNA polymerase. After this initial step, 20–28 cycles of PCR were performed. Each PCR cycle

consisted of heating at 95 °C for 30 s, denaturing at 56–68 °C for 30 s, and annealing for 30 s at 72 °C. Incubation was prolonged by 5 s in each cycle. Every experiment was amplified with at least two different numbers of cycles to ensure that amplification was at the exponential phase of PCR. We found that 25–30 cycles for each target gene and 20–25 cycles for β -actin were in this range. Under these conditions, we also found a linear dose–response of the PCR product to increase doses of cDNA. Then, 15 μ l of each sample containing amplified cDNA were loaded on an agarose gel (2%) containing ethidium bromide (0.5 μ g/ml). A DNA size marker was run on the same gel (100 bp ladder, Life Technologies, BRL). The PCR products were quantified densitometrically using Flurochem software (Alpha-Inno-tech, San Leandro, CA, USA). To correct differences in loading, we corrected densitometric values of each target gene cDNAs with corresponding values of β -actin cDNA and the ratios were calculated.

Western blot analysis

Heart tissue was homogenized on ice with a polytron (Kinetica, Littau, Switzerland) in lysis buffer (50 mM Tris (pH 7.4), 0.2% Triton X-100) containing 20 mM sodium pyrophosphate, 100 mM NaF, 4 mM EGTA, 4 mM Na₃VO₄, 2 mM phenylmethylsulphonyl fluoride, 0.25% aprotinin, and 0.02 mg/ml leupeptine. Extracts were centrifuged for 20 min at 17 000 *g* at 4 °C and the supernatants were collected and frozen. For the detection of heart AT1R, total and phospho-ERK1/2, poly (ADP-ribose) polymerase (PARP), and total and phospho-JNK homogenates were mixed with 5 $\times$ sample buffer and boiled for 5 min. Then, 200 μ g portions of sample protein were loaded in each gel lane and subjected to 10% SDS polyacrylamide gel and electroblotted into nitrocellulose membranes. Blots were blocked for 1 h in TBS buffer (10 mM Tris (pH 7.4), 138 mM NaCl) containing 5% nonfat dehydrated milk, followed by overnight incubation with the following antibodies (all diluted in TBS containing 5% dry milk): rabbit polyclonal antibody against AT1R (Santa Cruz Biotechnology, Santa Cruz, CA, USA), rabbit polyclonal antibody against ERK (Santa Cruz Biotechnology), mouse monoclonal anti-phospho-ERK1/2 (Sigma), rabbit

Table 1 PCR-specific primers

Gene	Sense	Antisense
β -MHC	CAAGTCAGCCTACCTCATGGG	TGACTCGAGGGTGGCACAA
ANP	CAAGAGAGAGCAGGACACCATCGC	TATGCAGACCCTCGGTGCTCACCT
Skeletal α -actin	GCGCAAGTACTCAGTGTGGATCGG	CACCTGCAACCATAGCACGATG
Type III collagen	ATATCAAACACGCAAGGC	GATTAAAGCAAGAGGAACAC
TGF- β	GGCACCATCCATGACATGAA	CAGGTGTTGAGCCCTTTCCA
ACE	CACCGGCAAGGTCTGCTT	CTTGGCATAGTTTCGTGAGGAA
ACE 2	GTGCACAAAGGTCACAATGG	TGTTTCATCATGAGGCAGAGG
β -Actin	GACGAGGCCAGAGCAAGAG	GGGCCGGACTCATCGTACTC

polyclonal anti-PARP (Cell Signaling), rabbit polyclonal antibody against Jun-NH₂-protein kinase (JNK; Santa Cruz Biotechnology), and rabbit polyclonal anti-phospho-JNK (Cell Signaling, Danvers, MA, USA). After washing thrice for 15 min in TBST (0.05% Tween-20), the blots were incubated with a secondary anti-rabbit antibody conjugated to horseradish peroxidase or a secondary anti-mouse antibody conjugated to horseradish peroxidase for 1 h at room temperature, and then washed again thrice. The band antibody was visualized by enhanced chemiluminescence (Amersham, Life Sciences Inc.) and exposed to Kodak-BioMax film (Eastman Kodak). Protein expression was quantitated densitometrically using FluorChem software (Alpha-Innotech).

Apoptosis was evaluated by immunoblot detection of cleaved PARP, a well-accepted marker of apoptosis in heart tissue (Los *et al.* 2002), using rabbit polyclonal antibody against PARP. This antibody detects full-length PARP (116 kDa), as well as the large (89 kDa) and small (24 kDa) fragments of PARP produced by caspase cleavage (Cell Signaling).

Statistical analysis

Six to eight animals were used in each experimental group. Differences within groups were determined using one-way ANOVA for non-repeated measurements followed by a *post hoc* analysis (Scheffe) to determine the source of significance. We set statistical significance at $P < 0.05$. Data are provided as means $\pm$ S.E.M.

Results

Blood glucose levels

STZ induced a diabetic phenotype that is consistent with that previously described in STZ-treated animals. Average glucose levels in D and DS groups were 21.3 ± 1.2 and 20.1 ± 1.8 mmol/l respectively. For comparison, blood glucose levels in the C and CS groups were 6.3 ± 0.2 and 6.1 ± 0.2 mmol/l respectively. We did not detect ketonuria in any of the diabetic animals during the study period.

Blood pressure

During 6 weeks of salt loading, CS and DS developed hypertension, BP beginning to rise as of the third week of the study period (Fig. 1). Upon termination of the study, average systolic BP was 175 ± 1 mmHg in CS and 181 ± 2 mmHg in DS ($P = \text{NS}$); in C rats, BP was 139 ± 1 mmHg in C and 128 ± 2 mmHg in D ($P < 0.001$).

Body weight

The diabetic animals gained less BW during the course of the study period than control animals, as shown in Fig. 2 (upper panel). Upon termination of the study, BW in D and DS was

Figure 1 Tail-cuff systolic BP in controls (C), control hypertensive (CS), diabetic (D), and hypertensive diabetic (DS) animals after 6 weeks of experimentation. SBP was significantly decreased in D in comparison with C, beginning in the second week of the experiment. SBP increased significantly in both CS and DS, but there was no difference between these two groups.

223 ± 15 and 216 ± 13 respectively, as opposed to 295 ± 10 and 270 ± 7 in C and CS respectively. Interestingly enough, the lesser gain in weight in diabetic animals was despite greater food intake. Daily food intake, as measured 4 weeks after the induction of diabetes, was 94.7 ± 6.9 and 105.3 ± 6.5 g/kg in C and CS respectively ($P = \text{NS}$), but was as high as 180.4 ± 8.2 and 157.2 ± 7 g/kg in D and DS respectively ($P < 0.001$ versus C).

Heart weight

The advent of hypertension in the non-diabetic C animals was associated with a 17% increase in heart weight (HW) from 1052 ± 38 mg in C to 1227 ± 42 mg in CS ($P < 0.01$). In contrast, in the diabetic D animals, the development of hypertension was not associated with any increase in HW, which was 1071 ± 69 mg in D and 1032 ± 77 mg in DS ($P = \text{NS}$). However, when adjusted for BW, the HW/BW ratio was similarly and significantly elevated in all the three experimental groups when compared with the C group ($127 \pm 6\%$ in CS, $132 \pm 5\%$ in D, and $135 \pm 6\%$ in DS, $P < 0.01$ versus C), as shown in Fig. 2 (lower panel). Histological analysis of heart tissue did not reveal any significant changes between the experimental groups.

Echocardiographic measurements

Absolute left ventricular fractional shortening was normal and similar in all study groups (66 ± 1 , 64 ± 2 , 61 ± 4 , and $65 \pm 1\%$ in C, CS, D, and DS, $P = \text{NS}$). Absolute LVIDD values were not significantly different between study groups. However, when corrected to BW, there was a significant increase in the LVIDD/BW ratio in the D and DS animals (2.6 ± 0.2 and 2.7 ± 0.1 in D and DS versus 1.9 ± 0.01 and 2.2 ± 0.1 in C and CS respectively, $P < 0.05$ versus C; Fig. 2). A similar

Figure 2 Body weight (upper panel), heart weight/body weight (HW/BW; middle panel) depicted as percentage of control animals; and left ventricular internal dimension during diastole (LVIDD)/BW (lower panel). * $P < 0.05$ versus C and CS.

significant difference between the study groups was seen for LVIDS/BW (0.65 ± 0.02 , 0.8 ± 0.07 , 0.99 ± 0.1 , and 0.95 ± 0.09 in C, CS, D, and DS respectively, $P < 0.05$). There was no similar significant difference between the study groups on the LV fractional shortening, E/A ratio, or acceleration and deceleration times of E waves.

Apoptosis

The ratio of cardiac cleaved (89 kDa) PARP to full-length PARP (116 kDa) protein levels, as evaluated by immunoblot, was not different between the experimental groups: 102 ± 6 , 106 ± 12 , and $98 \pm 14\%$ of C in CS, D, and DS respectively.

Gene expression

As shown in Fig. 3, β -MHC mRNA was 193 ± 28 and $178 \pm 23\%$ of C in D and DS respectively ($P < 0.05$); skeletal α -actin mRNA was 209 ± 26 and $210 \pm 25\%$ of C in D and DS respectively ($P < 0.05$); and ANP mRNA was 232 ± 48

and $230 \pm 53\%$ of C in D and DS respectively ($P < 0.05$). As shown in Fig. 4, type III collagen mRNA in CS was $157 \pm 4\%$ of C, but more notably so 189 ± 15 and $255 \pm 19\%$ of C in D and DS respectively ($P < 0.05$). TGF- β mRNA was increased in D to 161 ± 19 and in DS to $156 \pm 9\%$ of C ($P < 0.05$). These increases in gene expression in the hearts of D and DS are indicative of ongoing cardiac remodeling.

The renin–angiotensin system

As the balance between ACE and ACE2 expressions may determine the levels of free angiotensin II in the heart. ACE mRNA was increased in D to $618 \pm 91\%$ of C and DS to $569 \pm 76\%$ of C. ACE2 mRNA was decreased in D to $66 \pm 11\%$ of C and DS to $74 \pm 10\%$ of C. This difference was further accentuated if depicted as ACE/ACE2 ratio for each animal, as shown in Fig. 5. Cardiac AT1R protein levels were not different between the experimental groups: 92 ± 5 , 97 ± 5 , and $94 \pm 12\%$ of C in CS, D, and DS respectively.

Intracellular signalling

To further elucidate the mechanism involved in the observed expression of ACE and ACE2, signaling pathways of mitogen-activated protein kinases were studied by western blot analysis of protein extracts from heart tissue. As shown in Fig. 6, cardiac phospho-ERK1/2 to ERK1/2 ratio was increased in D and DS, 1.6 ± 0.2 and 1.3 ± 0.2 , respectively, compared with 0.53 ± 0.2 and 0.7 ± 0.1 in C and CS respectively ($P < 0.05$). In addition, increased activation of phospho- $p46$ JNK and phospho- $p55$ JNK to total JNK ratio was observed in D and DS, 1.7 ± 0.2 and 1.5 ± 0.15 , respectively, compared with 0.96 ± 0.1 and 0.75 ± 0.1 in C and CS respectively ($P < 0.05$).

Discussion

In the present study, we found evidence for early cardiac remodeling, both in animals with DM alone and in those with concurrent DM and hypertension. Our evidence for the occurrence of cardiac remodeling consists of a relative increase in heart size, along with an increase in LVIDD and LVIDS by echocardiography. We found no evidence for increased cardiac apoptosis. However, we found an increased expression of structural myocardial response genes, namely β -MHC, ANP, and skeletal α -actin, which have long been considered as markers of hypertrophy of the rat heart (Boluyt *et al.* 1995, Swynghedauw *et al.* 1995, Barbosa *et al.* 2005). We have also been able to demonstrate that DM induces similar changes in the expression of these genes without any additional effect for hypertension.

Our findings suggest that the occurrence of diabetes alone is sufficient to initiate cardiac remodeling. These findings are consistent with a previous study, during which a more prolonged period (18 weeks) of IDDM (without hypertension)

Figure 3 Cardiac β -myosin heavy chain (β -MHC; upper panel), α -skeletal actin (middle panel), and ANP (lower panel) mRNA levels in controls (C), control hypertensive (CS), diabetic (D), and hypertensive diabetic (DS) animals after 6 weeks of experimentation. * $P < 0.05$ versus C and CS.

incurred not only right ventricular remodeling but also overt resting left ventricular systolic dysfunction, accompanied by pathological changes of capillaries and cardiomyocytes including actin filaments (Nemoto *et al.* 2006). In another study, diastolic dysfunction was detected in type I diabetic mice after 30 days of DM (Kajstura *et al.* 2001).

Our findings also suggest that hypertension is not required in our diabetic model for cardiac remodeling to be evoked. Hypertension has indeed been shown to be a known risk factor for cardiac remodeling in non-diabetics, leading to hypertrophy and later cardiomyocyte loss and heart failure. Previous studies have shown that 6 weeks of hypertension is sufficient to induce early cardiac remodeling (Burla *et al.* 2007). Our present findings indicate that 6 weeks of salt-loading *per se* (without diabetes) and the rise in BP over 4 weeks increased the HW/BW ratio in group CS (Fig. 2), but were not enough to induce those markers of cardiac remodeling (Fig. 3) and fibrosis (Fig. 4), which were only

found in both diabetic groups. These findings suggest that for early cardiac remodeling to occur, the occurrence of DM suffices without the need for hypertension to develop. However, it is quite possible that the contribution of hypertension to cardiac remodeling in diabetic patients with long-standing hypertension may become significant on the long term, as previously shown (Nagata *et al.* 2006).

What are the mechanisms that lead to early cardiac remodeling? Most of the information that is currently known regarding cardiac remodeling stems from the models of myocardial infarction rather than of diabetic cardiomyopathy. Sustained cardiomyocyte apoptosis, left ventricular enlargement, increased cardiac fibrosis, and enhanced profibrogenic connective tissue growth factor expression have all been demonstrated after myocardial infarction in experimental models of DM (Backlund *et al.* 2004). However, no information is available whether such changes also occur in DM alone, without contributing infarction. Apoptosis is

Figure 4 Cardiac type III collagen (upper panel) and TGF- β (lower panel) mRNA levels in controls (C), control hypertensive (CS), diabetic (D), and hypertensive diabetic (DS) animals after 6 weeks of experimentation. * $P < 0.05$ versus C and CS.

orchestrated by the proteolytic activation of cysteine proteases known as caspases. Distinct pathways of apoptosis converge for the activation of executioner caspase-3, which cleaves multiple downstream cellular targets. An important target of caspase-3 is PARP, whose cleavage and inactivation maintain

the cellular ATP stores indispensable for apoptosis to proceed properly. The cleavage of PARP into 89 and 24 kDa fragments inactivates the enzyme by destroying its ability to respond to DNA strand breaks, thereby preventing the loss of cellular ATP associated with PARP activation and maintaining the cellular energy essential for the execution of apoptosis. Recent evidence has emerged that apoptosis (as measured by PARP levels) is an important mechanism of cardiomyocyte cell death in conditions such as myocardial reperfusion, cardiomyopathy, and heart failure (Pacher *et al.* 2002). In DM, evidence for increased apoptosis in heart tissue has been shown in patients and in a model of long-term disease (Chowdhry *et al.* 2007). Therefore, it could be that our negative findings reflect the early remodeling changes, still not associated with increased apoptosis.

Is AngII an important culprit in diabetic cardiomyopathy? AngII could be one of the important mediators of cardiac remodeling that antedates heart failure (Fiordaliso *et al.* 2000). Kajstura *et al.* (2001) have shown an increase in cardiac angiotensinogen and renin in diabetic mice after 30 days of DM. TGF- β may be a sign of AngII-mediated signaling in myocardium (Schultz *et al.* 2002). TGF- β 1 is up-regulated by AngII and induction of TGF- β 1 causes cardiac fibrosis (Lim & Zhu 2006). JNKs are implicated in cardiac diseases and are preferentially activated by stress signals, hypertrophic stimuli such as stretch and AngII, leading to the activation of transcription factor, activator protein (AP)-1. Increased cardiac JNK and AP-1 DNA-binding activities in stroke-prone hypertensive rats were reduced by losartan. Levels of phospho-p46JNK and phospho-p55JNK were elevated in the LVs of obese mice (Zaman *et al.* 2004). ACEI and angiotensin II receptor blockers (ARB) decreased the activation of these JNK isoforms. As JNKs are activated by AngII and genes involved in remodeling, such as TGF- β 1, collagen, and skeletal α -actin, have AP-1 consensus sequence in their promoter regions, it may be possible that JNK, partially mediated by AP-1, can participate in the AngII-mediated increased gene expression in obese mice. Our data consisting of increased expression of phosphorylated JNK, pERK 1/2, and fibrosis-related genes (including TGF- β) are entirely consistent with early cardiac fibrosis driven by AngII. We could not demonstrate such histological fibrotic changes in this early stage of disease, but it cannot be ruled out that a longer follow-up study would have revealed these changes.

Although we did not measure AngII peptide levels in the present study, the low ACE2 and elevated ACE expressions in the myocardium of the diabetic animals are consistent with the elevated AngII levels. In addition, elevation of TGF- β as well as phosphorylated ERK1/2 and JNK, both downstream signaling molecules in the AT1R signaling pathway are consistent with an increase in local AngII synthesis and action. However, more markers of specific intracellular AT1R downstream signaling need to be established to support this hypothesis. A recent study by Tsutsui *et al.* (2007) reports on

Figure 5 Cardiac ACE (upper panel), ACE2 (middle panel), and ACE/ACE2 ratio (calculated for each animal) (lower panel) in controls (C), control hypertensive (CS), diabetic (D), and hypertensive diabetic (DS) animals after 6 weeks of experimentation. * $P < 0.05$ versus C.

Figure 6 Western blot analysis of cardiac pERK1/2-to-ERK1/2 ratio (upper panel) and pJNK/JNK ratio (lower panel), using 200 µg tissue lysates, in the control (C), control hypertensive (CS), diabetic (D), and hypertensive diabetic (DS) animals after 6 weeks of experimentation. Shown are blots representative of four independent experiments. The graph shows a densitometry analysis (represented in arbitrary densitometric units) summarizing the four independent experiments for each peptide. * $P < 0.05$ versus C and CS.

the beneficial effects of the AT1R blocker candesartan on diastolic dysfunction as well as myocardial interstitial fibrosis and TGF- β -CTGF expression in diabetic mice. This study supports our findings on the central role of AngII in mediating the structural and functional abnormalities of the diabetic heart, in addition to the previously described role of increased oxidative stress in diabetic complications in general (Griendling *et al.* 1994) and the diabetic heart in particular (Fiordaliso *et al.* 2004).

We conclude that early cardiac remodeling occurs in the diabetic rat without the need for hypertension to develop, and that activation of the renin-angiotensin system may play an important pathophysiological role. What are the potential implications of our findings? It is already established that ACE inhibitors and ARBs reduce disease progression and mortality in patients with overt heart failure (Carr *et al.* 2005). Our findings suggest that blockage of the renin-angiotensin system, whether with ACE inhibitors or ARBs, might be in place in the early phases of diabetic cardiomyopathy as well, irrespective of the level of BP and even before the development of overt heart failure.

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Disclosure

None of the co-authors has any conflict of interest that would prejudice this work's impartiality.

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