Atheroprotective Effects of Neuronal Nitric Oxide Synthase in Apolipoprotein E Knockout Mice

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Objective—All 3 isoforms of the nitric oxide synthase (NOS) are expressed in atherosclerotic lesions. To test whether neuronal NOS (nNOS) deficiency affects atherosclerosis, we studied apoE/nNOSα double knockout (DKO) and apolipoprotein E (apoE) knockout (KO) control mice.

Methods and Results—Lesion area was significantly increased in male DKO (66%) mice after 14 weeks and in female DKO animals (31%) after 24 weeks of “western” diet. Moreover, mean arterial blood pressure was significantly reduced in female DKO animals. Immunohistochemistry revealed nNOS expression in the neointima of KO mice. In DKO animals, residual nNOS staining was caused by the presence of nNOS splice variants. Whereas nNOSα was present in vessels of KO and absent in DKO animals, nNOSγ was expressed in KO and DKO mice.

Conclusion—nNOSα protects against atherosclerosis as nNOSα deletion leads to an increase in plaque formation in apoE/nNOSα DKO mice. Female DKO mice showed a significant reduction in mean arterial blood pressure. Additionally, we found expression of nNOS splice variants in vessels of apoE KO mice. Our data highlights nNOSα overexpression as a potential therapeutic strategy and naturally occurring splice variants that lack exon 2 of the nNOS gene as a potential risk factor for vascular disease. (Arterioscler Thromb Vasc Biol. 2006;26:1539-1544.)

Key Words: nitric oxide ■ nitric oxide synthase ■ arteriosclerosis ■ blood pressure ■ gene expression

Neuronal nitric oxide synthase (nNOS) is expressed in early and advanced human atherosclerotic lesions. Immunolocalization and in situ hybridization revealed nNOS expression in endothelial cells, macrophages, and smooth muscle cells. In addition, nNOS expression is found in perivascular nitrinergic neurons. Although nNOS and endothelial nitric oxide synthase (eNOS) are termed constitutive NOS isoforms, nNOS is only detectable in intact human vessels using supersensitive methods, suggesting that expression may be induced in atherosclerosis. The nNOS gene produces multiple mRNA splice variants through various mechanisms, namely alternate promotor usage, alternative splicing, cassette insertion and deletions, and varied sites of 3’-UTR cleavage and polyadenylation. These mechanisms lead to 4 different peptides, of which 2 have a PDZ domain that anchors them to the sarcoplasmic reticulum, whereas 2 lack the PDZ-domain, localizing them to the cytosol. Schwarz et al reported the presence of small amounts of brain-type nNOSα and muscle-type nNOSµ in the media and adventitia of rat aorta and showed that nNOS may exert an inhibitory effect against a vasoconstrictive response. Recent studies in a mouse carotid artery ligation model, as well as a rat model of balloon induced vascular injury, demonstrated that nNOS is expressed after vascular injury and inhibits intima proliferation, pointing toward a vasculoprotective role of nNOS. So far, the relevance of nNOS expression in spontaneous plaque formation has not been addressed. To study the relative contribution of nNOS to lesion formation, we combined a genetic model of chronic nNOS deficiency (nNOS KO) with a mouse model of atherosclerosis, the hypercholesterolemic apolipoprotein E (apoE) KO mouse. ApoE KO mice develop progressive endothelial dysfunction which is more pronounced in “western” diet-fed animals. As previously published, deletion of eNOS leads to a dramatic increase in lesion formation in apoE KO atherosclerosis. Whereas eNOS deletion gave rise to an array of vascular complications including abdominal aneurysms, aortic dissections, and ischemic heart disease, deletion of the inducible NOS (iNOS) decreased atherosclerosis and plasma levels of lipid peroxides in apoE KO animals. The latter suggests that iNOS-derived NO formation is proatherogenic, partly through increasing oxidative stress. The current study tests the hypothesis that nNOS expression in atherosclerotic lesions inhibits plaque progression. In this case, genetic deficiency of nNOS would accelerate atherosclerosis. Additionally, we studied gender dependence of lesion formation, the influence of nNOS deletion on blood pressure regulation, and the expression of nNOS splice variants in atherosclerotic plaques.
Methods

All procedures performed conformed with the policies of the University of Würzburg, the National Institutes of Health guidelines and an independent governmental committee for care and use of laboratory animals.

Mice

nNOS KO mice were generated by targeted deletion in the laboratory of Paul Huang.12 The mice carry a deletion of the flanking region of exon 2, ablating translational start of the full-length brain-spliced nNOSα. ApoE KO animals were purchased from the Jackson Laboratories (Bar Harbor, ME, USA). All mice were backcrossed for ten generations to the C57BL/6 strain. nNOS KO and apoE KO animals were crossed to generate double heterozygous mice. From the F2-mating, apoE KO animals heterozygous for nNOS, were selected and the offspring were genotyped for nNOS by southern blotting and apoE using a polymerase chain reaction (PCR) protocol provided by the Jackson Laboratories.12 ApoE KO animals, wild-type or KO for nNOS, were weaned and started on a Western diet (42% of total calories from fat; 0.15% cholesterol; Harlan Teklad) at 6 weeks of age and for a period of 14 or 24 weeks.

Lesion Assessment

The aorta was dissected and analyzed as previously described.10,11 Animals were anesthetized with pentobarbital (80 µg/kg intraperitoneally) and perfused with 0.9% NaCl and dissected from the aortic valve to the iliac bifurcation. Adventitial tissue was removed and the aorta was opened longitudinally and pinned onto a black wax surface using micro needles (Fine Science Tools). Serial images of the submerged vessels were captured with a black and white video camera (COHU) mounted on a stereomicroscope (Leica). Lipid-rich intraluminal lesions were stained with Sudan IV. Sections were taken from the aortic valve and aortic arch and fixed in acetone before staining. Sections were stained with hematoxylin/eosin (H&E), Masson’s trichrome and Picric acid. Immunostaining was performed using primary antibodies (BD Biosciences) and antigen retrieval (citrate buffer 7.4) and incubated overnight at 4°C for the following antibodies: anti-smooth muscle actin (MOMA-2; Chemicon International); A polyclonal anti-nNOS antibody (1:200 dilution; Chemicon International); anti-HPRT (1:200 dilution; BioRad); anti-collagen IV (1:200 dilution; Chemicon International). All sections were counterstained with hematoxylin.

Histomorphometry

Photomicrographs of the aortic valve and aortic arch were taken with a Leitz-Camera mounted on a light microscope (Leica). All images were analyzed at 100-fold magnification. Areas of positive staining for smooth muscle cells (SMCs), macrophages (n=3 apoE/nNOS DKO and 3 apoE KO mice, respectively), and collagen (Picric acid staining, n=4 apoE/nNOS DKO and apoE KO mice) were measured in multiple plaques per animal and results were expressed as percent positive staining plaque area.

Reverse-Transcription PCR

First-strand cDNA was synthesized from 2 µg of total aortic RNA using random primers (Fermentas). PCR amplification was performed for 35 cycles at primer annealing temperature of 56°C. A 240-bp fragment of DNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a control (sense primer: 5′-TGA TGA CAT CAA GAA GGG GGA A-3′; antisense primer: 5′-TCC TTG GAG GCC ATG TGG GC CAT-3′). Published primers for splice variants were used as indicated or designed according to known cDNA sequences of nNOS variants.13 A 553-bp nNOSα fragment was amplified using sense: 5′-ATT AGG ATC CCT TTA CAT CAC ACC TGG AGA CCA C-3′ and antisense: 5′-ATT ATT CCA AGA CTG TCT GGT CCC CAG CCA CCT TGC CTT-3′. A 387-bp nNOSβ fragment was amplified using sense: 5′-GTG AGC AGC GTG ATC ATC CAC AGC-3′ and antisense: 5′-GAT ATC TTC TGA CTT CCG TAT GTG TC-3′. A 159-bp nNOSγ fragment was amplified with sense: 5′-CCC CAG CCA CCT TGC CTT-3′ and antisense: 5′-GAC TGT TCG TTC TCT GAA TAC GG-3′ primers. A 527-bp nNOS-µ was amplified with sense: 5′-GTC TTC CAC CAG GAG ATG-3′ and antisense: 5′-ACC AGA CTG TGG GCT TCA GA-3′. PCR fragments were analyzed by agarose gel electrophoresis.

Quantification of nNOS mRNA

mRNA expression of nNOSα and nNOSγ variants was quantified by real-time PCR (iCycler; Bio-Rad Laboratories). PCR amplification was performed for 40 cycles at primer annealing of 60°C. nNOS expression was normalized to HPRT signal. nNOS-α sense: 5′-CCC ACC AAA GCT GTC GAT CT-3′ and antisense: 5′-GGAG GTG TGG TCT TCG TAT TT. nNOS-γ sense: 5′-CAC CTC GTC TTC CAG AGA CCT-3′ and antisense: 5′-GAC TGT TCG TTC TCT GAA TAC GG-3′. HPRT sense: 5′-GTG GGA TAC AGG CCA GAC TTT GT and antisense: 5′-CCA CAG GAC TAG AAC ACC.

Figure 1. Sudan IV-stained, longitudinally opened aortas from female apoE KO (KO) and apoE/nNOS DKO (DKO) animals fed a “western” diet for 24 weeks. Luminal side facing up, displaying lipid rich (red) atherosclerotic lesions.
Fluorogenic probes for nNOS-α: 5′-6FAM-CAC ACC ATT AGC CTG GGA GAC TGA GCC-DB; nNOS-γ: 5′-6FAM-CCC CAC AGA TCA TTG AAG ACT: CGA TCA T-TMR; HPRT: 5′-6FAM-CTC GTA TTT GCA GAT TCA ACT TGC GC (TIB Molbiol, Germany) were used.

Western Blot Analysis

Aortas were dissected and samples were snap-frozen in LN₂. Western blots were performed using a polyclonal anti-eNOS antibody (1:200; BD 30020; Becton Dickinson) and anti-iNOS antibody (1:200; sc-650; Santa Cruz). To confirm equal loading of protein, an anti-actin antibody (sc-8432; Santa Cruz Biotechnologies) was used.

Blood Pressure Measurements

Mice were intubated and ventilated with a rodent respirator (stroke volume 250 μL, rate 90 minutes⁻¹). The common carotid artery was cannulated and a Millar catheter (1.4 F) was advanced into the left ventricle. Blood pressure was recorded under light isoflurane anesthesia.

Lipids and Lipoprotein Characterization

Lipoprotein cholesterol distribution of plasma samples was evaluated after fractionation by fast protein liquid (FPLC) gel filtration. Plasma (200 μL) was fractionated on 2 serial superose-6 columns using an Äkta-FPLC (Amersham Pharmacia Biotech) system. Total cholesterol of plasma samples and fast protein liquid chromatography fractions were measured using Sigma kit 352 and a Spectra MAX 250 photometer (Molecular Devices).

Statistical Analysis

Two-way ANOVA was used for repeated measures, followed by Scheffe F test (Stat View 4.51; Abacus Concepts Inc, Berkley, Calif). *P*≤0.05 was considered significant. Student t test was used for unpaired data. Survival of diet-fed animals was expressed by the Kaplan Meier method and survival curves were compared by the log-rank test (Prism 3; GraphPad Software Inc, San Diego, Calif).

Results

Inspection of the “en face” dissected aortas revealed that apoE KO and apoE/nNOS DKO animals develop atherosclerotic lesions in areas of disrupted laminar flow, namely in areas of branching off vessels and curvature (Figure 1). Atherosclerotic lesions of “western” diet-fed apoE KO animals stained nNOS-positive by immunohistochemistry, documenting that nNOS is present in lesions after 14 weeks of diet (Figure 2). However, plaques from apoE/nNOS DKO animals revealed residual nNOS staining (Figure 2). Reverse-transcription PCR from total aortic RNA documented the presence of nNOSα variant in C57BL6 and apoE KO vessels and its absence in apoE/nNOS DKO mice (Figure 3). In contrast, the nNOSγ variant was present in vessels from C57BL6, apoE KO, and apoE/nNOS DKO mice, whereas the nNOSβ and nNOSμ variant were not detectable in either group. Real-time PCR revealed low but equal expression of nNOSα in C57BL6 and apoE KO mice and nNOSγ in vessels from all 3 genotypes (Figure 3). After 14 weeks of “western” diet, lesion area in male apoE/nNOS DKO animals (15.56±1.14%, n=10) showed a 66% increase com-

Figure 2. Immunohistochemical staining for nNOS (left, upper row) of an aortic root lesion from an apoE KO animal. Second antibody alone, revealing no background staining (right, upper row). nNOS staining of the common carotid artery of apoE KO (left, lower row) compared with apoE/nNOS DKO mice (right, lower row) showing residual nNOS immunoreactivity in DKO animals.

Figure 3. RT-PCR for nNOS splice variants showing expected products for nNOSα, β, γ, and μ in control RNA. apoE KO aortas express nNOSα and nNOSγ variants, whereas nNOSβ and nNOSμ are absent. In apoE/nNOS DKO aortas only nNOSγ is expressed. Real-time PCR reveals equal nNOSα expression in C57BL6 and apoE KO mice (upper box blot) and of nNOSγ in C57BL6, apoE KO, and apoE/nNOS DKO mice (lower box blot).
pared with lesions in apoE KO control animals (9.35±1.32%, n=9; P=0.002; Figure 4a). Lesion area in female apoE/nNOS DKO (n=42) animals showed a significant 31% increase in lesion area (apoE/nNOS DKO: 31.8±2.3%, n=9; apoE KO: 24.26±1.1%, n=13, P=0.033) (Figure 4b), whereas the increase in lesion area in male animals did not reach statistical significance (apoE/ nNOS DKO: 31.64±3.5%, n=11; apoE KO: 24.89±3.5%, n=8, P=0.63). Kaplan Meier analysis revealed a pronounced increase in mortality of DKO animals by log rank test comparison of survival curves (apoE KO: n=31, apoE/nNOS DKO: n=42; P=0.0003). The increase in mortality in apoE/nNOS DKO mice affected male and female animals and was only present after 24 weeks of “western” diet (Figure 4c).

At both time points, total aortic area and total body weight did not differ between animals of either genotype. In addition, total plasma cholesterol (Table) and FPLC analysis of lipoprotein profiles (data not shown) did not differ among study groups.

Interestingly, mean arterial blood pressure of female apoE/ nNOS DKO animals was significantly reduced compared with control apoE KO animals (apoE/nNOS DKO: 84.1±2.73 mm Hg, n=9; apoE KO: 96.8±2.3 mm Hg, n=10, P=0.013), whereas blood pressure in male animals of both genotypes did not differ (Table). To rule out that nNOS deletion modulated expression levels of iNOS or eNOS, Western blot analysis was performed. eNOS protein was present in both genotypes at comparable levels. The iNOS protein level in female mice was higher compared with male animals, but no difference was observed between the 2 genotypes (Figure I, available online at http://atvb.ahajournals.org).

To check for differences in plaque composition, serial cryosections were taken from the aortic valve area and aortic arch and used for histochemistry and immunohistochemistry. All groups of animals studied developed complex lesions with a variable necrotic core and content of fibrous tissue (Figure IIa and IIb, available online at http://atvb.ahajournals.org). No statistical difference of picric acid positive areas (Figure IIa and IIb). Staining for vascular SMCs revealed a high degree of variability within each animal, regardless of the experimental groups. However, the strongest αSMC-actin staining was seen in apoE/nNOS DKO animals (apoE/nNOS DKO: 11.4% ±3.1 SE, n=18 plaques; apoE KO: 6.16% ±0.95 SE, n=14 plaques, P=0.16, Figure IIb and IIc).

**Discussion**

Nitric oxide has potential atheroprotective effects because it inhibits smooth muscle cell proliferation, leukocyte endothelial interactions, leukocyte adhesion and aggregation of platelets, and endothelial exocytosis of granules that mediate vascular inflammation and thrombosis.15–19 However, NO has proathero- genetic properties, because it reacts with superoxide to form peroxinitrite, which can further react to an array of oxygen and nitrogen radicals.20 Moreover, each NOS isoform is capable of generating superoxide under conditions of substrate and cofactor deficiency.21–24 To dissect the role of each isoform in lesion formation, we generated apoE/NOS DKO mice to test the relevance of the absence of each isoform on lesion development.
Our previously published results show that eNOS is atheroprotective, and apoE/eNOS DKO animals developed a dramatic increase in lesion formation. In contrast, genetic deficiency of iNOS decreases atherosclerosis and lowers plasma lipid peroxides, a marker of oxidative stress, in apoE KO animals.

In the present study we demonstrate that genetic deficiency of nNOS accelerates atherosclerotic lesion progression. The location of lesions was similar in apoE KO control and apoE/nNOS DKO animals. Western blot analysis revealed higher iNOS expression in female compared with male animals. However, iNOS expression levels in the female and the male group were similar and do not explain the differences in lesion formation within these gender groups. eNOS expression levels were equal in all experimental groups.

Lesion progression was significantly accelerated in male apoE/nNOS DKO compared with male apoE KO animals after 14 weeks of western-type diet. In female apoE/nNOS DKO mice, there was a significant increase in plaque area after 24 weeks of western diet. After 24 weeks of diet, however, the increase in lesion area in male apoE/nNOS DKO animals did not reach statistical significance, which may be explained by a higher mortality in apoE/nNOS DKO mice at this time point (Figure IIc).

The slower increase in lesion area in female apoE/nNOS DKO compared with male apoE KO animals was associated with a reduction in mean arterial blood pressure in female DKO mice. Because blood pressure elevation is an independent risk factor for atherosclerosis, the reduced blood pressure in female DKO animals renders a possible explanation for the delayed lesion progression in this group.

The reduction in blood pressure in female animals is not likely to occur at the level of the vessel wall because vascular nNOS would likely cause vasodilation and thus nNOS deletion would be expected to raise the blood pressure. Instead, nNOS deletion could affect blood pressure regulation in the central nervous system, at the level of the baroreceptors, or through endocrine mechanisms. In this respect, NO, regardless of its source, is known to be involved in the regulation of renin secretion. Whereas acute inhibition of nNOS does not seem to affect blood pressure, chronic pharmacological inhibition of nNOS using 7-nitroindazole (7-NI) significantly increased blood pressure in one study. In contrast, acute and chronic nNOS inhibition in eNOS KO animals caused a decrease in blood pressure, which may be caused by nNOS-dependent mechanisms at the level of the central nervous system or the baroreceptor pathway. And although nNOS KO animals were reported to have a tendency toward hypotension, gender differences in nNOS-dependent blood pressure regulation have not been reported so far.

To evaluate potential differences in plaque composition, we stained lesions by immunohistochemistry and histochemistry. Animals of both genotypes developed complex lesions with fibrous caps and necrotic cores. Both genotypes showed strong immunoreactivity for macrophages without an obvious difference between groups. Lesions with the strongest alpha smooth muscle actin staining were seen in apoE/nNOS DKO animals (Figure IIA and IIb). In the past, NO has been shown to suppress smooth muscle cell proliferation. Recent data indicated that angiotensin can induce nNOS expression in vascular smooth muscle cells and that this nNOS expression is sufficient to suppress SMC proliferation in vitro. However, neointimal alpha smooth muscle actin staining in our study was highly variable between animals and even among lesions within each mouse (Figure IIc), preventing statistical significance of our observation. The occurrence of plaques in apoE/nNOS DKO mice that lack any SMC staining suggests that the absence of nNOS does not by itself trigger SMC proliferation. Instead, the occurrence of pronounced SMC proliferation in single plaques of apoE/nNOS DKO mice let us speculate that nNOS serves to counterbalance local and systemic factors that would foster SMC proliferation.

It is important to note that nNOSα (exon 2) KO animals used in this study express nNOSβ and nNOSγ mRNA splice variants in brain tissue by using alternate translation start sites. Therefore, we investigated whether nNOS splice variants are present in vessels of C57BL6, apoE KO, and apoE/nNOS DKO animals. We find residual nNOS immunoreactivity in the neointima of apoE/nNOS DKO mice and similarly low expression levels of the nNOSy splice variant in aortas of C57BL6, apoE KO, and apoE/nNOS DKO animals. The N-terminal truncated nNOSγ variant lacks the PDZ domain and is localized to the cytosolic fraction. In vitro assays indicate that nNOSγ has low catalytic activity (3% of nNOSα), whereas nNOSβ possesses activity comparable to nNOSα. However, nNOSβ and nNOSμ variants were not detectable in mouse aortas in our study. Our study differs from a study by Schwartz et al who reported the presence of small amounts of nNOSμ in the media and adventitia of intact rat aorta and the study by Morishita et al, who reported absence of nNOS expression in carotid arteries of untreated C57BL6 mice. These latter differences may be explained by the use of different species, the study of the arteria carotis versus the aorta and the use of injury models as opposed to a model of spontaneous atherosclerosis. The nNOS KO animals used in this study are not expected to have membrane targeted nNOS expression caused by deletion of the PDZ targeting motif contained in exon 2. Interestingly, splice variants that lack exon 2 are reported to occur spontaneously in mice, rats, and humans. Our data highlight nNOSα overexpression as a potential therapeutic strategy. Moreover, the prevalence of splice...
variants that lack exon 2 of the nNOS gene within each individual may be a risk factor for vascular disease.

In summary, we provide first evidence for the presence of nNOS splice variants in atherosclerotic vessels and show that genetic deficiency of nNOSε increases atherosclerosis development in apoE KO animals. Therefore, our study indicates that nNOSα protects from atherosclerosis. In addition, systemic blood pressure was reduced in female apoE/nNOS DKO mice and nNOS deletion led to a significant increase in mortality in DKO animals.

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Disclosure(s)
None.

References
13. Matsushita K, Morrell CN, Cambien B, Yang SX, Yamakuchi M, Bao C, Hara MR, Quick RA, Cao W, O’Rourke B, Lowenstein JM, Pevsner J, Wagner DD, Lowenstein CJ. Nitric oxide regulates eicosytosis by S-nitrosylation of N-ethylmaleimide-sensitive factor. Cell. 2003;115:139–150.
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Fig. I

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Female          Male

eNOS
iNOS
α-sm-actin
Fig. IIa
Fig. IIb

trichrome

macrophages

smooth muscle cells

500µm
Fig. IIc
Figure I. (please see www.ahajournals.org) Western blots of total aortic lysates from male and female “western-type” diet fed apoE KO and apoE/nNOS DKO animals. eNOS protein is present at equal levels in animals of both genotypes and genders (140 kDa). iNOS-immunoreactivity is more pronounced in female than male animals without a difference between genotypes. Equal loading was confirmed by immunoblotting an anti-actin antibody with the lower part of the same membrane (43 kDa). Results were confirmed in duplicate experiments.

Figure II. Aortic arch sections of “western-type” diet fed apoE KO (6a) and apoE/nNOS DKO (6b) animals. Topographic view upper left. Masson’s trichrome staining (upper right). Immunohistochemical staining for macrophages (lower left). Smooth muscle actin (lower right) staining revealed a high variability of SMC positive plaques when comparing lesions within one animal or between different mice. Pronounced SMC positive plaques were found in DKO animals but variability prevented statistical significance (6c). Magnifications 6a and 6b: Upper left x 12.5, remaining sections x 50.