

Late chronic catechin antioxidant treatment is deleterious to the endothelial function in aging mice with established atherosclerosis

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Gendron M, Théorêt J, Mamarbachi AM, Drouin A, Nguyen A, Bolduc V, Thorin-Trescases N, Merhi Y, Thorin E. Late chronic catechin antioxidant treatment is deleterious to the endothelial function in aging mice with established atherosclerosis. *Am J Physiol Heart Circ Physiol* 298: H2062–H2070, 2010. First published April 9, 2010; doi:10.1152/ajpheart.00532.2009.—Various antioxidants, including polyphenols, prevent the development of atherosclerosis in animal models, contrasting with the failure of antioxidants to provide benefits in patients with established atherosclerosis. We therefore tested in a mouse model the hypothesis that although catechin is atheroprotective in prevention, catechin brings no global vascular protection when initiated after established atherosclerosis, because aging associated with dyslipidemia has induced irreversible dysfunctions. To this end, LDLr^{-/-}; hApoB^{+/+} atherosclerotic (ATX, 9 mo old) and pre-ATX (3 mo old) male mice were treated with catechin (30 mg·kg⁻¹·day⁻¹) up to 12 mo of age. Vascular function and endothelium/leukocyte interactions were studied at 12 mo old. The renal artery endothelium-dependent dilations were impaired with age whereas adhesion of leukocytes onto the native aortic endothelium was increased ($P < 0.05$). Aortic oxidative stress [reactive oxygen species (ROS)] increased ($P < 0.05$) at 3 mo in ATX and at 12 mo in wild-type mice. Aorta mRNA expression of NADPH oxidase increased, whereas that of manganese superoxide dismutase decreased in 12-mo-old ATX mice only. In mice with established ATX, catechin (from 9 to 12 mo) reduced ($P < 0.05$) by ~60% ROS without affecting plaque burden. Notably, catechin worsened endothelial dysfunction and further increased leukocyte adhesion ($P < 0.05$) in ATX mice. In contrast, the same catechin treatment reversed all age-related dysfunctions in wild-type mice. On the other hand, in pre-ATX mice treated for 9 mo with catechin, plaque burden was reduced by 64% ($P < 0.05$) and all vascular markers were normalized to the 3-mo-old values. These results demonstrate that an antioxidant treatment is deleterious in mice with established atherosclerosis.

endothelium; splenocyte; renal artery reactivity

ENDOTHELIAL DYSFUNCTION and inflammation are hallmarks of atherosclerosis. With age, risk factors for cardiovascular diseases (CVD) such as hypercholesterolemia accelerate endothelial damages, leading to inflammation and an excess production of reactive oxygen species (ROS) (9). The known consequences are an increased adhesiveness of the leukocytes onto the endothelium (31) and a reduced vasodilatory function accompanied by an augmented vascular production of vasoconstricting factors (14). All these changes point to a reduction in antioxidant defenses that yield to the development of atherosclerotic lesions (38, 39). The rationale for using antioxidants in atherosclerotic humans is based on the well-known

beneficial effects of a lifelong balanced diet on the cardiovascular system (27). Nonetheless, although an uncontrolled ROS metabolism is deleterious, antioxidant therapies have failed to reveal benefits in clinical trials (28, 29, 42). Failure of benefits from antioxidants in patients with CVD may be partly due to the fact that antioxidant therapies were initiated too late (28).

Antioxidants of various origins, including polyphenols like catechin (16, 23) and resveratrol (4, 40), or α -lipoic acid (45) and apocynin (9), have demonstrated their efficacy at preventing atherosclerosis in healthy animals fed a high-fat or high-carbohydrate diet. Polyphenols, for example, which are abundant in fruits, cocoa, vegetables, green tea, and red wine, have beneficial effects on cardiovascular health that are not questioned (2, 5, 10, 24, 25). They act as direct ROS scavengers (24), likely contributing to increasing the efficacy and/or the production of endothelium-derived relaxing factors (30) and improving the efficacy of endogenous antioxidants (25). We previously showed that a chronic catechin treatment can prevent the rise in endogenous thromboxane A₂ release associated with age and the subsequent production of ROS (14). Beneficial effects of polyphenols have been reported in cardiovascular diseases, stroke, and cancer (see Ref. 2 for review), and particularly in animal models of atherosclerosis (1, 3, 11, 16, 23, 33, 36, 44).

The objective of this study was therefore to address the discrepancy in the efficacy of antioxidant treatment between human and animal studies. We would like to propose that the lack of benefit of an antioxidant therapy in patients with coronary artery diseases (CAD) is not due to the nature of the antioxidant but rather because of the delay in the intervention. We therefore tested the hypothesis that, whereas catechin in primary prevention is beneficial, a similar treatment initiated after established atherosclerosis brings no global vascular protection because the vascular dysfunction induced by the combination of aging and risk factors for CVD is irreversible by a sole antioxidant treatment.

MATERIAL AND METHODS

Experimental groups. All experiments were performed in male wild-type (WT) C57B16 and C57B16-knockout/transgenic atherosclerotic LDLr^{-/-}; hApoB^{+/+} (ATX) mice. ATX mice were kindly provided by Dr. Hobbs (University of Texas Southwestern, Dallas, TX), and the colony was established at the animal facility of the Montreal Heart Institute (26). ATX mice were randomly assigned to the following three groups: 1) treatment with the polyphenol catechin (30 mg·kg⁻¹·day⁻¹) (14) in the drinking water from 3 to 12 mo (CAT9; prevention), or 2) from 9 to 12 mo (CAT3; intervention), or 3) no treatment (control). WT mice were only assigned to CAT3. At 12 mo, blood was collected for lipid quantification, renal arteries and the aorta were harvested and either used for vascular reactivity studies

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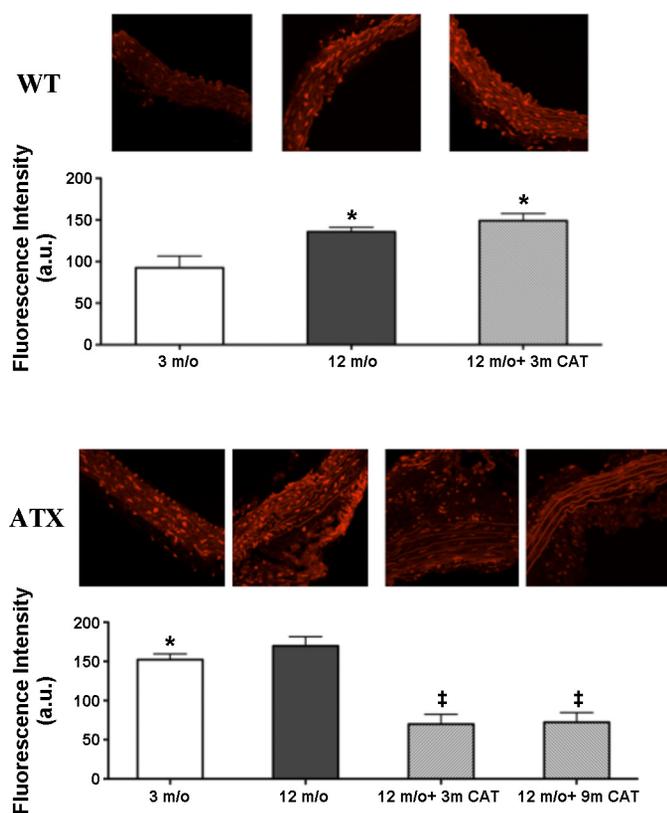


Fig. 1. Superoxide [dihydroethidium (DHE)] staining in aortic segments isolated from 3- and 12-mo-old wild-type (WT) and atherosclerotic (ATX) mice treated or not with catechin for 3 or 9 mo. Data were expressed as fluorescence intensities; $n = 3$ of triplicates analysis in each group. CAT n , number of months mice were chronically treated with catechin added in the drinking water. * $P < 0.05$ compared with WT 3-mo-old; ‡ $P < 0.05$ compared with untreated 12-mo-old ATX mice.

or snap frozen for histology or total RNA extraction. The aorta was also prepared for the leukocyte adhesion studies and the spleen was used for splenocyte and leukocyte isolation. The procedures and protocols were reviewed and approved by our institutional independent review board and followed the guidelines for the Care and Use of Laboratory Animals of Canada.

Superoxide expression. The oxidative fluorescent probe dihydroethidium (DHE; Sigma-Aldrich Canada, Oakville, ON, Canada) was used to evaluate in situ O_2^- production on renal and aortic histological sections (19). DHE is a cell-permeable dye that is oxidized by O_2^- to ethidium bromide, which subsequently intercalates with DNA and is trapped within cell nuclei. DNA counterstaining was performed by use of ToPro-3 (Molecular Probes, Burlington, ON, Canada). Frozen artery segments were cut into 20- μ m thick sections. Sections were mounted on positively charged slides (Fisher Scientific, Ottawa, ON, Canada), and double stained with a mixture of 5 μ mol/l DHE and 2 μ mol/l To-Pro-3 for 15 min. Fluorescence was visualized using a scanning confocal microscope LMS 510 (Carl Zeiss, Oberkochen, Germany) with a $\times 40/1.4$ Plan-Apochromat objective (λ_{Ex} : 490 nm; λ_{Em} : 520 nm). Acquisition settings of the camera were identical for all images. Computer-based analysis was performed with Image J software and calculated by the following equation: $I = \sum I/(A/N)$, where I is the fluorescence intensity, $\sum I$ is the summation of all nuclei intensity, A is the total area of the nuclei, and N is the number of nuclei used. Data are expressed as an average of total nuclei fluorescence quantified in triplicate of $n = 3$ mice (Fig. 1 and Supplemental Fig. S2 in the online supplement available online at the *American Journal of Physiology Heart and Circulatory Physiology* website).

Plaque quantification. Freshly dissected thoracic aortas were cut longitudinally and fixed in a petri dish, endothelium faced up. The complete aorta was pictured, and plaque area was measured with use of Adobe Photoshop 7.0 (Fig. 2).

Vascular reactivity studies. Experiments were conducted in isolated and pressurized (100 mmHg) mouse renal arteries as previously described (15). Arterial segments were precontracted with phenylephrine (30 μ mol/l) and concentration-response curves to acetylcholine (ACh; 0.001 to 30 μ mol/l) were obtained. Inhibition of NO and prostanooids production was achieved by N^G -nitro-L-arginine (L-NNA; 10 μ mol/l) (14) and indomethacin (Indo; 10 μ mol/l) (14), respectively. Since aortas cannot be pressurized, ACh-induced dilations of isolated aortas and renal arteries from 3-mo-old WT mice and aged-matched ATX mice were compared using a wire myograph (see Fig. S1 in the online supplement).

Splenocyte adhesion studies. The thoracic aorta was isolated, gently longitudinally opened, and pinned in a petri dish, the endothelium faced up. Then, the aorta was cut in segments, covered with a physiological salt solution (PSS) and kept at 37°C. The spleen was harvested, immersed in RPMI, disrupted by rubbing and the cell suspension was centrifuged. Contaminating erythrocytes were lysed with distilled water. Splenocytes were labeled with ^{51}Cr (activity of 100 μCi) for 1 h at 37°C, with gentle agitation every 15 min. Splenocytes were then centrifuged and washed twice. Splenocytes were resuspended and cell count was adjusted to 10^6 cell/ml. Segments of aorta were first exposed to PSS alone or stimulated with PSS containing histamine (0.1 μM). Labeled ^{51}Cr -splenocytes were allowed to adhere onto the native endothelium. The number of splenocytes adhering to the endothelium was expressed per surface area of the segment (splenocytes/ mm^2) (Fig. 5).

Flow cytometry studies. Splenocyte suspension (5×10^6 cell/ml) were incubated with different monoclonal antibodies (CD18- or CD62L-phycoerythrin-conjugated, from Serotec, Raleigh NC) or their isotype-matched control IgGs. Reaction was stopped with 1% formalin and samples were analyzed on an Altra flow cytometer (Beckman Coulter, Mississauga, ON, Canada). Antibody binding was determined as the percentage of positive splenocytes or the mean fluorescence intensity (MFI). The binding index (% of positive cells \times MFI) was then calculated (Fig. 5). Between 3,000 and 5,000 splenocytes were analyzed from each sample.

Quantification of gene expression by real-time PCR. Total RNA extraction from aorta and renal arteries (see online supplement) and reverse transcriptase reaction (5 ng/ μ l total RNA) were performed as previously (14) by using an RNeasy mini-kit (Qiagen) and the Moloney murine leukemia virus reverse transcriptase (200 U, Invitrogen), respectively. Quantitative PCR (qPCR) was performed as previously (14) with 2 ng of cDNA template containing the appropriate primer concentration specific for several vascular- and oxidation-related genes and SYBR Green PCR master mix (Stratagene). mRNA samples were calculated relative to cyclophilin A, for which level of

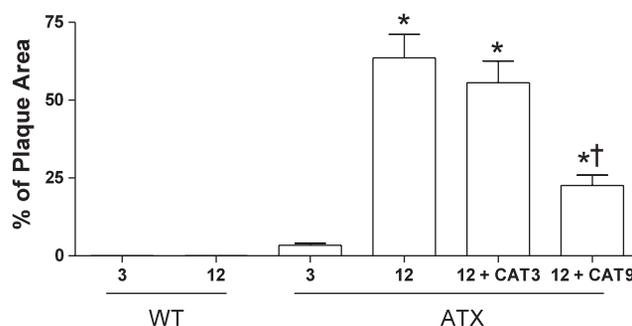


Fig. 2. Plaque area quantification. Data were expressed in percentage of total aortic area; $n = 5-8$ in each group. * $P < 0.05$ compared with 3 mo old; † $P < 0.05$ compared with 12 mo old.

Table 1. Sequences of the primers for real-time quantitative PCR

Genes	Forward (5'-3')	Reverse (5'-3')
Mouse SIRT-1	GAGCAGGTTGCAGGAATCCA	CCTGATTAATAATGTCTCCACGAA
Mouse eNOS	CACGAGGCACTGGTGTGGT	CTTGCGCCGCCAAGAGGATA
Mouse p22 ^{phox}	GCTGCCCTCCACTTCCT	CTCCTTGGGTTTAGGCTCAATG
Mouse MnSOD	GGCCAAGGGAGATGTTACAA	GCTTGATAGCCTCCAGCAAC
Mouse COX-1	ACTCAGCGCATGACTACATC	CTTCTCAGCAGCAGCTGTTG
Mouse COX-2	GAACATGGACTCACTCAGTTGTTG	CAAAGATAGCATCTGGACGAGGT
Mouse cyclophilin A	CCGATGACGAGCCCTTGG	GCCGCCAGTGCATTATG

expression did not vary between groups (Fig. 6 and Supplemental Fig. S3 in the online supplement). The sequence specificity of each primer was verified with the *Blast* program derived from the National Center for Biotechnology Information (<http://www.ncbi.nlm.nih.gov>). The primers used are shown in Table 1.

PCR products were purified, sequenced, and confirmed to be the genes of interest.

Statistical analysis. In every case, *n* refers to the number of animals used in each protocol. Continuous variables are expressed as means \pm SE. Half-maximum effective concentration (EC₅₀) of ACh was measured from individual concentration-response curves. The *pD*₂ value, the negative log of the EC₅₀, was obtained. At the end of the protocol, the maximal diameter (D_{max}) was determined by changing the PSS to a Ca²⁺-free PSS containing sodium nitroprusside (10 μ mol/l). In these passive conditions, the maximal diameter of the vessels was not different between groups, suggesting a normal smooth muscle reactivity to an exogenous NO donor. ACh-induced dilation is expressed as a percentage of the D_{max}. ANOVA studies followed by a Bonferroni correction were performed to compare E_{max} and *pD*₂ as well for adhesion and qPCR studies. Unpaired *t*-tests were performed for flow cytometry studies. Differences were considered to be statistically significant for a *P* value <0.05.

RESULTS

Plasma lipids. Total cholesterol levels (mmol/l) increased significantly with age in ATX mice (Table 2). LDL, but not HDL, cholesterol and triglycerides increased with age in ATX mice. Total, HDL, and LDL cholesterol increased with age in WT 12-mo-old mice, whereas triglycerides did not change. Catechin had no effect on plasma lipids (data not shown).

Superoxide production. Superoxide production was significantly greater in the aorta of ATX compared with WT mice at 3 mo old (Fig. 1). With age, DHE staining increased in the aorta isolated from WT mice only, to similar levels quantified in ATX mice. Catechin reduced DHE staining in ATX mice treated for either 3 or 9 mo (Fig. 1). In contrast, superoxide production was not reduced by catechin intervention (CAT3) in WT mice.

Table 2. Plasma levels of total, HDL, and LDL cholesterol and triglycerides of WT and ATX mice

Conditions	Cholesterol, mmol/l	HDL, mmol/l	LDL, mmol/l	Triglycerides, mmol/l
WT3	2.1 \pm 0.2	1.3 \pm 0.1	0.5 \pm 0.2	0.7 \pm 0.1
WT12	4.5 \pm 0.2*	1.8 \pm 0.1*	2.5 \pm 0.2*	0.4 \pm 0.1
ATX3	16.6 \pm 2.0*	1.7 \pm 0.1*	10.3 \pm 1.6*	6.2 \pm 0.5*
ATX12	23.3 \pm 3.4 ^b	2.1 \pm 0.3	15.2 \pm 2.2 ^b	9.5 \pm 0.9 ^b

Data are means \pm SE. WT, wild-type; ATX, atherosclerotic. Numbers represent age in months. **P* < 0.05 compared with WT3; ^b*P* < 0.05 compared with WT12.

Plaque formation. Plaque area increased significantly at 12 mo compared with 3-mo-old ATX mice (Fig. 2). As expected, primary prevention (CAT9) efficiently limited plaque formation, whereas intervention with catechin (CAT3) had no significant effect. WT mice did not develop plaque in the aorta.

Vascular reactivity in the renal artery. Precontraction levels did not change significantly between the different groups (data not shown). The first marker of endothelial function tested was the dilatory capacity and sensitivity to acetylcholine.

Effect of age. In healthy WT animals, aging was associated with a decline in the endothelial function, illustrated by a decrease in the maximal dilation induced by ACh (Fig. 3C) combined with a lower sensitivity (Table 3), and an age-dependent reduction in the L-NNA-sensitive component of the dilatory response (Fig. 3, B and C). In 3-mo-old WT mice the dilation observed in the presence of L-NNA was not further altered by indomethacin (Fig. 3B; Table 3), suggesting that a non-NO/non-PGI₂ dilatory mechanism [likely endothelium-derived hyperpolarizing factor (EDHF)] was responsible for this dilation; in contrast indomethacin inhibited ACh-induced dilation at 12 mo (Fig. 3C), likely preventing the production of dilatory PGI₂. Hence, the EDHF component was reduced with aging but compensated by a cyclooxygenase (COX) derivative in WT mice.

In young ATX mice, a premature endothelial dysfunction was already present compared with age-matched WT mice, illustrated by a decrease in the maximal dilation induced by ACh (Fig. 4B) combined with a lower sensitivity (Table 3). In addition, in 3-mo-old ATX mice, whereas L-NNA did not significantly reduce dilation, addition of indomethacin decreased dilation (Fig. 4B), as observed in renal arteries isolated from 12-mo-old WT mice. In old ATX mice, indomethacin no longer reduced the dilation induced by ACh in the presence of L-NNA (Fig. 4C), suggesting that the COX component, expressed at 3 mo in ATX and at 12 mo in WT mice, was no longer functional in old ATX mice.

Effect of catechin treatment. In WT mice, the age-dependent endothelial dysfunction was fully prevented by the treatment with catechin initiated at the age of 9 mo, with the prevention of the decrease in the maximal ACh-induced dilation and the prevention of the inhibitory effect of Indo (Fig. 3D; Table 3). In ATX mice, initiation of catechin at 3 mo of age normalized endothelial function to that of arteries isolated from 12-mo-old WT mice treated for 3 mo with catechin (Fig. 4E; Table 3); most conspicuously, neither L-NNA nor indomethacin reduced the maximal dilation induced by ACh (Fig. 4E) only slightly decreasing sensitivity (Table 3), strongly suggesting that catechin favored the expression of EDHF.

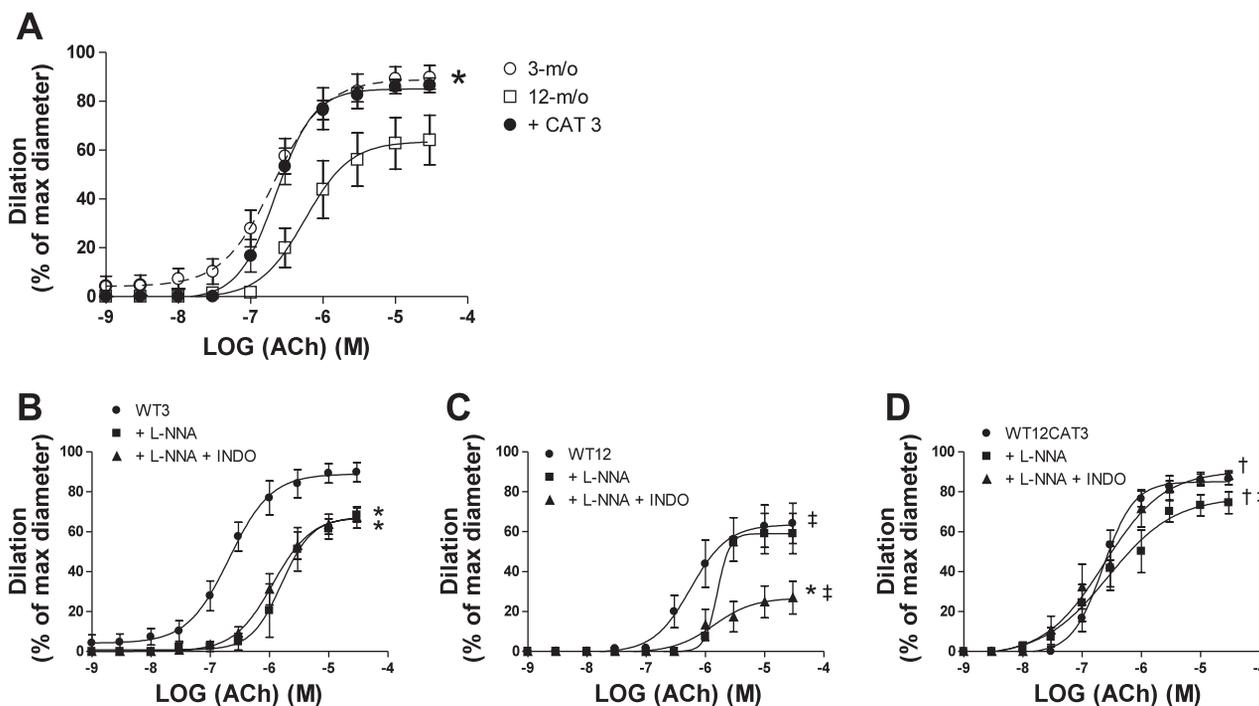


Fig. 3. Endothelial function of renal arteries in WT mice. A: acetylcholine-induced dilation of renal arteries isolated from 3- and 12-mo-old WT mice treated with catechin for the last 3 mo. Acetylcholine-induced dilation of renal arteries isolated from WT mice in the absence or in the presence of N^G -nitro-L-arginine (L-NNA, 10 μ M) or indomethacin (Indo, 10 μ M) in 3- (WT3; B), 12- (WT12; C), and 12-mo-old WT mice treated with catechin (WT12CAT3; D) during the last 3 mo. Data are means \pm SE (for n numbers, please refer to Table 3). * P < 0.05 compared with control conditions; ‡ P < 0.05 compared with untreated 12 mo old.

By opposition, initiation of catechin at 9 mo of age (3-mo treatment) further reduced the vascular sensitivity to ACh by one log unit in vessels isolated from 12-mo-old ATX mice despite a catechin-dependent expression of a non-NO/non-PGI₂ dilatory mechanism (likely EDHF) (Fig. 4D; Table 3).

Splenoctye adhesion onto the endothelium. Splenoctye adhesion onto the native autologous aortic endothelium significantly increased with age (Fig. 5). Age-dependent activation of both the endothelium and the splenoctyes accounted for this increase in adhesion: compared with the adhesion splenoctyes on the endothelium from 3-mo-old

mice (127 ± 10 cells/mm²; $n = 29$), the adhesion of splenoctyes isolated from 3-mo-old WT mice is increased when exposed to the endothelium of 12-mo-old mice (239 ± 18 cells/mm²; $P < 0.05$). Likewise, the adhesion of splenoctyes isolated from 12-mo-old WT mice is increased when exposed to the endothelium of 3-mo-old mice (228 ± 14 cells/mm²; $P < 0.05$).

Histamine increased adhesion in WT at 3 mo only. As expected, age was associated with an augmented expression of CD18 (MAC-1) and shedding of CD62L (L-selectin) (Fig. 5B). Catechin normalized all these parameters while preventing histamine-induced adhesion of splenoctyes, suggesting that histamine stimulates ROS production that promotes adhesion.

Autologous adhesion of splenoctyes from 12-mo-old ATX is further increased compared with 12-mo-old WT mice (Fig. 5C). The expression of MAC-1 at 3 mo was higher than in WT but did not vary with age (Fig. 5D). In contrast, shedding of L-selectin was not magnified in ATX, although it tended to increase at 12 mo of age. Catechin, in prevention, had no significant impact besides decreasing the expression of MAC-1 (CD18; Fig. 5D). In contrast, catechin in intervention therapy magnified splenoctye adhesion at baseline or following histamine stimulation (Fig. 5C) and strongly increased L-selectin shedding (Fig. 5D).

Quantification of gene expression of eNOS, COX-1 and -2, p22^{phox}, manganese superoxide dismutase (MnSOD), and sirtuin-1 (SIRT-1) in mouse aorta. Not surprisingly, aging in WT mice was associated with a reduction in endothelial NO synthase (eNOS) and SIRT-1 expression and an increase in COX-2 expression (Fig. 6). More surprising was the observation that catechin given in the drinking water from the age of

Table 3. Vascular sensitivity of renal arteries isolated from WT and ATX mice

Conditions	Control		L-NNA (10 μ M)		L-NNA + Indo (10 μ M)	
	pD ₂	n	pD ₂	n	pD ₂	n
WT3	6.7 \pm 0.1	7	5.8 \pm 0.1 ^a	5	5.9 \pm 0.1 ^a	7
WT12	6.3 \pm 0.1 ^b	7	5.8 \pm 0.1 ^a	5	5.9 \pm 0.3 ^a	6
WT12CAT3	6.6 \pm 0.1 ^c	11	6.6 \pm 0.2 ^{b,c}	9	6.6 \pm 0.1 ^{b,c}	9
ATX3	6.2 \pm 0.2 ^d	10	5.6 \pm 0.3	9	5.4 \pm 0.7	6
ATX12	6.6 \pm 0.2	14	6.0 \pm 0.2 ^a	13	6.5 \pm 0.2 ^e	10
ATX12CAT3	5.4 \pm 0.4 ^{e,f}	7	5.7 \pm 0.3 ^f	5	5.8 \pm 0.2 ^{e,f}	5
ATX12CAT9	7.1 \pm 0.1 ^b	10	6.6 \pm 0.2 ^{a,b}	9	6.6 \pm 0.2 ^a	9

Data are means \pm SE. L-NNA, N^G -nitro-L-arginine; Indo, indomethacin; pD₂, negative log of half-maximum effective concentration; CAT n , number of months mice were chronically treated with catechin added in the drinking water. ^a P < 0.05 compared with Control condition; ^b P < 0.05 compared with 3 mo old; ^c P < 0.05 compared with 12 mo old; ^d P < 0.05 compared with WT3; ^e P < 0.05 compared with WT12; ^f P < 0.05 compared with WT12CAT3.

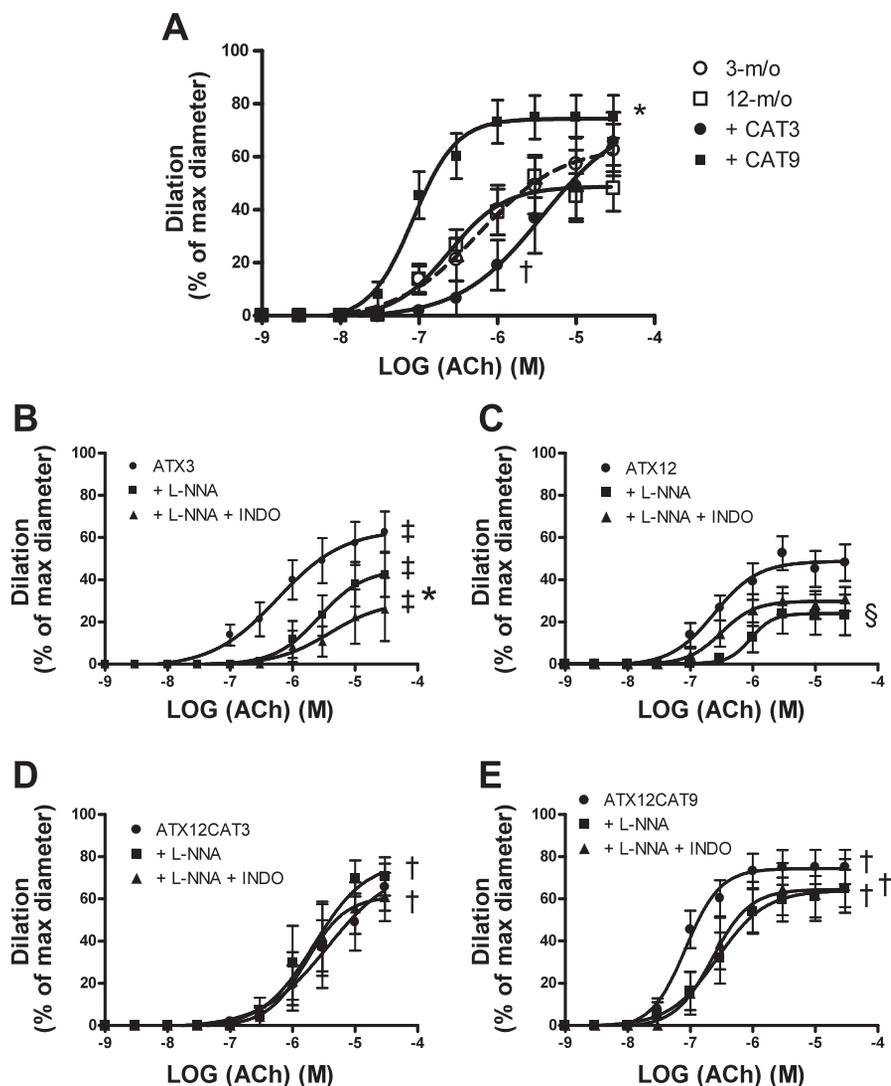


Fig. 4. Endothelial function of renal arteries in ATX mice. *A*: acetylcholine-induced dilation of renal arteries isolated from 3- and 12-mo-old ATX mice treated with catechin for 3 or 9 mo. Acetylcholine-induced dilation of renal arteries isolated from ATX mice in the absence or in the presence of L-NNA (10 μ M) or Indo (10 μ M) in ATX3 (*B*), ATX12 (*C*), ATX12CAT3 (*D*), treated with catechin for the last 3 mo, and ATX12CAT9 (*E*), treated with catechin for 9 mo. Data are means \pm SE (for *n* numbers, please refer to Table 3). * P < 0.05 compared with WT3; † P < 0.05 compared with untreated 12-mo-old; § P < 0.05 compared with ATX3.

9 to 12 mo (intervention treatment) did not correct the reduction in eNOS expression while preventing all other changes, i.e., the rise in COX-2 and decline in SIRT-1 expression.

In ATX mice, the impact of age on gene expression was completely different except for SIRT-1: its expression decreased at 12 mo but was prevented by catechin in prevention (Fig. 6). eNOS expression was maintained with age, whereas both COX-1 and COX-2 increased dramatically. Finally, MnSOD expression was upregulated at 3 mo of age but dropped at 12 mo, whereas the opposite occurred for p22^{phox}, increasing dramatically at 12 mo. The imbalance in the expression of these two latter enzymes certainly accounts for the changes in the redox environment observed in the aorta from ATX mice (Fig. 1).

The effects of catechin given in prevention were as expected: eNOS and SIRT-1 expression increased, whereas the level of mRNA expression of COX-1 and -2, p22^{phox}, and MnSOD were normalized compared with those of age-matched WT mice. When catechin was given for the last 3 mo, p22^{phox} expression was not normalized and the expression of COX-1 was reduced below 3-mo-old levels.

DISCUSSION

Treatments with antioxidants in animals, including polyphenols like catechin (16, 23), are known to be antiatherogenic and to protect the vasculature. In these studies, young animals were treated or the treatment was initiated when introducing the high-fat or high-carbohydrate diets. In contrast, antioxidants in interventional clinical studies were overall of no benefit to patients with established CAD (28, 29, 42). This led to two major conclusions: some questioned the premises that free radicals are involved or responsible for atherogenesis and others suggested that the wrong antioxidant had been used at the wrong dose and in the wrong patients (28, 42). We propose that these positions did not consider the context of the preclinical studies telling us that 1) all antioxidants tested have been successful in prevention and 2) none have been tested in a model of established atherosclerosis. In this context, our study aimed at testing the hypothesis that with age severe dyslipidemia leads to the accumulation of severe endothelial damages that cannot be reversed by an antioxidant treatment. In short, prevention with antioxidants is beneficial, whereas intervention is not.

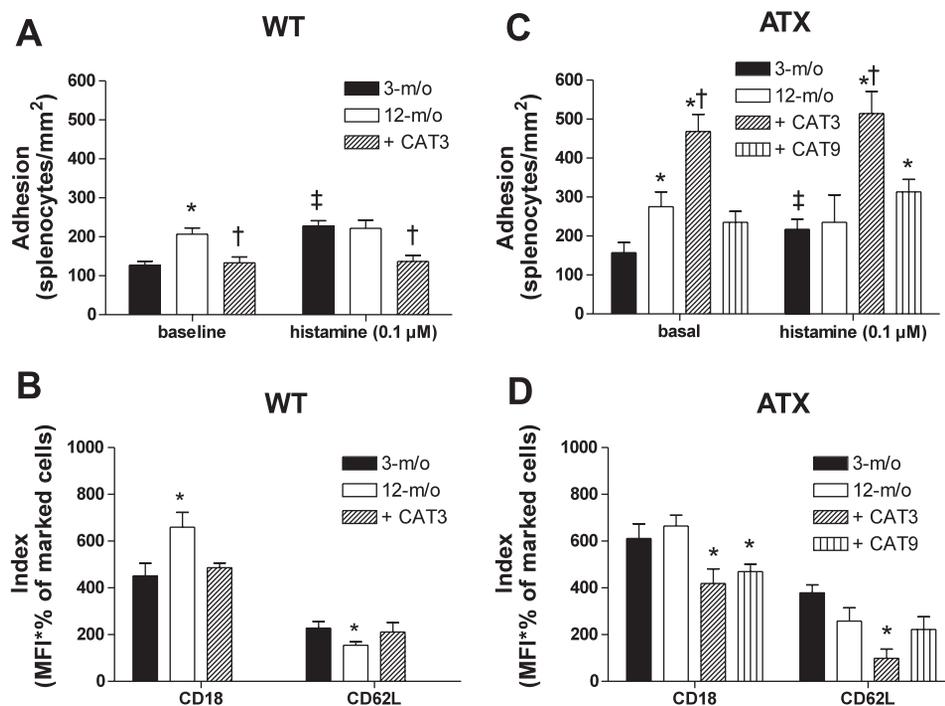


Fig. 5. Splenocyte adhesion on the native endothelium of aortic segments isolated from 3-mo-old, 12-mo-old, and 12-mo-old catechin-treated WT (A) and ATX (C) mice. Responses were obtained either in basal conditions (baseline) or following stimulation of the endothelium with histamine (0.1 μM). Data are means ± SE; $n = 4-6$. * $P < 0.05$ compared with 3-mo-old; † $P < 0.05$ compared with 12-mo-old; ‡ $P < 0.05$ compared with basal. Expression of cell adhesion molecules on the surface of splenocytes isolated from 3-mo-old, 12-mo-old, and 12-mo-old catechin-treated WT (B) and ATX (D) mice, measured by flow cytometry. Data are means ± SE; $n = 5-11$. * $P < 0.05$ to 12 mo old.

Aging leads to a vascular dysfunction, which is most commonly referred by a reduction in dilatory function associated with a reduced NO availability and a rise in oxidative stress (21). In addition, the imbalance toward an increased release and/or functional impact of endothelium-derived contracting factors on the vascular function is also associated with endothelial dysfunction (14, 20, 32). The production of endothelium-derived contracting factors has been documented in aging and in several human diseases such as essential hypertension (35, 37). In the present study, we observed that, at a young age, a risk factor such as dyslipidemia accelerated renal artery endothelial dysfunction via a decrease in the L-NNA-sensitive component of the dilatory response, suggestive of a lower eNOS activity, and a lower inhibitory effect of Indo, suggestive of a lower compensatory COX activity. Concomitantly, there was a rise in aortic oxidative stress despite a major increase in MnSOD expression, likely a compensatory mechanism (12). We would like to propose that these limited, but specific, oxidative stress-related changes are at the basis of the long-term protection induced by catechin when started in prevention at 3 mo of age in ATX. Catechin being an antioxidant (16, 24, 25), it is anticipated that it will prevent free radical-induced damages. In the early phases of dyslipidemia, damage is likely reversible, as demonstrated by a complete recovery of the endothelial dilatory function by catechin when started at 3 mo of age. This observation is not unique (16, 23), but it confirms that catechin, when used in prevention, is protective in this mouse model of atherosclerosis as well. The concept of prevention is at the basis of the beneficial effect of a balanced diet associated with regular exercise on the cardiovascular system in humans (2, 8, 27, 30, 34).

With age, dyslipidemia adds on numerous divergences between ATX and WT mice: 1) old dyslipidemic mice have a very significant atherosclerotic plaque; 2) aortic DHE staining, a marker of oxidative stress, is similar in old WT and ATX

mice, but the superoxide-generating $p22^{phox}$ enzyme is only increased in aortas from ATX, suggestive of a different origin of oxidative stress; 3) the renal artery endothelial dysfunction is different, since the inhibitory effect of L-NNA on the dilation induced by ACh is only maintained in ATX mice; 4) indomethacin decreased renal artery dilation to ACh only in WT, whereas COX-1 expression rose only in aortas from ATX. These differences must therefore be at the basis of the opposite response to catechin intervention treatment between WT and ATX mice: when initiated at 9 mo, although catechin decreased aortic DHE staining in ATX mice, it had no impact on plaque burden and it strongly reduced vascular sensitivity to ACh, almost doubled splenocyte adhesion, and promoted CD62L (L-selectin) shedding. The same treatment schedule in WT mice was highly protective, restoring endothelial function and normalizing cell adhesion and splenocyte adhesion molecule profile as well as gene expression except for eNOS, which remained lower than at 3 mo of age. The effects of catechin in WT closely resemble those of catechin in ATX when used in prevention. This reinforces the concept that catechin, and antioxidants in general, protect a minimally damaged vasculature but not after long-term exposure to a risk factor. This would certainly explain the overall lack of beneficial effect of antioxidant treatment in atherosclerotic patients (29, 42), although catechin has not been tested directly in a randomized clinical trial. This, however, does not mean that there is no hope for antioxidant therapy in patients with CAD: epidemiological studies reveal that the selection of the patient population is essential to gain from antioxidant therapies (29, 42). Antioxidants will likely be poorly effective in patients with the longest history of risk factors for atherosclerosis, hypertension and smoking being the most aggressive, since this will likely be associated with the highest level of damage (13, 38, 39).

Another important result of the present study is that catechin did not reduce aortic DHE staining in WT, demonstrating that

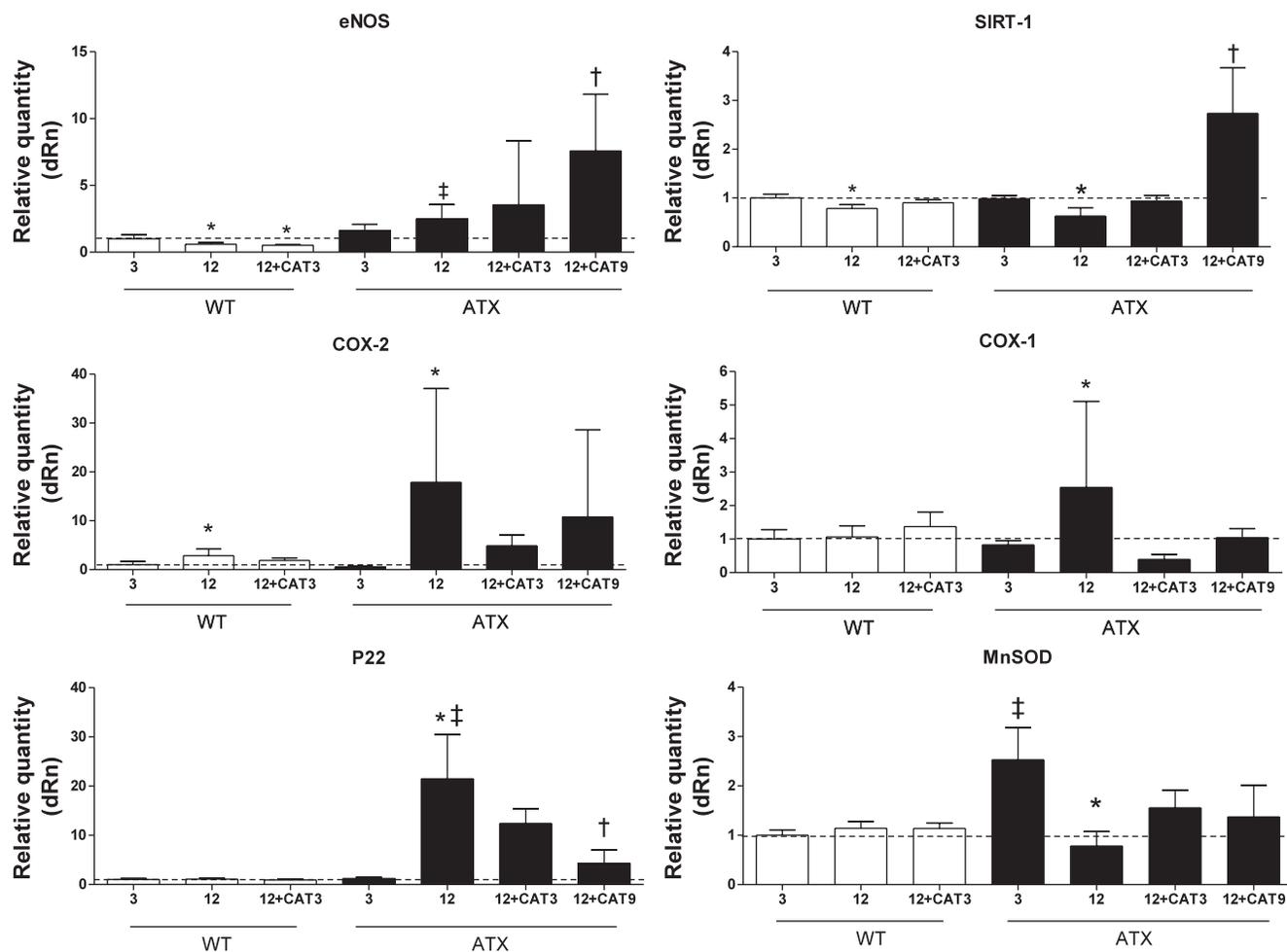


Fig. 6. Gene expression from total RNA isolated from the aorta of WT and ATX mice of 3 and 12 mo old treated or not by catechin. Gene expressions of endothelial NO synthase (eNOS), cyclooxygenase (COX)-1, COX-2, sirtuin-1 (SIRT-1), p22^{phox}, and manganese superoxide dismutase (MnSOD) were measured by quantitative RT-PCR. Data are means \pm SE ($n = 4-6$). * $P < 0.05$ compared with 3 mo old; † $P < 0.05$ compared with 12 mo old; ‡ $P < 0.05$ compared with WT. dRn, baseline: corrected and normalized fluorescence.

the increase in free radical production associated with age is different when combined with dyslipidemia. It is further supported by the response to catechin of aortic gene expression: COX-1 expression increased in ATX and was normalized by catechin. Hence COX-1 expression was sensitive to free radicals associated with dyslipidemia but not aging alone. The same applies to p22^{phox}. It is therefore likely that the background on which catechin was applied has determined its efficacy.

Catechin was deleterious in intervention, 6 mo after the development of atherosclerotic plaques. The most dramatic negative effect is on the promotion of splenocyte adhesion, which is known to contribute to inflammation and plaque growth (31). The endothelial function was also impaired by late catechin treatment, as revealed by the reduction in vascular sensitivity to ACh. Altogether, these data demonstrate that 1) free radicals may remain the last resources for cellular communication and function (7) in conditions of extensive damages, and 2) it is the background health status that governs the response to both the risk factor and the antioxidant. This is likely at the basis of the overall lack of benefit of antioxidants tested in atherosclerotic patients.

Finally, we used the increase in SIRT-1 mRNA expression as a response marker to catechin, a characteristic of all polyphenols (5, 17, 18, 43). In mammalian cells, SIRT-1 appears to control the cellular response to stress by regulating the FOXO family of Forkhead transcription factors (6) and may potentiate FOXO's ability to detoxify ROS and to repair damaged DNA (22, 41). Our data suggest that SIRT-1 mRNA expression decreases with age independently of dyslipidemia. Aging appears to downregulate SIRT-1 expression, whereas catechin upregulates it, suggesting a direct activation by polyphenols on SIRT-1 gene expression (17, 43).

Limitations of the study: we used two different vascular beds for the reactivity studies (pressurized renal arteries) and the biochemical assays (aortic superoxide levels and aortic gene expression), and thus any correlation has to be cautious. Renal artery is a first branch of the abdominal artery, and we verified that dilations to ACh were similar, in isometric conditions, in both vessels (Supplemental Fig. S1). We believe, however, that pressurized conditions are more suited to *in vitro* experimental conditions to study physiological changes in vasoreactivity throughout aging and atherosclerosis; this determined our choice of the renal artery. In parallel, endothelial splenocyte

adhesion was quantified in the aorta and compared with aortic superoxide levels and aortic gene expression. Our aim when measuring superoxide levels in the aorta was to reflect in vivo global estimation of oxidative stress, since this vessel displays large visible atherosclerotic lesions. However, we also performed DHE staining directly on renal arteries and found that superoxide levels are also significantly higher (by 1.6-fold) in ATX than in WT mice, although slightly later (at 6 mo old) than in the aorta (Supplemental Fig. S2). We also observed that the MnSOD mRNA expression was significantly higher (by 1.7-fold) in renal arteries from 3-mo-old ATX compared with WT mice, whereas the expression of p22 was not different, suggesting that aortic gene expression is representative of renal gene expression (Supplemental Fig. S3). However, it is possible that the kinetic of the changes in gene expression may differ between the two tissues.

In summary, although a preventive antioxidant treatment with catechin is beneficial in renal endothelial function, in aortic splenocyte adhesion, oxidative stress, and gene expression, a late treatment cannot reverse established vascular damages as well as the endothelium-dependent regulation of vascular tone. Hence, late catechin treatment was deleterious to both the renal and the aorta endothelium. To conclude, oxidative stress promotes atherogenesis and antioxidants can limit its progression. The arguments against this statement come from the lack of conclusive clinical results. Our data demonstrate that these clinical results are reproducible in animals if the experimental paradigm mimics the clinical setting, i.e., if the antioxidant treatment is initiated when atherosclerosis is established. Irreversible damages have then occurred: antioxidants prevent free radical damage, but since they do not stimulate repair, they are of no benefit in these conditions. When introduced in prevention, however, antioxidants facilitate the normal cellular repair functions and increase life span (4). This is a point in favor of early intervention to prevent early damage.

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DISCLOSURES

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

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