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# Endothelium-dependent nitroxyl-mediated relaxation is resistant to superoxide anion scavenging and preserved in diabetic rat aorta

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ABSTRACT

The aim of the study was to investigate whether diabetes-induced oxidant stress affects the contribution of nitroxyl (HNO) to endothelium-dependent relaxation in the rat aorta. Organ bath techniques were employed to determine vascular function of rat aorta. Pharmacological tools (3 mM L-cysteine, 5 mM 4-aminopyridine (4-AP), 200 μM carboxy-PTIO and 100 μM hydroxocobalamin, HXC) were used to distinguish between NO and HNO-mediated relaxation. Superoxide anion levels were determined by lucigenin-enhanced chemiluminescence. In the diabetic aorta, where there is increased superoxide anion production, responses to the endothelium-dependent relaxant ACh were not affected when the contribution of NO to relaxation was abolished by either HXC or carboxy-PTIO, indicating a preserved HNO-mediated relaxation. Conversely, when the contribution of HNO was inhibited with L-cysteine or 4-AP, the sensitivity and maximum relaxation to ACh was significantly decreased, suggesting that the contribution of NO was impaired by diabetes. Furthermore, whereas HNO appears to be derived from eNOS in normal aorta, in the diabetic aorta it may also arise from an eNOS-independent source, perhaps derived from nitrosothiol stores. Similarly, exposure to the superoxide anion generator, pyrogallol (100 μM) significantly reduced the sensitivity to the NO donor, DEANONOate and ACh-induced NO-mediated relaxation but had no effect on responses to the HNO donor, Angeli's salt and ACh-induced HNO-mediated relaxation in the rat aorta. These findings demonstrate that NO-mediated relaxation is impaired during oxidative stress but the HNO component of relaxation is preserved under those conditions.
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## 1. Introduction

The endogenous production of nitric oxide (NO) is well recognized as an important contributor to vascular homeostasis. The vascular action of NO predominately involves the activation of soluble guanylate cyclase (sGC), which catalyses the conversion of guanosine triphosphate (GTP) to cyclic guanosine monophosphate (cGMP), leading to vasodilatation [1]. NO may not be the only endothelium-derived nitrogen species, with nitroxyl (HNO), the one electron reduced and protonated form of NO, likely to have a role in modulating vascular tone as there is evidence that it is formed endogenously and contributes to endothelium-dependent relaxation in both conduit [2,3] and resistance vessels [4].

HNO can be produced by several distinct pathways in the vascular endothelium. Biochemical studies indicate that HNO can be synthesized via endothelial NO synthase (eNOS)-dependent and independent pathways [5]. HNO can be synthesized by eNOS itself as an intermediate product during the conversion of L-arginine to NO. Subsequently, HNO is oxidized to NO by superoxide dismutase [6]. Moreover, the production of HNO by eNOS could occur under conditions of reduced levels of the eNOS cofactor, tetrahydrobiopterin ($BH_4$) [7] or where there is oxidation of NOS intermediates, N-hydro-L-arginine [8] and hydroxylamine [9]. Furthermore, HNO can be produced from NOS-independent sources, including the reduction of NO by mitochondrial cytochrome c [10] and xanthine oxidase [11]. Finally, S-nitrosothiols are also known to generate HNO via S-thiolation, a reaction between S-nitrosothiols and other thiol species [12].

*Abbreviations:* ACh, acetylcholine; AS, Angeli's salt; 4-AP, 4-aminopyridine; $BH_4$, tetrahydrobiopterin; cGMP, cyclic guanosine monophosphate; DEA, DEANONOate; DPI, diphenylene iodonium; EA, ethacrynic acid; EDHF, endothelium-derived hyperpolarizing factor; eNOS, endothelial nitric oxide synthase; GTP, guanosine triphosphate; HXC, hydroxocobalamin; HNO, nitroxyl; L-NNA, N-nitro-L-arginine; NO, nitric oxide; ODQ, 1H-[1,2,4]-oxadiazolo[4,2-a]quinoxalin-1-one; $pEC_{50}$, negative logarithm of the half maximal effective concentration; $R_{max}$, maximum relaxation; PHMBA, p-hydroxymercuribenzoic acid; SNP, sodium nitroprusside; sGC, soluble guanylate cyclise; SOD, superoxide anions dismutase.

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Like NO, HNO has the ability to cause vascular relaxation via the generation of cGMP but HNO has additional, distinct pharmacological actions in comparison to NO [13]. For example, Miranda et al. (2002) demonstrated that, unlike NO the HNO donor Angeli's salt does not react with superoxide anions to form peroxynitrite [14], suggesting that HNO-mediated vasodilatation could be preserved under oxidative stress conditions. Despite this, Ellis et al. (2001) reported that Angeli's salt-induced relaxation of the rat aorta was significantly attenuated by the presence of a superoxide anion generator, pyrogallol [15], suggesting that HNO does react with superoxide anions. Thus, it remains unclear whether HNO-induced vascular relaxation is affected by superoxide anions in the vasculature.

It is well defined that the overproduction of reactive oxygen species is an important mechanism in vascular diseases such as diabetes, leading to impaired NO synthesis/activity and endothelial dysfunction [16–18]. It is not known, however, whether the vascular actions of endothelium-derived HNO are affected by oxidative stress, a condition which occurs in diabetes. Thus, the aim of this study was to investigate the effects of pyrogallol-induced superoxide anion production on HNO-induced relaxation in rat isolated aorta. Most importantly, we also evaluated the vascular actions of endothelium-derived HNO in diabetes where there is chronic oxidative stress.

## 2. Methods

All procedures were approved by the Animal Experimentation Ethics Committee of RMIT University and conformed to the National Health and Medical Research Council of Australia code of practice for the care and use of animals for scientific purposes (AEC approval number 0711).

### 2.1. Induction of diabetes

Type I diabetes was induced in rats as previously described [19]. Briefly, male Sprague-Dawley rats weighing approximately 200 g (Animal Resource Centre, Perth, WA, Australia) were randomly divided into two groups: normal and diabetic. Type I diabetes was induced by a single injection of streptozotocin (50 mg/kg) into the tail vein after the rats were fasted overnight. Ten weeks after streptozotocin, rats were asphyxiated by $CO_2$ inhalation, followed by decapitation, at which point blood was collected. Blood glucose was measured using a one touch glucometer (Roche, Sydney, NSW, Australia). Induction of diabetes was considered successful when the glucose level was higher than 25 mM.

### 2.2. Superoxide anion measurement in diabetes

Superoxide anion production in the aorta was determined by lucigenin-enhanced chemiluminescence assay as previously described [18]. Briefly, the aortae were preincubated for 45 min at 37 °C in Krebs–HEPES buffer containing diethylthiocarbamic acid (1 mM) and NADPH (100 μM). 300 μL of Krebs-HEPES buffer, containing lucigenin (5 μM) and the appropriate treatments were placed into a 96-well Optiplate, which was loaded into a Polarstar Optima photon counter (BMG Labtech, Melbourne, VIC, Australia) to measure background photon emission at 37 °C. After background counting was completed, a single ring of aorta was added to each well and photon emission was re-counted. The background reading was subtracted from the superoxide anion counts and normalized with dry tissue weight.

### 2.3. Preparation and equilibration of rat aortic rings

Male Sprague-Dawley rats were asphyxiated by $CO_2$ inhalation, followed by decapitation, and their chests opened to isolate the thoracic aortae. After the removal of superficial connective tissues, the aorta was cut into ring segments, of approximately 2–3 mm in length. The aortic rings were then mounted between two stainless steel wires, one of which was linked to an isometric force transducer (model FT03, Grass Medical Instruments, Quincy, MA, USA) connected to MacLab/8 (model MKIII, AD Instrument Co., Sydney, Australia), and the other end anchored to a glass rod submerged in a standard 10 mL organ bath. The organ bath was filled with Krebs-bicarbonate solution [composition (mM): NaCl, 118.0; KCl, 4.7; $KH_2PO_4$, 1.2; $MgSO_4 \cdot 7H_2O$, 1.2; D-glucose, 11.0; $NaHCO_3$, 25.0; $CaCl_2 \cdot 2H_2O$, 2.5; EDTA 0.026 [20]. The bath medium was maintained at 37 °C, pH 7.4 and continuously aerated with 95% $O_2$, 5% $CO_2$. Aortic rings were equilibrated for 1 h at a resting tension of 1 g, and then were precontracted with an isotonic, high potassium physiological salt solution (KPSS, 122.7 mM), to achieve maximal contraction. After re-equilibration, the rings were sub-maximally contracted with phenylephrine (PE, 0.01–0.3 μM) and endothelial integrity was tested by a single dose of acetylcholine (ACh, 10 μM). All rings responded to ACh (>80% relaxation).

### 2.4. Vascular reactivity in diabetes

To examine the effect of diabetes on NO and HNO-mediated relaxation, aortic rings were isolated from normal and diabetic rats. The effect of the treatment on relaxant responses was determined by cumulative concentration-response curves to ACh (0.1 nM–10 μM). In addition, responses to ACh were examined after 20 min incubation with different combinations of N-nitro-L-arginine (L-NNA, 100 μM), a non-selective nitric oxide synthase (NOS) inhibitor, 1H-[1,2,4]oxadiazolo[4,3-a]quinoxalin-1-one (ODQ, 10 μM), a soluble guanylate cyclase (sGC) inhibitor, 4-aminopyridine (4-AP, 5 mM), a voltage-gated potassium channel ($K_V$) blocker which has been demonstrated to inhibit HNO-mediated responses, but not NO-mediated responses [4,13,21,22], hydroxocobalamin (HXC, 100 μM) and carboxy-PTIO (200 μM), a selective NO scavenger [13,23,24] and L-cysteine (3 mM, which was incubated for 3 min), a selective HNO scavenger [13,23,25].

### 2.5. Assessment of nitrosothiols in diabetes

The role of nitrosothiols in diabetes was determined as previously described [26,27]. Briefly, aortic rings were submaximally precontracted with PE (0.01–0.3 μM). After stabilization of contraction, the aortic rings were incubated with either L-NNA alone or in the presence of one of the nitrosothiol depleting agents, ethacrynic acid (EA, 50 μM) [28,29] or p-hydroxymercuribenzoic acid (PHMBA, 10 μM)[28,29]. After 20 min incubation, the aortic rings were exposed to ACh (10 μM).

### 2.6. Vascular reactivity under conditions of oxidative stress

To examine the effect of superoxide anions on NO and HNO-mediated relaxation, responses to the HNO donor, Angeli's salt (AS), the NO donor, DEANONOate (DEA) or ACh were examined in the presence of pyrogallol. Aortic rings were isolated from male Sprague-Dawley rats (~8 weeks of age, 250–300 g) and mounted in standard 10 mL organ baths containing Krebs-bicarbonate solution as described above. After aortic rings were equilibrated, the maximum responses established, and the endothelium integrity tested, rings were incubated for 20 min with pyrogallol (100 μM), a superoxide anion generator, HXC (100 μM), L-cysteine (3 mM, which was incubated for 3 min) or a combination of these

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