Endothelial nitric oxide synthase enhancer reduces oxidative stress and restores endothelial function in *db/db* mice

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Aims	Endothelial dysfunction is caused by reduced nitric oxide (NO) bioavailability and/or over-produced reactive oxygen species (ROS). The present study investigated a vascular benefit of AVE3085, an endothelial nitric oxide synthase (eNOS) enhancer, in preserving endothelial function in diabetic mice and the mechanisms involved.
Methods and results	Male *db/db* and *db/m*$^+$ mice were orally administered AVE3085 for 7 days (10 mg kg^{-1} day^{-1}). Vascular reactivity of arteries was studied via myography under both isometric and isobaric conditions. ROS levels in aortas were determined using dihydroethidium fluorescence dye and electron paramagnetic resonance spin trapping. Chronic treatment with AVE3085 reduced blood pressure, enhanced endothelium-dependent relaxations (EDR) to acetylcholine in aortas, mesenteric, and renal arteries, lowered oxidative stress, and augmented the attenuated flow-dependent dilatation in mesenteric resistance arteries from *db/db* mice. Incubation of aortas from C57BL/6J mice in high glucose (30 mmol L^{-1}) culture medium for 48 h impaired EDR and elevated ROS generation, and these effects were reversed by co-treatment with AVE3085 (1 μmol L^{-1}). Benefits of AVE3085 were abolished by the transcription inhibitor actinomycin D, the NOS inhibitor N^G-nitro-L-arginine methyl ester, and in *eNOS*$^{-/-}$ mice. NO production in primary endothelial cells from mouse aortas was detected with a NO-sensitive fluorescence dye. Protein expression was assayed by western blotting. Treatment with AVE3085 enhanced NO production in endothelial cells and eNOS expression in aortas.
Conclusion	AVE3085 ameliorates endothelial dysfunction in *db/db* mice through increased NO bioavailability, which reduces oxidative stress in the vascular wall. Targeting eNOS and NO production may be a promising approach to combat diabetic vasculopathy.
Keywords	Endothelial NOS enhancer • Oxidative stress • Endothelium • Diabetes

1. Introduction

Endothelial dysfunction is a critical initiator for developing macro- and micro-vascular disease which is the principal cause of morbidity and mortality in type 2 diabetic patients. This is a condition resulting from imbalance in the production and release between endothelium-derived relaxing factors (EDRFs) and contracting factors, pro- and anti-inflammatory, and pro- and anti-thrombotic molecules.[1,2] A key

EDRF, nitric oxide (NO), generated by endothelial nitric oxide synthase (eNOS), regulates vascular tone[3] and remodelling,[4] angiogenesis,[5] and the functional activity of endothelial progenitor cells;[6] inhibits vascular smooth muscle cell proliferation,[7] platelet aggregation,[8] and leucocyte adhesion;[9] and may also confer mitochondrial oxidative stress protection.[10]

In type 2 diabetes, NO bioavailability is decreased because of the reduced NO production and the increased inactivation of NO.[11]

Diminished capacity of eNOS to form NO has been demonstrated in diabetic vascular dysfunction,[11,12] accompanied by an increased oxidative stress.[13] Superoxide reacts with NO, resulting in greater formation of peroxynitrite; the latter in turn oxidizes eNOS cofactor, tetrahydrobiopterin, to dihydrobiopterin, leading to eNOS uncoupling, and thus producing superoxide anions instead of NO.[14] In addition, hyperglycaemia-induced production of superoxide anions[15] and peroxynitrite[16] is found to decrease the eNOS expression and activity in endothelial cells.

Against this background, we hypothesized that directly increasing the eNOS expression and activity by eNOS enhancers could be an effective approach to ameliorate endothelial dysfunction in diabetes. AVE compounds were developed to increase eNOS transcription. For example, AVE9488 elevates the eNOS expression and activity, reverses the eNOS uncoupling,[17,18] protects the heart against ischaemia reperfusion,[19] and improves left ventricular remodelling after myocardial infarction.[20] Another analogue, AVE3085 elevates the eNOS expression and attenuates vascular dysfunction and inflammation in the hindlimbs of diabetic rats.[21] AVE3085 also improves endothelial function and reduces blood pressure in spontaneously hypertensive rats (SHRs).[22]

The present study investigated whether AVE3085 could restore the impaired endothelial function and reduce oxidative stress under high glucose condition and/or in diabetic *db/db* mice *in vitro* and *in vivo*. The results should help to verify the effectiveness of eNOS enhancers against diabetes-related endothelial dysfunction.

2. Methods

2.1 Artery preparation

This study was approved by the Animal Experimentation Ethics Committee, Chinese University of Hong Kong (CUHK). Male C57BL/6J, *db/m*[+], *db/db* and *eNOS*^{−/−} mice of 12−18 weeks old were supplied by the CUHK Laboratory Animal Service Centre. This investigation conformed to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health.

Sixteen-week old *db/db* mice were treated with 10 mg kg^{-1} day^{-1} AVE3085 suspended in 0.5% methyl cellulose or 0.5% methyl cellulose (vehicle) by oral gavages for 7 days. After treatment, mice were sacrificed by CO_2 inhalation. Mouse aortas, mesenteric and renal arteries were dissected and cut into 2-mm ring segments.[11] Aortas from *db/db* mice were incubated for 18 h in DMEM (Invitrogen, CA, USA) containing 10% FBS, 100 μg mL^{-1} streptomycin, and 100 U mL^{-1} penicillin, with and without co-treatment with AVE3085 (1 μmol L^{-1} for all organ culture experiments) or transcription inhibitor actinomycin D (2 μmol L^{-1}).

2.2 Measurement of isometric force in wire myograph

Aortic rings were suspended in wire myograph (Danish Myo Technology, Denmark) as described[11] for isometric tension measurement. The organ chamber was filled with Krebs solution (mmol L^{-1}): 119 NaCl, 4.7 KCl, 2.5 $CaCl_2$, 1 $MgCl_2$, 25 $NaHCO_3$, 1.2 KH_2PO_4, and 11 D-glucose, oxygenated with 95% O_2-5% CO_2, and kept at 37°C (pH ~7.4). Each ring was stretched to an optimal tension of 3 mN for aortic rings and 1 mN for mesenteric and renal artery segments, equilibrated for 1 h, and contracted by 60 mmol L^{-1} KCl-containing Krebs solution. Endothelium-dependent relaxations (EDR) to acetylcholine (ACh, 0.003−10 μmol L^{-1}) were determined in phenylephrine-contracted rings. Some rings were incubated with 100 μmol L^{-1} NOS inhibitor N^G-nitro-L-arginine methyl ester (L-NAME), 1 mmol L^{-1} DL-propargylglycine (cystathionine γ-lyase CSE inhibitor), or 1 mmol L^{-1} aminooxyacetic acid (cystathionine β-synthase CBS inhibitor) for 30 min to determine the role of NO. The endothelium-independent relaxations to sodium nitroprusside (SNP, 0.0003−1 μmol L^{-1}) were also examined. ACh-induced relaxations in C57BL/6J mouse aortas with intact endothelium were abolished, while the relaxations in resistance mesenteric arteries were only moderately inhibited by L-NAME. Therefore, mesenteric arteries from *eNOS*^{−/−} mice were used to verify that the effect of AVE3085 on EDR because the aortas from *eNOS*^{−/−} mice did not relax in response to ACh.

2.3 Primary culture of mouse aortic endothelial cells

Endothelial cells were isolated and cultured. In brief, isolated mouse aortas were cut open in PBS and digested with 0.2% collagenase (Type 1A, Sigma, MO, USA) at 37°C for 10 min. The suspension after was centrifuged at 600 g for 5 min and cells were re-suspended in DMEM. After 1-h incubation at 37°C, the medium was replaced to remove unattached cells, and cultured in an incubator with 5% CO_2 at 37°C till 70% confluence. The identity of the primary cultured endothelial cells was verified by a positive staining of eNOS (BD Transduction laboratory, Franklin Lakes, NJ, USA) and a negative staining of α-smooth muscle actin (Abcam, Cambridge, UK).

2.4 Measurement of NO production by laser confocal fluorescence microscopy

Fluorimetric measurement in endothelial cells was performed on an Olympus FV1000 laser scanning confocal system (Olympus, Tokyo, Japan) as described[23]. Intracellular NO level was determined using a NO-sensitive dye, 4-amino-5-methylamino-2′,7′-difluorofluorescein diacetate (DAF-FM DA) (excitation at 495 nm, emission filter at 505−525 nm). Cultured endothelial cells seeded on glass coverslips were incubated at 37°C for 10 min with 1 μmol L^{-1} DAF-FM diacetate (Invitrogen, USA) in normal physiological saline solution (NPSS) containing (mmol L^{-1}) 140 NaCl, 5 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 glucose, and 5 HEPES (pH ~7.4). Changes in intracellular NO production in response to Ca^{2+} ionophore A23187 (1 μmol L^{-1}, Tocris Bioscience, Bristol, UK) were analysed by Fluoview software (Olympus), and expressed as a ratio (F1/F0) comparing the fluorescence before (F0) and after (F1) adding A23187.

2.5 Western blotting of eNOS and phosphorylated eNOS protein

Aortic rings were frozen in liquid nitrogen and homogenized in ice-cold RIPA lysis buffer containing 1 μg mL^{-1} leupeptin, 5 μg mL^{-1} aprotinin, 100 μg mL^{-1} PMSF, 1 mmol L^{-1} sodium orthovanadate, 1 mmol L^{-1} EGTA, 1 mmol L^{-1} EDTA, 1 mmol L^{-1} NaF, and 2 mg mL^{-1} β-glycerolphosphate. The lysates were centrifuged at 20 000 g for 20 min and supernatants were collected. The protein concentration was determined by the Lowry method (BioRad, CA, USA). Protein sample (25 μg) was electrophoresed through the SDS−polyacrylamide gel and transferred to an immobilon-P polyvinylidene difluoride membrane (Millipore, Billerica, MA, USA). The membranes were blocked with 5% non-fat milk or 1% BSA and incubated with primary antibodies against phosphorylated eNOS at Ser1176 (p-eNOS^{Ser1176}) (Abcam) and at Thr495 (p-eNOS^{Thr495}) (Millipore), eNOS (BD Transduction laboratory), and GAPDH (Ambion, Austin, TX, USA) at 4°C overnight, followed by horseradish peroxidase-conjugated secondary antibodies (DakoCytomation, Glostrup, Denmark), developed with an enhanced chemiluminescence detection system (ECL reagents; Amersham Pharmacia Biotech, Buckinghamshire, UK). Densitometry was performed using a

documentation programme (Flurochem, Alpha Innotech Corp., San Leandro, CA, USA), and analysed with QuantityOne (Biorad).

2.6 Detection of intracellular oxidant formation by dihydroethidium (DHE) fluorescence

DHE (Invitrogen), a cell-permeable dye oxidized to elicit fluorescence in the presence of oxidants and then trapped by intercalation into DNA.[24] Frozen aortic segments were cut into sections at 10 μm-thickness using a Leica CM 1000 cryostat, and incubated with DHE (5 μmol L^{-1}) in NPSS at 37°C for 15 min.[11] Images were obtained by measuring the fluorescence intensity (excitation: 515 nm; emission filter: 565–605 nm) with an Olympus FV1000 laser scanning confocal system.

2.7 Detection of ROS by electron paramagnetic resonance (EPR) spin trapping

Intracellular reactive oxygen species (ROS) productions in aortic segments were also examined with the spin trap (TEMPONE-H) 1-hydroxy-2,2,6,6-tetramethyl-4-oxo-piperidine hydrochloride (100 μmol L^{-1}, Alexis Co., Bingham, UK), with diethylenetriaminepentaacetic acid to remove transition metal ions.[25] All EPR samples were suspended in Krebs solution and placed in 200-μl glass tubes, and EPR spectra were recorded at room temperature using a EMX EPR spectrometer (Bruker, Karlsruhe, Germany) as described.[26]

2.8 Measurement of flow-mediated dilatation in pressurized resistance arteries

The second-order mesenteric arteries of vehicle and AVE3085-treated db/db mice were cut into segments. As described,[27] each segment was cannulated between two glass cannulas in a chamber filled with Krebs solution oxygenated by 95% O_2/5% CO_2 and maintained at 37°C (pH ~7.4). Both cannulating pipettes were filled with Krebs solution with a pressure regulator controlling the intraluminal pressure. A video camera attached to a light-inverted microscope (Zeiss, LLC, USA) was employed to visualize alterations in the external diameter of cannulated vessels. The external diameter and intraluminal pressure were recorded simultaneously using an Myo-View software (Danish Myo Technology). After stabilizing at 80–100 mmHg intraluminal pressure, flow-mediated dilatation was induced by pressure difference of 20 mmHg in phenylephrine-precontracted vessels. Passive tension was obtained at the end of the experiment with Ca^{2+}-free Krebs solution containing 2 mmol L^{-1} EGTA.

2.9 Chemicals

ACh, aminooxyacetic acid, DL-propargylglycine, hypoxanthine, L-NAME, phenylephrine, SNP, and xanthine oxidase (Sigma); A23187 and actinomycin D (Tocris Bioscience). AVE3085 was generously provided by Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany. AVE3085, actinomycin D, and hypoxanthine were dissolved in dimethyl sulfoxide (DMSO), while others in water. DMSO at 0.1% (v/v) did not affect the vascular reactivity.

2.10 Data analysis

Results are means ± SEM from different mice. The relaxation was presented as percentage of the evoked contraction. Data were analysed using Graphpad Prism. The negative logarithm of the dilator concentration that caused 50% of the maximum response (pD_2) and the maximum relaxation (E_{max}%) were calculated. Student's t-test was performed between two curves or groups. $P < 0.05$ was considered statistically significant.

3. Results

3.1 AVE3085 restores the endothelium-dependent relaxation impaired by high glucose

Exposure of control C57BL/6J mouse aortas to 30 mmol L^{-1} glucose (48 h) attenuated EDR to ACh (pD_2: 6.93 ± 0.06, E_{max}%: 67.6 ± 2.0) compared with those to normal glucose (5 mmol L^{-1}) (pD_2: 7.42 ± 0.08, E_{max}%: 82.8 ± 2.5) in both sensitivity and maximum relaxation ($P < 0.05$, Figure 1A and B). Osmotic control with mannitol did not affect ACh-induced relaxations in control mouse aortas.[11] Co-treatment with 1 μmol L^{-1} AVE3085 reversed high glucose-induced reduction in the relaxation (pD_2: 7.33 ± 0.08, E_{max}%: 83.9 ± 2.5) and this effect was abolished by co-incubation with 50 nmol L^{-1} actinomycin D (pD_2: 6.77 ± 0.16, E_{max}%: 72.6 ± 6.6, Figure 1A and B). In contrast, neither high glucose nor AVE3085 affected the relaxations in response to SNP (Figure 1C). ACh-induced relaxations were abolished in the presence of L-NAME in C57BL/6J mouse aortas (Supplementary material online, Figure SA), or in aortas from eNOS$^{-/-}$ mice (Supplementary material online, Figure SB). In the mesenteric arteries from eNOS$^{-/-}$ mice, ACh-induced relaxations were partly mediated by endothelium-derived hyperpolarizing factors (EDHFs) and were unaffected neither by high glucose nor by AVE3085 (Figure 1D). Moreover, in the presence of 15 mmol L^{-1} K$^+$-containing Krebs solution to abolish any effect of EDHFs (Supplementary material online, Figure SC), DL-propargylglycine (CSE inhibitor, 1 mmol L^{-1}) and aminooxyacetic acid (CBS inhibitor, 1 mmol L^{-1}) to inhibit H_2S synthesis (Supplementary material online, Figure SD), did not affect the effect of AVE. All these data strongly supported that AVE3085-induced improvement of relaxation is mediated by eNOS.

3.2 AVE3085 improves the endothelium-dependent relaxation in db/db mouse aortas
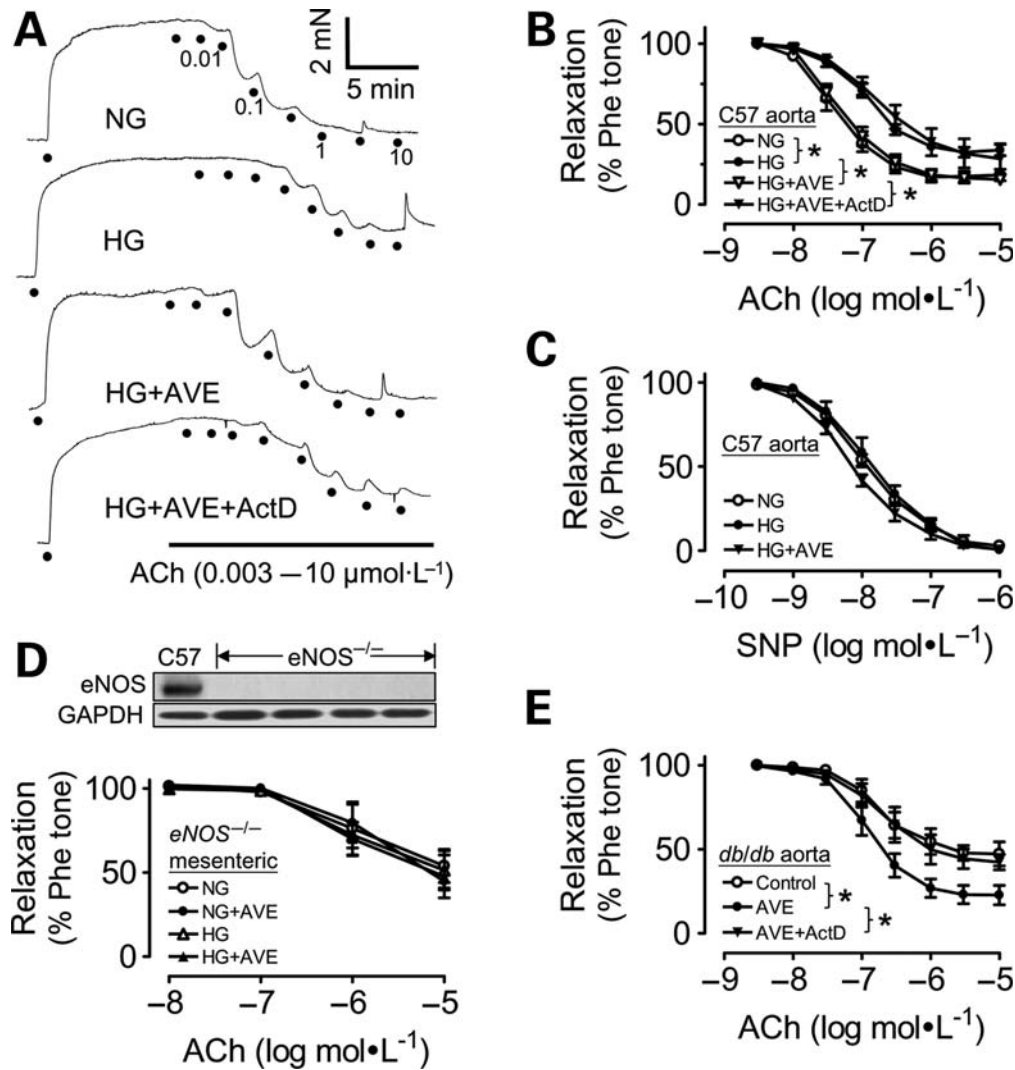
The EDR were impaired in db/db mouse aortas (pD_2: 6.73 ± 0.13, E_{max}%: 52.5 ± 4.2), which was improved by AVE3085 (1 μmol L^{-1}, 18 h) (pD_2: 7.00 ± 0.11, E_{max}%: 79.4 ± 4.4). The improvement was abolished by 2 μmol L^{-1} actinomycin D (pD_2: 6.69 ± 0.15, E_{max}%: 58.12 ± 5.4, Figure 1E). Again, AVE3085 did not modulate SNP-induced relaxations (Supplementary material online, Figure SE).

3.3 AVE3085 increases NO production in endothelial cells

To confirm whether AVE3085 could increase the bioavailability of NO in the vascular wall, NO production in primary endothelial cells from mouse aortas stimulated by A23187 (1 μmol L^{-1}) was measured. Treatment with 30 mmol L^{-1} glucose reduced the NO generation, which was reversed by co-incubation with AVE3085 (1 μmol L^{-1}) (Figure 2A and B). The NO level in normal-glucose medium was unchanged by AVE3085 treatment (Figure 2A and B).

3.4 AVE3085 increases eNOS expression in mouse aortas

Incubation with high glucose (48 h, 30 mmol L^{-1}) moderately reduced the eNOS level, while co-treatment with AVE3085 (1 μmol L^{-1}) reversed eNOS down-regulation in C57BL/6J mouse

Figure 1 (*A*) Traces showing endothelium-dependent relaxation to acetylcholine in C57BL/6J mouse aortas following 48-h exposure to normal glucose (NG, 5 mmol L^{-1}), high glucose (HG, 30 mmol L^{-1}), AVE3085 (AVE, 1 μmol L^{-1}), and actinomycin D (ActD, 50 nmol L^{-1}). Concentration−response curves for ACh (*B*) or sodium nitroprusside (SNP, *C*) under different treatments. (*D*) ACh-induced relaxation and eNOS expression in mesenteric arteries from *eNOS$^{-/-}$* mice. (*E*) Endothelium-dependent relaxations in *db/db* mouse aortas after 18-h organ culture with AVE (1 μmol L^{-1}) and ActD (2 μmol L^{-1}). Results are means $\pm$ SEM of four to eight experiments. Scale bars apply to all traces. Statistical difference between curves is indicated. *$P < 0.05$.

aortas. The effect of AVE3085 on the eNOS expression was inhibited by 50 nmol L^{-1} actinomycin D (*Figure 2C*). The eNOS uncoupling can be indicated by the ratio of eNOS dimer to monomer which was decreased upon high glucose treatment but was not improved by co-treatment with AVE (Supplementary material online, *Figure SF*). The eNOS level could also be elevated in AVE3085-treated *db/db* mouse aortas (18 h, 1 μmol L^{-1}); this effect was also sensitive to inhibition by 2 μmol L^{-1} actinomycin D (*Figure 2D*).

3.5 AVE3085 inhibits ROS generation induced by high glucose in mouse aortas

The ROS level measured by DHE fluorescence elevated in mouse aortas exposed to 30 mmol L^{-1} glucose for 48 h, which was inhibited by co-treatment of 1 μmol L^{-1} AVE3085 (*Figure 3A*). Again,

AVE3085-induced inhibition was abolished by actinomycin D (*Figure 3A*). AVE3085 did not affect the basal ROS levels in aortas treated in normal glucose medium (data not shown).

In *eNOS$^{-/-}$* mouse aortas, 48-h exposure to high glucose also increased ROS accumulation but the inhibitory effect on ROS generation by AVE3085 disappeared (*Figure 3B*). Instead, NO donor SNP (0.1 μmol L^{-1}) lowered the ROS level in high glucose-treated *eNOS$^{-/-}$* mouse aortas (*Figure 3B*). The ROS level was higher in aortas from *db/db* mice than those from control mice, which was reduced by AVE3085 treatment (18 h), and also reversed by actinomycin D (*Figure 3C*).

Thirty-minute incubation of control mouse aortas with hypoxanthine plus U mL^{-1} xanthine oxidase increased the ROS level (*Figure 3D*), which was unaffected by co-treatment of 10 μmol L^{-1} AVE3085 (*Figure 3D*).
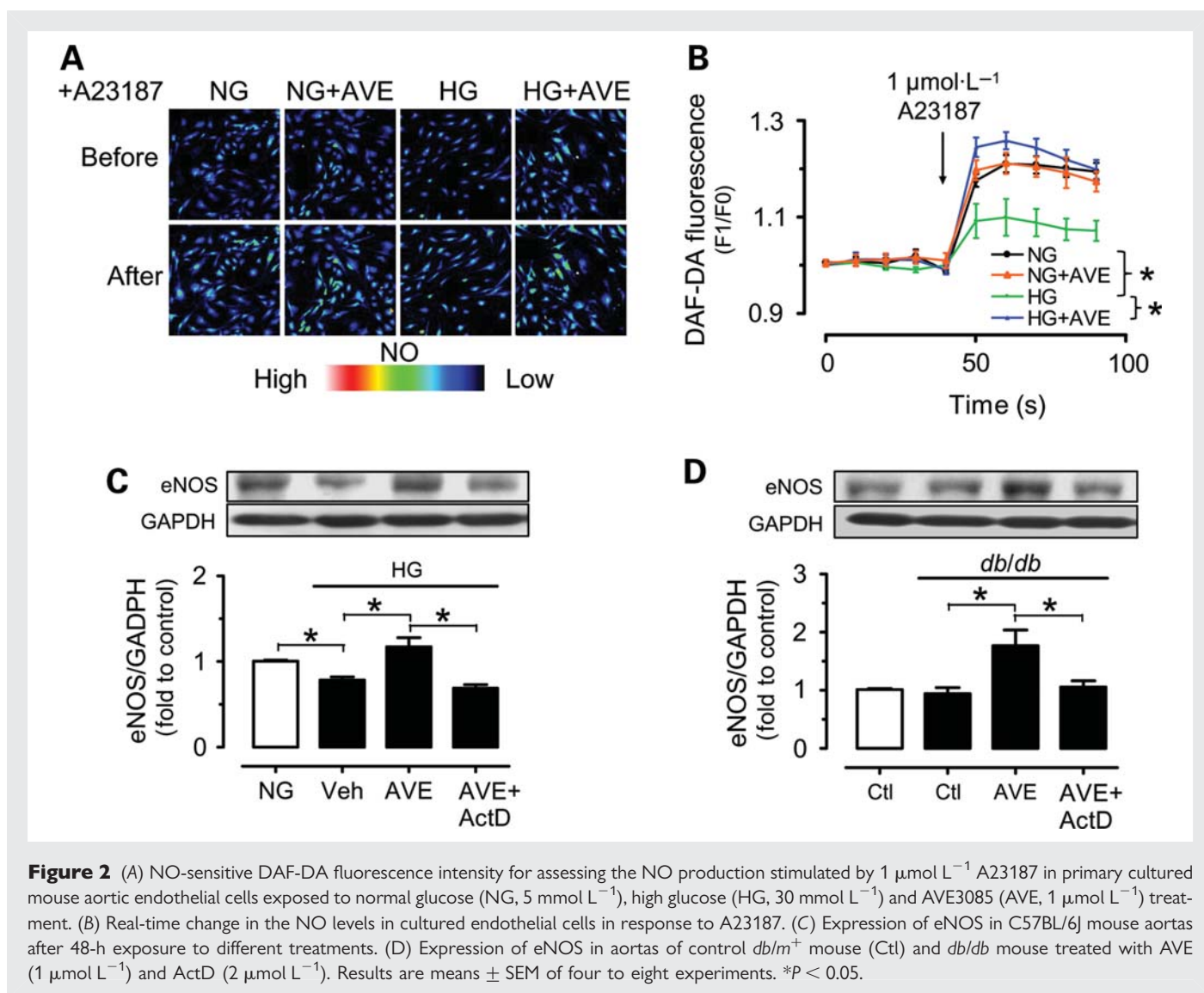
Figure 2 (*A*) NO-sensitive DAF-DA fluorescence intensity for assessing the NO production stimulated by 1 μmol L^{-1} A23187 in primary cultured mouse aortic endothelial cells exposed to normal glucose (NG, 5 mmol L^{-1}), high glucose (HG, 30 mmol L^{-1}) and AVE3085 (AVE, 1 μmol L^{-1}) treatment. (*B*) Real-time change in the NO levels in cultured endothelial cells in response to A23187. (*C*) Expression of eNOS in C57BL/6J mouse aortas after 48-h exposure to different treatments. (*D*) Expression of eNOS in aortas of control *db/m*$^+$ mouse (Ctl) and *db/db* mouse treated with AVE (1 μmol L^{-1}) and ActD (2 μmol L^{-1}). Results are means $\pm$ SEM of four to eight experiments. *$P < 0.05$.

To further confirm the inhibitory effect of AVE3085, another quantitative method, the EPR spin trapping was used to detect ROS generation (*Figure 4A*). Likewise, AVE3085 could reduce high glucose-stimulated ROS increase in aortas from control mice but not in those from eNOS$^{-/-}$ mice (*Figure 4B* and *C*).

3.6 AVE3085 treatment *in vivo* improves endothelial function in *db/db* mice

Oral administration of AVE3085 (10 mg kg^{-1} day^{-1}) for 1 week did not affect body weight (vehicle: 52.4 $\pm$ 1.5 g, AVE3085 treated: 55.0 $\pm$ 2.9 g), or 8-h fasting glucose level (vehicle: 26.1 $\pm$ 1.7 mmol L^{-1}, AVE3085 treated: 26.6 $\pm$ 2.2 mmol L^{-1}) in *db/db* mice; but reduced blood pressure (vehicle: 105.3 $\pm$ 0.8 mmHg, AVE3085 treated: 88.84 $\pm$ 0.8 mmHg) (*Figure 5A*). AVE3085 treatment enhanced the blunted ACh-induced relaxations in aortas (*Figure 5B*), mesenteric (*Figure 5C*), and renal arteries (*Figure 5D*) from *db/db* mice. In addition, AVE3085 treatment improved the impaired flow-mediated dilatation in mesenteric resistance arteries from *db/db* mice (*Figure 5E*).

ROS accumulation was higher in aortas from *db/db* mice than those from vehicle; the increased ROS level was markedly reduced after oral AVE3085 treatment (*Figure 5F*). The eNOS level and the ratio of p-eNOS^{Ser1176} to total eNOS were lower in aortas from *db/db* mice than those from *db/m*$^+$ mice. AVE3085 treatment increased eNOS level (*Figure 6A* and *D*) but did not change the level of p-eNOS^{Ser1176} to total eNOS (*Figure 6A* and *C*). The ratio of p-eNOS^{Thr495} to eNOS was not changed in *db/db* mice when compared with control or by AVE3085 treatment (*Figure 6A* and *B*).

4. Discussion

The present study demonstrates that *in vivo* and *in vitro* treatment with an eNOS enhancer AVE3085 improved endothelial function in diabetic *db/db* mice. AVE3085 prevented the effects of high glucose on the EDR in mouse aortas and NO production in cultured mouse endothelial cells. In addition, AVE3085 up-regulated eNOS and inhibited ROS production induced by high glucose or in *db/db* mice.
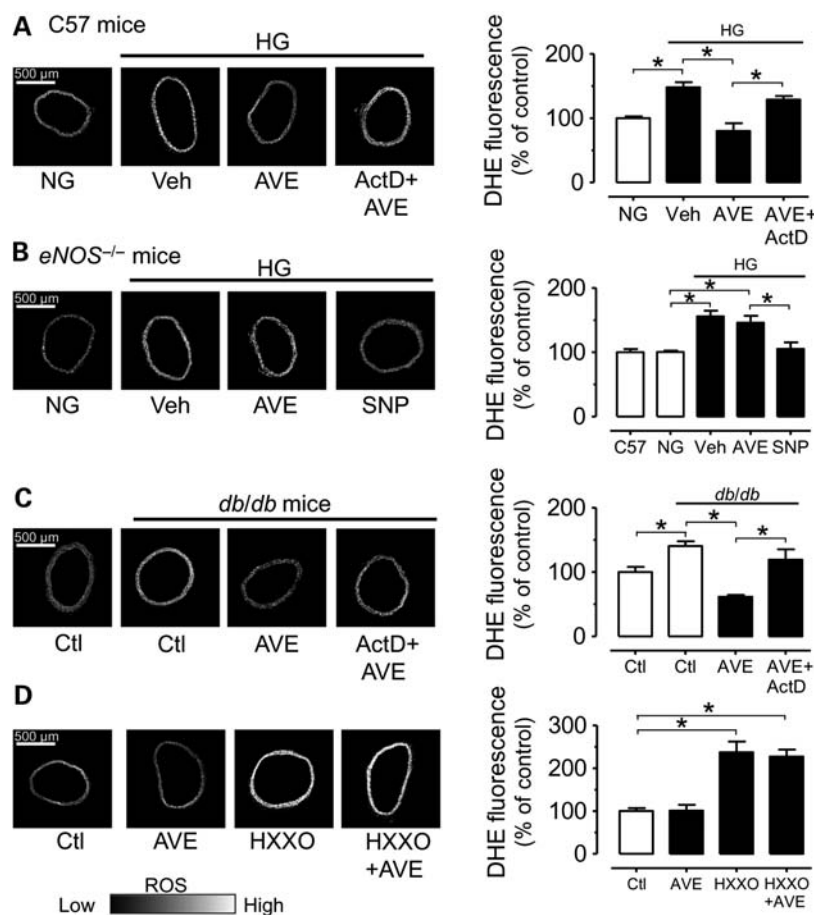
Figure 3 Images showing the ROS fluorescence in mouse aortas under different treatments; ROS level in aortas of (A) C57BL/6J mice and (B) eNOS$^{-/-}$ mice after 48-h exposure to normal glucose (NG, 5 mmol L^{-1}), high glucose (HG, 30 mmol L^{-1}), AVE3085 (AVE, 1 μmol L^{-1}), actinomycin D (ActD, 50 nmol L^{-1}), and sodium nitroprusside (SNP, 10 nmol L^{-1}). (C) ROS level in aortas of control (ctl) and db/db mice treated with AVE (1 μmol L^{-1}) and ActD (2 μmol L^{-1}) for 18 h. (D) Acute effect of AVE (10 μmol L^{-1}, 30 min) on ROS generation induced by hypoxanthine and xanthine oxidase (HXXO). Results are means ± SEM of three to five experiments. *$P < 0.05$.

Hyperglycaemia-associated vascular dysfunction involves multiple pathways such as the activation of the polyol pathway, non-enzymatic glycation, redox potential alterations, and stimulation of protein kinase C.[28] Glucose greatly promotes the production of superoxide anions and thus the inactivation of NO.[29] There are several sources of ROS production induced by hyperglycaemia, including NADPH oxidases in the vascular wall that are activated in diabetes, which diminishes NO bioavailability.[30–32] In consistence with these previous findings, the present study showed that high glucose impaired EDR in arteries, and reduced the NO formation in endothelial cells, decreased the eNOS expression, and increased oxidative stress, and such changes were reversed by AVE3085 treatment. Improvement of EDR by AVE3085 was abolished by L-NAME and in eNOS$^{-/-}$ mice but not affected by 15 mmol L^{-1} K$^+$-containing Krebs solution nor enzyme inhibitors for H$_2$S synthesis, thus excluding the role of EDHFs such as epoxy- and dihydroxy-eicosatrienoic acids (EETs and DHETs) and H$_2$S. It is clear that the vascular benefit of AVE3085 is closely associated with enhanced eNOS transcription and increased NO bioavailability.

ROS play physiological functions by involving the normal cellular redox signalling.[33,34] However, an imbalance occurring between

ROS production and detoxification of reactive intermediates results in excessive oxidative stress.[2] The present study shows that AVE3085 was effective to reduce high glucose-stimulated ROS demonstrated by two methods. AVE3085 did not have a direct ROS-scavenging effect because AVE3085 did not affect ROS induced by hypoxanthine plus xanthine oxidase. The three observations, (i) AVE3085-induced inhibition of ROS was inhibited by actinomycin D, (ii) external NO donor also inhibited ROS, and (iii) the effect of AVE3085 is absent in eNOS$^{-/-}$ mice, suggest that the effect of AVE3085 to reduce ROS is primarily through increasing eNOS expression and endothelium-derived NO production. NO can act as an antioxidant.[35] It has been shown to protect membranes and lipoproteins from oxidation by interacting with lipid peroxyl radicals or by inhibiting lipoxygenase activity.[36,37]

Previous studies showed that eNOS enhancer AVE9488 was able to up-regulate the eNOS expression and activity by re-coupling oxygen reduction to NO synthesis catalyzed by eNOS.[18] Since eNOS uncoupling is an important source of ROS production in diabetes,[38] AVE3085 might also be capable of reversing eNOS uncoupling. Endothelial cells exposed to high glucose or arteries from diabetic mice have disruption of eNOS dimers.[39] However,
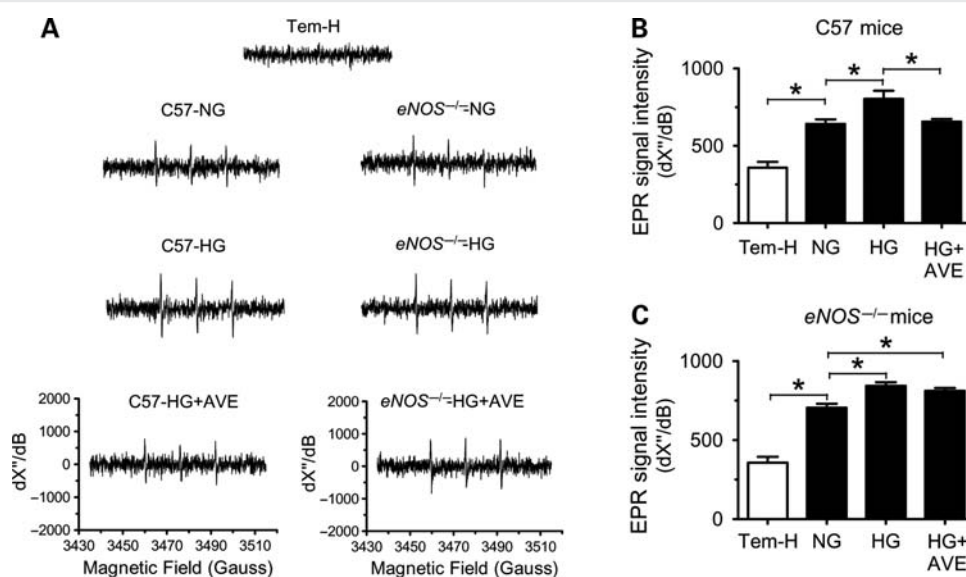
Figure 4 (*A*) Representative EPR spectrum of radicals detected by spin trap. EPR signal intensity in aortas of (*B*) C57BL/6J mice and (*C*) $eNOS^{-/-}$ mice after 48-h exposure to normal glucose (NG, 5 mmol L^{-1}), high glucose (HG, 30 mmol L^{-1}), and AVE3085 (AVE, 1 μmol L^{-1}). The negative control contains only the EPR spin trapping agent, tempone-H (Tem-H). Results are means $\pm$ SEM of three to five experiments. *$P < 0.05$.

AVE3085, unlike AVE9488, cannot reverse the reduced eNOS dimer to monomer ratio in mouse aortas exposed to high glucose, indicating that the effect of AVE3085 to reduce ROS is unlikely mediated through inhibiting eNOS uncoupling, but by increasing the NO bioavailability.

The phosphorylation of eNOS at Ser1176 is reduced in endothelial cells exposed to high glucose and in arteries from diabetic mice;[40,41] while the phosphorylation of eNOS at Thr495 is not changed.[42] We obtained similar results by showing that chronic AVE treatment did not alter the ratio of phosphorylation at Thr495 or Ser1176 to total eNOS in aortas of diabetic mice. In contrast, AVE3085 is shown to enhance eNOS phosphorylation at Ser1176 in SHRs,[22] in which phosphorylated eNOS is compared with GAPDH. Since the eNOS expression is increased, the phosphorylated eNOS might also be increased proportionally. This might not be a direct effect of AVE3085 to increase eNOS phosphorylation. We therefore measured the ratio of phosphorylated eNOS to total eNOS and suggested that AVE3085 does not affect the eNOS activity.

In the present study, we found that eNOS expression decreased in aortas from *db/db* mice. The expression of eNOS in diabetic condition differs in several previous reports. The expression level of eNOS increases in the aortas of diabetic Goto-Kakizaki rats.[43] The eNOS mRNA also increases at 1–2 weeks after streptozotocin treatment but decreases at 4 weeks in the aortas of streptozotocin-treated rats.[44] However, reductions in eNOS mRNA and protein expression are found in patients with type 2 diabetes.[45] In addition, acute exposure (4 h) of human aortic endothelial cells to 25 mmol L^{-1} glucose moderately increases eNOS activity and eNOS mRNA and protein expression which, in contrast, reduce after chronic high glucose exposure (7 days).[15] It is possible that eNOS expression may change with different pathological states and duration of

disease process. It is yet to be determined whether the increased eNOS expression may possibly compensate for the diminished availability of NO. However, chronic treatment with AVE3085 increased the eNOS expression, accompanied by improved endothelial function and ROS reduction in diabetic mouse arteries, indicating that AVE3085 is functionally effective to up-regulate eNOS and to protect endothelial function in diabetic mice.

Furthermore, we also provide novel evidence by demonstrating the benefit of AVE3085 treatment on resistance arteries under an isobaric condition. Chronic administration of AVE3085 to *db/db* mice partially augmented the impaired flow-mediated dilatation in mesenteric resistance arteries. It also reduced blood pressure in *db/db* mice. The present results are consistent with a recent report describing that AVE3085 can lower blood pressure in SHRs.[22] In view of the importance of resistance arterial tone in the control of peripheral resistance to flow and thus blood pressure, the present results show that AVE3085 can improve endothelial function in both conduit and resistance vessels as well as decrease blood pressure.

In conclusion, the novel synthetic agent, eNOS transcription enhancer AVE3085 is able to restore the impaired endothelial function in diabetic mice. This vascular benefit is achieved by enhancing NO generation which in turn reduces oxidative stress (endothelial dysfunction initiator) in the vascular wall. Both *in vitro* and *in vivo* experiments provide solid evidence for the therapeutic potential of AVE3085 for combating against diabetes-associated vascular dysfunction in both macro- and micro-vascular beds.

Supplementary material

Supplementary material is available at *Cardiovascular Research* online.
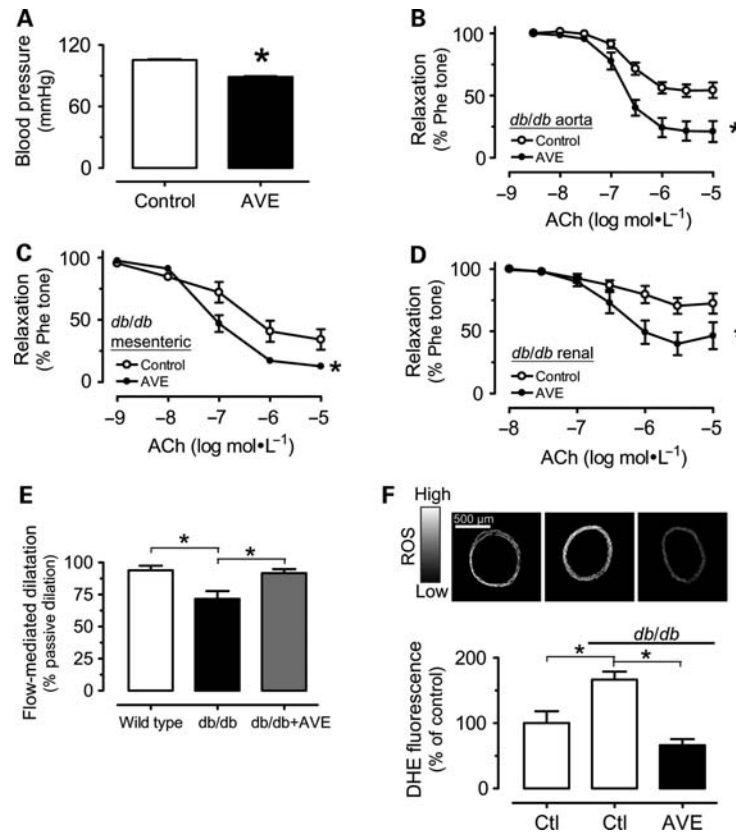
Figure 5 (A) Blood pressure and endothelium-dependent relaxations to acetylcholine (ACh) in *db/db* mouse aortas (B), mesenteric arteries (C), and renal arteries (D) after 1-week oral administration of AVE3085 (AVE, 10 mg kg^{-1}·day^{-1}). (E) Flow-mediated dilatation in small mesenteric arteries in wild type *db/m$^+$* and *db/db* mice with chronic AVE treatment. (F) ROS level in *db/db* mouse aortas after chronic AVE treatment compared with control *db/m$^+$* mice (Ctl). Results are means $\pm$ SEM of four to seven experiments. *$P < 0.05$.
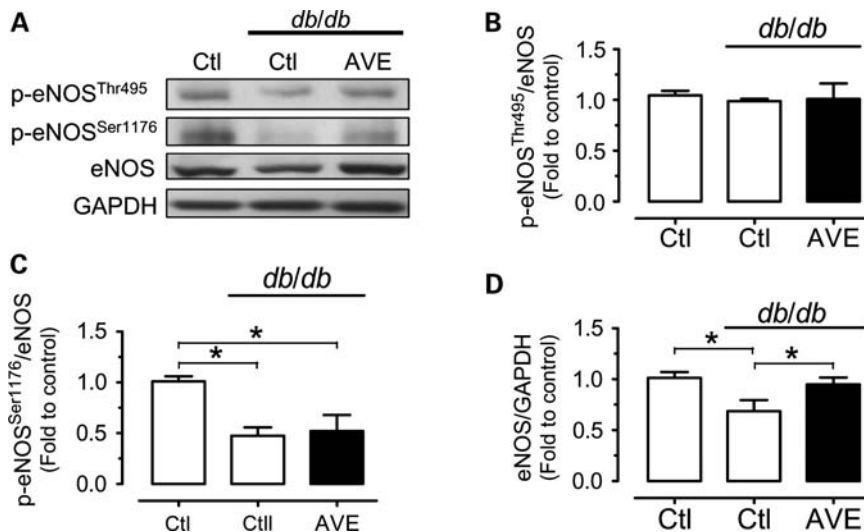

Figure 6 (A) Western blots of phosphorylated eNOS at Thr495 (p-eNOS^{Thr495}) and at Ser1176 (p-eNOS^{Ser1176}), total eNOS and GAPDH in *db/db* mouse aortas after 1-week oral administration of AVE3085 (AVE, 10 mg kg^{-1} day^{-1}) compared with control *db/m$^+$* mice (Ctl). (B) Ratio of p-eNOS^{Thr495} to total eNOS, (C) ratio of p-eNOS^{Ser1176} to total eNOS, and (D) eNOS expression in mouse aortas after chronic AVE treatment. Results are means $\pm$ SEM of four experiments. *$P < 0.05$.

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References

1. Calles-Escandon J, Cipolla M. Diabetes and endothelial dysfunction: a clinical perspective. *Endocr Rev* 2001;**22**:36–52.
2. Wong WT, Wong SL, Tian XY, Huang Y. Endothelial dysfunction: the common consequence in diabetes and hypertension. *J Cardiovasc Pharmacol* 2010;**55**:300–307.
3. Rees DD, Palmer RM, Moncada S. Role of endothelium-derived nitric oxide in the regulation of blood pressure. *Proc Natl Acad Sci U S A* 1989;**86**:3375–3378.
4. Rudic RD, Shesely EG, Maeda N, Smithies O, Segal SS, Sessa WC. Direct evidence for the importance of endothelium-derived nitric oxide in vascular remodeling. *J Clin Invest* 1998;**101**:731–736.
5. Ziche M, Morbidelli L, Masini E, Amerini S, Granger HJ, Maggi CA et al. Nitric oxide mediates angiogenesis in vivo and endothelial cell growth and migration *in vitro* promoted by substance P. *J Clin Invest* 1994;**94**:2036–2044.
6. Aicher A, Heeschen C, Mildner-Rihm C, Urbich C, Ihling C, Technau-Ihling K et al. Essential role of endothelial nitric oxide synthase for mobilization of stem and progenitor cells. *Nat Med* 2003;**9**:1370–1376.
7. Cornwell TL, Arnold E, Boerth NJ, Lincoln TM. Inhibition of smooth muscle cell growth by nitric oxide and activation of cAMP-dependent protein kinase by cGMP. *Am J Physiol* 1994;**267**:C1405–1413.
8. Yan ZQ, Yokota T, Zhang W, Hansson GK. Expression of inducible nitric oxide synthase inhibits platelet adhesion and restores blood flow in the injured artery. *Circ Res* 1996;**79**:38–44.
9. Belenky SN, Robbins RA, Rennard SI, Gossman GL, Nelson KJ, Rubinstein I. Inhibitors of nitric oxide synthase attenuate human neutrophil chemotaxis *in vitro*. *J Lab Clin Med* 1993;**122**:388–394.
10. Borniquel S, Valle I, Cadenas S, Lamas S, Monsalve M. Nitric oxide regulates mitochondrial oxidative stress protection via the transcriptional coactivator PGC-1alpha. *FASEB J* 2006;**20**:1889–1891.
11. Wong WT, Tian XY, Xu A, Ng CF, Lee HK, Chen ZY et al. Angiotensin II type 1 receptor-dependent oxidative stress mediates endothelial dysfunction in type 2 diabetic mice. *Antioxid Redox Signal* 2010;**13**:757–768.
12. Salvolini E, Rabini RA, Martarelli D, Moretti N, Cester N, Mazzanti L. A study on human umbilical cord endothelial cells: functional modifications induced by plasma from insulin-dependent diabetes mellitus patients. *Metabolism* 1999;**48**:554–557.
13. Giugliano D, Ceriello A, Paolisso G. Oxidative stress and diabetic vascular complications. *Diabetes Care* 1996;**19**:257–267.
14. Cosentino F, Hishikawa K, Katusic ZS, Luscher TF. High glucose increases nitric oxide synthase expression and superoxide anion generation in human aortic endothelial cells. *Circulation* 1997;**96**:25–28.
15. Srinivasan S, Hatley ME, Bolick DT, Palmer LA, Edelstein D, Brownlee M et al. Hyperglycaemia-induced superoxide production decreases eNOS expression via AP-1 activation in aortic endothelial cells. *Diabetologia* 2004;**47**:1727–1734.
16. El-Remessy AB, Tawfik HE, Matragoon S, Ali TK, Caldwell RB, Caldwell RW. Peroxynitrite mediates diabetes-induced endothelial dysfunction by reducing eNOS expression: possible role of Rho kinase (ROCK) activation. *Circulation* 2006;**114**:II.330.
17. Sasaki K, Heeschen C, Aicher A, Ziebart T, Honold J, Urbich C et al. Ex vivo pretreatment of bone marrow mononuclear cells with endothelial NO synthase enhancer AVE9488 enhances their functional activity for cell therapy. *Proc Natl Acad Sci U S A* 2006;**103**:14537–14541.
18. Wohlfart P, Xu H, Endlich A, Habermeier A, Closs EI, Hubschle T et al. Antiatherosclerotic effects of small-molecular-weight compounds enhancing endothelial nitric-oxide synthase (eNOS) expression and preventing eNOS uncoupling. *J Pharmacol Exp Ther* 2008;**325**:370–379.
19. Frantz S, Adamek A, Fraccarollo D, Tillmanns J, Widder JD, Dienesch C et al. The eNOS enhancer AVE 9488: a novel cardioprotectant against ischemia reperfusion injury. *Basic Res Cardiol* 2009;**104**:773–779.
20. Fraccarollo D, Widder JD, Galuppo P, Thum T, Tsikas D, Hoffmann M et al. Improvement in left ventricular remodeling by the endothelial nitric oxide synthase enhancer AVE9488 after experimental myocardial infarction. *Circulation* 2008;**118**:818–827.
21. Riad A, Westermann D, Van Linthout S, Mohr Z, Uyulmaz S, Becher PM et al. Enhancement of endothelial nitric oxide synthase production reverses vascular dysfunction and inflammation in the hindlimbs of a rat model of diabetes. *Diabetologia* 2008;**51**:2325–2332.
22. Yang Q, Xue HM, Wong WT, Tian XY, Huang Y, Tsui SKW et al. AVE3085, an enhancer of endothelial nitric oxide synthase, restores endothelial function and reduces blood pressure in spontaneously hypertensive rats. *Br J Pharmacol* 2011;**163**:1078–1085.
23. Hercule HC, Schunck WH, Gross V, Seringer J, Leung FP, Weldon SM et al. Interaction between P450 eicosanoids and nitric oxide in the control of arterial tone in mice. *Arterioscler Thromb Vasc Biol* 2009;**29**:54–U149.
24. Beswick RA, Dorrance AM, Leite R, Webb RC. NADH/NADPH oxidase and enhanced superoxide production in the mineralocorticoid hypertensive rat. *Hypertension* 2001;**38**:1107–1111.
25. Dikalov S, Grigor'ev IA, Voinov M, Bassenge E. Detection of superoxide radicals and peroxynitrite by 1-hydroxy-4-phosphonooxy-2,2,6,6-tetramethylpiperidine: quantification of extracellular superoxide radicals formation. *Biochem Biophys Res Commun* 1998;**248**:211–215.
26. Wong WT, Tian XY, Chen Y, Leung FP, Liu L, Lee HK et al. Bone morphogenic protein-4 impairs endothelial function through oxidative stress-dependent cyclooxygenase-2 upregulation: implications on hypertension. *Circ Res* 2010;**107**:984–991.
27. Chan YC, Leung FP, Wong WT, Tian XY, Yung LM, Lau CW et al. Therapeutically relevant concentrations of raloxifene dilate pressurized rat resistance arteries via calcium-dependent endothelial nitric oxide synthase activation. *Arterioscler Thromb Vasc Biol* 2010;**30**:992–999.
28. Ishii H, Koya D, King GL. Protein kinase C activation and its role in the development of vascular complications in diabetes mellitus. *J Mol Med* 1998;**76**:21–31.
29. Graier WF, Posch K, Fleischhacker E, Wascher TC, Kostner GM. Increased superoxide anion formation in endothelial cells during hyperglycemia: an adaptive response or initial step of vascular dysfunction? *Diabetes Res Clin Pract* 1999;**45**:153–160.
30. Guzik TJ, Mussa S, Gastaldi D, Sadowski J, Ratnatunga C, Pillai R et al. Mechanisms of increased vascular superoxide production in human diabetes mellitus: role of NAD(P)H oxidase and endothelial nitric oxide synthase. *Circulation* 2002;**105**:1656–1662.
31. Gao L, Mann GE. Vascular NAD(P)H oxidase activation in diabetes: a double-edged sword in redox signalling. *Cardiovasc Res* 2009;**82**:9–20.
32. Dagher Z, Park YS, Asnaghi V, Hoehn T, Gerhardinger C, Lorenzi M. Studies of rat and human retinas predict a role for the polyol pathway in human diabetic retinopathy. *Diabetes* 2004;**53**:2404–2411.
33. Fialkow L, Wang YC, Downey GP. Reactive oxygen and nitrogen species as signaling molecules regulating neutrophil function. *Free Rad Biol Med* 2007;**42**:153–164.
34. Zmijewski JW, Landar A, Watanabe N, Dickinson DA, Noguchi N, Darley-Usmar VM. Cell signalling by oxidized lipids and the role of reactive oxygen species in the endothelium. *Biochem Soc Trans* 2005;**33**:1385–1389.
35. Beligni MV, Lamattina L. Nitric oxide protects against cellular damage produced by methylviologen herbicides in potato plants. *Nitric Oxide Biol Chem* 1999;**3**:199–208.
36. Patel RP, Levonen AL, Crawford JH, Darley-Usmar VM. Mechanisms of the pro- and anti-oxidant actions of nitric oxide in atherosclerosis. *Cardiovasc Res* 2000;**47**:465–474.
37. Hummel S, Fischer A, Martin S, Schafer F, Buettner G. Nitric oxide as a cellular antioxidant: a little goes a long way. *Free Radical Biol Med* 2006;**40**:501–506.
38. Oak JH, Cai H. Attenuation of angiotensin II signaling recouples eNOS and inhibits nonendothelial NOX activity in diabetic mice. *Diabetes* 2007;**56**:118–126.
39. Zou MH, Shi CM, Cohen RA. Oxidation of the zinc-thiolate complex and uncoupling of endothelial nitric oxide synthase by peroxynitrite. *J Clin Invest* 2002;**109**:817–826.
40. Matrougui K, Belmadani S, Palen DI, Gonzalez-Villalobos RA, Boulares HA. Elevated epidermal growth factor receptor phosphorylation induces resistance artery dysfunction in diabetic db/db mice. *Diabetes* 2008;**57**:1629–1637.
41. Wang DW, Xu XZ, Zhao CX, Wang LY, Tu L, Fang XS et al. Increased CYP2J3 Expression Reduces Insulin Resistance in Fructose-Treated Rats and db/db Mice. *Diabetes* 2010;**59**:997–1005.
42. Musicki B, Kramer MF, Becker RE, Burnett AL. Inactivation of phosphorylated endothelial nitric oxide synthase (Ser-1177) by O-GlcNAc in diabetes-associated erectile dysfunction. *Proc Natl Acad Sci U S A* 2005;**102**:11870–11875.
43. Kazuyama E, Saito M, Kinoshita Y, Satoh I, Dimitriadis F, Satoh K. Endothelial dysfunction in the early- and late-stage type-2 diabetic Goto-Kakizaki rat aorta. *Mol Cell Biochem* 2009;**332**:95–102.
44. Oyadomari S, Gotoh T, Aoyagi K, Araki E, Shichiri M, Mori M. Coinduction of endothelial nitric oxide synthase and arginine recycling enzymes in aorta of diabetic rats. *Nitric Oxide* 2001;**5**:252–260.
45. Okon EB, Chung AW, Rauniyar P, Padilla E, Tejerina T, McManus BM et al. Compromised arterial function in human type 2 diabetic patients. *Diabetes* 2005;**54**:2415–2423.