Endothelial nitric oxide synthase enhancer reduces oxidative stress and restores endothelial function in \( \text{db/db} \) mice

Wai San Cheang\(^1\), Wing Tak Wong\(^1\)*, Xiao Yu Tian\(^1\), Qin Yang\(^2\), Hung Kay Lee\(^3\), Guo-Wei He\(^4,5\), Xiaoqiang Yao\(^1\), and Yu Huang\(^1\)*

\(^{1}\)Li Ka Shing Institute of Health Sciences, Institute of Vascular Medicine, and School of Biomedical Sciences, Chinese University of Hong Kong, Shatin, N.T., Hong Kong, China; \(^{2}\)Department of Surgery, Chinese University of Hong Kong, Hong Kong, China; \(^{3}\)Department of Chemistry, Chinese University of Hong Kong, Hong Kong, China; \(^{4}\)TEDA International Cardiovascular Hospital, Medical College, Nankai University, Tianjin, China; and \(^{5}\)Department of Surgery, Providence Heart and Vascular Institute, Albert Starr Academic Center, Oregon Health and Science University, Portland, OR, USA

Received 24 February 2011; revised 9 August 2011; accepted 24 August 2011; online publish-ahead-of-print 29 August 2011

Time for primary review: 45 days

**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 \( \text{db/db} \) and \( \text{db/m}^{+} \) 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 \( \text{db/db} \) 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 \mu mol L\(^{-1}\)). Benefits of AVE3085 were abolished by the transcription inhibitor actinomycin D, the NOS inhibitor \( \text{N}^{\text{G}}\)-nitro-L-arginine methyl ester, and in \( \text{eNOS}^{--} \) 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 \( \text{db/db} \) 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.\textsuperscript{11,12} accompanied by an increased oxidative stress.\textsuperscript{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.\textsuperscript{14} In addition, hyperglycaemia-induced production of superoxide anions\textsuperscript{15} and peroxynitrite\textsuperscript{16,17} 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,\textsuperscript{17,18} protects the heart against ischaemia reperfusion,\textsuperscript{19} and improves left ventricular remodelling after myocardial infarction.\textsuperscript{20} Another analogue, AVE3085 elevates the eNOS expression and attenuates vascular dysfunction and inflammation in the hindlimbs of diabetic rats.\textsuperscript{21} AVE3085 also improves endothelial function and reduces blood pressure in spontaneously hypertensive rats (SHRs).\textsuperscript{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/6, db/db, and db/db and eNOS\textsuperscript{-/-} 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\textsuperscript{-1} day\textsuperscript{-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\textsubscript{2} inhalation. Mouse aortas, mesenteric and renal arteries were dissected and cut into 2-mm ring segments.\textsuperscript{23} Aortas from db/db mice were incubated for 18 h in DMEM (Invitrogen, CA, USA) containing 10% FBS, 100 μg mL\textsuperscript{-1} streptomycin, and 100 μg mL\textsuperscript{-1} penicillin, with and without co-treatment with AVE3085 (1 μmol L\textsuperscript{-1} for all organ culture experiments) or transcription inhibitor actinomycin D (2 μmol L\textsuperscript{-1}).

2.2 Measurement of isometric force in wire myograph

Aortic rings were suspended in wire myograph (Danish Myo Technology, Denmark) as described\textsuperscript{24} for isometric tension measurement. The organ chamber was filled with Krebs solution (mmol L\textsuperscript{-1}): 119 NaCl, 4.7 KCl, 2.5 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 25 NaHCO\textsubscript{3}, 1.2 KH\textsubscript{2}PO\textsubscript{4}, and 11 glucose, oxygenated with 95% O\textsubscript{2}-5% CO\textsubscript{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 arterial segments, equilibrated for 1 h, and contracted by 60 mmol L\textsuperscript{-1} KCl-containing Krebs solution. Endothelium-dependent relaxations (EDR) to acetylcholine (ACH, 0.003–10 μmol L\textsuperscript{-1}) were determined in phenylephrine-contracted rings. Some rings were incubated with 100 μmol L\textsuperscript{-1} NOS inhibitor N\textsuperscript{2}-nitro-L-arginine methyl ester (L-NAME), 1 mmol L\textsuperscript{-1} dl-propargyglycine (cystathionine γ-lyase CSE inhibitor), or 1 mmol L\textsuperscript{-1} aminooxycetic 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\textsuperscript{-1}) were also examined. ACh-induced relaxations in C57BL/6 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\textsuperscript{-/-} mice were used to verify that the effect of AVE3085 on EDR because the aortas from eNOS\textsuperscript{-/-} 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\textsubscript{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\textsuperscript{25}. Intracellular NO level was determined using a NO-sensitive dye, 4-aminophenylglycine-2′,7′-dihydrofluorescein 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\textsuperscript{-1} DAF-FM diacetate (Invitrogen, USA) in normal physiological saline solution (NPSS) containing (mmol L\textsuperscript{-1}): 140 NaCl, 5 KCl, 1 CaCl\textsubscript{2}, 1 MgCl\textsubscript{2}, 10 glucose, and 5 HEPES (pH ≏ 7.4). Changes in intracellular NO production in response to Ca\textsuperscript{2+} ionophore A23187 (1 μmol L\textsuperscript{-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\textsuperscript{-1} leupeptin, 5 μg mL\textsuperscript{-1} aprotinin, 100 μg mL\textsuperscript{-1} PMSF, 1 mmol L\textsuperscript{-1} sodium orthovanadate, 1 mmol L\textsuperscript{-1} EGTA, 1 mmol L\textsuperscript{-1} EDTA, 1 mmol L\textsuperscript{-1} NaF, and 2 mg mL\textsuperscript{-1} β-glycerophosphate. 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\textsuperscript{Ser1176}) (Abcam) and at Thr495 (p-eNOS\textsuperscript{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...
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 an 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% O₂/5% CO₂ 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-prescavaged vessels. Passive tension was obtained at the end of the experiment with Ca²⁺/-free Krebs solution containing 2 mmol L⁻¹ EGTA.

2.9 Chemicals

ACh, aminoxyacetic acid, DL-propargylglycine, hypoxanthine, L-NNAME, 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 (pD₂) and the maximum relaxation (Eₘₐₓ%) 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 (pD₂: 6.93 ± 0.06, Eₘₐₓ%: 67.6 ± 2.0) compared with those to normal glucose (5 mmol L⁻¹) (pD₂: 7.42 ± 0.08, Eₘₐₓ%: 82.8 ± 2.5) in both sensitivity and maximum relaxation (P < 0.05, Figure 1A and B). Osmotic control with mannnitol 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 (pD₂: 7.33 ± 0.08, Eₘₐₓ%: 83.9 ± 2.5) and this effect was abolished by co-incubation with 50 nmol L⁻¹ actinomycin D (pD₂: 6.77 ± 0.16, Eₘₐₓ%: 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-NNAME 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 aminoxyacetic acid (CBS inhibitor, 1 mmol L⁻¹) to inhibit H₂S 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 (pD₂: 6.73 ± 0.13, Eₘₐₓ%: 52.5 ± 4.2), which was improved by AVE3085 (1 μmol L⁻¹, 18 h) (pD₂: 7.00 ± 0.11, Eₘₐₓ%: 79.4 ± 4.4). The improvement was abolished by 2 μmol L⁻¹ actinomycin D (pD₂: 6.69 ± 0.15, Eₘₐₓ%: 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
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).
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<sup>−/−</sup> 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<sup>−1</sup> day<sup>−1</sup>) for 1 week did not affect body weight (vehicle: 52.4 ± 1.5 g, AVE3085 treated: 55.0 ± 2.9 g), or 8-h fasting glucose level (vehicle: 26.1 ± 1.7 mmol L<sup>−1</sup>, AVE3085 treated: 26.6 ± 2.2 mmol L<sup>−1</sup>) in db/db mice; but reduced blood pressure (vehicle: 105.3 ± 0.8 mmHg, AVE3085 treated: 88.8 ± 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 vehilce; the increased ROS level was markedly reduced after oral AVE3085 treatment (Figure 5F). The eNOS level and the ratio of p-eNOS<sup>Ser1176</sup> to total eNOS were lower in aortas from db/db mice than those from db/m<sup>+</sup> mice. AVE3085 treatment increased eNOS level (Figure 6A and D) but did not change the level of p-eNOS<sup>Ser1176</sup> to total eNOS (Figure 6A and C). The ratio of p-eNOS<sup>Thr495</sup> 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.
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. Glucose greatly promotes the production of superoxide anions and thus the inactivation of NO. 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. 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 knockout 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. However, an imbalance occurring between ROS production and detoxification of reactive intermediates results in excessive oxidative stress. 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 knockout 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. It has been shown to protect membranes and lipoproteins from oxidation by interacting with lipid peroxyl radicals or by inhibiting lipoxygenase activity.

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. Since eNOS uncoupling is an important source of ROS production in diabetes, 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. However,
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; while the phosphorylation of eNOS at Thr495 is not changed. 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, 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. The eNOS mRNA also increases at 1–2 weeks after streptozotocin treatment but decreases at 4 weeks in the aortas of streptozotocin-treated rats. However, reductions in eNOS mRNA and protein expression are found in patients with type 2 diabetes. 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). 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. 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 ± SEM of four to seven experiments. *$P < 0.05$.

Figure 6  (A) Western blots of phosphorylated eNOS at Thr495 (p-eNOSThr495) and at Ser1176 (p-eNOSSer1176), 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-eNOSThr495 to total eNOS, (C) ratio of p-eNOSSer1176 to total eNOS, and (D) eNOS expression in mouse aortas after chronic AVE treatment. Results are means ± SEM of four experiments. *$P < 0.05$. 
Acknowledgements
Sanofi-Aventis Deutschland GmbH, Frankfurt, Germany is thanked for providing AVE3085.

Conflict of interest: none declared.

Funding
This work was supported by Hong Kong General Research Fund (CUHK 466110); RGC direct grant (2041581); CUHK Focused Investment Scheme (1903025); and National Basic Research Program, China (2012CB517805). W.S.C. is supported by Li Ka Shing Institute of Health Sciences, Chinese University of Hong Kong.

References