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Reduction of Blood Pressure, Plasma Cholesterol, and Atherosclerosis by Elevated Endothelial Nitric Oxide*

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In the vascular system, nitric oxide is generated by endothelial NO synthase (eNOS). NO has pleiotropic effects, most of which are believed to be atheroprotective. Therefore, it has been argued that patients suffering from cardiovascular disease could benefit from an increase in eNOS activity. However, increased NO production can cause oxidative damage, cell toxicity, and apoptosis and hence could be atherogenic rather than beneficial. To study the *in vivo* effects of increased eNOS activity, we created transgenic mice overexpressing human eNOS. Aortic blood pressure was ~20 mm Hg lower in the transgenic mice compared with control mice because of lower systemic vascular resistance. The effects of eNOS overexpression on diet-induced atherosclerosis were studied in apolipoprotein E-deficient mice. Elevation of eNOS activity decreased blood pressure (~20 mm Hg) and plasma levels of cholesterol (~17%), resulting in a reduction in atherosclerotic lesions by 40%. We conclude that an increase in eNOS activity is beneficial and provides protection against atherosclerosis.

Endothelial nitric oxide synthase (eNOS)[1] plays an important role in the regulation of vascular tone, vascular biology, and hemostasis. For example, NO produced by eNOS causes vasodilation. Thus, eNOS knockout mice are hypertensive (1), whereas eNOS transgenic mice have hypotension (2). In addition, NO reduces the activation and aggregation of platelets (3, 4), attenuates adhesion of leukocytes to the endothelium (5–7), reduces the permeability of the endothelium, and inhibits proliferation and migration of vascular smooth muscle cells (8). Impaired activity of eNOS is associated with endothelial cell dysfunction (9). For these reasons eNOS has been proposed to modulate atherosclerotic disease (10–12). Indeed, impairment or deficiency of eNOS gives rise to accelerated atherosclerosis in animal models (10, 12–14), indicating that physiological levels of eNOS are anti-atherogenic. This suggests that patients at increased risk of atherosclerotic vascular disease could possibly benefit from an increase of eNOS activity by pharmacological means or (local) gene therapy. However, eNOS-derived NO also has detrimental effects (15), such as the generation of superoxides (16), making it difficult to predict whether increased eNOS activity is beneficial or harmful (17). To determine whether increased eNOS activity may be beneficial, we created transgenic mice that express the human *eNOS* gene. These mice were crossbred to apolipoprotein E-deficient (apoE0) mice in order to evaluate the effects of a constitutive increase in eNOS activity on the development of atherosclerosis.

EXPERIMENTAL PROCEDURES

Mice

A DNA fragment containing the human *eNOS* gene was isolated from a homemade human genomic cosmid library (18) using eNOS cDNA (kindly donated by Dr. S. Janssens, Leuven, Belgium; Ref. 19) as a probe. In addition, the DNA fragment contained ~6 kb of 5′-natural flanking sequence, including the native *eNOS* promoter, and ~3 kb of 3′-sequence to the gene. Vector sequences were removed by restriction endonucleases. A solution of 1–2 μg/ml DNA was used for microinjection of fertilized oocytes from FVB donor mice and transplanted into the oviducts of pseudopregnant B10 × CBA mice. Founder mice and offspring were genotyped by PCR on DNA isolated from tail biopsies. Primers used were sense, 5′-GTCCTGCAGACCGTGCAGC-3′, and antisense, 5′-GGCTGTTGGTGTCTGAGCCG-3′. Mice were backcrossed to C57Bl6 for at least five generations (>96% C57Bl6). All eNOS transgenic mice were hemizygous. ApoE0 mice were obtained from The Jackson Laboratory, Bar Harbor, ME. Male mice were used in all experiments. All animal experiments were performed in compliance with institutional (Erasmus Medical Center, Rotterdam, The Netherlands) and national (Ministry of Health, Welfare, and Sport, The Hague, The Netherlands) guidelines.

Western Blotting and Immunohistochemistry

Aortas were collected and homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and 20 mM CHAPS. Western blotting was performed as described previously (20). 25 μg of protein (BCA protein assay kit; Pierce) was applied to each lane. Anti-eNOS was obtained from Santa Cruz Biotechnology Inc., Santa Cruz, CA. This antibody was also used for immunohistochemistry experiments, which were performed according to Bakker *et al.* (21).

Hemodynamic Measurements

Baseline Blood Pressure Measurements—Mice were weighed, anesthetized with ketamine (100 mg/kg intraperiteoneal) and xylazine (20 mg/kg intraperitoneal), intubated, and ventilated with a mixture of O_2 and N_2 (1/2 v/v) with a pressure-controlled ventilator (Servo ventilator 900C, Siemens-Elema, Sweden). The ventilation rate was set at 100 strokes/min with a peak inspiration pressure of 18 cm H_2O and a positive end expiration pressure of 6 cm H_2O. After intubation the mice were placed on a heating pad to maintain body temperature at 37 °C, and a polyethylene catheter (PE 10) was inserted into the right carotid artery and advanced into the aortic arch for the measurement of aortic pressure. In the first part of the study we used 12 eNOS-Tg2 and 5 eNOS-Tg3 mice for screening of eNOS expression and compared them to 33 wild type mice. Ten minutes after a second intraperitoneal bolus

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[1] The abbreviations used are: eNOS, endothelial nitric oxide synthase; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonic acid; LDL, VLDL, and HDL, low, very low, and high density lipoproteins, respectively. L-NAME, N^G nitro-L-arginine methyl ester.

of anesthetics (100 mg/kg ketamine and 20 mg/kg xylazine), baseline blood pressure recordings were obtained.

Effect of L-NAME on Systemic Vascular Resistance—Subsequently, we chose the eNOS-Tg2 line to determine whether the lower aortic blood pressure was the result of a NO-mediated decrease in systemic vascular resistance. For this purpose, in 17 eNOS-Tg2 mice and 17 wild type mice a polyethylene catheter (PE 10) was inserted into the right carotid artery and advanced into the aortic arch for the measurement of aortic pressure, while another PE 10 catheter was introduced into the right external jugular vein and advanced into the superior caval vein for infusion of L-NAME. After thoracotomy through the second right intercostal space, the ascending aorta was exposed and a transit-time flow probe (ID 1.5 mm; T206; Transonics Systems Inc.) was placed around the aorta for measuring aorta flow. Ten minutes after a second intraperitoneal bolus of 100 mg/kg ketamine and 20 mg/kg xylazine, baseline recordings were obtained. A continuous 10-min intravenous infusion of L-NAME (100 mg/kg) was started; 10 min after completion of the infusion, measurements were repeated.

Effects of Dietary Suppletion of L-Arginine on Baseline Hemodynamics—In the eNOS-Tg2 line we studied whether L-arginine deficiency contributed to the modest effects of eNOS overexpression on systemic vascular resistance. For this purpose, in six eNOS-Tg2 male mice and seven wild type male mice, L-arginine was supplemented in their drinking water (2.5% w/v). One week later, animals were instrumented as described above, and hemodynamic measurements were performed under baseline conditions.

Data Analysis—Hemodynamic data were recorded and digitized using an online 4-channel data acquisition program (ATCODAS, Dataq Instruments, Akron, OH), for later analysis with a program written in MatLab (Mathworks Inc, Natick, MA). Fifteen consecutive beats were selected for determination of heart rate, aortic pressure, and aorta blood flow.

eNOS Activity Assay

Aortas were collected and homogenized in 50 mM Tris-HCl, pH 7.4, containing 1 mM EDTA, 0.25 M sucrose, and 20 mM CHAPS. eNOS activity assays were performed by measuring L-arginine to L-citrulline conversion using a nitric oxide synthase assay kit (Calbiochem, La Jolla, CA; catalogue no. 482700) according to the manufacturer's instructions. Protein content was measured by the BCA protein assay kit (Pierce).

Lipid Measurements

Blood was collected via orbital puncture after an overnight fasting period. Plasma was frozen freshly or subjected to ultracentrifugation in a Beckman 42.2 Ti rotor (42,000 rpm, 3 h, 12 °C) at $d = 1.063$ g/ml. Tubes were sliced, and two fractions were collected: very low density lipoprotein (VLDL) + low density lipoprotein (LDL), $d < 1.063$ g/ml; and high density lipoprotein (HDL), $d > 1.063$ g/ml. Cholesterol was measured with the F-chol kit (Roche Molecular Biochemicals) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Roche Molecular Biochemicals).

Atherosclerosis

Atherosclerosis experiments were performed in age- and sex-matched mice. Male mice of 8 weeks were fed a Western type diet containing 15% (w/w) cacoa butter and 0.25% (w/w) cholesterol (diet W, Hope Farms, Woerden, The Netherlands) for 6 weeks, which leads to appreciable atherosclerosis (22–24). Animals were anesthetized using isoflurane, and *in situ* fixation was performed via the left ventricle of the heart using phosphate-buffered formalin (4%, v/v). A Sony digital camera was used to obtain images of sections of the aortic root. These were analyzed by Scion Image processing and analyzing software (available from www.scioncorp.com). Atherosclerosis was quantified in five sections per mouse with 80-μm intervals using the method of Paigen *et al.* (25).

Data Analysis

Analysis of data was performed using two- or one-way analysis of variance followed by the Scheffé test, as appropriate. Statistical significance was accepted when $p < 0.05$ (two-tailed). Data are presented as mean ± S.E.

RESULTS AND DISCUSSION

For the generation of eNOS transgenic mice, we used a DNA fragment that comprised the complete human *eNOS* genomic sequence, including all exons and introns as well its natural flanking sequences. Therefore, our mice are different from those described by Ohashi *et al.* (2), which overexpressed bovine *eNOS* cDNA, driven by the murine preproendothelin-1 promoter. Our approach was chosen to preserve the natural regulation of the gene and to prevent ectopic expression. For example, the endothelium enhancer element that is located 4.9 kilobases upstream from the transcription start site of the *eNOS* gene (26) is included in this construct. By using this construct we mimic the human situation in terms of regulation and tissue distribution of eNOS as much as possible.

Two independent lines with appreciable overexpression of the transgene, as measured by RT-PCR (not shown), were selected and arbitrarily designated eNOStg2 and eNOStg3. Production of human eNOS protein was demonstrated by Western blotting of aorta homogenates (Fig. 1*A*). Subsequently, eNOS activity in aortas from control (wild type) and eNOS overexpressing mice was measured, using the L-arginine to L-citrulline conversion assay (27). In aorta, the level of active eNOS enzyme was 10-fold increased in eNOStg2 mice and 7.5-fold in eNOStg3 mice when compared with wild type animals (Fig. 1*B*). Expression of human eNOS was also investigated by immunohistochemistry. There was no human eNOS staining of aortas of wild type mice, whereas the endothelial layer of the aorta was clearly stained in both human eNOS transgenic lines (Fig. 1*C*).

The expression pattern of the human *eNOS* transgene was investigated by immunohistochemistry in heart (Fig. 2*A*), liver (Fig. 2*B*), kidney (Fig. 2*C*), adrenal (Fig. 2*D*), and testis (Fig. 2*E*). Sections from wild type controls show virtually no immunostaining (not shown). The lining of the larger vessels is clearly stained (Fig. 2, *A*, *D*, and *E*). Staining of capillaries is visible in the heart between the cardiomyocytes. Immunoreactivity is observed in the sinusoids in the liver and in the kidney in the peritubular capillaries as well as in the capillaries from the glomeruli. In the adrenal, the cortical capillaries as well as medullary capillary sinusoids and veins are stained. In the testis, only blood vessels between the seminiferous tubules are stained. No appreciable immunoreactivity is perceptible in the parenchyma cells of any of these organs. Similar results were found with sections from eNOStg3 mice (not shown).

Our results show that the genomic sequences included in the DNA fragment we used are sufficient for expression in endothelial cells. Although eNOS activity is tightly regulated at the post-translational level (28, 29), there is also extensive regulation at the transcriptional level (30, 31). The present study shows that our construct results in high level expression of human eNOS that is not prevented by a feedback mechanism.

To study the effect of increased eNOS activity on blood pressure and vascular tone, we performed hemodynamic studies (32). Heart rates were similar for wild type controls and eNOS transgenic lines, although the eNOS transgenic lines each exhibited a 20–25-mm Hg lower mean aortic blood pressure compared with littermate controls (Fig. 3*A*). Subsequent hemodynamic studies were performed in eNOStg2, the transgenic mouse line with the highest expression. These experiments showed that the lower aortic blood pressure was the result of a 30% lower systemic vascular resistance, because mean aortic blood flow and heart rate were similar in both groups (Fig. 3*B*). Subsequent infusion of the NOS inhibitor, L-NAME, increased systemic vascular resistance and abolished the difference between control and eNOStg2 mice. We therefore conclude that the lower basal systemic vascular resistance in eNOStg2 mice is the result of increased NO production (Fig. 3*B*). This is corroborated by the significantly larger increase in blood pressure in response to L-NAME in the transgenic mice. Suppletion of L-arginine had no effect on the already lower mean aortic

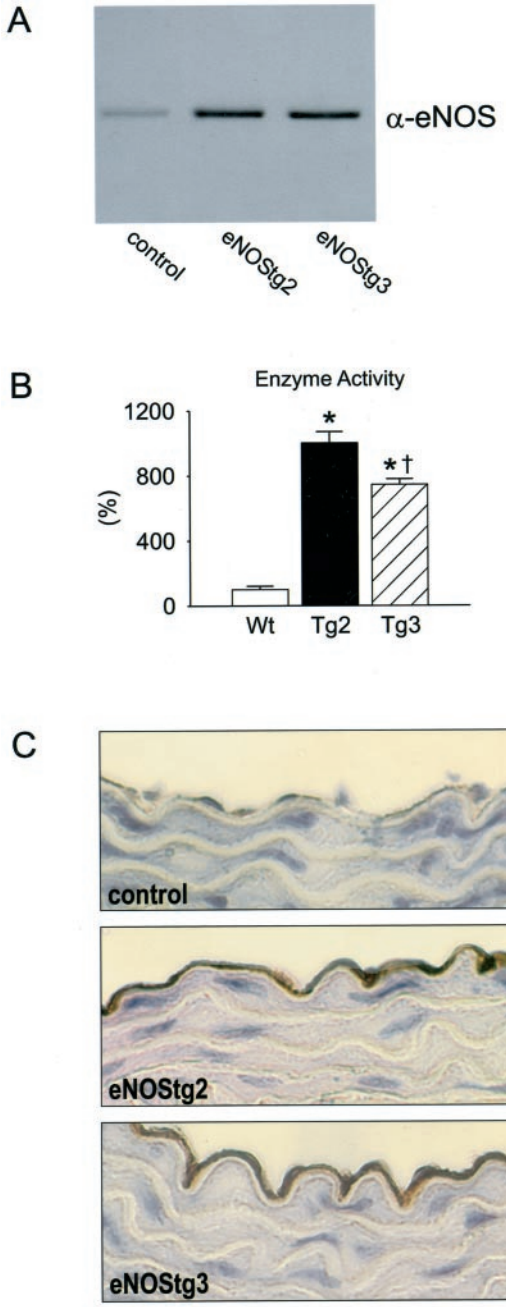

FIG. 1. **Expression of eNOS in transgenic mice.** *A*, Western blot analysis of aortas from control (wild type littermates), eNOStg2, or -3 mice. 25 μg of homogenate was applied per lane. The blot was probed with antihuman eNOS antibody. A single protein band of the expected molecular size (~130 kDa) was detected. *B*, eNOS activity was measured in aortas from control (*Wt*), eNOStg2 (*Tg2*), or -3 (*Tg3*) mice by the L-arginine to L-citrulline conversion assay using a nitric oxide synthase assay kit (Calbiochem). Activity is expressed as percentage of the activity in controls, which was 1.56 ± 0.31 pmol/μg/min. Each value represents the mean ± S.E. of three animals. One of three experiments is shown. *, $p < 0.05$ *versus* controls; †, $p < 0.05$ *versus* eNOStg2 mice (analysis of variance followed by the Scheffé test). *C*, immunohistochemistry on aortas from control, eNOStg2, or -3 mice. Aortas were collected after *in situ* fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. Original magnification, ×630.

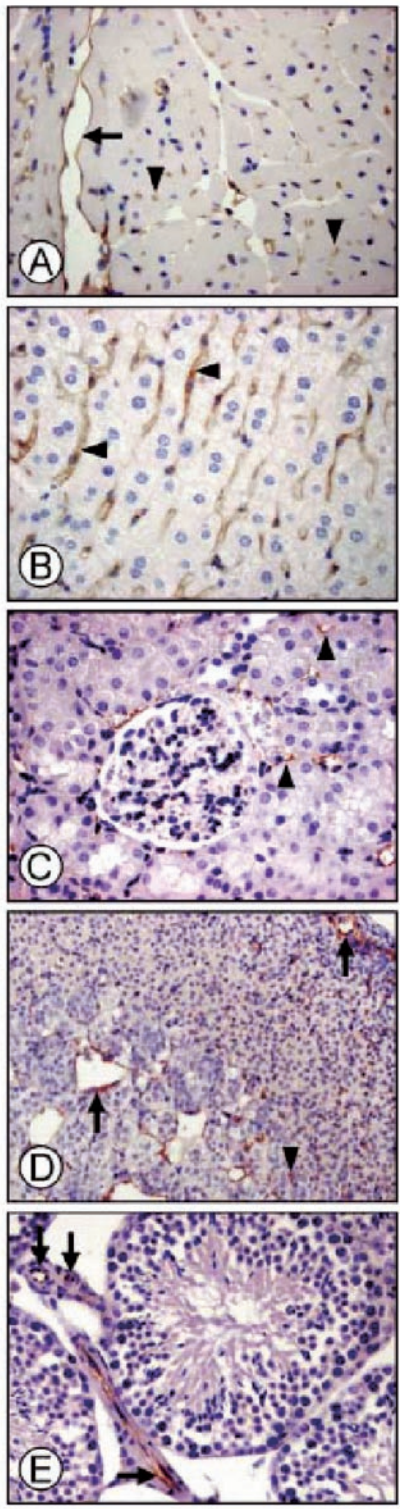

FIG. 2. **Expression pattern of human eNOS in transgenic mice.** Organs from eNOStg2 mice were collected after *in situ* fixation. Paraffin sections were incubated with antihuman eNOS antibody and a peroxidase-conjugated secondary antibody. *A*, heart. *B*, liver. *C*, kidney. *D*, adrenal. *E*, testis tissue. *Arrowheads* indicate representative immunoreactive capillaries; *arrows* indicate larger blood vessels. Original magnification, ×400.

blood pressure and heart rate in the transgenic mice (Fig. 3*C*). We therefore also conclude that the blood pressure-lowering effect of eNOS overexpression was not limited by a shortage of substrate.

The present study shows that the lower blood pressure associated with eNOS overexpression (as reported in Ref. 2) is the result of a lower systemic vascular resistance. Thus eNOS activity was not only increased in the larger blood vessels but also in the microcirculation. Although eNOStg2 and -3 mice showed a slight variation in eNOS activity (Fig. 1*B*), the degree

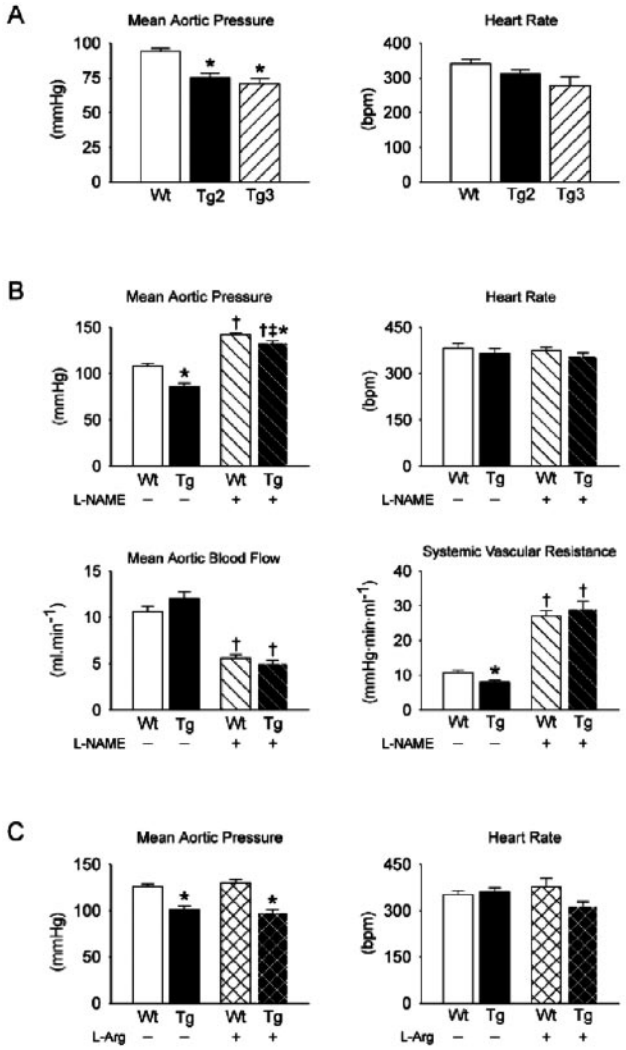

FIG. 3. **Hemodynamic measurements.** *A*, mean aortic pressure and heart rate were measured in anesthetized control (wild type littermates, *Wt*), eNOStg2 (*Tg2*), or -3 (*Tg3*) mice. *B*, mean aortic pressure, heart rate, mean aortic blood flow, and systemic vascular resistance were analyzed in Wt or eNOStg2 mice before and following infusion of L-NAME. *C*, mean aortic pressure and heart rate were measured in anesthetized Wt or Tg2 mice following 1 week of drinking water with or without L-arginine supplementation (L-Arg, 2.5% w/v). Each value represents the mean ± S.E. of ≥ five animals. *, $p < 0.05$ *versus* Wt. †, $p < 0.05$ *versus* baseline (before infusion of L-NAME). ‡, $p < 0.05$ *versus* Wt after infusion of L-NAME (analysis of variance followed by the Scheffé test).

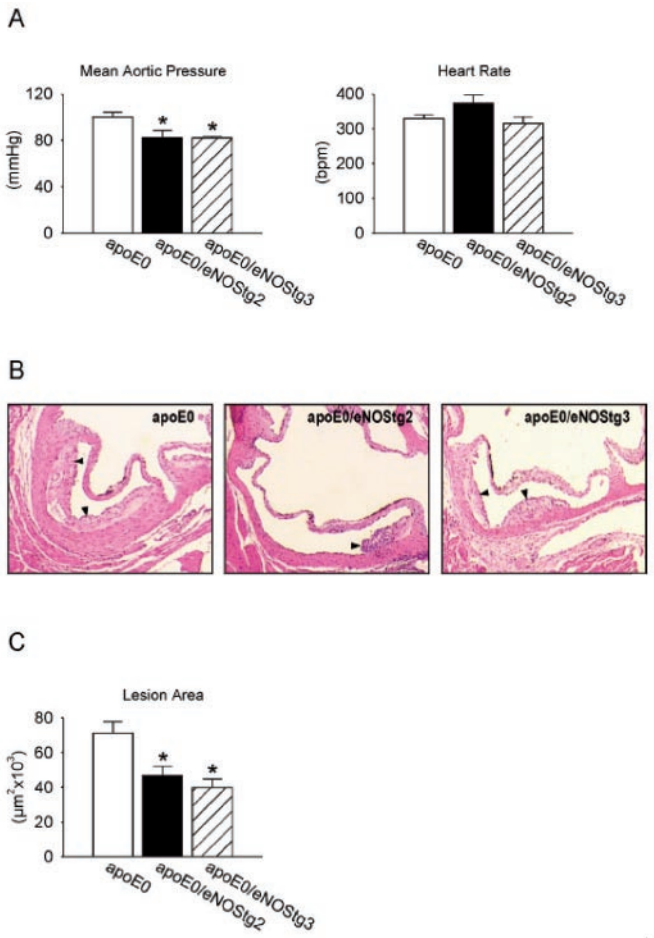

FIG. 4. **Analysis of eNOS transgenic/apoE-deficient mice.** *A*, mean aortic pressure and heart rate were measured in anesthetized *apoE0*, $n = 15$; *apoE0/eNOStg2*, $n = 8$; or *apoE0/eNOStg3*, $n = 8$ mice. *B*, representative photomicrographs of hematoxylin and eosin-stained paraffin sections with lesion areas in the aortic valves (*arrowheads*). Original magnification, ×25. *C*, lesion area in the aortic root of *apoE0*; $n = 32$; *apoE0/eNOStg2*, $n = 22$; or *apoE0/eNOStg3*, $n = 19$ mice. Areas were measured in five sections with 80-μm intervals and expressed as μm^2 per section per animal.

to which blood pressure was lowered was not different. This suggests that another rate-limiting factor or one or more compensatory mechanisms prevented a further decrease in blood pressure in the eNOStg2 mice.

To study whether increased expression of eNOS protects the mice against the development of diet-induced atherosclerosis, eNOS transgenic mice were cross-bred to apoE0 mice, which represent a well known mouse model for studying atherosclerosis (33, 34). The animals were fed a Western type diet for 6 weeks. As shown in Fig. 4*A*, overexpression of eNOS also caused a decrease in systemic blood pressure under these conditions, although heart rates were similar. Plasma cholesterol levels were measured before the start of the atherogenic diet (*i.e.* on normal chow diet) and at the end of the experiment (Table I). Both eNOS transgenic lines showed a decrease in plasma cholesterol of ~15% when compared with apoE0 mice when fed a normal chow diet. As expected, the Western diet resulted in a dramatic increase (~3-fold) in plasma cholesterol

TABLE I
Plasma lipid and lipoprotein analysis

Total cholesterol concentrations in plasma from apoE-deficient control (apoE0), apoE0/eNOStg2, or apoE0/eNOStg3 mice after feeding a normal chow diet or an atherogenic diet. Cholesterol concentrations were determined by enzymatic methods. VLDL + LDL and HDL fractions were isolated by ultracentrifugation. TC, total cholesterol (mM); VLDL + LDL, cholesterol in VLDL and LDL (mM); HDL, cholesterol in HDL (mM).

	n	Chow TC	Atherogenic diet		
			TC	VLDL + LDL	HDL
apoE0	32	10.9 ± 0.3	28.4 ± 0.9	27.3 ± 0.8	0.4 ± 0.02
apoE0/eNOStg2	22	9.2 ± 0.4[a]	23.3 ± 1.1[a]	21.9 ± 1.1[b]	0.4 ± 0.02
apoE0/eNOStg3	19	9.0 ± 0.2[b]	24.4 ± 1.1[c]	24.3 ± 1.0[c]	0.4 ± 0.02

[a] $p < 0.01$.
[b] $p < 0.001$.
[c] $p < 0.05$.

levels, whereas the total cholesterol concentration remained ~16% lower in the eNOS transgenic animals when compared with apoE0 controls. This difference was because of variations in VLDL and LDL, which contain the bulk of the plasma cholesterol under these conditions: HDL-cholesterol concentration in plasma was 0.4 mM and did not differ between the groups (Table I). These findings indicate that elevated eNOS activity results in a slightly more favorable (*i.e.* less athero-

genic) lipoprotein profile, because VLDL and LDL contain the atherogenic portion of plasma cholesterol (35), whereas HDL is protective against the development of atherosclerosis (36, 37).

To study the effect of eNOS overexpression on atherosclerosis, the atherosclerotic lesion areas in the aortic roots were measured. Fig. 4*B* shows representative examples of histological sections. Compared with apoE0 mice, we observed a decrease in atherosclerosis in both lines of eNOS transgenic mice studied (Fig. 4*C*).

Several studies have described a relation between plasma cholesterol and eNOS activity (38–40). However, these investigations exclusively focused on the effects of changes in plasma cholesterol levels on eNOS activity. Hypercholesterolemia is associated with decreased eNOS activity, probably via an interaction of oxidized LDL with caveolae, the plasma membrane domains in which eNOS resides (41). Cholesterol synthesis inhibitors (statins) have been reported to increase eNOS activity in addition to their cholesterol lowering effects, but these actions appear to be independent (38). In the present study we observed that the level of eNOS expression affects the level of plasma cholesterol: plasma levels of cholesterol were about 15% lower in eNOStg/apoE0 mice as compared with apoE0 controls. A similar difference in plasma cholesterol levels was found after feeding the mice a Western type diet for 6 weeks, indicating that eNOS overexpression alleviates diet-induced hypercholesterolemia. This effect is not caused by ectopic expression in organs involved in lipid metabolism, *e.g.* the liver, because the expression pattern is restricted to endothelial cells in all organs that were examined (Fig. 2). Recently it was shown that hypercholesterolemia in mice results in CD36-mediated cholesterol depletion of caveolae, followed by translocation of eNOS from caveolae and subsequent inactivation of the enzyme (42). Thus, eNOS activity is directly related to the cholesterol content of caveolae. Possibly, the moderate decrease in plasma cholesterol that we observed in our eNOS transgenic mice is caused by a recruitment of plasma cholesterol by the endothelial cells in order to handle the increased level of eNOS protein. This small decrease in plasma cholesterol likely contributed, at least in part, to the observed lower susceptibility to diet-induced atherosclerosis.

Although it has been proposed that elevation of eNOS activity would attenuate atherosclerosis (11), serious doubts have also been expressed as to whether an increase in eNOS activity *in vivo* would have beneficial effects, because high levels of NO (*e.g.* as produced by inducible NO synthase) have been implicated in cell toxicity and apoptosis (15, 43). During the preparation of our report, Ozaki *et al.* (44) reported the unexpected observation that relatively modest overexpression of eNOS resulted in *increased* atherosclerosis. Based on our findings, we conclude that this observation cannot be generalized, taking into account the following considerations. First, because enzyme activity is ± 1.5-fold increased, the level of eNOS overexpression in the mice used by Ozaki *et al.* is relatively low when compared with the much higher NO production levels by activated inducible NO synthase. A 1.5-fold increase is also rather low in terms of possible drug or gene therapy applications. Second, the observed increase in atherosclerosis is explained by measurements indicating that the overexpressed eNOS enzyme is dysfunctional in the mouse model used by Ozaki *et al.* This finding is not unexpected, given the moderate level of overexpression in their mice, because it has been previously reported that endogenous eNOS is indeed dysfunctional in terms of NO production in apoE0 mice fed a Western type diet (45). The results from the Ozaki *et al.* study are probably (at least in part) explained by the construct used, which consists of cDNA (often leading to low expression levels) and a heterologous promoter. In contrast, our results demonstrate that overexpression of human eNOS in endothelial cells indeed results in decreased atherosclerosis, most likely via lowering blood pressure and plasma cholesterol. Our study therefore suggests that elevation of eNOS activity could be beneficial for patients at risk of developing atherosclerotic disease.

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