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Joshi, A; Woodman, Owen

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**INCREASED NITRIC OXIDE ACTIVITY COMPENSATES FOR INCREASED
OXIDATIVE STRESS TO MAINTAIN ENDOTHELIAL FUNCTION IN RAT AORTA IN
EARLY TYPE 1 DIABETES**

A.Joshi¹ and O.L. Woodman^{1,2*}

¹ Department of Pharmacology, University of Melbourne, Victoria, Australia.

Email: anjali.jo@gmail.com

²School of Medical Sciences, RMIT University, Bundoora, Victoria, Australia.

Email: owen.woodman@rmit.edu.au

*Correspondence:

Professor Owen Woodman

School of Medical Sciences

RMIT University

PO Box 71 Bundoora

Victoria 3083 Australia

Tel: +61(03) 9925 7305

Fax: +61(03) 9925 7063

Email: owen.woodman@rmit.edu.au

RUNNING TITLE: Oxidative stress in diabetic endothelial dysfunction

ABSTRACT

Purpose: Hyperglycaemia and oxidative stress are known to acutely cause endothelial dysfunction *in vitro*, but in the initial stages of diabetes endothelium-dependent relaxation is preserved. The aim of this study was to investigate how endothelium-dependent relaxation is maintained in the early stages of type-1 diabetes.

Methods: Diabetes was induced in Sprague-Dawley rats with a single injection of streptozocin (48 mg/kg, i.v.) and after 6 weeks endothelium-dependent and -independent relaxation was examined in the thoracic aorta *in vitro*. Lucigenin-enhanced chemiluminescence was used to measure superoxide generation from the aorta.

Results: Diabetes increased superoxide generation by the aorta (2180 ± 363 vs 986 ± 163 AU/mg dry tissue weight). ACh-induced relaxation was similar in aortae from control (pEC_{50} 7.36 ± 0.09 , R_{max} $95 \pm 3\%$) and diabetic rats (pEC_{50} 7.33 ± 0.10 , R_{max} $88 \pm 5\%$). The ACh-induced relaxation was abolished by the combined presence of the NOS inhibitor L-NNA ($100 \mu M$) and an inhibitor of soluble guanylate cyclase, ODQ ($10 \mu M$) in control rats but under the same conditions the diabetic aortic rings showed significant relaxation to ACh (pEC_{50} 6.75 ± 0.15 , R_{max} $25 \pm 4\%$, $p < 0.05$). In diabetic aortae the addition of haemoglobin, which inactivates nitric oxide, to L-NNA+ODQ abolished the response to ACh. The addition of the potassium channel blockers, apamin and TRAM-34 to L-NNA+ODQ also abolished the relaxation response to ACh. Diabetes significantly elevated plasma total nitrite/nitrate and increased expression of eNOS and calmodulin in aortae.

Conclusions: These data indicate that after 6 weeks of diabetes, despite increased oxidant stress, endothelium-dependent relaxation is maintained due to the increased eNOS expression resulting in increased NO synthesis. In diabetic arteries NO acts both through, and independently of, cGMP pathways to cause relaxation.

KEY WORDS:

Diabetes, endothelium derived hyperpolarising factor, endothelium, nitric oxide, nitrosothiols, potassium channels.

INTRODUCTION

Cardiovascular complications are the major cause of morbidity and mortality in diabetic patients (Lopes-Virella et al., 2008). The pathogenic role of glucose in cardiovascular disease is increasingly apparent, as confirmed by studies carried out both on diabetic and non-diabetic patients (Bartnik et al., 2007). Acute hyperglycaemia (3-6 hrs) induces endothelial dysfunction of the aorta as well as of the renal circulation of non-diabetic rabbits (Gomes et al., 2004) and numerous studies report that long-term hyperglycaemia leads to endothelial dysfunction in various animal models of diabetes (Pieper et al., 1998, Pieper, 1999, Csanyi et al., 2007) and in humans (Johnstone et al., 1993). Hyperglycaemia both acute (Goel et al., 2007) and chronic (Karasu, 2000) is known to induce oxidative stress and it has been proposed that oxidative stress leads to endothelial dysfunction (Ammar et al., 2000, Hink et al., 2001, Jay et al., 2006). It is surprising then to note that there are several reports that endothelial function is normal after a short duration (2-4 weeks) of diabetes despite the presence of hyperglycaemia (Orie et al., 1993, Pieper, 1999, Shen et al., 2003).

Pieper (1999) reported a triphasic effect of diabetes on endothelial function. Initially endothelium-dependent relaxation was increased after 1 week of diabetes followed by a normal response after 2 weeks and impaired response after 8 weeks of diabetes. While the mechanism of endothelial dysfunction has been extensively studied in long-term diabetes, it still remains to be elucidated how endothelium-dependent relaxation is maintained in the early stages of type-1 diabetes.

The endothelium produces vasodilators such as nitric oxide, prostacyclin and a still unidentified, endothelium-derived hyperpolarising factor (EDHF) (Bryan et al., 2005). Nitric oxide is considered to be the main endothelium-derived relaxing factor in larger arteries (Clark and Fuchs, 1997). The major source of nitric oxide in the endothelium is endothelial nitric oxide synthase (eNOS) but a small amount of nitric oxide may also be released from preformed endothelial nitrosothiol stores (Alencar et al., 2003, Lima et al., 2010).

There are several possible mechanisms that may contribute to the maintenance of endothelial function in short-term diabetes e.g. increased production of nitric oxide (Stadler et al., 2003), increased expression of eNOS (Hink et al., 2001) or a compensatory upregulation of the release of other vasodilators (Shi et al., 2006, Malakul et al., 2008, Nacci et al., 2009). Several studies have shown increased nitric oxide production in the early stages of diabetes. Stadler et al. (2003) reported increased production of nitric oxide in aortae from diabetic rats for a limited time after induction of diabetes (up to 7 weeks). There are conflicting reports regarding expression of eNOS at various stages of diabetes. Hink et al. (2001) showed an increased expression of eNOS but decreased basal nitric oxide in aortae after 2 weeks of diabetes leading the authors to propose that eNOS became uncoupled and shifted towards superoxide rather than nitric oxide synthesis whereas Bojunga et al. (2004) reported a decreased expression of eNOS in diabetic aorta but increased production of nitric oxide. On the other hand, increased expression of inducible NOS has also been demonstrated in diabetic rat aorta and heart (Bojunga et al., 2004, Jesmin et al., 2006). Overall it has been demonstrated that whereas diabetes induces endothelial dysfunction by decreased synthesis or increased breakdown of nitric oxide (Beckman and Crow, 1993, Jay et al., 2006) in the longer term but it is yet not clear what changes take place in early diabetes to maintain endothelial function despite hyperglycaemia and oxidant stress.

As there has been limited investigation of the mechanism of relaxation in early diabetes (Shen et al., 2003, Abboud et al., 2009) the purpose of this study was to gain better understanding of mechanism of endothelium-dependent relaxation in the early stages of type-1 diabetes before acetylcholine-induced relaxation is impaired with a focus on the determination of the role of various endothelium-derived vasodilators in maintaining endothelial function in the presence of increased oxidative stress.

METHODS

Animals

Male Sprague-Dawley rats (200-230 g, 8 weeks, Animal Resource Centre, Perth, WA) were housed 2-3 per cage, fed standard rat chow and had free access to food and water. All the experiments were performed according to the guidelines of the National Health and Medical Research Council of Australia and approved by the Animal Ethics Committees of the University of Melbourne and RMIT University.

Induction of diabetes

Diabetes was induced in rats by a single injection of streptozotocin (48 mg/kg) dissolved in 0.1 M citrate buffer (pH 4.5) into the tail vein after an overnight fast. Control rats received an injection of citrate buffer. Blood glucose was measured at the time of experiment for all groups using an Accu-Check Advantage II monitor (Roche, Mannheim, Germany). The rats showing blood glucose of >20 mmol/L were considered to be diabetic.

Preparation of aortic rings

The rats were killed 6 weeks after streptozotocin or vehicle injection by exposure to a mixture of halothane and oxygen for 5 min. The chest was opened and the thoracic aorta was isolated. After removing superficial fat and connective tissue, the thoracic aorta was cut into 2-3 mm long rings and mounted in organ baths in Krebs bicarbonate solution (118 mM NaCl, 4.7 mM KCl, 1.18 mM MgSO₄, 1.2 mM KH₂PO₄, 25 mM NaHCO₃, 11.1 mM D-glucose, and 1.6 mM CaCl₂), bubbled with 5% CO₂ in O₂ at 37°C to assess vascular function. Blood plasma was collected for NOx assay and from some of the rats, the thoracic aorta was isolated and immediately frozen for western blotting.

Functional experiments

The aortic rings, mounted in organ baths at a resting tension of 1g, were equilibrated for 60-90 min with the bath medium changed every 15 min. The tissues were maximally contracted by exposure to physiological saline solution containing a high potassium concentration of 123 mM. Endothelial integrity was determined by measuring the relaxation caused by exposure to acetylcholine (10^{-5} M) in phenylephrine-precontracted rings and the endothelium was considered to be intact if the preparation relaxed by greater than 80%. Cumulative concentration response curves to acetylcholine and sodium nitroprusside were determined using rings submaximally contracted with phenylephrine (10^{-8} to 10^{-7} M). Responses to acetylcholine and sodium nitroprusside were also tested in the presence or absence of N-nitro-L-arginine (L-NNA, 100 μ M) a nitric oxide synthase (NOS) inhibitor, 1H-[1,2,4] oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μ M) a soluble guanylate cyclase (sGC) inhibitor, indomethacin (10 μ M) a cyclooxygenase inhibitor, apamin (1 μ M) a small conductance calcium activated potassium channel (SK_{Ca}) blocker, TRAM-34 (1 μ M) an intermediate conductance calcium activated potassium channel (IK_{Ca}) blocker, haemoglobin (20 μ M) a nitric oxide scavenger, carboxy-PTIO (20 mM) a scavenger of $NO^{\cdot}$ and L-cysteine (1 mM) a scavenger of $NO^{\cdot}$.

Lucigenin-enhanced chemiluminescence assay

Lucigenin-enhanced chemiluminescence was used to measure superoxide release from aortic rings that were not used in the functional experiments, using a previously described procedure (Chan et al., 2003). At the conclusion of the assay, tissues were dried for 48 h at 65°C to allow superoxide production to be normalized to dry tissue weight. The results were expressed as superoxide counts per mg tissue.

Western blot analysis

Western blots were performed as described previously (Woodman et al., 2004) with the following modifications. The aortic tissues were homogenized and the total protein concentration of the

samples was quantified using a Bradford assay. Equal amounts of protein homogenate were subjected to SDS-PAGE and western blot analysis with mouse antibodies against eNOS, inducible-NOS, caveolin-1 (Transduction Laboratories), calmodulin (Millipore), NADPH oxidase (NOX)-2 and heme oxygenase-1 (AbCAM). To normalize for the amount of protein loaded on the gels, membranes were reprobed with a loading control antibody (β -tubulin, AbCAM). All proteins were detected by enhanced chemiluminescence (Amersham) after incubation with anti-mouse secondary antibody. All protein bands were quantified by densitometry (Biorad Chemidoc) and expressed as a ratio of the loading control.

Nitrite estimation

The level of nitric oxide metabolites (NO_x, ie. nitrite + nitrate) was measured in plasma using a colorimetric assay kit (Roche Diagnostics). Plasma samples were stored at -80°C until assayed. The concentration of NO_x was measured in samples of plasma after converting all nitrates to nitrite with nitrate reductase. Total nitrite levels were measured in duplicate by absorbance at 560 nm using the Griess reaction. The calibration curve was prepared using sodium nitrite as a standard.

Drugs

Acetylcholine perchlorate (BDH Chemicals), apamin (Sigma), L-cysteine (Sigma), p-hydroxymercuribenzoic acid (Sigma) and phenylephrine hydrochloride (Sigma) were all dissolved in distilled water. Haemoglobin (Sigma) and sodium nitroprusside (Sigma) were dissolved in Krebs solution. Indomethacin (Sigma) was dissolved in 0.1M sodium carbonate and L-NNA (Sigma) was dissolved in 0.1 M sodium bicarbonate. Ethacrynic acid (Sigma), ODQ (Cayman), carboxy-PTIO (Cayman) and TRAM-34 (Sigma) were dissolved in dimethyl sulfoxide. The final concentrations of DMSO in the bath were 0.0001%-0.01% while performing the functional experiments which has been shown to have no effect on vasorelaxation in these concentrations.

Analysis and statistics

Concentration-response data were fitted to a sigmoidal plot using GraphPad Prism version 5.0, which estimated the pEC_{50} . Statistical analysis was carried out using Student's unpaired t-test or multiple comparisons were performed using analysis of variance (ANOVA) followed by a Bonferroni post test. $p < 0.05$ was considered statistically significant. All values are presented as $mean \pm SEM$. Each n represents the number of animals per group.

RESULTS

Blood glucose and body weight

The control and diabetic animals had a similar initial body weight but diabetic animals had a significantly lower body weight at the end of 6 weeks compared to control animals (Table 1). All of the diabetic animals exhibited hyperglycaemia at the time of experiment.

Vascular superoxide

Superoxide counts measured from segments of thoracic aorta were significantly higher in diabetic animals compared to control animals using the lucigenin-enhanced chemiluminescence assay.

Diphenyliodonium, a non-selective inhibitor of NADPH oxidase, reduced the amount of superoxide generation in both groups (Table 1).

Plasma nitrite concentrations

The diabetic animals showed significantly higher plasma NO_x concentration compared to control animals (Table 1).

Endothelium-dependent and endothelium-independent relaxation

6 weeks after streptozotocin injection, endothelium-dependent and endothelium-independent relaxation to acetylcholine (10^{-10} to 10^{-5} M) and sodium nitroprusside (10^{-11} to 10^{-5} M) respectively were similar in aortae from control and diabetic animals (Figure 1).

Effect of L-NNA, ODQ and indomethacin on relaxation

The presence of L-NNA or ODQ completely abolished acetylcholine-induced relaxation in control aortae (Figure 2). In the aortic rings from diabetic animals the relaxation was partially inhibited, but not abolished, by L-NNA or ODQ (Figure 2b). Pretreatment with indomethacin had no effect on relaxation to either ACh or SNP in normal or diabetic animals (Figure 2). The diabetic animals showed a higher sensitivity to the nitric oxide donor sodium nitroprusside in the presence of L-NNA and ODQ compared to control animals.

Effect of nitric oxide scavengers on relaxation

The presence of the nitric oxide scavenger haemoglobin in addition to L-NNA+ODQ abolished the relaxation response to acetylcholine in diabetic aortae (Figure 3a). The presence of haemoglobin significantly decreased the pEC_{50} to sodium nitroprusside in control as well as diabetic aortae. Carboxy-PTIO and L-cysteine also significantly decreased the pEC_{50} and R_{max} to acetylcholine in diabetic aorta when used in combination with L-NNA (Figure 3). The pEC_{50} and R_{max} values for acetylcholine and sodium nitroprusside in the presence of these inhibitors are shown in Tables 2 and 3.

Effect on relaxation of agents depleting nitrosothiol stores

The role of preformed nitrosothiol stores in relaxation response to acetylcholine in diabetic aorta was examined using nitrosothiol depleting agent ethacrynic acid (EA) and p-hydroxymercuribenzoic acid (PHMBA). The aortic rings were incubated for 20 minutes with L-NNA with or without ethacrynic acid or PHMBA. After the first exposure of acetylcholine, the rings incubated with ethacrynic acid or PHMBA showed significantly less relaxation compared to L-NNA incubated rings (Figure 4). After the second exposure to acetylcholine, the ethacrynic acid and PHMBA incubated rings showed contraction indicating a complete depletion of nitrosothiol stores.

Effect of potassium channel blockers on relaxation

The presence of apamin plus Tram-34 had no effect on vasorelaxation to acetylcholine or to sodium nitroprusside in control aortae (Figure 2c) whereas in diabetic animals they significantly decreased the maximum relaxation to acetylcholine (Figure 2d) but had no effect on sodium nitroprusside responses (Table 3). In diabetic animals the relaxation to acetylcholine observed in the presence of ODQ plus L-NNA was abolished by addition of apamin and TRAM-34 (Figure 2d) and the maximum response to sodium nitroprusside was significantly decreased compared to control aortae (Table 3).

Protein expression by western blot analysis

The expression of eNOS, calmodulin and NADPH oxidase-2 was found to be significantly increased in aortae from the diabetic group compared to control (Figure 5). The eNOS dimer-monomer ratio was significantly decreased in diabetic aorta. The expression of caveolin and heme oxygenase-1 was not altered by diabetes. Inducible NOS expression was not detected in either of the groups (data not shown).

DISCUSSION

Our study showed that, despite an increase in oxidative stress, endothelium-dependent and endothelium-independent relaxation of the rat aorta was unaltered in the early stages of diabetes, but the mechanism of endothelium-dependent relaxation had been changed. We found that endothelium-dependent relaxation of diabetic aortae was resistant to inhibition by a NOS inhibitor and/or inhibition of sGC and that the remaining component of relaxation was sensitive to SK_{Ca} and IK_{Ca} channel blockers as well as nitric oxide scavengers. In diabetic arteries the nitrosothiol depleting agents also abolished the relaxation response to acetylcholine that persisted in the presence of L-NNA. Western blot analysis showed increased expression of eNOS and calmodulin in diabetic aortae, which may explain the increase in nitric oxide synthesis, indicated by the elevation in plasma nitrite levels. These results suggest that despite increased oxidative stress, endothelial function was maintained in early diabetes due to a compensatory increased activity of nitric oxide which acted primarily through activating soluble guanylate cyclase and partly through the opening of potassium channels. In diabetes, nitrosothiol stores acted as the additional source of nitric oxide in addition to eNOS.

Hyperglycaemia increases superoxide production by several mechanisms, including autooxidation of glucose, activation of polyol pathways, and generation of advanced glycation end products (Jay et al., 2006). Several enzymes are involved in the production of ROS at a vascular level such as cyclooxygenase, NADPH oxidase and xanthine oxidase (Ellis and Triggle, 2003). In vascular disease, uncoupling of NOS also accounts for the increased ROS production. In this study diabetic animals showed an increase in oxidative stress measured by production of superoxide counts using lucigenin-enhanced chemiluminescence. We also found increased expression of NADPH oxidase -2 in diabetic rat aorta compared to control animals (Figure 5). Diphenyliodonium, a non-selective inhibitor of NADPH oxidase enzyme, blocked the production of superoxide in lucigenin assay (Table 1) suggesting that NADPH oxidase is the major source of superoxide. The dimer-monomer ratio of eNOS was decreased in diabetic animals (Figure 5) suggesting eNOS was

uncoupled form and therefore may also contribute to superoxide production. These results are consistent with other studies which have shown increased oxidative stress (Bitar et al., 2005, Ding et al., 2007) as well as increased activity/expression of NADPH oxidase, p22 phox and p67phox subunits in diabetes (Wendt et al., 2005, Guo et al., 2007).

Hyperglycaemia, both acute and chronic, has been widely reported to cause endothelial dysfunction. Rat aortic rings showed a diminished response to endothelium-dependent vasodilator when incubated with high glucose for 2-6 hrs (Gomes et al., 2004, Goel et al., 2007, Fang et al., 2009). By contrast, diabetic animals have shown variable endothelium-dependent relaxation responses depending upon the duration of diabetes (Pieper, 1999). In the early stages of diabetes (1-6 weeks of induction), it has been reported that endothelium-dependent relaxation is unaltered (Orie et al., 1993, Garcia et al., 1999) or increased in rats (Altan et al., 1989) or mice (Shen et al., 2003). By contrast, there are numerous studies which have reported decreased relaxation responses in various animal models of long-term diabetes (8 weeks or more) (Oyama et al., 1986, Garcia-Pascual et al., 1995, Bitar et al., 2005, Alper et al., 2006). Whilst the mechanism of endothelial dysfunction has been widely explored in long-term diabetes, it is yet to be established why endothelium-dependent relaxation is either unchanged or increased in early diabetes.

In aortae, the endothelium-dependent relaxation is normally mediated entirely by nitric oxide through the activation of sGC (Clark and Fuchs, 1997). This was confirmed by complete blockade of acetylcholine-induced relaxation by the NOS inhibitor, L-NNA or the sGC inhibitor ODQ in control animals. By contrast, aortae from the diabetic animals showed significant relaxation in the presence of L-NNA, ODQ or the combination of both. We postulated that this might be due to increased nitric oxide activity or that there might be a compensatory up-regulation of another non-nitric oxide pathway of relaxation. We investigated the possibility of increased nitric oxide activity by using nitric oxide scavengers, in combination with L-NNA and ODQ. The L-NNA and ODQ resistant relaxation to acetylcholine was found to be sensitive to haemoglobin (Figure 3a) and was also significantly decreased by L-cysteine, a scavenger of NO⁻ or carboxy-PTIO (scavenger of

NO[•]), suggesting an increased nitric oxide activity in diabetes as well as advocating potential contribution of both NO[•] and NO⁻ in acetylcholine-induced relaxation in diabetes. Haemoglobin has also been shown to bind nitrosothiol (Kim-Shapiro et al., 2006) and previous studies have shown that pre-formed stores of nitric oxide could exist in vascular tissue in the form of nitrosothiols and nitric oxide in the form of NO[•]/NO⁻ could be released from these stores by acetylcholine even in the presence of a NOS inhibitor (Ng et al., 2007). Nitrosothiol-derived compounds have been suggested to be potent vasodilators (Myers et al., 1990, Bates et al., 1991) which may cause relaxation mediated by both sGC and the opening of SK_{Ca} and IK_{Ca} channels (Batenburg et al., 2009) and recent reports suggest a physiological role for NO⁻ in spreading vasodilation (Yuill et al., 2011). It is also interesting to note that vasorelaxant responses to nitroxyl donors are maintained in the presence of oxidant stress (Bullen et al., 2011) supporting the possibility that NO⁻ might contribute to relaxation in diabetes. Our results using nitrosothiol depleting agents (Figure 4) also support a contribution of nitric oxide released from pre-formed nitrosothiol stores towards endothelium-dependent relaxation in early diabetes. This is consistent with the observations of Ng et al. (2007) who found concluded that relaxation caused by ultraviolet light involved release of pre-formed NO stores that was unaffected by type 2 diabetes.

In our study diabetic animals showed a significant increase in total plasma nitrite/nitrate levels (Table 1) consistent with earlier reports that demonstrated increased nitrite levels in short term (4 weeks) diabetes (Bojunga et al., 2004). Stadler et al. (2003) found increased nitric oxide levels in diabetic aorta starting from 2 weeks up to 7 weeks after diabetes induction. We also observed increased expression of eNOS protein in diabetic aorta (Figure 5) but inducible NOS was not detected. Several studies in diabetes had reported an upregulated, but dysfunctional, uncoupled NOS leading to increased oxidative stress and endothelial dysfunction (Hink et al., 2001, Satoh et al., 2005) and we also found a lower expression for the eNOS dimer and higher expression for the eNOS monomer indicating uncoupling and the potential to generate superoxide rather than nitric oxide. A further possibility is that in the presence of elevated oxidant stress the consequent

depletion of BH₄ could promote the synthesis of NO⁻ rather than NO by eNOS as previously reported (Rusche et al., 1998, Adak et al., 2000). We also investigated expression of two proteins that modulate eNOS activity (i.e. calmodulin and caveolin-1). The constitutively expressed eNOS is catalytically inactive when bound to caveolin-1, however, calmodulin displaces caveolin-1 in the presence of calcium and binds to eNOS and thereby activates eNOS to produce nitric oxide (Frank et al., 2007) . We found that the expression of calmodulin was significantly increased while expression of caveolin-1 remained unchanged in diabetes. Takahashi and Mendelsohn (2003) reported that calmodulin causes concentration dependent increases in eNOS activity *in vitro* and we, and others, have reported that an increase in calmodulin expression *in vivo* is associated with an increase in eNOS activity (Sobey et al., 2004, Woodman et al. 2004, Vera et al., 2007). This suggested that eNOS activity may be increased due to increased calmodulin binding despite a decreased dimer formation in diabetic aorta. Therefore, diabetic animals may produce more nitric oxide due to increased eNOS activity, to resist endothelial dysfunction caused by increased oxidative stress. There have been conflicting findings regarding eNOS regulation in diabetic animals with reports of both an increased (Stockklauser-Färber et al., 2000) or decreased (Komers et al., 2006) expression. Stockklauser-Färber et al. (2000) found that initially cardiac NOS expression and activity increased to a maximum after 4-6 weeks of diabetes and then decreased afterwards. They suggested that high glucose could lead to induction of eNOS in the early stages of diabetes in response to increased ROS production as the increase in eNOS could be blocked by antioxidant treatment. In a recent study, Riad et al. (2008) found a marked decrease in eNOS expression in diabetic skeletal muscle after 8 weeks of streptozotocin injection and stimulation of eNOS production by eNOS transcription enhancer AVE3085 improved endothelial function and reduced oxidative stress and vascular inflammatory responses. Taken together, our results suggested a compensatory increase in basal nitric oxide levels to combat increased oxidative stress in diabetic aorta.

The endothelial SK_{Ca} and IK_{Ca} channels have been reported to contribute to ACh-induced endothelial hyperpolarization and NO-mediated relaxation in large arteries (Stankevicius et al., 2006). It is also reported that opening of SK_{Ca} and IK_{Ca} channels decreases myogenic tone and increases ACh-induced relaxation in rat cremaster arterioles (Sheng et al., 2009) and restores attenuated EDHF-type relaxation in mesenteric small arteries from Zucker diabetic fatty rats (Brondum et al., 2010). Also recent studies have suggested that SK_{Ca} and IK_{Ca} channel opening is associated with the activation of NO synthase and NO production (Stankevicius et al., 2006, Sheng and Braun, 2007, Brahler et al., 2009, Sheng et al., 2009, Dalsgaard et al., 2010). The production of endothelium-dependent relaxing factors generally involves an increase in the intracellular Ca²⁺ concentration [Ca²⁺]_i. An increase in endothelial [Ca²⁺]_i will open calcium-activated potassium channels of small (SK_{Ca}) and intermediate (IK_{Ca}) conductance, thereby hyperpolarizing the endothelial cell. A recent study showed that the SK_{Ca} and IK_{Ca} channel opener NS309-induced calcium influx that contributed to the formation of NO (Stankevicius et al., 2011). However, further studies are required to clarify how activation of these mechanisms is coupled to increased formation of NO in endothelial cells.

CONCLUSION

Previous studies have suggested that even acute hyperglycaemia (Bartnik et al., 2007, Cameron and Cruickshank, 2007) or long term diabetes i.e. 8 weeks or more (Hink et al., 2001, Peredo et al., 2006) could lead to endothelial dysfunction due to increased oxidative stress and decreased nitric oxide bioavailability. We suggest that, in diabetes, an initial increased synthesis of nitric oxide by increased activity of eNOS might be able to compensate for increased oxidative stress leading to preserved endothelial function at the start of diabetes followed by a final reduction in endothelium response as the disease progresses leading to endothelial dysfunction. In the light of these findings, we can conclude that despite marked hyperglycaemia and increased oxidative stress, endothelium-dependent and -independent relaxation was maintained after 6 weeks of streptozotocin-induced diabetes due to increased nitric oxide bioavailability. The increase in nitric

oxide bioavailability could be attributed to increased expression of eNOS and calmodulin and/or release from nitrosothiol stores. The nitric oxide could be acting through sGC as well as a sGC-independent pathway through activation of SK_{Ca} and IK_{Ca} channels to cause relaxation in diabetic rat aorta. Therefore our results suggest that increased nitric oxide bioavailability is a compensatory mechanism to counteract increased oxidative stress in the early stages of diabetes to maintain endothelial function in aorta.

ABBREVIATIONS:

EDHF, endothelium-derived hyperpolarising factor; eNOS, endothelial nitric oxide synthase; sGC, soluble guanylate cyclase; ODQ, 1H-(1,2,4)-oxadiazolo(4,2-a)quinoxalin-1-one; L-NNA, N-nitro-L-arginine; TRAM-34, 1-[(2-chlorophenyl)(diphenyl)methyl]-1H-pyrazole;; IK_{Ca}, intermediate-conductance calcium activated potassium channel; SK_{Ca}, small-conductance calcium-activated potassium channel; ROS, reactive oxygen species; PHMBA, p-hydroxymercuribenzoic acid.

COMPETING INTEREST

The authors declare that they do not have any competing interests.

AUTHORS' CONTRIBUTIONS

AJ made substantial contributions to conception and design of experiments, performed the experiments, analysed the data and wrote the manuscript. OLW made substantial contributions to conception and design of experiments, edited the manuscript and contributed to the discussion. All authors read and approved the final manuscript.

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LEGENDS FOR FIGURES:

Figure 1: Effect of 6 weeks of diabetes on endothelium-dependent and endothelium-independent relaxation responses to (a) acetylcholine (10^{-10} to 10^{-5} M) and (b) sodium nitroprusside (10^{-11} to 10^{-5} M) in phenylephrine (10^{-8} to 10^{-7} M) precontracted aortic rings. The data is expressed as mean $\pm$ SEM. The pEC₅₀ and R_{max} values calculated from the data are shown in Table 2.

Figure 2: Concentration response curve to ACh in a) control b) diabetic animals in the presence of (●) vehicle, (◆) N-nitro-L-arginine (L-NNA), (□) ODQ, (Δ) indomethacin and c) control d) diabetic animals in the presence of (●) vehicle, (◇) L-NNA+ODQ, (■) Apamin+TRAM-34, (▽) L-NNA+ODQ+Apamin+TRAM-34 in phenylephrine (10^{-8} to 10^{-7} M) precontracted aortic rings. The data is expressed as mean $\pm$ SEM. The pEC₅₀ and R_{max} values calculated from the data are given in Tables 2 and 3.

Figure 3: Concentration response curve to acetylcholine (ACh) in diabetic animals in the presence of a) (●) vehicle, (◇) L-NNA+ ODQ, (◆) Hb+ODQ, (□) Hb+L-NNA+ODQ and b) (●) vehicle, (◆) L-NNA, (□) L-NNA+carboxy-PTIO, (Δ) L-NNA+L-cysteine in phenylephrine (10^{-8} to 10^{-7} M) precontracted aortic rings. All the experiments were performed in the presence of indomethacin. The data is expressed as mean $\pm$ SEM. The pEC₅₀ and R_{max} values calculated from the data is given in Tables 3 and 4.

Figure 4: Effect of the nitrosothiol depleting agents ethacrynic acid (50 μ M) and p-hydroxymercuribenzoic acid (10 μ M) on relaxation responses to three consecutive exposures to ACh (10 μ M) in the presence of L-NNA (100 μ M). All experiments were performed in the presence

of indomethacin (10 μ M). * Significantly different compared to L-NNA treated rings (one way ANOVA, Bonferroni post test, $p<0.05$). $n=4$ for each data set.

Figure 5: Protein expression of (a) eNOS, (b) calmodulin (c) NOX2 and (d) eNOS dimer-monomer in the normal and diabetic rat aorta determined by Western blot analysis. Diabetes significantly increased the expression of all the proteins in the rat aorta. Results are shown as mean $\pm$ s.e.m.
*Significantly different from control rats (Student's unpaired t-test, $p<0.05$).

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Table1: Initial and final body weights, blood glucose, superoxide counts from aortic rings and plasma nitrite levels in control and diabetic animals.

	Control	Diabetic
Initial body weight (g)	268±13	245±8
Final body weight (g)	451±7	306±14*
Blood glucose (mmol/L)	9.8±1	>33*
Superoxide counts (AU/mg dry tissue weight)	986±163	2180±363*
Superoxide counts in the presence of DPI (AU/mg dry tissue weight)	112±18	177±53
Plasma nitrite levels (μmol/L)	114±16	272±68*

The data is expressed as mean±SEM.

*Significantly different from control group (Student's unpaired t-test, $p < 0.05$).

Table 2: Parameters for endothelium-dependent and independent relaxation in response to ACh and SNP in aortic rings from control and diabetic rats. The effects of L-NNA (100 μ M), ODQ (10 μ M) and indomethacin (Indo, 10 μ M) on responses to ACh and SNP are shown.

	Control			Diabetic		
	n	pEC ₅₀	R _{max}	n	pEC ₅₀	R _{max}
ACh						
Vehicle	7	7.36 $\pm$ 0.1	92 $\pm$ 4	7	7.34 $\pm$ 0.11	88 $\pm$ 5
L-NNA	7	ND	10 $\pm$ 6*	7	6.78 $\pm$ 0.1*	54 $\pm$ 7* [#]
ODQ	7	ND	6 $\pm$ 4*	7	6.77 $\pm$ 0.15*	46 $\pm$ 13* [#]
Indo	7	7.43 $\pm$ 0.1	91 $\pm$ 4	7	7.48 $\pm$ 0.12	93 $\pm$ 2
SNP						
Vehicle	7	8.13 $\pm$ 0.14	100 $\pm$ 0	7	8.57 $\pm$ 0.27	101 $\pm$ 2
L-NNA	7	8.74 $\pm$ 0.24*	101 $\pm$ 1	7	9.35 $\pm$ 0.16* [#]	101 $\pm$ 2
ODQ	7	5.80 $\pm$ 0.34*	50 $\pm$ 11*	7	6.54 $\pm$ 0.07* [#]	55 $\pm$ 6*
Indo	7	8.10 $\pm$ 0.19	95 $\pm$ 4	7	8.59 $\pm$ 0.15	101 $\pm$ 2

The data is expressed as mean $\pm$ SEM.

n= number of experiments

ND= not determined

* Significantly different compared to vehicle in the same treatment group (one way ANOVA, Bonferroni post test, p<0.05).

[#]Significantly different compared to the control group (Student's unpaired t-test, p<0.05).

Table 3: The effect of various treatments on responded to ACh and SNP in aortic rings from control and diabetic rats. All the experiments were performed in the presence of indomethacin. The combination of apamin+TRAM-34 had no effect on relaxation in any of the group. ODQ+L-NNA+ apamin +TRAM-34 treatment completely abolished relaxation in both the groups. Responses to ACh in the diabetic group showed a resistance to inhibition by L-NNA+ODQ, but were abolished by additional presence of Hb.

	Control			Diabetic		
	n	pEC ₅₀	R _{max}	n	pEC ₅₀	R _{max}
ACh						
Vehicle	6	7.49± 0.1	90±1	6	7.45±0.2	93±5
L-NNA +ODQ	6	ND	1±1*	6	6.75±0.15*	25±4* [#]
Apamin+Tram-34	6	7.34± 0.13	89±2	6	7.43±0.11	81±5*
L-NNA +ODQ+	6	ND	2±2*	6	ND	6±4*
Apamin+Tram-34						
Hb+ODQ	6	ND	0*	6	ND	15±5*
Hb+ODQ+ L-	6	ND	4±4*	6	ND	0*
NNA						
SNP						
Vehicle	6	8.32± 0.15	99.4±0.4	6	8.33±0.17	98±3
L-NNA +ODQ	6	5.94± 0.10*	61±3*	6	6.76±0.19* [#]	56±6*
Apamin+Tram-34	6	8.03 ± 0.21	99.6±0.7	6	8.30±0.18	100±0.7
L-NNA +ODQ+	6	5.93 ± 0.09*	57±4*	6	6.28±0.09*	33±10* [#]
Apamin+Tram-34						
Hb+ODQ	6	6.56±0.12*	85±7*	6	6.46±0.05*	68±22*
Hb+ODQ+ L-	6	6.46±0.10*	84±4*	6	6.61±0.12*	85±5
NNA						

The data is expressed as mean±SEM. n= number of experiments

ND= not determined

* Significantly different compared to vehicle in the same treatment group (one way ANOVA,

Bonferroni post test, p<0.05). # Significantly different compared to the same treatment group from

the control animals (Student's unpaired t-test, p<0.05).

Table 4: The effect of the NO scavenger carboxy-PTIO and L-cysteine on pEC_{50} and R_{max} in aortic rings from diabetic rats for ACh concentration response curve. All these experiments were performed in the presence of indomethacin.

Treatment		Diabetic	
	n	pEC_{50}	R_{max}
ACh			
Vehicle	7	7.19±0.08	93±3
L-NNA	7	6.69±0.14*	44±6*
L-NNA+PTIO	6	6.33±0.06*	26±4* [#]
L-NNA +L-cys	5	6.23±0.23*	15±6* [#]

The data is expressed as mean±SEM. n= number of experiments

* Significantly different compared to vehicle (one way ANOVA, Bonferroni post test, $p<0.05$).

Significantly different compared to L-NNA (one way ANOVA, Bonferroni post test, $p<0.05$).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5