European Journal of Pharmacology 611 (2009) 53–59

Contents lists available at ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Cardiovascular Pharmacology

Chronic inhibition of inducible nitric oxide synthase ameliorates cardiovascular abnormalities in streptozotocin diabetic rats

Prabhakara Reddy Nagareddy, John H. McNeill, Kathleen M. MacLeod *

Faculty of Pharmaceutical Sciences, 2146 East Mall, The University of British Columbia, Vancouver, BC, Canada V6T 1Z3

ARTICLE INFO

Article history:
Received 13 November 2008
Received in revised form 11 March 2009
Accepted 23 March 2009
Available online 1 April 2009

Keywords:
Inducible nitric oxide synthase
Nitric oxide
Endothelial dysfunction
Cardiovascular abnormalities
Diabetes

ABSTRACT

Previous studies from our lab have demonstrated cardiovascular abnormalities such as depressed mean arterial blood pressure and heart rate, endothelial dysfunction and attenuated pressor responses to vasoactive agents in streptozotocin diabetic rats. We investigated whether these abnormalities are due to diabetes-associated chronic activation of inducible nitric oxide synthase (iNOS). Control and streptozotocin (60 mg/kg, *iv*) diabetic rats were treated with either vehicle or N6-(1-Iminoethyl)-L-lysine dihydrochloride (L-NIL, 3 mg/kg/day, p.o), a specific inhibitor of iNOS for 8 weeks. At the end of treatment, the mean arterial blood pressure and heart rate were measured in freely moving conscious rats. Further, pressor responses to bolus doses of methoxamine were determined. Endothelial nitric oxide synthase (eNOS) and iNOS expression as well as nitrotyrosine (NT) levels were assessed in the heart and superior mesenteric arteries by western blot and immunohistochemistry. Untreated diabetic rats showed depressed mean arterial blood pressure and heart rate and exhibited vascular hyporeactivity that were significantly improved by treatment with L-NIL. Further, decreased eNOS expression and increased iNOS expression and activity were associated with increased NT levels in the heart and superior mesenteric arteries of untreated diabetic rats. L-NIL treatment of diabetic rats normalized the expression of eNOS and NT levels without any effect on iNOS expression in the heart and superior mesenteric arteries. The results of our study suggest that induction of iNOS in cardiovascular tissues contributes significantly to the depressed mean arterial blood pressure, heart rate and pressor responses to vasoactive agents. Chronic inhibition of iNOS in diabetes may prove beneficial in the treatment of cardiovascular abnormalities.

1. Introduction

Cardiovascular abnormalities, manifested by depressed mean arterial blood pressure, heart rate, cardiac output, endothelial dysfunction and attenuated pressor responses to vasoactive agents are the key pathophysiological events associated with hyperglycemia, particularly in animal models of type 1 diabetes (Cheng et al., 2004; Smith et al., 1997). Although the exact mechanisms by which chronic hyperglycemia contributes to these abnormalities are currently unknown, a number of mechanisms have been proposed including activation of protein kinase C, increased activity of the polyol pathway, formation of non-enzymatic advanced glycosylation end products, oxidative stress and/or possibly by induction of nitric oxide synthase (Ido et al., 1997; Williamson et al., 1993). Studies from our lab and elsewhere have demonstrated an increased expression of iNOS in cardiac, vascular and renal tissues of streptozotocin diabetic rats (Bardell and MacLeod, 2001; Bojunga et al., 2004; Nagareddy et al., 2006, 2005).

Physiological concentrations of nitric oxide (NO) maintain the vasculature in a state of active vasodilatation and regulate regional blood flow to tissues such as heart and kidney in response to local environmental changes (Lane and Gross, 1999; Moncada et al., 1991). NO in large quantities however, is toxic to the cardiovascular system, particularly when associated with increased levels of reactive oxygen species such as superoxide anions (Ceriello et al., 2001, 2002). Both NO and superoxide anions are highly reactive and can rapidly form peroxynitrite [$ONOO^-$], a cytotoxic compound (Beckman and Koppenol, 1996; Ferdinandy et al., 2000). In fact, peroxynitrite is a favored product under conditions of hyperglycemia where cellular production of both NO and reactive oxygen species are increased (Ceriello et al., 2001, 2002). Further, peroxynitrite has been shown to cause severe hypotension, profound vasodilatation, cardiac depression and multiple organ failure in various models of septic shock (Ferdinandy et al., 2000; Parrillo, 1993). Previous studies from our lab demonstrated that acute inhibition of iNOS improves vascular reactivity but not depressed mean arterial blood pressure and heart rate in streptozotocin diabetic rats (Nagareddy et al., 2005). In the present study we hypothesized that long-term inhibition of iNOS is necessary to

* Corresponding author. Tel.: +1 604 822 3830; fax: +1 604 822 3035.
E-mail address: kmm@interchange.ubc.ca (K.M. MacLeod).

0014-2999/$ – see front matter © 2009 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2009.03.061

prevent endothelial dysfunction and depression of mean arterial blood pressure, heart rate and pressor responses to vasoactive agents.

2. Methods

2.1. Study design and induction of diabetes

This study conforms to the Canadian Council on Animal Care Guidelines on the Care and Use of Experimental Animals. Sixty-four male Wistar rats weighing between 280 and 300 g were obtained from Charles River Laboratories Inc., Quebec and allowed to acclimatize to the local *vivarium*. They were randomly divided into four equal groups: Control (C), Control treated (CT), Diabetic (D) and Diabetic treated (DT). Diabetes was induced by a single tail vein injection of 60 mg/kg streptozotocin. The presence of diabetes was confirmed by hyperglycemia (>20 mmol/L) 72 h after streptozotocin administration. Plasma glucose was measured by an enzymatic colorimetric assay kit (Roche Diagnostics, Laval, Quebec) using a Beckman Glucose Analyzer. One week after the induction of diabetes, CT and DT groups received the selective iNOS inhibitor, N6-(1-Iminoethyl)-L-lysine dihydrochloride (L-NIL) at a dose of 3 mg/kg/day by oral gavage. L-NIL is a potent and relatively a selective iNOS inhibitor with an IC50 of 5.9 μM for iNOS compared to an IC50 of 138 μM for eNOS and 35 μM for nNOS (Hallinan et al., 2002). A dose of 3 mg/kg/day was selected based on our previous study in which we were able to inhibit iNOS derived NO production in heart tissues from streptozotocin diabetic rats (Soliman et al., 2008). After 8 weeks of treatment each animal was surgically prepared for measurement of mean arterial blood pressure and heart rate.

2.2. Surgical procedures

Rats were anesthetized with halothane and fluid-filled (heparinised saline, 20 U/ml) catheters were placed in the left carotid artery (PE 50) and jugular vein (PE 10) for measurements of blood pressure and for drug administration respectively. All the catheters were exteriorized at the nape of the neck, passed through a harness and tether and connected to swivels (Instec Lab Inc., PA) mounted above the cage for free movement of the animal. The arterial catheter was connected to a disposable pressure transducer (Viggo-Spectramed, CA) mounted on the cage exterior at the level of the rat. Mean arterial blood pressure and heart rate were simultaneously recorded on a Gould TA 2000 Thermal Array Recorder (Gould Instrument System Inc., OH) and a computer, using custom-made data acquisition software. In order to achieve normalization of cardiac baroreflexes, the animals were allowed to recover from anesthesia and surgery for at least 4 h before recording mean arterial blood pressure and heart rate (Hebden et al., 1987).

Four hours after surgery, basal mean arterial blood pressure and heart rate were measured in all rats. Subsequently, dose response curves (dose response curve) to isovolumic bolus doses of methoxamine (100–300 nmol/kg) were constructed in control and diabetic rats. Dose response curves were constructed by measuring mean arterial blood pressure in response to each bolus dose of methoxamine, allowing sufficient time (or 10 min) for mean arterial blood pressure to return to normal between each dose. At termination, the heart and superior mesenteric artery or whole mesenteric arterial bed were immediately removed and placed in ice-cold Krebs solution (120 mM NaCl, 5.9 mM KCl, 25 mM $NaHCO_3$, 11.5 mM glucose, 1.2 mM NaH_2PO_4, 1.2 mM $MgCl_2$, 2.5 mM $CaCl_2$) containing 0.1 μM water-soluble dexamethasone to prevent induction of iNOS *in vitro*. The tissues were cleaned of all adherent tissue, snap frozen in liquid nitrogen and stored at −70 °C for Western blot and iNOS activity (in superior mesenteric artery) measurements.

2.3. Western blot studies

Protein expression and NT levels in superior mesenteric arteries and hearts were determined by western blot analysis using specific antibodies directed against eNOS, iNOS and NT. Briefly, aliquots of the pulverized superior mesenteric artery from each rat were homogenized in modified RIPA buffer containing 50 mM Tris–HCl pH 7.4, 150 mM NaCl, 1 mM PMSF, 1 mM EDTA, 5 μg/ml aprotinin, 5 μg/ml leupeptin, 1% Triton x-100, 1% Sodium deoxycholate and 0.1% SDS at 4 °C, using a homogenizer. The homogenate was centrifuged at 10,000× *g* at 4 °C for 15 min. Total protein content of the supernatant was determined using the Bio-Rad protein assay, which is based on Bradford method (Bradford, 1976). Aliquots of protein were separated by SDS-7.5% (for eNOS and iNOS) or SDS-10% (for NT) polyacrylamide gel electrophoresis. Immunoblotting was performed either with a mouse monoclonal anti-iNOS (1:200, BD Transduction Labs), anti-NT (1:500, Cayman Chemical Company) or rabbit polyclonal anti-eNOS antibody (Upstate Biotechnology). HRP conjugated goat anti-rabbit and anti-mouse secondary antibodies were obtained from Santa Cruz Biotechnology. The immune complexes were detected using enhanced chemiluminescence dye (ECL, Amersham Pharmacia Biotech, Piscataway, NJ). The intensity of the bands was determined using image J software from NIH.

2.4. Immunohistochemistry of iNOS and NT in the heart and superior mesenteric artery sections

The heart and superior mesenteric artery tissues were fixed in 10% NBF overnight and transferred to 70% ethanol. This was followed by paraffin processing through increasing grades of ethanol, xylene and paraplast (Fischer Scientific, ON). Paraffin embedded tissue blocks were sectioned at 3 μm and mounted on slides. The sections were deparaffinized, rehydrated, washed with PBS and blocked with 5% normal goat serum (NGS) in PBS for 60 min. The slides were subsequently incubated with primary rabbit polyclonal anti-iNOS (1:100, Abcam) or mouse monoclonal anti-NT (1:200, Cayman Chemicals) antibodies in PBS containing 1% NGS overnight at 4 °C in a humidity chamber. After washing off the primary antibody with PBS, the sections were incubated with biotinylated goat anti-rabbit or anti-mouse secondary antibody (1 drop in 10 mL PBS, Vectastain ABC kit, Vector Laboratories) for 1 h followed by avidin-biotinylated HRP complex and color development performed using 3,3-diaminobenzidine. Some sections incubated without primary antibodies served as negative controls. Using a high power microscope and digital imaging system all images were observed individually and photographed (10× or 100×). In each section, 4–5 random fields were selected and staining intensity analyzed using a scale ranging from 1 to 5.

2.5. iNOS activity assay in the mesenteric arterial bed

iNOS activity in the mesenteric arterial bed was measured by monitoring the biochemical conversion of [^{14}C]L-arginine to [^{14}C]L-citrulline using a commercially available NOS assay kit (Cayman Chemical, Ann Arbor, MI). Briefly, mesenteric arterial bed homogenates in buffer were centrifuged, and supernatant (80 μg protein) was added to a calcium free reaction mixture composed of buffer, 10 mM NADPH, and 10 μl of [^{14}C] L-arginine (50 μCi/μl). The reaction was maintained for 60 min at room temperature and stopped using stop buffer. The remaining [^{14}C] L-arginine was removed from the solution using an equilibrated resin. Finally, the radioactivity was measured using a liquid scintillation counter.

2.6. Statistical analysis

All values are expressed as mean ± S.E.M. "n" denotes the sample size in each group. Statistical analysis was performed using one-way analysis of variance (ANOVA) followed by the Newman–Keuls test for

Table 1
General characteristics of rats following 8 weeks of L-NIL treatment.

Parameters ($n=16$)	C	D	CT	DT
Body weight (g)				
Before treatment	365 ± 21.4	338 ± 16.1	357 ± 20.2	335 ± 14.6
After treatment	580 ± 14.9	384 ± 10.8[a]	571 ± 9.6	408 ± 9.3
Blood glucose (mM)	7.88 ± 0.13	25.45 ± 1.2[a]	7.4 ± 0.13	26.28 ± 0.64

Data represent means ± S.E.M. Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test.
[a] Significantly different from C and CT.

multiple comparisons. GraphPad Prism (GraphPad Software, CA) software program was used for statistical analysis. For all results the level of significance was set at $P<0.05$.

3. Results

3.1. General characteristics

The general characteristics of rats following 8 weeks of L-NIL treatment are shown in Table 1. All streptozotocin-injected rats were hyperglycemic (>20mM) compared to their age matched controls. In addition, body weights of both untreated and treated diabetic rats were significantly lower than their age matched controls. Treatment of diabetic rats with L-NIL did not change either the body weight or blood glucose in any of the groups.

3.2. Effect of L-NIL on mean arterial blood pressure and heart rate

Untreated diabetic rats showed significantly lower mean arterial blood pressure (Fig. 1A) and heart rate (Fig. 1B) than matched control rats. Treatment with L-NIL however, improved the depression in both mean arterial blood pressure and heart rate in diabetic rats without affecting these parameters in control animals.

3.3. Effect of L-NIL treatment on pressor responses to methoxamine

Administration of bolus doses of methoxamine (Fig. 2) increased mean arterial blood pressure in both control and diabetic rats in a dose-dependent manner. Compared to the corresponding age matched control rats, the responses to methoxamine were significantly attenuated in untreated diabetic rats. However, treatment of diabetic rats with L-NIL significantly augmented pressor responses without affecting the pressor responses in control rats.

3.4. Effect of L-NIL treatment on eNOS, iNOS and NT expression in the heart and superior mesenteric artery

Decreased eNOS expression was observed both in the heart (Fig. 3A) and superior mesenteric artery (Fig. 3B) of untreated diabetic rats compared to C and CT rats. L-NIL treatment of diabetic rats improved the expression of eNOS to that in the control rats. On the contrary, untreated diabetic rats exhibited increased expression of iNOS both in the heart (Fig. 4A) and superior mesenteric artery (Fig. 4B) as determined by immunoblotting. Further, immunohistochemical analysis revealed increased localization of iNOS in the medial and advential layers of superior mesenteric arteries (Fig. 5) from untreated diabetic rats which is consistent with our previous findings (Bardell and MacLeod, 2001). As opposed to the dense immunostaining in the medial and advential layers, very little staining can be observed in the tunica intima or endothelium, suggesting that the major source of iNOS is media and adventia. As expected treatment with L-NIL did not change the expression of iNOS (Fig. 4) but its activity was significantly reduced in superior mesenteric arteries from diabetic rats (Fig. 6).

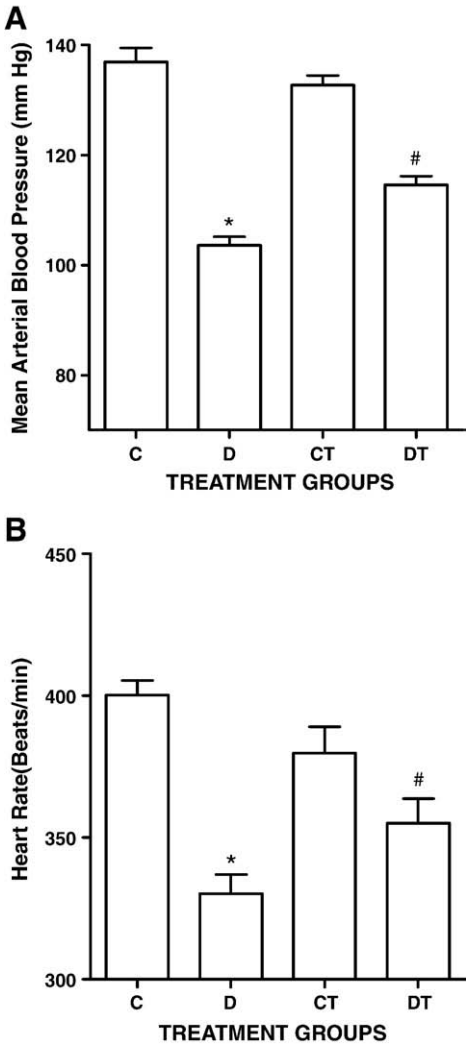

Fig. 1. Effect of 8 weeks of L-NIL treatment on mean arterial blood pressure (A) and heart rate (B) in freely moving conscious rats ($n=16$). Data represent means ± S.E.M. Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C, CT and DT groups; # different from C, D and CT groups ($P<0.05$).

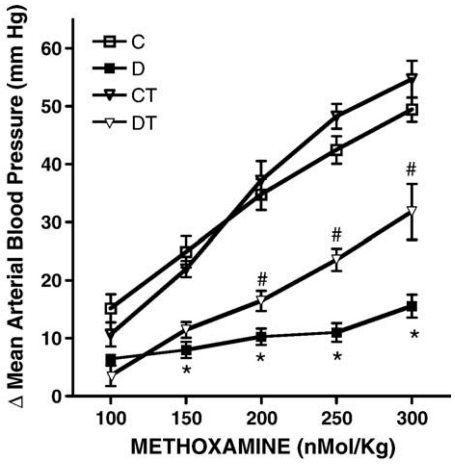

Fig. 2. Effect of 8 weeks of L-NIL treatment on pressor responses to bolus doses of methoxamine (100–300 nmol) in freely moving conscious rats ($n=8$). Data represent means ± S.E.M. Δmean arterial blood pressure represents change in blood pressure (compared to basal blood pressure) in response to every bolus dose of methoxamine. Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C and CT groups; # different from D group ($P<0.05$).

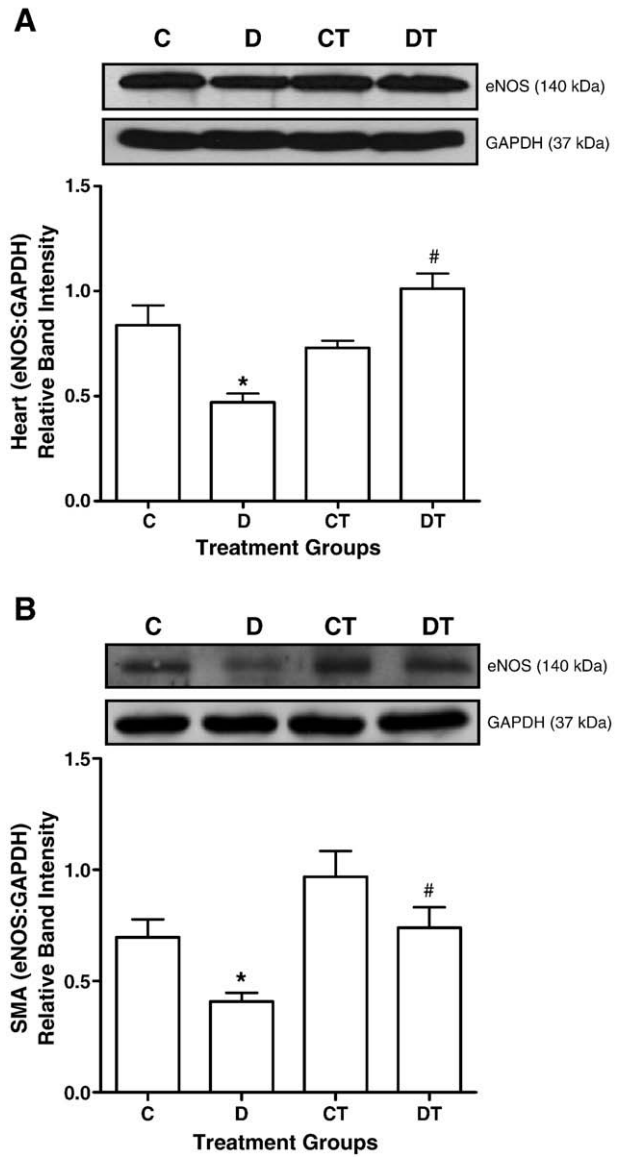

Fig. 3. Effect of L-NIL treatment on eNOS expression in the heart (A) and superior mesenteric artery (SMA) (B). Western blot analysis showing expression of eNOS and the corresponding GAPDH protein in the heart and superior mesenteric artery. Densitometric analysis of eNOS expression (corrected by normalizing to the corresponding GAPDH expression) in the heart and superior mesenteric artery ($n=8$). Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C, CT and DT groups; # different from D group ($P<0.05$).

Similarly, Western blot analysis of NT, an indirect marker of peroxynitrite formation, indicated an increase in the nitration of proteins in the hearts (Fig. 7A) and superior mesenteric artery (Fig. 7B) of untreated diabetic rats. Among various nitrated proteins, an unidentified protein with a molecular weight of 80 kDa showed increased nitration both in the hearts and superior mesenteric arteries. Further, the detection of elevated levels of NT in whole hearts (Fig. 8) suggests that cardiomyocytes rather than inflammatory or endothelial cells are the major sites of iNOS expression and probably the source for increased peroxynitrite formation in the diabetic heart. As shown, there is a substantial increase in the levels of NT in the diabetic heart (Fig. 8; panel B) and pictures of higher magnification ($\times 100$) clearly demonstrate that the immunostaining is predominantly located in cardiomyocytes which are histologically characterized by their elongated brick-like appearance, relatively large size and striations (Fig. 8; panel E). Treatment with L-NIL significantly decreased the formation of NT in the DT group compared to untreated diabetic rat not only in superior mesenteric artery but also heart (Fig. 7).

4. Discussion

The major findings of the present study are that chronic inhibition of iNOS attenuated the depression of mean arterial blood pressure and heart rate that is found in streptozotocin diabetic rats. This was associated with normalization of the expression of vascular eNOS, reduced formation of NT and improved pressor responsiveness to α_1-adrenoceptor stimulation. These data, together with the results of our previous studies, highlight an important role for iNOS in mediating cardiovascular depression in the streptozotocin diabetic rat.

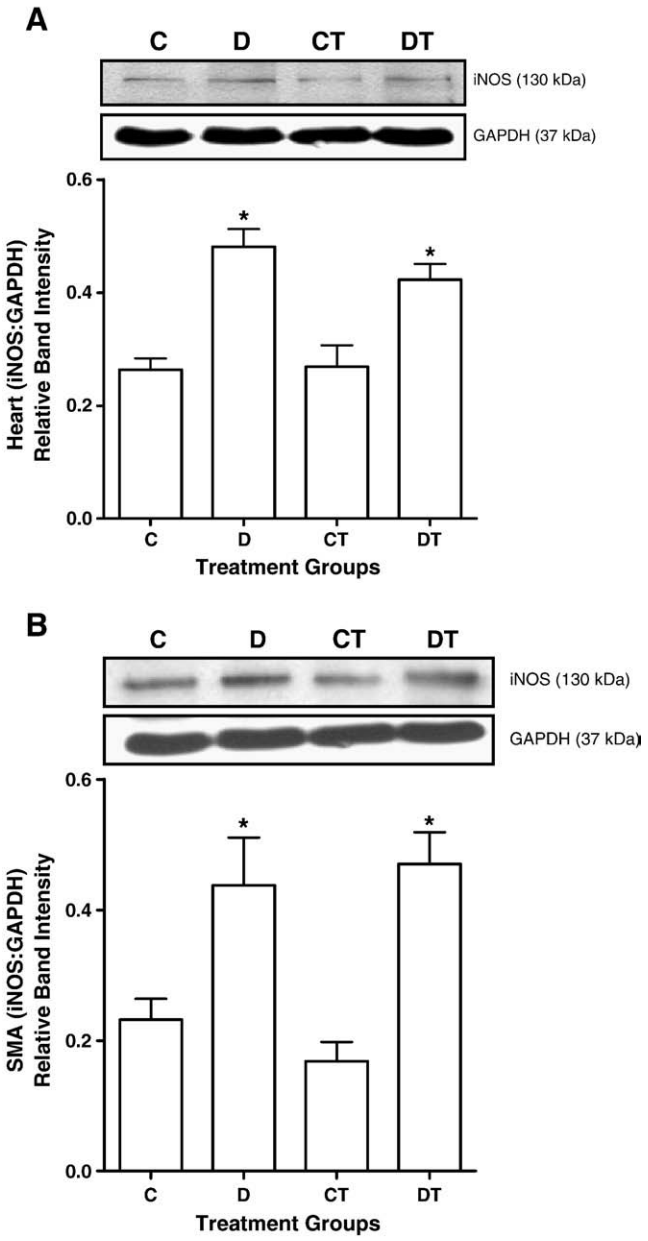

Fig. 4. Effect of L-NIL treatment on iNOS expression in the heart (A) and superior mesenteric artery (SMA) (B). Western blot analysis showing expression of iNOS and the corresponding GAPDH protein in the heart and superior mesenteric arteries. Densitometric analysis of iNOS expression (corrected by normalizing to the corresponding GAPDH expression) in the heart and superior mesenteric arteries ($n=8$). Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C, CT and DT groups; # different from D group ($P<0.05$).

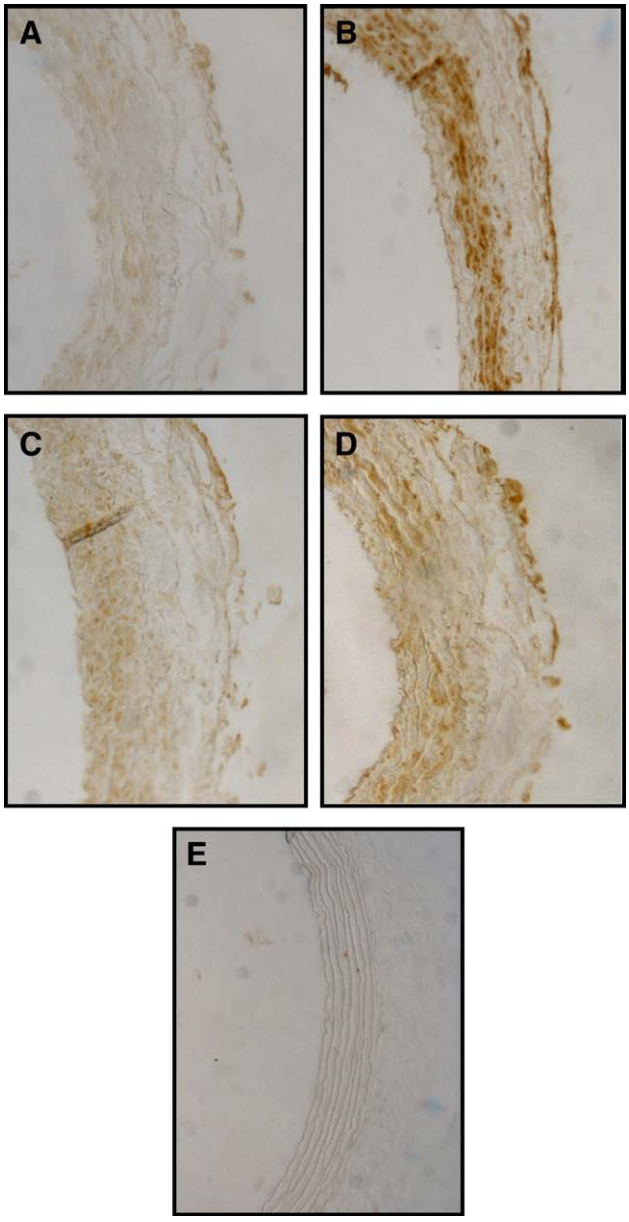

Fig. 5. Photomicrographs (×10) illustrating the immunohistochemical localization of iNOS in sections of superior mesenteric arteries of control (A), diabetic (B), Control treated (C) and diabetic treated (D) rats. As shown, there is an increase in the expression of iNOS in the medial and advential layers of the untreated diabetic superior mesenteric artery. E represents a negative control (where the section was incubated with NGS instead of the iNOS antibody) showing no staining, indicating that the secondary antibody at the concentration used is devoid of any non-specific binding.

Administration of streptozotocin to rats results in the classical symptoms of diabetes such as hyperglycemia, decreased body weight gain, polyuria, and increased food and fluid intake (data not shown). In addition, diabetic rats also demonstrate lower mean arterial blood pressure, heart rate and attenuated pressor responses compared to their age matched control animals. The mechanisms underlying the depressed mean arterial blood pressure and bradycardia in streptozotocin diabetic rats are not well understood. However, previous studies from our lab have shown that although the expression of eNOS is reduced and that of nNOS is unchanged, the expression and activity of iNOS in heart, aorta and superior mesenteric artery from streptozotocin diabetic rats are increased (Bardell and MacLeod, 2001; Nagareddy et al., 2005). Since NO plays an important role in cardiovascular homeostasis, we hypothesized that its over-production in diabetes contributes to the depressed mean arterial blood pressure and heart rate. However, we have previously shown that acute inhibition of iNOS, while restoring pressor responses to vasoactive agents, had no effect on the depressed mean arterial blood pressure and heart rate in streptozotocin diabetic rats (Nagareddy et al., 2005). Therefore, in the present study we investigated the possibility that chronic inhibition of iNOS would prevent the depression of mean arterial blood pressure and heart rate in streptozotocin diabetic rats.

If iNOS is responsible for the depressed mean arterial blood pressure and heart rate observed in streptozotocin diabetic rats, then why did acute inhibition of iNOS not improve resting mean arterial blood pressure and heart rate? Although NO from iNOS does not seem to directly depress mean arterial blood pressure and heart rate, changes secondary to increased expression of iNOS and over-production of NO in cardiovascular tissue may contribute to depressed mean arterial blood pressure and heart rate in these rats. These include increased formation of peroxynitrite in cardiovascular tissue. Increased formation of reactive oxygen species, which occurs in hyperglycemic conditions, can scavenge NO resulting in the formation of peroxynitrite. We previously have found altered levels of plasma and tissue markers of oxidative stress including decreased total antioxidant and superoxide dismutase and increased free 15-F2t-Isoprostane levels in streptozotocin diabetic rats (Xia et al., 2007). Our present data demonstrating increased levels of NT (a marker of peroxynitrite formation) both in the heart and superior mesenteric artery suggests increased levels of nitrosative stress in diabetic rats. Further, an improvement in blood pressure and heart rate by L-NIL was associated not only with normalization of iNOS expression and activity, but with attenuation of NT levels in the heart and superior mesenteric artery of diabetic rats. These data suggest that the depressed mean arterial blood pressure and heart rate in untreated diabetic rats may be due to the actions of peroxynitrite on cardiovascular tissues.

In addition to reduced peroxynitrite formation, normalization of eNOS expression or iNOS activity in the heart by L-NIL treatment may explain in part, the improvement in bradycardia in DT rats. Studies have demonstrated that knockout of eNOS results in decreased basal heart rate, leading to the suggestion of a positive chronotropic effect of eNOS (Shesely et al., 1996). Further, low and physiological concentrations of NO have been shown to increase heart rate in vitro by activating cAMP-dependent protein kinase. On the other hand, high concentrations of NO can also reduce heart rate by activating cGMP-dependent protein kinase (Kojda et al., 1996). Our recent studies demonstrated that increased iNOS expression is associated with elevated NO*x* levels in the hearts of untreated diabetic rats (Soliman et al., 2008). This is consistent

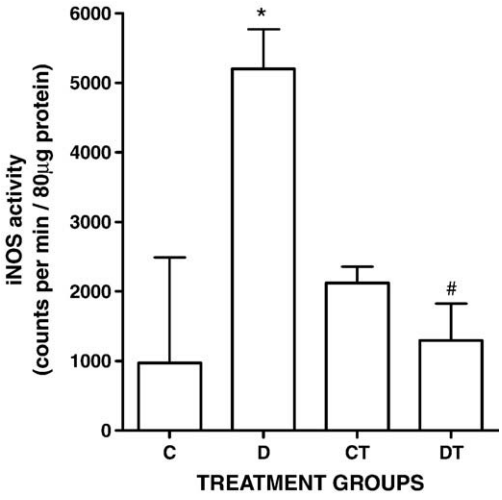

Fig. 6. Effect of L-NIL treatment on iNOS activity in the mesenteric arterial bed ($n=8$). Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C, CT and DT groups; # different from D group ($P<0.05$).

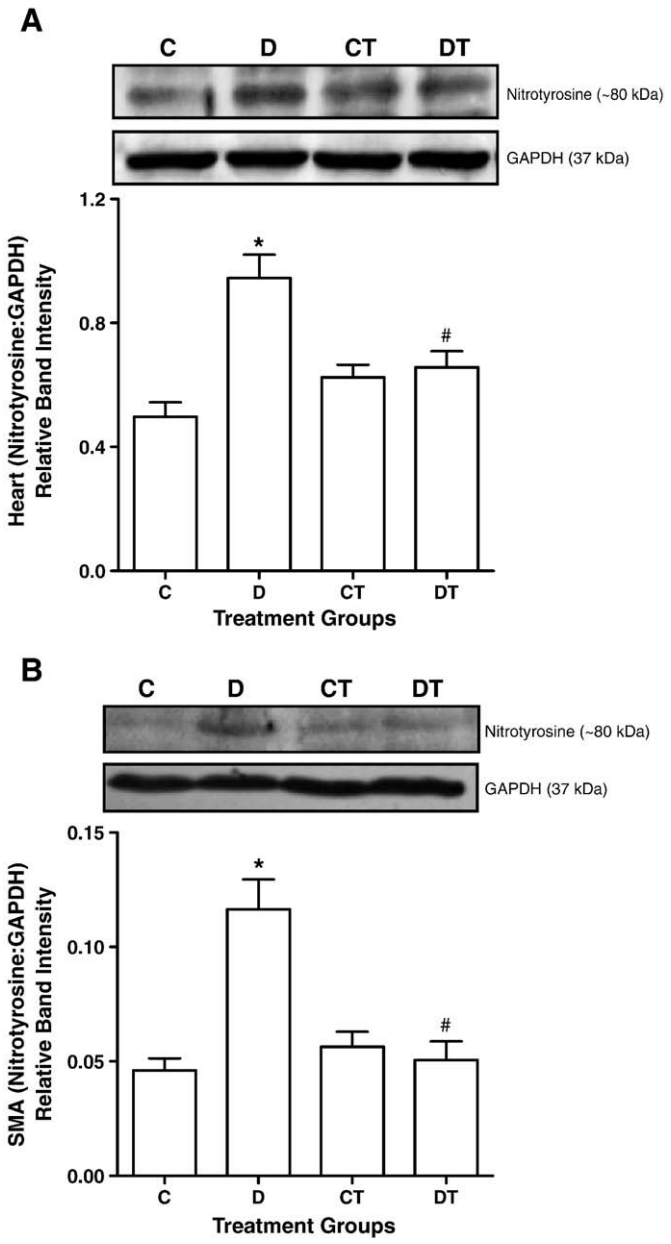

Fig. 7. Effect of L-NIL treatment on NT levels in the heart (A) and superior mesenteric artery (SMA) (B). Western blot analysis showing NT (a representative protein at ~80 kDa that was significantly elevated) and the corresponding GAPDH protein in the heart and superior mesenteric arteries. Densitometric analysis of NT levels (corrected by normalizing to the corresponding GAPDH expression) in the heart and superior mesenteric arteries ($n=8$). Data were analyzed using One-Way ANOVA with Newman–Keuls post hoc test. *Different from C, CT and DT groups; # different from D group ($P<0.05$).

with the possibility that high concentrations of locally produced NO in the heart can cause bradycardia.

Increased formation of peroxynitrite may reduce the bioavailability of functional NO from eNOS and cause endothelial dysfunction (Zou et al., 2004). Previous studies from our lab have reported an improvement in endothelial function when iNOS was inhibited acutely (Nagareddy et al., 2005). The data from the present study demonstrate decreased expression of eNOS in the superior mesenteric arteries of untreated diabetic rats that was also improved by pretreatment with L-NIL. Although we do not have any direct evidence to show that peroxynitrite *per se* is involved in the decreased expression of eNOS, it is possible that L-NIL treatment, by inhibiting the formation of NO, prevented the pro-oxidant actions of peroxynitrite on the eNOS protein. A recent study reported a positive correlation between decreased eNOS expression and endothelial dysfunction with that of an imbalance in [NO]/[$ONOO^-$] concentrations (Heeba et al., 2007). In human umbilical vein endothelial (HUVEC) cells, using real time measurements of NO and $ONOO^-$, the authors reported that a shift in the [$NO/ONOO^-$] towards a high production of $ONOO^-$ causes decreased eNOS protein expression. Further, peroxynitrite can oxidize the zinc thiolate center in eNOS, a modification that results in reduced NO bioactivity and enhanced endothelial O^{2-} production. This modification can result in the generation of more peroxynitrite and eNOS uncoupling. Another mechanism of eNOS uncoupling by peroxynitrite is by direct oxidation of tetrahydrobiopterin (BH4), an essential and critical cofactor for NO synthesis (Kuzkaya et al., 2003). Thus peroxynitrite formation represents an important complement in the mechanisms of endothelial dysfunction.

Treatment of diabetic rats with L-NIL also improved pressor responses to methoxamine. These data, in addition to that of our previous study (Nagareddy et al., 2005) suggest that NO from iNOS contributes significantly to the vascular hyporeactivity in diabetic rats. Although we believe that improvements in pressor responses to methoxamine are due to the direct effects of L-NIL on iNOS, it is possible that L-NIL also protects the vascular tissues from the harmful effects of peroxynitrite by preventing its formation. In the vasculature, it has been reported that peroxynitrite causes direct oxidation of catecholamines (Takakura et al., 2003) and reduces the binding capacity of α-adrenergic receptors, thereby decreasing the vascular reactivity to vasoactive agents (Takakura et al., 2002). It is therefore possible that L-NIL by inhibiting iNOS and formation of peroxynitrite preserves the pressor responses in diabetic rats.

Increasing evidence suggests that many of the cardiovascular abnormalities in diabetic rats can be prevented by inhibiting the formation of peroxynitrite (Cuzzocrea et al., 2001; Nassar et al., 2002). For instance, previous studies from our lab (Nagareddy et al., 2006;

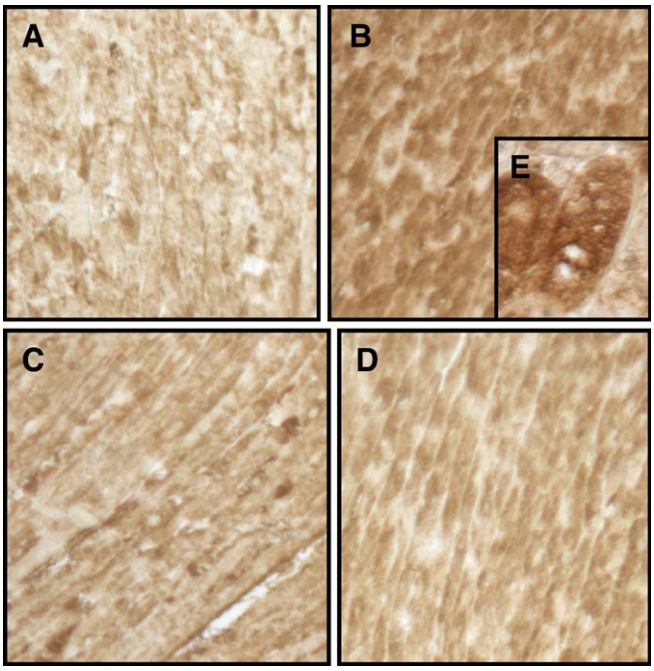

Fig. 8. Photomicrographs (×10) illustrating the immunohistochemical localization of NT in sections of heart from control (A), diabetic (B), control treated (C) and diabetic treated (D) rats. As shown, there is a substantial increase in nitrotyrosine immunostain in the diabetic heart section (B) compared to the control (A) and L-NIL treated control (C). E represents a photomicrograph of higher magnification (×100) clearly demonstrating NT immunostain predominantly in cardiomyocytes.

Xia et al., 2007) showed that inhibition of peroxynitrite using antioxidants such as *n*-acetylcysteine and ascorbic acid improves cardiac performance, mean arterial blood pressure and heart rate in streptozotocin diabetic rats. At the same time, the inability of L-NIL treatment to completely restore the depressed mean arterial blood pressure and heart rate in streptozotocin diabetic rats suggests the possibility of other mechanisms in the etiology of these abnormalities. Recent studies from our lab suggest the involvement of PKCβ, since treatment of streptozotocin diabetic rats with a selective inhibitor of PKCβ for 3 weeks also improved mean arterial blood pressure, heart rate and pressor responses (unpublished observations).

Recent clinical studies have shown that increased generation of NO occurs in patients with type 1 diabetes, and is associated with enhanced peroxynitrite production and lipid peroxidation (Hoeldtke, 2003). Furthermore, a correlation between increased plasma NOx levels and endothelial dysfunction, lower blood pressure and sympathetic nerve dysfunction in type 1 diabetes has also been found (Ceriello et al., 2007; Hoeldtke, 2003). Although a large body of evidence suggests the involvement of peroxynitrite/nitrotyrosine in the etiology of hypotension, many reports have also linked nitrotyrosine with hypertension in both humans and animal models of diabetes, particularly of type 2 diabetes (Frustaci et al., 2000; Martina et al., 2008). This is most likely due to decreased bioavailability of physiological NO because of its quenching by increased levels of reactive oxygen species leading to attenuation of endothelium-dependent vasorelaxation. Further, post-translational modifications of proteins such as nitration by peroxynitrite can also play an important role in the etiology of hypertension (Tyther et al., 2007). Increasing evidence now suggests that the relative levels of reactive oxygen and nitrogen species and a critical balance between the cellular concentration of NO and antioxidants may dictate the hemodynamic outcome in diabetes. However in conditions of abnormal expression and activation of iNOS such as in the present study, increased formation of peroxynitrite is associated with the pathological features of hypotension.

In summary, the results of the present study suggest that induction of iNOS contributes significantly to the depressed mean arterial blood pressure, heart rate, endothelial dysfunction and pressor responses to vasoactive agents in type 1 diabetes. Thus therapeutic strategies aimed at inhibiting iNOS may be a rational approach to prevent some of the cardiovascular abnormalities that occur in this condition.

Acknowledgements

Supported by an operating grant (to KMM) and a Program Grant from the Heart and Stroke Foundation of BC & Yukon. PRN is a recipient of a Doctoral Research Award from the Heart and Stroke Foundation of Canada and a Senior Graduate Studentship from the Michael Smith Foundation for Health Research.

References

Bardell, A.L., MacLeod, K.M., 2001. Evidence for inducible nitric-oxide synthase expression and activity in vascular smooth muscle of streptozotocin-diabetic rats. J. Pharmacol. Exp. Ther. 296, 252–259.

Beckman, J.S., Koppenol, W.H., 1996. Nitric oxide, superoxide, and peroxynitrite: the good, the bad, and ugly. Am. J. Physiol. 271, C1424–C1437.

Bojunga, J., Dresar-Mayert, B., Usadel, K.H., Kusterer, K., Zeuzem, S., 2004. Antioxidative treatment reverses imbalances of nitric oxide synthase isoform expression and attenuates tissue-cGMP activation in diabetic rats. Biochem. Biophys. Res. Commun. 316, 771–780.

Bradford, M.M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. 72, 248–254.

Ceriello, A., Mercuri, F., Quagliaro, L., Assaloni, R., Motz, E., Tonutti, L., Taboga, C., 2001. Detection of nitrotyrosine in the diabetic plasma: evidence of oxidative stress. Diabetologia 44, 834–838.

Ceriello, A., Quagliaro, L., D'Amico, M., Di Filippo, C., Marfella, R., Nappo, F., Berrino, L., Rossi, F., Giugliano, D., 2002. Acute hyperglycemia induces nitrotyrosine formation and apoptosis in perfused heart from rat. Diabetes 51, 1076–1082.

Ceriello, A., Piconi, L., Esposito, K., Giugliano, D., 2007. Telmisartan shows an equivalent effect of vitamin C in further improving endothelial dysfunction after glycemia normalization in type 1 diabetes. Diabetes Care 30, 1694–1698.

Cheng, X., Cheng, X.S., Kuo, K.H., Pang, C.C., 2004. Inhibition of iNOS augments cardiovascular action of noradrenaline in streptozotocin-induced diabetes. Cardiovasc. Res. 64, 298–307.

Cuzzocrea, S., Riley, D.P., Caputi, A.P., Salvemini, D., 2001. Antioxidant therapy: a new pharmacological approach in shock, inflammation, and ischemia/reperfusion injury. Pharmacol. Rev. 53, 135–159.

Ferdinandy, P., Danial, H., Ambrus, I., Rothery, R.A., Schulz, R., 2000. Peroxynitrite is a major contributor to cytokine-induced myocardial contractile failure. Circ. Res. 87, 241–247.

Frustaci, A., Kajstura, J., Chimenti, C., Jakoniuk, I., Leri, A., Maseri, A., Nadal-Ginard, B., Anversa, P., 2000. Myocardial cell death in human diabetes. Circ. Res. 87, 1123–1132.

Hallinan, E.A., Tsymbalov, S., Dorn, C.R., Pitzele, B.S., Hansen Jr., D.W., Moore, W.M., Jerome, G.M., Connor, J.R., Branson, L.F., Widomski, D.L., Zhang, Y., Currie, M.G., Manning, P.T., 2002. Synthesis and biological characterization of L-N(6)-(1-iminoethyl)lysine 5-tetrazole-amide, a prodrug of a selective iNOS inhibitor. J. Med. Chem. 45, 1686–1689.

Hebden, R.A., Bennett, T., Gardiner, S.M., 1987. Pressor sensitivities to vasopressin, angiotensin II, or methoxamine in diabetic rats. Am. J. Physiol. 253, R726–734.

Heeba, G., Hassan, M.K., Khalifa, M., Malinski, T., 2007. Adverse balance of nitric oxide/peroxynitrite in the dysfunctional endothelium can be reversed by statins. J. Cardiovasc. Pharmacol. 50, 391–398.

Hoeldtke, R.D., 2003. Nitrosative stress in early Type 1 diabetes. David H. P. Streeten Memorial Lecture. Clinic. Auton. Res. 13, 406–421.

Ido, Y., Kilo, C., Williamson, J.R., 1997. Cytosolic NADH/NAD^+, free radicals, and vascular dysfunction in early diabetes mellitus. Diabetologia 40 (Suppl 2), S115–S117.

Kojda, G., Kottenberg, K., Nix, P., Schluter, K.D., Piper, H.M., Noack, E., 1996. Low increase in cGMP induced by organic nitrates and nitrovasodilators improves contractile response of rat ventricular myocytes. Circ. Res. 78, 91–101.

Kuzkaya, N., Weissmann, N., Harrison, D.G., Dikalov, S., 2003. Interactions of peroxynitrite, tetrahydrobiopterin, ascorbic acid, and thiols: implications for uncoupling endothelial nitric-oxide synthase. J. Biol. Chem. 278, 22546–22554.

Lane, P., Gross, S.S., 1999. Cell signaling by nitric oxide. Semin. Nephrol. 19, 215–229.

Martina, V., Masha, A., Gigliardi, V.R., Brocato, L., Manzato, E., Berchio, A., Massarenti, P., Settanni, F., Della Casa, L., Bergamini, S., Iannone, A., 2008. Long-term *N*-acetylcysteine and L-arginine administration reduces endothelial activation and systolic blood pressure in hypertensive patients with type 2 diabetes. Diabetes Care 31, 940–944.

Moncada, S., Palmer, R.M., Higgs, E.A., 1991. Nitric oxide: physiology, pathophysiology, and pharmacology. Pharmacol. Rev. 43, 109–142.

Nagareddy, P.R., Xia, Z., McNeill, J.H., MacLeod, K.M., 2005. Increased expression of iNOS is associated with endothelial dysfunction and impaired pressor responsiveness in streptozotocin-induced diabetes. Am. J. Physiol. Heart Circ. Physiol. 289, H2144–H2152.

Nagareddy, P.R., Xia, Z., MacLeod, K.M., McNeill, J.H., 2006. *N*-acetylcysteine prevents nitrosative stress-associated depression of blood pressure and heart rate in streptozotocin diabetic rats. J. Cardiovasc. Pharmacol. 47, 513–520.

Nassar, T., Kadery, B., Lotan, C., Da'as, N., Kleinman, Y., Haj-Yehia, A., 2002. Effects of the superoxide dismutase-mimetic compound tempol on endothelial dysfunction in streptozotocin-induced diabetic rats. Eur. J. Pharmacol. 436, 111–118.

Parrillo, J.E., 1993. Pathogenetic mechanisms of septic shock. N. Engl. J. Med. 328, 1471–1477.

Shesely, E.G., Maeda, N., Kim, H.S., Desai, K.M., Krege, J.H., Laubach, V.E., Sherman, P.A., Sessa, W.C., Smithies, O., 1996. Elevated blood pressures in mice lacking endothelial nitric oxide synthase. Proc. Natl. Acad. Sci. U. S. A. 93, 13176–13181.

Smith, J.M., Paulson, D.J., Romano, F.D., 1997. Inhibition of nitric oxide synthase by L-NAME improves ventricular performance in streptozotocin-diabetic rats. J. Mol. Cell. Cardiol. 29, 2393–2402.

Soliman, H., Craig, G.P., Nagareddy, P., Yuen, V.G., Lin, G., Kumar, U., McNeill, J.H., Macleod, K.M., 2008. Role of inducible nitric oxide synthase in induction of RhoA expression in hearts from diabetic rats. Cardiovasc. Res. 79, 322–330.

Takakura, K., Taniguchi, T., Muramatsu, I., Takeuchi, K., Fukuda, S., 2002. Modification of alpha1-adrenoceptors by peroxynitrite as a possible mechanism of systemic hypotension in sepsis. Crit. Care Med. 30, 894–899.

Takakura, K., Xiaohong, W., Takeuchi, K., Yasuda, Y., Fukuda, S., 2003. Deactivation of norepinephrine by peroxynitrite as a new pathogenesis in the hypotension of septic shock. Anesthesiology 98, 928–934.

Tyther, R., Ahmeda, A., Johns, E., Sheehan, D., 2007. Proteomic identification of tyrosine nitration targets in kidney of spontaneously hypertensive rats. Proteomics 7, 4555–4564.

Williamson, J.R., Chang, K., Frangos, M., Hasan, K.S., Ido, Y., Kawamura, T., Nyengaard, J.R., van den Enden, M., Kilo, C., Tilton, R.G., 1993. Hyperglycemic pseudohypoxia and diabetic complications. Diabetes 42, 801–813.

Xia, Z., Kuo, K.H., Nagareddy, P.R., Wang, F., Guo, Z., Guo, T., Jiang, J., McNeill, J.H., 2007. *N*-acetylcysteine attenuates PKCbeta2 overexpression and myocardial hypertrophy in streptozotocin-induced diabetic rats. Cardiovasc. Res. 73, 770–782.

Zou, M.H., Cohen, R., Ullrich, V., 2004. Peroxynitrite and vascular endothelial dysfunction in diabetes mellitus. Endothelium 11, 89–97.