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Role of Myocardial Nitric Oxide in Diabetic Ischemia-Reperfusion Dysfunction: Studies in Mice with Myocyte-Specific Overexpression of Endothelial Nitric Oxide Synthase

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ABBREVIATIONS:

1400W, N-(3-(aminomethyl)benzyl)acetamidine; ESR, electron spin resonance; L-NAME, N^{ω}-nitro-L-arginine methyl ester; L-NNA, N^{ω}-nitro-L-arginine; LVDevP, left ventricular developed pressure; LVEDP, left ventricular end-diastolic pressure; NO, nitric oxide; NOS, nitric oxide synthase; ROS, reactive oxygen species; TG, transgenic; WT, wild-type

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ABSTRACT

We investigated the role of nitric oxide (NO) in myocardial ischemia-reperfusion injury of diabetic mice with myocyte-specific overexpression of endothelial NO synthase (eNOS). Four weeks after diabetes induction with streptozotocin (blood glucose ~29 mM), isolated isovolumic heart function and cellular NO metabolites in response to brief normothermic ischemia-reperfusion were determined. Under normoxic conditions transgenic (TG) hearts from non-diabetic and diabetic animals generated less left-ventricular developed pressure (LVDevP) compared to wild-type (WT) control hearts, and this abnormality was unaffected by NOS inhibition. During ischemia, the rise in end-diastolic pressure was less in the TG than WT group of non-diabetic hearts, whereas the transgene had no effect in the diabetic group. Similarly, the transgene also improved reperfusion systolic and diastolic function in non-diabetic but not in diabetic hearts. NOS inhibition worsened reperfusion function in diabetic hearts. Post-ischemic nitrite and cGMP formation were higher in non-diabetic TG than WT hearts, but in diabetic hearts cGMP was no longer elevated. The formation of reactive oxygen species (superoxide and peroxynitrite) during early reperfusion, measured by electron spin resonance spectroscopy, was similar in non-diabetic WT and TG hearts, but significantly higher in diabetic TG hearts. Stimulating endogenous NO production with bradykinin (10 μM) more strongly reduced myocardial O_2 consumption in diabetic TG than diabetic WT hearts perfused in normoxia, whereas there was no difference after ischemia-reperfusion. Thus, providing additional endogenous NO is sufficient to protect non-diabetic hearts against ischemia-induced injury, but for a similar protection in diabetic hearts, effective scavenging of ROS is also important.

Introduction

Cardiovascular complications are among the most common causes of morbidity and mortality in diabetic patients. The cardiac complications of diabetes involve decreased cardiac muscle function independent of atherosclerotic coronary artery disease, which has led to the recognition of "diabetic cardiomyopathy" as a major impairment that can progress toward overt heart failure, a leading cause of death for diabetic patients (LeWinter, 1996; Adeghate, 2004).

The oxygen radical nitric oxide (NO) plays a crucial role in coronary and cardiac physiology and appears to be important in diabetic cardiomyopathy as well. Long-term administration of L-arginine, the substrate of NO synthase (NOS), to diabetic rats reduced the severe alterations in myocardial structure and function, implying that endothelial NOS (eNOS) of cardiac myocytes is dysregulated in the course of diabetes (Okruhlicova et al., 2002). Further, the bioavailability of NO may be reduced in diabetes due to oxidative stress factors (Stockklauser-Färber et al., 2000). However, several studies using NO donor drugs or NOS inhibitors have yielded contradictory results, partly even when using the same model and similar protocols (Smith et al., 1997; Joffe et al., 1999). Therefore, many aspects of NO function in diabetic hearts need to be clarified, including the importance of the different NOS isoforms, the mechanisms of altered eNOS regulation, and the functions of NO metabolites.

Experimental diabetes mellitus is often induced with streptozotocin administration which leads to pancreatic islet cell damage and insulin deficiency (Wohaieb and Godin, 1987). This approach has been used mostly with larger animals such as rats, but recently was extended to normal and genetically modified mice to study specific aspects of diabetic myocardial dysfunction, including Ca^{2+} transport (Trost et al., 2002), chronic oxidant exposure (Kajstura et al., 2001; Liang et al., 2002) and the role of inducible NOS

(iNOS) in modulating defensive responses against ischemia and reperfusion injury (Marfella et al., 2004). Our laboratory has developed a transgenic model that expresses eNOS specifically in cardiomyocytes (Brunner et al., 2001). This model may be suitable to test the significance of myocardial NO in the cardiac complications of diabetes because the transgene is overexpressed permanently and specifically in the heart. Using mice overexpressing eNOS (transgenic genotype) and wild-type control mice with normal enzyme levels, we investigated the role of NO in diabetic cardiomyopathy in normoxic hearts and in hearts subjected to ischemia-reperfusion. We determined myocardial and coronary function, the formation of reactive oxygen species (ROS), nitrite and cGMP at reperfusion, and measured the modulation of NO-mediated myocardial oxygen uptake.

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Materials and methods

Animals. We have previously described the development of mice with myocyte-specific overexpression of eNOS (Brunner et al., 2001). Hearts from transgenic (TG) line 23 were used in this study. This line shows a more than 50-fold overexpression of eNOS in cardiomyocytes. Wild-type (WT) littermates served as controls. NOS activities, determined as ^{3}H-L-citrulline formation, are about 50-fold higher in this TG line than in WT hearts (Brunner et al., 2001). Hearts from animals of either sex of the following 4 experimental groups were studied: 1) WT control; 2) TG control; 3) WT diabetic, and 4) TG diabetic. A total of 4 sets of hearts, each comprising all 4 groups, was used: set I for functional studies, set II for measurement of ROS and nitrite in coronary effluent, set III for measurement of cGMP in coronary effluent, and set IV for myocardial O_2 uptake measurements (see Results). The protocols for which sets II and III were used required the coinfusion of the spin trap and of IBMX (isobuthyl-methyl-xanthine, a phosphodiesterase inhibitor), respectively which could affect baseline function; set IV served to determine oxygen uptake prior to ischemia/reperfusion (oxygen uptake post ischemia/reperfusion was determined with set I). All animals received care in accordance with the "Austrian Law on Experimentation with Laboratory Animals" (last amendment, 2004) which is based on the principles of laboratory animal care as adopted by the American Heart Association and the Declaration of Helsinki.

Diabetes induction. Diabetes was induced by use of a single intraperitoneal injection of streptozotocin (200 mg/kg) dissolved in citrate buffer (pH 4.5). This model is well characterized and contains a high oxidant component and no autoimmune involvement (Wohaieb and Godin, 1987; Kajstura et al., 2001). The dose of 200 mg/kg was chosen, because lower doses did not result in consistent hyperglycemia from the outset, i.e. after about 3-5 days; on the other hand, ~20 % of hyperglycemic animals were lost during the four weeks of diabetes duration irrespective of genotype. Control

animals were injected with citrate buffer alone. Animals were maintained on normal chow and did not receive supplemental insulin injections. Diabetes was confirmed by the presence of hyperglycemia (see Results). Non-fasted blood glucose was measured early in the morning using OneTouch Ultra glucose test strips (Lifescan, Neckargemünd, Germany). The animals were studied at 12 (range: 12-14) months (n=30), when they show least ischemic tolerance (Willems et al., 2005). All animals were killed 4 weeks (28 days) after streptozotocin administration and the experiments were done on the same day.

NOS catalytic activity. NOS catalytic activity was measured by assaying the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline as previously described (Wölkart et al., 2006). Control assays were done in the presence of 100 µM N^{ω}-nitro-L-arginine (L-NNA; NOS subtype-nonselective inhibitor), 5 mM EDTA (Ca^{2+}-free condition, blocking constitutive (= endothelial + neuronal) NOS activity) or 10 µM N-(3-(aminomethyl)benzyl)acetamidine (1400W) (Garvey et al., 1997), blocking inducible NOS (iNOS) activity. The enzyme inhibitors L-NNA, 1400W and EDTA (or distilled water in assays measuring total activity) were added to the supernatant for 15 min at 37 °C prior to starting the assay.

Heart perfusion and experimental protocol. Hearts were isolated and transferred to a Langendorff set-up for contractile studies in isolated mouse hearts as previously described (Brunner et al., 2001). In brief, hearts were removed from anesthetized mice and immersed in ice-cold perfusion buffer. After cannulation of the aorta using a head-lens, hearts were perfused retrogradely at constant flow (2 ml/min) at 37 °C with a modified Krebs-Henseleit buffer containing 1.25 mM Ca^{2+} and 11 mM glucose. A small fluid-filled balloon made of household cellophane was inserted into the left-ventricular cavity and coupled to a pressure transducer. The balloon was inflated

until the end-diastolic pressure reached 5 mm Hg. Hearts were perfused without pacing. Digitized recordings of the ventricular developed pressure (LVDevP; = peak systolic minus diastolic pressure), left ventricular end-diastolic pressure (LVEDP), maximum speed of ventricular contraction and relaxation (+dp/dt and -dp/dt), heart rate and coronary perfusion pressure were analyzed from the recordings and compared between experimental groups. The experimental protocol comprised an equilibration phase (30 min; baseline), followed by 20 min of no-flow ischemia at 37 °C and reperfusion for 30 min at 2 ml/min (total duration of experiments: 80 min). Some diabetic WT and TG mice were treated with N^{ω}-nitro-L-arginine methyl ester added to the drinking water at 50 mg/kg per day for the last two days before sacrifice (days 27 and 28 after streptozotocin administration). This regimen effectively blocks NOS without affecting blood pressure as evident from the complete inhibition of acetylcholine-induced coronary and aortic relaxation that were specifically tested in each L-NAME-treated mouse. Further details were reported previously (Brunner et al., 2001). Some hearts were perfused at normoxic conditions for the same duration (time-matched controls), which showed that the preparation is stable as judged by the parameters LVDevP and heart rate (maximum reduction, -10%).

HPLC analysis of nitrite in coronary effluent. Nitrite concentration in the coronary effluent was measured by examining the conversion of 2,3-diaminonaphthalene (DAN) to its fluorescent product, 1-(H)-naphthotriazole which is stable in alkaline solution (Gharavi and El-Kadi, 2003). 100 µl of effluent (taken from the collections gathered for ROS analyses, i. e. the second set of hearts) was mixed with 15 µl of freshly prepared DAN (17 µg/ml in 0.62 M HCl) and incubated at 37 °C for 30 min in the dark. The reaction was terminated with 10 µl of NaOH (2.8 M). After 10 min the samples were centrifuged at 16,000 g for 3 min to remove any precipitate and the supernatant was used for chromatographic analysis (Merck-Hitachi D-6000; Vienna,

Austria) The mobile phase (53 % Na_2HPO_4 15 mM, pH 7.5, 47 % methanol) was pumped through a Lichrospher column (RP 18,5 µm) at a flow rat of 1 ml/min. Samples were measured with a fluorescence detector (Hitachi F-1050) at 380 nm excitation and 405 nm emission. Effluent nitrite concentration was obtained from a standard curve (50 nM-1 µM) for known concentrations of sodium nitrite. The nitrite concentration of the perfusion buffer itself in the absence of an attached heart was 177±14 nM (20 determinations).

Measurement of cGMP concentration in coronary effluent. A third set of hearts was used to determine cGMP levels in coronary effluents. In these experiments, hearts were pre-treated with, isobuthyl-methyl-xanthine (IBMX; 100 µM) to assure quantitative recovery of cGMP. All details have been reported previously (Brunner et al., 2003).

ESR measurements. ROS in coronary effluents were quantified using the spin trap 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CP-H) and electron spin resonance (ESR) spectroscopy. CP-H has been used for the quantitative determination of superoxide (and possibly other) radicals in vitro and in vivo (Dikalov et al., 1997; Matsumoto et al., 2003). To this purpose, hearts were perfused as described above, coronary effluent was collected and transferred to 50 µl-glass capillaries (Bartelt, Graz, Austria) and analyzed immediately in a MiniScope MS 100 spectrometer (Magnettech, Berlin, Germany) at room temperature. CP radical typically generates a triple-line spectrum. ESR instrumental settings were as follows: microwave frequency, 9.48 GHz; microwave power, 15.8 mW; field center, 3370 G; sweep width, 50 G; sweep time, 30 s; modulation amplitude, 2.0 G; receiver gain, 9×10^2. For quantification, the low-field component (first of the three peaks) of the ESR spectrum was used. The amplitude of this component was divided through the receiver gain to standardize values (expressed in arbitrary units).

In a series of in vitro validation experiments, superoxide radical was generated using the hypoxanthine/xanthine oxidase system and the suitability of CP-H to detect the radical was tested. Hypoxanthine (1 mM) and xanthine oxidase (1.25 mU/ml) were incubated in heart perfusion buffer with 65 µM DTPA (pH 7.4) for 10 min at room temperature together with CP-H (100 µM). CP radical formation increased steadily as a result of superoxide radical generation which was verified in separate experiments using the cytochrome c method (Lass et al., 2002). In addition, using chemically synthesized peroxynitrite (0.01-1 mM) (Brunner and Wölkart, 2003), we also verified the scavenging potential of CP-H for this oxidant. The suitability of the probe to detect these ROS was tested with superoxide dismutase (SOD; 50 U/ml) and urate (50 µM) (see Results).

Effluent sampling protocol for ESR. Hearts from non-diabetic WT and TG animals and diabetic WT and TG animals were isolated and perfused as described above. After equilibration (15 min) hearts were subjected to 20 min of global ischemia, followed by 30 min of reperfusion. CP-H was administered directly into the aortic cannula via a lateral port at an infusion rate of 165 µl/min (6.5 % of the coronary flow rate). The infusion pump was switched on 50 sec before intended sample collection, thus ensuring a final CP-H concentration of 100 µM. For each heart, 1 sample was obtained during equilibration and 5 samples during the first 2 min of reperfusion. For each sample, effluent was collected over 10 sec (~300 µl/sample). CP-H exerted slightly negative effects at baseline and during initial reperfusion, whereas at the end of reperfusion myocardial function tended to be improved. The stability of the CP-H solution for the entire duration of the experiment was strictly controlled by ascertaining that it registered a zero-line spectrum at the beginning and at the end of the experiment. Finally, we also verified that CP radical once formed inside the heart is quantitatively recovered in the coronary effluent. This was tested by adding CP radical at 100 µM final

concentration via the sideline as described for CP-H, followed by measuring the resulting ESR signal and comparing it to the signal in the absence of the heart obtained after dismounting the heart. There was no difference between the signals, indicating that the CP radical was quantified correctly.

Oxygen uptake measurements. Tissue oxygen uptake was determined in vitro essentially as previously described (Zhao et al., 2000). Briefly, ventricles (from set 1 of hearts) were cut with a McIlwain mechanical tissue chopper (Mickle Laboratory Engineering, Gomshall, UK) to sections of about 0.3-0.5 mm width, and 20-30 mg (wet weight) were incubated at room temperature in phosphate buffered saline containing 10 mM glucose, the pieces were equilibrated with room air at 37 °C (5 min), and transferred to an air-tight bottle. The bottle contained 1.8 ml of air-saturated buffer, a stirrer and a Clarke-type O_2 electrode (ISO 2 World Precision Instruments, Mauer, Germany) and was made air-tight with a rubber septum. Oxygen tension was recorded on-line as voltage (mV). After recording baselines over 5 min, bradykinin (10 µM final concentration) or diethylamino nitric oxide (DEA/NO; 5 µM) were injected into the vial through the septum to evaluate the inhibitory role of endogenous and exogenous NO production, respectively. The typical observation time for each drug was 5 min. Sodium cyanide (1 mM) was added at the end of each respiration measurement to confirm that the change in O_2 consumption was from mitochondrial sources (total duration of experiment: ~20 min). Basal O_2 uptake was calculated as the rate of decrease in O_2 concentration after the addition of muscle segments assuming an initial O_2 concentration of 207 µM. The change (inhibition) in O_2 consumption induced by the two NO drugs was derived from the change in the voltage signal observed after addition of test drugs, compared to the preceding baseline signal. The inhibitory effect was expressed as reduction from baseline O_2 consumption. O_2 uptake was measured in pieces of myocardium from all 4 experimental groups (WT and TG controls and WT and

TG diabetic hearts), both after normoxic perfusion and at the end of the ischemia/reperfusion protocol.

Reagents. Hypoxanthine, xanthine oxidase (from bovine milk), SOD (from bovine erythrocytes), diethyltetraaminopentaacetic acid (DTPA), bradykinin, cyanide, IBMX, and buffer reagents were obtained from Sigma (Vienna, Austria). 1-hydroxy-3-carboxy-2,2,5,5-tetramethylpyrrolidine (CP-H) was supplied by Noxygen Science Transfer & Diagnostics (Elzach, Germany), 3-carboxy-2,2,5,5-tetramethyl-1-pyrrolidine-1-oxyl free radical (CP radical) and DEA/NO were purchased from Alexis (Vienna, Austria). Stock solutions of CP-H (1.5 mM) were prepared in 9.5 mM phosphate-buffered saline saturated with nitrogen. DTPA (1 mM) was added to decrease the auto-oxidation of CP-H possibly catalyzed by traces of transition metal ions. Stock solutions of CP-H were freshly prepared before each experiment and kept in a closed and chilled syringe to prevent air contact. DEA/NO was prepared and stored frozen as 1 mM stock solution in 10 mM NaOH (NO release commences with dilution in buffer at pH 7.4).

Data analysis and calculations. Data are reported as arithmetic mean ± S.E. Two-way analysis of variance (ANOVA) was used to analyze differences in functional parameters between groups and differences between means were tested by post-hoc analysis using Student´s unpaired t-test (see also figure legends). Significance was assumed at $P<.05$.

Results

Baseline and model characteristics. Streptozotocin-treated mice had severe hyperglycemia and reduced body and heart weights compared to non-diabetic animals (Table 1). These changes were not different between the WT and TG genotype (P=NS). Baseline characteristics were similar in all 4 sets of hearts used in this study (Table 1). None of the animals had any clinical evidence of infection during the four weeks of observation.

NOS catalytic activity. To determine NOS catalytic activity and the possible contribution of cardiac iNOS in the present experiments, we measured constitutive and iNOS activity in the hearts of all groups by assaying the conversion of [^{3}H]L-arginine to [^{3}H]L-citrulline in tissue homogenates (Table 2). Both WT and TG hearts contained detectable amounts of catalytic activity that is wholly blocked by L-NNA or in the absence of Ca^{2+}. 1400W, an iNOS-selective antagonist, had no significant effect, indicating that the bulk of NOS activity in hearts from non-diabetic and diabetic animals is due to the constitutive isoform.

Normoxic ventricular function. Overexpression of eNOS *per se* resulted in a reduction of baseline LVDevP by ~30 % compared to wild-type controls (P<.05) (Fig. 1A). Similar results were obtained for +dP/dt and -dP/dt (Fig. 1B, C). Streptozotocin treatment reduced LVDevP in WT, but had no effect in TG hearts; blockade of NOS with L-NAME had no significant effect.

Ischemic heart function. During 20 min of ischemia, heart beat quickly stopped and LVEDP rose to 38±1 mm Hg in WT and to 23±2 mm Hg in TG non-diabetic hearts (P<.05). In addition, time-to-onset of ischemic contracture was slightly prolonged from 10.9±0.9 min in WT to 13.5±0.7 min in TG hearts (P<.05), whereas peak contracture

during ischemia was not different between genotypes (38±2 % in TG and 44±2 % in WT hearts; P=NS). Similar results were obtained in diabetic hearts; in these hearts NOS blockade significantly increased peak ischemic contracture (WT, 1.7-fold; TG, 1.6-fold) and abbreviated time-to-onset of ischemic contracture (WT, -34 %; TG, -35 %) (P<.05), consistently indicating a substantial protective effect of NO during ischemia.

Reperfusion heart function. The reperfusion contractile response is shown in Fig. 2. LVDevP recovered to 57±2 mm Hg in TG hearts and to 51±1 mm Hg in hearts from WT non-diabetic animals (Fig. 2A). Thus, the transgene marginally improved contractility in absolute terms (+6 mm Hg; P<.05). In diabetic hearts, absolute recovery was not different between WT and TG groups (55±3 mm Hg; P=NS), and L-NAME significantly reduced recovery in both genotypes (Fig. 2B). +dp/dt and -dp/dt were similarly improved in non-diabetic TG hearts but not in diabetic hearts (data not shown). During reperfusion diastolic pressure in non-diabetic hearts was always significantly lower in TG than WT hearts (mean, ~9 mm Hg (Fig. 3A), whereas in hearts from diabetic animals the effect of the transgene was less pronounced (mean, 5 mm Hg; P=NS; Fig. 3B). Importantly, L-NAME significantly raised LVEDP in diabetic hearts by 10-20 mm Hg in both genotypes (Fig. 3B).

Coronary function and heart rate. Baseline coronary perfusion pressure was identical in WT and TG hearts of both non-diabetic and diabetic animals (see baseline in Fig. 4). This is expected because the transgene is restricted to the heart. Post-ischemia, perfusion pressure exceeded baseline pressure, reflecting ischemia/reperfusion vascular injury, and the pressure rise was more pronounced in diabetic than in non-diabetic hearts . Heart rate was not different between groups at baseline or during reperfusion, and L-NAME was without effect (data not shown).

Cardiac nitrite release. To obtain a measure of cardiac NO formation, the nitrite concentration in coronary effluents was determined (Fig 5). Nitrite concentration did not differ between groups at baseline (the reason for this is unclear), but was increased in TG hearts after reperfusion. Diabetes *per se* did not change coronary nitrite concentrations in WT or in TG hearts (Fig. 5A, B). Nitrate release was not determined because the nitrate content of the commonly available buffer constituents is rather high compared to cardiac nitrate levels which would lead to substantial overestimations of cardiac nitrite/nitrate production.

Cardiac cGMP formation. We also measured cGMP levels in coronary effluent as an index of NO-induced cGMP activity. In non-diabetic TG hearts cGMP efflux into the coronary effluent was significantly higher than in WT hearts (Fig 6A). This was the case both at baseline and during reperfusion. Increases of this magnitude are known to exert substantial smooth muscle effects (Brunner and Wölkart, 2001). In diabetic hearts, cGMP levels were not different between WT and TG hearts, implying that in diabetes its stimulation by NO was less than in non-diabetic hearts (Fig 6B). The cGMP content of TG hearts was higher in non-diabetic hearts, but similar in diabetic hearts at the end of reperfusion (Fig 6C).

Cardiac ROS formation. Pre-ischemic and post-ischemic measurements of ROS formation were performed on the coronary effluent of hearts exposed to the spin trap CP-H. This probe readily detects superoxide and peroxynitrite as evident from the inhibitory effects of SOD and urate, respectively (Fig 7A). Whereas prior to ischemia, only a trace signal was observed in the effluents of hearts perfused with CP-H, following reperfusion after 20 min of global ischemia a prominent triplet EPR spectrum was observed due to the formation of the superoxide-CP and/or the peroxynitrite-CP radical. Measurements performed in a series of hearts consistently demonstrated an increase in

the CP signal during the first 15 sec of reflow, followed by a rapid decline over the first min (Fig. 7B, C). In non-diabetic hearts, the CP radical signal was of similar magnitude both in WT and TG hearts (Fig. 7B), but in diabetic hearts, the signal was significantly higher in the TG group of hearts (Fig. 7C).

Modulation of myocardial O_2 consumption. We tested to what extent O_2 consumption by cardiac muscle in normoxia and following ischemia-reperfusion was modulated by NO. In non-diabetic hearts, baseline O_2 uptake was not different between genotypes (373±52 and 348±61 pmol/mg per min in WT and TG hearts (n=15 and 13 determinations; P=NS). After streptozotocin treatment, baseline O_2 uptake was ~ 30 % lower (253±24 and 254±30 pmol/mg per min; P<.05). Bradykinin, a stimulator of endogenous NO formation, more potently inhibited specific O_2 uptake in TG than in WT hearts of both non-diabetic and diabetic animals (Fig. 8A). After ischemia-reperfusion, bradykinin still inhibited O_2 uptake, but the differences between genotypes were abolished (Fig. 8B). The effect of exogenous NO on O_2 consumption was tested with a maximally active dose of DEA/NO (5 µM), a NO donor releasing NO with a half-time of several minutes (Sampson et al., 2001). The agonist decreased O_2 uptake by 60-70 %, irrespective of genotype, perfusion conditions (normoxia, ischemia-reperfusion) or diabetes induction (n=15 determinations based on 5 hearts per group; data not shown).

Discussion

In the present study, the additional generation of NO from constitutive eNOS in TG hearts improved ischemic and reperfusion function in non-diabetic hearts whereas in diabetic hearts this protection was largely lost. Biochemical analyses in non-diabetic TG hearts showed increased cardiac nitrite and cGMP generation, whereas in diabetic hearts the final NO effector cGMP was no longer different from WT hearts, possibly due to increased ROS generation (despite similar cardiac nitrite formation). Thus, in diabetic myocardial ischemia-reperfusion, simply providing additional endogenous NO is insufficient to restore myocardial function to normalcy; rather, effective scavenging of ROS is also important.

Previous investigations have implicated NO as one of the mediators used by the immune system to damage and destroy the ß-cells following streptozotocin treatment (Kolb et al., 1991). Because this effect appears to be mediated by NO derived from iNOS and the deleterious actions of cytokines (Flodström et al., 1999), we specifically studied the role of myocardial NO, derived from eNOS, in the functional aberrations as they occur in this widely used model of type 1 diabetes. Overexpressing this enzyme created ideal conditions to study the role of cardiac NO in the myocardial complications of diabetes development, because eNOS is only weakly expressed in WT cardiac myocytes (Massion et al., 2003). There was no apparent difference in the time-course of hyperglycemia development between genotypes: Three to five days after injection of streptozotocin, all surviving animals started developing hyperglycemia that reached ~29 mM (fed state) after 4 weeks, similar to previous reports for this model (Kajstura et al., 2001). Cardiac iNOS is not implicated in the present experiments, as we detected no change in NOS catalytic activity in the presence of 1400W, a commonly used iNOS-selective inhibitor.

Following diabetes induction, basal ventricular contractility was reduced in WT hearts, but NO may not play a significant role in this reduction as L-NAME was without effect (Fig. 1A-C). The reduced basal contractility in TG hearts was previously shown to be due to desensitization of the contractile elements toward Ca^{2+} (Brunner et al., 2001) and was not further affected by diabetes induction. Together, these data suggest that eNOS may not contribute substantially to the depressed function in unstressed normoxic-perfused diabetic hearts. In this regard, the extent of inhibition of O_2 uptake is higher in TG than WT hearts and similarly unaffected by diabetes (Fig. 8), reflecting the well-known O_2-uptake-inhibitory effect of NO and the intactness of this metabolic pathway in normoxic diabetic mouse hearts (Tada et al., 2000).

In the setting of ischemia-reperfusion, TG diabetic hearts performed consistently better than WT hearts only during ischemia, whereas in non-diabetic hearts the TG conferred protection both during ischemia and reperfusion. The NO-induced cardioprotection we observed in non-diabetic hearts is similar to previous observations using exogenous NO (Lefer et al., 1993; Pabla and Curtis, 1995) and conforms to exacerbated ischemia-reperfusion injury induced by NOS inhibitors (Pabla et al., 1995) or after genetic ablation of eNOS (Jones et al., 1999). The origin and mechanisms of ischemic contracture are incompletely understood, but may relate to altered rates of glucose flux or delivery (King et al., 1995) or elevated intracellular Ca^{2+} and ATP depletion (Koretsune and Marban, 1990; Steenbergen et al., 1990). NO is known to limit the rise in cellular Ca^{2+} levels following stimulation of cardiomyocytes with different agents (Dhalla et al., 1996), and this mechanism may play a role in the anti-contracture effects during ischemia in the diabetic as well as the non-diabetic state.

Recovery of reperfusion contractile function in WT hearts was ~57 % of pre-ischemic function, identical to a previous report (Cross et al., 2002), although both lower and

higher recoveries after 20 min of ischemia have also been observed. In diabetic hearts, recovery was 76 %, mainly due to the lower baseline pre-ischemia reflecting diabetic-induced ventricular dysfunction. Reperfusion systolic and diastolic performance was improved in the non-diabetic state by the transgene, confirming the well-known beneficial effects of NO in this setting (Jones and Bolli, 2006). In diabetic hearts, however, TG hearts developed identical wall pressure and diastolic compliance as WT hearts, reflecting the selective loss of NO-mediated tissue protection (Fig. 2B and 3B). Because nitrite levels in coronary effluents of TG diabetic hearts were not reduced compared to non-diabetic hearts, impaired formation of NO is unlikely to account for the loss of functional protection. An important hint is provided by the reduced cGMP levels observed in TG diabetic hearts. The reduced formation of this mediator likely reflects a curtailed efficacy of guanylyl cyclase activation, as would be the case if NO were inactivated prior to its action, e. g. by reperfusion-generated ROS. In this regard, NO is known to react with superoxide anion to yield peroxynitrite which exerts a number of tissue-toxic effects (Beckman and Koppenol, 1996; Ferdinandy and Schulz, 2003). In the present study, we provide direct ESR evidence for increased formation of ROS immediately on reperfusion in TG diabetic hearts. Because CP-H, the ESR probe we used, recognizes superoxide anion (Fig 7A), it is likely that the impaired NO protection in our TG diabetic hearts is partly due to reaction of NO with superoxide to form peroxynitrite, which results in protein nitration and myocardial injury (Zweier et al., 2001). There is also evidence that eNOS may be uncoupled in the diabetic state due to a lack of the NOS co-factor tetrahydrobiopterin (Pieper, 1997), which would increase the cellular superoxide formation and impair NO-dependent myocardial functions, particularly diastolic relaxation (Shah and MacCarthy, 2000). Taken together, this interpretation is in line with the broader view that diabetic complications are linked to oxidative processes and tissue injury (Rösen et al., 1998; Hinokio et al., 1999; Ye et al.,

2003). Therefore, to retain the cardioprotective effects of NO in diabetes, effective inactivation of cardiac ROS such as by radical scavengers appears to be necessary .

It was recently suggested that the decreased release of NO from the vascular endothelium contributes to the depressed modulation of O_2 consumption by endogenous NO after diabetes (Zhao et al., 2000). In the present study, bradykinin inhibited O_2 consumption to a greater extent in TG than WT muscle (from non-diabetic animals) (Fig. 8A). This is novel evidence for the control of cardiac respiration by endogenous, myocyte-derived NO in amounts generated by a physiological agonist, extending previous evidence for a similar role of endothelium-derived NO (Poderoso et al., 1998; Trochu et al., 2000). In reperfused (non-diabetic) ventricle, O_2 uptake was no longer preferentially inhibited in TG tissues, possibly indicating a metabolic compensation in the face of limited oxygen availability during ischemia, which might partly explain the improved recovery of reperfusion function in TG hearts (compare Fig. 8B and 2A). Our studies also showed that both exogenous NO- (DEA/NO) and bradykinin-stimulated NO formation exert inhibitory effects on O_2 consumption in the cardiac muscle from diabetic mice. Taken together, O_2 consumption measurements suggest that tonic control of the respiratory chain by myocardial NO guards the heart against metabolic stresses such as ischemia and reperfusion of short duration.

In summary, these studies demonstrated that increased formation of NO as a result of overexpressing eNOS in myocytes leads to energy-conserving cardiac contractile hyporesponsiveness and functional protection during short-term ischemia-reperfusion of non-diabetic hearts. In diabetic hearts, the enhanced formation of superoxide anion and/or peroxynitrite exhausted the salubrious effects of NO on myocardial diastolic and systolic reperfusion function. Thus, with increasing oxidant stress, both a high NO supply and effective antioxidants are necessary to guard against diabetic cellular injury.

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Footnotes

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FIGURE LEGENDS

FIG. 1. Ventricular function of WT and TG hearts in normoxic perfusion. Left ventricular developed pressure (LVDevP; A), maximum speed of ventricular contraction (+dp/dt, B) or relaxation (-dp/dt, C) of isolated perfused non-diabetic or diabetic wild-type (WT; open bars) and transgenic (TG; filled bars) hearts are shown. The diabetic+L-NAME group refers to diabetic mice treated with L-NAME over two days prior to the experiments. Data are mean ± S.E.; for number of experiments (n), see Table 1. * significantly different from WT; ‡ significantly different from non-diabetic hearts (unpaired t-test).

FIG. 2. Reperfusion systolic function. Left ventricular developed pressure (LVDevP) of non-diabetic (A) and diabetic (B) hearts from wild-type (WT; open symbols) and transgenic (TG; closed symbols) animals, either not treated (squares) or treated (diamonds) with L-NAME. Data are mean ± S.E.; for n, see Table 1. * significantly different from WT; #, significantly different from respective genotype of diabetic group (two-way ANOVA).

FIG. 3. Reperfusion diastolic function. Left ventricular end-diastolic pressure (LVEDP) of non-diabetic (A) and diabetic (B) hearts from wild-type (WT; open symbols) and transgenic (TG; closed symbols) animals, either not treated (squares) or treated (diamonds) with L-NAME. Data are mean ± S.E.; for n, see Table 1. * significantly different from WT; #, significantly different from respective genotype of diabetic group; P=NS for diabetic WT vs. TG group (two-way ANOVA).

FIG. 4. Reperfusion coronary function. Coronary perfusion pressure of non-diabetic and diabetic hearts from wild-type (WT; open symbols) and transgenic (TG; closed symbols) animals, either not treated (squares) or treated (diamonds) with L-NAME. Data are mean ± S.E.; for n, see Table 1. ‡ significantly different from non-diabetic hearts (baseline: unpaired t-test; curves: two-way ANOVA).

FIG. 5. Cardiac nitrite release in non-diabetic (A) and diabetic (B) hearts. The graph shows the release at baseline and during early reperfusion for wild-type (WT; open bars) and transgenic (TG; filled bars) hearts. Data are mean ± S.E.; for n, see Table 1. * significantly different from respective WT (two-way ANOVA).

Fig 6. Cardiac cGMP release in non-diabetic (A) and diabetic (B) hearts and cardiac cGMP tissue levels (C). The graph shows the release at baseline and during 30 min of reperfusion for wild-type (WT; open symbols) and transgenic (TG; filled symbols) hearts. Tissue levels were determined at the end of reperfusion. Data are mean ± S.E.; for n, see Table 1. * significantly different from respective WT group; NS=non-significant (A, B: two-way ANOVA; C: unpaired t-test).

Fig. 7. Cardiac ROS-release during reperfusion. A: ESR signal in vitro resulting from the reaction of superoxide anion ($O^{2-\bullet}$) in the absence and presence of SOD (50 U/ml), and of peroxynitrite ($ONOO^-$) in the absence and presence of urate (50 µM), respectively. B, C: ROS release rates in coronary effluents of non-diabetic and diabetic wild-type (WT; open bars) and transgenic (TG; filled bars) hearts, respectively. Data are mean ± S.E.; for n, see Table 1. * significantly different from respective WT group (two-way ANOVA).

FIG. 8. Inhibition of myocardial O_2 consumption by endogenous NO. In normoxia (A), the inhibitory effect of bradykinin (10 µM) was significantly greater in TG (closed bars) than WT (open bars) hearts, whereas there was no difference between genotypes at the end of reperfusion (B). Data are mean ± S.E. of 13-15 individual determinations. * significantly different from WT; NS=non-significant (unpaired t-test).

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Table 1: Baseline characteristics of control and diabetic groups

	Non-diabetic		**Diabetic**	
	wild-type	*transgenic*	*wild-type*	*transgenic*
Set I				
Blood glucose (mg/dL)	101±3	103±3	518±31*	520±45*
Body weight (g)	26.2±2.1	25.8±2.3	20.2±0.8*	21.1±0.9*
Heart weight (g)	0.135±0.006	0.127±0.005	0.121±0.002*	0.115±0.002*
Heart/body weight	0.0052±0.0002	0.0050±0.0003	0.0060±0.0003	0.0055±0.0002
Set II				
Blood glucose (mg/dL)	108±3	109±2	543±19*	565±35*
Body weight (g)	24.3±1.1	25.5±1.2	23.2±1.2*	24.2±0.7*
Heart weight (g)	0.122±0.004	0.119±0.004	0.117±0.005	0.117±0.003
Heart/body weight	0.0051±0.0002	0.0047±0.0002	0.0051±0.0002	0.0049±0.0002
Set III				
Blood glucose (mg/dL)	105±5	107±4	530±22*	512±31*
Body weight (g)	25.9±2.1	26.1±1.6	21.1±1.2*	20.8±1.4*
Heart weight (g)	0.133±0.008	0.124±0.007	0.118±0.006	0.114±0.007
Heart/body weight	0.0051±0.0003	0.0048±0.003	0.0056±0.004	0.0055±0.003
Set IV				
Blood glucose (mg/dL)	101±3	103±3	518±31*	520±45*
Body weight (g)	26.2±2.1	25.8±2.3	19.9±1.1*	20.8±1.5*
Heart weight (g)	0.135±0.006	0.127±0.005	0.119±0.001	0.115±0.004
Heart/body weight	0.0052±0.0002	0.0050±0.0003	0.0059±0.0003	0.0056±0.0003

Data are mean ± S.E. The number of animals was 5 (non-diabetic) and 10 (diabetic) in set I, 7 in set II, 4 in set III and 5 in set IV. *P<0.05 vs. corresponding non diabetic group, based on ANOVA analysis of data pooled from all 4 sets.

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Table 2: NOS activities in cardiac homogenates from the four experimental groups.

	Non-diabetic		**Diabetic**	
	wild-type	*transgenic*	*wild-type*	*transgenic*
Total activity	0.12±0.04	10.48±1.93	0.16±0.05	8.11±2.58
L-NNA (100 µM)	-0.02±0.01	0.07±0.03	-0.02±0.02	0.05±0.04
1400W (10 µM)	0.08±0.02	9.05±2.07	0.1±0.03	7.51±2.21
EDTA (5 mM)	0.0±0.0	0.0±0.0	0.0±0.0	0.0±0.0

Enzyme activity is given as pmol [^{3}H]L-citrulline formation per min and mg protein. Data are mean ± S.E. of 5 non-diabetic and 4 diabetic hearts. Activities obtained in the presence of 1400W were not significantly different from total activity (P=NS).

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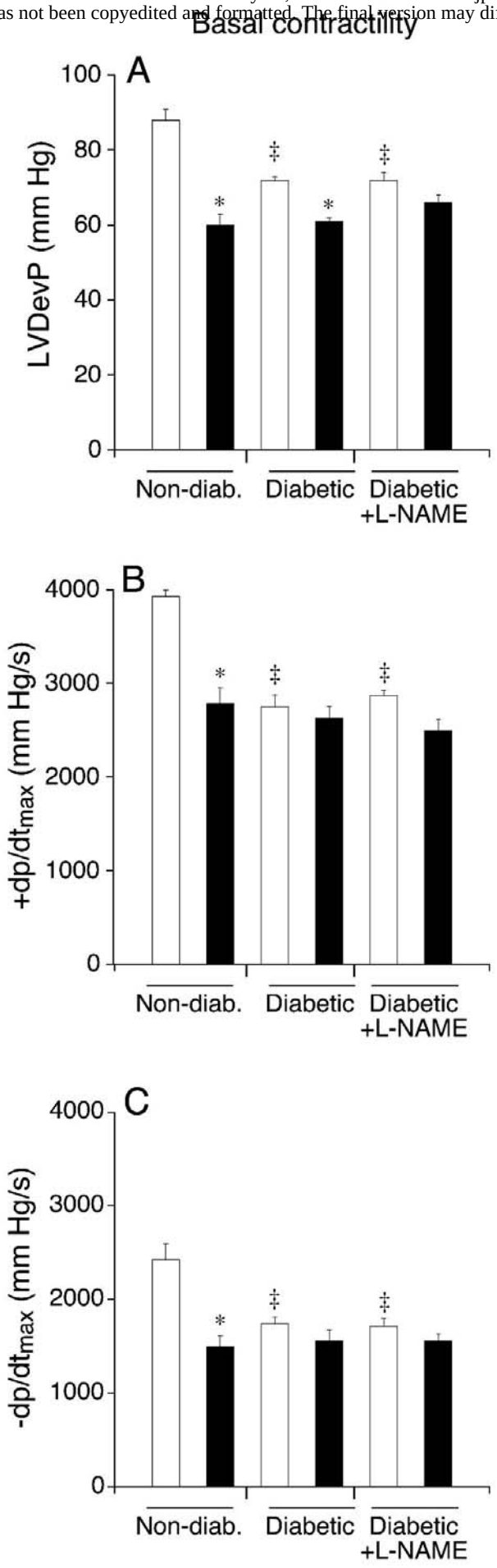

Figure 1

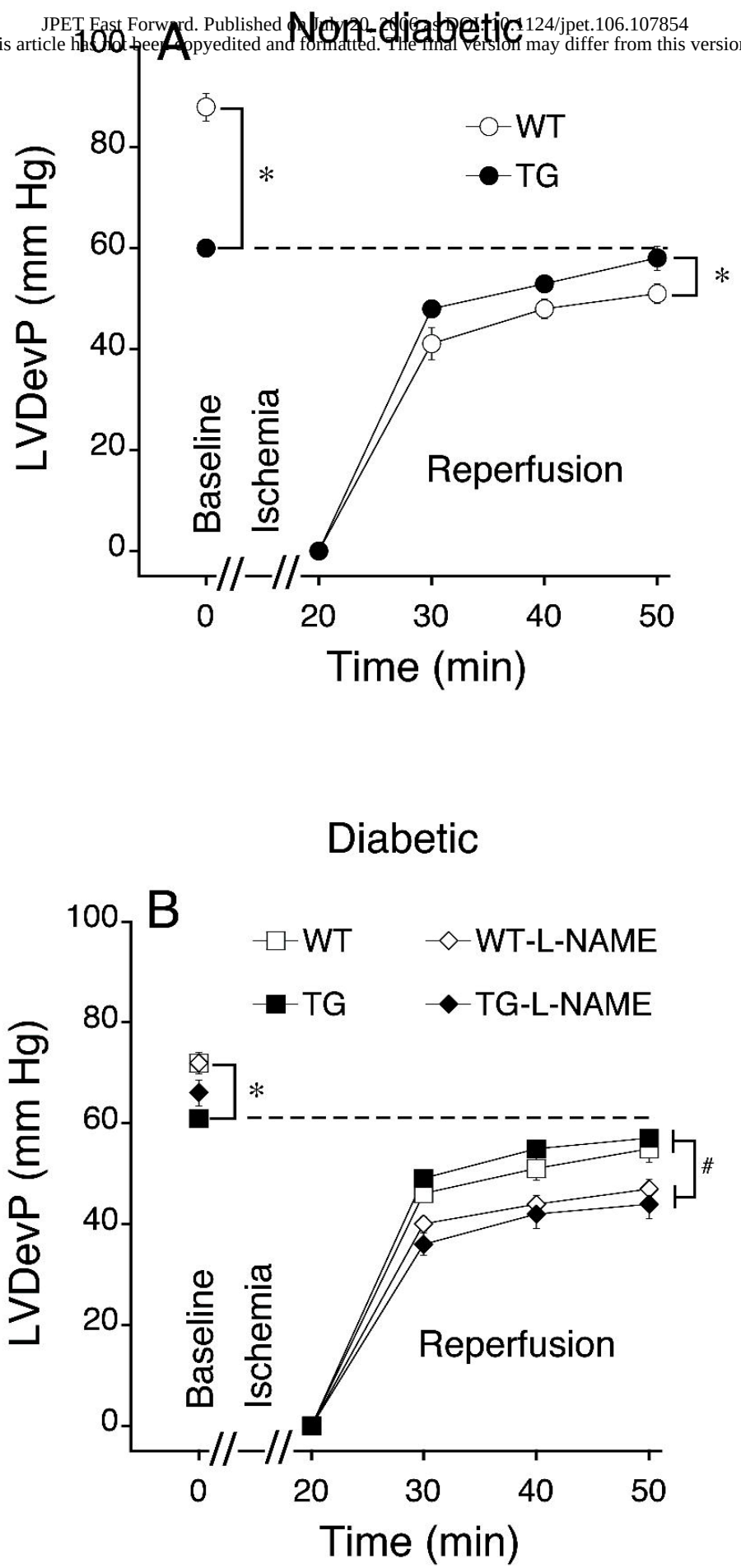

Figure 2

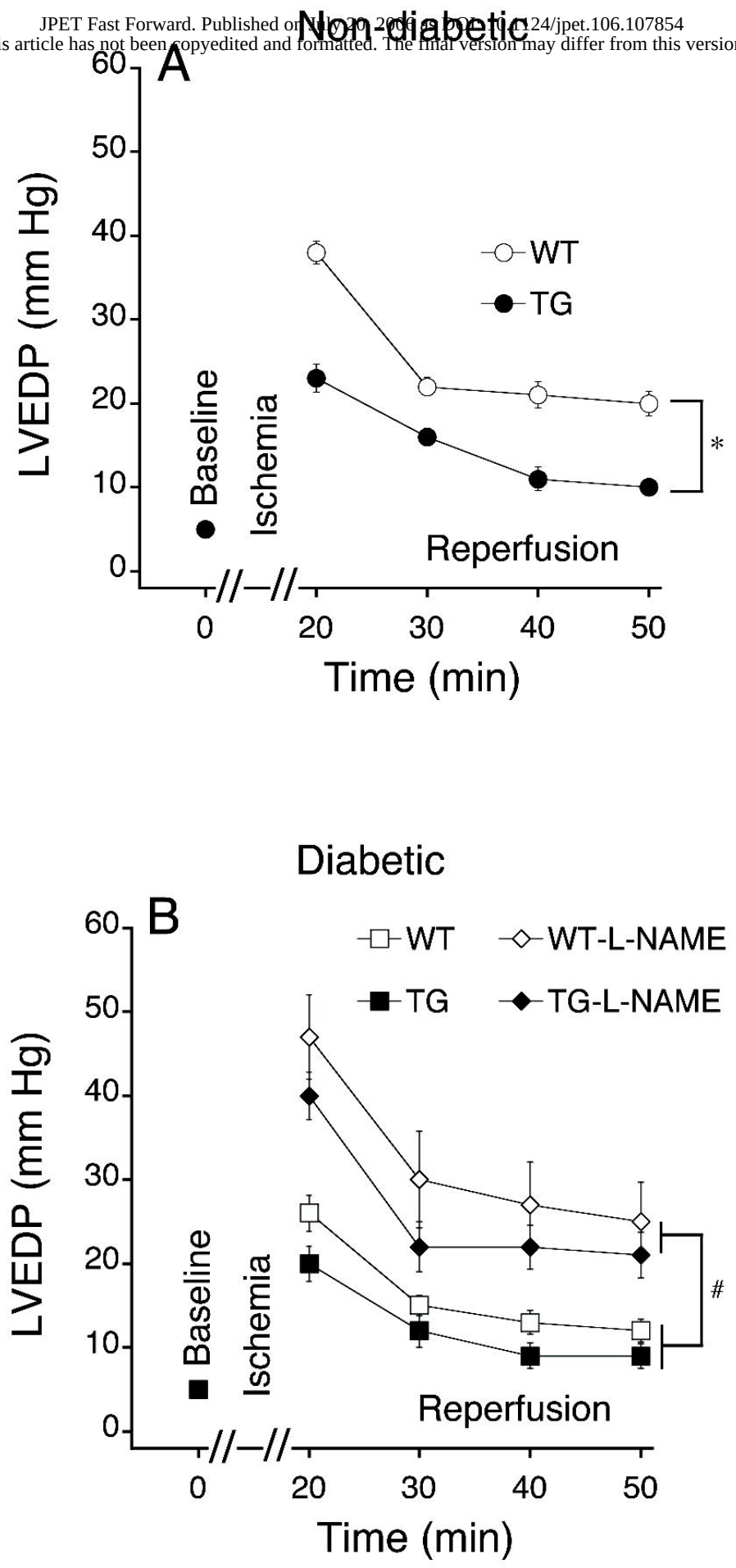

Figure 3

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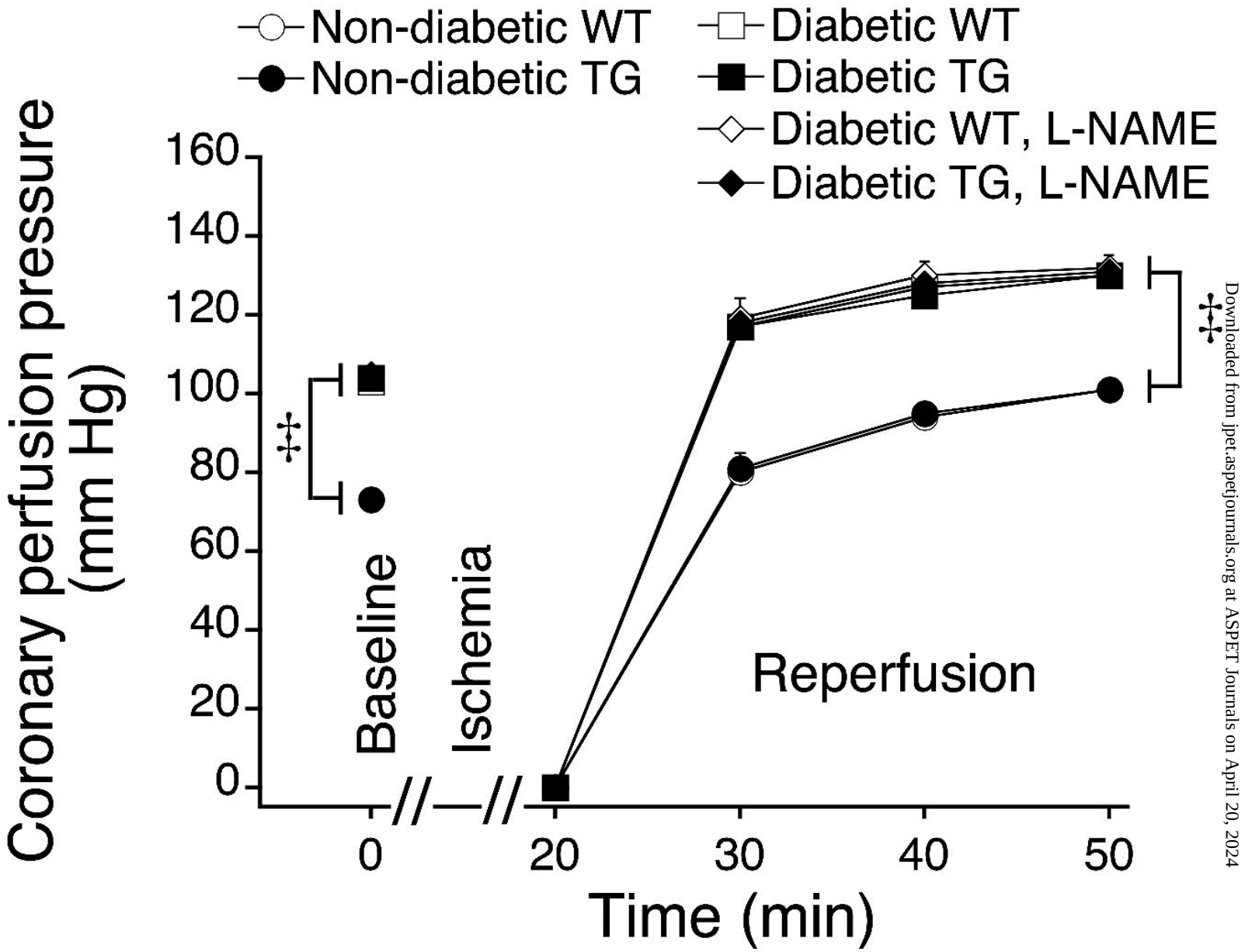

Figure 4

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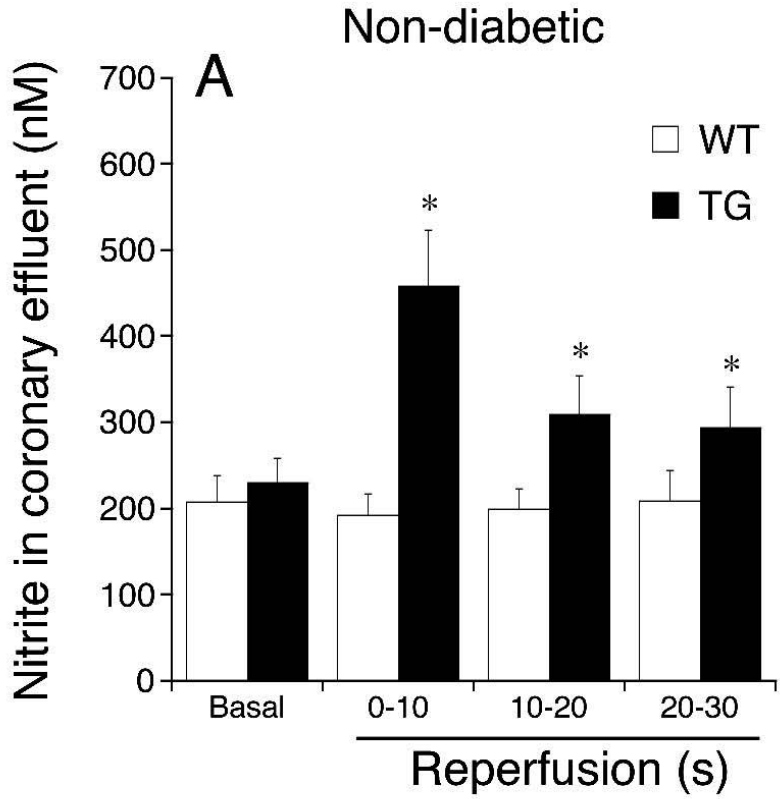


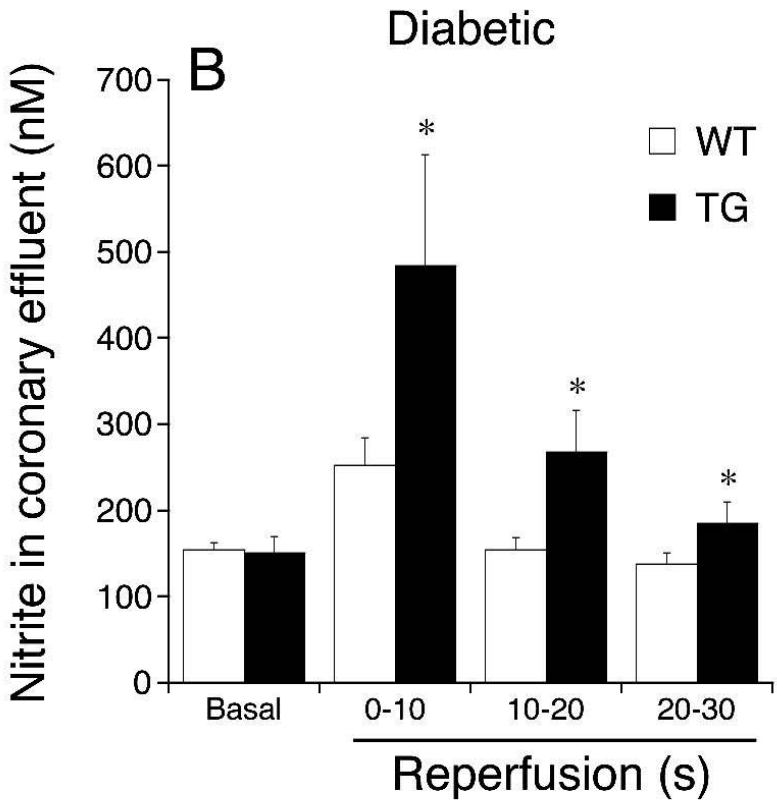
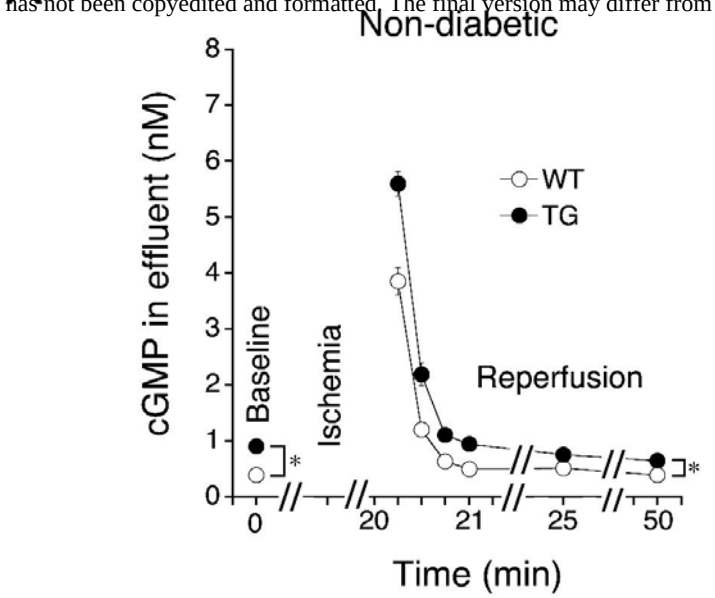
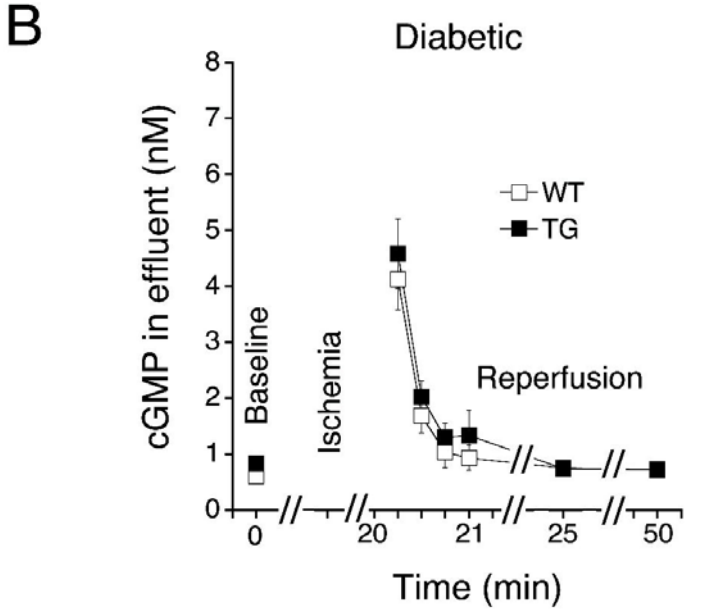


Figure 5





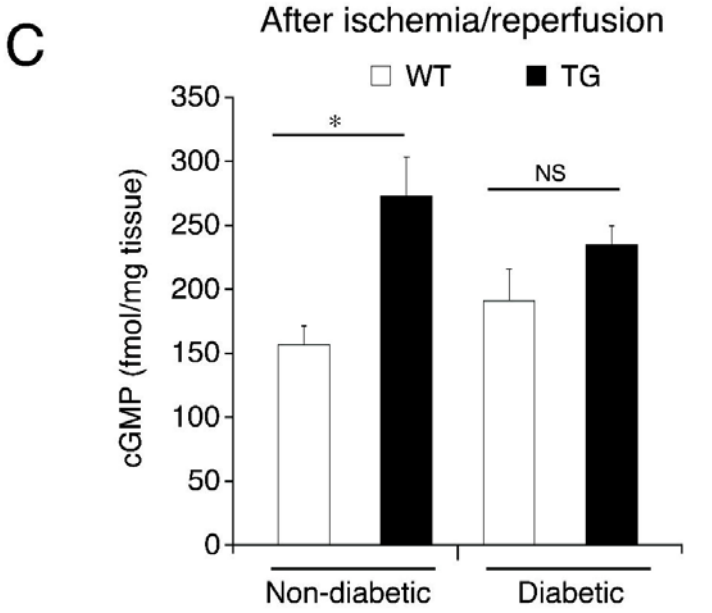


Figure 6

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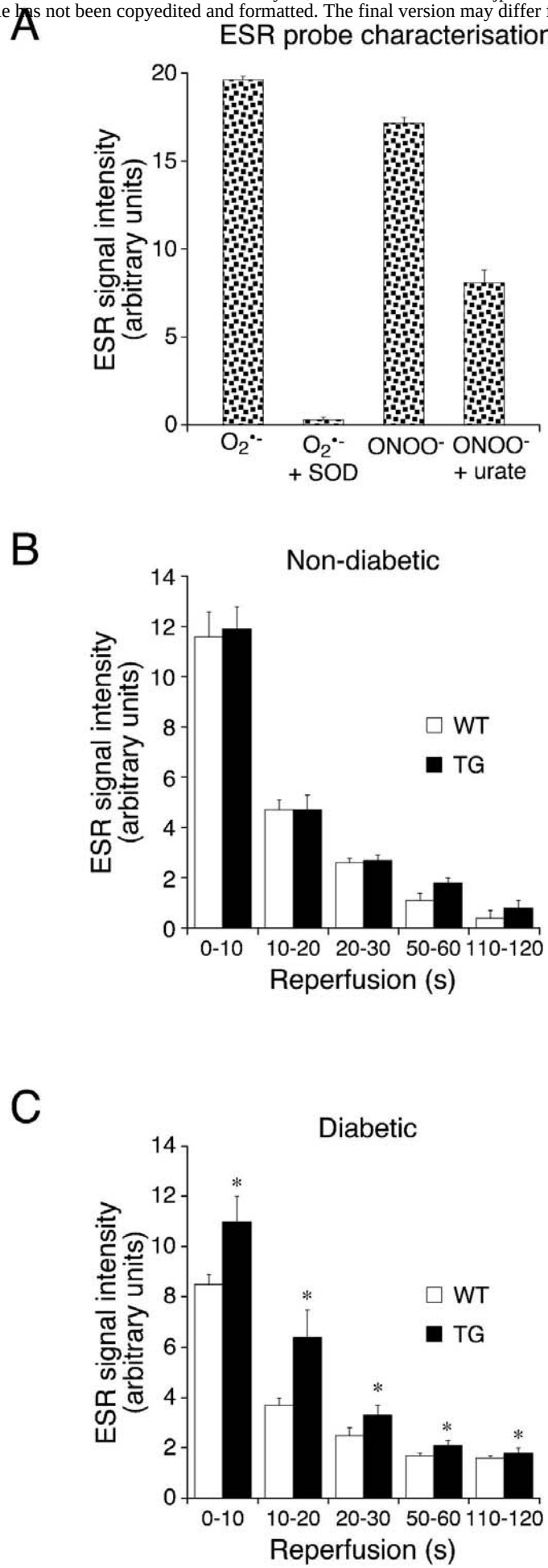

Figure 7

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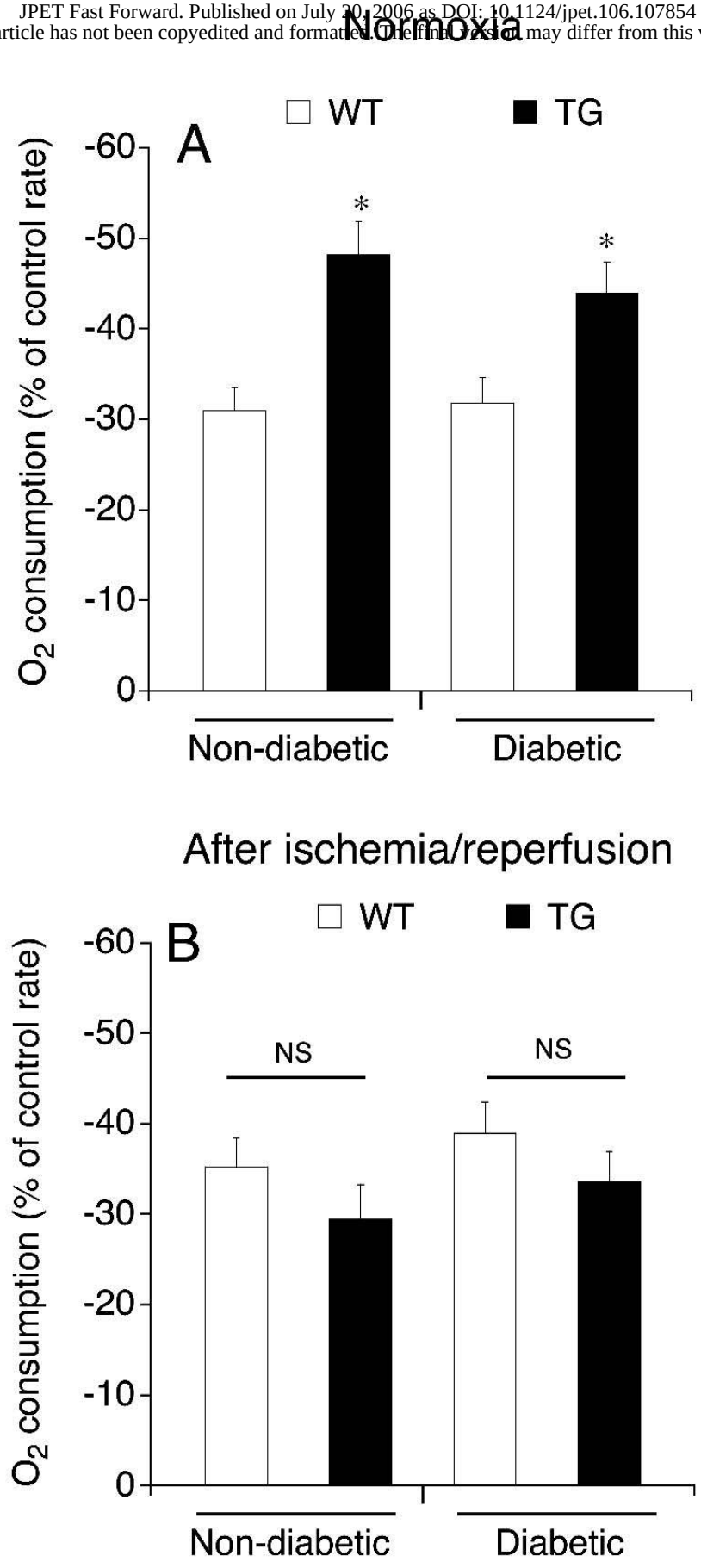

Figure 8