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Long-term treatment in vivo with NOX-101, a scavenger of nitric oxide, prevents diabetes-induced endothelial dysfunction

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Summary Substantial evidence exists that diabetes results in impaired endothelial dysfunction suggesting diminished nitric oxide production from diabetic endothelium. It is not known what factors contribute to the development of this defect. In this study, we tested whether chronic treatment in vivo with NOX-101, a water-soluble nitric oxide scavenger, prevents endothelial dysfunction in diabetes. Sprague-Dawley rats were made diabetic by an intravenous injection of streptozotocin. A subgroup of control or diabetic animals received twice daily subcutaneous injections of 80 mg/kg NOX-101 beginning at 48 h after streptozotocin was injected and throughout 8 weeks of diabetes. Body weights and glucose concentrations were monitored weekly. At the end of 8 weeks, blood glucose and glycosylated haemoglobin was raised in diabetic rats but serum insulin concentrations were reduced. Treatment with NOX-101 did not alter glucose or insulin concentrations in control or diabetic rats; however, total glycosylated haemoglobin was partially reduced compared with untreated rats. In a subgroup of 2-week diabetic and age-matched rats fasted for 24 h, NOX-101 abolished total urinary nitrate plus nitrite (an index of nitric oxide production in vivo). In isolated tissue baths, relaxation to the endothelium-dependent vasodilator, acetylcholine, was impaired in diabetic aortic rings and relaxation to nitroglycerin was unaltered. Treatment of control rats with NOX-101 did not alter maximum relaxation to acetylcholine but shifted the response curve slightly to the right. In contrast in diabetic rats, NOX-101 prevented the impairment in endothelium-dependent relaxation but had no effect on relaxation induced by nitroglycerin. These data suggest the possibility that diabetes-induced endothelial dysfunction in diabetes results, in part, from a paradoxical increase in nitric oxide production during the course of the disease. This suggests a novel pathway of vascular complications. [Diabetologia (1998) 41: 1220–1226]

Keywords Nitric oxide, endothelium, diabetes mellitus.

Considerable evidence using experimental models [1–5] have shown that diabetes mellitus is an independent risk factor for the development of impaired endothelium-dependent relaxation – that is, endothelial dysfunction. This defect has been confirmed in Type I (insulin-dependent) [6, 7] and Type II (non-insulin-dependent) [8] diabetic patients. The factor(s) which contribute to endothelial dysfunction in diabetic patients is not known but data derived in experimental models have suggested several possibilities including: (a) concurrent release of an endothelium-derived constricting factor arising from the cyclooxygenase pathway [9, 10]; (b) increase in protein kinase C [11]; (c) inappropriate utilization of arginine for nitric oxide (NO) synthesis [12, 13]; (d) an abnormal NO synthase (NOS) activity due to inadequate co-factor [14]; (e) NO quenching by advanced glycosylation end-products [15]; or (f) concomitant increased synthesis

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Abbreviations: NO, Nitric oxide; NOS, nitric oxide synthase; STZ, streptozotocin; $\bullet O_2^-$, superoxide anion radical; •OH, hydroxyl radical.

of superoxide anion radical ($\bullet O_2^-$) which interacts to destroy NO activity [16–20] including the possibility of increased NO synthesis but that the actual NO bioactivity is masked by enhanced $\bullet O_2^-$ production [16, 21].

In the studies cited above, experiments were designed to identify immediate factors which compromise endothelium-dependent relaxation in the acute setting. In contrast, little effort has been made to identify the underlying antecedent factors or pathways in vivo which contribute to the chronic development of diabetes-induced endothelial dysfunction. Recently, aminoguanidine, a proposed inhibitor of inducible NOS, prevented the early development of vascular permeability associated with diabetes mellitus [22, 23] suggesting that the aetiology of vascular permeability results from excessive NO production.

To explore this possibility further, we evaluated the efficacy of NOX-101, a water-soluble NO scavenger, belonging to the class of dithiocarbamate-based NO chelators to prevent diabetes-induced endothelial dysfunction. This class of compounds bind NO in solution forming iron-nitrosyl complexes and bind the excess NO produced during septic shock [24, 25].

Materials and methods

Male, Sasco Sprague-Dawley rats (Charles River Laboratory, Wilmington, Masss., USA) at 10–11 weeks of age were anaesthetized with sodium pentobarbital (60 mg/kg, i.p.). Diabetes was induced by tail-vein injection of streptozotocin (STZ) (55 mg/kg in pH 4.5 citrate buffer). Blood glucose was measured 2 days and 1 week later to verify hyperglycaemia and weekly throughout 8 weeks of diabetes. A subset of control or diabetic animals received twice daily subcutaneous injections in sterile saline of 80 mg/kg NOX-101 (Medinox, San Diego, Calif., USA) beginning 48 h after STZ and throughout the duration of diabetes.

At the conclusion of the 8-week period, blood glucose was determined from a drop of tail blood using an ExacTech glucometer and test strips (Medisense, Inc., Cambridge, Mass., USA). Serum insulin was determined using commercial radioimmunoassay kits (Diagnostic Products Corp., Los Angeles, Calif., USA; or from, Linco Research, Inc., St. Louis, MO, USA). To assess chronic glycemic control, blood was collected for analysis of total glycosylated haemoglobin (Sigma Diagnostics, St. Louis, MO, USA).

To evaluate the in vivo NO scavenging properties of NOX-101, an additional series of 2-week diabetic and age-matched control rats with or without treatment with NOX-101 were used to measure urinary nitrate plus nitrite concentrations using a commercial kit (Alexis Biochemicals, San Diego, Calif., USA). At the end of this time, animals were fasted for 24 h in metabolic cages to collect urine samples. Collection reservoirs contained 100 U/ml penicillin, 100 U/ml streptomycin and 0.25 μg/ml amphotericin B. Animals were fasted to avoid potential interference in urinary nitrate plus nitrite determinations by inadvertent food particle contamination.

On the day of vascular reactivity measurements, rats were anaesthetized with injections (i.p.) of 65 mg/kg sodium pentobarbital. Thoracic aortas were carefully isolated, removed from open-chest animals and placed in 4 °C Krebs-bicarbonate buffer (pH 7.4). The aortic segments were carefully cleaned of fat and loose connective tissue. The thoracic aortic segments were sectioned into 3 mm (in length) rings. Extreme care was taken to avoid stretching and contact with the luminal surface of the endothelium to avoid inadvertent damage during isolation.

Isolated vascular ring experiments. Aortic rings were suspended between parallel triangular hooks in 10-ml tissue baths which were maintained at 37 °C. The medium consisted of a modified Krebs-Henseleit bicarbonate buffer [14] which was maintained at pH 7.4 by oxygenation with 95 % O_2:5 % CO_2. The buffer also contained 0.8 μmol/l imipramine and 0.9 μmol/l propranolol to control for any potential differences in diabetes-induced changes in catecholamine uptake or β-adrenergic activity. The potential anticholinergic effect of imipramine has been shown previously in our laboratory to not significantly alter relaxation elicited by acetylcholine [14, 26]. Rings were stretched to an optimal resting tension of 2.0 g for both control and diabetic blood vessels. Changes in isometric tension were recorded on a Gould TA6000 recorder using Radnoti force-displacement transducers and amplifiers.

Individual protocols. After 90 min of equilibration, rings were precontracted with increasing concentrations of norepinephrine (1 nmol/l to 30 μmol/l) stock solutions of which contained ascorbate to prevent auto-oxidation (final concentration = 20 nmol/l). The pD_2 (-log EC_{50}) for response of rings to norepinephrine was determined for each ring experiment. After contraction, each ring was serially-washed and re-equilibrated to baseline. Rings were then contracted with a submaximum concentration of norepinephrine (usually 1 μmol/l). The concentration of agonist was varied in some instances so that equieffective concentrations of constrictor were used based upon the tensions derived in the initial norepinephrine concentration-response measurements.

At the plateau of contraction, relaxation responses to cumulative concentrations of vasodilators were done using acetycholine (endothelium-dependent vasodilator) or nitroglycerin (endothelium-independent vasodilator). We have shown previously that relaxation to acetylcholine in control and diabetic rat aorta is due to NO-dependent relaxation based upon the complete blockade of relaxation by arginine-based inhibitors of NOS [12, 14] and the lack of modification by incubation with indomethacin [27]. Only one type of vasodilator was used in each ring preparation.

All chemicals were of highest purity available. Most chemicals were obtained from Sigma Chemical Co. and dissolved in deionized water and diluted in buffer. Statistical analysis was performed using analysis of variance followed by Duncan's test for multiple group mean comparisons, unpaired *t* test when comparing two group means, or paired *t* test when comparing two group means in a repeated format. A value of *p* less than 0.05 was set as indicating statistical significance. All data are expressed as the mean ± SEM.

Results

Body weight and blood analysis. Body weight was increased ($p < 0.01$) at the end of the study compared with starting weight in control animals (untreated control: 350 ± 4 and 471 ± 12 g; NOX-101-treated control: 380 ± 7 and 512 ± 20 g, for intitial and final

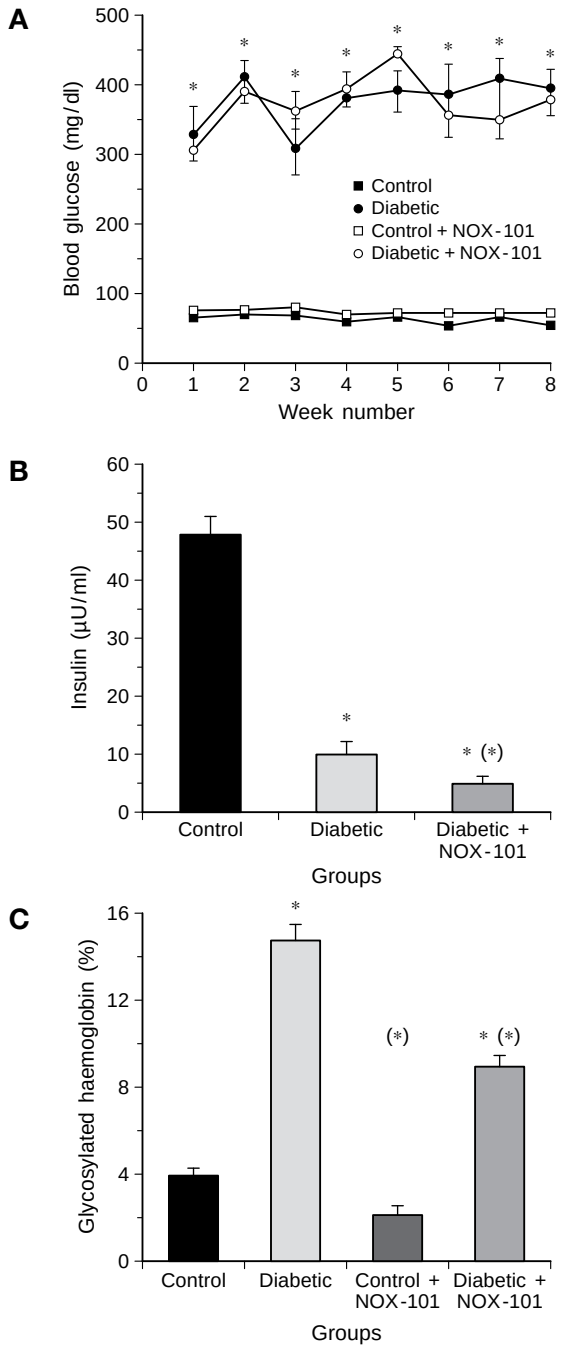

Fig. 1A–C. Final values (at the time of killing) for blood glucose (**A**, control: $n = 15$; NOX-101-treated control: $n = 8$; diabetic: $n = 9$; NOX-101-treated diabetic: $n = 8$), serum insulin (**B**, $n = 9$ each except for NOX-101-treated diabetic where $n = 7$) and total glycosylated haemoglobin (**C**, $n = 9$ each) for the various experimental groups. The value of insulin for control animals treated with NOX-101 (i.e. 129 ± 17 μU/ml) is not included in **B** as these samples were measured using the new highly-specific rat insulin kits (Linco Research, Inc.). Using the new Linco kits, measurement of insulin in an independent series of age-matched control rats but without treatment with NOX-101 showed no effect of drug treatment (e.g. 112 ± 30 μU/ml, $n = 4$; $p > 0.05$). $\neq p < 0.01$ compared with control group; $(\neq) p < 0.01$ compared with untreated diabetic group

weights, respectively). In contrast, no weight gain was observed in diabetic rats (untreated diabetic: 347 ± 4 and 291 ± 18 g; NOX-101-treated diabetic: 335 ± 5 and 284 ± 7 g, for initial and final weights, respectively). One of the drug-treated diabetic rats developed severe weight loss by week 5 and was removed from the study. Treatment with NOX-101 did not prevent weight gain in control rats and did not alter the weekly body weight of diabetic rats.

Prior to injection of STZ, blood glucose was not different between control and diabetic rats. At 48 h after STZ injection and before injection of NOX-101, blood glucose increased ($p < 0.01$) compared with age-matched control rats (untreated control: 75 ± 3 mg/dl; treated-control: 75 ± 5 mg/dl; untreated diabetic: 354 ± 17 mg/dl; treated diabetic: 311 ± 16 mg/dl). There was no significant difference in the blood concentration of glucose monitored weekly (beginning at 1 week) in drug-treated rats compared with untreated rats (not shown). At the end of 8 weeks, blood glucose remained raised in both diabetic groups with no difference in values between the untreated rats and rats treated with NOX-101 (Fig. 1). Serum insulin was decreased in untreated and treated diabetic rats (Fig. 1). Glycosylated haemoglobin was elevated in untreated diabetic rats and diabetic rats treated with NOX-101 (Fig. 1). Treatment with NOX-101 caused a reduction in glycosylated haemoglobin in both groups; however, concentrations in drug-treated diabetic rats were still raised above that seen in control rats.

In the 2-week experimental subgroups, there was no difference in total urinary nitrate plus nitrite arising from 24-h fasted control compared with diabetic rats (i.e. control, $n = 4$: 258 ± 83.3; diabetic, $n = 5$: 248.3 ± 62.2 nmol · 24 h^{-1} · 100 g^{-1} body weight, respectively). In contrast, urinary nitrate plus nitrite was essentially abolished by treatment with NOX-101 (i.e. 0.2 ± 0.1 and 11.9 ± 10.6 nmol · 24 h^{-1} · 100 g^{-1} body weight, for control and diabetic rats, respectively, $n = 6$ each).

Vascular studies. Concentration-response of aortic rings to norepinephrine showed no difference in the maximum tension normalized for cross-sectional areas (i.e. control: 2.00 ± 0.11 g/mm^2; drug-treated control: 2.07 ± 0.19 g/mm^2; diabetic: 1.82 ± 0.25 g/mm^2; drug-treated diabetic: 1.75 ± 0.18 g/mm^2). Furthermore, there was no difference in norepinephrine-induced contraction between each group (Fig. 2). For example, pD_2 was not altered (i.e. control: 6.8 ± 0.1; drug-treated control: 6.6 ± 0.1; diabetic: 6.7 ± 0.1; drug-treated diabetic: 6.7 ± 0.1).

Acetycholine produced concentration-dependent relaxations in control and diabetic aortic rings (Fig. 2). These relaxations were impaired in diabetic compared with control rings and were returned to normal in diabetic rats receiving long-term injections

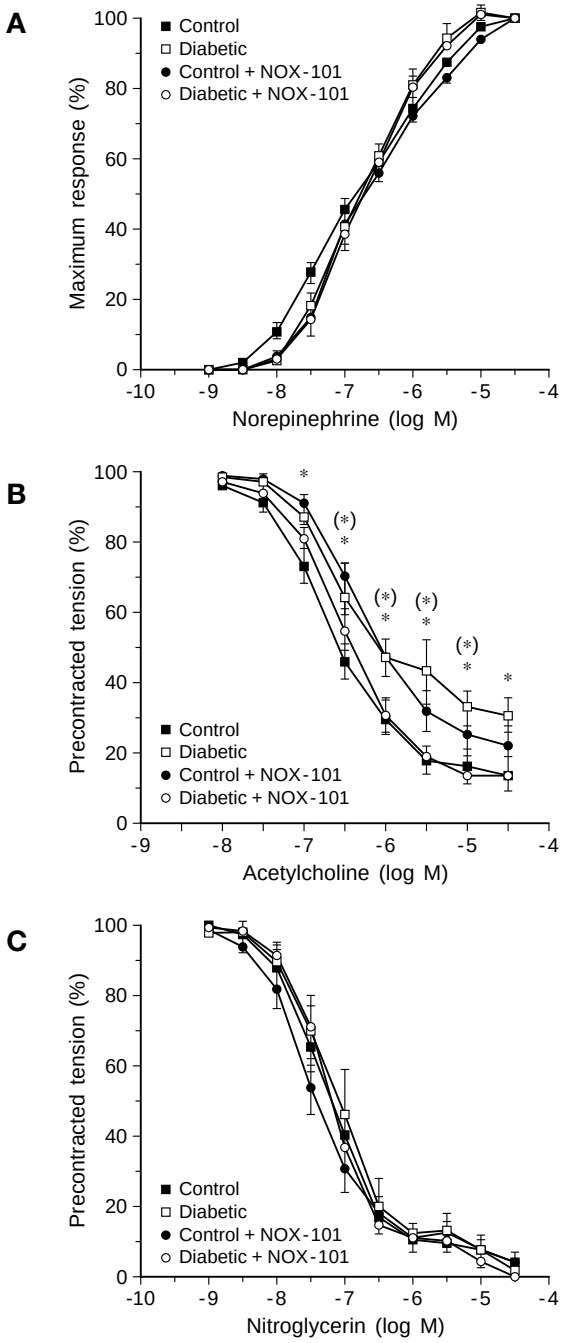

Fig. 2. **A** Concentration-dependent contraction to norepinephrine in aortic rings from control and diabetic animals without or with chronic treatment with NOX-101. (**B**). Concentration dependent relaxation to the endothelium-dependent vasodilator, acetylcholine, in aortic rings from animals without or with long-term treatment with NOX-101 $*p < 0.05$ compared with control group. (**C**). Concentration dependent relaxation to the endothelium-independent vasodilator, nitroglycerin, in aortic rings from animals without or with chronic treatment with NOX-101. Responses in **A**, **B** and **C** represent the mean ± SEM for untreated control ($n = 15$), treated control ($n = 8$), untreated diabetic ($n = 9$), treated diabetic ($n = 9$) except in **C** for untreated control ($n = 11$) and untreated diabetic ($n = 6$)

of NOX-101. Long-term treatment of control rats with NOX-101 did not alter maximum relaxation but shifted the curve to the right (i.e. $pD_{50} = 6.5 \pm 0.1$ and 6.1 ± 0.1 for untreated and treated groups, respectively. In contrast, endothelium-independent relaxation to nitroglycerin was not altered by diabetes (Fig. 2). Furthermore, chronic treatment with NOX-101 did not alter the dilator responses to nitroglycerin in either group.

Discussion

Most previous studies have focused on immediate or concurrent factors which contribute to the phenomenon of diabetes-induced endothelial dysfunction. In contrast, very few studies have focused on the antecedent factors in vivo which contribute to the development of the underlying dysfunction. A few studies using aldose reductase inhibitors [28, 29] or vitamin E [30, 31] have suggested that alterations in sorbitol pathway or oxygen-radicals contribute to diabetes induced endothelial dysfunction.

In the present study, we have shown that long-term treatment with NOX-101, a NO scavenger, prevents the impaired endothelium-dependent relaxation to acetylcholine and that this is not accounted for by a generalized improvement in diabetic vascular smooth muscle reactivity in that responses to the endothelium-independent vasodilator, nitroglycerin, were not altered by drug treatment. The finding that NOX-101 partially attenuated endothelium-dependent relaxation in the control group is similar to production of sciatic nerve blood flow deficits in control rats treated with L-nitroarginine presumably due to inhibiting normal constitutive NO bioactivity [32].

Our results using NOX-101 are consistent with evidence of increased NO production especially early in diabetes as described in recent reviews [5, 33]. Evidence supporting increased NO production include findings that endothelium-dependent relaxation is enhanced early after onset of STZ-induced diabetes in the rat renal artery [34], in rat mesenteric arteries [35] and in rat aorta [36]. Also, plasma or urinary nitrate plus nitrite (end-products of NO metabolism) or both are increased in diabetic rats [37–40]. The source including cell type for increased NO production is not known. The increase in urinary NO-derived products has been shown as early as 4 days [38] and as late as 32 weeks [41] of diabetes. Our analysis of urinary nitrate plus nitrite failed to show differences at 2-weeks of disease. It is possible that the fasting protocol in our study contributes to the return of nitrate/nitrite values to normal as pair-feeding of 6-week diabetic rats reduced but did not eliminate the increase in urinary nitrate plus nitrite [37].

Other studies have shown that aminoguanidine, a putative inhibitor of inducible NOS (iNOS), prevent-

ed diabetes-induced vascular albumin permeation and prevented the early increases in retinal blood flow [22]. These authors concluded that increased NO has a role in the aetiology of these defects. That this assumed increased NO arises from iNOS in vascular tissue is unclear as no data exists to support either increased mRNA or protein for iNOS. Furthermore, addition in vitro of aminoguanidine to aorta of STZ-diabetic (unpublished observations) and spontaneous diabetic BB [19] rats had no effect on contractile tension or relaxation to acetycholine.

In contrast, long-term treatment with aminoguandine prevented diabetes-induced endothelial dysfunction in rat aorta [15, 42] or rat mesenteric artery [43] but was ineffective in rat skeletal muscle arterioles [44]. These opposing findings might be explained by variances in experimental design, duration and severity of disease or that a much lower dosage of aminoguanidine was used in the latter study (i.e. 1/10th the dosage used in studies showing benefit). Furthermore, the putative benefits of aminoguanidine in one study [15] are inconclusive as this drug was given beginning 1 week before injection with STZ. In this regard, other NOS inhibitors are known to reduce the diabetogenic potential of STZ in both mice and rats [45–47].

Studies using aminoguanidine (and other NOS inhibitors) have limitations and present difficulties in interpretation as this agent can also inhibit constitutive NOS (cNOS) activity [48] which similarly could account for the right-shift in response to acetycholine in NOX-101-treated control rats. Effects of aminoguanidine in vivo are complex due to several potential actions including: inhibition of diamine oxidase [49], decreased formation of advanced glycosylation end products [50], antioxidant or prooxidant properties or both [51, 52], promotion of leucocyte adherence to normal blood vessels [53] and inhibition of glucose-stimulated insulin release from beta-cells [54, 55]. Nevertheless, one group concluded that renal protection provided by aminoguanidine to be mediated via inhibition of NOS and not advanced glycation products [41].

We elected to use an alternative approach to evaluate a potential role of excess NO production in diabetes-induced endothelial dysfunction by using the water-soluble NO scavenger, NOX-101. Dithiocarbamate-based NO scavengers similar to NOX-101 have been shown to bind NO in solutions in vitro and in animals in vivo and to trap the increased NO produced during sepsis [24 25]. This approach circumvents the potential adverse side-effects of current NOS enzyme inhibitors and avoids other nonspecific properties of aminoguanidine which might also account for its previously observed salient effects.

Our studies are consistent with the hypothesis that decreases in agonist-stimulated endothelium-dependent NO production as a consequence of diabetes can result from a paradoxical increase in systemic NO production during the course of the disease. This would be analogous to the observations after endotoxin treatment that increase in iNOS cause a reduction in cNOS activity [56] or the decrease in cNOS activity seen after long-term treatment with NO donor compounds [57]. Whether this reflects a negative feedback control [58] or downregulation in NOS pathway is not known with certainty.

We assume that the primary mechanism of salient action of NOX-101 arises from scavenging NO as iron-thiol based dithiocarbamate derivatives are known to bind NO coordinated to the iron moiety of the molecule. Indeed, our studies confirm the action of scavenging NO by the 95–99 % reduction of urinary nitrate plus nitrite concentrations. This is consistent with findings in our laboratory that NOX-101 reduces the increase in plasma nitrate plus nitrite during cardiac allograft rejection [59]. We believe that the property of NOX-101 to scavenge NO is relatively specific as electron spin resonance spectroscopy confirms that similar derivatives bind nitrite and nitrogen dioxide with only 2–3 % efficiency and do not bind nitrate or peroxynitrite [60].

It is possible that rather than binding peroxynitrite directly, long-term treatment with NOX-101 could potentially act by preventing peroxynitrite-mediated endothelial injury by excess NO production in the presence of $\bullet O_2^-$. It is known that rate constant of interaction of $\bullet O_2^-$ with NO is greater than dismutation by superoxide dismutase [61]. Peroxynitrite, the product of the reaction of $\bullet O_2^-$ with NO, is a very toxic radical species which decomposes to hydroxyl radicals (•OH). The observation that •OH are detected in plasma after 72 h of diabetes in the rat [62], that the chronic treatment with dimethylthiourea, a •OH scavenger, and that a unique form of iron chelator (which prevents metal ion-catalysed •OH formation) both prevent endothelial dysfunction [63, 64] are all consistent with this pathway for the aetiology of diabetes-induced endothelial dysfunction.

It is not possible from our studies to exclude the possibility that NOX-101 provided partial benefit by an antioxidant action. Although the kinetics of the molecular interaction of NOX-101 with reactive oxygen in vitro are not known, the rate constant for reaction of NO with the iron-thiol-based NO trapping agent, Fe (II)-proline dithiocarbamate is 1.1×10^8 M^{-1} S^{-1} [65]. Since the rate constant of reaction of $\bullet O_2^-$ with NO is 6.7×10^9 M^{-1} S^{-1}, this suggests that most, but not all, $\bullet O_2^-$ is likely to interact with NO rather than with NOX-101. Furthermore, the rate constant of reaction of sodium diethyldithiocarbamate with $\bullet O_2^-$ and H_2O_2 is 9×10^2 M^{-1} S^{-1} and 0.025 S^{-1}, respectively [66]. This interaction is with free thiol which is not the case of NOX-101 in which the thiol moeity is bound with iron. Another possibility is that reactive oxygen species such as H_2O_2 might alter the

redox state of thiol-bound iron (II to III). While ferric-thiolated compounds also actively bind NO, this binding occurs at a reduced efficiency [67].

The observation that NOX-101 partially reduced glycated haemoglobin in diabetic (and control) animals suggests that NOX-101 could benefit the diabetic endothelium, in part, by decreasing glycated protein formation. The mechanism for this action should be evaluated in more detail including studies of glycated tissue proteins especially since NOS inhibitors have been shown not to reduce glycated haemoglobin but dramatically reduce advanced glycation products in renal tissue [41].

The use of the NO scavenger, NOX-101, suggests new and, more direct, evidence that endothelial cell injury as a consequence to diabetes is mediated, at least in part, by excess NO production during the course of the disease. Accordingly, this opens the possibility of a novel approach for therapeutic intervention to prevent diabetes-induced endothelial dysfunction.

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