Research Paper

Journal of Vascular Research

J Vasc Res 2003;40:87–96
DOI: 10.1159/000070705

Received: May 3, 2002
Accepted after revision: January 15, 2003

Persistence of the Nitric Oxide Pathway in the Aorta of Hypercholesterolemic Apolipoprotein-E-Deficient Mice

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Key Words
Nitric oxide · Nitric oxide synthase · Atherosclerosis · Mice · Endothelial function

Abstract
The markers of the bioavailability of NO (endothelium-dependent relaxation to acetylcholine and cyclic GMP content) in the thoracic aorta of apolipoprotein-E-deficient (ApoE KO) mice, 20 weeks old with enriched cholesterol diet or 35 weeks old on regular chow, are not decreased, in contrast with other models of atherosclerosis. However, severe hypercholesterolemia, the presence of atherosclerotic lesions and increased NADPH oxidase activity have been reported as early as at 20 weeks of age. The present experiments were designed to test if an increased capacity of NO production in these mice explains this paradox. The expressions of the 3 isoforms of NO synthase (NOS) were compared in ApoE KO and C57Bl/6J mice using Western blot and localized by immunohistochemistry. No adaptive increase in the expression of NOS was detected in ApoE KO mice. NO bioavailability could also be preserved by upregulation of enzymes involved in redox status such as CuZn or Mn superoxide dismutase and catalase. However, these enzymes were less expressed in ApoE KO mice than in control mice. These results highlight that ApoE KO mice represent an atypical model of atherosclerosis.

Apolipoprotein-E-deficient (ApoE KO) mice are considered to be one of the most relevant models for atherosclerosis since they are hypercholesterolemic and develop spontaneous arterial lesions [1]. In many experimental models, as well as in humans, atherosclerosis is associated with a reduced bioavailability of NO [2]. In ApoE KO mice, endothelium-dependent relaxations are normal in early stages of the pathology [3]. Impairment is described at later stages (from 35 weeks), but only when mice are fed a western-type cholesterol-rich diet [4–7]. As in other models of hypercholesterolemia, an increase in the production of reactive oxygen species has been described as early as at 20 weeks of age [6, 8]. This excessive production of superoxide anion in the vascular wall should reduce the bioavailability of NO by inactivation but also by uncoupling of NO synthase (NOS) by peroxynitrite [6]. The coexistence of disturbances of redox homeostasis

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1018–1172/03/0402–0087$19.50/0

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with a normal endothelial function remains unexplained. An increase in the production of NO or an upregulation of enzymes involved in the control of the redox status could explain this paradox. The level of the constitutive endothelial NOS (eNOS) protein is either paradoxically increased in the aged ApoE KO aorta [6] or unchanged despite a reduction in acetylcholine-induced relaxation [4]. The present experiments were designed to study the expression of eNOS in ApoE KO mouse aorta with a normal bioavailability of NO but an increased degradation of the mediator. The expression of the inducible (iNOS) or neuronal form (nNOS) of NOS was also verified because they can provide NO under pathological conditions as in hypercholesterolemic rabbits [9, 10] and hypertensive rats [11]. The possibility that NO could be indirectly preserved by a limited production of superoxide anions was investigated by evaluating the expression of both superoxide dismutase (SOD) and catalase. Because both genetic factors and diet may play a role in the development of vascular disease, ApoE KO mice at 20 weeks fed with a hypercholesterolemic diet and 35-week-old animals fed normal chow which exhibit comparable aortic lesions were compared.

Methods

Animal Protocol and Diet

Male homozygous ApoE-deficient (Transgenic Alliance, L'Arbresle, France) and C57BL/6J mice (Iffa-Credo, L'Arbresle, France) were obtained at 8 weeks of age. After 1 week of acclimatization the control mice received regular mouse chow and the ApoE KO mice were fed either regular mouse chow or a western-type diet containing 0.15% cholesterol (Usine d'Alimentation Rationnelle (UAR), Epinay sur Orge, France). Some of the ApoE KO mice were treated chronically with the NOS inhibitor N^{ω}-nitro-*L*-arginine (0.1 g/l in the drinking water during 25 weeks). Mice were sacrificed either at 20 or 35 weeks of age. They were anesthetized with sodium pentobarbital (0.5 mg/kg i.p), and blood samples were collected by puncture of the jugular artery. All experimental protocols were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care.

Measurement of Blood Pressure

Before sacrifice, mice from groups treated with N^{ω}-nitro-*L*-arginine or its vehicle (water) were anesthetized with pentobarbital (6 mg/kg i.p.), and a catheter was implanted in the right carotid artery for the measurement of mean arterial pressure using a P23XL strain gauge transducer (Gould Inc., Ballainvilliers, France).

Measurement of Serum Cholesterol and Urinary Nitrite

Total cholesterol concentrations were determined enzymatically using a Cobas Mira autoanalyzer (Roche Diagnostics).

Nitrite was measured in urine, collected over 18 h, with the Griess colorimetric method (Cayman Chemical Company) and the creatinine level by using the Cobas Mira autoanalyzer.

Aortic Lesions

The aortic arch and the thoracic aorta were isolated and carefully dissected from periadventitial tissue and fat. The atherosclerotic lesions were visible and clearly distinguishable on the luminal surface of the vessels without staining. The plaque area was quantified by analyzing the luminal surface image of the aortic arch with a computerized Biocom morphometry system and expressed as a percentage of the total aortic arch area.

Vascular Reactivity

Thoracic aortas were quickly immersed in Krebs' solution containing (in m*M*): NaCl, 120.3; KCl, 4.8; $CaCl_2$, 2.5; KH_2PO_4, 1.2; $MgSO_4$, 1.3; $NaHCO_3$, 24.2; glucose, 11.1; EDTA 0.016; bubbled with a mixture of 95% O_2 and 5% CO_2 at pH 7.4. Four aortic rings were prepared from each aorta. Changes in isometric tension were recorded with an IT1-25 transducer (EMKA Technologies, France) in conventional 20-ml organ bath chambers.

Relaxations. Cumulative concentration-response curves to acetylcholine (10^{-8} to 10^{-5} *M*) in the presence of indomethacin (10^{-5} *M*) were obtained in rings contracted with phenylephrine at a concentration ($5 \cdot 10^{-7}$ *M*) inducing approximately 60–70% of the maximal contraction.

Basal Production of NO. Cumulative concentration-response curves to phenylephrine (10^{-8} to 10^{-5} *M*) were obtained in rings with or without endothelium in the absence or presence of N^{ω}-nitro-*L*-arginine (10^{-4} *M*), an inhibitor of the eNOS, or N-iminoethyl-*L*-lysine dihydrochloride (10^{-5} *M*), an inhibitor of the iNOS.

Cyclic GMP

After dissection, the thoracic aorta (including the arch) was frozen immediately in liquid nitrogen and stored at –80°C. Radioimmunoassay of cyclic GMP using an Amersham kit was performed in the presence of a nonspecific inhibitor of phosphodiesterases, 3-isobutyl-1-methylxanthine (10^{-4} *M*), on acetylated samples to increase the sensitivity of the assay. Data were expressed as picomoles per milligram protein (measured by the method of Bradford [12]).

Immunohistochemistry

The following antibodies were used: anti-e,i,nNOS rabbit polyclonal antibodies, (dilution 1/200) and isotype-matched rabbit IgG antibody (dilution 1/400) as control antibody, all from Santa Cruz Biotechnology. For cell identification, serial sections were stained with the specific macrophage marker MOMA-2 (dilution 1/50) and isotype-matched rat IgG2b (dilution 1/50) from Becton Dickinson Co.

Before harvested, aortas were perfused in situ with physiological serum via the left ventricle. Aortic arch and thoracic aorta were embedded in OCT compound and snap-frozen in liquid nitrogen. Tissue sections (7 μm) were fixed, and nonspecific immunoglobulin-binding sites were blocked. After incubation with primary antibody, the presence of antigen was revealed with a biotinylated goat antirabbit IgG for NOS and biotinylated antirat IgG2b for MOMA-2. This was followed by incubation with streptavidin-peroxidase complex and aminoethyl carbazole substrate (Zymed). Slides were counterstained with hematoxylin.

Peroxynitrite production was assessed by staining the stable end product of its interaction with cellular tyrosine residues, 3-nitrotyrosine (3-NT), using a polyclonal antibody from Upstate Biotechnology. Tissue sections were deparaffinized, incubated with blocking agent (goat serum) for 30 min and with the antinitrotyrosine anti-

Table 1. Comparison of the cholesterolemia, urinary nitrite, aortic lesions, aortic basal cGMP content and acetylcholine (ACh) relaxation of 20-week-old ApoE KO mice fed western diet (WD) and 35-week-old ApoE KO mice fed normal chow (NC) versus control mice

	20-week-old mice		35-week-old mice	
	ApoE KO WD (n = 10)	control NC (n = 10)	ApoE KO NC (n = 10)	control NC (n = 9)
Cholesterolemia, mmol/l	22.5 ± 1.7*	1.54 ± 0.06	19.8 ± 1.4*	2.0 ± 0.01
Urinary nitrite, μ*M*/mmol × l^{-1} creatinine	399.7 ± 71.2	399.3 ± 71.6	443.7 ± 43.8	424.5 ± 18.9
Aortic arch lesions, % lesion area/total aortic arch area	33.0 ± 2.5*	–	44.3 ± 3.2*	–
Aortic cGMP, pmol/mg protein	4.13 ± 1.00	3.62 ± 0.45	3.95 ± 0.55	3.59 ± 0.94
ACh relaxation, % maximal relaxation	87.7 ± 2.4	85.3 ± 4.7	89.9 ± 3.8	87.3 ± 4.9
IC_{50} ACh, *M*	1.7×10^{-7}	1.5×10^{-7}	1.4×10^{-7}	1.9×10^{-7}

Data are represented as means ± SEM for the number of mouse aortas indicated in parentheses. * $p < 0.05$ versus intact rings of the same group.

body (1/600) overnight at 4°C. The presence of the antibody was detected with a Vectastain ABC kit followed by Vector red (Vector Laboratories). The specificity of the staining was confirmed by replacing the primary antibody with a nonimmune rabbit IgG or by incubating the antinitrotyrosine antibody in the presence of 10 m*M* nitrotyrosine.

Western Blotting

Frozen aortas were homogenized in ice-cold phosphate buffer saline containing a protease inhibitor cocktail (Roche Diagnostics) and centrifuged at 12,000 rpm for 15 min. The protein concentration was quantified by the method of Bradford [12]. Fifty micrograms of samples were run out in 5% (NOS), 7.5% (catalase) or 15% (SOD) SDS-PAGE under denaturing conditions, subsequently electrotransferred to nitrocellulose membranes (Amersham Pharmacia Biotech) and blocked with 10% skimmed milk in 0.1% PBS-Tween 20 for 60 min at room temperature. The membranes were then incubated overnight at 4°C in 3% milk with the specific primary antibodies. Rabbit anti-eNOS polyclonal IgG (1/250), rabbit anti-iNOS polyclonal IgG (1/200) and rabbit anti-nNOS polyclonal IgG (1/200) were purchased from Santa Cruz Technology. Sheep anti-CuZn-SOD (1/400), sheep anti-Mn-SOD IgG (1/100) and rabbit anticatalase IgG (1/500) were obtained from Calbiochem. The antigen-antibody complexes were revealed using the appropriate secondary antibody peroxidase and the enhanced chemiluminescence detection system (Amersham Pharmacia Biotech). For actin, blots were rehybridized with goat polyclonal antiactin IgG (Santa Cruz).

Densitometric analyses were carried out using NIH Image.

Statistical Analysis

Numerical data were expressed as means ± SEM. Statistical comparisons were performed with an unpaired two-tailed Student's t test. Statistical significance was accepted at the 0.05 level of probability.

Results

Cholesterolemia, Aortic Lesions and Endothelial Function

Cholesterolemia, aortic lesions and endothelial function were evaluated in C57Bl/6J versus ApoE KO mice at 20 weeks with a hypercholesterolemic diet or 35 weeks with regular chow. Atherosclerotic lesions developed mainly in the aortic arch. Their size increased with age. Relaxation to acetylcholine in the presence of indomethacin remained normal at 20 and 35 weeks with or without the hypercholesterolemic diet, respectively. The aortic content in cyclic GMP and the urinary nitrite did not differ significantly in ApoE KO versus control mice at 20 or 35 weeks of age (table 1). In the same way, similar phenylephrine-induced contractions were obtained in both strains, and the increase in tension due to the removal of NO (removal of the endothelium or treatment with N^{ω}-nitro-*L*-arginine; table 2) was identical in aortas from the 4 groups.

Chronic Inhibition of NOS

Chronic treatment with N^{ω}-nitro-*L*-arginine began at 10 weeks of age and corresponded to 23.1 ± 4.5 mg/kg/day at the end of the 25-week treatment period. Mice receiving this treatment showed a 60% drop in aortic content of cyclic GMP and a 63% decrease in urinary nitrite. The treatment significantly increased the mean arterial pressure by 14.9 ± 9.0% (from 90.6 ± 4.1 to 104.1 ± 4.9 mm Hg). Cholesterolemia was not affected by treat-

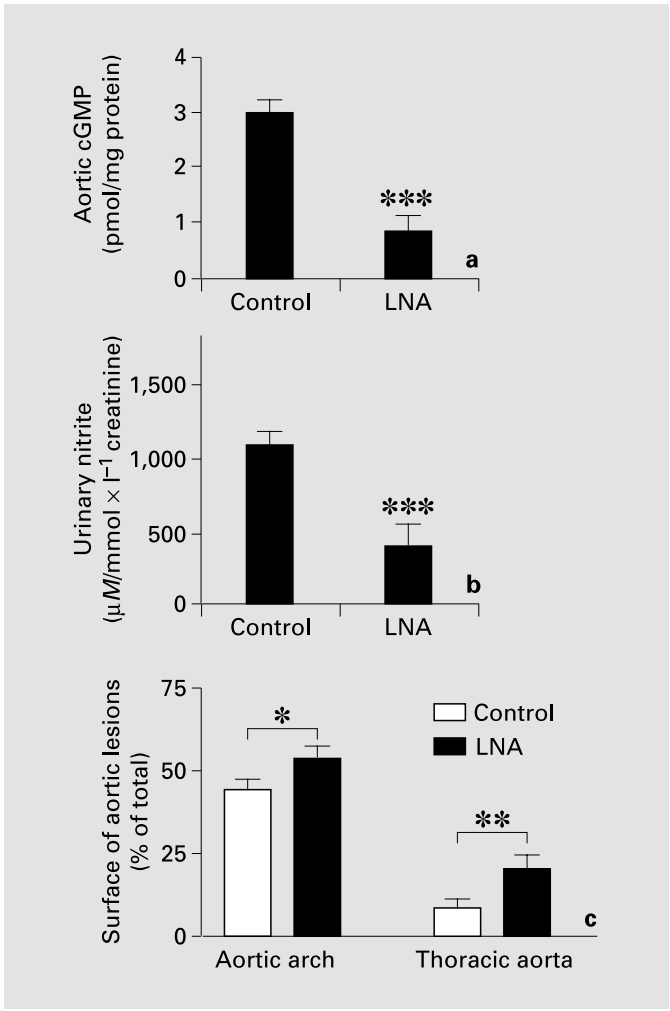

Fig. 1. Effects of a chronic treatment with N$^{\omega}$-nitro-*L*-arginine (LNA, 0.1 g/l/day) on aortic basal cGMP content (**a**), urinary nitrite (**b**) and the surface of aortic lesions (**c**) of 35-week-old ApoE KO mice fed normal chow (n = 7). *p < 0.05, **p < 0.001, ***p < 0.005 versus vehicle.

Table 2. Maximal contraction to phenylephrine (in grams) of aortic rings from 35-week-old ApoE KO and control mice fed normal chow

	ApoE KO	C57Bl/6J
Intact rings	0.56 ± 0.08 (11)	0.59 ± 0.07 (13)
Intact rings + LNA (10^{-4} *M*)	0.94 ± 0.11 (9)*	0.80 ± 0.13 (8)*
Endothelium-denuded rings	0.88 ± 0.96 (13)*	0.84 ± 0.05 (11)*

Data are represented as means ± SEM for the number of aortic rings indicated in parentheses. Phenylephrine-induced contraction in the intact or endothelium-denuded aortic rings is expressed as gram of change in basal tension. * p < 0.05 versus intact rings of the same group. LNA = N$^{\omega}$-nitro-*L*-arginine.

ment with N$^{\omega}$-nitro-*L*-arginine (19.8 ± 1.4 vs. 19.5 ± 5.1 mmol/l). Extensive fatty streak formation and advanced plaques were observed in many regions of the arterial tree. With chronic inhibition of NOS, the total surface of lesions was significantly increased in both the aortic arch and the thoracic aorta (fig. 1).

Expression of NOS

The expression of NOS was compared in the different groups of mice using Western blot and the enzymes localized by immunohistochemistry. The staining for eNOS was intense and found exclusively in the endothelial cells (fig. 2a, b). The immunodetection signal was of comparable intensity in all groups of mice. When lesions were present in ApoE KO mice, the endothelial cells covering the lesion were also stained (fig. 2b). The staining for iNOS was negative in control mice at both 20 and 35 weeks. It was positive in 35-week-old ApoE KO mouse aorta, but only in advanced lesions (fig. 3a), together with the presence of macrophages as shown by the use of a MOMA-2 antibody (data not shown). Whether they had lesions or not, mice did not show any staining for nNOS. The negative control used for the 3 isoforms of the enzyme NOS yielded no staining.

Western blot analysis showed no significant difference in the expression of eNOS (133 kD) between ApoE KO and control mice irrespective of age (fig. 2c). The inducible form of NOS (130 kD) was detected only in 35-week-old ApoE KO mice (fig. 3b). nNOS was not detected.

The basal production of NO by iNOS was evaluated in aortic rings without endothelium. The specific inhibitor of this isoform, N-iminoethyl-*L*-lysine dihydrochloride (10 μ*M*), did not affect the phenylephrine-induced contraction (fig. 3c).

Immunostaining of 3-NT

Staining was never observed in control mice or 20-week-old hypercholesterolemic mice while preincubation with 10 m*M* of free 3-NT gave an intense staining (fig. 4c). By contrast, in 35-week-old ApoE KO mice, a diffuse staining was detected (fig. 4a, b). Specificity of the anti-3-NT antibody was confirmed by using a nonimmune rabbit IgG isotypic control that gave no staining in any group of mice (fig. 4d).

Expression of SOD and Catalase

Western blot analysis revealed that the CuZn and Mn forms of SOD were expressed less in aortas of 35-week-old ApoE KO than in control mice. Similar findings were obtained for catalase (fig. 5).

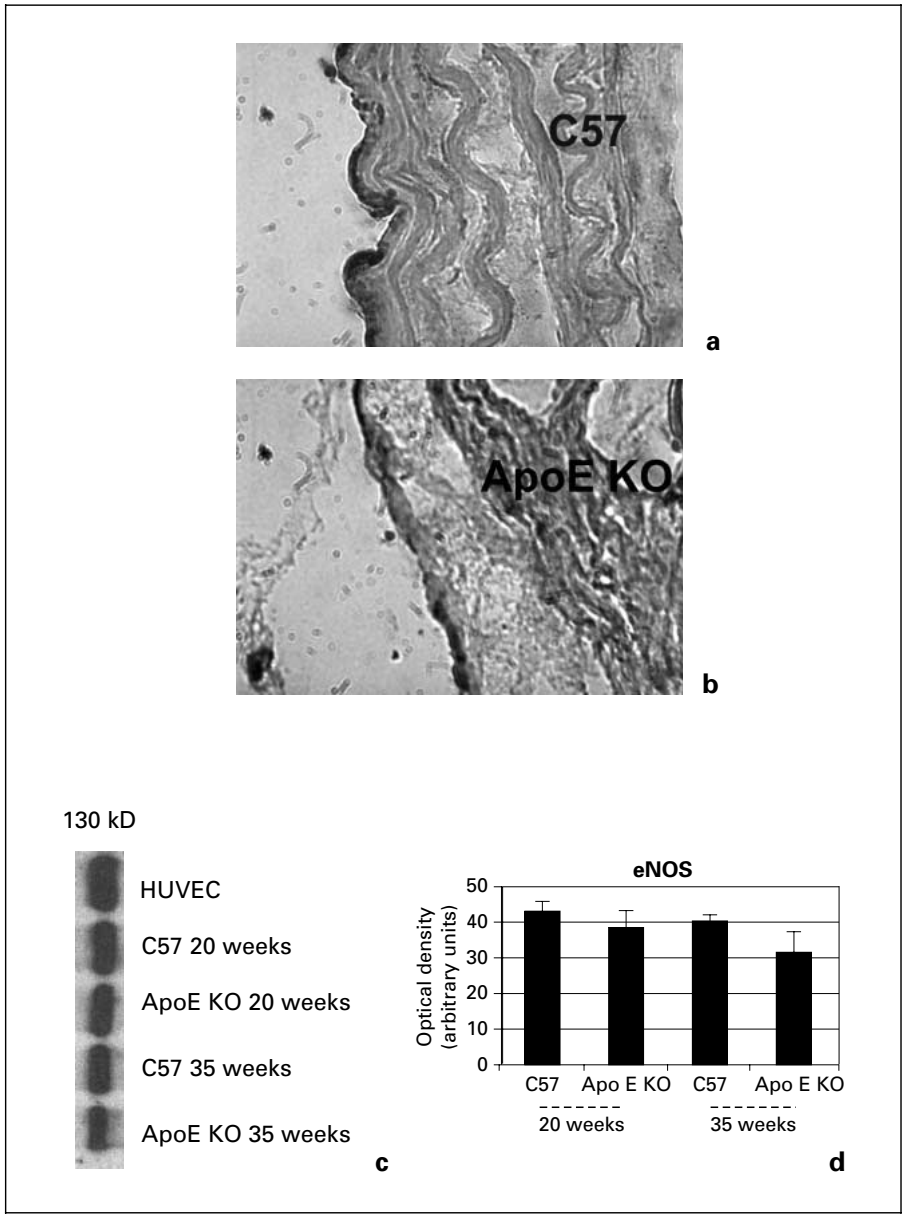

Fig. 2. Immunohistochemical staining for eNOS in frozen sections of thoracic aorta from control (C57; **a**) and 35-week-old ApoE KO mice (**b**) fed normal chow and representative Western blot showing eNOS expression in aortas from control (20 and 35 weeks) and 20- and 35-week-old ApoE KO mice fed a western diet and normal chow, respectively (**c**). Control staining was performed on human umbilical vein endothelial cells (HUVEC). Restaining with actin antibody ensured equal protein loading. The bar graph indicates results of a densitometric analysis (**d**). Results are means ± SEM. Experiments were repeated 6 times.

Discussion

Endothelial dysfunction is considered to be one of the early steps in atherosclerosis [13]. This study highlights that ApoE KO mice represent a particular model of atherogenesis because results are not consistent with this observation. Actually, at 20 weeks with a hypercholesterolemic diet or at 35 weeks on regular chow, no alteration in the markers of the production of NO was detected in the thoracic aorta: relaxation to acetylcholine, the content of cyclic GMP, N^{ω}-nitro-*L*-arginine-induced increase in phenylephrine contraction; urinary nitrite levels were normal. However, at that time, a severe hypercholesterolemia and the presence of lesions in the arterial wall were detected. As in other models of hypercholesterolemia [3, 6, 8], a modification of the vascular redox status was also observed [8], the result of an increased NADPH oxidase activity with a decreased expression of detoxifying enzymes such as SOD and catalase which should lead to an increased breakdown of NO by superoxide anion. An adaptive increase in the expression of constitutive eNOS does not explain the maintained bioavailability of NO.

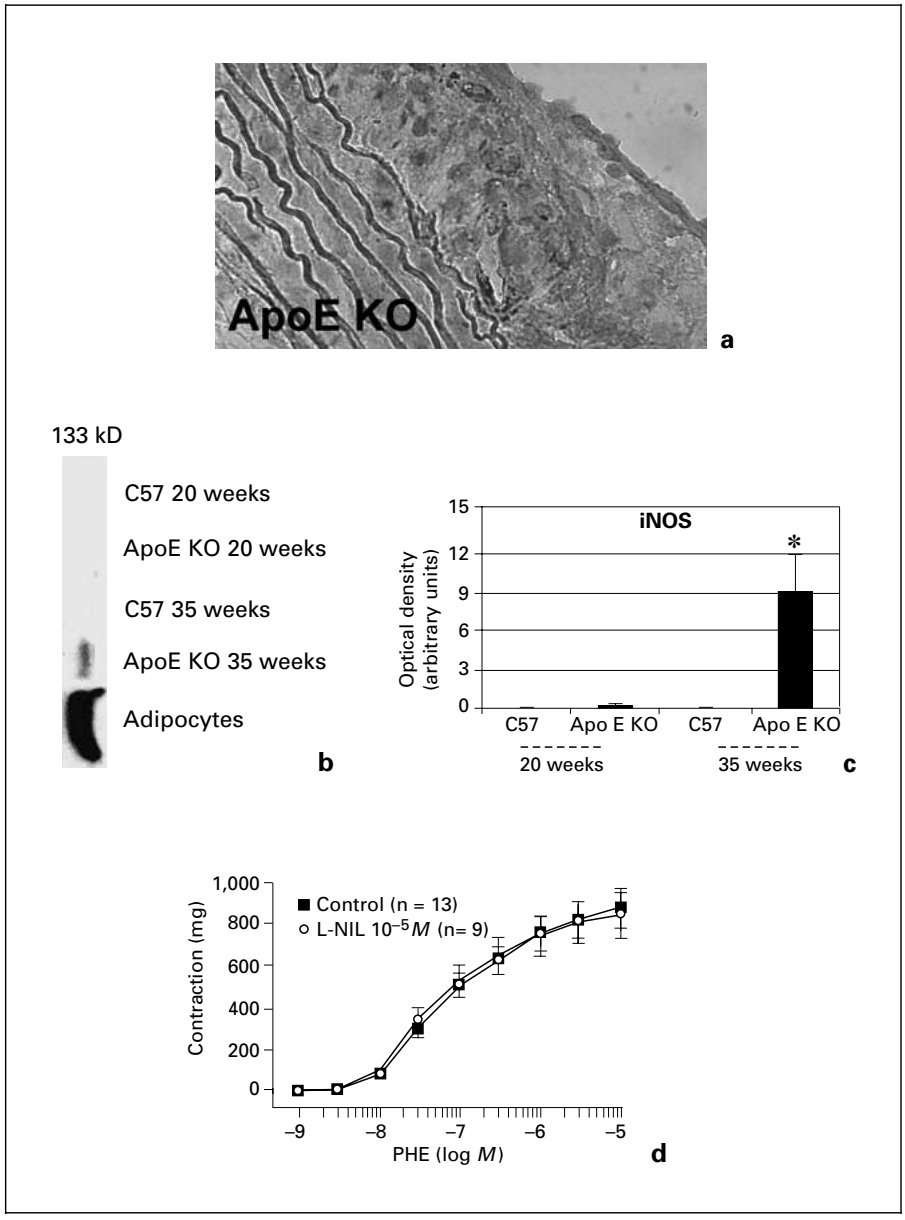

Fig. 3. Immunohistochemical staining for iNOS in frozen sections of thoracic aorta from 35-week-old ApoE KO mice (**a**) fed normal chow and representative Western blot showing iNOS expression in aortas from control (C57; 20 and 35 weeks) and 20- and 35-week-old ApoE KO mice fed a western diet and normal chow, respectively (**b**). Control staining was performed on adipocytes. Restaining with actin antibody ensured equal protein loading. The bar graph indicates results of a densitometric analysis (**c**). Results are means ± SEM. *$p < 0.001$ versus C57 mice. Experiments were repeated 4 times. **d** Effect of N-iminoethyl-*L*-lysine dihydrochloride (L-NIL) on the cumulative dose-response curve to phenylephrine (PHE) of 35-week-old ApoE KO mouse aorta without endothelium.

The absence of endothelial dysfunction in the ApoE KO mice, which generally occurs before vascular structural changes [14], is surprising. As the relaxation to acetylcholine can be due to different endothelium-dependent relaxing factors [15], the relative role of NO in this phenomenon was evaluated first. In control or ApoE KO mouse aortas, inhibitors of NOS abolished the relaxation to acetylcholine indicating that it is mediated by NO [16]. Moreover, a cyclooxygenase inhibitor was present during all the experiments in order to suppress the involvement of prostacyclin in the relaxation. One study reported that the basal but not the stimulated production of NO is decreased in 20-week-old ApoE KO mice [17], suggesting that separate pathways may regulate these processes. In the present experimental conditions, no decrease in basal NO production was evident at 20 or 35 weeks. This conclusion was based on measurements of cyclic GMP, a mediator of NO at the level of smooth muscle cells [15], evaluation of the effect of NO on the amplitude of contraction to phenylephrine and quantification of urinary nitrite, the stable metabolite of NO. An increase in the activity of soluble guanylate cyclase in the ApoE KO

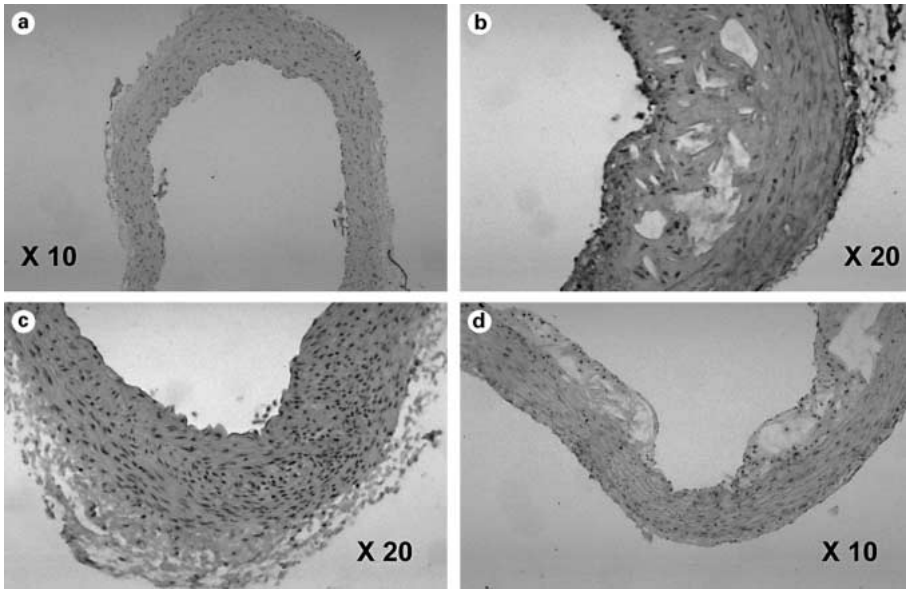

Fig. 4. Representative staining of 3-NT in fixed sections of thoracic aortas from 35-week-old control (**a**) and ApoE KO (**b**) mice fed normal chow. The specificity of anti-3-NT was confirmed by preincubation of control aorta with 10 m*M* of free 3-NT (**c**) or by using a nonimmune rabbit IgG isotypic control that gave no staining in any group of mice (**d**, ApoE KO mouse aorta). Experiments were repeated 3 times.

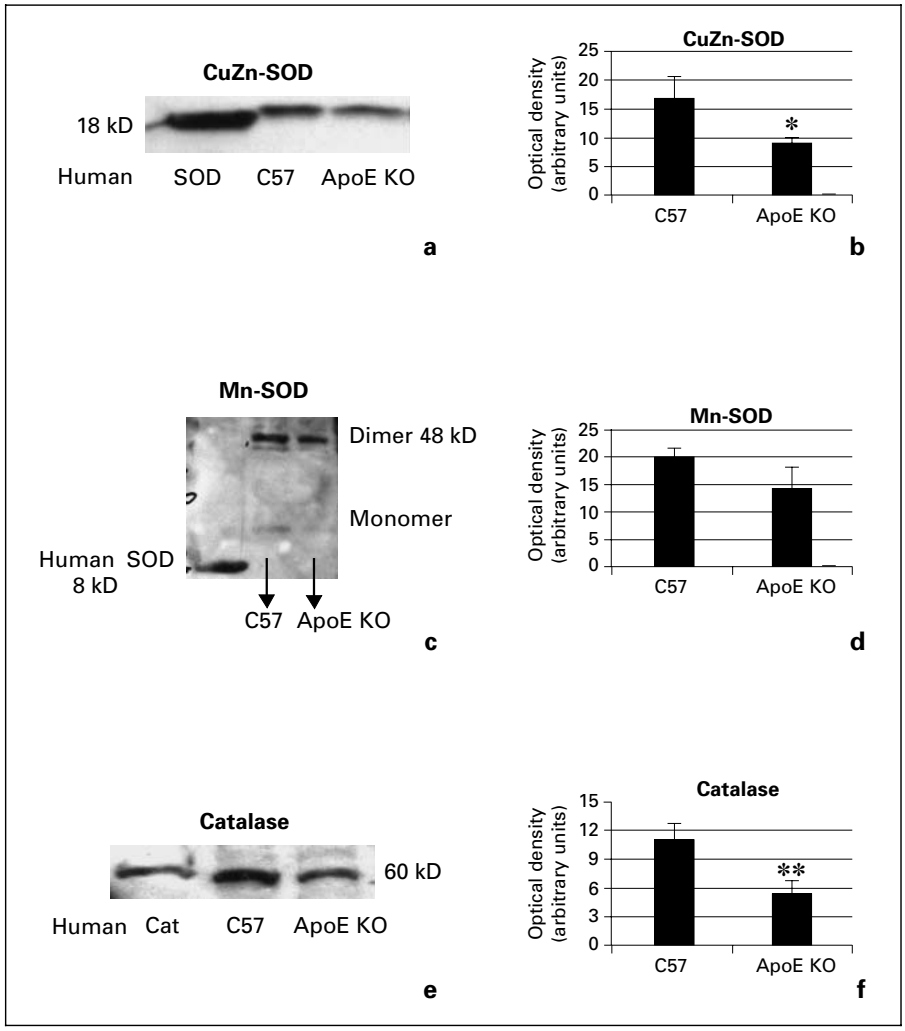

Fig. 5. Representative Western blot showing CuZn-SOD (**a**), Mn-SOD (**c**) and catalase (**e**) expression in aortas from 35-week-old control and ApoE KO mice fed normal chow. Control staining of human SOD and catalase was performed. Restaining with actin antibody ensured equal protein loading. The bar graphs indicate results of a densitometric analysis (**b**, **d**, **f**, respectively). Results are means ± SEM. * $p < 0.05$, ** $p < 0.001$ versus C57 mice. Experiments were repeated 6 times.

mouse aorta could also lead to similar relaxation despite a lesser production of NO, but relaxations to exogenous NO are maximal and comparable in the two strains [16]. Actually, in later stages of atherosclerosis, a reduced sensitivity to exogenous NO is observed rather than an increase [7, 18].

The normal bioavailability of NO probably inhibits several components of the atherogenic process [13]. Indeed, chronic treatment with a NOS inhibitor led to an increase in the development of atherosclerotic lesions, which is in line with findings in double ApoE KO/eNOS KO mice compared to ApoE KO mice [19].

A regional alteration in the relaxation to acetylcholine has been described, as well as the absence of correlation between lesions and dysfunction in ApoE KO mouse aorta [20]. This observation is in agreement with the present results, since the relaxation studies were performed in the thoracic part of the aorta, which is less susceptible than the abdominal aorta to the impairment of acetylcholine-induced relaxation.

The thoracic aorta of ApoE KO mice from 20 weeks exhibits an increased NADPH oxidase activity, leading to a greater production of superoxide anions [4, 7, 8] which may contribute to inactivation of locally produced NO [21]. A compensatory mechanism would then be expected in order to explain the persistence in bioavailability of NO. Thus, the normal endothelium-dependent response could be the result of an adaptive increase in the release of NO by eNOS, as reported in aged ApoE KO mouse aorta [4, 6], in atherosclerotic rabbit aorta [22, 23] or in endothelial cells after incubation with cholesterol [24], oxidized low-density lipoprotein or lysophosphatidylcholine [25]. However, in the present study, no modification in the expression or immunoreactivity of eNOS could be demonstrated in the hypercholesterolemic mouse aorta after 20 weeks of western-type diet or at 35 weeks with regular chow. eNOS protein expression was also unchanged in ApoE KO mice fed for 26–29 weeks with a hypercholesterolemic diet [7]. Actually, under pathophysiological conditions, the other isoforms of NOS can contribute to the vascular production of NO. In hypertensive blood vessels, a calcium-dependent nonendothelial NOS activity, attributed to neuronal NOS, has been located in the media [11]. But in ApoE KO mouse aorta, no expression of nNOS was revealed by Western blot or immunostaining. A weak expression of the inducible form of the enzyme was detected only in 35-week-old hypercholesterolemic mice. The staining of iNOS was associated with that for MOMA-2, specific for cells of macrophagic origin. Induction of iNOS has been described in old hypercholesterolemic rabbits [10] as well as in human advanced atherosclerotic arteries [26]. However, the present functional studies do not indicate that the iNOS protein detected in the ApoE KO mouse aorta is an important source of NO.

Since iNOS is also a potential source of superoxide anion, an increase in the expression of this isoform, combined with an increased activity of NADPH oxidase, can lead to formation of peroxynitrite. Measuring peroxynitrite is problematic in that it cannot be done directly. Therefore, the surrogate measures of its endproduct 3-NT footprints have been done. Actually, as in human [27] or rabbit atherosclerotic vessels [10], these measurements revealed the presence of peroxynitrite in the thoracic aorta of 35-week-old hypercholesterolemic mice, which correlated well with the temporal appearance of iNOS. Laursen et al. [28] have shown that peroxynitrite may oxidize tetrahydrobiopterin and alter the endothelium-dependent relaxation by uncoupling of eNOS. However, in our study, despite the increased staining for 3-NT, this deleterious role of peroxynitrite in the endothelial function of ApoE KO mice has not been demonstrated as relaxation to acetylcholine and cGMP levels were normal.

Another hypothesis, which could explain the maintained bioavailability of NO in hypercholesterolemic mice, is an increased activity of eNOS despite a normal expression of protein. Indeed, subtle changes in the eNOS subcellular location could play a compensatory part in the pathology. However, the activity of this enzyme, located in caveolae, is directly and negatively regulated by caveolin, especially in the hypercholesterolemic context [29, 30], and a decrease in the number of caveolae is described in the aorta of the hypercholesterolemic rabbits [31]. These two observations are always associated with an endothelial dysfunction.

Upregulation of enzymes involved in redox status such as SOD, catalase or gluthatione peroxidase could also represent a compensatory adaptive function by scavenging superoxide anion. However, the Western blot analysis of cytosolic CuZn- and Mn-SOD or catalase showed a reduced expression of these proteins in 35-week-old ApoE KO mouse aorta than in control mice as it has already been reported [5]. Actually, the increased production of superoxide anions in the atherosclerotic mouse aorta [8] can also be attributed to a reduced expression of SOD and catalase. The present results are at variance with other studies showing no alteration in the protein levels of these enzymes [4, 6, 7] despite an increase in the production of superoxide anions. This discrepancy may be related to the

difference in age and diet of mice. The specific activity and the protein expression of extracellular SOD are increased in ApoE KO mice, but the implication of these findings in atherosclerosis remains to be elucidated [32].

A decreased expression of SOD may lead to less dismutation of superoxide anions, which becomes more available to react with NO to form peroxynitrite, as demonstrated in 35-week-old ApoE KO mice. In 18-month-old ApoE KO mouse aorta, the decrease in endothelium-dependent relaxation by uric acid has been explained by the interaction of peroxynitrite with tetrahydrobiopterin [6]. This mechanism obviously does not appear in this present study, as endothelial function appeared normal.

Glutathione is also an important factor in the control of biological activity of NO [18]. However, an increase in its level is unlikely to explain the present findings because hypercholesterolemia reduces it [33].

In conclusion, despite a modification of the vascular redox status, no adaptive increase in NOS expression could explain the maintenance of a normal bioavailability of NO in ApoE KO mice. Moreover no upregulation of detoxifying enzymes like CuZn- or Mn-SOD or catalase was demonstrated. Additional studies in this model are now required in order to understand the mechanisms underlying this atypical persistence of the NO pathway in the hypercholesterolemic context. There are discrepancies between different studies on the same model, but it is important to keep in mind that the genetic background of ApoE KO mice may differentially influence the susceptibility to atherosclerosis [34]. Alteration of the lipid profile in different transgenic models of hypercholesterolemia could also be a source of discrepancies when studying endothelial function. Moreover, a cDNA array technique showed that expression of multiple nonlipoprotein genes was also markedly altered in transgenic hypercholesterolemic mice and needs to be considered [35].

Acknowledgements

We thank Cyril Briant, Denis Gransagne, Camille Decaux, Michel Suc, Loïc Vasseur, Fabien Guirado and Yann Mauclair for excellent technical assistance, Catherine De Montrion, Pierre Bougneux and Mr Boivin for expert advice.

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