Anti-atherosclerotic effects of small molecular weight compounds enhancing eNOS expression and preventing eNOS uncoupling

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Running title: eNOS transcription enhancement suppresses atherosclerosis

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Abbreviations:
apoE-KO, apolipoprotein E-knockout; apoE/eNOS-DKO, apoE/eNOS-double knockout; BH₄, (6R)-5,6,7,8-tetrahydro-L-biopterin; eNOS, endothelial NO synthase; eNOS-KO, eNOS-knockout; GCH1, guanosine triphosphate (GTP)-cyclohydrolase-I; HUVEC, human umbilical vein endothelial cells; L-NAME, N⁵-nitro-L-arginine methyl ester; NO, nitric oxide; SOD1, CuZn superoxide dismutase; sGC, soluble guanylate cyclase; SOD2, mitochondrial superoxide dismutase; SOD3, extracellular SOD; TBP, TATA box binding protein

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ABSTRACT

Many cardiovascular diseases are associated with reduced levels of bioactive NO and an uncoupling of oxygen reduction from NO synthesis in endothelial NO synthase (eNOS uncoupling). In human endothelial EA.hy 926 cells, two small molecular-weight compounds with related structures, AVE9488 and AVE3085, enhanced eNOS promoter activity in a concentration-dependent manner; with the responsible cis-element localized within the proximal 263bp of the promoter region. RNA interference-mediated knockdown of the transcription factor Sp1 significantly reduced the basal activity of eNOS promoter, but did not prevent the transcription activation by the compounds. Enhanced transcription of eNOS by AVE9488 in primary human umbilical vein endothelial cells was associated with increased levels of eNOS mRNA and protein expression, as well as increased bradykinin-stimulated NO production. In both wild type C57BL/6J mice and apolipoprotein E-knockout (apoE-KO) mice, treatment with AVE9488 resulted in enhanced vascular eNOS expression. In apoE-KO mice, but not in eNOS-knockout mice, treatment with AVE9488 reduced cuff-induced neointima formation. A 12-week treatment with AVE9488 or AVE3085 reduced atherosclerotic plaque formation in apoE-KO mice, but not in apoE/eNOS-double knockout mice. Aortas from apoE-KO mice showed a significant generation of reactive oxygen species. This was partly prevented by NOS inhibitor L-NAME indicating eNOS uncoupling. Treatment of mice with AVE9488 enhanced vascular content of the essential eNOS cofactor (6R)-5,6,7,8-tetrahydro-L-biopterin and reversed eNOS uncoupling. The combination of an upregulated eNOS expression and a reversal of eNOS uncoupling is likely to be responsible for the observed vaso-protective properties of this new type of compounds.
Introduction

Nitric oxide (NO) generated by endothelial NO synthase (eNOS) is physiologically important for vascular homeostasis. Blockade of NO synthesis with pharmacological inhibitors causes significant peripheral vasoconstriction and elevation of blood pressure. Similarly, mice with a disrupted eNOS gene are hypertensive and lack endothelium-dependent, NO-mediated vasodilatation (Huang et al., 1995). Besides its role in controlling blood pressure, NO protects the vasculature from thrombosis by inhibiting platelet aggregation and adhesion. In addition, endothelial NO possesses multiple anti-atherosclerotic properties, including inhibition of leukocyte adhesion and prevention of smooth muscle proliferation (Li and Forstermann, 2000a).

In agreement with this concept, pharmacological inhibition of eNOS causes accelerated atherosclerosis in rabbits (Cayatte et al., 1994) and in mice (Kauser et al., 2000), and genetic eNOS deficiency accelerates the development of atherosclerosis in apolipoprotein E-knockout (apoE-KO) mice (Kuhlencordt et al., 2001).

Although eNOS is a constitutively expressed enzyme, its expression is regulated by a number of biophysical, biochemical and hormonal stimuli, both under physiological conditions and in pathology (Li et al., 2002a; Li et al., 2002b; Searles, 2006). Physiological stimuli upregulating eNOS expression include shear stress produced by the flowing blood, growth factors as well as hormones such as estrogens (Li et al., 2002a; Li et al., 2002b; Searles, 2006). Pleiotropic effects of some cardiovascular drugs such as statins (Endres et al., 1998; Laufs et al., 1998), ACE inhibitors (Linz et al., 1999a; Linz et al., 1999b), AT1 angiotensin receptor blockers and dihydropyridine calcium channel blockers (Ding and Vaziri, 1998) also include an upregulation of eNOS expression.
Due to the anti-thrombotic, anti-atherosclerotic and anti-hypertensive properties of endothelial NO, the eNOS enzyme is an interesting target for the prevention or therapy of cardiovascular diseases.

A primary screening of chemical libraries for compounds increasing eNOS transcription yielded two small molecular weight compounds with related structures, namely AVE9488 (earlier designation C2431, (Wohlfart et al., 2002) and AVE3085. Experiments presented in the current paper provide evidence that AVE9488 and AVE3085 increase endothelial NO production by a simultaneous upregulation of eNOS expression and a reversal of eNOS uncoupling. In vitro pretreatment of marrow mononuclear progenitor cells (BMC) from patients with ischemic cardiomyopathy (ICMP) with AVE9488 significantly increased their eNOS expression. This was associated with an enhanced migratory capacity in vitro and improved neovascularization capacity of the infused BMC in vivo (Sasaki et al., 2006). In the present study, we provide evidence that the increased endothelial NO production induced by AVE9488 and AVE3085 was associated with reduced cuff-induced neointima formation and reduced formation of atherosclerotic plaques in apoE-KO mice.
METHODS

Chemicals and Reagents.

AVE9488 (4-fluoro-N-indan-2-y1-benzamide; CAS number 291756-32-6; empirical formula C_{16}H_{14}FNO) and AVE3085 (2,2-difluoro-benzo[1,3]dioxole-5-carboxylic acid indan-2-y1amide; CAS number 450348-85-3; empirical formula C_{17}H_{13}F_{2}NO_{3}) were synthesized at Sanofi-Aventis (Industriepark Höchst, Frankfurt, Germany). All other biochemical reagents were of highest analytical purity and purchased from Sigma (Deisenhofen, Germany).

Cell culture.

All cell culture experiments were performed in accordance with the German genetic engineering law and German bio-safety guidelines. Before use of primary human cell cultures, a respective protocol was submitted to and approved by a local bio-safety committee. Isolation of human umbilical vein endothelial cells (HUVEC) and measurements of intracellular cyclic GMP (cGMP) were performed as described (Wohlfart et al., 1997). Cell culture medium for HUVEC consisted of IMDM containing GlutaMax (Invitrogen, Karlsruhe, Germany) and supplemented with 100 U/ml penicillin, 100 µg/ml streptomycin and 20% fetal bovine serum. HUVEC-derived EA.hy926 endothelial cells were kindly provided by Dr. Cora-Jean Edgell (Chapel Hill, NC) and grown under 10% CO_{2} in Dulbecco’s modified Eagle’s medium (DMEM, Sigma) supplemented with 10% fetal bovine serum, 2 mM L-glutamine, 1 mM sodium pyruvate, 100 U/ml penicillin, 100 µg/ml streptomycin, and 1x HAT (hypoxanthine, aminopterin and thymidine) (Invitrogen) (Li and Forstermann, 2000b).

Analysis of eNOS promoter activity.

A stable EA.hy 926 cell line was generated by transfection of EA.hy 926 cells with pGL3-eNOS-Hu-3500-neo, which contains a neomycin-resistance gene and a 3.5 kb promoter fragment of human eNOS driving the luciferase reporter gene (Li et al., 1998). These EA.hy926 cells
were cultured in medium containing additionally 1 mg/ml G418 as selection reagent. Cells were plated in 96-wells at a density of 40,000 cells per well. One day later, confluent cells were incubated with AVE9488 and AVE3085 for 18h at the indicated concentrations. After washing once with phosphate-buffered saline (PBS), luciferase activity was determined using the Luciferase Assay System (Promega, Mannheim, Germany) in a Genios microplate reader (Tecan, Vienna, Austria). The luciferase activity, normalized for protein concentration of cell lysates, was used as a determinant of eNOS promoter activity (Li et al., 1998).

In order to localize the promoter region responsible for the transcription activation by the compounds, transient transfection experiments were performed in EA.hy 926 cells using the transfection reagent SuperFect (Qiagen, Hilden, Germany). In addition to pGL3-eNOS-Hu-3500 (Li et al., 1998) and pGL3-eNOS-Hu-1600 (Li and Forstermann, 2000b), pGL3-eNOS-Hu-954 and pGL3-eNOS-Hu-263 were also used. The latter two constructs contained the proximal human eNOS promoter sequence of 954bp and 263bp, respectively, and derived from pGL3-eNOS-Hu-1600 by progressive deletion of the promoter fragment. The plasmid pRL-SV40 (containing the renilla-luciferase gene driven by an SV40 promoter) was co-transfected for normalization. 24h after transfection, cells were incubated for further 18h with AVE9488 and AVE3085. Thereafter, the luciferase and renilla luciferase activities of the extracts were determined using the Dual-Luciferase System (Promega) as described (Li and Forstermann, 2000b).

**Electrophoretic mobility shift assay (EMSA).**

Binding activities of the transcription factors were determined by EMSA as previously described (Kleinert et al., 1998). EA.hy 926 cells were treated with 5 µM AVE9488 and nuclear proteins were extracted. Ten micrograms of nuclear protein was incubated with 32P-labeled double-stranded oligonucleotide containing either of the following binding motifs from the human eNOS promoter (Karantzoulis-Fegaras et al., 1999): GATA: 5’-GCTCCCACCTATCAGCCTCAGT-3’ (positions -239 to -218), Sp1/3-like: 5’-TTTAGAGCCTCCAGCCGGG-3’ (-153 to -134), Elf-1: 5’-AGCCGGGCTTGTCTGCCTGTC-3’
(-140 to -122), YY1: 5'-TCCCATTGTGTATGGGATA-3' (-123 to -105), Sp1: 5'-
GGATAGGGGCGGGGCGAGG-3' (-109 to -91), PEA3: 5'-
CTCCCTCTTCCTAAGGAAAAGGCC-3' (-44 to -20). DNA-protein complexes were analyzed
on 5% polyacrylamide gels (buffer 6.7 mM Tris/HCl, pH 7.5; 3.3 mM Na acetate; 1 mM EDTA).
The gels were dried and autoradiographed on x-ray film (Kleinert et al., 1998).

**siRNA-mediated knockdown of Sp1.**

EA.hy 926 cells stably transfected with the 3.5 kb human eNOS promoter luciferase construct were seeded at a density of 50,000 cells/well in 96-well-plates. After 24h the cells were washed twice with PBS. Lipofection of siRNA was performed according to the manufacturer’s instructions using Lipofectamin-2000 and OptiMEM-I medium (Invitrogen). To knockdown Sp1, commercially available siRNA was used (siGENOME SMARTpool, Perbio-Dharmacon, Schwerte, Germany) at a final concentration of 100 nM. A control siRNA of similar length served as control. After 6h incubation, siRNA was removed and cells were grown in normal growth medium for further 48h. Thereafter, cells were treated with AVE3085 and AVE9488 at the indicated concentrations for 18h for the analysis for eNOS promoter activity.

**Western blotting.**

After washing once in cold PBS, cells were lysed in Laemmli sample buffer containing a mixture of complementary inhibitors (Complete™, Roche, Mannheim, Germany) and the endonuclease Benzonase (Merck, Darmstadt, Germany). The samples were incubated at 37°C for 15 min to reduce viscosity and then denatured at 70°C for 20 min before electrophoresis.

Snap-frozen mice tissues were homogenized in liquid nitrogen using a 6750 Spex-Freezer-mill (SPEX CertiPrep, Metuchen, NJ) and extracted for 1 hour on ice with a Tris/SDS lysis buffer (10mM Tris-HCl, pH 7.4, 1% SDS and Complete™ protease inhibitors). After centrifugation at 4°C for 30 min at 100,000x g, supernatants were mixed with 5x Laemmli sample buffer and denatured at 70°C for 20 min before electrophoresis.
Electrophoresis and transfer to nitrocellulose membranes were carried out according the manufacturer’s instructions using pre-cast Criterion-SDS-polyacrylamide gels (4-15% gradient gels, Bio-Rad, Munich, Germany). Membranes were blocked overnight in TBST (10 mM Tris-HCl, 150 mM NaCl, 0.05% Tween-20, pH 7.6) containing 5% dry milk powder. After incubation with primary antibodies for 18h at 4°C in TBST containing 1% bovine serum albumin (1%) and three washing steps in TBST (each 10 min), samples were incubated with an alkaline phosphatase-conjugated secondary antibody (sheep anti-rabbit IgG, Zymed/Invitrogen) in TBST and 0.2% BSA for 1h at room temperature. After three additional washing steps, bound antibodies were detected using a chemi-fluorescence substrate (RPN-5785, Amersham Biosciences, GE Healthcare, Munich, Germany) on a Fluorimaget FI-595 (Molecular Dynamics, Amersham GE Healthcare, Munich, Germany).

In some cases (e.g. Sp1 protein expression), detection of secondary antibodies conjugated with near-infrared fluorescent dyes (LI-COR Biosciences) was performed on an ODYSSEE infrared-imaging system according to manufacturer’s instructions (LI-COR Biosciences). We observed in general no major differences in quantification using the different fluorescence detection systems. However, the ODYSSEE system allows for the simultaneous detection of two antigens (Sp1 and GAPDH) on the same blot with two secondary antibodies linked to different excitation wavelengths of $\lambda = 680$ and 800nm.

The following primary antibodies were used: a polyclonal rabbit antibody against eNOS (Santa Cruz Biotechnology, Santa Cruz, CA), a polyclonal rabbit antibody against Sp1 (Abcam, Cambridge, United Kingdom), a polyclonal rabbit antibody against $\beta$-tubulin (Santa Cruz Biotechnology) and a monoclonal mouse antibody against GAPDH (Chemicon).

**RNA isolation and real-time RT-PCR.**

RNA was isolated from HUVEC using the RNeasy Mini kit (Qiagen, Hilden, Germany). Quantitative real-time RT-PCR (qRT-PCR) analysis for eNOS mRNA expression was performed using an iCycler™ iQ System (Bio-Rad Laboratories) and the QuantiTect SYBR Green PCR Kit (Qiagen). Primers for human eNOS: 5’-CTGCACCTATGG-AGTCTGCTC-3’ (sense) and 5’-AGCCCTTGTCTCTCAATG-3’ (antisense). eNOS mRNA expression was normalized to
house-keeping gene GAPDH (primers: 5'-CAACGGATTTGGCTATT-3', sense, and 5'-ATATTGGAAC-ATGTAAACCATGTA-3', antisense).

mRNA expression of other genes was analyzed with the QuantiTect™ Probe RT-PCR kit (Qiagen). TaqMan Gene Expression Assays (pre-designed probe and primer sets) were obtained from Applied Biosystems (Foster City, CA, USA): Nox1 (assay ID Mm00549170_m1), Nox2 (assay ID Mm00432775_m1), Nox4 (assay ID Mm00479246_m1), p22phox (assay ID Mm00514478_m1), CuZn superoxide dismutase (SOD1, assay ID Mm01344233_g1), mitochondrial superoxide dismutase (SOD2, assay ID Mm00449726_m1), extracellular SOD (SOD3, Mm00448831_s1), GTP-cyclohydrolase I (GCH1, assay ID Mm00514993_m1), α1 and β1-subunit of the soluble guanylate cyclase (sGC, assay ID Hs00168325_m1 and Hs00168336_m1, respectively), and TATA box binding protein (TBP, for normalization, assay ID Hs00427620_m1).

Cuff-induced neointima formation.

All animal experiments were performed in accordance with the German animal protection law and the guidelines for the use of experimental animals as given by the Guide for the Care and Use of Laboratory Animals of the US National Institutes of Health.

Only male animals were included into the study. Neointima was induced by placing a non-occlusive polyethylene cuff around the left femoral artery in apoE-KO mice and eNOS-knockout (eNOS-KO) mice as described (Moroi et al., 1998). Both of these mice strains have been backcrossed to a C57BL/6J background to ensure genetic homogeneity. Although neointima formation can be induced in normal mice, apoE-KO mice were used in these experiments to ensure comparability with the subsequent atherosclerosis study. Three days before surgery, the animals were randomized into a placebo group and a group receiving AVE9488 via gavage (10 mg/kg/d, b.i.d.) over the whole study period (17 days). Prior to the surgery, mice were anesthetized with an intra-peritoneal injection of pentobarbital (60 mg/kg) followed by an intramuscular injection of xylazin (2 mg/kg). Two weeks after surgery neointima
formation was analyzed in the femoral artery (hematoxylin & eosin staining) and eNOS protein expression in the aorta (Western blotting).

Atherosclerosis studies.

Male apoE-KO mice at the age of 10 weeks mice were randomized to receive either standard rodent chow (Altromin, Lage, Germany) or chow supplemented with AVE9488 (10 and 30 mg/kg/d) for 12 weeks. At the end of the treatment, mice were anesthetized with an intra-peritoneal injection of pentobarbital (60 mg/kg) followed by an intramuscular injection of xylazin (2 mg/kg). Femoral arteries were taken for analysis of eNOS protein expression. Heart and aorta were fixed in 4% formaldehyde overnight. Thereafter, heart was cut into two halves. The lower half of the heart was discarded and the upper half was embedded in paraffin. Cross sections were cut and discarded until the 3-valve cusps at the junction of the aorta to the heart became visible. Once this section was located, 18 cross-sections (1 µm) were continuously cut and subjected to standard hematoxylin and eosin staining to determine the average lesion size per section. In order to determine the lesion area covering aortic surface, aortas were opened longitudinally along a lateral margin, stained with oil-red-O and mounted on slides with endothelium side up. The stained area was measured in relation to the total aortic surface by an observer who was blinded regarding the different treatment groups using an image analysis computer program (LeicaQWin, Leica Imaging Systems, Wetzlar, Germany).

In additional experiments, atherosclerosis studies were performed in male apoE-KO mice and apoE/eNOS-double knockout (DKO) mice. apoE/eNOS-DKO mice were generated by crossing apoE-KO with eNOS-KO mice and by subsequent intercrossing of heterozygous F1 animals. Male mice at the age of 10 weeks were treated with AVE9488 or AVE3085 (30 mg/kg/d, each) applied in Western-type diet (20% fat, 0.5% cholesterol; Altromin, Lage, Germany) for 12 weeks.

Measurement of reactive oxygen species (ROS) production by L-012 chemiluminescence.
Vascular ROS production was determined using the luminol derivate L-012 [8-amino-5-chloro-7-phenylpyrido[3,4-d]pyridazine-1,4(2H,3H)dione] (Li et al., 2006). Briefly, apoE-KO mice were treated for two weeks with AVE9488 (30 mg/kg/day pressed in chow). Aortas were isolated, dissected free from remaining connective and fat tissue and cut into 2-mm rings. Then, the rings were incubated for 30 min at 37°C on 96-well plates in Hanks' buffered salt solution containing 500 µM L-012, with or without L-NAME. L-012-derived chemiluminescence was measured using a microplate luminometer (Berthold, Bad Wildbad, Germany). The photon counts were normalized for the respective dry weight of aortic rings.

**Measurement of vascular content of (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄).**

After treatment of male apoE-KO mice with AVE9488 (30 mg/kg/day) for two weeks, aortas were isolated and homogenized in ice-cold lysis buffer (0.1 M Tris-HCl, pH 7.8, containing 5 mM EDTA, 0.3 M KCl, 5 mM 1,4-dithioerythritol, 0.5 mM Pefabloc, and 0.01% saponin). Samples were oxidized under either acidic conditions (with 0.2 M HCl containing 50 mM I₂) or alkaline conditions (with 0.2 M NaOH containing 50 mM I₂). Biopterin content was assessed using high-performance liquid chromatography with fluorescence detection (350 nm excitation, 450 nm emission). BH₄ concentration was calculated as fmol/µg protein by subtracting the biopterin peak resulting from alkaline oxidation (accounting for BH₂) from the biopterin peak resulting from acidic oxidation (accounting for both BH₂ and BH₄).

**Statistics.**

Data are expressed as mean ± SEM. Data were first analyzed for distribution of values. In case of a Gaussian distribution, statistical differences were measured by one-way analysis of variance followed by Newman-Keuls post-hoc test. In case of non-gaussian distribution, a non-parametric Kruskal-Wallis test was employed. For all statistical analyses, p<0.05 was considered significant.
RESULTS

Effects of AVE9488 and AVE3085 on eNOS transcription.

In the human endothelial cell line EA.hy 926 stably transfected with a 3.5kb human eNOS promoter fragment, two small molecular weight compounds with related structures, AVE9488 and AVE3085, enhanced eNOS promoter activity in a concentration-dependent manner (Fig. 1A). Simvastatin is a HMG CoA reductase inhibitor that upregulates eNOS by stabilizing eNOS mRNA and has little effect on eNOS promoter activity (Laufs et al., 1998). In our eNOS promoter study, simvastatin was used as a negative control (Fig. 1A). Consistent with previous findings, simvastatin did not change eNOS promoter activity up to 3 µM and showed an effect on eNOS promoter activity only at the high concentration (10 µM) (Fig. 1A).

In transient transfection experiments with eNOS promoter fragments progressively deleted from -3500bp to -263bp, AVE9488 and AVE3085 displayed an identical activation pattern (Fig. 1B). Even with the smallest eNOS promoter fragment of 263bp, both compounds remained active (Fig. 1B).

In order to identify the responsible transcription factor binding sites, we employed EMSA analysis with oligonucleotides derived from this 263bp promoter region of human eNOS: GATA (positions -239 to -218), Sp1/3-like (-153 to -134), Elf-1 (-140 to -122), YY1 (-123 to -105), Sp1 (-109 to -91), PEA3 (-44 to -20). Significant DNA-protein binding could be observed with all the above oligonucleotides. AVE9488 did not change the binding activity of nuclear proteins to Sp1 site or GATA site (Figs. 1C and 1D). Binding activity to binding sites of Sp1/3-like, Elf-1, YY1 or PEA3 was not changed by AVE9488, either (data not shown).

Treatment with Sp1 siRNA resulted in a complete knockdown of this transcription factor in EA.hy 926 cells (Fig. 1E), which was associated with a significant decrease of baseline eNOS promoter activity (Fig. 1F). However, the inducing effect by AVE9488 and AVE3085 on eNOS transcription was still present (Fig. 1F), indicating that Sp1 does not contribute to the eNOS-
stimulating effect of both compounds. Also siRNA-mediated knockdown of transcription factors ETS and GATA2 (with putative binding sites to PEA3 and GATA within eNOS promoter) could not prevent eNOS promoter activation by AVE9488 and AVE3085 (data not shown).

**AVE9488 and AVE3085 did not change eNOS mRNA stability.**

To determine the effect of AVE9488 and AVE3085 on the stability of eNOS mRNA, EA.hy 926 cells were pretreated with both compounds for 24h. Then, 60 µM of the RNA polymerase II inhibitor 5,6-dichlorobenzimidazole riboside (DRB) was added to stop gene transcription. eNOS mRNA was analyzed at 6h or 24h after DRB. eNOS mRNA showed a half-life of about 24h, which is consistent with previous findings (Li et al., 2004). Neither AVE9488 nor AVE3085 had an effect on eNOS mRNA stability (Fig. 2A). When gene transcription was first stopped by DRB pretreatment and AVE9488 or AVE3085 added 30min after DRB, both compounds could no longer increase eNOS mRNA expression (Fig. 2B).

**Effects of AVE9488 on eNOS expression and NO production in HUVEC.**

When primary HUVEC were incubated with AVE9488 for 18h, eNOS mRNA expression was increased, an effect comparable with simvastatin (Fig. 3A), which increases eNOS expression by stabilizing eNOS mRNA (Laufs et al., 1998). AVE9488 increases eNOS protein expression in a concentration-dependent manner (Fig. 3B).

Finally, to examine whether the enhanced eNOS expression resulted in enhanced release of NO, HUVEC pre-treated with AVE9488 for 18h were stimulated acutely with bradykinin. Intracellular cGMP content was measured as an indicator of NO production. Whereas the baseline levels of cGMP were not significantly affected by AVE9488, in bradykinin-stimulated cells, however, AVE9488 treatment significantly enhanced NO production (Fig. 3C). AVE9488 had no effect on expression of sGC subunits (Fig. 3D).
Sub-chronic effects of AVE9488 in mice.

AVE9488 was administered for 17 consecutive days to adult C57BL/6J mice at a dose of 30mg/kg/d pressed in the chow. Then, eNOS protein expression was analyzed by Western blotting in different tissues (Fig. 4). Significantly higher amounts of eNOS protein were detected in aortas and femoral arteries of AVE9488-treated animals as compared to controls (Fig. 4).

We then investigated the sub-chronic effects of AVE9488 in a mouse model of vascular neointima formation, in which the endothelium is left undamaged (Moroi et al., 1998). We applied cuff placements in three different mouse strains, wild type C57BL/6 and apoE-KO or eNOS-KO mice. Vascular pathologies were most strongly induced in the apoE-KO animals in which neointima became clearly visible consisting of up to 5 cell layers overlaying the lamina elastica interna (Fig. 5A). Treatment with AVE9488 (10 mg/kg, b.i.d., starting 3 days prior to the cuff placement) significantly reduced neointima formation (Figs. 5B and C). In parallel, an increased eNOS protein expression was detected in the aorta of AVE9488-treated animals (Fig. 5D). When eNOS-knockout mice were subjected to identical conditions of cuff-placement, no effect of AVE9488 on neointima formation could be observed (Fig. 5C), indicating that the inhibiting effect of AVE9488 on neointima formation was mediated by eNOS.

Anti-atherosclerotic effect of AVE9488 and AVE3085 in apoE-KO mice.

apoE-KO mice were randomized at the age of 10 weeks to receive either standard rodent chow or chow supplemented with AVE9488 at doses of 10 or 30 mg/kg/d for 12 weeks. In control animals, approximately 13.3% of the aortic surface was covered by atherosclerotic plaques, which is comparable with previous studies (Davis et al., 2001; Kauser et al., 2000). At 30 mg/kg/d, AVE9488 reduced plaque formation to 39% of control (Figs. 6A and B). The plaque area in the aortic root region close to the aortic valves was 32% lower in AVE9488-treated animals compared to placebo-treated mice (Figs. 6C and D). Accordingly, eNOS protein expression remained enhanced by AVE9488 treatment to the end of the study, as analyzed by
Western blot using protein samples from femoral arteries (not shown). The 12-week long-term treatment with AVE9488 did not affect plasma lipids or heart rate (Table 1). In our experimental setup, blood pressure tended to be lower in animals treated with AVE9488 (Table 1). Similarly, treatment of apoE-KO mice on Western-type diet with AVE9488 or AVE3085 (30 mg/kg/d, each, 12 weeks) resulted in a reduction (36% and 45%, respectively) of plaque formation (Fig. 6E). In contrast, the anti-atherosclerotic effect of both compounds could not be observed in apoE/eNOS-DKO mice (Fig. 6F). The aortic lesion area in placebo-treated apoE/eNOS-DKO mice was 149.4±15.4% (p < 0.05) of placebo-treated apoE-KO mice (set 100%).

Effects of AVE9488 on vascular ROS formation and eNOS functionality in apoE-KO mice.

apoE-KO mice were treated for 2 weeks with the anti-atherosclerotic dose of AVE9488 (30 mg/kg/d). Aortas from AVE9488-treated animals showed higher levels of the eNOS cofactor BH₄ in the aorta (Fig. 7A). ROS production in aortic rings was measured with L-012, a compound specifically reacting with superoxide and peroxynitrite, but not with NO (Sohn et al., 1999; Mollnau et al., 2003; Daiber et al., 2004). Aorta of apoE-KO mice showed significant basal ROS formation. This could be partially inhibited by the NOS-inhibitor L-NAME (Fig. 7B), indicating that part of ROS was produced by an uncoupled eNOS. Treatment of apoE-KO mice for two weeks with AVE9488 resulted in a significant reduction in aortic ROS production (Fig. 7B). Under these conditions, L-NAME did not further reduce vascular ROS production in AVE9488-treated animals (Fig. 7B), indicating a re-coupling of oxygen reduction and NO synthesis in eNOS.

Effect of AVE9488 on expression of genes important for vascular BH₄ content.

In order to determine how AVE9488 increase the vascular content of BH₄, we investigated its influence on the expression of various genes important for anabolism and catabolism of this co-factor. apoE-KO mice were treated orally for 2 weeks with AVE9488 (30 mg/kg/d). Thereafter RNA from the aortas was isolated via standard techniques and mRNA gene expression determined by real-time quantitative RT-PCR (Table 2). The rate-limiting step of
BH₄-biosynthesis is initial GTP-modification via the enzyme GTP-cyclohydrolase-I (GCH1). Aortic expression of GCH1 mRNA was not changed significantly by AVE9488. BH₄ is susceptible to oxidation by reactive oxygen species. Among NADPH-oxidase subunits, we detected significant levels of Nox2, Nox4 and p22phox mRNA in the aorta (Nox1 was undetectable). Aortic expression of these NADPH subunits was not modulated by AVE9488. All three superoxide-dismutating enzyme isoforms, SOD1, SOD2 and SOD3, could be detected in the aortas. Again, none of these factors was changed by AVE9488 on mRNA level.
DISCUSSION

In the present study, we have identified AVE9488 and AVE3085, two novel small molecular weight compounds with oral bioavailability, as eNOS-upregulating agents. In human endothelial cells, the two compounds increased the activity of human eNOS promoter both in stable (Fig. 1A) and in transient transfection (Fig. 1B) experiments. The shortest promoter fragment used (263 bp) was still responsive to AVE9488 and AVE3085 (Fig. 1B), indicating that the cis-elements responsible for the transcription activation by the two compounds are located in the proximal 263bp promoter region.

Within this promoter region, several binding sites have been shown to play an important role in controlling eNOS promoter activity. These include Sp1, Sp1/3-like, GATA, PEA3, Elf-1 and YY1 (Karantzoulis-Fegaras et al., 1999; Zhang et al., 1995). Mutation of Sp1, GATA, or PEA3 sites, for example resulted in a significant reductions in eNOS promoter activity (Cieslik et al., 1998; Karantzoulis-Fegaras et al., 1999; Zhang et al., 1995). In EMSA experiments, however, binding activity of nuclear proteins to any of these binding sites remained unchanged by AVE9488 (Figs. 1C and D). siRNA-mediated knockdown of Sp1 in EA.hy 926 cells resulted in reduction of basal eNOS promoter activity, but could not prevent eNOS promoter activation by AVE9488 or AVE3085 (Figs. 1E and F). Knockdown of GATA2 or ETS (which bind to GATA and PEA3 sites, respectively) could not prevent eNOS transcription activation by the two compounds, either (data not shown). These results indicate that Sp1, GATA2 and ETS are not required for the effect of AVE9488 and AVE3085 on eNOS expression. The responsible transcription factors/cis-elements, however, still remain to be identified.

As shown above, AVE9488 and AVE3085 stimulate eNOS transcription. These compounds are unlikely to be non-specific, broad-band gene regulators. In a microarray experiment performed in EA.hy 926 cells with the Affymetrix human genome U133 set (which contains approximately 33,000 genes/ESTs), only 24 genes/ESTs were upregulated (>2-fold) and 31 genes/ESTs downregulated (>50%) by AVE9488 (10 μM, 18h; data not shown). In a series
of cellular transcription factor assays, AVE9488 and AVE3085 did not activate liver-X-receptor isoforms (LXRα, LXRβ), retinoid acid receptor isoforms (RARα, RARβ), or retinoid-X-receptor isoforms (RXRα, RXRβ; data not shown).

AVE9488 and AVE3085 had no effect on eNOS mRNA stability (Fig. 2). Thus, both compounds seem to be pure eNOS transcription enhancers. Activation of eNOS transcription by AVE9488 resulted in enhanced eNOS mRNA (Fig. 3A) and protein (Fig. 3B) expression. The efficacy of AVE9488 was comparable to that of simvastatin (Fig. 3A), which increases eNOS mRNA by stabilizing eNOS mRNA and has little effect on eNOS transcription (Fig. 1A) (Laufs et al., 1998). Such an increase in eNOS protein levels may well have biological significance. Treatment of mice with simvastatin, for example, resulted in a stroke protection which was mediated by eNOS upregulation, because no such protection was seen in eNOS-KO mice (Endres et al., 1998).

AVE9488 increased eNOS protein expression also in the mouse in vivo (Figs. 4-5). Treatment via chow (30 mg/kg/d, Fig. 4) or via gavage (10 mg/kg b.i.d, Fig 5) resulted in similar plasma concentrations (data not shown) and a similar extent of eNOS upregulation.

In mice, in vivo treatment with AVE9488 resulted in inhibition of neointima formation (Fig. 5) and reduced atherosclerosis (Fig. 6). These protective effects observed in vivo are likely to be mediated by eNOS upregulation, because they were not seen in eNOS-KO mice (Fig. 5C) or apoE/eNOS-DKO mice (Fig. 6F). AVE9488 had no effects on plasma lipid levels (Table 1). Thus, the effects of AVE9488 on neointima and atherosclerotic plaque formation are unlikely to be related to hyperlipidemia.

Cuff-induced neointima formation is a nonocclusive vessel injury model. In these animals, the endothelium remains intact and eNOS-derived NO has been demonstrated to play a protective role (Moroi et al., 1998). Consistent with this concept, our results show that pharmacological upregulation of eNOS expression by AVE9488 reduced neointima formation in this model (Fig. 5). apoE knockout mice have been widely used as a model of hyperlipidemia.
and atherosclerosis (Meir and Leitersdorf, 2004). Treatment with AVE9488 or AVE3085 reduced atherosclerosis in apoE-KO mice (Figs. 6A though E) but not in apoE/eNOS-DKO mice (Fig. 6F). These results clearly indicate that the anti-atherosclerotic effect of both compounds is eNOS-dependent.

An anti-atherosclerotic role of endogenous eNOS has been demonstrated in apoE-KO mice (Chen et al., 2001; Kuhlencordt et al., 2001). apoE/eNOS-DKO mice displayed accelerated atherosclerosis and developed abdominal aortic aneurysm formation and ischemic heart disease compared with apoE-KO mice. Similarly, pharmacological inhibition of eNOS causes accelerated atherosclerosis in rabbits (Cayatte et al., 1994) and in mice (Kauser et al., 2000). Based on these data, one would expected that overexpression of eNOS protects against atherosclerosis. However, transgenic mice highly over-expressing eNOS on an apoE-KO background developed larger atherosclerotic lesions than apoE-KO mice alone (Ozaki et al., 2002). These data can be easily explained because the apoE-KO mouse is a model of vascular oxidative stress and eNOS tends to become dysfunctional under such pathophysiological conditions (Forstermann and Munzel, 2006). This has been shown in animal models of hypertension or diabetes (where eNOS was also upregulated, (Hink et al., 2001; Mollnau et al., 2002)). Under pre-existing oxidative stress, oxygen reduction by eNOS uncouples from NO synthesis, and the enzyme becomes a source of superoxide. This has been referred to as eNOS “uncoupling” (Forstermann and Munzel, 2006).

However, eNOS upregulated by AVE9488 remains functional, and the enhanced eNOS expression is associated with an elevation of bradykinin-stimulated cGMP generation in HUVEC (Fig. 3C). Because AVE9488 had no effect on sGC expression (Fig. 3D), the elevated cGMP content is likely to indicate increased production of bioactive NO

Untreated atherosclerotic apoE-KO mice showed a significant ROS production in their aortas, part of which was inhibited by NOS inhibitor L-NAME (Fig. 7B). This is consistent with previous findings (Alp et al., 2004), which demonstrated that eNOS is in an uncoupled state and
producing ROS in this pathological model. Treatment with AVE9488 resulted in a marked reduction in aortic ROS production to a level that could not be lowered any further by L-NAME (Fig. 7B). This suggests that eNOS was no longer producing ROS in AVE9488-treated apoE-KO mice, i.e. AVE9488 was able to reverse eNOS uncoupling.

The main reason for eNOS uncoupling is a deficiency of the essential eNOS cofactor BH₄ (Channon, 2004). Supplementation with BH₄ is capable of correcting eNOS dysfunction in several types of pathophysiology (Forstermann and Munzel, 2006). In isolated aortas from prehypertensive SHRs, BH₄ supplementation diminished the NOS-dependent generation of superoxide. Administration of BH₄ restored endothelial function in animal models of atherosclerosis, diabetes and insulin resistance, as well as in patients with hypercholesterolemia, diabetes mellitus, essential hypertension and in chronic smokers (reviewed in (Channon, 2004; Forstermann and Munzel, 2006; Schmidt and Alp, 2007)). Oral administration of BH₄ also slowed the progression of atherosclerosis in apoE-KO mice (Hattori et al., 2007).

In the present study, treatment with AVE9488 significantly enhanced vascular BH₄ content (Fig. 7A). This may be an important mechanism for the reversal of eNOS uncoupling in apoE-KO mice (Fig. 7B).

Intracellular BH₄ levels depend on the balance of its de novo synthesis and its oxidation/degradation. BH₄ is synthesized from GTP with GCH1 being the rate-limiting enzyme (Channon, 2004; Alp et al., 2004). BH₄ is one of the most potent naturally occurring reducing agents and susceptible to oxidation by ROS such as peroxynitrite (Laursen et al., 2001). Oxidation of BH₄ due to NADPH oxidase-mediated vascular oxidative stress may represent a major cause of BH₄ deficiency in many cases (Forstermann and Munzel, 2006; Landmesser et al., 2003). Suppression of oxidative stress by downregulating the expression or activity of vascular NADPH oxidase has been shown to increase vascular BH₄ levels (Li et al., 2006) and restore eNOS functionality (Hink et al., 2001; Li et al., 2006; Mollnau et al., 2002).
Treatment of AVE9488 resulted in elevated levels of vascular BH₄ (Fig. 7A). However, mRNA expression of the BH₄-generating enzyme GCH1 was not increased in response to AVE9488 treatment (Table 2). The expression of the major superoxide-producing enzyme NADPH oxidase was also not changed. In addition, the superoxide-depredating enzymes (SOD1, SOD2 and SOD3) were not changed (Table 2). Thus, the mechanisms for the increase in BH₄ by AVE9488 still remain unknown. In cultured EA.hy 926 cells, AVE9488 and AVE3085 had no effect on BH₄ content, whereas sepiapterin (a precursor of BH₄ synthesis via the salvage pathway), significantly increased BH₄ content (data not shown). The fact that AVE9488 increased vascular BH₄ content when administered in vivo, but not in endothelial cells in vitro, suggests that the elevation of BH₄ in vivo may be indirect (e.g. by an action of AVE9488 on cell types other than endothelial cells).

In conclusion, we have identified AVE9488 and AVE3085 as two novel small molecular weight compounds with vaso-protective properties in experimental atherosclerosis. The beneficial vascular effects of these compounds are likely to result from a combination of eNOS upregulation and a reversal of eNOS uncoupling. Such compounds have therapeutic potentials for the treatment of cardiovascular diseases.
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FOOTNOTES

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Legends for Figures

Fig. 1. AVE9488 and AVE3085 enhance eNOS transcription. Panel A: Human EA.hy 926 endothelial cells were stably transfected with a 3.5kb human eNOS promoter fragment driving a luciferase reporter gene. Cells were treated for 18h and luciferase activity was analyzed as a measurement of eNOS promoter activity. Panel B: EA.hy 926 cells were transiently transfected with human eNOS promoter fragments of different lengths (3500bp, 1600bp, 954bp or 263 bp). Cells were treated compounds at 10 µM for 18h and luciferase activity was analyzed. Panels C and D: Electrophoresis mobility shift assays with Sp1 and GATA binding sites in human eNOS promoter and nuclear proteins from EA.hy 926 cells treated with 5 µM AVE9488. Panels E and F: siRNA-mediated knockdown of Sp1 in EA.hy 926 cells stably transfected with the 3.5kb human eNOS promoter. Panel E shows a representative Western blotting gel 48h after siRNA transfection. Panel F demonstrates eNOS promoter activity. Cells were treated with compounds for 18h starting 48h after siRNA transfection. Shown are means ± SEM, n = 6 (*p< 0.05, compared with respective controls).

Fig. 2. AVE9488 and AVE3085 had no effect on eNOS mRNA stability. Panel A: Human EA.hy 926 endothelial cells were pretreated with AVE9488 or AVE3085 (5 µM each) for 24h, and then gene transcription was terminated by 5,6-dichlorobenzimidazole riboside (DRB, 60 µmol/L). eNOS mRNA was analyzed with Real-Time RT-PCR at indicated time points after adding DRB. eNOS mRNA levels at time zero of all groups were set 100%. Panel B: EA.hy 926 cells were pretreated with DRB (60 µM) and then treated with 5 µM AVE9488 or AVE3085 (added 30 min after DRB) for 24h. eNOS mRNA expression was analyzed with quantitative Real-Time RT-PCR. Symbols/columns represent mean ± SEM, n=6 (**p<0.01, compared with control without DRB).
Fig. 3. AVE9488 increases eNOS expression and NO production. Panel A: Human umbilical vein endothelial cells (HUVEC) were treated with AVE9488 (2 µM) or simvastatin (5 µM, as a positive control) for 18h and eNOS mRNA expression was analyzed with quantitative real-time RT-PCR. Panel B: HUVEC were treated with AVE9488 for 18h and eNOS protein expression was analyzed with Western blot using a polyclonal anti-eNOS antibody. GAPDH was shown for normalization. Panel C: After the 18h pre-treatment with AVE9488 (2 µM), cells were left untreated (basal) or stimulated with bradykinin (100 nM) for 3 min and intracellular cGMP content was determined with radioimmunoassay as an indicator of bioactive NO production. Panel D: (HUVEC) were treated with AVE9488 (2 µM) or simvastatin (5 µM) for 18h and mRNA expression of soluble guanylate cyclase (sGC) α1 or β1 was analyzed with quantitative real-time RT-PCR. Columns represent mean ± SEM, n = 4 (*p < 0.05, compared with control).

Fig. 4. AVE9488 increases eNOS protein expression in mice in vivo. Adult C57BL/6J mice were treated for 17 days with AVE9488 (30 mg/kg/d). Protein expression of eNOS was analyzed with Western blot in different vascular tissues using a polyclonal anti-eNOS antibody. Densitometric values were normalized to β-tubulin. Columns represent mean ± SEM, n = 6 (*p < 0.05; n.s. not significant).

Fig. 5. AVE9488 reduces neointima formation in apoE-KO mice but not eNOS-KO mice. Neointima formation was induced by a non-occlusive peri-vascular cuff around the femoral arteries of mice (panels A-C). Neointima formation in apoE-KO mice treated with placebo (panel A) or AVE9488 (10 mg/kg/d, b.i.d. for 17 days, panel B) (hematoxylin & eosin staining). Arrows indicate the inner elastic lamina. Panel C: quantification of neointima formation in wild type (WT) C57BL/6J mice, apoE-KO mice, and eNOS-KO mice, respectively. All mice were male, at C57BL/6J background and of similar age (10 weeks). Panel D: eNOS
protein expression in the aorta of apoE-KO mice analyzed with Western blot (normalized to β-tubulin). Columns represent mean ± SEM, n = 10 (*p< 0.05).

**Fig. 6. Long-term treatment with AVE9488 reduces atherosclerotic plaque formation in apoE-KO mice but not in apoE/eNOS-DKO mice.** Male apoE-KO mice were treated with AVE9488 supplemented in normal diet for 12 weeks (panels A through D; n=12). Panel A shows representative aortas stained with the lipophilic dye oil-red-O. Quantification of plaque area was demonstrated in panel B. Panels C and D illustrate cross-sections through the proximal aortic root and quantification of plaque area, respectively (hematoxylin & eosin staining). Panels E and F: male apoE-KO (E; n=15) or apoE/eNOS-DKO (F; n=7-13) mice on Western-type diet were treated with AVE9488 or AVE3085 (30 mg/kg/d, each) for 12 weeks; atherosclerotic plaques were quantified in oil-red-O-stained aorta. Columns represent mean ± SEM (*p< 0.05, n.s., not significant, compared with placebo).

**Fig. 7. AVE9488 increases vascular (6R)-5,6,7,8-tetrahydro-L-biopterin (BH₄) and reverses eNOS uncoupling** apoE-KO mice were treated for 2 weeks with AVE9488 (30mg/kg/d). Panel A shows aortic BH₄ levels as measured with high-performance liquid chromatography. Panel B shows vascular production of superoxide (and peroxynitrite) as determined with luminescence probe L-012. Experiments were performed in the absence or presence of the NOS inhibitor L-NAME (1 mM). Columns represent mean ± SEM, n=9 (*p< 0.05, **p< 0.01).
Table 1. Effect of the AVE9488 on blood pressure, heart rate and serum lipid profile after chronic treatment with AVE9488 (30 mg/kg/d, 12 weeks) in wildtype, apoE-KO, and eNOS-KO mice.

<table>
<thead>
<tr>
<th>Group</th>
<th>n</th>
<th>Blood pressure (mmHg)</th>
<th>Heart rate (bpm)</th>
<th>Total cholesterol (mM)</th>
<th>LDL (mM)</th>
<th>Triglycerides (mM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>apoE-KO</td>
<td>8</td>
<td>107 ± 5</td>
<td>635 ± 14</td>
<td>12.3 ± 0.6</td>
<td>8.9 ± 0.5</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>apoE-KO + AVE9488</td>
<td>8</td>
<td>99 ± 6</td>
<td>666 ± 18</td>
<td>12.8 ± 1.1</td>
<td>9.7 ± 0.8</td>
<td>1.2 ± 0.2</td>
</tr>
<tr>
<td>C57BL/6J</td>
<td>6</td>
<td>103 ± 7</td>
<td>673 ± 21</td>
<td>2.3 ± 0.4</td>
<td>1.7 ± 0.3</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>C57BL/6J + AVE9488</td>
<td>6</td>
<td>101 ± 8</td>
<td>645 ± 19</td>
<td>2.7 ± 0.3</td>
<td>2.1 ± 0.4</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>eNOS-KO</td>
<td>6</td>
<td>120 ± 4</td>
<td>598 ± 30</td>
<td>2.2 ± 0.5</td>
<td>1.6 ± 0.4</td>
<td>0.9 ± 0.2</td>
</tr>
<tr>
<td>eNOS-KO + AVE9488</td>
<td>6</td>
<td>123 ± 3</td>
<td>620 ± 16</td>
<td>2.4 ± 0.6</td>
<td>1.8 ± 0.3</td>
<td>0.8 ± 0.1</td>
</tr>
</tbody>
</table>
Table 2. Effect of the AVE9488 (30 mg/kg/d for 2 weeks) on aortic mRNA expression of genes important for production of BH₄ and reactive oxygen species in apoE-KO mice. Values are means ± SEM and expressed in percent expression of the placebo group.

<table>
<thead>
<tr>
<th>Gene</th>
<th>HUGO gene identifier</th>
<th>N</th>
<th>Placebo</th>
<th>AVE9488</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>GTP-cyclohydrolase-I</td>
<td>GCH1</td>
<td>6</td>
<td>100% ± 25%</td>
<td>92% ± 18%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>NADPH oxidase 1</td>
<td>NOX1</td>
<td>6</td>
<td>below</td>
<td>below</td>
<td></td>
</tr>
<tr>
<td>Cytochrome b-245 heavy chain (Nox2, gp91-phox)</td>
<td>CYPB</td>
<td>6</td>
<td>100% ± 42%</td>
<td>105% ± 14%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>NADPH oxidase 4</td>
<td>NOX4</td>
<td>6</td>
<td>100% ± 51%</td>
<td>117% ± 5%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Cytochrome b-245, alpha polypeptide (p22 phox)</td>
<td>CYBA</td>
<td>6</td>
<td>100% ± 20%</td>
<td>111% ± 4%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Soluble superoxide dismutase-1</td>
<td>SOD1</td>
<td>6</td>
<td>100% ± 14%</td>
<td>76% ± 7%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Soluble superoxide dismutase-2</td>
<td>SOD2</td>
<td>6</td>
<td>100% ± 24%</td>
<td>74% ± 6%</td>
<td>p&gt;0.05</td>
</tr>
<tr>
<td>Soluble superoxide dismutase-3</td>
<td>SOD3</td>
<td>6</td>
<td>100% ± 2%</td>
<td>121% ± 10%</td>
<td>p&gt;0.05</td>
</tr>
</tbody>
</table>
**Fig. 2**

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Fig. 4

This article has not been copyedited and formatted. The final version may differ from this version.
Fig. 5

A

B

C

D

Intima / media

AVE9488

WT

ApoE-/-

eNOS-/-

0.0

0.1

0.2

0.3

0.4

0.5

AVE9488

WT

ApoE-/-

eNOS-/-

0

50

100

150

200

250

eNOS protein (%)

Placebo

AVE9488

*
Fig. 6

A

Placebo

AVE9488

B

Plaque size

(% of placebo)

0

10

30

AVE9488 [mg/kg/d]

n.s.

*  

C

Placebo

AVE9488

D

Lesion area

(% of placebo)

0

50

100

AVE9488 [mg/kg/d]

n.s.

*  

E

Plaque size

(% of placebo)

0

50

100

200

Placebo

AVE3085

AVE9488

*  

F

Placebo

AVE3085

AVE9488

n.s.

n.s.
Fig. 7

A

BH4 (fmol/mg protein)

Placebo  AVE9488

B

Aortic ROS production (% of placebo)

Placebo  AVE9488  L-NAME

+  +  -  -  +  +  -  +