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**Superoxide-NO Interaction Decreases Flow- and Agonist-Induced Dilation of Isolated Coronary Arterioles of Mice with Type 2 Diabetes Mellitus**

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**Abstract**

Type 2 diabetes mellitus (T2-DM) markedly increases the incidence of ischemic heart disease (IHD), consequently mortality. However, the underlying mechanisms leading to IHD in T2-DM are not completely understood. We hypothesized that in T2-DM the regulation of coronary microvascular resistance by local mechanisms is altered. Thus, in coronary arterioles (diameter:  $\sim 80 \mu\text{m}$ ) isolated from male mice with T2-DM (C57BL/KsJ-db/db) and control littermates responses to changes in intraluminal pressure, flow and agonists with known mechanisms of action were studied. Increases in pressure (from 20-120 mmHg) resulted in similar myogenic responses of coronary arterioles of control and db/db mice, whereas dilations in response to cumulative concentrations of acetylcholine (ACh) and the NO donor NONOate were significantly decreased compared to those of control vessels. On the other hand, responses to adenosine were not different between vessels of control and db/db mice. Increases in flow (0-20  $\mu\text{L}/\text{min}$ ) resulted in dilations of control vessels (max:  $38 \pm 4\%$ ) that were inhibited by the NO synthase inhibitor L-NAME. In contrast, arterioles of db/db mice exhibited greatly reduced dilations to flow (max:  $4 \pm 6\%$ ) that were unaffected by L-NAME. In carotid arteries of db/db mice a superoxide dismutase (SOD)-sensitive, enhanced superoxide production was detected by dihydroethydyne staining and lucigenin enhanced chemiluminescence. Correspondingly, intraluminal administration of SOD significantly augmented flow-, ACh- and NONOate-induced dilations of diabetic arterioles, responses that could be inhibited by L-NAME. Collectively, these findings suggest that in T2-DM, due to an enhanced superoxide production, NO-mediation of agonist- and flow-induced dilation of coronary arterioles is reduced. This alteration in the regulation of coronary microvascular resistance may contribute to the development of ischemic heart disease in type 2 diabetes mellitus.

**Key words:** type 2 diabetes mellitus, coronary arteriole, flow-induced dilation, nitric oxide, superoxide

## INTRODUCTION

Diabetes mellitus (type 1 and type 2 DM) is associated with a markedly increased incidence of cardiovascular diseases accounting for approximately 66% of deaths in the diabetic population (27, 32, 38). Most of the previous studies, regarding the nature of changes in coronary function revealing the risk of developing cardiovascular, particularly ischemic heart disease (IHD), have been conducted in type 1 DM (16). However, although type 2 DM accounts for 90-95% of all diabetes (27), much less is known about the underlying mechanisms responsible for the elevated risk in this condition.

Clinical and experimental studies have demonstrated that cardiac pump function is compromised in type 2 DM and it has been suggested that alterations in cardiac tissue metabolism in the diabetic state are responsible for this impairment (4, 21). In addition to metabolic changes, it has been proposed that changes in coronary vessel function can lead to a mismatch of myocardial supply and demand (4, 21) thereby provoking ischemic episodes in the diabetic heart. Accordingly, in earlier studies decreased coronary perfusion, due to accelerated atherosclerosis of the large coronary arteries, was suspected to be the primary cause of cardiac pump dysfunction (5). However, recent clinical investigations have shown that coronary blood flow at rest is not compromised in the diabetic heart especially in the early phases of DM (12, 35).

Still, there could be significant changes in the response of the diabetic coronary circulation to metabolic and pharmacologic challenges. Indeed, recent studies indicate that the maximum coronary dilator capacity to pharmacological agents is significantly attenuated in type 2 diabetic compared to nondiabetic patients (17, 28, 29). In addition, these studies also concluded that

epicardial atherosclerosis is unlikely to be the primary cause of the abnormalities in coronary flow dilator reserve, but rather this is due to reduction of the dilator reserve of coronary microvessels (12).

Several mechanisms could be responsible for the reduced coronary microvascular dilator reserve (5). In type 2 DM decreased vasodilation of intestinal and skeletal muscle vessels to pharmacological agonist has been reported (17, 28). In addition to pharmacologically provoked reduction in vasodilation, alterations in local regulatory mechanisms, intrinsic to the vascular wall, such as pressure-sensitive myogenic and flow-sensitive endothelial mechanisms (14), may also contribute to the reduced dilation of coronary microvessels in type 2 DM. It has recently been reported that in peripheral (mesenteric, skeletal muscle, adipose tissue) arterioles of type 2 diabetic subjects, there is an enhanced pressure-sensitive myogenic constriction, which may adversely affect dilator mechanisms (6, 20). Another important physiologically relevant mechanism, which contributes to the regulation of coronary resistance, is flow-dependent dilation (19). In this context, a previous study of skeletal muscle arterioles from type 2 diabetic obese Zucker rats has found a decreased flow-dependent dilation (7).

On the basis of all of the above we hypothesized that type 2 DM significantly alters the regulation of coronary arteriolar function by local mechanisms. Thus, in the present study we aimed to characterize the alterations in pressure-sensitive myogenic constriction, flow-dependent dilation and agonist-induced responses of coronary arterioles of type 2 diabetic, db/db mice, an accepted model of type 2 diabetes mellitus.

## **METHODS**

### **Animal model of type 2 diabetes mellitus**

In order to study the dysfunction of coronary arterioles in type 2 DM, a mouse model of type 2 DM was chosen. The genetically diabetic mouse (C57BL/KsJ-db/db) has a mutation on chromosome 4 that inhibits the expression of leptin receptors. The loss of functional leptin receptors prolongs the ingestion time in homozygous (db/db) mice, which are obese and hyperglycemic, whereas heterozygous (control) mice cannot be distinguished morphologically or physiologically from normal mice (11). The syndrome of type 2 DM in db/db mice is similar to the type 2 DM developing in adult humans, which is also characterized by obesity, insulin resistance/hyperinsulinemia and hyperglycemia.

### **Experimental procedures, determination of serum glucose and insulin levels**

Twelve to 14 week old male control (n=20) and db/db (n=20) mice (purchased from Jackson Lab. Co, USA) were used. Animals were fed standard chow and given tap water ad libitum. Mice were housed in the animal care facility at the New York Medical College approved by the American Association for the Accreditation of Laboratory Animal Care. Mice were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg). Blood was collected from the femoral the artery and centrifuged immediately. Serum was stored at  $-80^{\circ}\text{C}$  for later biochemical assays. The heart and carotid arteries were excised and placed in a silicone-lined Petri dish containing cold ( $0-4^{\circ}\text{C}$ ) physiological salt (PS) solution composed of (in mmol/L): 110 NaCl, 5.0 KCl, 2.5  $\text{CaCl}_2$ , 1.0  $\text{MgSO}_4$ , 1.0  $\text{KH}_2\text{PO}_4$ , 5.5 glucose and 24.0  $\text{NaHCO}_3$  equilibrated with a gas mixture of 10%  $\text{O}_2$  and 5%  $\text{CO}_2$ , balanced with nitrogen, at pH 7.4. Serum glucose

concentrations were measured with commercial glucose assay kits (Sigma Co, USA). Serum insulin levels were determined by mouse ELISA kits (ALPCO Diagnostic Inc, USA) using a microplate reader (Bio-Tec Inc, USA).

### **Isolation of coronary arterioles**

Using microsurgery instruments and an operating microscope, a branch of the septal artery (~ 0.5 mm in length) running intramuscularly was isolated and transferred into an organ chamber containing two glass micropipettes filled with PS solution. Vessels were cannulated on both ends and micropipettes were connected with silicone tubing to an adjustable PS solution-reservoir. Inflow and outflow pressures were set to 80 mmHg and continuously measured by a pressure servo control system (Living Systems Instrumentation, VT, USA). Temperature was set at 37°C by a temperature controller (Grant Instruments, USA). The internal arteriolar diameter at the midpoint of the arteriolar segment was measured by videomicroscopy with a microangiometer (Texas Instruments, TX, USA). Changes in arteriolar diameter and intraluminal pressure were continuously recorded with the Biopac-MP100 system connected to a computer and analyzed with AcqKnowledge data acquisition software (Biopac Systems, Inc, CA, USA).

### **Coronary arteriolar responses to agonists**

Cumulative concentrations of the endothelium dependent dilator, acetylcholine ( $10^{-9}$ - $10^{-6}$  mol/L), the endothelium independent NO donor, (NONOate,  $10^{-9}$ - $10^{-6}$  mol/L) and adenosine ( $10^{-6}$ - $10^{-4}$  mol/L) were used to test the function of endothelium and smooth muscle of arterioles.

**Pressure-induce arteriolar responses**

Basal coronary arteriolar tone was established at 80 mmHg. Then, changes in diameter of arterioles were measured in response to step increases in intraluminal pressure from 20 to 120 mmHg. To obtain the passive diameter, arterioles were exposed to a  $\text{Ca}^{2+}$ -free solution containing EGTA (ethylene glycol-bis ( $\beta$ -aminoethylether)-N,N,N',N'-tetraacetic acid,  $10^{-3}$  mol/L) and  $10^{-4}$  mol/L sodium nitroprusside (SNP). Basal tone was calculated at each pressure step and expressed as a percentage of active (AD, in  $\text{Ca}^{2+}$  containing) and passive (PD, in  $\text{Ca}^{2+}$  free PS solution) diameters as follows:  $((\text{PD} - \text{AD})/\text{PD}) \times 100$ .

**Flow/Shear stress-induced arteriolar responses**

Coronary arteriolar responses were obtained to step increases in intraluminal flow (0-20  $\mu\text{L}/\text{min}$ ). Intraluminal flow was established at a constant intravascular pressure (80 mmHg) by changing inflow and outflow pressures to an equal degree, but in opposite directions, to keep midpoint luminal pressure constant. Intraluminal flow was measured with a ball flowmeter (Omega Engineering Inc, CT, USA). Step increases in flow were used and each flow rate was maintained for five minutes to allow the vessel to reach a steady state diameter. Flow-induced arteriolar responses were expressed as changes in arteriolar diameter as a percentage of the maximal dilation of the vessel, defined as the passive diameter at 80 mmHg intraluminal pressure in a  $\text{Ca}^{2+}$ -free medium. Wall shear stress (WSS) was calculated by the formula  $4\eta Q/r^3$ , where  $\eta$  is the viscosity of the perfusate (0.007 poise at 37 °C), Q is the perfusate flow, and r is the vessel radius.

**Endothelium removal and inhibition of endothelial NO synthesis in arterioles**

The endothelium of the arterioles was removed by perfusion of the vessel with air, as described previously (15). Endothelium denudation was ascertained by the loss of dilation to acetylcholine ( $10^{-7}$  mol/L) and the maintained dilation to the NO donor, NONOate ( $10^{-7}$  mol/L). To inhibit endothelial NO synthase isolated coronary arterioles were incubated with N<sup>o</sup>-nitro-L-arginine-methyl-ester (L-NAME,  $10^{-4}$  mol/L, for 20 min under no-flow conditions),

**Quantification of vascular superoxide production by lucigenin-enhanced chemiluminescence assay**

Vascular superoxide production was assessed from carotid arteries isolated from control and db/db mice by the lucigenin enhanced chemiluminescence method according to a modified protocol of Mohazzab et al. (26). A segment of carotid arteries was removed from mice, cleared of connective tissue, immersed in PS solution (37°C), and incubated for 60 minutes. Then, arteries were placed in scintillation vials containing HEPES buffered (10 mmol/L; pH 7.4) PS solution and lucigenin (10  $\mu$ mol/L, Calbiochem, Inc, USA) and chemiluminescence was measured in a liquid scintillation counter (Beckman LS-6000IC). Scintillation counts were obtained 20 minutes after addition of vessels and background corrected values were normalized to tissue weight.

**Detection of in situ vascular superoxide production by ethidium bromide fluorescence assay**

Dihydroethydine (DHE), an oxidative fluorescent dye, was used to localize superoxide production according to the modified protocol of Frisbee et al (6). Cells are permeable to DHE,

which in the presence of superoxide is oxidized to fluorescent ethidium bromide (EB). EB is trapped by intercalation with DNA and the number of fluorescent nuclei indicates the relative level of superoxide production. Thus, carotid arteries isolated from control and db/db mice were transferred to chambers containing PS solution and incubated for 60 minutes at 37°C. DHE ( $5 \times 10^{-6}$  mol/L; Molecular Probes, USA) was then added to the PS solution and incubated further for 10 minutes followed by 5 minutes of washing in cold PS solution to remove the non-intercalated EB molecules. Frozen sections of vessels were then visualized by fluorescence microscopy (Olympus, Inc, USA) and then stained with hematoxylin-eosin (HE). The separately obtained EB fluorescent and HE images were overlaid using computer image software and then the number of EB stained fluorescent nuclei was counted in five control vessels and five vessels of db/db mice.

#### **Use of superoxide dismutase (SOD)**

Coronary arterioles of control and db/db mice were incubated in the presence of superoxide-dismutase (SOD, 120 U/mL) for 30 min under zero-flow conditions, then coronary arteriolar responses were obtained again. In lucigenin-enhanced chemiluminescence assays, after obtaining control signals, carotid arteries were incubated in oxygenated PS solution for an additional 30 min at 37°C min in the presence of SOD and then assays were performed again. In case of dihydroethydyne staining, parallel experiments were carried out. Carotid arteries of control or db/db mice were incubated in oxygenated PS solution for 30 min and then SOD or vehicle (as control) were added to the separate solutions for an additional 30 min. Ethidium bromide fluorescent images were obtained of frozen sections of arteries.

**Statistics**

Data are expressed as means  $\pm$  S.E.M. Statistical analyses were performed by two-way analysis of variance for repeated measures (ANOVA) followed by the Tukey post hoc test or Student's t-test, as appropriate.  $P < 0.05$  was considered statistically significant.

## RESULTS

At twelve weeks of age the body weight, serum glucose and serum insulin of db/db mice were significantly increased compared to age matched lean control mice (Table 1).

### **Pressure-induced arteriolar responses**

In coronary arterioles isolated from control and db/db mice there were no significant differences between the active ( $79\pm 7$  and  $77\pm 5$   $\mu\text{m}$ , respectively) and passive (in  $\text{Ca}^{2+}$  free PS solution,  $99\pm 7$  and  $107\pm 7$   $\mu\text{m}$ ) arteriolar diameters developed to 80 mmHg intraluminal pressure (Fig. 1A). Also, there was no significant difference in active and passive arteriolar diameter (Fig. 1A) and the calculated myogenic tone (Fig. 1B) developed to stepwise increases in intraluminal pressure from 20 to 120 mmHg in the two groups.

### **Flow/shear stress-dependent arteriolar dilation**

Increases in wall shear stress elicited by increases in intraluminal flow (from 0 to 20  $\mu\text{L}/\text{min}$ ) resulted in substantial dilations of coronary arteries isolated from control mice, responses that were significantly reduced in arterioles of db/db mice (Fig. 2A). Endothelium removal abolished flow-dependent dilation in control arterioles, but did not affect arterioles of db/db mice (not shown). Inhibition of NO synthesis by L-NAME decreased flow-dependent dilation in control arterioles, but did not affect responses of diabetic arterioles (Fig. 2A). Flow-induced responses were also obtained after intraluminal administration of arterioles with superoxide-dismutase (SOD, 120 U/mL). Intraluminal administration of SOD enhanced flow-induced dilation in

coronary arterioles isolated from db/db mice (Fig 2B), responses that were inhibited by L-NAME.

### **Agonist-induced arteriolar dilation**

In coronary arterioles of db/db mice dilations in response to cumulative doses of acetylcholine (Fig. 3A) and the NO donor NONOate (Fig. 3B) were significantly decreased compared to those of control vessels. On the other hand, responses to adenosine were not different between vessels of control and db/db mice (Fig. 3C). Intraluminal administration of SOD enhanced ACh- and NONOate-induced dilation in coronary arterioles isolated from db/db mice, but did not affect adenosine-induced dilations (Fig 3).

### **Quantification and in situ detection of vascular superoxide production**

Vascular superoxide production was also assessed in carotid arteries of control and db/db mice by the lucigenin enhanced chemiluminescence method. Summarized data show that there was an enhanced lucigenin chemiluminescence in carotid arteries of db/db mice, which was inhibited by preincubation with SOD (Fig. 4A).

Dihydroethydine, an oxidative fluorescent dye, was used to localize superoxide production in situ in the wall of carotid arteries of five control and five db/db mice. Fluorescent photomicrograph of ethidium-bromide staining showed an increased number of fluorescence labeled nuclei indicating an enhanced superoxide production in diabetic carotid arteries ( $24 \pm 6$  nuclei/section), as compared to controls ( $8 \pm 4$  nuclei/section), which was significantly reduced by preincubation with SOD ( $11 \pm 4$  nuclei/section) (Fig. 4B). Overlaid ethidium-bromide and

hematoxylin-eosin stained photomicrographs showed that the enhanced superoxide production was mainly localized in the endothelial and subendothelial layers of the vessels.

## DISCUSSION

The new findings of the present study are that in coronary arterioles isolated from db/db mice, a model for type 2 diabetes mellitus (DM), NO-mediation of flow- and agonist-induced dilation is reduced, whereas pressure-induced myogenic tone is unaltered. The reduced NO-mediation in diabetic arterioles is due to an increased vascular production of superoxide anions.

Clinical and experimental studies have demonstrated that in type 2 DM in addition to specific metabolic changes, altered vasodilator mechanisms of coronary vessels can lead to a mismatch of myocardial supply and demand (4, 21, 29), thereby provoking ischemic episodes. Recently it has been reported that in diabetic mellitus, vasodilation to hypoxia of coronary microvessels is reduced due to an impaired  $K_{ATP}$ -channel activation (25). Furthermore, changes in local regulatory mechanisms, intrinsic to the vascular wall, such as pressure-sensitive myogenic and flow-sensitive endothelial mechanisms, have been also proposed to contribute to the decreased dilator capacity of coronary vessels in type 2 DM (6, 7).

In the present study we have found that in db/db mice at 12 weeks of age body weight, serum glucose and serum insulin are significantly increased compared to age matched lean control mice. Similar changes have been observed in type 2 DM in humans (Table 1).

Microvessels respond to an increase or decrease in transmural pressure by constriction and dilation, respectively. In type 2 diabetic db/db mice Lagaud et al have found that in mesenteric arterioles there is an enhanced pressure-induced myogenic tone due to the upregulation and activation of smooth muscle protein-kinase C (20). In obese Zucker rats Frisbee et al have also reported an enhanced myogenic tone in skeletal muscle arterioles (7). Since coronary vascular resistance is influenced by myogenic reactivity and an enhanced myogenic tone could adversely

affect vasodilator function of arterioles, in the present study, first, responses to increases in intraluminal pressure were obtained in coronary arterioles of db/db mice. We have found no significant difference between active and passive diameters of coronary arterioles of db/db and control mice, developed to 80 mmHg intraluminal pressure. Moreover, changes in diameter and the calculated myogenic tone of arterioles in response to stepwise increases in intraluminal pressure from 20 to 120 mmHg were also not significantly different in the two groups (Fig. 1A,B), indicating that in db/db mice an enhanced myogenic constriction is unlikely to be responsible for the decreased vasodilation of coronary arterioles. On the other hand, the unaltered basal myogenic tone of coronary arterioles of diabetic mice may support the hypothesis of earlier observations that in the diabetic state the basal coronary blood flow is unchanged (12, 35).

The endothelium continuously regulates vascular diameter by releasing vasodilator (nitric oxide, prostacyclin etc.) and vasoconstrictor (thromboxane, endothelin etc.) substances (13), thereby contributing to regulation of vascular resistance. One of the primary in vivo physiological stimuli for local regulation of arteriolar diameter is the presence of intraluminal blood flow (15, 18). Increases in intraluminal flow elicit endothelium-dependent vasodilation via release of vasodilator substances, such as nitric oxide and dilator prostaglandins (15). It has been reported that in vivo, in skeletal muscle microvessels flow-mediated dilation was significantly reduced in type 2 diabetic obese Zucker rats compared to controls (7). In the present study we have found that increases in wall shear stress via increases in intraluminal flow elicited substantial dilations of coronary arterioles of control mice that were significantly reduced in arterioles of db/db mice (Fig. 2A). One of the key mechanisms of decreased flow-induced dilation could be the reduced synthesis and/or availability of endothelium-derived NO. We have

found that inhibition of NO synthesis or endothelium removal abolished flow-induced dilation in control arterioles, but did not affect responses of arterioles of db/db mice (Fig. 2A) supporting the hypothesis that the decreased flow-induced dilation of coronary arteries of db/db mice is due to the decreased mediation of the response by endothelium-derived NO. On the other hand, Miura et al found that in human atrial coronary microvessels flow-induced dilation is mediated by hydrogen peroxide (24). This finding suggests that flow-induced dilation might be differently regulated in various regions of the myocardium. Recently, Lagaud et al (20) and Pannirselvam et al (30) have demonstrated that in mesenteric arteries of db/db mice dilations in response to acetylcholine were reduced suggesting an impaired endothelium dependent NO-mediated dilation. In the present study we have also found that in coronary arterioles of db/db mice there is a decreased dilation in response to acetylcholine compared to responses of control arterioles (Fig. 3A). Although the above-mentioned authors found an unaltered dilation in response to the NO donor SNP, we have found a decreased dilation in response to the NO-donor NONOate in coronary arterioles of db/db mice (Fig 3B). Others have also found a reduced endothelium independent dilation to the NO donor SNP (22, 39) or glycerine trinitrate in brachial artery of type 2 diabetic patients (22, 39). In contrast, dilations to adenosine were not different between vessels of control and db/db mice (Fig 3C). These observations suggest that in coronary arterioles of db/db mice, while endothelium-dependent responses and responses to NO are reduced, smooth muscle function may not be affected by the diabetic condition.

A reduced NO-mediated dilation could be due to a decreased synthesis of NO if, for example the eNOS substrate, L-arginine, is not available (31) or the level of tetrahydrobiopterin were reduced (36, 37), as suggested to occur both in type 1 (2) and in type 2 DM (30, 33). However, our finding that the dilation in response to the NO-donor NONOate was also decreased in

coronary arterioles of db/db mice suggests that an alteration in NO synthesis is unlikely to be the main cause of the decreased dilation. One of the other possible mechanisms, which could be responsible for impaired dilation is a reduced bioavailability of NO due to its interaction with reactive oxygen species (ROS). Indeed, it has been proposed that oxidative stress contributes to the development of vascular complications in type 2 DM (3, 8, 34). In diabetic patients, increased ROS production has been observed together with decreased levels of antioxidants such as ascorbic acid, vitamin E and glutathione (1, 23). Studies of various animal models of type 2 DM showed that administration of scavengers of ROS, such as superoxide dismutase and catalase, improved endothelium-dependent arteriolar dilations (7), suggesting an important role for elevated levels of ROS, which may interfere with mechanisms mediated by NO. In addition, in obese Zucker rats Frisbee et al have found that enhanced levels of ROS resulted in increased myogenic tone in gracilis arterioles, suggesting a role for ROS in pressure-induced vascular tone in type 2 DM (7). However, we have found no differences in myogenic reactivity of coronary arterioles in control and db/db mice.

In the present study, using two different methods (dihydroethydyne staining and lucigenin enhanced chemiluminescence), we have found an enhanced superoxide production in carotid arteries of db/db mice compared to controls, which was significantly reduced by preincubation of the vessels with SOD (Fig. 4A,B). The dihydroethydyne staining of carotid arteries demonstrates enhanced vascular production of superoxide anions both in endothelium and subendothelial smooth muscle cells layers. Our functional studies showed that intraluminal administration of SOD, which reduced superoxide production in carotid arteries, enhanced flow-, ACh- and NONOate-induced dilation in coronary arterioles of db/db mice supporting the hypothesis that an

enhanced vascular production of superoxide anions in coronary arterioles interferes with the mediation by NO of flow- and agonist-induced dilation in arterioles of db/db mice (Fig 2,3).

It has been suggested that the excess production of vascular superoxide may be derived from different ROS-producing systems, including NADPH oxidases, xanthine oxidase, and neuronal or endothelial NO synthase in the vascular wall itself (41). Recent investigations of DM have proposed a crucial role for vascular NAD(P)H oxidases in the enhanced production of ROS (9, 10). Regardless of the source of ROS (40, 42), it is likely that an excess production of ROS contributes importantly to the alterations in NO-dependent coronary microvascular responses in type 2 DM. Moreover, recent investigations suggest that NO, released from the coronary endothelium, besides its pivotal role in the mediation of vasodilator mechanisms, has an important role in the regulation of cardiac metabolism by reducing cardiac oxygen consumption (43). The reduced availability of cardiac NO by ROS could result in an increased oxygen consumption thereby further compromising the function of the diabetic heart (43).

In summary, the present study demonstrates that in coronary arterioles isolated from mice with type 2 diabetes mellitus, flow- and agonist-induced dilation is reduced due to enhanced superoxide production, which interferes with the mediation of the responses by NO, whereas pressure-induced myogenic tone remains unaltered. Although there may be no significant differences in basal coronary blood flow in diabetic hearts, the decreased mediation by NO of flow- and agonist-induced dilations could result in a significant reduction of the dilator capacity of coronary microvessels predisposing these hearts to ischemic episodes.

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**Table 1**

	control (n=15-18)	db/db (n=15-18)
body weight (g)	31.8±0.7	49.2±1.19*
serum glucose (mmol/L)	5.8±0.6	22.9±1.3*
serum insulin (pmol/L)	173±38	2673±315*

*Table 1.*

**Figure legends**

**Figure 1.** Changes in diameter of coronary arterioles isolated from control (n=9) and db/db (n=9) mice in response to step increases (20 to 120 mmHg) in intraluminal pressure in the presence or absence extracellular  $\text{Ca}^{2+}$  (**Panel B**). Calculated myogenic tone of coronary arterioles of control and db/db mice developed in response to step increases in intraluminal pressure (**Panel A**). Data are mean  $\pm$  S.E.M.

**Figure 2.** Changes in diameter of coronary arterioles isolated from control (n=9) and db/db (n=9) mice in response to step increases in wall shear stress before and after incubation with L-NAME (**Panel A**). Changes in diameter of coronary arterioles isolated from control (n=7) and db/db (n=7) mice in response to step increases in wall shear stress after incubation with SOD (**Panel B**). Data are mean  $\pm$  S.E.M. Asterisk indicates significant difference ( $p < 0.05$ ).

**Figure 3.** Dilatation of coronary arterioles isolated from control (n=6) and db/db (n=6) mice in response to cumulative doses of acetylcholine (ACh, **Panel A**), NONOate (**Panel B**) and adenosine (**Panel C**) before and after incubation with SOD. Data are mean  $\pm$  S.E.M. Asterisks indicate significant differences ( $p < 0.05$ ).

**Figure 4.** Summarized data of lucigenin enhanced chemiluminescence of carotid arteries of control (n=5) and db/db (n=5) mice before and after incubation with SOD (**Panel A**). Representative fluorescent photomicrograph of ethidium-bromide and hematoxylin-eosin staining of carotid arteries isolated from control and db/db mice

(n=5-5) before and after incubation with SOD (**Panel B**). Data are mean  $\pm$  S.E.M. Asterisks indicate significant differences ( $p < 0.05$ ).

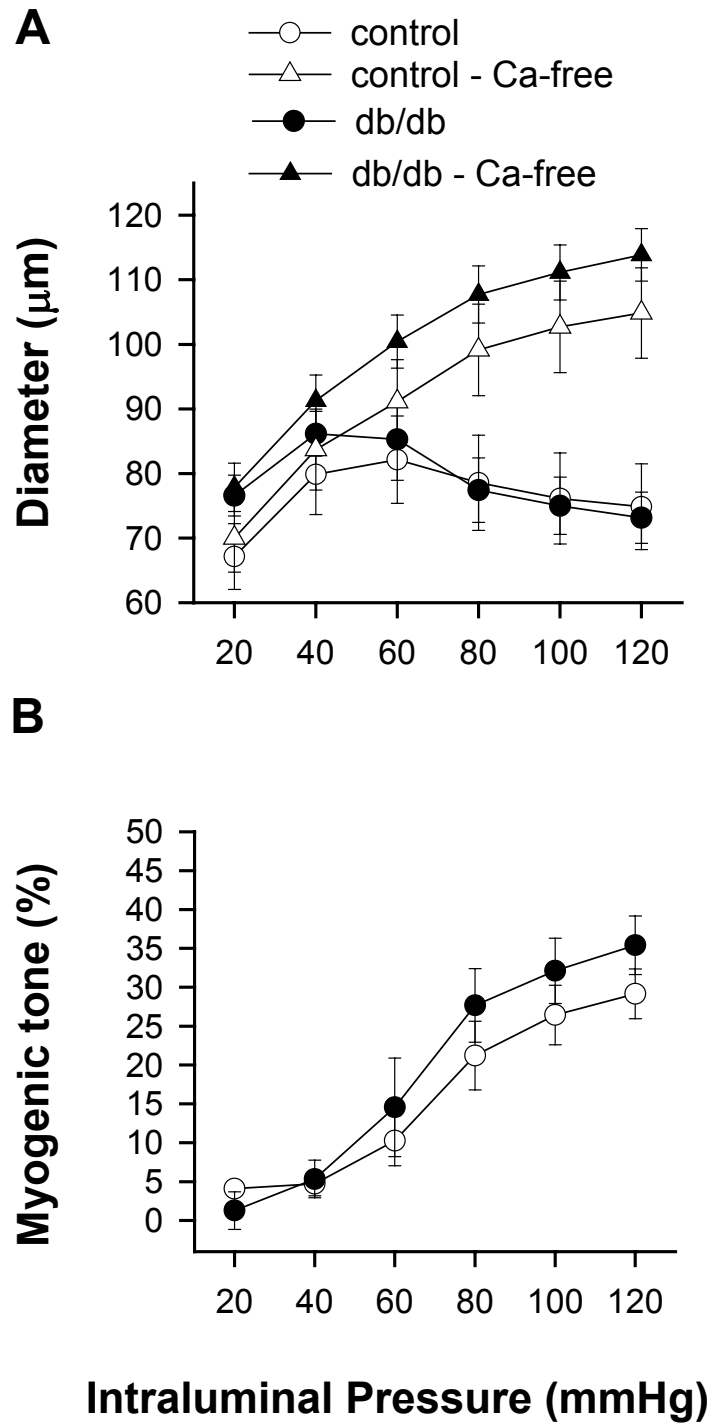


Figure 1.

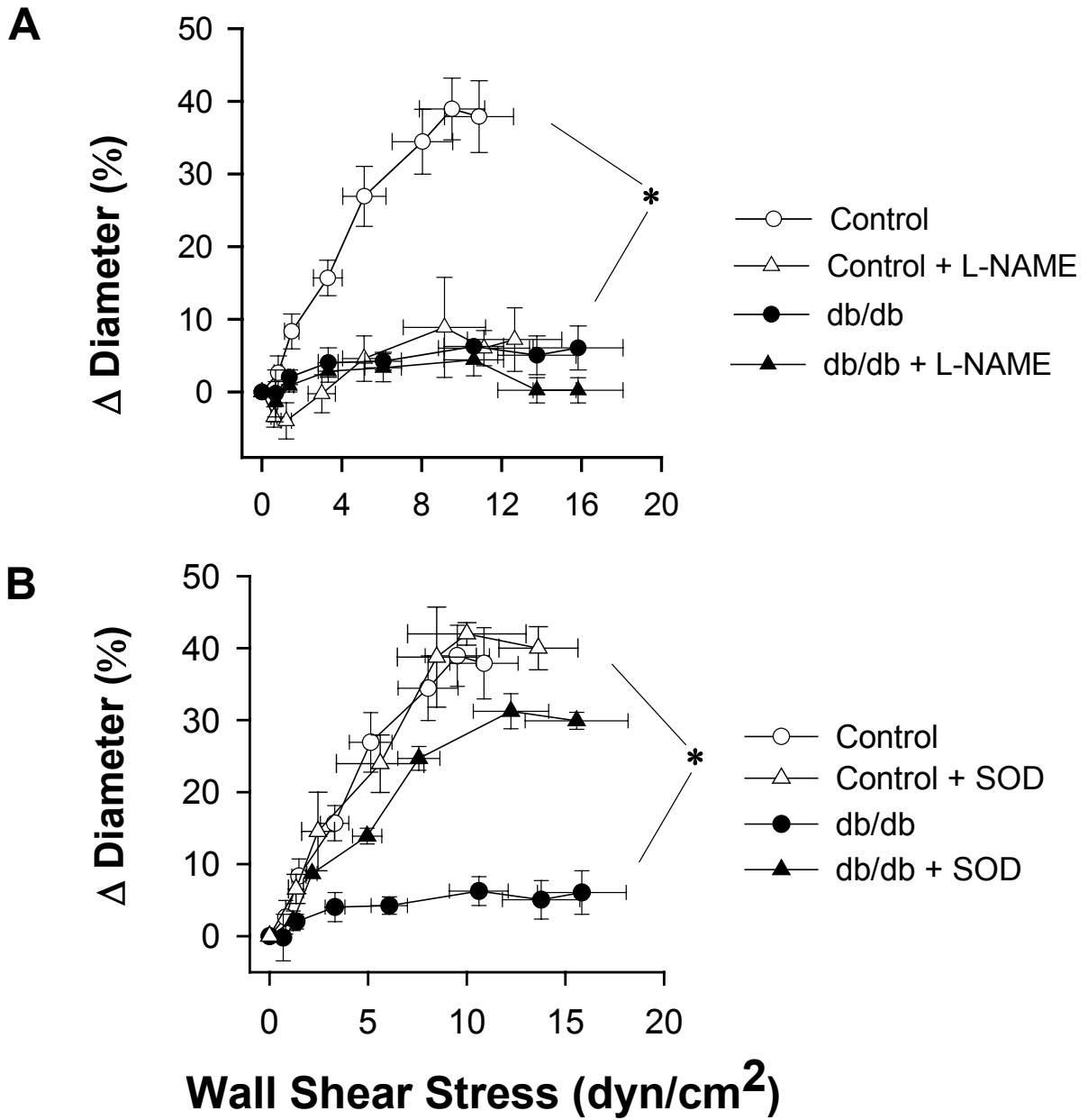


Figure 2.

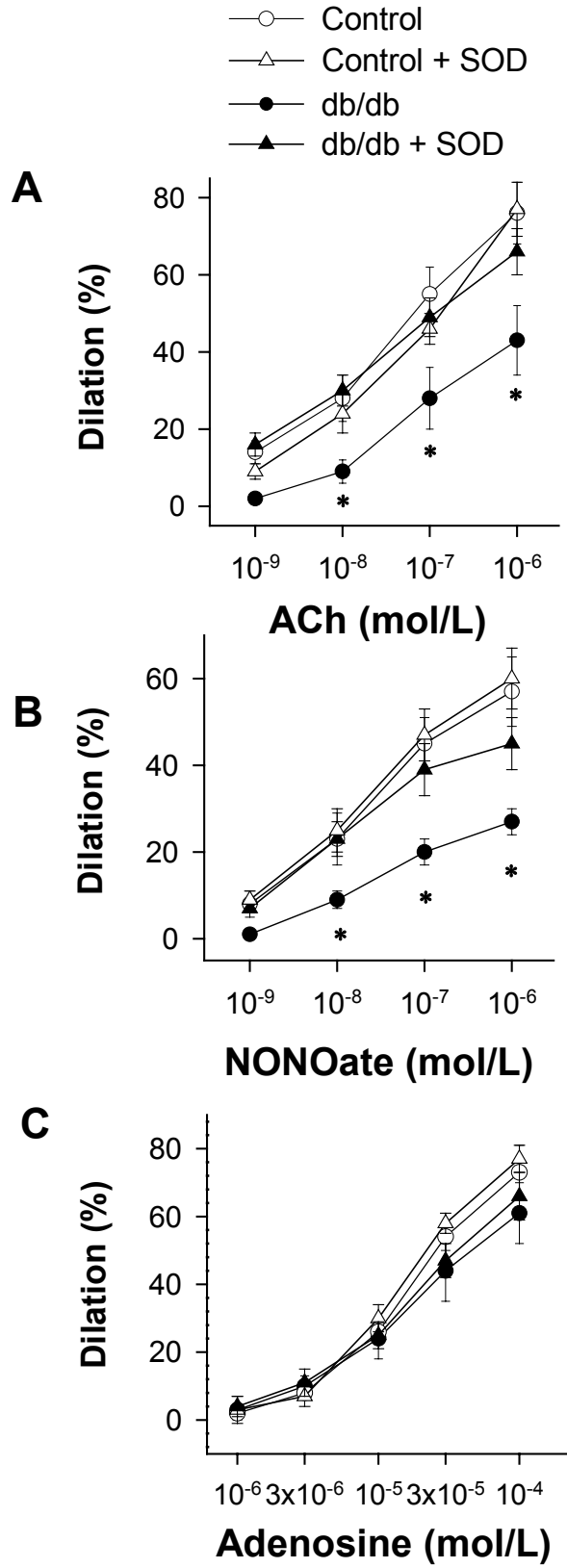


Figure 3.

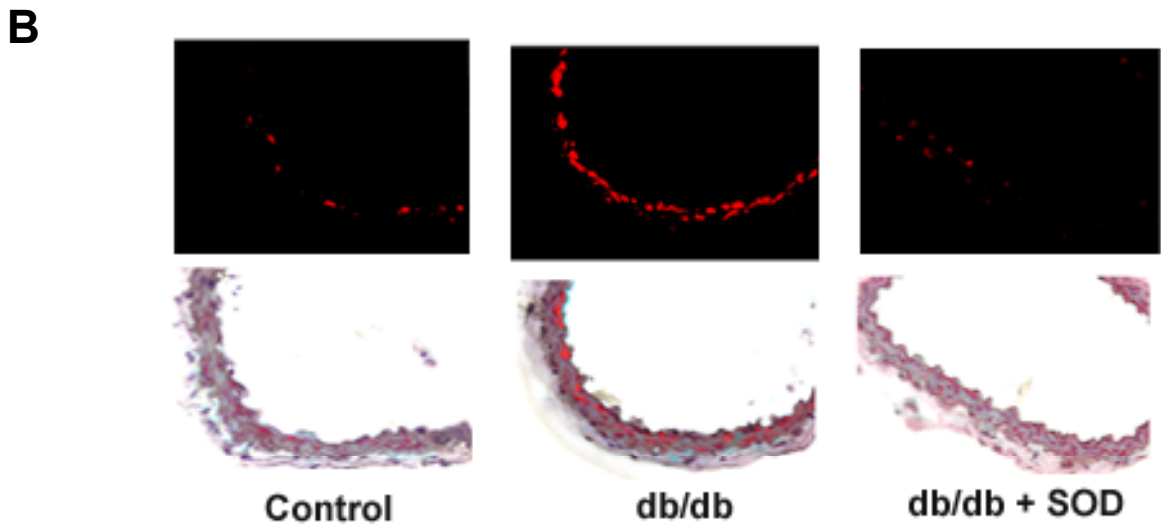
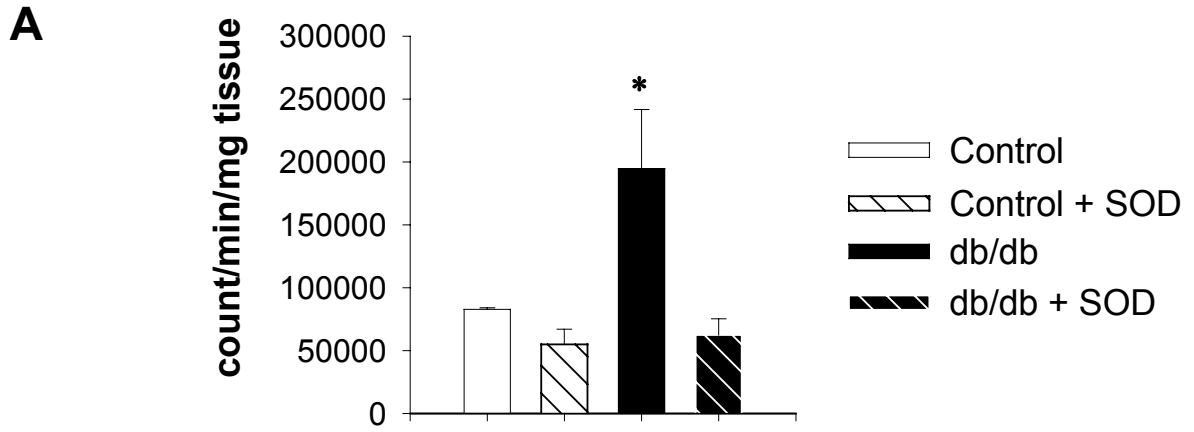


Figure 4.