Endothelin-Mediated Vasoconstriction in Early Atherosclerosis Is Markedly Increased in ApoE$^{-/-}$ Mouse but Prevented by Atorvastatin

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We have discovered that endothelin-1 (ET-1) vasoconstriction is significantly enhanced in aortas of young (8–16-week-old) apolipoprotein E–deficient (ApoE$^{-/-}$) mice devoid of atherosclerotic lesions (maximum response expressed as a percentage of the mean response to 100 m*M* KCl (E_{MAX}) = 55.7% ± 19.5% KCl, $n = 5$) compared to age-matched C57BL/6J control animals (E_{MAX} = 12.6% ± 2.5% KCl, $n = 8$), indicating that alterations in the endothelin system may contribute to disease progression, at least in this animal model. There was no difference in the potency of ET-1 to contract aorta from the two groups (C57BL/6J $pD_2 = 8.74 \pm 0.30$; ApoE$^{-/-}$ $pD_2 = 8.50 \pm 0.15$, $P > 0.05$). This increased response was specific to ET-1, as it was not observed with phenylephrine or U46619, nor was it due to a non–receptor mediated increase in contractile sensitivity, as there was no change in response to KCl between the two groups. [^{125}I]ET-1 bound with subnanomolar affinity (K_D) to aorta ($K_D = 0.018 \pm 0.002$ n*M*, $n = 4$) and, with an order of magnitude lower affinity, to heart ($K_D = 0.47 \pm 0.05$, $n = 5$) of C57BL/6J mice with binding densities (B_{MAX}) of 9.3 ± 2.4 fmol mg^{-1}protein and 100 ± 14 fmol mg^{-1} protein, respectively. Alterations in vascular reactivity to ET-1 could not be explained by increased endothelin receptor density or affinity, as these were not altered in aorta ($K_D = 0.011 \pm 0.003$ n*M*; $B_{MAX} = 10.1 \pm 3.9$ fmol mg^{-1}, $n = 4$) and heart ($K_D = 0.43 \pm 0.04$ n*M*; $B_{MAX} = 115 \pm 26$ fmol mg^{-1}, $n = 6$) of ApoE$^{-/-}$ animals. The ratio of ET_A to ET_B receptors in heart of control and ApoE$^{-/-}$ mice was similar, comprising 89% and 85% ET_A receptors, respectively. In isolated aorta from ApoE$^{-/-}$ mice on the Western diet, which more closely resembled more advanced stages of the disease in man, the augmented ET-1 vasoconstrictor response was maintained (E_{MAX} = 25.2% ± 6.8% KCl, $n = 9$); however, it was completely prevented in animals that had received 10 weeks of oral atorvastatin (30 mg kg^{-1} day^{-1}) (E_{MAX} = 4.0% ± 1.5% KCl, $n = 5$), a concentration that was chosen because it did not affect plasma cholesterol and triglyceride levels. Therefore, this protective prevention of enhanced ET-1 vasoconstriction in ApoE$^{-/-}$ mice by atorvastatin was independent of its lipid-lowering properties. Exp Biol Med 231:806–812, 2006

Key words: ApoE$^{-/-}$ mouse; endothelin-1; atherosclerosis; vasoconstriction; atorvastatin

Tissue and plasma levels of the vasoconstrictor endothelin-1 (ET-1) are upregulated in patients with atherosclerosis (1, 2), and we find that activity of the endothelin-converting enzyme is increased in human atherosclerotic coronary artery *in vitro* (3). The apolipoprotein E–deficient (ApoE$^{-/-}$) mouse has been proposed as a model for human atherosclerosis because of the spontaneous formation of atherosclerotic plaques (4). In concordance with observations in human disease, ET-1 is upregulated in the aorta of ApoE$^{-/-}$ mice with advanced lesions (5). However, it remains unclear if this is a cause or a consequence of disease progression. Therefore, the primary aim of this study was to investigate if there was any change in the endothelin system in young ApoE$^{-/-}$ mice with aortas devoid of atherosclerotic plaques. Additionally, it has been reported that ApoE$^{-/-}$ mice fed a high-fat diet more closely resemble the advanced stages of atherosclerotic disease in man. In these animals, the HMG-CoA reductase inhibitor atorvastatin, at concentrations that did not lower plasma lipid levels, had beneficial effects on aortic plaque composition (6, 7) that resulted in a decrease in the proportion of advanced plaques and an increase in plaque stability (7). We have therefore determined in ApoE$^{-/-}$ mice fed a high-fat diet whether treatment with atorvastatin had any effect against ET-1–mediated vasoconstriction in isolated aorta *in vitro* that may not be attributable to the lipid-lowering actions of this drug.

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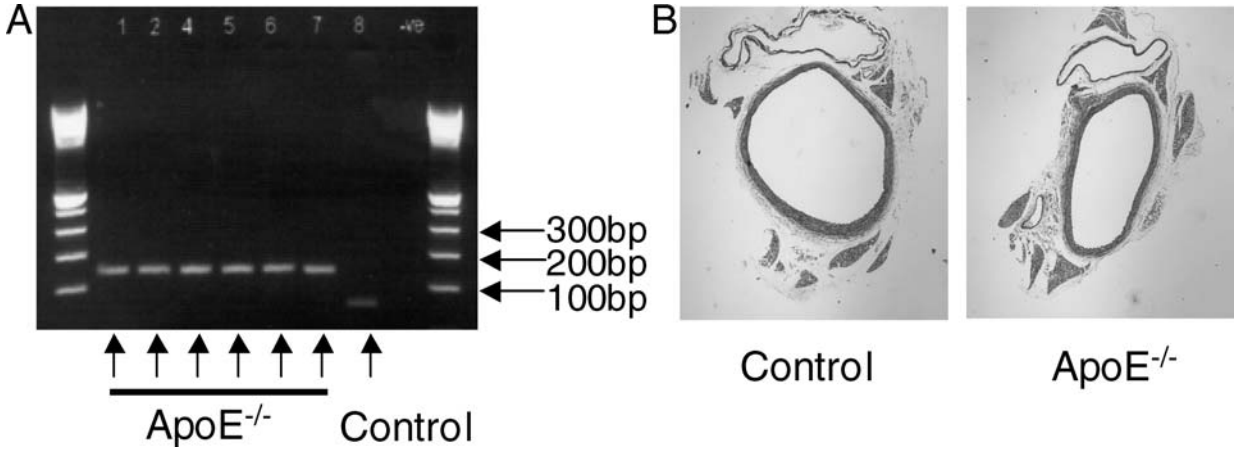

Figure 1. (A) Electrophoretic gel showing PCR products of the expected size for the ApoE gene in six ApoE$^{-/-}$ mice and one wild-type C57BL/6/J control. Each ApoE$^{-/-}$ mouse was homozygous for the disrupted gene. (B) Histology (Masson stain) showing examples of aorta from both the control and ApoE$^{-/-}$ mice devoid of atherosclerotic lesions.

Methods and Materials

Animals. The procedures used in this study were approved by the local animal ethical committee and were performed under UK Home Office Project Licence authority; the study conformed to the National Institutes of Health Guidelines for the Care and Use of Laboratory Animals. Young C57BL/6/J and ApoE$^{-/-}$ mice (8–16 weeks of age) were maintained on normal chow, with older ApoE$^{-/-}$ mice (20–25 weeks of age) maintained on the high-fat Western diet (Research Diets, Inc., New Brunswick, NJ; comprising, wt/wt, 20% protein, 50% carbohydrate, 21% fat, and 0.21% cholesterol) from age 8–10 weeks onward, with some receiving 30 mg kg^{-1} day^{-1} atorvastatin by oral gavage for 10 weeks. Mice (either sex, 25–35 g; Charles River, Margate, UK) were killed with CO_2 and aorta and heart removed.

Genotyping and Histology. The presence of ApoE genes disrupted by homologous recombination was confirmed using polymerase chain reaction (PCR) (Fig. 1A), and histologic analysis confirmed that aortas from young ApoE$^{-/-}$ mice were devoid of atherosclerotic lesions (Fig. 1B).

Plasma Lipid Profile. Plasma cholesterol and triglyceride levels were measured using colorimetric assays (cholesterol; Sigma-Aldrich, Poole, UK; triglyceride; Wako, Neuss, Germany). Levels of both were significantly higher in young ApoE$^{-/-}$ mice (438 ± 56 mg dl^{-1} and 118 ± 22 mg dl^{-1}, respectively, $n=6$) compared to control C57BL/6/J mice (36 ± 4 mg dl^{-1} and 46 ± 9 mg dl^{-1}, respectively, $n=4$), with a further elevation in older ApoE$^{-/-}$ mice on the Western diet (1107 ± 71 mg dl^{-1} and 118 ± 18 mg dl^{-1}, respectively, $n=8$). In ApoE$^{-/-}$ mice receiving atorvastatin, the dose chosen (30 mg kg^{-1} day^{-1}) did not affect plasma cholesterol or triglyceride levels (1032 ± 75 mg dl^{-1} and 110 ± 10 mg dl^{-1}, respectively, $n=5$).

Radioligand Binding. Binding assays were carried out as previously described (8). Saturation experiments were carried out to determine the affinity (K_D) and binding density (B_{MAX}) of ET-1 for its receptors. Briefly, 10-μm cryostat-cut sections of aorta and whole mouse heart were incubated at room temperature for 2 hrs in Hepes buffer (Hepes, 50 m*M*; $MgCl_2$, 5 m*M*; bovine serum albumin, 0.3%; pH 7.4) containing increasing concentration of [^{125}I]ET-1 (4 p*M*–4 n*M*). Competition binding experiments were carried out in sections of mouse heart to determine the relative ratio of ET_A to ET_B receptors in this tissue. Heart sections (10 μ*M*) were incubated in Hepes buffer containing 0.1 n*M* [^{125}I]ET-1 and increasing concentrations of the ET_A selective antagonist PD156707 (20 p*M*–100 μ*M*) for 2 hrs at room temperature. Nonspecific binding for all experiments was determined using 1 μ*M* ET-1, and binding was counted in a γ-counter. Data were analyzed using the KELL suite of programs (Biosoft, Cambridge, UK), and values of pooled K_D and B_{MAX} were expressed as mean ± SEM. For receptor autoradiography, heart sections were incubated as above with 0.1 n*M* [^{125}I]ET-1 in the absence (to label all ET receptors) or presence of either 0.2 μ*M* BQ3020 (to reveal ET_A receptors) or 0.1 μ*M* BQ123 (to reveal ET_B receptors). Sections were apposed to radiation-sensitive film for 5 days and analyzed using the Quantimet 970 system.

***In Vitro* Pharmacology.** Rings of aorta (<2 mm in length) were dissected and mounted in wire myographs for measurement of isometric tension, as previously described (9). The segments were bathed in oxygenated Krebs' solution at 37°C and were set to 90% of the internal diameter at 100 mm Hg. Maximal force (mN mm^{-1}) was measured three times with a potassium-rich Krebs' solution (100 m*M*) at 15-min intervals before constructing cumulative concentration-response curves to ET-1 (0.1 n*M*–300 n*M*), U-46619 (9,11-dideoxy-11α,9α-epoxymethanoprostaglandin $F_{2\alpha}$) (1 n*M*–3 μ*M*), phenylephrine (1 n*M*–3 μ*M*), and KCl (0.1 m*M*–0.3 *M*). Concentration response curves were expressed as a percentage of the mean of the three responses to KCl and were analyzed using a four-parameter

Table 1. Saturation Analysis of [^{125}I]ET-1 Binding in the Heart and Aorta from C57BL/6J Control and ApoE$^{-/-}$ Mice[a]

	Heart		Aorta	
	C57BL/6J	ApoE$^{-/-}$	C57BL/6J	ApoE$^{-/-}$
K_D (n*M*)	0.47 ± 0.05	0.43 ± 0.04	0.018 ± 0.002	0.011 ± 0.003
B_{MAX} (fmol mg^{-1} protein)	100 ± 14	115 ± 26	9.3 ± 2.4	10.1 ± 3.9
n	5	6	4	4

[a] Values are mean ± SEM from *n* mice.

logistic equation using FigP 2.98 Software (Biosoft) to obtain values of molar concentration producing 50% of the maximum response (EC_{50}) and maximum response expressed as a percentage of the mean response to 100 m*M* KCl (E_{MAX}). Potency values were normalized by logarithmic transformation to pD_2 values ($-\log_{10} EC_{50}$).

Statistical Analysis. All data are expressed as arithmetic mean ± SEM. *n* values refer to the number of mice from which tissues were obtained. Where appropriate, responses were compared using Student's two-tailed *t* test, with a *P* value of <0.05 considered significant.

Materials. ET-1 was obtained from the Peptide Institute (Osaka, Japan), and BQ3020 was obtained from Neosystem (Strasbourg, France). BQ123 was synthesized by solid-phase t-Boc chemistry. PD156707 was a gift from Dr. Annette Doherty (Parke-Davis Pharmaceutical Research Division, Ann Arbor, MI). [^{125}I]ET-1 (2000 Ci mmol^{-1}) was from Amersham Biosciences UK Ltd. (Chalfont St. Giles, UK). Other chemicals and reagents were from Sigma-Aldrich, unless otherwise stated. Krebs' solution comprised (in m*M*); NaCl, 90; $NaHCO_3$, 45; KCl, 5; $MgSO_4 \cdot 7H_2O$, 0.5; $Na_2HPO_4 \cdot 2H_2O$, 1; $CaCl_2$, 2.25; fumaric acid, 5; glutamic acid, 5; sodium pyruvate, 5; and glucose, 10 (pH 7.4, when gassed with 95% O_2/5% CO_2).

Results

Binding Assays. In saturation experiments, [^{125}I]ET-1 bound with comparable subnanomolar affinity and maximum binding density to aorta and heart from C57BL/6J control and ApoE$^{-/-}$ mice (Table 1). In both C57BL/6J control and ApoE$^{-/-}$ mice, the affinity of [^{125}I]ET-1 was an order of magnitude higher and binding density 10-fold lower in aorta compared to heart. In competition assays, PD156707 competed in a biphasic manner for [^{125}I]ET-1 binding in heart from both C57BL/6J and ApoE$^{-/-}$ mice, and it was apparent that the ratio of ET_A to ET_B receptors between the two groups was similar, with ET_A receptors comprising approximately 85%–90% of the total in each (Fig. 2). As expected, PD156707 competed with high affinity for ET_A receptors (K_D control 1.10 ± 0.20 n*M*, $n = 5$; K_D ApoE$^{-/-}$ 0.76 ± 0.18 n*M*, $n = 6$) and lower affinity for the small population of ET_B receptors (K_D control 480 ± 30 n*M*, $n = 5$; K_D ApoE$^{-/-}$ 260 ± 69 n*M*, $n = 6$). The relatively high density of ET_A receptors in mouse heart from both groups of animals was confirmed autoradiographically (Fig. 3).

In additional experiments, [^{125}I]ET-1 binding in control heart tissue was found to be unaffected by incubation with atorvastatin, confirming that any effect of the statin on ET-mediated vasoconstriction would not be due to competition of the drug for ET-1 binding to its receptor.

***In Vitro* Pharmacology.** The internal diameters of aorta from young C57BL/6J mice (1188 ± 104 μm, $n = 8$) were not significantly different ($P > 0.05$) from aorta from young ApoE$^{-/-}$ mice (1189 ± 139 μm, $n = 5$). Mean responses to KCl before construction of agonist concen-

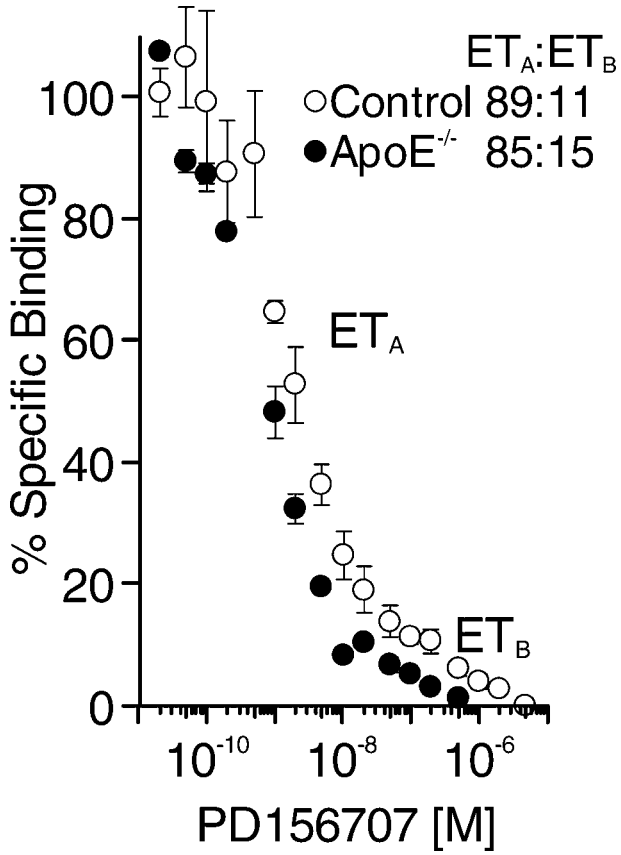

Figure 2. Competition binding curves for the ET_A selective antagonist PD156707 against 0.1 n*M* [^{125}I]ET-1 in sections of heart from C57BL/6J control and ApoE$^{-/-}$ mice. There was no difference in the ratio of ET_A to ET_B receptors between the two groups.

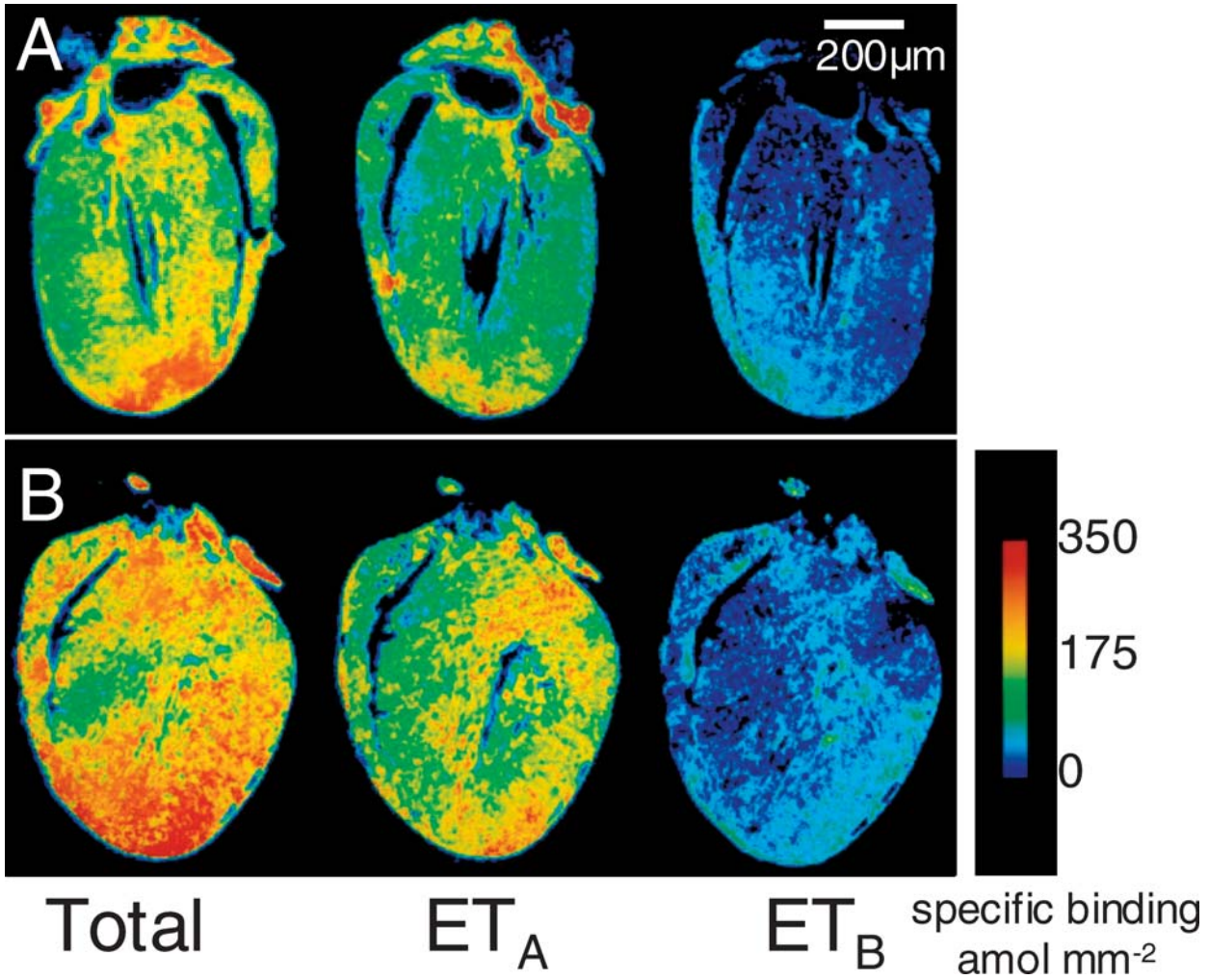

Figure 3. Color-coded images of specific [^{125}I]ET-1 binding in (A) C57BL/6/J control and (B) ApoE$^{-/-}$ mouse heart indicating the predominant expression of the ET_A compared to the ET_B receptor subtype in both. Total [^{125}I]ET-1 (0.1 n*M*) binding is obtained in the absence of competing ligands, ET_A receptors are revealed in the presence of the ET_B agonist BQ3020 (200 n*M*), and ET_B receptors identified in the presence of the ET_A antagonist BQ123 (100 n*M*). (Color figure available in on-line version.)

tration-response curves were not different ($P > 0.05$) between young C57BL/6/J (E_{MAX} KCl = 2.44 ± 0.15 mN mm^{-1}, $n = 8$) and ApoE$^{-/-}$ (E_{MAX} KCl = 2.01 ± 0.27 mN mm^{-1}, $n = 5$) animals. However, the maximum vasoconstrictor response to ET-1 was significantly enhanced in aorta from the ApoE$^{-/-}$ animals compared to C57BL/6/J controls ($P < 0.05$). Potency of ET-1 was not different between the two groups (Fig. 4A and Table 2). For other agonists (phenylephrine, U-46619, and KCl), there was no difference in either maximum response or potency in the young C57BL/6/J and ApoE$^{-/-}$ animals (Fig. 4B–D and Table 2).

In older ApoE$^{-/-}$ mice fed the Western diet, there was no significant difference in control mean responses to KCl between the untreated (2.23 ± 0.16 mN mm^{-1}, $n = 9$) and atorvastatin (1.80 ± 0.41 mN mm^{-1}, $n = 5$) groups. There was a trend for a reduction in maximum response to ET-1 in the older ApoE$^{-/-}$ animals compared to the younger ApoE$^{-/-}$ group, but this did not reach significance. However, while ET-1 vasoconstriction was detected in the older untreated ApoE$^{-/-}$ animals (pD_2 8.44 ± 0.21; E_{MAX} 25.2% ± 6.8% KCl, $n = 9$), this was significantly attenuated in the older ApoE$^{-/-}$ mice that had received 10 weeks of oral atorvastatin treatment (pD_2 9.10 ± 0.15; E_{MAX} 4.0% ± 1.5% KCl, $n = 5$) (Fig. 5).

Discussion

Increased expression of ET peptide, receptors, and converting enzyme activity is reported in human atherosclerosis (1–3) and in the aorta from ApoE$^{-/-}$ mice with advanced lesions (5, 10). In the present study we did not find any increase in ET receptor density in aorta and heart from ApoE$^{-/-}$ mice compared with C57BL/6/J control animals, nor did we detect any alteration in the ratio of ET_A to ET_B receptor subtypes in heart tissues in competition and autoradiographic experiments, which correlate well with our data for the ET system in human atherosclerosis (2). Our

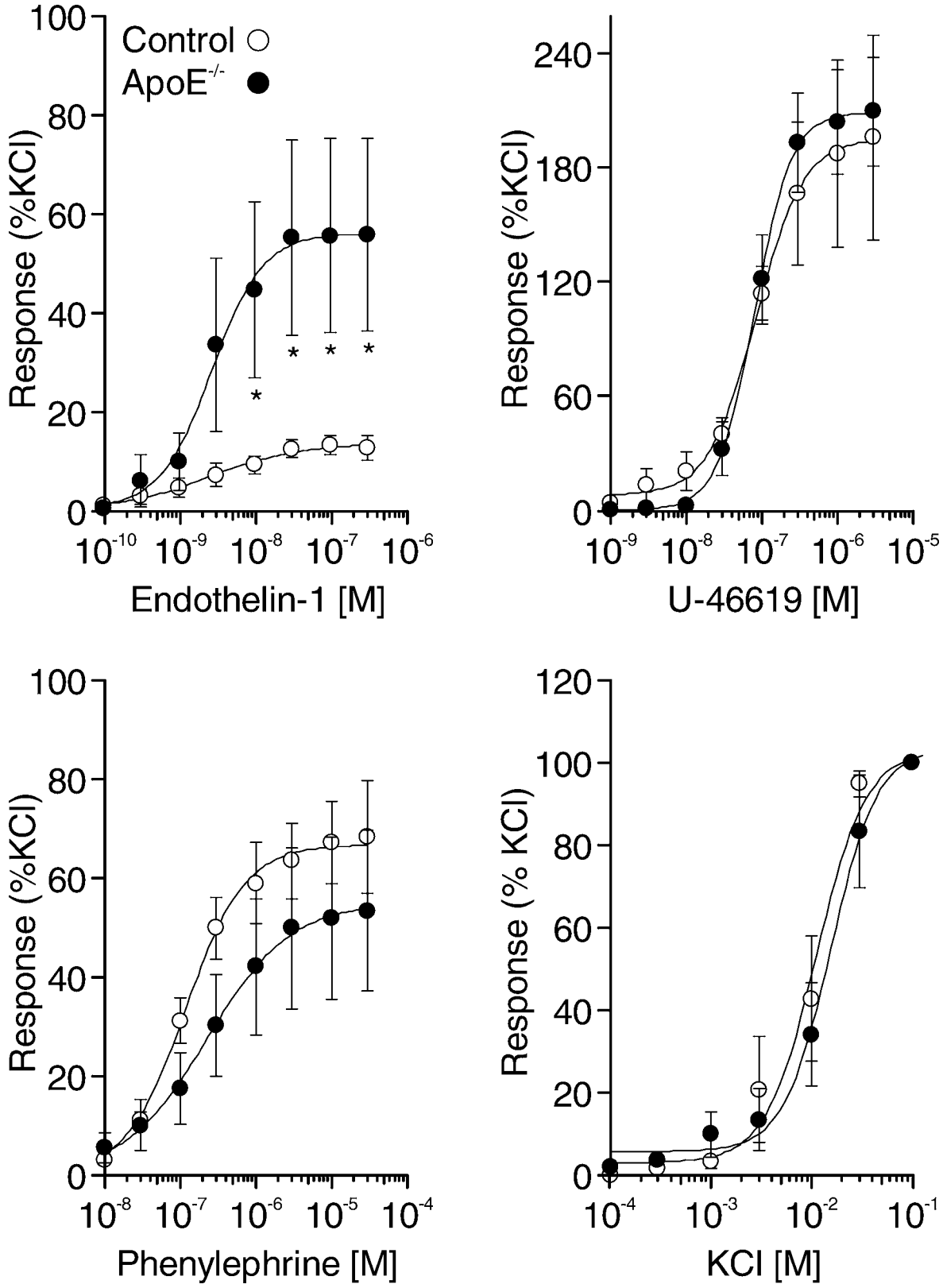

Figure 4. Concentration-response curves to agonists in isolated aorta from young (8–16-week-old) C57BL/6/J and ApoE$^{-/-}$ mice *in vitro*. Values are mean ± SEM from four to eight mice. *, Significantly different from control, Student's two-tailed *t* test ($P < 0.05$).

Table 2. Potency (pD_2) and Efficacy (E_{MAX}) of Vasoconstrictor Agonists in Isolated Aorta from Young C57BL/6J Control and ApoE$^{-/-}$ Mice *In Vitro*[a]

	C57BL/6J			ApoE$^{-/-}$		
	pD_2	E_{MAX}	*n*	pD_2	E_{MAX}	*n*
ET-1	8.74 ± 0.30	12.6 ± 2.5	8	8.50 ± 0.15	55.7 ± 19.5*	5
U-46619	7.47 ± 0.34	195.8 ± 53.5	5	7.10 ± 0.10	209.5 ± 28.6	7
Phenylephrine	6.87 ± 0.18	68.2 ± 11.4	4	6.58 ± 0.08	53.3 ± 16.3	7
KCl	1.86 ± 0.33	100 ± 0.0	5	2.17 ± 0.13	100 ± 0.0	6

[a] Values are mean ± SEM from *n* mice.
* Significantly different from C57BL/6J control ($P < 0.05$, Student's *t* test).

data in aorta appear to contrast with the report of an increase of smooth muscle ET_A receptors in aorta from ApoE$^{-/-}$ mice maintained on a high-fat diet (10). However, these animals were older (30 weeks) than the animals that we have used and were maintained on the diet for a longer period of time. One explanation may be that this may indicate a more pronounced upregulation of the ET system is apparent with disease progression.

We have demonstrated that vascular reactivity to ET-1 is significantly enhanced in the aorta of ApoE$^{-/-}$ mice that are devoid of atherosclerotic lesions. This implies that alterations in the endothelin system occur in the early stages of disease and may therefore contribute to, rather than occur as a consequence of, disease progression. It also indicates that the alteration in vascular reactivity is not due to changes in receptor density or ratio of receptor subtype expression *per se*, but may result from enhanced signaling at a point downstream of ET receptor activation.

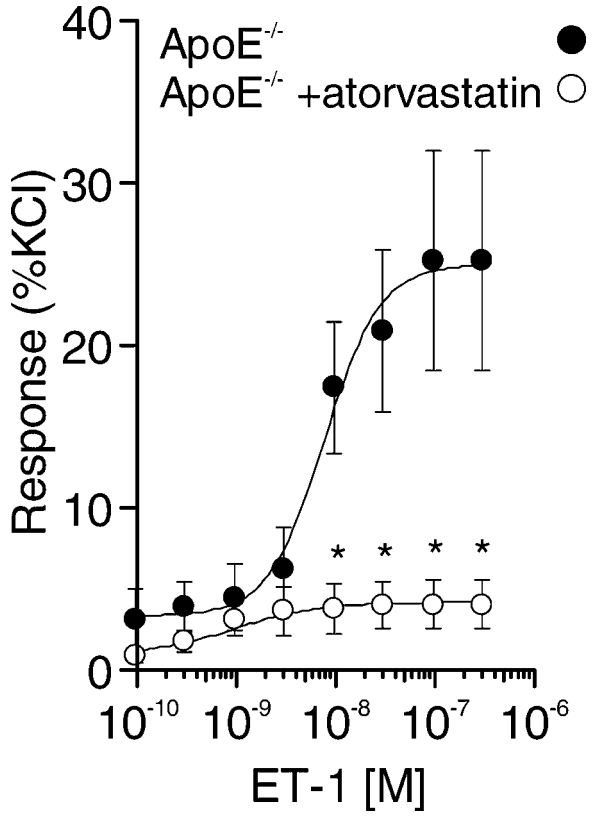

Figure 5. Concentration-response curves to ET-1 in isolated aorta from 22–25-week-old untreated ApoE$^{-/-}$ ($n = 9$) mice and those receiving oral atorvastatin, 30 mg kg^{-1} day^{-1}, for 10 weeks ($n = 5$). Values are mean ± SEM. *, Significantly different from ApoE$^{-/-}$ without atorvastatin treatment, Student's two-tailed *t* test ($P < 0.05$).

It has been suggested that oxidized low-density lipoprotein (oxLDL), a key mediator of endothelial dysfunction in atherosclerosis, may enhance vasoconstriction in disease *via* reduction in endothelial nitric oxide synthesis (eNOS) mRNA stability and, thus, *via* a loss of NO-induced dilator tone (11) and, additionally, *via* stimulating the activity of PKC isoforms (12) or directly increasing the sensitivity of the contractile apparatus of vascular smooth muscle cells to vasoconstrictors *via* a Rho and Rho-kinase–dependent mechanism (13). The relative contribution, if any, of these effects of oxLDL to the enhanced ET-1 vasoconstrictor response that we observe in aorta of ApoE$^{-/-}$ mice remains to be determined. Further experiments are also required to identify whether differences in intracellular signaling pathways activated by phenylephrine and the thromboxane mimetic U-46619 in mouse aorta, compared to ET-1, are sufficient to explain the apparent selective upregulation of response to ET-1 in the ApoE$^{-/-}$ animals. In mesenteric arteries from lipopolysaccharide-treated rats that show increased expression of Rho-kinase, a similar enhanced ET-1 constrictor response has been reported that is not observed with either phenylephrine or angiotensin-II (14).

Statins block the action of HMG-CoA reductase to reduce biosynthesis of cholesterol but have other important actions that include both an increase in eNOS expression and reduction in ET-1 synthesis, resulting in an improvement in endothelial function (15, 16). In our study we found that atorvastatin, administered at a concentration that did not reduce plasma lipid levels, prevented the augmented vasoconstrictor response to ET-1 in ApoE$^{-/-}$ mice that had been fed a Western diet. In addition to lipid lowering, statins inhibit the synthesis of isoprenoids, for example geranylgeranyl pyrophosphate, that are required for the activation of small GTP binding proteins such as Rho (17). Therefore, as Rho-kinase inhibitors are known to reverse or prevent ET-1 constriction, for example in human mammary

artery *in vitro* (18), this may be one mechanism by which atorvastatin prevents ET-1–augmented vasoconstriction in the ApoE$^{-/-}$ mouse model. This finding is consistent with the recent report that simvastatin is able to reverse the constrictor response to ET-1 in rat thoracic aorta *in vitro* and that this effect could be mimicked by the Rho-kinase inhibitor HA-1077 (19).

In conclusion, we have demonstrated that there is a marked increase in vasoconstrictor response to ET-1 in ApoE$^{-/-}$ mice that occurs before atherosclerotic plaque formation, implying a role for this peptide in the early stages of the disease. Prevention of this response may contribute to the "pleiotropic" effects of statins that are secondary to reduction of cholesterol biosynthesis.

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