

CALL FOR PAPERS | *Mechanisms of Diastolic Dysfunction in Cardiovascular Disease*

Left ventricular pressure-volume measurements and myocardial gene expression profile in type 2 diabetic Goto-Kakizaki rats

Sevil Korkmaz-Icöz,¹ Alice Lehner,¹ Shiliang Li,¹ Adrian Vater,¹ Tamás Radovits,² Maik Brune,³ Mihály Ruppert,^{1,2} Xiaoxin Sun,¹ Paige Brlecic,¹ Markus Zorn,³ Matthias Karck,¹ and Gábor Szabó¹

¹Department of Cardiac Surgery, University of Heidelberg, Heidelberg, Germany; ²Heart and Vascular Center, Semmelweis University, Budapest, Hungary; and ³Department of Internal Medicine I, University of Heidelberg, Heidelberg, Germany

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Korkmaz-Icöz S, Lehner A, Li S, Vater A, Radovits T, Brune M, Ruppert M, Sun X, Brlecic P, Zorn M, Karck M, Szabó G. Left ventricular pressure-volume measurements and myocardial gene expression profile in type 2 diabetic Goto-Kakizaki rats. *Am J Physiol Heart Circ Physiol* 311: H958–H971, 2016. First published August 12, 2016; doi:10.1152/ajpheart.00956.2015.—The Goto-Kakizaki (GK) rat, a non-obese model of type 2 diabetes mellitus (T2DM), was generated by the selective inbreeding of glucose-intolerant Wistar rats. This is a convenient model for studying diabetes-induced cardiomyopathy independently from the effects of the metabolic syndrome. We investigated the myocardial functional and structural changes and underlying molecular pathomechanisms of short-term and mild T2DM. The presence of DM was confirmed by an impaired oral glucose tolerance in the GK rats compared with the age-matched nondiabetic Wistar rats. Data from cardiac catheterization showed that in GK rats, although the systolic indexes were not altered, the diastolic stiffness was increased compared with nondiabetics (end-diastolic pressure-volume-relationship: 0.12 ± 0.04 vs. 0.05 ± 0.01 mmHg/ μ l, $P < 0.05$). Additionally, DM was associated with left-ventricular hypertrophy and histological evidence of increased myocardial fibrosis. The plasma pro-B-type natriuretic peptide, the cardiac troponin-T, glucose, and the urinary glucose concentrations were significantly higher in GK rats. Among the 125 genes surveyed using PCR arrays, DM significantly altered the expression of five genes [upregulation of natriuretic peptide precursor-A and connective tissue growth factor, downregulation of c-reactive protein, interleukin-1 β , and tumor necrosis factor (TNF)- α mRNA-level]. Of the altered genes, which were evaluated by Western blot, only TNF- α protein expression was significantly decreased. The ECG recordings revealed no significant differences. In conclusion, while systolic dysfunction, myocardial inflammation, and abnormal electrical conduction remain absent, short-term and mild T2DM induce the alteration of cardiac TNF- α at both the mRNA and protein levels. Further assessments are required to reveal if TNF- α plays a role in the early stage of diabetic cardiomyopathy development.

cardiac function; diabetic cardiomyopathy; gene expression profiling; Goto-Kakizaki rats; type 2 diabetes mellitus

electrical conduction, myocardial inflammation, and systolic dysfunction, cardiac gene/protein TNF- α expression was significantly altered and requires further research.

DIABETES MELLITUS (DM), a chronic, progressive metabolic disorder, is caused by impaired insulin production or by the produced insulin's ineffectiveness, thus leading to hyperglycemia. Type 2 diabetes mellitus (T2DM) (named noninsulin dependent) is the most common form and represents more than 90% of all diabetes cases. The global rate of mortality attributable to T2DM has been estimated at 5.2% of all deaths (26). Diabetic cardiomyopathy has been defined as ventricular dysfunction and abnormal myocardial structure in absence of changes in blood pressure and coronary artery disease. It is initially characterized by diastolic dysfunction (31), ventricular dilatation, enlargement of cardiomyocytes, and prominent interstitial fibrosis. The mechanisms leading to diabetic heart disease have been widely discussed. Metabolic abnormalities (hyperglycemia, hyperinsulinemia, and hyperlipidemia) that accompany diabetes and their cellular consequences (increased oxidative stress, endothelial dysfunction and inflammation and enhanced activity of tissue renin-angiotensin system), resulting in cardiac functional (diastolic and systolic dysfunction) and structural changes [left ventricular (LV) hypertrophy and cardiac fibrosis], seem to contribute to the development of diabetic cardiomyopathy (33).

The Goto-Kakizaki (GK) rat, a nonobese, nonhypertensive genetic model of T2DM, which like humans, shares a susceptibility locus on chromosome 10 (14), was developed by the selective inbreeding of glucose-intolerant Wistar rats (12). The characteristics of the disease in the GK rat include a reduced β -cell mass (up to 60% with markedly impaired glucose-induced insulin secretion), mild hyperglycemia, glucose intolerance, hepatic glucose overproduction, and/or a peripheral insulin resistance in muscle and adipose tissue (24). Additionally, GK rats are not associated with the development of obesity, hypertension, or hyperlipidemia. Previous studies have been conducted in diabetic GK rats, demonstrating echocardiographic signs of preserved cardiac systolic function or impaired systolic (17) and diastolic function (14) by 20 wk of age. However, the pathomechanisms leading to characteristic changes in the type 2 diabetic GK rat hearts have not been completely elucidated, hence needing further investigation.

In the present study, we sought to provide a detailed depiction of the LV's systolic and diastolic functions independently from the effect of hyperlipidemia, obesity, or hypertension in a

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To extend previous works we investigated early myocardial molecular signatures associated with short-term, mild type 2 diabetes mellitus-induced diastolic dysfunction and structural remodeling in Goto-Kakizaki rats. In the absence of abnormal

Address for reprint requests and other correspondence: S. Korkmaz-Icöz, Laboratory of Cardiac Surgery, Dept. of Cardiac Surgery, Univ. of Heidelberg, INF 326, 69120 Heidelberg, Germany (e-mail: korkmaz@uni-heidelberg.de).

rat model of mild T2DM, using a Millar pressure-volume conductance catheter system. Since diabetic cardiomyopathy is highly prevalent in asymptomatic patients with T2DM, screening at its earliest stage can reduce the risk of developing chronic heart failure. Therefore, the earlier identification of cardiac changes in short-term diabetes could be a better strategy to prevent the disease progressing to a more serious stage. To this aim, we compared the myocardial gene expression profiles relevant to apoptosis, oxidative stress, phosphoinositide 3-kinase (PI3K)/Akt signaling, inflammation, cardiac fibrosis, and hypertrophy between GK and control Wistar rats.

METHODS

Animals. Spontaneously diabetic male GK rats (Taconic, Ry, Denmark) and age- and sex-matched nondiabetic control Wistar (W) rats (Charles River, Sulzfeld, Germany) were housed in a standard animal facility with a constant temperature ($22 \pm 2^\circ\text{C}$), 12-h light-dark cycles, and free access to standard laboratory rat diet and water. The male GK rat develops characteristics of T2DM at around 14–16 wk of age (<http://www.taconic.com>). Therefore, in the present study, 16 wk of age was defined as the early onset of diabetes. The experiment conforms with the *Guide for the Care and Use of Laboratory Animals* published by the National Institutes of Health (NIH Publication No. 85-23, revised 1996) and the German animal protection code. All procedures and handling of animals during the investigations were reviewed and approved by the appropriate institutional review board (G-122/11). The experiments were performed at 16 wk of age.

Experimental groups. The rats were divided into two groups: 1) W: nondiabetic control Wistar rats ($n = 10$); and 2) GK: diabetic GK animals ($n = 8$).

Oral glucose test. An oral glucose tolerance test was performed as described elsewhere (39, 40) with slight modifications. Briefly, the animals fasted for 12 h before receiving intragastrically administered 40% glucose solution (2 g/kg body wt). Blood samples from the tail vein of conscious rats were obtained to determine blood glucose levels using a digital blood glucose meter and test strips (Accu-Chek Sensor; Roche, Mannheim, Germany) at 0, 15, 30, 60, 90, 120, 180, and 240 min after glucose administration.

Echocardiography. One day before final experiments, the rats were slightly anesthetized with 1.5–2.0% isoflurane by mask, the left side of the chest was shaved to obtain a clear image, and the animals were situated in the supine position on a warming pad. Transthoracic echocardiography was performed by using an HDI 5000 CV echocardiography machine (ATL Ultrasound; Philips, Bothell, WA) equipped with a 10-MHz linear probe. Two-dimensional parasternal short-axis images as well M-mode recordings at the mid-papillary muscle level were assessed. The following parameters were measured: LV internal end-diastolic dimensions (LVIDd) during diastole and LV anterior (AWT) and posterior wall thicknesses (PWT) in diastole (index: d). LV mass was calculated to estimate the myocardial weight using the Devereux formula: $\text{LV mass (g)} = \{[(\text{LVEDD} + \text{AWT}_d + \text{PWT}_d)^3 - \text{LVEDD}^3] \times 1.04\} \times 0.8 + 0.14$, where LVEDD is LV end-diastolic diameter (9). To exclude an influence of body weight differences, these parameters were normalized to body weight.

Electrocardiography. Before the hemodynamic measurements, the rats were anesthetized with sodium pentobarbital (60 mg/kg ip) and kept in a supine position on heating pads, maintaining their core temperature (measured via a rectal probe) at 37°C . Standard 12-lead electrocardiograms were monitored for 10 min and recorded using subcutaneously placed needle electrodes. All leads were connected to a standard direct-writing recorder (Mortara Instrument). The ECG analysis was evaluated in lead II including the following measurements: ST-segment elevation and PQ and QT intervals. The QT interval, measured from the onset of the QRS complex to the end of the T wave, was corrected using the normalized Bazett's formula

adjusted for rats [$\text{nQTc} = \text{QT}/(\text{RR}/\text{f})^{1/2}$] (RR corresponding to cardiac cycle length and f to frequency) (18). The electrocardiography was analyzed in a blinded fashion.

Hemodynamic measurements: LV pressure-volume analysis. After the ECG recordings, the rats were tracheotomized, intubated, and artificially ventilated with ambient air. A polyethylene catheter was inserted into the left external jugular vein for fluid administration purposes. A 2-F microtip pressure-volume catheter (SPR-838; Millar Instruments, Houston, TX) was inserted into the right carotid artery and advanced into the ascending aorta. After a 5-min stabilization period, the arterial blood pressure was recorded, and the catheter was advanced into the left ventricle under pressure control. With the use of a special pressure-volume analysis program (PVAN; Millar Instruments), heart rate, systolic and diastolic blood pressures, mean arterial pressure, LV end-diastolic pressure (LVEDP), stroke volume, ejection fraction, cardiac output, maximal slope of the systolic pressure increment ($\text{dP}/\text{d}t_{\text{max}}$), maximal slope of the diastolic pressure decrement ($\text{dP}/\text{d}t_{\text{min}}$), and systemic vascular resistance were computed and calculated. To exclude an influence of body weight differences, cardiac output and stroke volume were normalized to body weight (cardiac index and stroke volume index). Ventricular relaxation was assessed by the time constant of LV pressure decay (Tau), calculated by the Glantz method (Tau-g; regression of $\text{dP}/\text{d}t$ vs. pressure). LV pressure-

Table 1. Gene table: cardiac fibrosis, inflammation, and hypertrophy

Subcategory/Gene Symbol	Official Full Name
Cardiac Fibrosis	
Profibrotic	
Agt	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
ccl11	Chemokine (C-C motif) ligand 11
Ctgf	Connective tissue growth factor
Grem1	Gremlin 1, cysteine knot superfamily, homolog (<i>Xenopus laevis</i>)
Remodeling enzymes	
Mmp1a	Matrix metalloproteinase 1alpha
Mmp2	Matrix metalloproteinase 2
Growth factors	
Agt	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
Ctgf	Connective tissue growth factor
Signal transduction	
Grem1	Gremlin 1, cysteine knot superfamily, homolog (<i>Xenopus laevis</i>)
Tgfb1	Transforming growth factor, beta 1
Inflammation	
Chemokine	
Ccl5 (Rantes)	Chemokine (C-C motif) ligand 5
ccl11	Chemokine (C-C motif) ligand 11
Ifng	Interferon gamma
Tnf	Tumor necrosis factor (TNF superfamily, member 2)
Vegfa	Vascular endothelial growth factor A
Interleukins	
Il1b	Interleukin 1 beta
Il6	Interleukin 6
Other	
crp	C-reactive protein, pentraxin-related
Hypertrophy	
Acta1	Actin, alpha 1, skeletal muscle
c-fos	FBJ osteosarcoma oncogene
Myl2	Myosin, light polypeptide 2, regulatory, cardiac, slow
Nppa	Natriuretic peptide precursor A
Prkca	Protein kinase C, alpha
Tgfb1	Transforming growth factor, beta 1

Table 2. *Gene table: apoptosis*

Subcategory/Gene Symbol	Official Full Name
Induction of apoptosis	
Death domain receptors	
Casp8ap2	Caspase 8 associated protein 2
DNA damage	
Ab11	C-abl oncogene 1, receptor tyrosine kinase cell death-inducing DFFA-like effector a
Cidea	Cell death-inducing DFFA-like effector a
Cideb	Cell death-inducing DFFA-like effector b
Tp53	Tumor protein p53
Tp73	Tumor protein p73
Extracellular signals	
Casp8ap2	Caspase 8 associated protein 2
Cflar (Casper)	CASP8 and FADD-like apoptosis regulator Death associated protein kinase 1
Dapk1	Death associated protein kinase 1
Other	
Bax	Bc/II-associated X protein
Casp3	Caspases 3
Casp6	Caspases 6
Tp53	Tumor protein p53
Bax	Bc/II-associated X protein
Antiapoptosis	
Bax	Bc/II-associated X protein
Bc/II	B-cell CLL/lymphoma 2
Bcl211	Bc/II-like 1
Cflar (Casper)	CASP8 and FADD-like apoptosis regulator Death associated protein kinase 1
Regulation of apoptosis	
Negative regulation	
Bc/II	B-cell CLL/lymphoma 2
Bcl211	Bc/II-like 1
Casp3	Caspases 3
Cflar (Casper)	CASP8 and FADD-like apoptosis regulator Death associated protein kinase 1
Cidea	Cell death-inducing DFFA-like effector a
Dapk1	Death associated protein kinase 1
Tp53	Tumor protein p53
Tp73	Tumor protein p73
Positive Regulation	
Bax	Bc/II-associated X protein
Casp6	Caspases 6
Cideb	Cell death-inducing DFFA-like effector b
DEATH domain proteins	
Dapk1	Death associated protein kinase 1
Caspases and regulators	
Caspases	
Casp3	Caspases 3
Casp6	Caspases 6
Cflar (Casper)	CASP8 and FADD-like apoptosis regulator Death associated protein kinase 1
Caspase activators	
Bax	Bc/II-associated X protein
Casp8ap2	Caspase 8 associated protein 2
Tp53	Tumor protein p53
Others	
Sfrp4	Secreted frizzled-related protein 4

volume relations were assessed by transiently compressing the inferior vena cava. The slope $E_{\max}$ of the LV end-systolic pressure-volume relationship (ESPVR), preload recruitable stroke work (PRSW), and the slope of $dP/dt_{\max}/\text{end diastolic volume}$ ($dP/dt_{\max}/\text{EDV}$) relationship were calculated as a load-independent index of LV contractility. The slope of the end-diastolic pressure-volume relationship (EDPVR) was calculated as a reliable index of LV stiffness.

Biochemical measurements. After hemodynamic measurements were completed, blood samples from the abdominal aorta were collected in Lithium-Heparin-Gel Monovette, EDTA, and serum tubes. Then, after centrifugation (4,500 g, 15 min, 4°C), plasma and serum samples were obtained. The levels of high-sensitive cardiac troponin-T and NH₂-terminal pro-B-type natriuretic peptide (BNP) were determined in the central laboratory of the Heidelberg University Clinic with ECLIA on Cobas E411 (Roche Diagnostics), and the levels of plasma glucose, triglycerides, and total cholesterol were determined on an ADVIA 2400 chemistry analyzer (Siemens Healthineers). Serum aldosterone levels were analyzed by radioimmunoassay (RIA) and plasma renin activity via generation of angiotensin I by RIA. The levels of serum insulin were measured via Ultrasensitive Rat Insulin ELISA kit (Mercodia, Uppsala, Sweden). Additionally, bladder urine was collected by needle puncture, urinary glucose, and norepinephrine levels were determined.

Histopathology. After the blood sample collection, all rats were killed by bleeding and the hearts were explanted. Pieces of the LV myocardial tissue were fixed in buffered paraformaldehyde solution (4%) and embedded in paraffin. Then, 5- μm thick sections were

Table 3. *Gene table: oxidative stress*

Subcategory/Gene Symbol	Official Full Name
Antioxidants	
Glutathione peroxidases	
Gpx1	Glutathione peroxidases 1
Gpx2	Glutathione peroxidases 2
Gpx3	Glutathione peroxidases 3
Gpx4	Glutathione peroxidases 4
Gpx5	Glutathione peroxidases 5
Gpx6	Glutathione peroxidases 6
Gpx7	Glutathione peroxidases 7
Gstk1	Glutathione S-transferase kappa 1
Gstp1	Glutathione S-transferase pi 1
Other eroxidase	
Cat	Catalase
Other antioxidants	
Alb	Albumin
Gsr	Glutathione reductase
Sod1	Superoxide dismutase 1, soluble
Sod3	Superoxide dismutase 3, extracellular
Txnrd1	Thioredoxin reductases 1
Txnrd2	Thioredoxin reductases 2
Genes involved in reactive oxygen species metabolism	
Superoxide dismutases	
Sod1	Superoxide dismutase 1, soluble
Sod2	Superoxide dismutase 2, mitochondrial
Sod3	Superoxide dismutase 3, extracellular
Oxidative stress responsive genes	
Cat	Catalase
Gpx1	Glutathione peroxidases 1
Gpx2	Glutathione peroxidases 2
Gpx3	Glutathione peroxidases 3
Gpx4	Glutathione peroxidases 4
Gpx5	Glutathione peroxidases 5
Gpx6	Glutathione peroxidases 6
Gpx7	Glutathione peroxidases 7
Gsr	Glutathione reductase
Sod1	Superoxide dismutase 1, soluble
Srxn1	Sulfiredoxin 1 homolog (<i>S. cerevisiae</i>)
Txnrd1	Thioredoxin reductases 1
Others	
Acad5b	Acyl-Coenzyme A dehydrogenase, short/branched chain
	Selenoprotein S
Xdh	Xanthine dehydrogenase

Table 4. *Gene table: PI3K/Akt signaling*

Gene Symbol	Official Full Name
AKT and PI3K family members and their regulators	
Akt1	V-akt murine thymoma viral oncogene homolog 1
Akt2	V-akt murine thymoma viral oncogene homolog 2
Akt3	V-akt murine thymoma viral oncogene homolog 3
Btk	Bruton agammaglobulinemia tyrosine kinase
Grb10	Growth factor receptor bound protein 10
Grb2	Growth factor receptor bound protein 2
Hspb1	Heat shock protein 1
Ilk	Integrin-linked kinase
Mtcp1	Mature T-cell proliferation 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Pdpk1	3-phosphoinositide dependent protein kinase-1
Pik3ca	Phosphoinositide-3-kinase, catalytic, alpha polypeptide
Pik3cg	Phosphoinositide-3-kinase, catalytic, gamma polypeptide
Pik3r1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
Pik3r2	Phosphoinositide-3-kinase, regulatory subunit 2 (beta)
Prkca	Protein kinase C, alpha
Prkcb	Protein kinase C, beta
Prkcg	Protein kinase C, gamma
Pten	Phosphatase and tensin homolog
Tcl1a	T-cell leukemia/lymphoma 1A
IGF-1 signaling pathway	
Grb2	Growth factor receptor bound protein 2
Hras	Harvey rat sarcoma virus oncogene
Igf1r	Insulin-like growth factor 1 receptor
Irs1	Insulin receptor substrate 1
Map2k1	Mitogen activated protein kinase kinase 1
Mapk3	Mitogen activated protein kinase 3
Shc1	SHC (Src homology 2 domain containing) transforming protein 1
Sos1	Son of sevenless homolog 1 (<i>Drosophila</i>)
PTEN-dependent cell cycle arrest and apoptosis	
Akt1	V-akt murine thymoma viral oncogene homolog 1
Ilk	Integrin-linked kinase
Mapk1	Mitogen activated protein kinase 1
Mapk3	Mitogen activated protein kinase 3
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Pten	Phosphatase and tensin homolog
Shc1	SHC (Src homology 2 domain containing) transforming protein 1
Sos1	Son of sevenless homolog 1 (<i>Drosophila</i>)
BAD phosphorylation and antiapoptotic pathways	
Akt1	V-akt murine thymoma viral oncogene homolog 1
Bad	BCL2-associated agonist of cell death
Hras	Harvey rat sarcoma virus oncogene
Igf1r	Insulin-like growth factor 1 receptor
Irs1	Insulin receptor substrate 1
Map2k1	Mitogen activated protein kinase kinase 1
Mapk1	Mitogen activated protein kinase 1
Mapk3	Mitogen activated protein kinase 3
Rps6ka1	Ribosomal protein S6 kinase polypeptide 1
Shc1	SHC (Src homology 2 domain containing) transforming protein 1
Sos1	Son of sevenless homolog 1 (<i>Drosophila</i>)
Ywhah	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, eta polypeptide
Genes involved in the mTOR signaling pathway	
Akt1	V-akt murine thymoma viral oncogene homolog 1
Eif4b	Eukaryotic translation initiation factor 4B
Eif4e	Eukaryotic translation initiation factor 4E
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1
Eif4 g1	Eukaryotic translation initiation factor 4 gamma, 1
Fkbp1a	FK506 binding protein 1a
Mtor	Mechanistic target of rapamycin (serine/threonine kinase)
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Pten	Phosphatase and tensin homolog
Rheb	Rheb
Rps6kb1	Ribosomal protein S6 kinase, polypeptide 1
Tsc1	Tuberous sclerosis 1
Tsc2	Tuberous sclerosis 2
Regulation of eIF4e and p70 S6 kinase	

Continued

Table 4.—*Continued*

Gene Symbol	Official Full Name
Akt1	V-akt murine thymoma viral oncogene homolog 1
Eif4e	Eukaryotic translation initiation factor 4E
Eif4ebp1	Eukaryotic translation initiation factor 4E binding protein 1
Eif4 g1	Eukaryotic translation initiation factor 4 gamma, 1
Irs1	Insulin receptor substrate 1
Mapk1	Mitogen activated protein kinase 1
Mapk3	Mitogen activated protein kinase 3
Mtor	Mechanistic target of rapamycin (serine/threonine kinase)
Pdk1	Pyruvate dehydrogenase kinase, isozyme 1
Pdk2	Pyruvate dehydrogenase kinase, isozyme 2
Prkca	Protein kinase C, alpha
Pten	Phosphatase and tensin homolog
Rps6kb1	Ribosomal protein S6 kinase, polypeptide 1

stained with hematoxylin and eosin. Cardiomyocyte cross-sectional areas were calculated on a microscope using the Cell $\hat{A}$ software (Olympus Soft Imaging Solutions). Masson's trichrome staining was performed to determine the extent of myocardial fibrosis. Four sections per heart were inspected under light microscopy and rated according to the following scoring system: grade 0 indicates normal tissue showing no fibrotic region; grade 1 indicates mild fibrosis; grade 2 indicates moderate fibrosis, and grade 3 indicates severe fibrosis. The histological evaluation was conducted by an analyst unaware of the experimental groups.

Terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling staining. We performed terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) staining to detect DNA-strand breaks as previously described (20). Sections were deparaffinized with xylene and passed through decreasing concentrations of ethanol and then washed with phosphate buffered saline (PBS, $\times 1$) for 3×5 min. Hereafter DNase-free Proteinase K was used to digest tissue sections for 20 min to retrieve antigenic epitopes. Sections were washed with PBS ($\times 1$) for 2×5 min and treated with 3% hydrogen peroxide at room temperature for 5 min to block the endogenous peroxidase activity. After being washed with PBS ($\times 1$) for 2×5 min, the sections were incubated with 50 μ l of terminal deoxynucleotidyl transferase (TdT) enzyme and TUNEL reaction mixture for 1 h at 37°C in the dark (Roche Diagnostics). The sections were then washed with PBS ($\times 1$) for 3×5 min. The slides were mounted using 4',6-diamidino-2-phenylindole (DAPI)-Fluoromount-GTM (SouthernBiotech, Birmingham, AL), covered with coverglass and analyzed under a fluorescent microscope. The number of TUNEL-positive cells was expressed as the ratio of DAPI-TUNEL double-labeled nuclei to the total number of nuclei stained with DAPI. Cells were counted in four fields characterizing each specimen, and an average value was calculated for each experimental group. The evaluation was conducted by an analyst blinded to the experimental groups.

Measurement of capillary density. To visualize the capillaries in the myocardium, endothelial cells were stained with CD31. Briefly, deparaffinized tissue sections were blocked with 1% horse serum for 60 min at 37°C. Primary mouse monoclonal antibody against rat CD31 (Abcam, Cambridge, UK, 1:100) was used for 90 min. After primary antibody incubation, the sections reacted with a secondary antibody. Capillaries were visualized by ABC as brown precipitate and counterstained using hematoxylin. Then, to determine capillary density, the microvessels were counted in four random microscope fields using $\times 200$. Results were expressed based on the average number of capillaries per $\times 4$ field of each section ($n = 8-10$).

Nitrotyrosine immunohistochemical staining. Nitrotyrosine is a product of the tyrosine nitration mediated by reactive oxygen species. In brief, deparaffinized tissue sections were blocked with 1% horse serum for 60 min at 37°C. Primary rabbit polyclonal antibody against rat nitrotyrosine (Abcam, Cambridge, UK; 1:1,000) was used for 90 min. After primary antibody incubation, the sections reacted with a

secondary antibody. After initial evaluation of all corresponding tissue sections ($\times 100$ magnification), the tissue section with the most intense labeling signals was used as a reference for maximum labeling intensity. All other tissue sections were comparatively evaluated. The colors were measured using densitometry and put in four color classes: one class for background staining and three classes for positively stained areas. On the basis of the measured intensity, the color classes were coupled with score values as follows: 0: no positive staining, 1: weak staining, 2: intermediate staining, and 3: extensive staining. Using the program, we measured the area of the objects in each class in each field, assigned an area score (1 = up to 10% positive cells, 2 = 11–50% positive cells, 3 = 51–80% positive cells, and 4 = >80%-positive cells), and calculated an average score for the whole picture (intensity score multiplied by area score, 0–12). Finally, each specimen was characterized with the average of the four adjacent fields.

Gene expression analysis using RT² Profiler PCR arrays. After blood sample collection, pieces of the LV myocardial tissue were snap-frozen in liquid nitrogen and stored at -80°C . Using RT² Profiler PCR Arrays (CAPRI12307E), we determined the expression patterns of 125 genes. The official full name of these genes are presented in Tables 1, 2, 3, 4, and 5. Total RNA and microRNA were extracted from LV myocardial samples with a miRNeasy Mini Kit (Qiagen, Hilden, Germany) and were reverse-transcribed into cDNA using the RT² First Strand Kit, mixed with RT² qPCR Master Mix, containing SYBR Green, according to manufacturer's instructions (Qiagen, Hilden, Germany). In this Custom Array, the following nonregulated genes (genes-of interest) were used for normalization in the fold change expression data calculations: Parp1, Enpp1, Gsk3b, Nos3, Tgfb1, Ikbkb, Mapk8, Casp8ap2, Dapk1, Bax, Gpx1, Gpx3, Gpx4, Gstp1, Gsr, Sels, Txnrd2, Sod1, Sod2, Bad, Hras, Map2k1, Mapk1, Mapk3, Shc1, Sos1, Ywhah, Eif4b, Eif4e, Eif4g1, Fkbp1a, Mtor, Rheb, Rps6kb1, Tsc1, Tsc2, Akt1, Akt2, Akt3, Grb10, Grb2, Ilk, Pdpk1, Pik3ca, Prkca, Pten, and Myl2. The data were analyzed with the RT² Profiler PCR Array Data Analysis Template available on the manufacturer's website. Genes with fold changes of less than twofold at $P < 0.05$ were considered as significantly altered.

Western blotting. Proteins were extracted from the heart tissue in a $1 \times$ RIPA buffer (Melford, Ipswich, UK). The protein concentration was determined with Bradford assay. Total protein homogenates 20 μ g/30 μ l were denatured, separated on sodium dodecyl sulfate polyacrylamide electrophoresis gels, and transferred to a polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). The membrane was blocked with 2.5% BSA in Tris-buffered Saline Tween 20 for 1 h, before being incubated overnight at 4°C with NPPA (1:1,000; Cell Signaling Technology, Cambridge, UK), CTGF (1:100; Santa Cruz Biotechnology, Heidelberg, Germany), CRP (1:1,000; Cell Signaling Technology), IL1 β (1:1,000; Cell Signaling Technology), and TNF α (1:1,000; Cell Signaling Technology). After the blots were washed to remove excessive primary antibody binding, the blots were incubated

Table 5. Gene table: diabetes

Gene Symbol	Official Full Name
Metabolic enzymes	
Ace	Angiotensin I converting enzyme (peptidyl-di-peptidase A) 1
Acly	ATP citrate lyase
Dpp4	Dipeptidylpeptidase 4
Enpp1	Ectonucleotide pyrophosphatase/phosphodiesterase 1
Fbp1	Fructose-1,6-bisphosphatase 1
G6pc	Glucose-6-phosphatase, catalytic subunit
Gck	Glucokinase
Gpd1	Glycerol-3-phosphate dehydrogenase 1 (soluble)
Gsk3b	Glycogen synthase kinase 3 beta
Hmox1	Heme oxygenase (decycling) 1
Ide	Insulin degrading enzyme
Nos3	Nitric oxide synthase 3, endothelial cell
Pygl	Phosphorylase, glycogen, liver
Parp1 (Adprt)	Poly (ADP-ribose) polymerase 1
	dipeptidylpeptidase 4
Sod2	Superoxide dismutase 2, mitochondrial
Receptor, transport, channels	
Gcg	Glucagon
Secreted factors	
Ag1	Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)
Ccl5 (Rantes)	Chemokine (C-C motif) ligand 5
Ifng	Interferon gamma
Il6	Interleukin 6
Il10	Interleukin 10
Il12b	Interleukin 12b
Ins1	Insulin 1
Retn	Resistin
Tgfb1	Transforming growth factor, beta 1
Tnf	Tumor necrosis factor (TNF superfamily, member 2)
Vegfa	Vascular endothelial growth factor A
Signal transduction	
Akt2	V-akt murine thymoma viral oncogene homolog 2
Dusp4	Dual specificity phosphatase 4
Igfbp5	Insulin-like growth factor binding protein 5
Ikbkb (IKKbeta)	Inhibitor of kappa light polypeptide gene enhancer in B-cells, kinase beta
Inpp1 (SHIP2)	Inositol polyphosphate phosphatase-like 1
Irs2	Insulin receptor substrate 2
Mapk14 (p38 MAPK)	Mitogen activated protein kinase 14
Mapk8 (JNK1)	Mitogen-activated protein kinase 8
Pik3cd	Phosphoinositide-3-kinase, catalytic, delta polypeptide
Pik3r1	Phosphoinositide-3-kinase, regulatory subunit 1 (alpha)
Ptpn1 (PTP-1B)	Protein tyrosine phosphatase, nonreceptor type 1
Trib3 (Skip3)	Tribbles homolog 3 (<i>Drosophila</i>)

for 1 h with a horseradish peroxidase conjugated secondary antibody at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), a housekeeping protein, was used for loading control and protein normalization. The immunoreactive protein bands were developed using the Enhanced Chemiluminescence system (PerkinElmer, Rodgau-Juegesheim, Germany). The intensity of the immunoblot bands was detected with Chemismart 5100.

Statistical analysis. All data are expressed as mean $\pm$ SE. Student *t*-test was used to analyze the differences between the groups. *P* < 0.05 was considered statistically significant.

RESULTS

Oral glucose test tolerance. The diabetic GK rats showed an overt glucose intolerance during the oral glucose tolerance test

compared with the nondiabetic Wistar rats (Fig. 1). Fifteen minutes after glucose administration, the glycemia was 13.2 ± 1.4 mmol/l, and high glycemia was displayed after 30 min (19.5 ± 0.9 mmol/l).

Biochemical parameters. All basic biochemical parameters are given in Table 6. We found no difference in plasma triglyceride levels between the experimental groups, and GK rats showed a nonsignificant increase in serum insulin levels. However, high levels of urinary glucose, plasma glucose, high-sensitive cardiac troponin-T, and cholesterol have been observed in diabetic rats compared with the controls.

Cardiac hypertrophy, structural remodeling, and myocardial inflammation. Compared with the corresponding age-matched control rats, the body weight was significantly lower (GK: 326 ± 7 g vs. W: 452 ± 7 g, *P* < 0.05), while there was no significant difference in the absolute heart weight values between the groups (W: 1.32 ± 0.05 g vs. GK: 1.22 ± 0.03 g, *P* > 0.05). However, the heart-to-body weight ratio was higher (GK: 3.75 ± 0.10 g/kg vs. W: 2.92 ± 0.09 g/kg, *P* < 0.05) in the diabetic animals. Echocardiography was performed and all anatomical dimensions were indexed to body weight. Compared with nondiabetic rats, GK rats display a significant increase in LV mass index and, during diastole, increased LV posterior and anterior wall indexes and LV internal dimension. All together, these changes indicate LV hypertrophy (Table 7). Furthermore, the histological examination revealed that the cardiomyocyte transverse cross-sectional area in the GK rats was significantly increased in the hematoxylin and eosin-stained sections compared with the nondiabetic animals (Fig. 2A). A remarkable increase in the mRNA expression of the natriuretic peptide precursor A (Nppa; fold change +6.3), a candidate gene in pathological cardiac hypertrophy, was found in the GK rats compared with nondiabetic animals, whereas acta-1, c-fos, myl-2, Prkca, and Tgf- β 1 mRNA levels were unchanged (Fig. 3 and Tables 1 and 8). NPPA protein expression was unaltered (Fig. 4A). The plasma pro-BNP concentration was also significantly higher in the GK rats compared with the Wistar animals without changes in plasma renin activity, serum aldosterone, or urinary norepinephrine concentrations

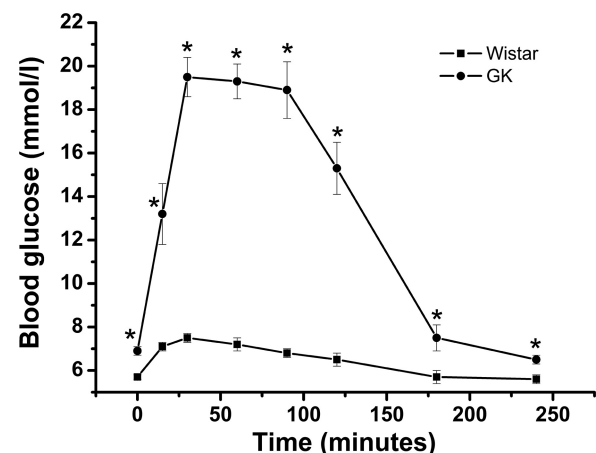


Fig. 1. Oral glucose tolerance test. Animals fasted for 12 h and then a 40% glucose solution (2 g/kg body weight) was administered intragastrically. Blood glucose levels were measured by a digital blood glucose meter and test strips (Accu-Chek Sensor; Roche) at 0, 15, 30, 60, 90, 120, 180, and 240 min after administration. GK, Goto-Kakizaki; W, Wistar. Data are presented as means $\pm$ SE. **P* < 0.05 vs. W; *n* = 9–12.

Table 6. Biochemical parameters in nondiabetic control and diabetic Goto-Kakizaki rats

	Wistar	GK
P-pro-BNP, ng/l	328 ± 40	3,416 ± 585*
P-glucose, mM	8.3 ± 0.6	12.4 ± 1.8*
P-cardiac troponin-T, pg/ml	755 ± 135	3044 ± 750*
P-cholesterol, mg/dl	66 ± 9	96 ± 7*
P-triglycerides, mg/dl	64 ± 10	60 ± 11
S-insulin, µg/l	0.76 ± 0.09	1.48 ± 0.46
S-aldosterone, ng/dl	142 ± 27	131 ± 33
P-renin activity, ng AI·ml ⁻¹ ·h ⁻¹	2.8 ± 0.7	4.8 ± 1.9
P-aldosterone/P-renin activity, ng/dl per ng AI·ml ⁻¹ ·h ⁻¹	68 ± 13	38 ± 10
U-glucose, g/l	0.12 ± 0.01	0.86 ± 0.31*
U-norepinephrine, nmol/l	2,348 ± 373	2,743 ± 883

Data are expressed as mean ± SE GK, Goto-Kakizaki; pro-BNP, NH₂-terminal pro-B-type natriuretic peptide. **P* < 0.05 vs. W.

(Table 6). Additionally, the Masson's trichrome stained sections showed myocardial fibrosis in the GK rats compared with the nondiabetic animals (Fig. 2B), along with the activation of the connective tissue growth factor (ctgf; fold change +3.7), a key player in fibrogenic pathways (Fig. 3 and Tables 1 and 8). Nevertheless, myocardial CTGF protein expression was unaltered (Fig. 4B). However, Agt, Ccl11, Grem-1, Mmp-1α, Mmp-2, and Tgf-β1 gene expressions were identical for both groups. As the histological examination with hematoxylin and eosin staining of the myocardial tissue exemplified, the short-term and mild diabetes did not lead to an inflammatory reaction. However, genes related to the inflammation, such as the c-reactive protein (Crp; fold change -2.1) and genes linked to diabetes, including interleukin (IL)-1β (fold changes -3.2) and tumor necrosis factor (Tnf)-α (fold change -3.1), were significantly downregulated. The cardiac ccl5, ccl11, ifng, Vegfa, and Il6 mRNA expressions, on the other hand, were unchanged between the groups (Fig. 3 and Tables 1 and 8). Myocardial protein expression of CRP and IL-1β were unaltered in GK animals compared with the control rats (Fig. 4, C and D). However, TNF-α1 protein expression was significantly decreased in diabetic hearts compared with the controls (Fig. 4E). Figure 3A shows a volcano plot presenting upregulated and downregulated genes, which also show statistical significance in one plot.

Cardiac pump function. The heart rate did not differ between the experimental groups. The systolic and diastolic blood pressures and the mean arterial pressure in GK rats were significantly lower compared with the control animals (Table 7). Additionally, Wistar rats showed a tendency toward increased systemic vascular resistance compared with the GK rats but without reaching the level of statistical significance (*P* = 0.064). (Table 7).

The LV systolic function, as characterized by both classical (ejection fraction, cardiac output, cardiac index, stroke volume, stroke volume index, and dP/dt_{max}) and sensitive PV loop derived load-independent functional parameters (E_{max}, PRSW, and dP/dt_{max}/EDV), were not significantly impaired in the GK animals compared with controls (Table 7 and Fig. 5). There was a strong tendency toward a decreased dP/dt_{min}, prolonged Tau-g, and increased LVEDP in diabetic GK rats compared with the nondiabetic animals (Table 7). These differences however, did not reach a level of statistical significance. In

contrast, the LV stiffness index (slope of the EDPVR) was significantly increased in the diabetic rats compared with the control animals (Fig. 5).

ECG patterns. On ECG recordings, ECG patterns such as ST segment (0.05 ± 0.01 vs. 0.05 ± 0.01 mV, *P* > 0.05), PR interval (41 ± 2 vs. 42 ± 2 ms, *P* > 0.05), QRS complex duration (22 ± 1 vs. 22 ± 1 ms, *P* > 0.05), QT interval (66 ± 3 vs. 65 ± 4 ms, *P* > 0.05), and corrected QT interval for heart rate (68 ± 5 vs. 65 ± 3 ms, *P* > 0.05) in the diabetic GK rats were similar to the ECG patterns in the nondiabetic Wistar animals.

DNA-strand breaks and apoptosis. Compared with the control group, diabetes had no effect on cardiomyocyte DNA-strand breakage, as reflected by the quantitative assessment of TUNEL-positive cell nuclei (W: 18 ± 5% vs. GK: 13 ± 4%, *P* < 0.05). Additionally, no significant changes in expression were found in the cardiac muscle for the tested apoptotic/antiapoptotic genes [Ab11, Bax, Bcl2, Bcl2l1, caspase-3, caspase-6, Casp8ap2, Cflar (Casper), cidea, cideab, Dapk1, Sfrp4, Tp53, and Tp73; Tables 2 and 8].

Myocardial capillary density. Capillary density, expressed as number of CD31 positive cells per field in ventricular muscle, was increased in diabetic animals compared with control (Fig. 6).

Cardiac markers of oxidative stress, pro-survival phosphatidylinositol-3-kinase-akt signaling pathway. From the 125 genes surveyed, no significant changes in expression were found in the cardiac muscle of diabetic animals for genes

Table 7. Cardiac catheterization and echocardiography parameters in nondiabetic control and diabetic Goto-Kakizaki rats

	Wistar	GK
Cardiac catheterization parameters		
Heart rate, beats/min	461 ± 12	476 ± 18
Systolic blood pressure, mmHg	150 ± 5	127 ± 5*
Diastolic blood pressure, mmHg	120 ± 4	93 ± 5*
Mean arterial pressure, mmHg	130 ± 5	104 ± 5*
Ejection fraction, %	61 ± 3	66 ± 11
Cardiac output, ml/min	27 ± 4	32 ± 6
Cardiac index, ml/min/g	60 ± 10	97 ± 21
Stroke volume, µl	60 ± 8	65 ± 11
Stroke volume index, µl/g	0.13 ± 0.02	0.20 ± 0.04
Arterial elastance, Ea	1.58 ± 0.20	1.26 ± 0.12
Systemic vascular resistance, mmHg·ml ⁻¹ ·min ⁻¹	3.43 ± 0.33	2.49 ± 0.23
dP/dt _{max} , mmHg/s	11,765 ± 568	10,429 ± 568
dP/dt _{min} , mmHg/s	-16,149 ± 1,287	-12,940 ± 1,112
Tau-g, ms	7.95 ± 0.54	8.15 ± 0.19
PRSW, mmHg	84 ± 9	73 ± 6
LV end-diastolic pressure, mmHg	4.8 ± 0.3	5.7 ± 0.4
dP/dt _{max} /EDV, mmHg·s ⁻¹ ·µl ⁻¹	82 ± 12	104 ± 10
Echocardiography parameters		
LV mass index, g/kg	2.52 ± 0.17	3.41 ± 0.17*
LVPWid, mm/kg	6.04 ± 0.27	8.85 ± 0.31*
LVAWid, mm/kg	4.94 ± 0.15	7.31 ± 0.22*
LVIDd, mm/kg	12.55 ± 0.25	15.29 ± 0.51*

Data are expressed as mean ± SE. GK, Goto-Kakizaki; dP/dt_{max}, maximal slope of the systolic pressure increment; dP/dt_{min}, maximal slope of the diastolic pressure decrement; PRSW, preload recruitable stroke work; LV, left-ventricular; dP/dt_{max}/EDV, dP/dt_{max}/end-diastolic volume; LVPWid, left ventricular posterior wall index diastole; LVAWid, left ventricular anterior wall index during diastole; LVIDd, left ventricular internal dimension index during diastole. **P* < 0.05 vs. W.

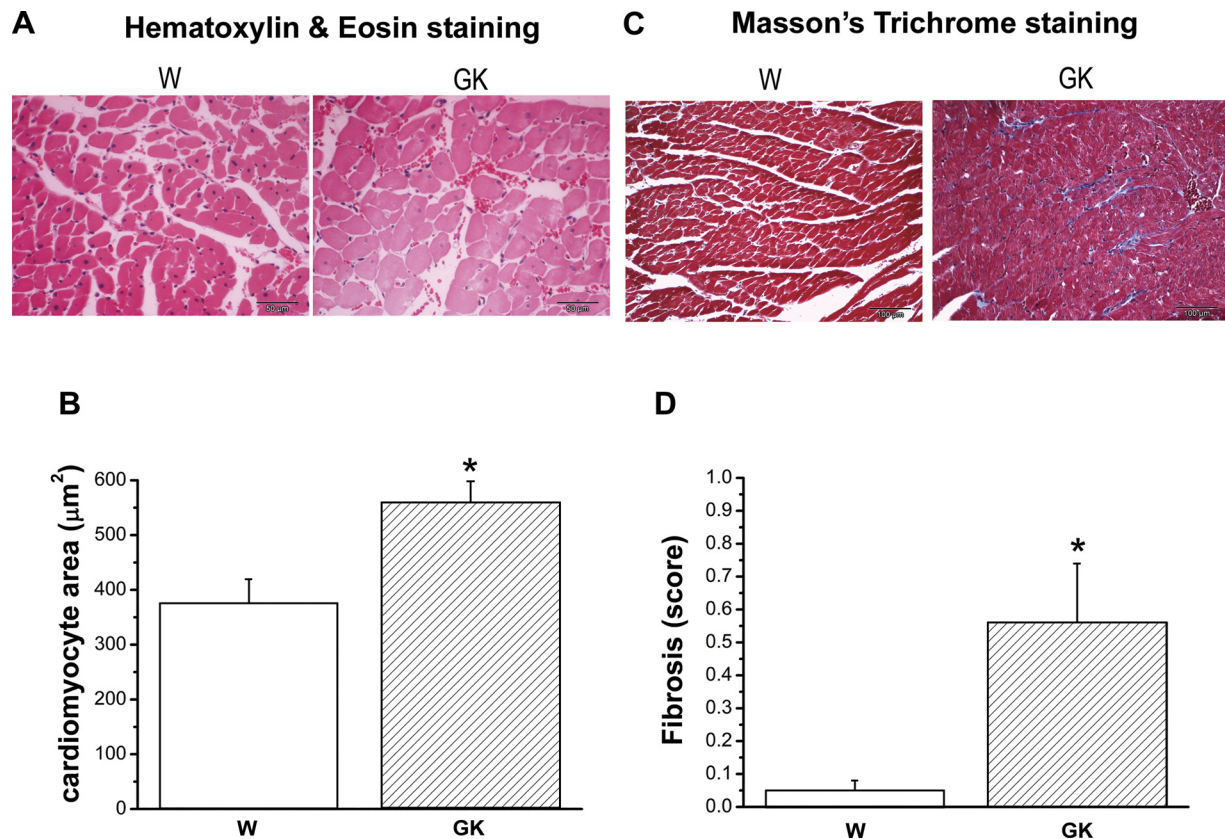


Fig. 2. Histological analysis. *A*: hematoxylin and eosin staining micrographs of transverse sections of myocardium (magnification: $\times 400$; scale bar = $50 \mu\text{m}$). *B*: quantitative analysis of cardiomyocyte cross-sectional area using measurements of ~ 20 cardiomyocytes in each group; $n = 8-11$. *C*: Masson's trichrome staining micrographs of transverse sections of myocardium (magnification: $\times 100$; scale bar = $100 \mu\text{m}$). *D*: fibrosis score (grade 0 indicates normal tissue showing no fibrotic region; grade 1 indicates mild fibrosis; grade 2 indicates moderate fibrosis, and grade 3 indicates severe fibrosis); $n = 8-10$. GK, Goto-Kakizaki; W, Wistar. Data are presented as means $\pm$ SE. $*P < 0.05$ vs. W.

related to oxidative stress and antioxidant defense (Acadsb, Alb, catalase, Gpx-1, Gpx-2, Gpx-3, Gpx-4, Gpx-5, Gpx-6, Gpx-7, Gstk2, Gstp1, Sels, Sod1, Sod2, Sod3, Srxn1, Txnrd1, and Txnrd2; Tables 3 and 8). These findings were supported by

the immunohistochemical staining of paraffin-embedded heart tissue for nitrotyrosine as a nitro-oxidative stress marker showing no significant alterations between GK and Wistar rats (Fig. 7). Additionally, the expression of genes involved in the

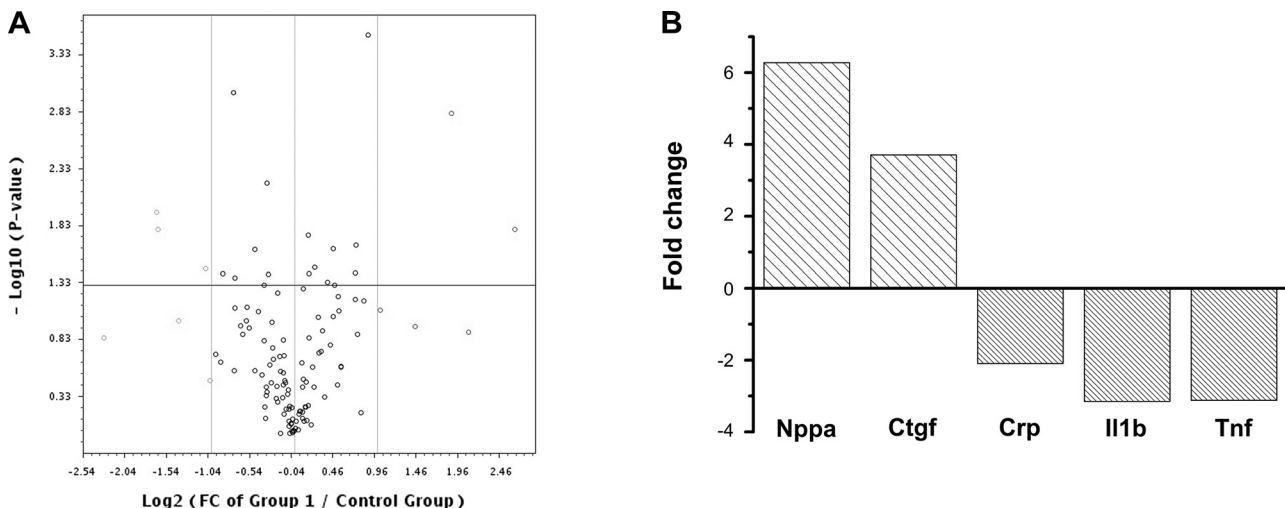


Fig. 3. Cardiac gene expression profile. *A*: volcano plot. The x-axis plots the $\log_2$ of the fold differences, while the y-axis plots their P values based on student's t -test. The red symbols readily identify upregulated genes, and the green symbols downregulated genes. *B*: fold changes in mRNA expression of the mediator of hypertrophy [natriuretic peptide precursor A (Nppa)], fibrosis [connective tissue growth factor (ctgf)], and inflammatory reaction [c-reactive protein (crp), interleukin-1 β (Il1b), and Tnf; tumor necrosis factor (Tnf)] in the diabetic Goto-Kakizaki heart tissue vs. age-matched nondiabetic Wistar heart. RNA from $n = 4/\text{group}$ rats were isolated and expression analyzed using PCR array from SA Biosciences (Qiagen).

Table 8. Fold regulation and associated *P* value for all tested genes

Genes	Fold Regulation	<i>P</i> Value	Comments	Genes	Fold Regulation	<i>P</i> Value	Comments
Ace	-1.26	0.430	—	Map2k1	1.03	0.921	—
Acly	1.66	0.039*	—	Mapk1	-1.08	0.344	—
Parp1	-1.05	0.416	—	Mapk3	1.05	0.639	—
Dpp4	-1.81	0.039*	—	Rps6ka1	-1.05	0.861	—
Enpp1	1.12	0.567	—	Shc1	1.05	0.635	—
Fbp1	1.10	0.585	C	Sos1	-1.13	0.285	—
G6pc	1.07	0.645	B	Ywhah	1.04	0.671	—
Gck	-1.04	0.573	B	Eif4b	1.06	0.238	—
Gpd1	-1.07	0.614	—	Eif4e	1.13	0.145	—
Gsk3b	-1.10	0.486	—	Eif4ebp1	1.38	0.093	—
Hmox1	1.47	0.258	—	Eif4 g1	-1.06	0.453	—
Ide	-1.26	0.006*	—	Fkbp1a	-1.15	0.058	—
Nos3	1.40	0.0499*	—	Mtor	1.13	0.039*	—
Pygl	-1.64	0.078	—	Pdk1	-1.10	0.152	—
Agt	1.15	0.839	B	Rheb	1.26	0.126	—
Ccl5	-1.45	0.118	—	Rps6kb1	-1.02	0.813	—
Gcg	1.10	0.585	C	Tsc1	-1.00	0.960	—
Ifng	-1.40	0.281	B	Tsc2	-1.10	0.294	—
Il10	2.73†	0.115	B	Akt1	1.16	0.261	—
Il12b	1.10	0.585	C	Akt2	1.08	0.333	—
Ins1	1.10	0.585	C	Akt3	-1.29	0.153	—
Retn	-4.90†	0.144	B	Btk	-1.93	0.201	A
Tgfb1	1.10	0.355	—	Grb10	-1.27	0.389	—
Tnf	-3.12†	0.016*	—	Grb2	1.08	0.054	—
Dusp4	1.18	0.392	—	Hspb1	1.69	0.134	—
Igfbp5	1.67	0.022*	—	Ilk	-1.20	0.176	—
Ikbkb	-1.05	0.611	—	Mtcp1	-1.54	0.135	—
Inpp1	-1.24	0.040*	—	Pdk2	-1.044	0.784	—
Irs1	-1.49	0.103	—	Pdpk1	1.07	0.391	—
Irs2	1.85	0.0003*	—	Pik3ca	-1.09	0.207	—
Mapk8	1.23	0.196	—	Pik3cg	-1.85	0.236	—
Mapk14	1.11	0.774	—	Pik3r1	-1.27	0.462	—
Pik3cd	-1.29	0.0499*	—	Pik3r2	-1.35	0.085	—
Ptpn1	1.08	0.776	—	Prkca	-1.23	0.251	—
Trib3	1.43	0.374	A	Prkcb	-2.63†	0.103	A
Ab1	1.48	0.260	—	Prkcz	-1.27	0.738	B
Cidea	1.45	0.084	—	Pten	-1.08	0.359	—
Cideb	2.04†	0.082	B	Tcl1a	1.10	0.585	C
Tp53	1.35	0.167	—	Il1b	-3.15†	0.011*	A
Tp73	1.10	0.585	C	Il6	4.25†	0.129	A
Casp8ap2	1.22	0.096	—	Vegfa	-1.28	0.586	—
Cflar	1.18	0.035*	—	Crp	-2.10†	0.036*	—
Dapk1	-1.09	0.678	—	Ctgf	3.71†	0.002*	—
Sfrp4	-1.12	0.998	B	Ccl11	-1.65	0.280	—
Bcl1	-1.21	0.361	—	Mmpl1a	1.25	0.190	B
Bcl2l1	1.66	0.066	—	Mmp2	1.31	0.047*	—
Bax	-1.03	0.827	—	Grem1	-1.02	0.593	B
Casp3	-1.16	0.384	—	Prkcg	-1.01	0.980	B
Casp6	-1.66	0.001*	—	Nppa	6.28†	0.016*	—
Gpx1	-1.19	0.224	—	Acta1	-1.16	0.492	—
Gpx2	1.07	0.739	B	Myl2	1.38	0.024*	—
Gpx3	-1.15	0.529	—	Fos	1.74	0.653	—
Gpx4	-1.02	0.743	—	RGDC	1.10	0.585	C
Gpx5	1.10	0.585	C	RTC	-1.47	0.145	—
Gpx6	1.10	0.585	C	PPC	1.28	0.415	—
Gpx7	-1.70	0.114	—	Hras	1.12	0.018*	—
Gstk1	-1.21	0.106	—	Igf1r	1.44	0.062	—
Gstp1	-1.04	0.991	—	Xdh	-1.49	0.078	—
Alb	-2.02†	0.344	B	Bad	-1.13	0.208	—
Gsr	-1.01	0.940	—	Acadsb	-1.64	0.043*	—
Sels	-1.02	0.960	—	Cat	-1.39	0.024*	—
Srxn1	1.79	0.069	—	Sod1	1.02	0.778	—
Txnrd1	1.29	0.476	—	Sod2	-1.10	0.377	—
Txnrd2	1.01	0.903	—	Sod3	-1.31	0.304	—

See Tables 1–5 for genes abbreviation. Comments “A,” “B,” and “C” describe experimental limitations. A: this gene’s average threshold cycle is relatively high (> 30) in either the control or the test sample. B: this gene’s average threshold cycle is relatively high (> 30), meaning that its relative expression level is low, in both control and test samples, and the *P* value for the fold change is either unavailable or relatively high (*P* > 0.05). C: this gene’s average threshold cycle is either not determined or greater than the defined cut-off value (default 35), in both samples meaning that its expression was undetected, making this fold change result erroneous and uninterpretable. **P* value < 0.05. †Fold-regulation values > 2 and less than -2.

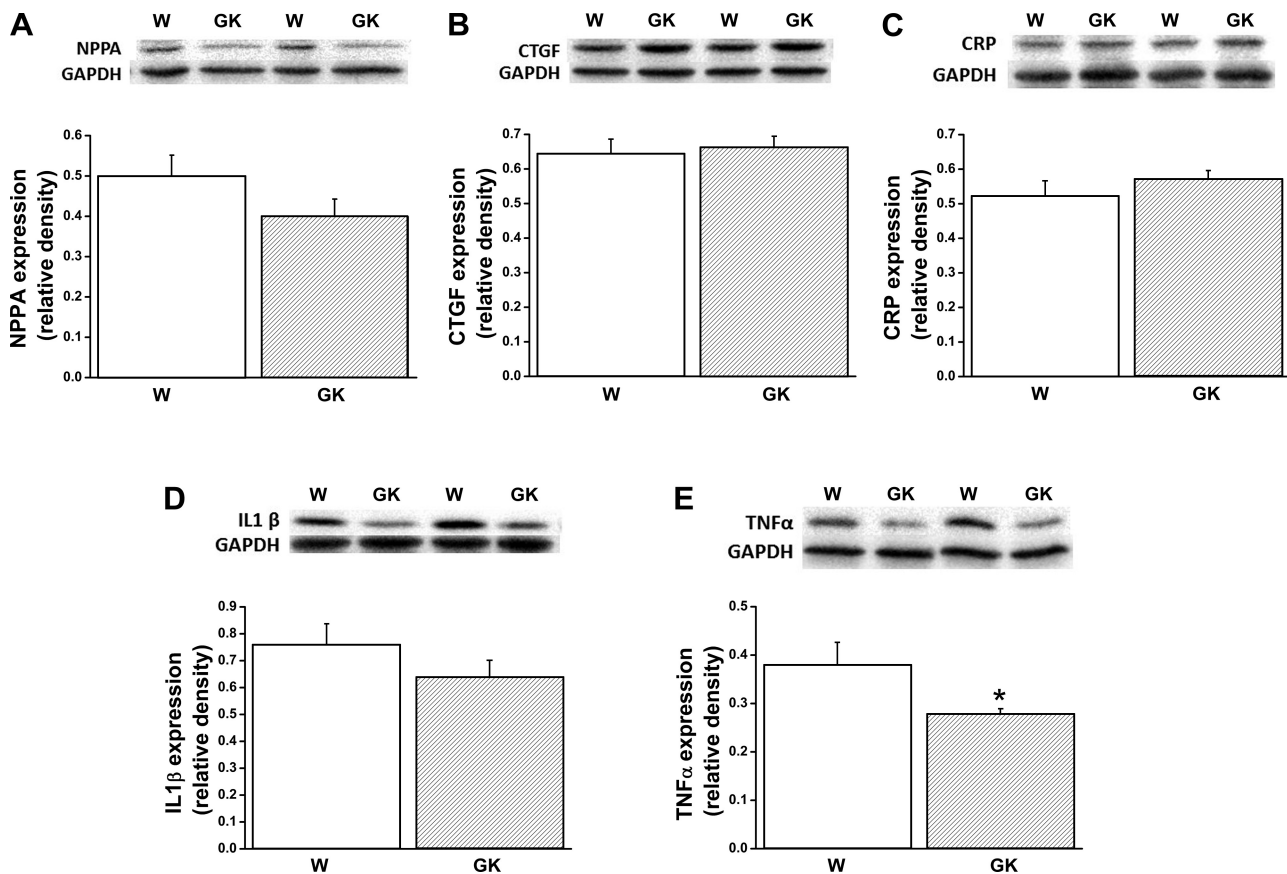


Fig. 4. Myocardial protein expression of natriuretic peptide precursor A (NPPA), connective tissue growth factor (CTGF), c-reactive protein (CRP), interleukin (IL)-1 β , and tumor necrosis factor (TNF)- α . Immunoblot analysis of NPPA (A), CTGF (B), CRP (C), IL-1 β (D), and TNF- α (E) protein band densities in the myocardium. Glyceraldehyde-3-phosphate dehydrogenase (GADPH), a housekeeping protein, was used as reference. GK, Goto-Kakizaki; W, Wistar. * $P < 0.05$ vs. W; $n = 6-7$.

prosurvival PI3K/Akt signal transduction pathway (Akt1, Akt2, Akt3, Bad, Btk, Eif4e, Eif4ebp1, Eif4g1, Grb10, Grb2, Hras, Hspb1, Igflr, Irs1, Ilk, Map2k1, Mapk1, Mapk3, Mtcp1, Mtor, Pdk1, Pdk2, Pdpk1, Pik3ca, Pik3cg, Pik3r1, Pik3r2, Prkca, Prkcb, Prkcg, Pten, Rps6kal, Rps6kb1, Shc1, Sos1, Tcl1a, Ywah; Tables 4 and 8) and genes related to diabetes (Ace, Acly, Agt, Akt2, Ccl5, Dpp4, Disp4, Enpp1, Fbp1, G6pc, Gcg, Gck, Gpd1, Gsk3b, Hmox1, Ide, Ifng, Igfbp5, Ikbkb, Il10, Il10b, Il6, Inpp11, Ins1, Irs2, Map14, Mapk8, Nos3, Parp1, Pik3cd, Pik3r1, Ptpn1, Pygl, Retn, Sod2, Tgfb1, Tnf, Trib3, and Vegfa; Tables 5 and 8) wsd unaltered between the two experimental groups.

DISCUSSION

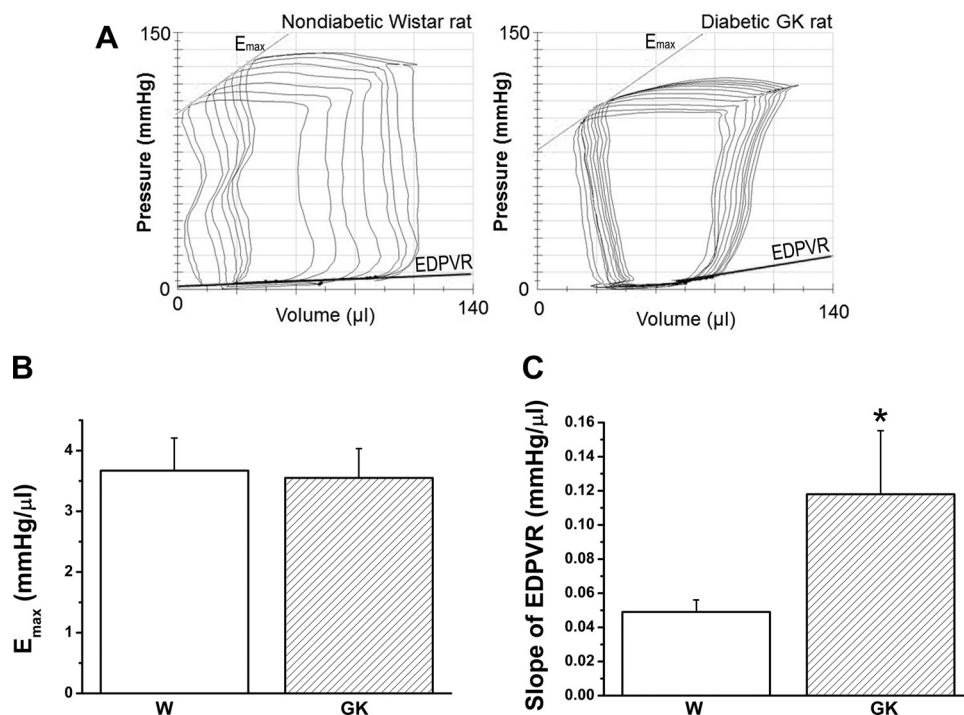
The goal of this study was to identify early molecular signatures associated with short-term and mild T2DM-induced diastolic dysfunction and structural remodeling in GK rats, thus expanding previous studies and hoping to prevent the disease from progressing to a more serious stage. Our results showed that in the absence of abnormal electrical conduction, myocardial inflammation, or systolic dysfunction, myocardial TNF- α at both the mRNA and the protein levels was significantly altered in the diabetic GK rat at 16 wk of age. Structurally we have demonstrated LV hypertrophy, on the cellular level cardiomyocyte hypertrophy and at a molecular level, increased transcriptional expression of the well-known patho-

logical hypertrophy marker *Nppa* and the important profibrotic molecule *ctgf*. Additionally, increased myocardial capillary density has been observed in diabetic rats. However, myocardial mRNA levels were not changed significantly by any of the tested genes related to apoptosis, oxidative stress, or PI3K/Akt signaling.

Diabetic cardiomyopathy is a clinical condition defined by the presence of myocardial functional and structural abnormalities in patients with diabetes, while coronary atherosclerosis, hypertension, and significant valvular disease remain absent (1). Asymptomatic LV diastolic dysfunction often progresses to cardiac hypertrophy and fibrosis, leading to both myocardial diastolic and systolic dysfunction. Multiple pathophysiological mechanisms have been proposed to explain this matter, but hyperglycemia is considered to be one of the main pathogenic mechanisms in diabetic cardiomyopathy. Metabolic abnormalities present in diabetes (hyperinsulinemia, hyperlipidemia, and hyperglycemia) might be the initial cause of oxidative stress, endothelial dysfunction, inflammation, and apoptosis, leading to the impairment of cardiac function (systolic and diastolic dysfunction) and structure (LV hypertrophy and cardiac fibrosis). During the early stage of diabetes, the existence of early cardiac alteration has been suggested, but the data are limited because of the coexistence of other perturbations.

A number of studies in both humans and animals have shown structural changes in diabetic hearts in the absence of

Fig. 5. Left-ventricular cardiac function. *A*: representative original pressure-volume loops registered during transient occlusion of the inferior vena cava. *B*: the slope of end-systolic pressure-volume relationship ($E_{\max}$). *C*: end-diastolic pressure-volume relationship (EDPVR). GK, Goto-Kakizaki; W, Wistar. $*P < 0.05$ vs. W; $n = 6-8$.



hypertension, coronary artery disease, and any other known cardiac disease. Hypertrophy is a form of myocyte growth in the adult heart. Another proposed mechanism to explain cardiac alterations in diabetic cardiomyopathy is related to the development of myocardial fibrosis. Cardiac fibrosis is characterized by an increase in collagens and other extracellular collagen matrix components, leading to the increased stiffening

and reduced relaxation of the ventricles. Thus cardiomyocyte hypertrophy, as well as myocardial fibrosis, is found in diabetic cardiomyopathy (5, 19). TGF- β plays a key role in hypertrophic and fibrotic remodeling of the heart and has been implicated in cardiomyocyte growth, fibroblast activation, and collagen and extracellular matrix production. It has been shown that ventricular remodeling occurs during mild hyperglycemia before the onset of type 2 diabetes (7). The presence of the remodeling of the ventricular myocardium has been confirmed morphologically in 8-wk old GK rats by marked myocyte hypertrophy, increased extracellular matrix deposition, and fibrosis, culminating in an increased heart size (7). In agreement with these previously published reports, the present study's echocardiographic and histological evaluations suggest structural remodeling of the myocardium. These authors clearly demonstrated that the level of hyperglycemia in prediabetes increased levels of BNP and cellular α -skeletal actin and was associated with elevated TGF- β 1 expression (7). Additionally, we have shown structural vascular remodeling in the form of angiogenesis of capillaries within the diabetic myocardial tissue. Hypertrophic responses increase oxygen demand and promote myocardial angiogenesis to diminish the hypoxic situation and to maintain cardiac contractile function. In the present study, the increased troponin T levels confirms the hypoxic/ischemic condition in the diabetic heart. This may in part explain the preserved LV systolic function and increased myocardial capillary density (as an adaptive response reaction) in the diabetic animal at the early stage. The expression of the angiogenic growth factors in myocytes in response to adaptive hypertrophic stimuli may increase, whereas pathological hypertrophy is correlated with a reduction in capillary density (37). Accordingly, in GK type 2 diabetic rats, myocardial capillary density may initially increase in the early phase but has been shown to be decreased during the transition from cardiac hypertrophy to heart failure (10). However, we do not

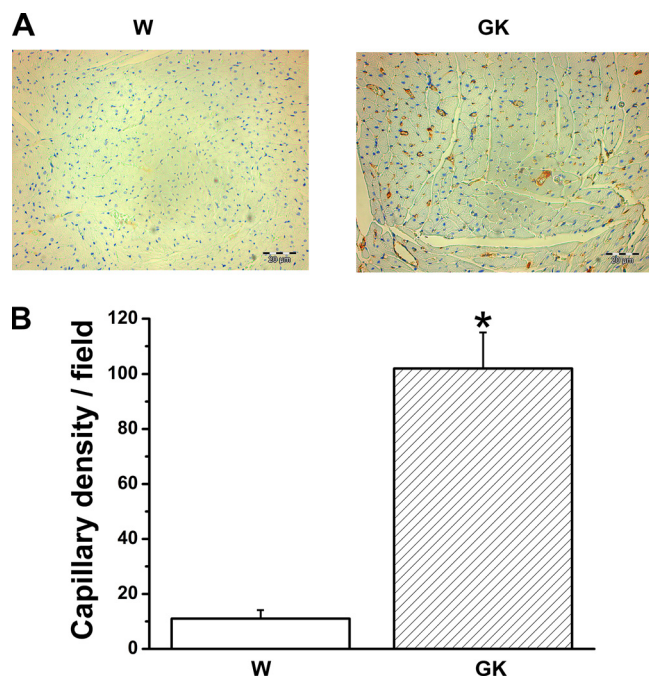


Fig. 6. Myocardial capillary density. *A*: representative photographs showing immunohistochemical staining ($\times 200$) of myocardial tissue with anti-CD31 monoclonal antibody. *B*: quantitative analysis of capillary density. GK, Goto-Kakizaki; W, Wistar. $*P < 0.05$ vs. W; $n = 8-10$.

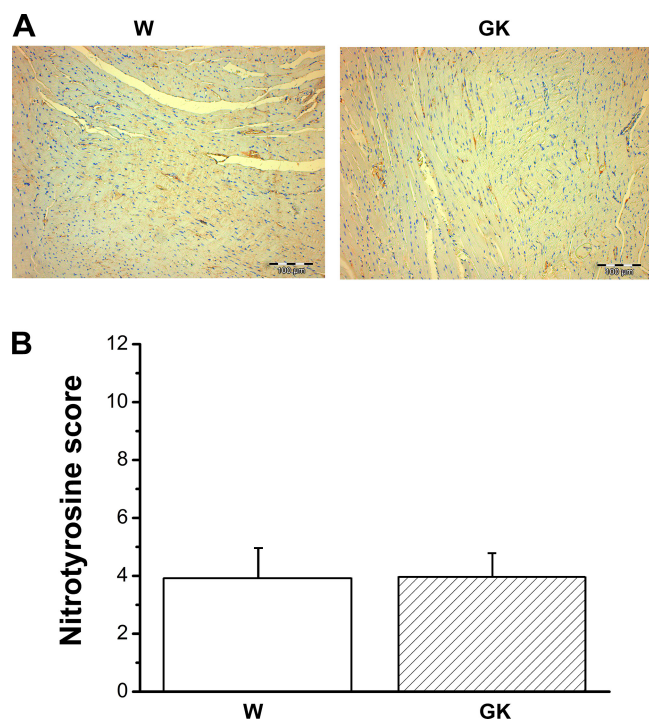


Fig. 7. Myocardial nitro-oxidative stress. A: representative photographs showing immunohistochemical staining ($\times 100$) of myocardial tissue with anti-nitrotyrosine polyclonal antibody. B: semiquantitative histomorphological scoring of nitrotyrosine immunostaining; $n = 8-10$. GK, Goto-Kakizaki; W, Wistar.

have evidence for this point. In the present study, hyperglycemia had no effects on acta-1, c-fos, Myl-2, Prka, Agt, ccl11, Grem-1, Mmp-1a, Mmp-2, Agt, Grem-1, and tgf- β 1 gene expression. However, it may contribute to cardiac fibrosis, by stimulating profibrotic gene expression, such as connective tissue growth factor (ctgf) with myocardial levels that have been shown to be increased after diabetes (7), and to cardiomyocyte hypertrophy, by atrial natriuretic peptide (ANP) expression. The protein encoded by nppa belongs to the natriuretic peptide family. Other peptides in this family include C-natriuretic peptide, ANP, and BNP. In the present study, the hypertrophy of cardiomyocytes in the GK rat heart was also accompanied by an increase in cardiac load confirmed by the increase in the plasma pro-BNP levels, as an indicator of pressure/volume overload. It has also been shown that ANP prohormone mRNA is increased in the left ventricle of the heart of type 2 diabetic GK rats (13) and ANP is increased in the circulation two- to sixfold in GK rats compared with nondiabetic Wistar rats (36). Thus our data are in agreement with these reports. Additionally, there were no differences between the two groups in circulating plasma renin activity, aldosterone concentrations or urinary norepinephrine levels, which is in accordance with previous works on 12-wk-old (34) and 20-wk-old (35) GK rats compared with age-matched nondiabetic Wistar rats.

Previous studies are lacking important functional data and the investigation of the electrical remodeling in ventricular dysfunction in prediabetes. It is widely accepted that the diabetic heart is associated with LV diastolic dysfunction, often prior to LV systolic dysfunction, in diabetic animals (22,

27, 28) and diabetic patients (2) lacking clinical manifestations of congestive heart failure. Changes in the diastolic dysfunction can be due to alterations in relaxation and/or myocardial stiffness (25). We studied systolic and diastolic function in prediabetic and age-matched control rats using the Millar pressure-volume conductance catheter system at different preloads, extending previous studies and concluding that prediabetic cardiac morphological changes, termed remodeling (a maladaptive process), and altered gene expression contribute to further LV dysfunction and the progression of heart failure. The pressure-volume analysis is a useful approach for studying the intact chamber function, in vivo independently from loading conditions. This study demonstrated preserved LV systolic function, as characterized by both classical and sensitive, pressure-volume loop-derived contractility parameters, in type 2 diabetic GK rats at 16 wk of age. However, in a previous study Grönholm et al. (14) showed echocardiographic signs of cardiac systolic dysfunction by the age of 20 wk. Based on the results of our pressure-volume analysis, the diabetic diastolic dysfunction is mainly characterized by a decreased LV compliance in type 2 diabetic GK rats, as evidenced by the increased slope of the EDPVR, a robust index of LV stiffness. Even though we did not detect significant differences regarding the indexes of active LV relaxation (Tau and dP/dt_{min}) and LVEDP, a load-dependent parameter of LV stiffness, there was a tendency toward an impaired myocardial relaxation. Our observations are in line with a ventricular diastolic dysfunction in GK rats characterized by echocardiography (14). According to our findings, myocardial stiffness and to a lesser extent impaired LV active relaxation seem to predominate this stage of diabetes. However, contrary to the literature showing a small, but significant, increase in mean arterial pressure and systolic blood pressure in GK rats compared with their Wistar control (6), our results demonstrated modest low blood pressures in diabetic animals.

Recently, 67 cardiac muscle genes involved in the process of excitation-contraction coupling have been investigated in the hearts from 8- to 10-wk-old GK and age-matched control rats (30). The results showed that of the tested genes the expression of nine genes encoding some membrane pumps, exchangers, and calcium, sodium, and potassium channel proteins was significantly altered even at the early stage of diabetes (30). Additionally, long-term ECG recording has already been described with the telemetric system (16). The authors have shown that there was no significant difference in the normalized heart rate and QT-interval between GK and Wistar control rats at 2, 7, or 15 mo (16). In line with these observations, our findings also reveal similar QT intervals between 4-mo old diabetic and age-matched nondiabetic rats. Furthermore, even though Howarth et al. (16) have shown that the QRS complex duration was significantly longer at 2 and 7 mo in GK rats compared with the control animal, our results demonstrated no significant difference at 4 mo of age.

Cardiac overexpression of TNF- α has been associated with cardiac hypertrophy and fibrosis, as well as with LV dysfunction (41). In our type 2 diabetes model we found that of 39 tested genes involved in diabetes, only Tnf- α has been down-regulated. Additionally, its protein expression levels in myocardial tissue were also reduced. Our observations are in line with the study of Devanathan et al. (8), who have shown that mRNA expression of Tnf- α was significantly down-regulated

in 24-wk-old GK rats. Resistin (for “resistance to insulin”), a newly discovered adipocyte-secreted factor, has been shown to be link among obesity, type 2 diabetes, and insulin resistance (32). It has been observed that the action of resistin is modulated, at least in part, by TNF- α (38). In the present study, the mRNA expression of resistin was not significantly downregulated (-4.9 fold). Decreased resistin gene expression was also demonstrated by Devanathan et al. (8). The long-term complications of diabetes are supposed to be due to acute cardiac responses at the early stage of the disease; therefore, further research is required to assess if TNF- α plays a role in the early stage of diabetic cardiomyopathy development.

The inflammatory process also plays a key role in the onset and progression of diabetic cardiomyopathy by upregulating proinflammatory cytokines and chemokines and by activating various leukocyte populations (15). However, its presence and influence differs in the early and the long-standing stage of the disease. CRP has been found in the circulation of and in the diabetic hearts of type-1 DM patients (23). In the present study, even though crp and Il-1 β mRNA levels were significantly downregulated, their protein expression, evaluated by Western blot, was not altered. Additionally, no inflammatory cell infiltration, evidenced by HE staining, was observed in the diabetic heart.

Cardiac hypertrophy and fibrosis are usually associated with apoptosis, a well-known form of programmed cell death (29). Increased cardiomyocyte apoptosis has been associated with the transition from the compensated to decompensated hypertrophic state in the diabetic heart (11). In both clinical (11) and experimental (4) settings, it has been shown that cardiomyocyte apoptosis plays an important role in the development of diabetic cardiomyopathy. In the present study, no significant changes in expression were found in the diabetic hearts amongst the tested apoptosis/antiapoptosis-related genes, which was also confirmed by our *in situ* DNA fragmentation (based on the TUNEL assay) staining, which results from the apoptotic signaling cascade. Hyperglycemia-induced oxidative stress has been recognized to play an important role in the development of diabetic cardiomyopathy (3). Oxidative stress occurs when reactive oxygen species, and/or reactive nitrogen species production, exceed the degradation capacities of antioxidant defenses. Even though oxidative stress plays a critical role in the pathogenesis of diabetic cardiomyopathy (21), in the present study, myocardial mRNA levels were not changed significantly by any of the tested genes related to oxidative stress. These observations were supported by immunohistochemical analysis demonstrating no significant alterations of nitrotyrosine expression in the heart tissue. As cardiac contractile dysfunction is likely to arise in some part as a consequence of oxidative stress, through posttranslational modifications of contractile proteins, preserved contractile function in GK type 2 diabetic rats at 16-wk of age may provide an explanation for the absence of nitro-oxidative stress.

PI3K, a key component of the insulin signaling pathway, and its downstream targets, including serine/threonine protein kinase Akt (also known as protein kinase B), mammalian target of rapamycin (mTOR), glycogen synthase kinase (GSK), et al., play important roles in cardiac adaptation, including protein synthesis, inhibition of apoptosis, and fatty acid and glucose metabolism, as well as gene expression and cell survival. The activation of the PI3K pathway increases serine phosphoryla-

tion of protein kinase B, resulting in glucose uptake by inducing the translocation of GLUT-4 protein to the cell membrane. Despite the beneficial trait of the PI3K/Akt pathway's acute activation, a chronic activation of the PI3K/Akt pathway can, under some circumstances, be detrimental, causing cardiomyopathy. In the present study, members of the AKT and PI3K families and their regulators, genes involved in BAD phosphorylation and anti-apoptotic pathway, IGF-1, mTOR pathways, regulators of eIF4e and p70 S6 kinase, PTEN-dependent cell cycle arrest, and apoptosis were screened. In our setting and at this stage of diabetes PI3K/Akt pathway seems not to be involved.

In conclusion, collectively, the data reported in this study have shown that short-term and mild T2DM, in the absence of cardiac systolic dysfunction, myocardial inflammation, or abnormal electrical conduction of the heart, induces the alteration of TNF- α at both mRNA and protein levels in myocardial tissue, leads to LV hypertrophy, and cardiac fibrosis and decreases LV compliance. Therefore, the preexisting alterations in cardiac gene/protein expression of TNF- α require further research to determine if they represent an important component of this model of prediabetes' genetic background.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS

S.K.-I., T.R., M.K., and G.S. conception and design of research; S.K.-I., A.L., S.L., A.V., T.R., M.B., M.R., X.S., and M.Z. performed experiments; S.K.-I., A.L., S.L., A.V., T.R., M.R., P.B., and M.Z. analyzed data; S.K.-I., A.L., S.L., A.V., T.R., M.R., and P.B. interpreted results of experiments; S.K.-I., A.L., S.L., and A.V. prepared figures; S.K.-I., S.L., T.R., M.R., P.B., M.K., and G.S. drafted manuscript; S.K.-I., T.R., M.Z., M.K., and G.S. approved final version of manuscript; M.B. edited and revised manuscript.

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