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Mild type 2 diabetes mellitus improves remote endothelial dysfunction after acute myocardial infarction

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ABSTRACT

Aims: Myocardial infarction (MI) is a common cause of mortality in patients with diabetes mellitus (DM) and vascular dysfunction is a major component of diabetic cardiomyopathy. We investigated the systemic influence of acute MI on the diabetes-induced pathogenic changes in the rat aorta.

Methods: Nondiabetic Wistar (W) and type-2 diabetic Goto-Kakizaki (GK) rats underwent 45 min of left anterior descending coronary artery occlusion followed by 24 h of reperfusion. Isometric force was measured using organ bath.

Results: Plasma glucose-levels were significantly higher in diabetic rats (GK + sham: 13 ± 2 mM; GK + MI: 19 ± 2 mM) compared to nondiabetic rats (W + sham: 8 ± 0 mM; W + MI: 8 ± 1 mM). Acetylcholine-induced relaxation was significantly weaker in rings from W + MI and GK + MI rats compared to corresponding sham-operated animals. Myocardial reperfusion injury was smaller in GK + MI than W + MI rats, and the concentration-response curves to acetylcholine were significantly enhanced in rings from GK + MI than W + MI rats. Nevertheless, the relaxation response to acetylcholine was similar in W + sham and GK + sham. Densitometric analysis of bands for endothelial nitric oxide synthase showed a significant decrease in W + MI rats compared to W + sham and GK + sham animals. Aortas from both GK + sham and GK + MI rats showed impaired contractile responses to phenylephrine in comparison with the nondiabetics.

Conclusions: For the first time we showed that short-term and mild type-2 DM improved remote endothelial dysfunction after reperfused acute MI.

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1. Introduction

Diabetes mellitus (DM) is a chronic metabolic disorder characterized by inappropriate hyperglycemia due to a lack of, or resistance to insulin. More than 90% of diabetic patients are afflicted with type 2 diabetes dominated by hyperinsulinemia, hyperglycemia and dyslipidemia. DM can affect cardiac structure and function independent of hypertension, coronary artery disease, or hyperlipidemia, a condition called diabetic

cardiomyopathy. However, DM not only affects the myocardium, but also leads to vascular complications characterized by a hyperreactivity to vasoconstrictors and/or impaired vascular relaxation, which contribute to an increased incidence of cardiovascular disease. Of the many metabolic disturbances due to diabetes, hyperglycemia has been suggested to be the main cause of vascular dysfunction. Enhanced vascular reactivity to the α_1 -adrenoreceptor agonist, noradrenaline, was demonstrated in vascular beds from diabetic animals (Abebe, Harris, & MacLeod, 1990). However, unchanged (Heygate, Lawrence, Bennett, & Thurston, 1995) or attenuated (Oyama, Kawasaki, Hattori, & Kanno, 1986) responses have also been described.

The structural alterations that diabetes cause on vessels results in an impaired large arterial function (Giannattasio, Failla, Piperno, et al., 1999). The aorta, having an elastic structure, functions not only as a conduit delivering oxygenated blood from the heart to the tissues but also plays an important role in the modulation of the cardiovascular system. Thus, the intermittent pulsatile output from the left ventricle is converted to a steady blood flow to capillary beds (Luchsinger, Sachs, & Patel, 1962). Therefore, aortic dysfunction is one of the major

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components of diabetic cardiomyopathy and is also closely related to functional impairment of the myocardium (Karamitsos, Karvounis, Didangelos, et al., 2008). Consequently, macrovascular disease in diabetes is responsible for a high incidence of vascular diseases such as myocardial infarction (MI) which occurs as a result of an imbalance between coronary blood supply and the myocardial demand. It has been demonstrated that during the post-infarction remodelling process in the absence of diabetes the change of vascular reactivity can occur with non-cardiac vessels such as the large conduit artery (Lee et al., 2009). Previous in vitro studies with non-diabetic rat aorta support the assertion that vascular contractile responses in a large conduit artery can be decreased at the early stage after myocardial ischemic reperfusion injury or acute MI (Lee, 2009, Kim et al., 2014). It has been suggested that enhanced endothelial nitric oxide synthase (eNOS) activity can be attributed to the decreased vasocontractile response in the rat thoracic aorta after MI (Lee et al., 2009).

The Goto–Kakizaki (GK) rat, a model of type 2 diabetes, has been established from normal Wistar rats by selectively breeding the animals with signs of impaired glucose tolerance (Goto, Kakizaki, & Masaki, 1976). The GK rat develops mild hyperglycemia and hyperinsulinemia, and is not associated with the development of obesity, hypertension, or hyperlipidemia. The spontaneous diabetic GK rat thus offers a convenient model for studying the pathogenesis of diabetic vasculopathy independent from the effect of hyperlipidemia, obesity or hypertension.

Although several studies have examined vascular dysfunction in patients with diabetes or in diabetic animal models, experimental data describing vascular damage induced by diabetes after acute MI are limited. In the present study, we therefore investigated the systemic influence of acute MI on vascular function in thoracic aortic rings, as a model of conductance vessel, obtained from type 2 diabetic and nondiabetic control rats.

2. Methods

2.1. Animals

Spontaneously diabetic male GK rats (Taconic, Ry, Denmark) and age-matched nondiabetic male Wistar control rats (Charles River, Sulzfeld, Germany) were purchased at 4–9 weeks of age, and studied at the age of 16 weeks. The animals were housed at a constant room temperature ($22 \pm 2^\circ\text{C}$) and 12 h light/dark cycles, with free access to standard laboratory rat diet and water. The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996). All procedures with and handling of the animals during the investigations were reviewed and approved by the appropriate institutional review committee.

2.2. Induction of myocardial infarction

A model was constructed by occluding the left anterior descending artery (LAD) for 45 minutes followed by 24 hours of reperfusion. In brief, rats were anesthetized with a mixture of ketamine (100 mg/kg) and xylazine (3 mg/kg) intraperitoneally. An intratracheal tube was inserted, and the animals were artificially ventilated using a rodent ventilator (Föhr Medical Instruments GmbH, Seeheim-Ober Beerbach, Germany). The body temperature was maintained at 37°C with a controlled heating pad. The chest was opened via a left thoracotomy, followed by a pericardiotomy. A 6–0 single silk suture was passed around the LAD coronary artery, and the ends of the tie were pulled through a small pledget to form a snare and then tightened. After 45 minutes of ischemia, reperfusion was achieved by releasing the snare. After surgery the thorax was closed, the skin was sutured and the rats were allowed to recover on a heating pad. Sham-operated

animals were subjected to the same surgical procedures, except that the suture under the LAD coronary artery was not tied.

2.3. Experimental groups

The experimental groups were as follows: (1) Sham-operated Wistar rats (W + sham, $n = 11$); (2) Sham-operated GK rats (GK + sham, $n = 7$); (3) myocardial infarcted Wistar rats (W + MI, $n = 10$), and (4) myocardial infarcted GK rats (GK + MI, $n = 8$).

2.4. Measurement of arterial blood pressure and heart rate

Twenty-four hours after the sham operation or the induction of MI, rats were anesthetized with sodium pentobarbital (60 mg/kg, i.p.), placed on controlled heating pads maintaining their core temperature (measured via a rectal probe) at 37°C , tracheotomized, and intubated to facilitate breathing. A 2 F microtip pressure-volume catheter (SPR-838, Millar Instruments, Houston, TX, USA) was inserted into the right carotid artery and advanced into the ascending aorta. Systolic and diastolic blood pressures, and heart rate were measured, the mean arterial blood pressure was calculated.

2.5. Determination of area at risk and infarct size

After hemodynamic measurements, the hearts were excised and quickly hung on a Langendorff apparatus. After the heart was perfused with 0.9% NaCl solution to wash out blood from the coronary vessels, the coronary artery was reoccluded. Then 1.5 ml of Evans blue dye (1% w/v) was injected into the aorta and coronary arteries in order to demarcate the ischemic risk (non-stained) or non-risk (stained) area of the heart. Heart tissue was cut, and transverse slices were incubated with 1% 2,3,5-triphenyltetrazolium chloride (TTC) for 30 minutes at 37°C . The viable myocardium was stained with brick red due to the formation of a precipitate that resulted from a reaction of TTC with dehydrogenase enzymes. The loss of these enzymes from the infarcted myocardium prevents the formation of the precipitate, and the infarcted area within the region at risk remains pale yellow. The tissue samples were then fixed in 4% formalin solution for at least 24 hours. Individual slices were photographed in colour, and using the Cell^A software (Olympus Soft Imaging Solutions GmbH, Germany) the extent of myocardial necrosis and the area at risk were quantified by an investigator blinded to the experimental groups.

2.6. Biochemical estimation

Blood was collected 24 hours after the sham operation or induction of MI in Lithium–Heparin–Gel Monocuvette® tubes, immediately centrifuged, and the plasma was separated. Levels of plasma glucose and high-sensitive cardiac troponin T were determined.

2.7. In vitro organ bath experiments

Isolated aortic rings (in each group, 7–11 independent experiments) were mounted on stainless steel hooks, and placed in an oxygenated (95% O_2 and 5% CO_2) organ bath (Radnoti Glass Technology, Monrovia, CA, USA) with 25 ml of Krebs–Henseleit solution at 37°C as previously described (Korkmaz, Radovits, Barnucz, et al., 2009). Special attention was paid during the preparation to avoid damaging the endothelium. Tissues were equilibrated for 60 minutes under a resting tension of 2 g. During this period, the tension was periodically adjusted to the desired level, and the Krebs–Henseleit solution was replaced every 30 minutes as a precaution against interfering metabolites. At the beginning of each experiment, maximal contraction forces to potassium chloride (KCl, 80 mM) were determined, and the aortic rings were washed until the resting tension was again obtained. Aortic preparations were precontracted with an α_1 -adrenergic receptor agonist, phenylephrine

(10^{-9} – 5×10^{-5} M) until a stable plateau was reached, and relaxation responses were examined by adding cumulative concentrations of an endothelium-dependent vasorelaxant acetylcholine (10^{-9} – 10^{-4} M). For testing the relaxing responses of smooth muscle cells, a direct nitric oxide donor, sodium nitroprusside (10^{-10} – 10^{-5} M) was used. The tensions were recorded using isometric force transducers of a myograph (159901A, Radnoti Glass Technology, Monrovia, CA, USA), digitized, stored and displayed with the IOX Software System (EMKA Technologies, Paris, France). Contractile responses are expressed in g, in phenylephrine's case also as percent of the maximal contraction induced by KCl 80 mM). Half-maximal response (EC_{50}) values were obtained from individual concentration-response by fitting experimental data to a sigmoidal equation using Origin 7.0 (Microcal Software, Northampton, USA). The sensitivity to vasorelaxants was assessed by $pD_2 = -\log EC_{50}$ (M), vasorelaxation (and its maximum (R_{max})) is expressed as percent of the contraction induced by phenylephrine (5×10^{-5} M).

2.8. Histopathological process

Aortic segments were fixed in a buffered paraformaldehyde solution (4%) and embedded in paraffin. Then, 5- μ m thick sections were placed on adhesive slides. The intima-media width of the aorta was examined using hematoxylin & eosin staining in 4×3 fields characterizing each specimen, and an average value was calculated and then normalized to body weight (Dileepan, Johnston, Li, Tawfik, & Stechschulte, 2004) for each experimental group. The evaluation was conducted by an investigator blinded to the experimental groups.

2.9. Western blotting

Proteins from the aorta were extracted in a 2x RIPA buffer (Melford, Ipswich, UK). The protein concentration was determined with Bradford assay. Total protein homogenates 20 μ g/30 μ l was denatured, separated on a sodium dodecyl sulfate polyacrylamide electrophoresis gels and transferred to a polyvinylidene fluoride membrane (Millipore, Darmstadt, Germany). The membrane was blocked with 5% BSA in Tris-Buffered Saline Tween 20 for 1 hour before being incubated overnight at 4 °C with an eNOS antibody (1:1000 in 2.5% BSA/TBS-T, abcam Cambridge, UK). After washing the blots to remove excessive primary antibody binding, the blots were incubated for 1 hour with horseradish peroxidase-conjugated secondary antibody at room temperature. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH), housekeeping proteins was used as loading control and protein normalization. After stripping with stripping buffer phospho-eNOS^{Ser1177} was determined on the same way like eNOS. The immunoreactive protein bands were developed using the Enhanced Chemiluminescence system (PerkinElmer, Rodgau-Juegesheim, Germany). The intensity of the immunoblot bands was detected with a Chemismart 5100.

2.10. Chemical reagents

Phenylephrine, acetylcholine, sodium nitroprusside and KCl were dissolved in 0.9% NaCl and were bought from Sigma-Aldrich, Steinheim,

Germany. Sodium pentobarbital was purchased from Merial GmbH (Halbergmoos, Germany), Evans Blue and TTC from Sigma-Aldrich, (Steinheim, Germany).

2.11. Statistical analysis

All data is expressed as mean $\pm$ standard error of the mean (SEM). Intergroup comparisons were performed by using one-way analysis of variance followed by a Student's unpaired t-test with Bonferroni's correction for multiple comparisons. A value of $p < 0.05$ was considered statistically significant.

3. Results

3.1. Plasma glucose and body weight

The plasma glucose level was significantly higher in diabetic rats (GK + sham: 13 ± 2 mM and GK + MI: 19 ± 2 mM) as compared to the nondiabetic animals (W + sham: 8 ± 0 mM and W + MI: 8 ± 1 mM), indicating the manifestation of an overt diabetes. Additionally, diabetic rats with myocardial infarction had higher glucose levels when compared to the GK + sham animals. Compared with the corresponding control aged-matched rats, their body weight was significantly lower, and heart-to-body weight ratio was higher in the diabetic animals (Table 1).

3.2. Myocardial infarct size and plasma high sensitive cardiac troponin T

Myocardial infarct size (infarct zone/area at risk) was smaller in diabetic hearts than in nondiabetic hearts (GK + MI: $24 \pm 6\%$ vs. W + MI: $58 \pm 9\%$, $p < 0.05$). After 24 hours of reperfusion, the levels of plasma high sensitive cardiac troponin T in the ischemia/reperfusion groups (W + MI: 7870 ± 815 ; GK + MI: 7200 ± 1192 pg/ml) were significantly increased compared with the sham-operated animals (W + sham: 807 ± 126 ; GK + sham: 3313 ± 488 pg/ml).

3.3. Heart rate and arterial blood pressure

Heart rate values did not differ between the experimental groups (Table 1). The systolic blood pressure in GK + MI animals and the diastolic blood pressure and mean arterial pressure in GK + sham and GK + MI rats were significantly lower compared to the W + sham animals (Table 1).

3.4. Endothelium-dependent vasorelaxation of aortic rings

In aortic rings precontracted with 10^{-6} M phenylephrine, 10^{-9} M– 10^{-4} M acetylcholine induced a concentration-dependent relaxation (Fig. 1A). Relaxation responses to acetylcholine were similar in W + sham compared to GK + sham rats. In post-MI rats, ACh-induced relaxation was significantly weaker in rings from both W + MI and GK + MI animals compared to the corresponding sham-operated animals. However, after MI, the concentration-response curves and maximal

Table 1
Physiological characteristics.

	W	GK	W + MI	GK + MI
Body weight [g]	453 $\pm$ 7	330 $\pm$ 7* #	447 $\pm$ 10	319 $\pm$ 6* #
Heart-to-body weight ratio (10^{-3})	2.9 $\pm$ 0.1	3.7 $\pm$ 0.1#	3.6 $\pm$ 0.1#	4.1 $\pm$ 0.1* #
Heart rate [beats/min]	456 $\pm$ 12	457 $\pm$ 24	449 $\pm$ 9	454 $\pm$ 4
Systolic blood pressure [mmHg]	146 $\pm$ 5	123 $\pm$ 5	138 $\pm$ 7	121 $\pm$ 7#
Diastolic blood pressure [mmHg]	118 $\pm$ 5	90 $\pm$ 5#	114 $\pm$ 7	93 $\pm$ 7#
Mean arterial pressure [mmHg]	127 $\pm$ 5	101 $\pm$ 5#	122 $\pm$ 7	102 $\pm$ 7#

Data is represented as mean $\pm$ SEM. n = 7–11 rats. * $p < 0.05$ vs. W + MI; # $p < 0.05$ vs. W. W indicates Wistar, GK Goto–Kakizaki, MI myocardial infarction.

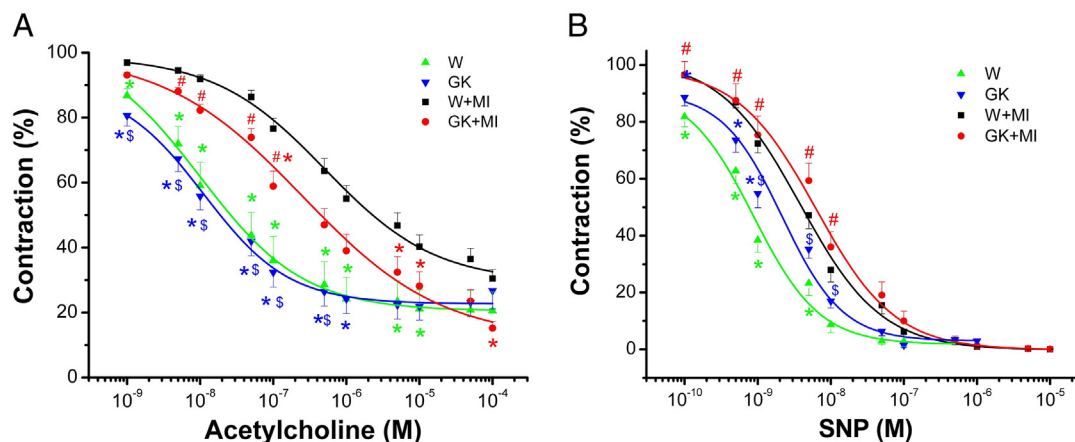


Fig. 1. Concentration-response curves for (A) acetylcholine-induced endothelium-dependent and for (B) sodium nitroprusside (SNP)-induced endothelium-independent vasorelaxations of aortic rings from nondiabetic and type-2 diabetic rats. Data is represented as mean $\pm$ SEM. In each group, 7–11 independent experiments ($n = 18$ –36 aortic rings/group). * $p < 0.05$ vs. W + MI; # $p < 0.05$ vs. W; \$ $p < 0.05$ vs. GK + MI. W indicates Wistar, GK Goto-Kakizaki, MI myocardial infarction.

relaxation to acetylcholine were significantly enhanced, and pD_2 values were increased in aortic rings from GK + MI compared to those of the age-matched control (W + MI). Additionally, pD_2 values for acetylcholine were significantly increased in GK + sham rats when compared with the GK + MI and W + MI animals and decreased in W + MI rats compared with the W + sham rats.

3.5. Endothelium-independent vasorelaxation of aortic rings

Fig. 1B shows concentration-dependent relaxations induced by 10^{-10} M– 10^{-5} M sodium nitroprusside, an endothelium-independent vasodilator. Acute MI caused a significant shift of the sodium nitroprusside concentration-response curves to the right in rings precontracted with phenylephrine without any alterations in the R_{max} . Moreover, pD_2 values to sodium nitroprusside in W + MI and GK + MI rats were significantly decreased compared with the W + sham animals (Table 2).

3.6. Contractile responses of aortic rings

Exposure of aortic segments to phenylephrine (10^{-9} M– 5×10^{-5} M), an α_1 -adrenergic receptor agonist led to a concentration-dependent increase in tension in all experimental groups (Fig. 2). Aortas from both GK + sham and GK + MI rats showed impaired contractile responses to phenylephrine compared to the nondiabetic rats (Fig. 2, Table 2). Moreover, pD_2 values to phenylephrine were significantly increased in

GK + sham rats compared to those of both W + MI and GK + MI animals (Table 2). Regarding the contractions of the aortic rings induced by phenylephrine, expressed as percent of the maximal contraction induced by 80 mM KCl, the GK + sham group showed decreased maximal contraction forces compared to W + sham and W + MI rats (Table 2). Additionally, the contractile responses to high K^+ -induced depolarization were decreased in GK + sham and GK + MI rats in comparison to W + MI animals.

3.7. Thickness of the intima-media

The aortas showed a typical hematoxylin & eosin staining pattern with smooth muscle cells and elastic layers stained pink (lighter and darker, respectively) while nuclei were purple. The aortic intima-media width (normalized to body weight) was significantly higher in GK + MI rats when compared to the controls (W + sham and W + MI animals) (Fig. 3).

3.8. Endothelial NOS protein expression in the thoracic aorta

The densitometric analysis of bands for eNOS showed a significant decrease in W + MI rats compared to W + sham and GK + sham animals (Fig. 4A). Furthermore, eNOS expression in GK + MI rats did not significantly differ when compared to the sham-operated animals. Additionally, we observed no difference on the Ser¹¹⁷⁷ phosphorylation status (Fig. 4B). Therefore, the phospho-eNOS^{Ser1177} to total eNOS

Table 2
Quantitative analysis of vascular function.

	W	GK	W + MI	GK + MI
Phenylephrine (g)	2.32 $\pm$ 0.15	1.27 $\pm$ 0.13 ^{*,#}	2.35 $\pm$ 0.12	1.49 $\pm$ 0.10 ^{*,#}
Phenylephrine (% of KCl)	99 $\pm$ 11	59 $\pm$ 5 ^{*,#}	88 $\pm$ 4	78 $\pm$ 5
pD_2 to phenylephrine	7.05 $\pm$ 0.06	7.25 $\pm$ 0.09 ^{*,§}	6.67 $\pm$ 0.10	6.73 $\pm$ 0.10
KCl (g)	2.6 $\pm$ 0.2	2.2 $\pm$ 0.1 [*]	2.7 $\pm$ 0.1	1.9 $\pm$ 0.1 ^{*,#}
R_{max} to acetylcholine (%)	79 $\pm$ 6	73 $\pm$ 6	70 $\pm$ 3	85 $\pm$ 3 [*]
pD_2 to acetylcholine	7.65 $\pm$ 0.24	8.02 $\pm$ 0.18 ^{*,§}	6.08 $\pm$ 0.22 [#]	6.95 $\pm$ 0.21 [*]
R_{max} to SNP (%)	97 $\pm$ 0	97 $\pm$ 0	97 $\pm$ 1	97 $\pm$ 1
pD_2 to SNP	9.18 $\pm$ 0.13	8.78 $\pm$ 0.19	8.40 $\pm$ 0.09 [#]	8.23 $\pm$ 0.18 [#]

Data is represented as mean $\pm$ SEM. In each group, 7–11 independent experiments ($n = 18$ –36 aortic rings/group). W indicates Wistar, GK Goto-Kakizaki, MI myocardial infarction. Values of maximal relaxation (R_{max} , %) and pD_2 to the vasorelaxant action of acetylcholine (10^{-4} M) and sodium nitroprusside (SNP, 5×10^{-7} M), and contraction induced by phenylephrine (5×10^{-5} M) and potassium chloride KCl (80 mM) in thoracic aortic rings.

* $p < 0.05$ vs. W + MI.
$p < 0.05$ vs. W.
§ $p < 0.05$ vs. GK + MI.

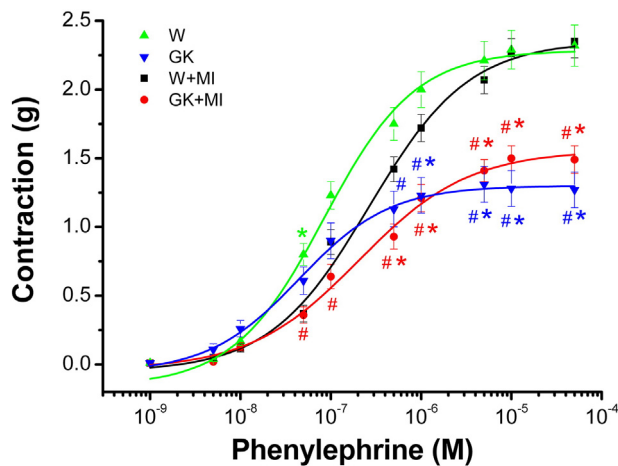


Fig. 2. Concentration-response curves for phenylephrine-induced vasoconstriction of aortic rings from nondiabetic and type-2 diabetic rats. Data is represented as mean $\pm$ SEM. In each group, 7–11 independent experiments ($n = 18$ –36 aortic rings/group). * $p < 0.05$ vs. W + MI; # $p < 0.05$ vs. W. W indicates Wistar, GK Goto-Kakizaki, MI myocardial infarction.

ratio was found to be increased in the W + MI group compared to the GK + sham group (Fig. 4B).

4. Discussion

In the present study, we explored the systemic influence of an acute MI on diabetes-induced pathogenic changes in the large vessel. To the best of our knowledge, this is the first paper showing that short-term and mild type 2 DM improves remote endothelial dysfunction after reperfusion acute MI. We also found that type 2 DM is associated with preserved endothelium-dependent and -independent vasorelaxations along with a marked impairment of contractile function of the vascular smooth muscle.

The vascular endothelium plays a key role in the maintenance of vascular homeostasis, mediated by a number of endothelium-derived factors. Vasodilator factors produced by the endothelium, such as nitric oxide (NO) (Furchgott & Vanhoutte, 1989; Ignarro, Buga, Wood, Byrns, & Chaudhuri, 1987), prostacyclin (PGI₂) (Moncada, Gryglewski, Bunting, & Vane, 1976), and endothelium-derived hyperpolarizing factor (EDHF) (Griffith, 2004) underlie this activity and are the most significant endothelial mediators working co-operatively. NO, synthesized from L-arginine by NOS diffuses from the endothelium to the underlying smooth muscle and activates guanylyl cyclase generating cyclic guanosine monophosphate (cGMP). Increased intracellular cGMP levels inhibit the influx of calcium into the vascular smooth muscle, thereby decreasing intracellular calcium concentration, thereby playing a fundamental role in the relaxation process. NO also activates potassium channels, which leads to hyperpolarization and relaxation. PGI₂ is synthesized by cyclooxygenase (COX) isozymes and elicits smooth muscle relaxation by activating specific cell-surface receptors that are G-protein-coupled to adenylyl cyclase and thereby elevate cyclic adenosine monophosphate (cAMP) levels. The identity of EDHF remains uncertain, although several candidates have been proposed including potassium channels, cAMP, cytochrome P450 2C products, hydrogen peroxide (H₂O₂), and most recently C-type natriuretic peptide (Ahluwalia & Hobbs, 2005). Endothelial function was evaluated in thoracic aortic segments by performing acetylcholine concentration-response curves in rings precontracted with phenylephrine. In the present study, acute MI significantly impaired the endothelial function in both diabetic and nondiabetic rats. These results are in agreement with the findings of Lee et al. which showed a decreased relaxation response in the non diabetic rat thoracic aorta 3 days after MI (Lee et al., 2009). Their data demonstrated that

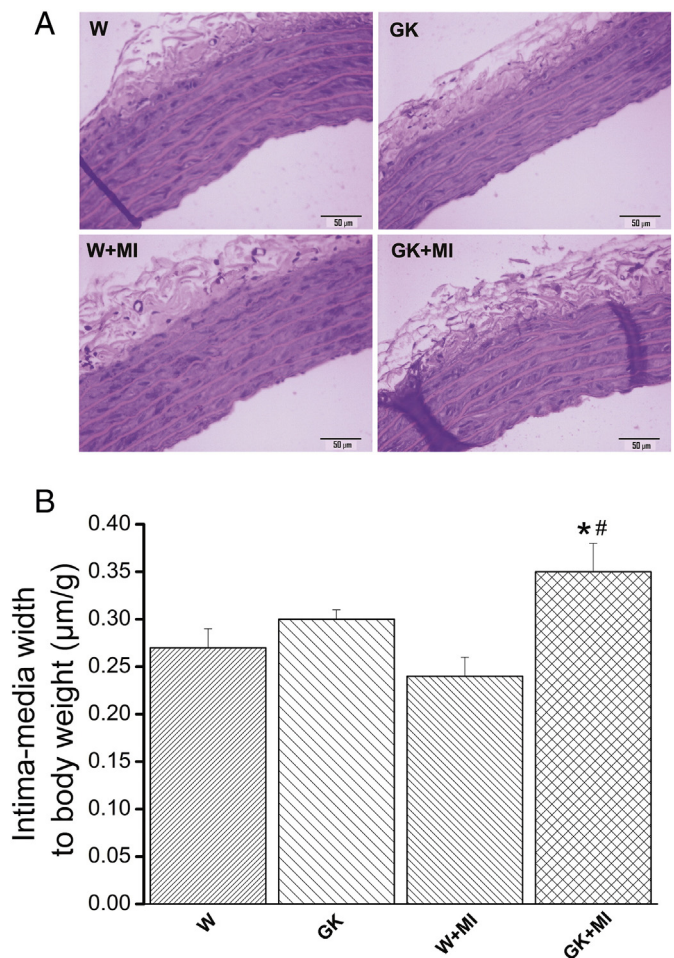


Fig. 3. Intima-media width in the aorta of nondiabetic and type-2 diabetic rats. (A) Hematoxylin and eosin stained sections (magnification $\times 100$, bar = 50 μ m), (B) intima-media width normalized to the body weight. Data is represented as mean $\pm$ SEM. $n = 5$ –6 rats/group. * $p < 0.05$ vs. W. W indicates Wistar, GK Goto-Kakizaki, MI myocardial infarction.

endothelial dysfunction after an acute MI occurs despite an increased eNOS activity and the up-regulation of the NO-cGMP pathway (Lee et al., 2009). Although clinical trials showed that DM increased the myocardial sensitivity to ischemia/reperfusion injury (Alegria, Miller, Gibbons, Yi, & Yusuf, 2007; Marso, Miller, Rutherford, et al., 2007), infarct size studies in diabetic animal models showed contradictory results. Some studies have shown that the diabetic heart is more sensitive to ischemic injury (Desrois, Clarke, Lan, et al., 2010; Di Filippo, Marfella, Cuzzocrea, et al., 2005; Marfella, D'Amico, Di Filippo, et al., 2002); others found no difference (Bulhak et al., 2009; Matsumoto, Cho, Tosaka, et al., 2009). It has also been demonstrated that the myocardium of animals with DM compared to non diabetic animals paradoxically is more resistant to ischemia, resulting in smaller MI (Feuvey & Lopaschuk, 1997; Hadour et al., 1998). In the present work, the infarct size after the LAD coronary artery occlusion and reperfusion in diabetic rats was smaller than in non diabetic control animals, demonstrating that hearts from diabetic rats are protected against ischemic damage. The major indicator of the transition from reversible to irreversible ischemia/reperfusion injury is the release of intracellular cardiac enzymes or markers such as troponin-T, lactate dehydrogenase, and creatine kinase into the circulation (Imamura, 2011) reflecting major cellular membrane damage and/or death of cardiomyocytes (Ravkilde, Nissen, Horder, & Thygesen, 1995). Even though our study demonstrated smaller myocardial infarct size in diabetic hearts, the increased plasma levels of high sensitive cardiac troponin-T were not reduced by short term and

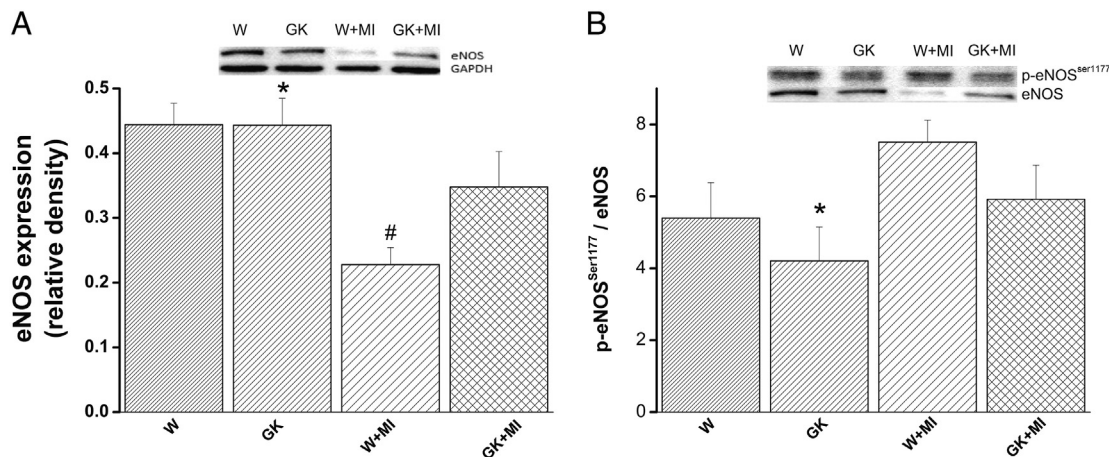


Fig. 4. Endothelial nitric oxide synthase (eNOS) and phosphor-eNOS (Ser1177) expression in the thoracic aorta. Representative western blot analysis of (A) eNOS and glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and of (B) p-eNOS^{Ser1177} to total eNOS. Data is represented as mean $\pm$ SEM. $n = 5$ rats/group. * $p < 0.05$ vs. W + MI; # $p < 0.05$ vs. W. W indicates Wistar, GK Goto-Kakizaki, MI myocardial infarction.

mild diabetes. It should be noted that, the high values of cardiac troponin T in GK + sham rats without MI may be associated to the presence of diabetic cardiomyopathy. Additionally, even though the diabetic rats had smaller myocardial infarct size compared to the nondiabetics, they had the same level of troponin releasing, indicating established diabetes-induced damage in cardiomyocytes. Taken together, mild hyperglycaemic environment rescues the cardiac cells in the ischemic reperfused heart (by decreasing the infarct size) but has no protective effect on the established diabetic cardiomyopathy (diabetic infarcted rats showed elevated levels of plasma troponin T). This is supported by previous studies, which reported that the infarct size was smaller in both obese Zucker diabetic fatty (ZDF) and lean GK type 2 diabetic rats than in non-diabetic animals (Kristiansen, Lofgren, Stottrup, et al., 2004; Kristiansen, Lofgren, Nielsen, et al., 2011). Several factors have been proposed to explain the vulnerability of diabetic hearts to myocardial ischemia/reperfusion injury. These include the duration and severity of the diabetic state, the ischemic insults and the metabolic profiles after diabetic induction. The persistence of a hyperglycemic environment prior to ischemia/reperfusion injury may induce a myocardial adaptation (Kristiansen, et al., 2004) nevertheless the exact mechanisms responsible for this have yet to be identified. However, it has been shown that the preischemic myocardial glycogen content is increased in the diabetic heart (Kristiansen et al., 2011; Lajoie, Calderone, Trudeau, et al., 2004) and high glycogen reserves protect from ischemic damage resulting in smaller infarcts in the diabetic rat hearts (Kristiansen et al., 2011). The main finding of the present study is that after MI, the acetylcholine-induced relaxation was enhanced in the aorta from diabetic animals in comparison to controls, suggesting that smaller infarcts may protect against myocardial ischemia/reperfusion injury induced endothelial dysfunction. eNOS-derived NO plays an important role in vascular relaxation. Although we did not examine endothelial NO-production, a reduced protein expression of eNOS in the thoracic aorta was observed in nondiabetic myocardial infarcted rats, suggesting that mild hyperglycemic environment may preserve the eNOS expression in the vascular wall and thereby enhanced endothelium-dependent relaxation after an acute MI. Additionally, five phosphorylation sites in eNOS have been identified, and its enzyme activity is mainly regulated by Akt-dependent Ser1177 phosphorylation (Dimmeler et al., 1999). In the present study, the phospho-eNOS^{Ser1177} values were similar among the experimental groups. Nevertheless, in the present work endothelium-derived NO-mediated relaxation was preserved in 16 week-old diabetic rats as compared to that of nondiabetics. This seems surprising, because in a previous study Kobayashi et al. showed that in the early stages of diabetes (12 weeks), endothelium-dependent relaxation is enhanced in helical

strips of the aorta from GK rats compared to those from age-matched control rats (Kobayashi, Matsumoto, Oishi, & Kamata, 2004). These authors found an increased eNOS expression in diabetic rats which presumably increases NO production, thus increasing the relaxation response to acetylcholine (Kobayashi et al., 2004). In contrast, at a later stage (36 weeks), the diabetic aortas exhibited decreases in the endothelium-dependent relaxation due to reductions in both NO production and NO responsiveness (Kobayashi et al., 2004). However, Kazuyama et al. showed that in both early- (12-week-old) and late-stages (70-week-old) type 2 diabetic GK rats' aortas had a significant decrease in acetylcholine-induced relaxation compared to age-matched controls (Kazuyama et al., 2009). Reduced expressions of eNOS, inducible NOS, and neuronal NOS mRNAs were observed in the old GK rats compared to the age-matched Wistar rats, resulting in a decline of relaxation directly via the smooth muscle (Kazuyama et al., 2009). However, in our study, the endothelial function remained unchanged in 16-week-old GK rat's aorta, which could correspond with middle-stage diabetes. The conflicting results may be due to differences in experimental techniques, the phase of the diabetes and the conditions used because in literature, even with the same vascular bed of the same animal, there are many differences between the reported findings.

Vasorelaxation is possibly induced by endothelium-independent mechanisms mediated by stimulation of NO, as well as by opening of potassium channels which stimulates the release of NO within smooth muscle cell. For example, nitrodonors (such as sodium nitroprusside) are drugs that stimulate endogenous NO action by releasing NO or forming NO within tissues. These drugs act directly on the vascular smooth muscle to cause relaxation and therefore serve as endothelium-independent vasodilators. The mechanisms that impair the relaxation by the direct NO-donor sodium nitroprusside in diabetes are unclear. In the present study, an acute MI impaired both endothelium-dependent and -independent vascular relaxation. However, even though after an acute MI, acetylcholine-induced endothelium-dependent relaxation was enhanced, SNP-induced endothelium-independent vasorelaxation was not improved in the aorta from diabetic animals compared to controls. For the different regulation observed between endothelium-dependent and endothelium-independent vasorelaxations, we can speculate that an enhanced eNOS expression in diabetic rats with MI (Kobayashi et al., 2004) which presumably increases the NO content could play an important role in improving the NO bioavailability, responsible for the increased relaxation response to acetylcholine. We can also hypothesize that an increase in muscarinic receptor density and/or affinity in the diabetic aorta from MI rats may also enhance the

endothelium-dependent relaxation. In future works, it would be interesting to measure the eNOS activity and investigate the muscarinic receptor affinity, number or post-receptor pathways.

In the present study, type 2 DM is associated with a marked impairment of the contractile function of the vascular smooth muscle. It has been reported that an activation of the α_2 -adrenoceptors located on endothelial cells stimulates the release of NO, an action that would tend to attenuate the vasoconstriction mediated by vascular α_1 -adrenoceptors (Angus, Cocks, & Satoh, 1986; Vanhoutte & Miller, 1989). The decreased norepinephrine-induced contraction observed in diabetic rat helical aorta strips has already been shown to be due to an overexpression of α_{2D} -adrenoceptor mRNA (Kobayashi et al., 2004). In diabetic rats a decreased phenylephrine- and KCl-induced aortic rings contractions reflects an altered adrenergic response and smooth muscle damage respectively. Increased intima-media thickening has been reported in children and adolescents with short-term diabetes (Atabek, Kurtoglu, Pirgon, & Baykara, 2006) and in adult diabetic patients (Gomez-Marcos, Recio-Rodriguez, Rodriguez-Sanchez, et al., 2011). Aortic intima-media thickness has been demonstrated to be an early marker of vascular changes in the diabetic population (Harrington et al. 2010). When normalized for the body weights, the mean aortic intima-media thickness was significantly higher in myocardial infarcted diabetic rats than in controls, as a marker of vascular changes. Even though LAD coronary artery occlusion was associated with a decreased contractile response in the rat thoracic aorta 3 days after (Lee et al., 2009), our results in contrast showed that acute MI does not alter the aortic response to phenylephrine.

A limitation of this study was that the vascular reactivity of the thoracic aortic rings was studied *ex vivo* in a standard tissue bath. Therefore, confirmation of these observations *in vivo* with the involvement of non aortic tissue, blood flow, and leucocytes activation is essential. Moreover, a detailed mechanistic understanding of these functional alterations at the molecular level would have been interesting to investigate. Future works examining the impact of ischemia and/or reperfusion durations on systemic influence of an acute MI on diabetes-induced vascular changes need to be examined.

In conclusion, we demonstrated, that short-term and mild type-2 DM has a protective effect, not only against myocardial ischemia/reperfusion injury (smaller infarcts), but also against the associated systemic endothelial dysfunction.

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