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Elevated systemic TGF- β impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling, and increased plaque formation in apoE^{-/-} mice

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¹Institute of Pathophysiology and ²Institute of Human Physiology and Clinical Experimental Research, Semmelweis University, Budapest, Hungary; ³Department of Physiology, New York Medical College, Valhalla, New York; and ⁴Department of Pathology, University of Heidelberg, Heidelberg, Germany

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Buday A, Órsy P, Godó M, Mózes M, Kökény G, Lacza Z, Koller A, Ungvári Z, Gross ML, Benyó Z, Hamar P. Elevated systemic TGF- β impairs aortic vasomotor function through activation of NADPH oxidase-driven superoxide production and leads to hypertension, myocardial remodeling, and increased plaque formation in apoE^{-/-} mice. *Am J Physiol Heart Circ Physiol* 299: H386–H395, 2010. First published May 28, 2010; doi:10.1152/ajpheart.01042.2009.—The role of circulating, systemic TGF- β levels in endothelial function is not clear. TGF- β ₁ may cause endothelial dysfunction in apolipoprotein E-deficient (apoE^{-/-}) mice via stimulation of reactive oxygen species (ROS) production by the NADPH oxidase (NOX) system and aggravate aortic and heart remodeling and hypertension. Thoracic aorta (TA) were isolated from 4-mo-old control (C57Bl/6), apoE^{-/-}, TGF- β ₁-overexpressing (TGF β ₁), and crossbred apoE^{-/-} \times TGF β ₁ mice. Endothelium-dependent relaxation was measured before and after incubation with apocynin (NOX inhibitor) or superoxide dismutase (SOD; ROS scavenger). Superoxide production within the vessel wall was determined by dihydroethidine staining under confocal microscope. In 8-mo-old mice, aortic and myocardial morphometric changes, plaque formation by en face fat staining, and blood pressure were determined. Serum TGF- β ₁ levels (ELISA) were elevated in TGF β ₁ mice without downregulation of TGF- β -I receptor (immunohistochemistry). In the aortic wall, superoxide production was enhanced and NO-dependent relaxation diminished in apoE^{-/-} \times TGF β ₁ mice but improved significantly after apocynin or SOD. Myocardial capillary density was reduced, fibrocyte density increased, aortic wall was thicker, combined lesion area was greater, and blood pressure was higher in the apoE^{-/-} \times TGF β vs. C57Bl/6 mice. Our results demonstrate that elevated circulating TGF- β ₁ causes endothelial dysfunction through NOX activation-induced oxidative stress, accelerating atherosclerosis and hypertension in apoE^{-/-} mice. These findings may provide a mechanism explaining accelerated atherosclerosis in patients with elevated plasma TGF β ₁.

aorta; oxidative stress; nicotinamide adenine dinucleotide phosphate; transforming growth factor- β ; apolipoprotein E-deficient mice

TRANSFORMING GROWTH FACTOR (TGF)- β ₁ is a pleiotropic cytokine with proinflammatory and inflammation inhibitory (34) as well as profibrotic effects (37). The inflammation inhibitory effects of TGF- β ₁ are attributed to T-cell regula-

tion by local TGF- β ₁ production from infiltrating regulatory T lymphocytes (34).

Vascular effects of systemic TGF- β ₁ are less characterized. TGF- β ₁ is known to induce nicotinamide adenine dinucleotide phosphate (NADPH) oxidase (NOX) activity in the vessel wall in endothelial (26) and smooth muscle cells (60). We hypothesized that TGF- β ₁ leads to vascular endothelial dysfunction by stimulation of NOX-mediated superoxide production. Growing evidence indicates that chronic and acute overproduction of reactive oxygen species (ROS) plays a causal role in vascular endothelial dysfunction (43). One important source of ROS is the NOX enzyme family. NOXs are important initial enzymes of the ROS-producing enzyme cascade, responsible for superoxide (O₂⁻) production (33). ROS has been demonstrated to induce proinflammatory phenotype of endothelial cells by upregulating proinflammatory mediator production and adhesion molecule expression, leading to vascular endothelial dysfunction (14). Endothelial dysfunction has been associated with the pathogenesis of several chronic diseases, including hypertension and atherosclerosis (44).

Elevated circulating TGF- β ₁ due to the TGF β ₁ transgene did not alter endothelial function significantly in C57Bl/6 mice, a strain not developing endothelial dysfunction or hypertension normally (55, 56). Since apolipoprotein E-deficient (apoE^{-/-}) \times TGF β ₁ crossbred mice develop significant systemic hypertension by the age of 8 mo, we hypothesized that TGF- β ₁ may induce vascular endothelial dysfunction only in the atherosclerotic surrounding of apoE^{-/-} mice.

To investigate the hypothesis that elevated circulating TGF- β ₁ induces vascular endothelial dysfunction in the atherosclerotic surrounding, apoE^{-/-} mice (29) bearing the TGF β ₁ transgene (apoE^{-/-} \times TGF β ₁) were generated. TGF β ₁ mice were crossed to apoE^{-/-} mice because both genetic modifications were on C57Bl/6 background.

We examined the effects of elevated serum TGF- β levels and the role of NOX on nitric oxide (NO)-dependent dilatation of the aorta and consequent myocardial and aorta morphology and blood pressure. Double-gene-modified animals with apoE^{-/-} and TGF β ₁ transgene (apoE^{-/-} \times TGF β ₁) had both elevated TGF- β plasma levels and apoE deficiency with consequent atherosclerosis.

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METHODS

Mice. ApoE-deficient mice (C57Bl/6J-apoe^{tm1Unc}) were purchased from the Jackson Laboratory (Bar Harbor, ME); C57Bl/6 controls were purchased from Charles River Laboratories (Sulzfeld, Germany). TGF β 1 transgenic mice (CBA.B6-TgAlb/TGF β 1 F₂) were obtained from S. Thorgeirsson (National Cancer Institute/National Institutes of Health, Bethesda, MD) (59). Overexpression of TGF- β 1 is ensured by the transgene construct of porcine TGF- β 1 selectively expressed in hepatocytes under the control of murine albumin promoter and enhancer.

TGF β 1 mice on CBA \times C57Bl/6 F₂ background were backcrossed to C57Bl/6 strain for 10 generations. The newly generated B6-TgAlb/TGF β 1 mice have homogenous C57Bl/6 genetic background but elevated plasma TGF- β 1 levels (Mózes M and Kókény G, unpublished data).

B6-TgAlb/TGF β 1 mice were crossed to apoE^{-/-} mice to generate apoE^{-/-} mice bearing the TGF β 1 transgene (apoE^{-/-} \times TGF β 1). TGF β 1 male mice were mated with apoE^{-/-} female mice. F₂ mice were genotyped for the presence of the TGF β 1 transgene and the absence of both apoE alleles by PCR and were then used in the studies.

All mice were bred in our laboratory and were housed under standard conditions with access to standard rodent chow and tap water ad libitum. Aortic function was investigated in 4-mo-old male mice, whereas blood pressure, atherosclerotic lesions, and morphology were examined in 8-mo-old male mice. All animal protocols were approved by the Semmelweis University Ethical Committee for Animal Welfare.

Perfusion fixation and tissue sampling. All animals at the age of 4 mo were euthanized under deep ether anesthesia (Sigma Aldrich, Steinheim, Germany) supplemented with 100 IU heparin (Biochemie Austria; 304219, 500 IU/ml) intraperitoneally. Body weight was measured, and mice were bled and perfused transcardially with 10 ml of heparinized (10 IU/ml) Krebs solution (25) for assessment of ex vivo aortic function and superoxide detection. After organ harvest, the distal part of the thoracic aorta (TA) was prepared under an operating microscope (Wild, Heerburg, Switzerland). For morphometric investigations, animals were perfused with Hank's balanced salt solution and consequently with 3% glutaraldehyde. After perfusion, the heart and aorta of each animal were removed. The heart and left ventricular weight and volume were determined. Hearts from each experimental group were weighed and prepared according to the orientator method (20) or dried in an oven at 65°C for 24 h and reweighed to determine the wet-to-dry weight ratio of the myocardium.

Ex vivo determination of NO-dependent relaxation of TA. TA segments of 3 mm from each experimental group were mounted on stainless steel vessel holders (200 mm in diameter) of a conventional myograph setup (610-M Multi Myograph System; Danish Myo Technology, Aarhus, Denmark). The organ chambers of the myographs were filled with 8 ml of Krebs solution [in mM: 119 NaCl, 4.7 KCl, 2.5 CaCl₂·2H₂O, 1.17 MgSO₄·7H₂O, 20 NaHCO₃, 1.18 KH₂PO₄, 0.027 EDTA, and 11 glucose (Sigma Aldrich)] and aerated with 5% CO₂ balanced with O₂ (Linde, Répcelak, Hungary). The bath solution was warmed to 37°C, and the resting tension of TA rings was adjusted to 15 mN, according to previous studies (25).

Segments were exposed to 124 mM K⁺ Krebs solution to elicit reference contraction. Thirty minutes later, precontraction was induced by 10 mM prostaglandin F_{2 α} (PGF_{2 α} ; Cayman Chemicals, Ann Arbor, MI) (55, 56). After each precontraction and administration of the vasorelaxant agents, the vessels were washed and allowed a 30-min recovery period. Aortic relaxation was tested after a stable plateau of contraction had been reached. Carbachol-mediated relaxation was registered to compare endothelial function within the experimental groups. After preincubation with the NOX inhibitor apocynin (100 μ M) or superoxide dismutase (SOD, 200 U/ml; Sigma Aldrich), a second precontraction and carbachol responses were reg-

istered and compared with the first responses. Relaxation responses were expressed as the percentage of relaxation from the precontraction produced by PGF_{2 α} . The isometric tension recording of the TA segments was made with the MP100 system, and the recorded data were analyzed with AcqKnowledge 3.7.3 software (BIOPAC Systems, Goleta, CA). Vasoactive substances (PGF_{2 α} , carbachol, apocynin, and SOD) were dissolved in distilled water. All concentrations are expressed as the final concentration of each vasoactive substance in the organ bath.

Immunohistology and plasma levels. Paraffin sections of aorta were deparaffinized (xylol, 3 \times 5 min), rehydrated (100–96–70%), and incubated in trypsin (0.17%); sections were then stained with TGF- β 1 (sc-146, Santa Cruz Biotechnology; 1:100) and TGF- β receptor I antibodies (sc-398, Santa Cruz Biotechnology; 1:50) and finally counterstained with hematoxylin. The slides were evaluated by two blinded observers using semiquantitative scores described in detail elsewhere (2). Scoring was as follows: 0, no staining; 1, faint/barely perceptible/incomplete staining in >10% of cells; 2, weak to moderate staining in >10% of cells; and 3, strong complete staining in >10% of cells (13).

Before termination of the experiment, blood samples were collected from retroorbital puncture into siliconized tubes containing EDTA at a final concentration of 1 mg/ml. Plasma TGF- β 1 levels were determined in all animals with the Quantikine TGF- β ELISA kit (MB100B; R&D Systems, Minneapolis, MN). Plasma was prepared and TGF- β 1 levels were determined according to the manufacturer's protocol.

Superoxide detection. Production of O₂⁻ was determined in segments of the aortas that were used for functional studies. Hydroethidine, an oxidative fluorescent dye, was used to localize superoxide production in situ as previously reported (42). In brief, unfixed, frozen aorta rings were cut into 30- μ m-thick segments and incubated with hydroethidine (2 \times 10⁻⁶ M at 37°C for 30 min) (53). The aortas were then washed three times, and the endothelial layer of en face preparations was visualized using a Zeiss LSM 510 Meta confocal microscope (Zeiss, Göttingen, Germany). Fluorescent images were captured at \times 10 magnification. Hoechst 33258 dye (Sigma Aldrich) was used to visualize nuclei. Intensity of nuclear hydroethidine staining was analyzed using ImageJ imaging software. Ten to fifteen entire fields per vessel were analyzed with one image per field. The mean fluorescence intensities of ethidium-stained nuclei in the endothelium were calculated for each vessel. Thereafter, these intensity values for each animal in the group were averaged. Average values were divided by the control C57Bl/6 group's average to obtain fold changes. Unstained aortas and vessels preincubated with polyethylene glycol (PEG)-SOD were used for background correction and negative control, respectively.

Heart and aorta morphometry. Heart samples were obtained and stained according to the orientator method (20). Uniformly random sampling of the myocardium was achieved by preparing a set of equidistant slices of the left ventricle and the interventricular septum with a random start. Two slices were selected and processed. Eight pieces of the left ventricular myocardium, including the septum, were

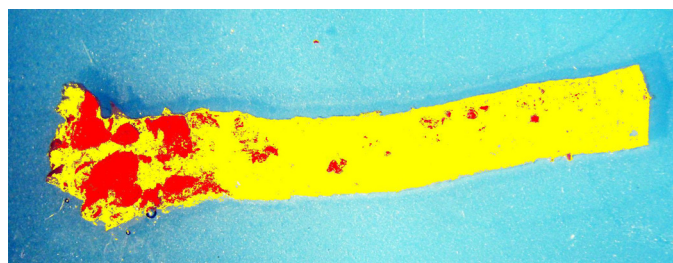


Fig. 1. Quantification of stained plaques by microscopy and computer-aided morphometry (ImagePro Plus, Media Cybernetics) shown as a percentage of plaque per total area.

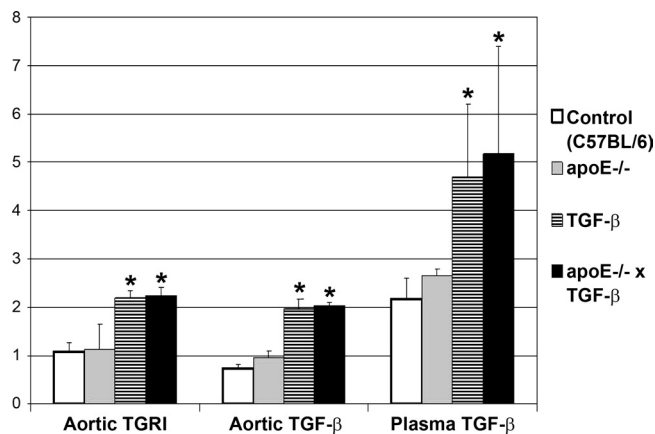


Fig. 2. Transforming growth factor- β_1 receptor I and TGF- β_1 immunostaining (scores) in aortic tissue ($n = 6$ /group) and plasma TGF- β_1 concentration (ng/ml) values ($n = 10$ /group). * $P < 0.05$ vs. control (C57BL/6) mice. TGRI, TGF- β_1 receptor I; apoE^{-/-}, apolipoprotein E-deficient mice.

prepared and embedded in Epon-Araldite. Semithin sections (0.8 μ m) were stained with methylene blue and basic fuchsin and examined by light microscopy with oil immersion and phase contrast at $\times 1,000$ magnification.

Stereological analysis was performed on eight random samples of the left ventricular myocardium from each animal. Length density (LV) of capillaries, that is, the length of capillaries per unit tissue volume, and the volume density (VV) of cardiac capillaries, defined as the volume of capillaries per unit myocardial tissue volume, were measured in eight systematically subsampled areas per section using a Zeiss eyepiece with 100 points for point counting. The length density of myocardial capillaries (LV) was determined using the equation $LV = 2 \text{ QA}$, where QA is area density, or the number of capillary transects per area of myocardial reference tissue. Total

capillary length (L) per heart was calculated using the volume of the left ventricle (V) according to the formula $L = LV \times V$. Intercapillary distance (i.e., the distance between the centers of two adjacent intramyocardial capillaries) was calculated according to a modification of the formula of Henquell and Honig as described previously by Gross et al. (20). Volume density (VV) of the capillaries, interstitial tissue, and myocytes was obtained using the point counting method according to the equation $PP = VV$, where PP is point density. Reference volume was the total myocardial tissue (exclusive of noncapillary vessels, that is, arterioles and veins).

One-millimeter-thick sections of the TA were cut perpendicular to the vessel axis, embedded in paraffin, and stained with hematoxylin-eosin to visualize the lamina elastica interna and externa of the aorta. Pictures were taken with a camera (Olympus U-TVO.5XC-2, Shinjuku-ku, Japan) attached to a microscope (Olympus BX50F-3) at a high-power magnification ($\times 400$). The ratio of wall thickness (intima + media) to lumen diameter was analyzed using a semiautomatic image analysis system (Scion, NIH, Bethesda, MD) (3). All investigations were performed in a blinded manner.

Analysis of atherosclerotic lesions. Atherosclerotic lesion severity was assessed using en face preparations of descending aortas as previously described (47). Briefly, dissected aortas were washed in distilled water and then in 100% propylene glycol and stained with 1% oil red-O (Sigma, Budapest, Hungary) for 10 min at room temperature. Aortas were further washed in 85 and 50% propylene glycol and distilled water, mounted on glass slides, and coverslipped using an aqueous medium (Aquatex, Merck). Stained plaques were quantified by microscopy and computer-aided morphometry (ImagePro Plus, Media Cybernetics) and given as a percentage of plaque per total area (Fig. 1).

In vivo blood pressure. For the determination of long-term consequences of elevated circulating TGF- β on endothelial function, blood pressure was determined in 8-mo-old mice. For blood pressure measurement, mice were anesthetized with ketamine (150 mg/kg)-xylazine (15 mg/kg). Narkosis induced significant hypotension compared

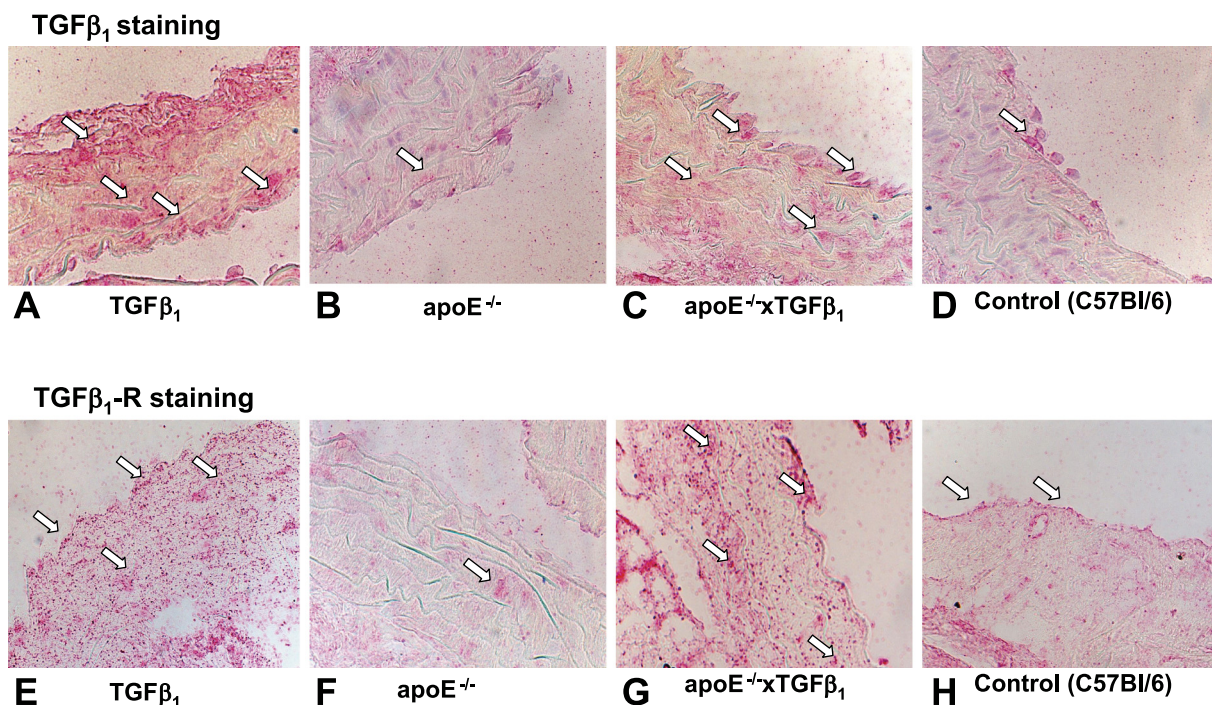


Fig. 3. TGF- β_1 (A–D) and TGF- β_1 receptor (TGF- β_1 R) staining in aorta (E–H) of TGF β_1 transgene (A and E), apoE^{-/-} (B and F), apoE^{-/-} \times TGF β_1 (C and G), and control (C57BL/6) mice (D and H). Both TGF- β_1 and TGF- β_1 R staining were more intense in TGF β_1 transgene-bearing mice. Arrows indicate endothelial cells and smooth muscle cells with positive immunostaining for TGF- β_1 and TGF- β receptor.

with conscious animals as described in previous studies (6). The left common carotid artery was cannulated for measurement of arterial blood pressure. Systolic and diastolic blood pressure were determined with a Cardiosys CO-104 system (Experimetria, Budapest, Hungary).

Statistics. All data are means \pm SD. Comparisons were made between the different experimental groups using the Kruskal-Wallis test and Mann-Whitney *U*-test, with Tukey's post hoc test. A value of $P \leq 0.05$ was considered significant.

RESULTS

TGF- β plasma levels and immunohistology. TGF- β_1 immunostaining appeared in the endothelial and muscular layers of aortas (Figs. 2 and 3, A–D). Staining was significantly stronger in aorta of TGF- β transgene-bearing (TGF β_1 and apoE $^{-/-}$ \times TGF β_1) mice compared with C57Bl/6 animals. Plasma TGF- β_1 levels (Fig. 2) of apoE $^{-/-}$ mice as measured by ELISA did not differ significantly from that of C57Bl/6 controls. Despite higher plasma and tissue TGF- β_1 expression, TGF- β receptor I staining (Figs. 2 and 3, E–H) was stronger in apoE $^{-/-}$ \times TGF β_1 and TGF β_1 mice compared with C57Bl/6 controls.

Superoxide detection. Images obtained with confocal microscopy demonstrated practically no dihydroethidine (red) staining in control (C57Bl/6) animals (Fig. 4, A–D), demon-

strating a lack of O $_2^{\cdot -}$ production. Mild staining, similar to that in C57Bl/6 mice, was observed in aortas from TGF β_1 and apoE $^{-/-}$ animals. Intense red staining in the interstitium and yellow nuclei with a 1.6-fold increase (Fig. 4E) in fluorescence intensity of dihydroethidine staining was observed in the double-gene-modified apoE $^{-/-}$ \times TGF β_1 mice compared with C57Bl/6 mice.

Ex vivo determination of NO-dependent relaxation of TA. Endothelium-mediated aortic relaxation induced by carbachol was similar in the single-gene-modified (TGF β_1 and apoE $^{-/-}$) mice compared with the control (C57Bl/6) mice (Table 1). However, carbachol-induced relaxation was impaired in the apoE $^{-/-}$ \times TGF β_1 (double gene modified) mice compared with controls.

After incubation of aortic rings with apocynin (*experimental protocol 1*) or SOD (*experimental protocol 2*), there was no significant improvement in control (C57Bl/6) apoE $^{-/-}$ and TGF β_1 mice. In the apoE $^{-/-}$ \times TGF β_1 mice, both apocynin and SOD significantly improved carbachol-induced relaxation.

Myocardial hypertrophy and body weight. Despite similar body weight at harvest, heart weight was higher in all apoE $^{-/-}$ animals compared with C57Bl/6 mice (Table 2). TGF- β_1 alone did not elevate relative heart weight significantly. However,

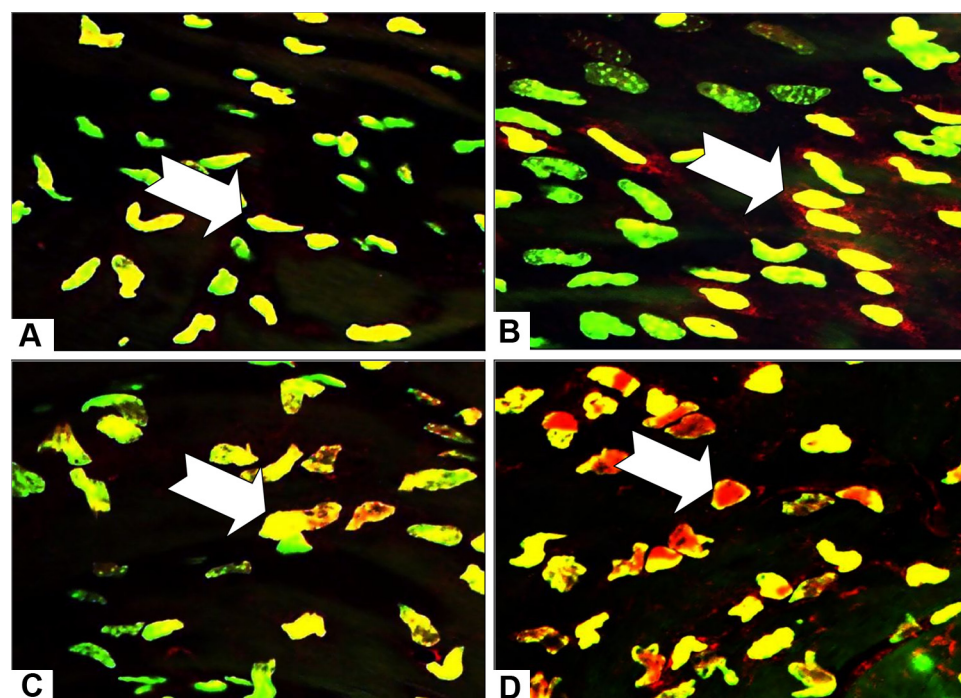


Fig. 4. Representative confocal images of dihydroethidine staining (red fluorescence) in endothelial cells of en face aortas from C57Bl/6 (A), TGF β_1 (B), apoE $^{-/-}$ (C), and apoE $^{-/-}$ \times TGF β_1 mice (D). Original magnification, $\times 1,000$. Hoechst counterstaining of endothelial cell nuclei is shown as pseudocolor green. Nuclei of cells that exhibit significant superoxide production appear in yellow (red + green). Bar graph (E) shows summary data for fluorescence intensity of dihydroethidine staining (fold change values compared with control C57Bl/6). Data are means \pm SE. * $P < 0.05$.

Table 1. Carbachol-induced relaxation of thoracic aorta

	Protocol 1		Protocol 2	
	CBH response	After apocynin	CBH response	After SOD
Control (C57Bl/6)	84 \pm 5	87 \pm 6	82 \pm 3	83 \pm 5
apoE ^{-/-} \times TGF β ₁	61 \pm 6 [†]	76 \pm 3*	59 \pm 4 [†]	69 \pm 5*
apoE ^{-/-}	75 \pm 5	82 \pm 4	73 \pm 4	77 \pm 5
TGF β ₁	81 \pm 6	87 \pm 4	75 \pm 4	80 \pm 4

Values are means \pm SE ($n = 10$ /group) of carbachol (CBH) responses before and after apocynin (protocol 1) and before and after superoxide dismutase (SOD; protocol 2) in control (C57Bl/6) mice, apolipoprotein-deficient (apoE^{-/-}) mice bearing transforming growth factor- β ₁ transgene (apoE^{-/-} \times TGF β ₁), apoE^{-/-} mice, and TGF β ₁ mice. * $P < 0.05$ vs. control (C57Bl/6). [†] $P < 0.05$, before vs. after incubation.

high TGF- β ₁ levels induced further significant heart weight gain in apoE^{-/-} \times TGF β ₁ compared with apoE^{-/-} mice. The wet-to-dry weight ratio was similar in all groups, demonstrating that the observed differences were not due to differences in blood removal or perfusion of the hearts.

Morphometry. In hearts of control (C57Bl/6) mice, lack of fibrotic changes was demonstrated by normal capillary density per surface area without significant appearance of fibrocytes in the interstitium (Table 3 and Fig. 5). Density of capillary profiles did not differ significantly in apoE^{-/-} and TGF β ₁ mice compared with those of C57Bl/6 mice. Loss of myocardial capillarization was observed as significantly lower capillary length density and lower relative capillary volume in apoE^{-/-} \times TGF β ₁ mice compared with C57Bl/6 mice. Relative fibrocyte volume was normal in C57Bl/6 and apoE^{-/-} mice. Expansion of the nonvascular myocardial interstitium with elevation of relative fibrocyte volume was significant in the apoE^{-/-} \times TGF β ₁ and TGF β ₁ mice compared with apoE^{-/-} and C57Bl/6 mice.

Aortic wall thickness was $\sim 5\%$ of the lumen in control (C57Bl/6) mice. Aortic wall thickness did not differ significantly from C57Bl/6 values in single-gene-modified apoE^{-/-} and TGF β ₁ mice. This ratio was significantly elevated only in the apoE^{-/-} \times TGF β ₁ group.

En face fat staining in aorta. Oil red-O en face staining was practically negative in aortic arches and roots isolated from nonatherosclerotic TGF β ₁ and control (C57Bl/6) mice (Fig. 6). Significant staining was observed in apoE^{-/-} mice. Plaque area and staining intensity demonstrated more prominent atherosclerotic lesions in double-gene-modified apoE^{-/-} \times TGF β ₁ mice compared with apoE^{-/-} animals.

In vivo blood pressure. Intra-aortic blood pressure was similar in TGF β ₁ mice compared with control (C57Bl/6) mice (Table 4). Blood pressure was significantly elevated in apoE^{-/-} mice compared with control (C57Bl/6) mice. Double-gene-modified apoE^{-/-} \times TGF β ₁ animals had the highest blood pressure, significantly higher compared with that of apoE^{-/-} mice.

DISCUSSION

TGF- β ₁ has long been known to play multifaceted roles in vascular pathophysiology. On one hand, TGF- β ₁ is a profibrotic cytokine when produced in a scarring niche (50). On the other hand, it also exerts important inflammation inhibitory effects as the main cytokine of regulatory T cells (34). Our study adds a new dimension to these results, demonstrating that systemic overexpression of TGF- β ₁ is associated with increased vascular O₂⁻ production and endothelial dysfunction in a mouse model of atherosclerosis. Furthermore, we also have demonstrated that NOX activation contributes to TGF- β ₁-induced endothelial impairment associated with more severe atherosclerotic plaque formation, more severe hypertension, and hypertrophy and fibrosis of the heart.

Although much is known about the profibrotic and inflammation inhibitory effects of local TGF- β production, data on direct vascular effects of systemic TGF- β ₁ levels are scarce. TGF- β is expressed in most cell types and tissues such as lung, kidney, bone, macrophages, smooth muscle cells, and platelets, among others. Focusing on the vasculature, platelets contain high concentrations of TGF- β , and on degranulation at a site of injury, platelets release TGF- β into surrounding tissues (4). In addition, in murine lung vasculature, high expression of all three TGF- β isoform mRNA transcripts was detected in smooth muscle cells of large vessels (48). Furthermore, both β ₁- and β ₂-isoforms were detected in vascular endothelial cells (10).

Regulation of TGF- β expression in the vasculature is not clearly understood. Mechanical stretch or vascular injury may stimulate TGF- β mRNA and protein expression in vascular parenchymal cells and infiltrating lymphoid cells, particularly vascular smooth muscle cells and macrophages (5). Angiotensin II (ANG II) can induce TGF- β expression in vascular smooth muscle cells (28) and can activate the Smad pathway independently of TGF- β . Furthermore, ANG II shares many intracellular signaling elements with TGF- β , implicated in fibrosis (54). These data suggest a possible cross talk or

Table 2. Bodyweight and heart weight at time of experiment termination in 4-mo-old mice

Genotype	Body weight, g	Heart weight wet, mg	Heart weight dry, mg	Wet/Dry Ratio
Control (C57Bl/6)	27 \pm 3.25	118 \pm 12	28 \pm 3	4.2 \pm 0.24
apoE ^{-/-} \times TGF β ₁	31 \pm 3.2*	181 \pm 11* [†]	44 \pm 5*	4.1 \pm 0.33
apoE ^{-/-}	30 \pm 3.45*	165 \pm 8*	38 \pm 4*	4.3 \pm 0.45
TGF β ₁	28 \pm 4.2	142 \pm 14	32 \pm 3	4.4 \pm 0.56

Values are means \pm SE. * $P < 0.05$ vs. control (C57Bl/6). [†] $P < 0.05$ vs. apoE^{-/-}

Table 3. Myocardial and aortic morphometry

	Myocardium			
	Relative capillary volume, %	Relative fibrocyte volume, %	Capillary length density, mm ² /mm ³	AortaWall/Lumen, %
Control (C57Bl/6)	4.1 ± 0.8	0.8 ± 0.05	6,484 ± 917	5.5 ± 0.4
apoE ^{-/-} × TGF β ₁	2.5 ± 0.9*	1.1 ± 0.2*†	4,783 ± 394*	6.9 ± 0.5*
apoE ^{-/-}	3.0 ± 0.8	0.8 ± 0.2	5,328 ± 540	6.3 ± 0.3
TGF β ₁	2.9 ± 0.9	1.0 ± 0.1*†	5,415 ± 814	6.6 ± 0.5

Values are means ± SE (*n* = 8/group). **P* < 0.05 vs. control (C57Bl/6). †*P* < 0.05 vs. apoE^{-/-}.

parallel effects of ANG II and TGF- β in fibrotic and vascular processes, including atherosclerosis.

Furthermore, TGF- β is able to induce its own production, in a self-perpetuating manner, which may be an important factor of the progressive nature of chronic scarring and fibrosis. Furthermore, TGF- β -induced TGF- β expression may be responsible for the observed overexpression of TGF- β in our isolated aortas (Fig. 3).

Profibrotic effects of TGF- β ₁ can be mediated indirectly by several growth factors. TGF- β ₁ stimulates plasminogen activator inhibitor-1 (PAI-1) expression in vascular smooth muscle cells. Increased PAI-1 level was associated with increased occurrence of thrombosis, progression of fibrotic processes, and remodeling (57). Furthermore, TGF- β stimulates platelet-derived growth factor-B (PDGF-B) synthesis by endothelial cells, causing fibroblast growth factor (FGF) and connective tissue growth factor (CTGF) release from the subendothelial matrix, and promotes vascular endothelial growth factor (VEGF) synthesis. Endothelial cell-derived PDGF-B and FGF influence the proliferation and migration of neighboring cells. Together, these mediators control angiogenesis and vascular repair. Thus endothelial cells and TGF- β actions on the endothelium play important roles during both the initial phase of

immune injury and the later remodeling phase of atherosclerosis (49).

Studies emphasizing the inflammation inhibitory effects of TGF- β ₁ have concluded that inflammation inhibitory effects are protective (18, 19), whereas studies focusing on the profibrotic effects have concluded that plaque stabilization by TGF- β ₁ is beneficial to prevent complications (3, 30, 38). These studies in favor of the protective cytokine hypothesis investigated local effects of TGF- β in the vessel wall (18, 50) or disruption of TGF- β production in T cells (39, 51). These studies examined local, endogenous production of TGF- β ₁ and focused on inflammation inhibitory effects of this cytokine.

A recent study investigated the role of TGF- β ₁ in atherosclerosis and aortic aneurysm formation in 4.5-mo-old female apoE^{-/-} mice on a Western diet with doxycycline-regulated overexpression of TGF- β ₁ in the heart. After doxycycline withdrawal, elevated cardiac TGF- β ₁ expression was accompanied by fewer atherosclerotic lesions in the aorta, less aortic root dilation, and fewer pseudoaneurysms. In the background of fewer lesions, TGF- β ₁ did not affect plasma lipids but reduced T lymphocytic infiltration of the aortas and reduced inflammatory cytokine expression. In the background of fewer aneurysm formations, metalloproteinase inhibition was dem-

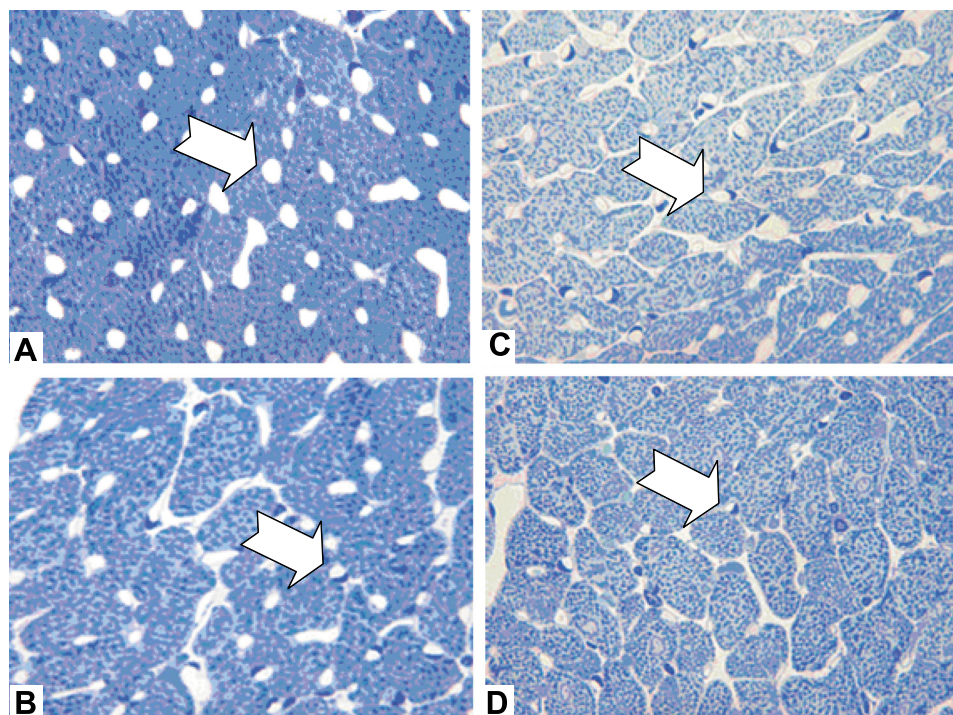
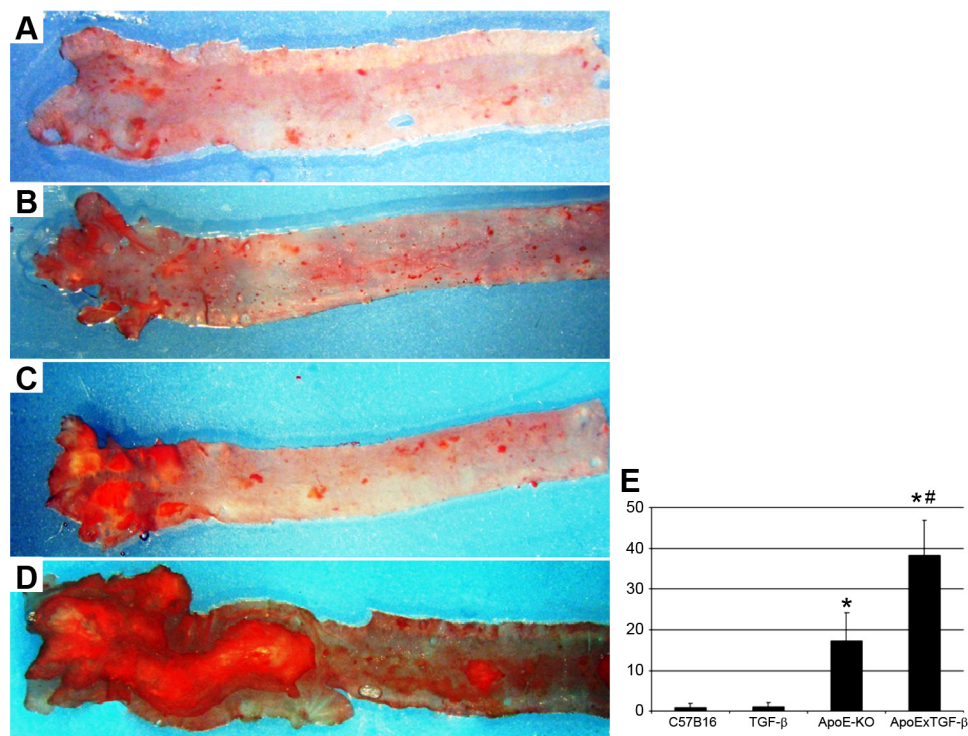


Fig. 5. Representative morphometric images. Capillarization of the heart is shown in semithin sections from control C57Bl/6 (A), apoE^{-/-} (B), TGF β ₁ (C), and apoE^{-/-} × TGF β ₁ mice (D). Original magnification, 1:1,200.

Fig. 6. Representative oil red-O-stained images (A–D). Bar graph (E) shows quantification of atherosclerotic lesions and plaques in the wall of aortic arches and roots. * $P < 0.05$ vs. control (C57Bl/6) mice. # $P < 0.05$ vs. apoE^{-/-} mice.



onstrated. This study thus investigated local inflammation inhibitory and fibrosis-promoting effects of TGF- β_1 overexpressed in the heart, but vasoactive effects of circulating TGF- β_1 were not investigated. Furthermore, local TGF- β_1 producing regulatory T lymphocytes in sites of atherosclerotic lesions were not investigated either (16). No previous studies examined the effects of high systemic TGF- β_1 levels on endothelial function and oxidative balance of the vessel wall.

Previous studies already have suggested that TGF- β may induce NOX enzyme synthesis and consequent superoxide production in vascular endothelial cells. Recent studies provided novel evidence for TGF- β -induced ROS production and cytoskeletal alterations in human endothelial cells, mediated by a NOX-4-dependent pathway (26, 40), and indicated that oxidative stress can lead to vascular endothelial damage and dysfunction (32). Further data suggest a role of NOX4-Smad2/3 signaling in cardiac fibroblast differentiation to myofibroblasts and extracellular matrix (ECM) production in response to TGF- β (11). In addition to NOX stimulation, further indirect effects of TGF- β on the vasculature have been described acting through factors regulating vascular remodeling and endothelial function. TGF- β_1 , secreted by activated plate-

lets, is involved in wound healing and regulation of local vascular tone by stimulating endothelin-1 (ET-1) production in endothelial cells. Thus TGF- β_1 overexpression may contribute to high blood pressure through stimulation of endothelial NOX or ET isoforms (32a).

In this study, isolated aortic rings of 4-mo-old apoE^{-/-} mice with elevated plasma TGF- β level had impaired endothelial relaxation, suggesting that endothelial dysfunction of apoE knockout mice is exacerbated by elevated systemic TGF- β_1 . Overexpression of TGF- β_1 was ensured by the transgene construct of porcine TGF- β_1 selectively expressed in hepatocytes under the control of murine albumin promoter and enhancer. Furthermore, NOX inhibition with apocynin and scavenging ROS by SOD partly improved endothelial function, suggesting that the stimulated NOX system is responsible for endothelial dysfunction of apoE^{-/-} × TGF β_1 mice.

Similar findings have been demonstrated previously. TGF- β_1 has been found to stimulate ROS production in a variety of cell types such as human pulmonary smooth muscle cells, (60), lung fibroblasts (31, 61), and hepatoma cells (58), including endothelial cells (22). NOX complexes have been demonstrated to be the main source of ROS in the vessel wall (7a). This redox-sensitive signaling in human endothelial cells (17) is stimulated and may become overactivated by TGF- β_1 (26).

In the background of significantly inhibited endothelium-dependent vascular relaxation in aortas of apoE^{-/-} × TGF β_1 animals, we observed enhanced superoxide formation. The most probable pathomechanism is that NO-dependent relaxation was inhibited due to NO scavenging of superoxide by peroxynitrite (PON) formation (46). The NO-dependent relaxation of the vascular wall is highly vulnerable to ROS as reduced bioavailability of NO leads to endothelial dysfunction. Our data provide evidence that NOX-induced ROS production

Table 4. *In vivo* blood pressure measured in anesthetized 8-mo-old mice

	Blood Pressure, mmHg	
	Systole	Diastole
Control (C57Bl/6)	77 ± 8	58 ± 7
apoE ^{-/-} × TGF β	121 ± 10*†	90 ± 11*†
apoE ^{-/-}	97 ± 7*	75 ± 8
TGF β_1	78 ± 8	64 ± 11

Values are means ± SE ($n = 8$ /group). * $P < 0.05$ vs. control (C57Bl/6). † $P < 0.05$ vs. apoE^{-/-}.

is responsible for endothelial dysfunction in the case of high systemic TGF- β_1 levels.

Interestingly, in our study high systemic TGF- β_1 levels alone were not sufficient to significantly inhibit carbachol-induced relaxation of the aorta in non-apoE^{-/-} C57Bl/6 control mice. A possible explanation is that an ongoing vascular wall injury in apoE^{-/-} mice, such as oxidized LDL (oxLDL) and other lipid deposition and consequent inflammation may be necessary for the deleterious effects of TGF- β_1 to develop.

Regarding our model, the finding that TGF- β receptor I expression was even stronger in mice carrying the TGF β_1 transgene than in C57Bl/6 controls may have been a consequence of receptor upregulation due to elevated systemic TGF- β_1 levels, as has been demonstrated previously (41).

Vascular endothelial dysfunction plays a pivotal role in the pathogenesis of atherosclerosis and hypertension (27, 44). In our study, overproduction of circulating TGF- β_1 elevated blood pressure and markedly increased plaque formation in apoE^{-/-} mice. TGF- β_1 is also well known for its profibrotic effects. TGF- β_1 causes interstitial fibrosis by inducing epithelial-to-myofibroblast transformation (EMT). Myofibroblasts are a key source of increased ECM synthesis in fibrotic processes (45). EMT contributes to renal fibrosis in mice (35). TGF- β also may be responsible for age-related functional and structural changes in the kidney. Age-related changes in the activity and responsiveness of the renin-angiotensin system (RAS) may contribute to stimulation of TGF- β production, leading to NOX-dependent ROS generation, vascular and mesangial cell proliferation, and hypertrophy (12).

TGF- β_1 also mediated proliferation of pulmonary arteriolar smooth muscle cells through NOX4 activation by increasing ROS production (60). Our findings demonstrate that elevated circulating TGF- β_1 enhanced aortic endothelial superoxide production, impairing endothelial function and leading to aortic wall thickening and aggravated plaque formation in apoE^{-/-} \times TGF β_1 mice.

In addition to direct profibrotic effects of TGF- β_1 , redox-sensitive signaling pathways also mediate TGF- β_1 -induced cardiac hypertrophy, fibrosis, and structural remodeling in chronic disease states (7, 23). Several cardiac diseases are associated with an increased expression of TGF- β , particularly during the transition from stable cardiac hypertrophy to heart failure. TGF- β_1 induces cardiomyocyte contractile dysfunction associated with enhanced ROS production and oxidative alterations in Ca²⁺ handling proteins (36). In the pathomechanism of cardiac hypertrophy, besides myocyte hypertrophy, cardiac interstitial fibroblast proliferation, and ECM synthesis are involved (1). Similarly to previous studies, which demonstrated TGF- β -induced cardiac hypertrophy in mice (52), our study demonstrates that overexpression of TGF- β_1 promotes NOX-mediated myocardial hypertrophy and fibrosis.

Our present study may explain some recent findings in human subjects, where atherosclerosis has been associated with elevated systemic TGF- β_1 levels. Substantial evidence from recent studies demonstrates that elevated systemic TGF- β_1 levels may exacerbate atherosclerosis. Specimens retrieved from the superficial femoral artery of patients undergoing atherectomy for primary or recurrent atherosclerotic disease demonstrated an essential role of TGF- β_1 through Smad3 signaling in smooth muscle cell (SMC) proliferation, leading to restenosis in humans. Restenosis was associated

with higher Smad3 expression and more SMCs in restenotic lesions. Overexpression of Smad3 enhanced, whereas inhibition of Smad3 reduced, SMC proliferation (15). Furthermore, TGF- β_1 was positively correlated with body mass index and creatinine clearance in patients with essential hypertension (62). Also, women with gestational diabetes mellitus and obesity were found to have significantly higher plasma TGF- β_1 levels than healthy controls, and serum TGF- β_1 levels correlated with postprandial glucose, age, and body mass index in these patients (63).

In conclusion, elevated circulating TGF- β_1 levels in apoE^{-/-} mice, but not in C57Bl/6 normal mice, induced endothelium-mediated vasomotor dysfunction through stimulation of NADPH oxidase-derived oxidative stress. Furthermore, high circulating TGF- β_1 was associated with aortic wall thickening, acceleration of plaque formation, and consequent hypertension, with myocardial hypertrophy and fibrosis. Thus TGF- β_1 may exacerbate the ongoing inflammation in an atherosclerotic surrounding in apoE^{-/-} mice. This mechanism may provide a link between systemic overproduction of TGF- β_1 and acceleration of atherosclerosis.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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