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Original Article

Vascular hypercontractility and endothelial dysfunction before development of atherosclerosis in moderate dyslipidemia: role for nitric oxide and interleukin-6

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Abstract: Atherosclerosis is a chronic disease that affects peripheral arteries and the aorta. Several inflammatory processes are required until the production of an atheroma. Before the atheroma appears, endothelial dysfunction is a key event. We hypothesized that endothelial dysfunction occurs in a mouse model of mild dyslipidemia, the mouse deficient in apolipoprotein E (apoE$^{+/-}$). Using aortic rings preparation, we found that apoE$^{+/-}$ mice showed increased developed tension in response to KCl 60 mM when using a range a pre-loads from 0.5 to 2.0 grams ($p = 0.038$). Next, we tested the vasorelaxant capacity of apoE$^{+/-}$ aortas (pre-contracted with phenylephrine) in response to acetylcholine, an endothelium-dependent vasodilator. ApoE$^{+/-}$ aortas showed diminished vasorelaxation in a range of Ach concentrations ($p = 0.0032$). Next we assessed the levels of plasma NO metabolites, nitrite plus nitrate. These were significantly reduced, along with a significant decrease of the endothelial nitric oxide synthase in ApoE$^{+/-}$ mice. When we analyzed the morphology of the aortas in apoE$^{+/-}$ mice, these showed no signs of atheroma. In addition, we analyzed the levels of inflammatory cytokines, TNF-alpha, MCP-1 and interleukin 6 (Il-6). While TNF-alpha was similar in both groups, (18.3 ± 2 pg/mL in wild type vs. 17.5 ± 2 pg/mL in apoE$^{+/-}$), MCP-1 was increased in ApoE deficient mice (71.5 ± 0.8 pg/mL in wild type vs. 85.1 ± 7.4 pg/mL in ApoE$^{+/-}$ mice, $p = 0.006$), along with IL-6 (24.7 ± 1.7 pg/ml in wild type vs. 47.1 ± 12.5 in ApoE mice, $p = 0.0055$). These results suggest that mild dyslipidemia produces a pro-inflammatory state, associated with diminished NOS and NO production, which produces endothelial dysfunction.

Keywords: Atherosclerosis, endothelial dysfunction, nitric oxide, eNOS, apoE

Introduction

Atherosclerosis is a chronic inflammatory disease that affects peripheral arteries and the aorta [1]. Several inflammatory processes are required until the production of an atheroma [2]. Before the atheroma appears, endothelial dysfunction is a key event, as the subclinical state of the disease [3]. This is associated with increased expression of adhesion molecules, decreased nitric oxide (NO) bioavailability, and increased platelet adhesion. At the vascular wall, nitric oxide regulates leukocyte adhesion, vessel tone [4], vascular permeability [5] and smooth muscle proliferation.

This alterations may be influenced by several factors such as genetics, age, smoking diabetes mellitus and hypercholesterolemia [6]. These factors eventually lead to a pro-inflammatory state, associated to oxidative stress and endothelial dysfunction, which is key process that leads to the development of atherosclerosis [7].

Endothelial dysfunction corresponds to the impairment of capacity of endothelial cells to regulate vessel tone, fibrinolyisis, leukocyte adhesion, platelet aggregation and smooth muscle proliferation. Typically this state is characterized by reduced NO bioavailability. This may be due to increase in oxidative stress, increased nitric oxide synthase (eNOS) uncoupling, increase in eNOS inhibitors, and reduced eNOS transcription, among other factors [7, 8].

Several cytokines have been described to participate in the pathogenesis of atherosclerosis

[9, 10]. Interleukin 1, 6, tumor necrosis factor-alpha (TNF-α), monocyte chemo-attracting protein 1 (MCP-1) and several others, are produced by macrophages, T-cells, monocytes, endothelial and vascular smooth muscle cells. In smooth muscle cells, cytokines activate protein kinase C, Rho-kinase, and MAPK pathways, which promote cell growth, migration, and contractility.

Endothelial dysfunction has been considered as a predicting factor for the development of atherosclerosis [11]. The aim of this study was to analyze the state of vascular endothelium in a model of chronic mild dyslipidemia and the role of eNOS and pro-inflammatory cytokines.

Materials and methods

Animals

Mice heterozygous for apolipoprotein E ($ApoE^{+/-}$, n = 15) and controls C57B1/6 (n = 23) between 8 y 12 weeks of age, males and females, were used. Animals were maintained in the animal facility of Universidad de Talca, with food and water *ad libitum*, at room temperature (22 ± 5°C) and cycles of 12 hrs light/darkness. The diet used was obtained from Champion® (20.5% crude protein, 5% fiber, 4% fat). All procedures were performed in accordance with the guidelines of the institutional Animal Care and Use Committee; in conform to the NIH *Guide for the Care and Use of Laboratory Animals.*

Aorta dissection

Mice were anesthetized with an intraperitoneal injection of ketamine 10% (Ketostop; Drogas Pharma-Invetec, Santiago-Chile), xylazine 2% (Xylavet; Alfasan International BV, Holland), acepromazine 1% (Drag Pharma) and pre-medicated with 100 UI i.p of heparin. After deep anesthesia, a midline incision was made and the aorta obtained in block, and immersed in Krebs modified NaCl 115 mM, KCl 4.7 mM, $CaCl_2$ 2.5 mM, $MgSO_4$ 1.2 mM, KH_2PO_4 1.2 mM, $NaHCO_3$ 25 mM, EDTA 0.01 mM and glucose 11.1 mM. The heart was removed and immersed in NaCl 0.9%.

Vascular reactivity

Once the aorta was cleaned, it was cut into 3-4 mm sections and one end of the vascular strip was attached to a nickel-covered steel hook using a thread loop and the other end was connected to a Grass force transducer (FT03; Grass instruments, Quincy, Massachusetts). Vascular strips were allowed to equilibrate for 1 hour in a water-jacketed, temperature controlled tissue bath filled with 10 mL Krebs solution continuously bubbled with 95% O_2 5% CO_2 at 37°C. The changes in isometric tension were recorded on a Grass polygraph (Model 79D Grass Instruments).

To study the contraction at different stretching loads, the aortic rings were stretched to reach 0.5, 1, 1.5 and 2 g of developed tension. For this, they were stabilized for 60 min at each tension and then challenged with KCl 60 mM. After reaching a maximal developed tension, the chamber was washed until maximal relaxation was reached. The procedure was repeated with another stretching tension.

To test the phenylephrine contraction, the viability of the ring was first tested with a KCl challenge. For this, the vessels were stretched to develop 2 g of active tension. After viability was corroborated, KCl was washed and after reaching 2 g of tension, phenylephrine (5 μM) was applied to bath solution. To test for endothelium-dependent relaxation, the vessels were pre-contracted with phenylephrine (5 μM) at 2 g of developed tension. After reaching a plateau contraction, increasing doses of acetylcholine were applied to the bath to reach 0.01, 0.1, 1, 1.5 and 10 μM. The results are expressed as the percentage of relaxation induced by acetylcholine.

Western blotting

Cardiac tissue was homogenized in 1 ml of lysis buffer (Tris 50 mM, SDS 0.1%, NaCl 30 mM, EDTA 2 mM) supplemented with 10 μl of proteases inhibitors cocktail (MP Biomedicals, Solon, OH, EE.UU.) using an ultraturrax (3 cycles of 15 s). Then the homogenate was centrifuged at 4000 rpm for 10 min at 3°C. The supernatant was removed and mixed with loading buffer. Total protein concentration from hearts was determined using the BCA method, with the BCA Protein Assay de Thermo Scientific, Pierce Biotechnology (Rockford, IL, EE.UU.). Protein samples of each fraction (100 μg) were separated by 7% SDS-PAGE and blotted onto a nitrocellulose membrane (Bio-Rad, Hercules, CA). The membrane was incubated with a primary antibody overnight at 4°C and then with an

Table 1. Lipid profile of plasma from wild type and Apo$^{+/-}$ mice.

	Wild type	ApoE$^{+/-}$	*p* value (T test)
n	15	23	
Body weight (g)	30.4 ± 1.1	31.5 ± 1.6	0.56
Total cholesterol (mg/dL)	62.1 ± 5.9	102 ± 13.3	0.0147
Triglycerides (mg/dL)	80.5 ± 4.6	155.3 ± 17.9	0.0009
HDL (mg/dL)	66.1 ± 2.7	42.1 ± 4.2	0.0001
VLDL (mg/dL	19.0 ± 2.4	45.2 ± 6.2	0.0015
Glucose (mg/dL)	152.9 ± 10.4	132.1 ± 7.6	0.1184

HDL: high density lipoprotein, VLDL: very low density lipoprotein.

appropriate secondary antibody (2 hours at room temperature). The primary antibodies were used at the following dilutions: Anti-eNOS 1:2000; anti-tubulin1 1:2500. The protein bands were visualized with standard methods, using Western Lighting™ Chemiluminescence reagent (Perkin Elmer, Boston, MA), SuperSignal® West Femto (Pierce, Rockford, IL). Western blots were scanned and evaluated by densitometry analysis using Image J software.

Nitrite and nitrate determination

Nitrate (NO_3^-) and nitrite (NO_2^-) concentrations in plasma from ApoE$^{+/-}$ and controls (wild type) mice were determined using the Griess reaction. Briefly, plasma were filtered using Amicon Ultra 3K, PR03711, (Merck Millipore, Ireland) filters. The ultrafiltrate was analyzed using Griess reagents from Cayman Chemical Company (Ann Arbor, MI, EE. UU.) After the reaction took place, absorbance was measured at 570 nm.

Histology

Aortas were fixed in 10% formalin at pH 7.4. Once the samples were fixed and included in paraffin, sections of 5 μm were obtained using a rotating Microm HM325 microtome, then, were mounted on xylanized slides. Sections of hydrated and de-paraffinized tissues were stained with haematoxylin-eosin (HE) and Masson's trichrome.

Lipid profile

Blood was obtained from the inferior cava. After centrifugation, plasma glucose triglycerides, total cholesterol and HDL concentrations were determined by enzymatic methods, using commercially available reagents (Valtek, Santiago, Chile), following the manufactures instructions and using a spectrophotometer (Rayleigh UV 9200).

Cytokines

Inflammatory cytokines interleukin 6 (IL-6), TNF-α y MCP-1 were measured from plasma using commercial ELISA kits (Invitrogen).

Statistical analysis

Results are expressed as mean ± standard error. Comparisons between 2 groups were analyzed using *t* student test. For analysis of curves, two-way ANOVA was used. Statistical significance was set at a value of $p < 0.05$. These analyses were performed using GraphPad Prism 5 software for Windows (San Diego, California).

Results

First, we determined the lipid profile of ApoE$^{+/-}$ mice fed on a normal diet, to check for dyslipidemia (**Table 1**). We observed that total cholesterol, VLDL and triglycerides were increased in ApoE$^{+/-}$ mice compared to wild type. HDL on the other hand was significantly reduced in ApoE-deficient mice. These results confirm that the heterozygote mice for ApoE develop dyslipidemia, at least in our conditions of study, similar to was originally described for ApoE heterozygous mice [12, 13].

KCl-stimulated contraction at different stretching loads

Next, we evaluated vascular reactivity by determining the developed tension in response to KCl at different stretching loads (**Figure 1**). We found a significant increase in the tension developed by ApoE-deficient aortas compared to wild type. ($p = 0.0383$) in the whole range of pre-loads, being maximal at 1.5 grams. This result suggests increased contractility in the ApoE-deficient aortas.

Phenylephrine-induced contraction

Next, we assessed the degree of contraction in response to phenylephrine, a α1 agonist (**Figure 2A**). Phenylephrine-induced contractions (5 μM) for ApoE$^{+/-}$ aortas were not different among both groups 0.50 ± 0.09 in ApoE and 0.48 ± 0.06 g for WT (**Figure 2**) ($P = 0.83$).

Endothelium-dependent vasorelaxation

To assess the vascular response to an endothelium dependent vasodilator, we treated the

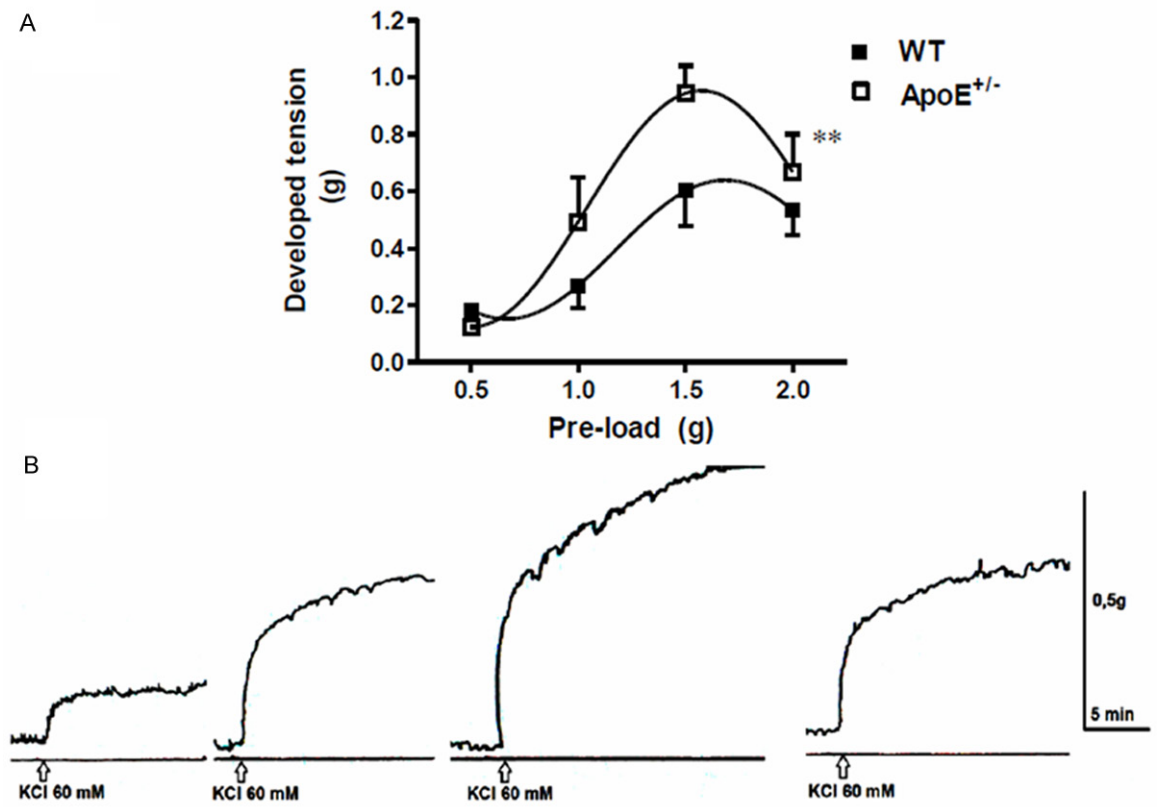

Figure 1. A. Contraction curves of aortas stimulated with KCl 60 mM to increasing stretching loads. (n = 3-6 WT, n = 4-14 ApoE$^{+/-}$). Asterisk indicates $p = 0.0383$ for the curves of WT vs. ApoE$^{+/-}$ (two-way ANOVA). B. Representative traces of KCl-stimulated contractions at different stretching loads.

aortas with increasing concentrations of acetylcholine (**Figure 2B**). For this, the aortic rings were stretched to develop 2 grams of tension, since at this tension both groups behave similarly, and were pre-contracted with 5 μM phenylephrine, that also was similar in both groups. In this condition, ApoE$^{+/-}$ vessels showed a diminished relaxant response to acetylcholine, compared to wild type ($P = 0.0055$) (**Figure 3**), with a -logEC_{50} of 5.81 ± 0.4 for ApoE-deficient mice vs. 6.91 ± 0.2 in wild type ($p < 0.0025$).

NO_2^- + NO_3^- content

Since ApoE-deficient aortas displayed diminished response to acetylcholine, we tested for the activity of the endothelial nitric oxide synthase (eNOS), a mayor mediator for the vasodilatatory effects of Ach. For this, we analyzed the levels of nitrate and nitrite, the main metabolites of NO in the blood (**Figure 3A**). We measured the concentration of these metabolites in the plasma of both strains. ApoE mice showed decreased concentration of NO_2^- + NO_3^-, 38.9 ± 4.3 μM in ApoE plasma vs. 70.2 ± 8.1 in wild type ($p = 0.0017$).

eNOS expression

Since the endothelium-dependent vasorelaxation and NO metabolites were diminished in ApoE$^{+/-}$ mice, we looked for eNOS expression in the vasculature. For this, we performed Western blot analysis form cardiac homogenates (**Figure 3B**). From these we observed decreased levels of eNOS (140 KDa) normalized to the expression of a housekeeping protein, tubulin (55 kDa) (1.63 ± 0.20 in wild type vs. 0.84 ± 0.15 arbitrary units, $p = 0.0314$).

Pro-inflammatory cytokines

Next, we measured the plasma levels of three important pro-inflammatory cytokines involved in the process of atherogenesis (**Figure 4**). While the levels of the tumor necrosis factor alpha (TNF-α) were not different between both groups (18.3 ± 3 pg m/mL in WT vs. 17.5 ± 2.4 in ApoE-deficient mice, $p = 0.8135$), the levels of interleukin 6 (Il-6) in ApoE deficient mice doubled those in wild type (24.0 ± 1.7 pg/mL WT vs. 47.1 ± 12.5 in ApoE$^{+/-}$, $p = 0.0055$). The levels of the monocyte chemo-attracting protein 1

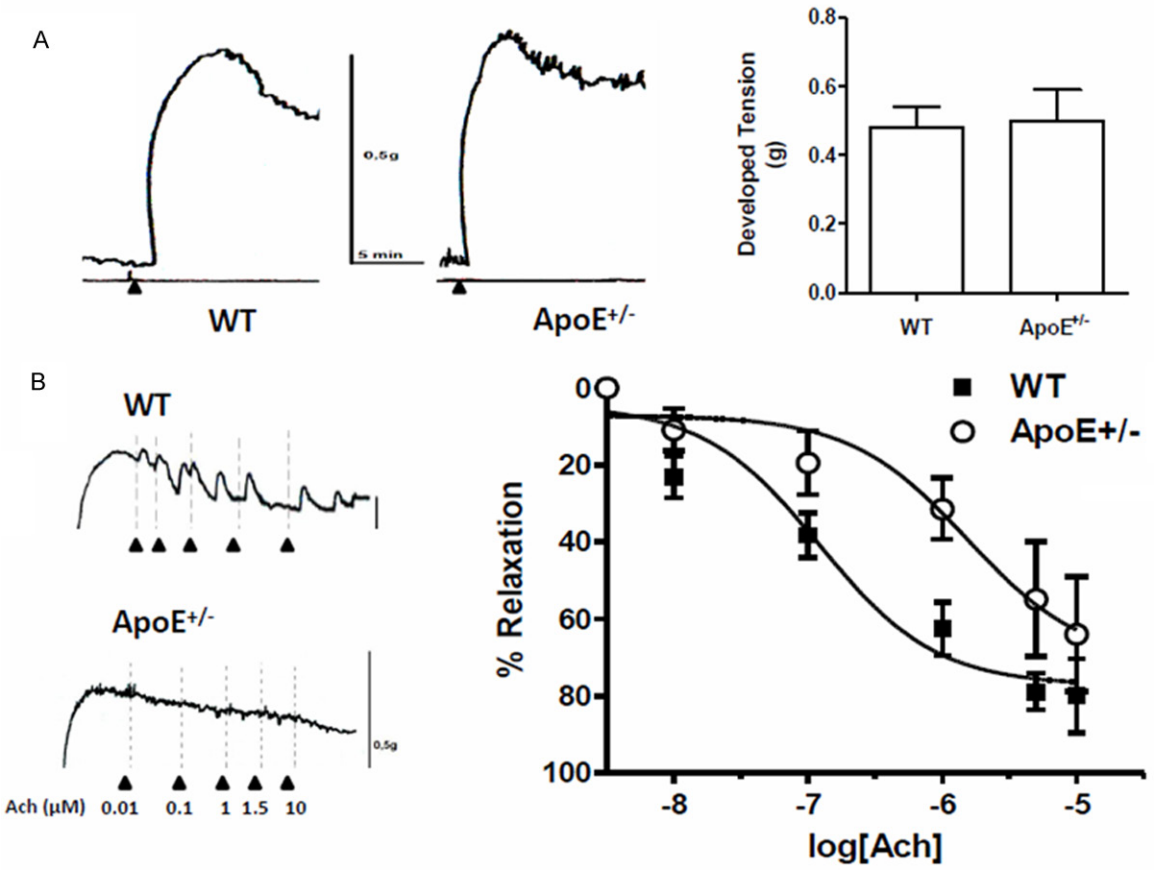

Figure 2. Endothelium-dependent vasorelaxation. A. Representative traces of phenylephrine (5 μM) - induced vasoconstrictions in WT and ApoE$^{+/-}$ aortas. The black arrow indicates the moment of application of the drug. The bars graph depicts the average ± SE for both groups. (n = 8 WT, n = 7 ApoE$^{+/-}$). B. Acetylcholine-induced vasorelaxation in WT and ApoE$^{+/-}$ aortas. Left, representative traces for the response to acetylcholine (Ach) in both groups, the black arrows indicate the time of application of Ach to reach the different concentrations. Right, the graph depicts the concentration-response to Ach in both groups. Double asterisk indicates P = 0.0032 WT vs. ApoE$^{+/-}$, n = 7 WT, n = 8 ApoE$^{+/-}$.

(MCP-1) were also increased, but modestly (71.1 ± 1.1 pg/mL wild type vs. 85.1 ± 7.4 in ApoE-deficient mice), but which is consistent with an Il-6 induced release of MCP-1.

Histological analysis

When we analyzed the histology of the aortas, by hematoxilin-eosin and Masson's trichrome we found a normal morphology in both groups, with absence of atheroma (**Figure 5**), ruling out the possibility that the endothelial dysfunction may have been produced by altered histoarquitecture of the vessels.

Discussion

Atherosclerosis is a pathological state of the vasculature that progress as dyslipidemia produces a state of endothelial dysfunction that ultimately leads to the accumulation of lipids and inflammatory cells in the arterial wall. Since the development of endothelial dysfunction is a key step in this process, we studied these initial events in an animal model of moderate dyslipidemia. The apoE heterozygous mouse feed a normal diet (4% fat) presented altered plasma lipids, in a similar way as a subclinical state of atherosclerosis. These mice presented endothelial dysfunction, evidenced by a hypercontractility of the aorta and a diminished response to an endothelial-dependent vasodilator, but not signs of atherosclerosis. This endothelial dysfunction was associated with reduced nitric oxide bioavailability, confirming previous observations [14, 15]. These reduced NO levels were accompanied by lower levels of eNOS. This differs from other studies in ApoE homozygous knockout mice, where no differences in eNOS were found, but increases in reactive-oxygen species (ROS) that quenched NO [15, 16].

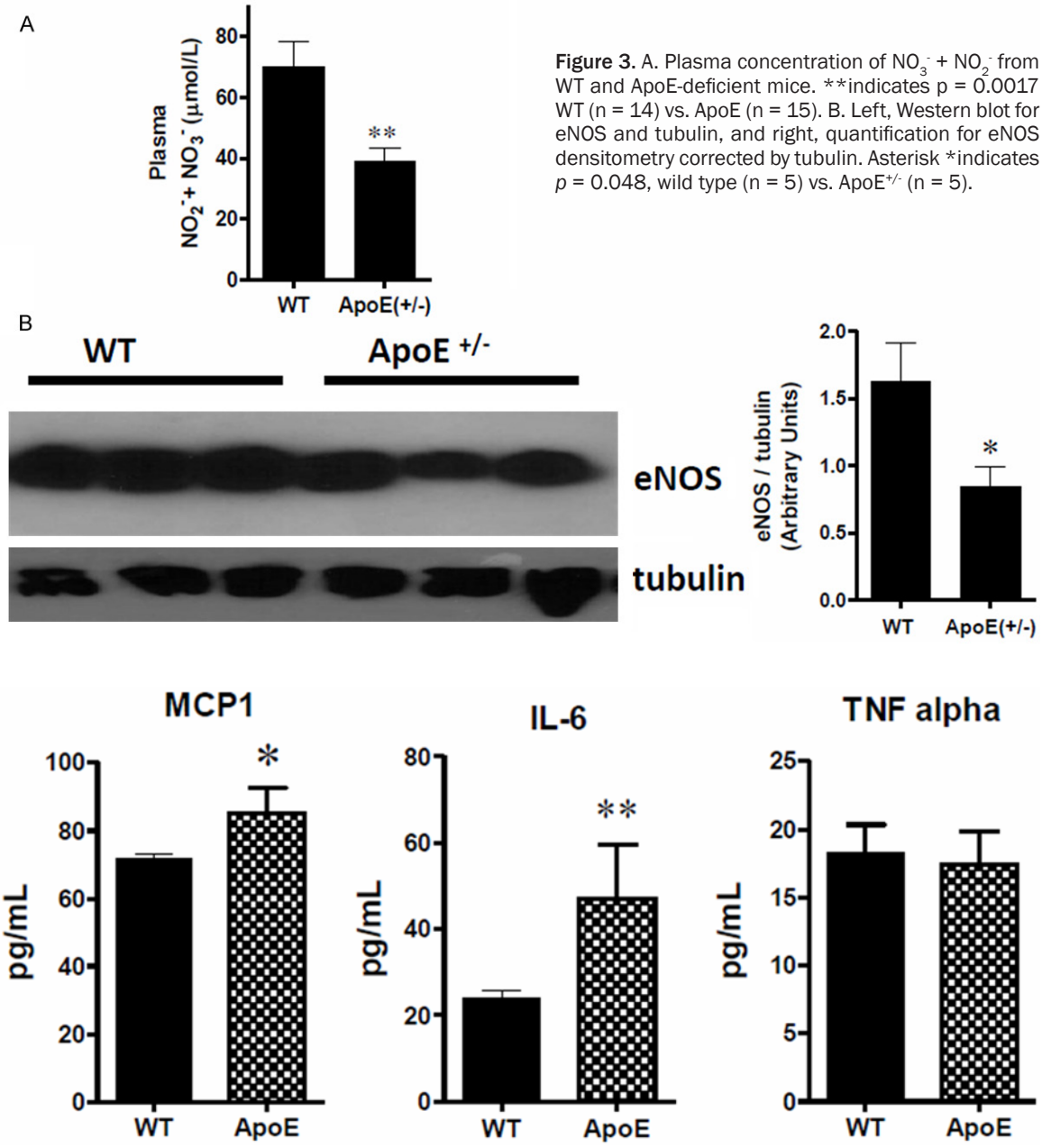

Figure 3. A. Plasma concentration of NO_3^- + NO_2^- from WT and ApoE-deficient mice. **indicates p = 0.0017 WT (n = 14) vs. ApoE (n = 15). B. Left, Western blot for eNOS and tubulin, and right, quantification for eNOS densitometry corrected by tubulin. Asterisk *indicates p = 0.048, wild type (n = 5) vs. $ApoE^{+/-}$ (n = 5).

Figure 4. Pro-inflammatory cytokines. Plasma levels of MCP-1, Interleukin-6 (Il-6) and tumor necrosis factor alpha (TNF-α) from wild type (WT, n = 8-13) and ApoE deficient ($ApoE^{+/-}$) mice (n = 4). *, p=0.019, **, p=0.0055 WT vs. ApoE.

In our study, the reduced eNOS levels were associated with increased plasma levels of IL-6, a pro-inflammatory cytokine that has been shown to be increased in subclinical atherosclerosis [17] and in patients with unstable coronary artery disease, circulating IL-6 is a strong independent marker of increased mortality [18].

Interestingly, IL-6 has been shown to reduce the expression of eNOS in endothelial cells by activation of the signal transducer and transactivator-3 (Stat-3) [19]. In addition, it has been shown that IL-6 is able to reduce NO production by inhibiting eNOS acutely [20]. IL-6 increases the association between eNOS and caveolin-1, a caveolae scaffold protein that inhibits eNOS tonically. Apparently, both mechanisms may operate *in vivo*, the reduction in expression and activity, which is consistent with our observation of a moderate, but significant decrease of eNOS but a clear decrease in NO metabolites.

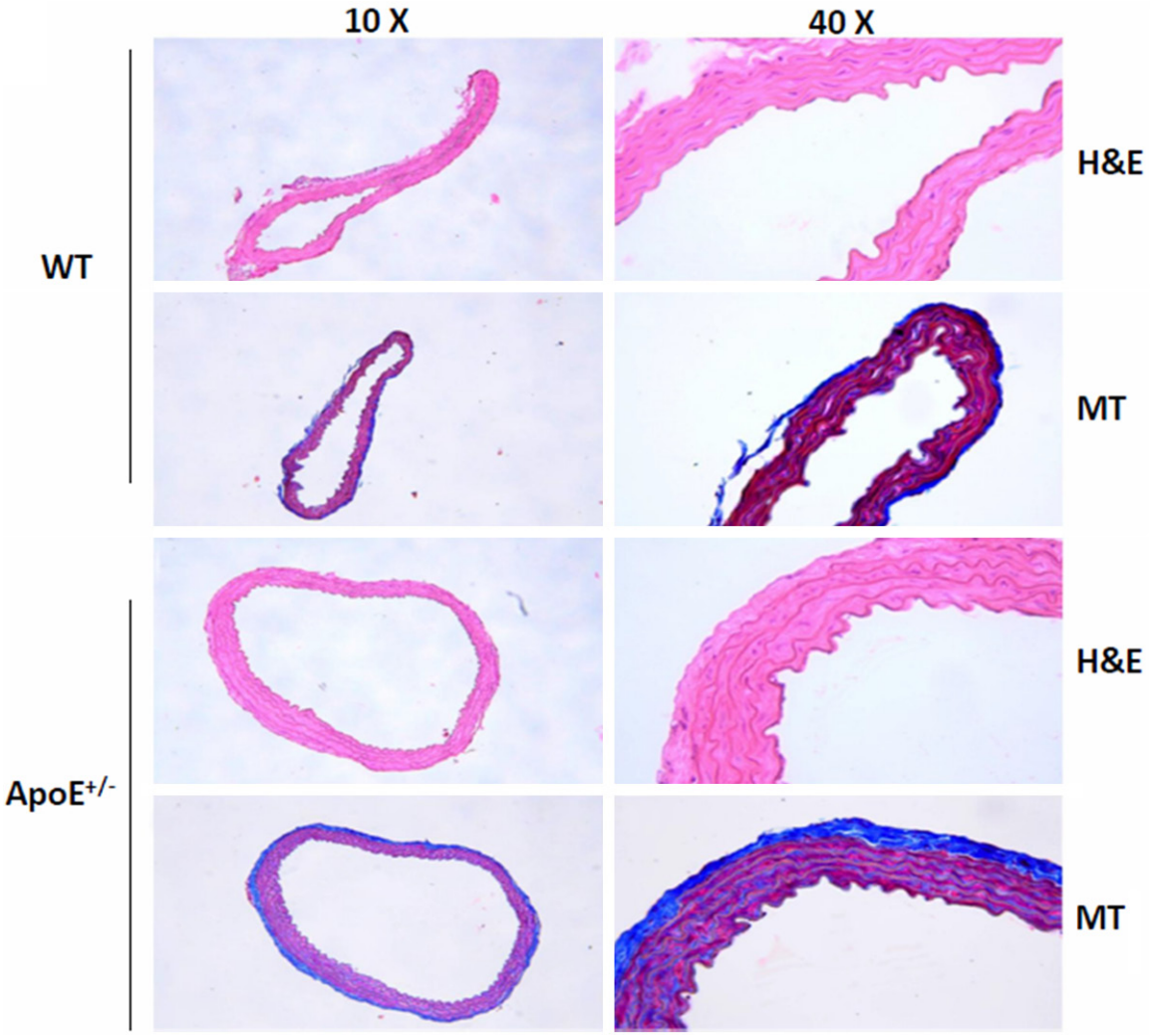

Figure 5. Histology of transversal sections of aortas from wild type (WT) and ApoE$^{+/-}$ mice. A normal histo-architecture is observed in both strains, with the absence of atheromata or inflammatory cells. (A) 10 x magnification, complete view, (B) 40 x magnification: intimae and media layers detailed. H&E, hematoxilin-eosin, MT, Masson's trichrome. Representative of three aortas for each group.

Nevertheless, we do not discard the possibility of increased oxidative stress is involved in this scenario.

The source of increased IL-levels might correspond to endothelial cells, monocytes and smooth muscle cells. This cytokine is a major activator of the acute phase response that induces hepatocytes to produce C - reactive protein, fibrinogen and sICAM-1. For this, IL-6 binds its receptor, which is composed of two sub units: an 80 kDa IL-6 binding protein and a 130-kDa transmembrane signal transducing component (gp130). Upon this binding, gp130 dimerizes and activates the Jak-Stat pathway. It has been reported that Il-6 is able to induce the production of MCP-1 in macrophages, which is consistent with our observation of increased MCP-1 levels in ApoE deficient mice.

The hypercontractile state of ApoE vessels in response to stretching might be a direct consequence of reduced NO production. In addition, it has been described increased force production in response to phenylephrine in ApoE knockout, associated to lower NO production in endothelial cells that might account for this effect [21]. Van Assche et al suggested that this hypercontractile response to phenylephrine is due to higher levels of calcium in the smooth muscle cells of ApoE knockout mice [22]. According to these authors, this higher

level of calcium in the smooth muscle of knockout mice is a consequence of increased calcium influx through store-operated calcium channels. We suggest that a similar phenomenon may occur in ApoE$^{+/-}$ mice.

In conclusion, this study shows that endothelial dysfunction is present in a murine model of dyslipidemia, before the appearance of atheroma plaques. This dysfunction presents stretch-induced hypercontractility and diminished endothelium-dependent vasorelaxation. This is accompanied by decreased levels of NO and eNOS, and increased plasma levels of IL-6, a proinflammatory cytokine that reduces eNOS levels and activity.

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Disclosure of conflict of interest

None.

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