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Up-regulation of thromboxane A_2 impairs cerebrovascular eNOS function in aging atherosclerotic mice

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Abstract

We previously reported that in healthy mouse cerebral arteries, endothelial nitric oxide synthase (eNOS) produces H_2O_2, leading to endothelium-dependent dilation. In contrast, thromboxane A_2 (TXA_2), a potent pro-oxidant and pro-inflammatory endogenous vasoconstrictor, is associated with eNOS dysfunction. Our objectives were to elucidate whether (1) the cerebrovascular eNOS–H_2O_2 pathway was sensitive to oxidative stress associated with aging and dyslipidemia and (2)

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TXA_2 contributed to cerebral eNOS dysfunction. Atherosclerotic (ATX= $LDLR^{-/-}$; $hApoB^{+/+}$) and wild-type (WT) control mice were used at 3 and 12 months old (m/o). Three-m/o ATX mice were treated with the cardio-protective polyphenol catechin for 9 months. Dilations to ACh and the simultaneous eNOS-derived H_2O_2 production were recorded in isolated pressurized cerebral arteries. The age-associated decrease in cerebral eNOS–H_2O_2 pathway observed in WT was premature in ATX mice, decreasing at 3 m/o and abolished at 12 m/o. Thromboxane synthase inhibition by furegrelate increased dilations at 12 months in WT and at 3 and 12 months in ATX mice, suggesting an anti-dilatory role of TXA_2 with age hastened by dyslipidemia. In addition, the non-selective NADP(H) oxidase inhibitor apocynin improved the eNOS–H_2O_2 pathway only in 12-m/o ATX mice. Catechin normalized the function of this pathway, which became sensitive to L-NNA and insensitive to furegrelate or apocynin; catechin also prevented the rise in TXA_2 synthase expression. In conclusion, the age-dependent cerebral endothelial dysfunction is precocious in dyslipidemia and involves TXA_2 production that limits eNOS activity. Preventive catechin treatment reduced the impact of endogenous TXA_2 on the control of cerebral tone and maintained eNOS function.

Keywords

Cerebral artery; Aging; Dyslipidemia; Endothelial function; Endothelium-derived constricting factors

Introduction

We previously reported that in mouse cerebral arteries, endothelial nitric oxide synthase (eNOS), cyclooxygenase (COX), and an endothelium-derived hyperpolarizing factor (EDHF) are important regulators of vascular tone in response to ACh and flow [7, 8]. We also reported that eNOS stimulation of healthy mouse cerebral arteries leads to the production of vasodilatory H_2O_2 and not the release of NO, showing that eNOS produces physiologically relevant levels of free radicals leading to H_2O_2-dependent dilation [7, 8]. This eNOS–H_2O_2 pathway represents a non-traditional mechanism for healthy vessels and is still a subject of ongoing debate. A similar vasodilatory pathway, however, has been described in peripheral mouse and human arteries [27, 33] and human coronary arterioles [25]. The generation by eNOS of H_2O_2 rather than NO is usually associated with eNOS uncoupling and endothelial dysfunction, and reported in a pathological situation [11]. With the brain being highly susceptible to oxidative damage [1], one would therefore expect the arterial eNOS–H_2O_2 pathway to be highly sensitive to the *redox* environment.

There is overwhelming evidence that a rise in oxidative stress is associated with aging and multiple cardiovascular diseases (CVD). Although an “apparent” protection of intracranial arteries from atherosclerosis has been reported based on the fact that atherosclerotic lesions are rarely observed in cerebral arteries from animal models [5, 49], it is now clear that endothelial dysfunction also occurs in the cerebral circulation: atherosclerosis in $ApoE^{-/-}$ mice is associated with NOS dysfunction and an increase in NADP (H) oxidase activity, leading to the rise in oxidative stress and cerebral endothelial damage [20, 31, 32]. In peripheral arteries, it has been proposed that the impairment of eNOS function by oxidative

stress could promote TXA_2 production, a potent vasoconstrictor with inflammatory properties, which would further contribute to the rise in ROS and endothelial dysfunction [13, 14, 57]. Serum levels of TXA_2 and thromboxane receptor expression are elevated in patients with several vascular and ischemic diseases [37], including cerebral ischemia [2, 21, 43, 55]. In addition, a specific polymorphism in the *TXA_2 synthase-1* gene has been reported in some patients with cerebral infarction [40]. TXA_2 is produced by brain tissues [9] and cerebral arteries, both in vitro [15] and in vivo [42], and the release of TXA_2 in cerebral arteries is 10-fold higher than in coronary, mesenteric, or saphenous arteries [48]. Although reports on the contribution of TXA_2 in cerebral arteries with atherosclerosis are scarce [10], they support a role of TXA_2 in endothelial dysfunction and the pathological control of cerebrovascular tone [42].

Despite the demonstration that inhibition of TXA_2 synthase [28] and COX activity [24] is neuroprotective in rodent models of stroke and neurodegenerative diseases, and that cardio-protective polyphenols with antioxidant properties reduce the incidence of ischemic stroke [23, 50], the exact link between cerebral eNOS dysfunction, a rise in oxidative stress, and TXA_2 has not been defined. We hypothesized that in a pro-oxidant environment associated with aging and atherosclerosis, a dysfunction of cerebral eNOS function reduces the dilatory role of H_2O_2, increases the TXA_2 synthase activity, thus perpetuating the vicious circle of oxidative stress and accelerating endothelial damage. To validate this hypothesis, chronic treatment with the polyphenol catechin [12, 50, 54] and the effects of acute pharmacological TXA_2 synthase inhibition were tested in cerebral arteries isolated from middle-aged severely dyslipidemic mice. The results support the concept that with aging and dyslipidemia, eNOS dysfunction mirrors the rise in TXA_2 synthase activity, promoting cerebral endothelial dysfunction. Long-term preventive catechin treatment minimizes the decrease in eNOS activity and the rise in TXA_2 synthesis.

Materials and methods

The procedures and protocols were performed in accordance with our institutional guidelines and the Guide for the Care and Use of Laboratory Animals of Canada. We used 3- and 12-m/o C57Bl/6 male mice (WT, 29±1 g and 46±3 g, respectively, n=20; Charles River Laboratories, St-Constant, QC, Canada) and 3- and 12-m/o atherosclerotic mice (ATX, 25±1 g and 39±2 g, respectively, n=20). ATX mice are knockout for the low density lipoprotein receptor (LDLR) and express the human apolipoprotein B-100 gene ($LDLR^{-/-}$; $hApoB^{+/+}$ [6, 44]). Founders of the colony were generous gifts from Dr. H. Hobbs (University of Texas Southwestern, Dallas, TX, USA). An additional group of ATX mice received catechin (CAT, 98% purity; Sigma-Aldrich, Oakville, ON, Canada) for 9 months (from 3 to 12 m/o, n=20) in the drinking water (ATX+CAT; 30 mg/kg/day), as previously described [12] and to provide an appropriate dietary intake of ≈0.75 mg/day and per mouse of catechin ([18, 26, 34, 56]; see also comment in the supplementary data). All mice were fed with a normal standard diet (Harlan Laboratories, Tecklad 2014S, Montreal, QC, Canada) since ATX mice spontaneously develop atherosclerotic lesions.

Systemic pressure and plasma parameters

At the age of 3 and 12 months, mice were sacrificed by exsanguination under deep anesthesia (isoflurane) after monitoring heart rate and blood pressure using a Millar catheter inserted into the left carotid artery. Isoflurane anesthesia has no impact on vascular reactivity in isolated cerebral arteries (data not shown). Plasma was collected from blood sample and frozen at −80°C. Total cholesterol, LDL, and triglycerides were measured in the hospital biochemical laboratory. Plasma levels of the stable metabolite of TXA_2 (11-dehydro-thromboxane B_2, TXB_2) were quantified using an ELISA according to the protocol provided by the manufacturer (Cayman Chemical, Ann Arbor, MI, USA).

Plaque area quantification

Thoracic aortas were removed from the mice and carefully dissected from surrounding tissues. The vessels were opened, pinned, and a picture was taken. Plaque area quantification was calculated with Photoshop software and expressed in percentage of total aortic area [6].

Quantification of oxidative stress

Concomitant with aortic plaque quantification, the oxidative fluorescent probe dihydroethidium (DHE, 5 μmol/l; Sigma-Aldrich Canada) was used to evaluate global in situ oxidative stress on 14-μm sections of unfixed frozen aorta [12]. In addition, brain sections containing the basilar artery were processed similarly to evaluate cerebrovascular oxidative stress [6]. DNA counterstaining was performed using ToPro-3 (2 μM; Molecular Probe, Burlington, ON, Canada). Computer-based analysis was performed using Image J software and data are expressed as average fluorescence per nucleus.

Experimental design for reactivity studies

The brain was rapidly removed from the cranial cavity and placed in ice-cold physiological salt solution (PSS) of the following composition (mM): NaCl 130, KCl 4.7, $CaCl_2$ 1.6, $MgSO_4$ 1.17, $NaHCO_3$ 14.9, KH_2PO_4 1.18, EDTA 0.026, and glucose 10. As previously described [6–8], cerebral arteries from the circle of Willis were isolated, cannulated at both ends (average internal diameter of 136±2 μm; n=60), and pressurized at 60 mmHg in no-flow condition in PSS oxygenated by a gas mixture containing 12% O_2, 5% CO_2, and 83% of N_2, generating a pO_2 of 150±10 mmHg. An equilibration period of 40 min was allowed before starting the experiments, time during which myogenic tone (MT) develops. The vessels were contracted with phenylephrine (PE; 10 to 30 μmol/l) to obtain a similar pre-constriction level (of 40–50%). Then, a single cumulative concentration–response curve to acetylcholine (ACh, 0.1 nM to 30 μM) was performed on each segment. In some protocols, the arteries were pre-treated for 30 min with (1) N^w-nitro-L-arginine (L-NNA; 10 μmol/l), a NOS inhibitor; (2) indomethacin (INDO; 10 μmol/l), a COX inhibitor; (3) furegrelate (FUR; 10 μmol/l), a potent TXA_2 synthase inhibitor [13]; or (4) apocynin (APO; 10 μmol/l), a non-specific NADP(H) oxidase inhibitor. PE, ACh, INDO, FUR, APO, and L-NNA were purchased from Sigma-Aldrich Canada Ltd. The maximal diameter (D_{max}) was determined by changing the PSS to a Ca^{2+}-free PSS; MT and dilations were expressed as percentage of the D_{max} [6–8]. Half-maximum effective concentration (EC_{50}) of ACh was measured from each individual concentration–response curve using a logistic curve-fitting program (Allfit;

Dr André De Léan, Université de Montreal, QC, Canada). Vascular sensitivity (pD_2) values, the negative log of the EC_{50}, were obtained.

Fluorescent measurement of eNOS-derived H_2O_2

We previously demonstrated that in mouse cerebral arteries, ACh-induced dilation is principally mediated by eNOS-derived H_2O_2 [8] and we reported the method to measure simultaneously the dilation and the production of H_2O_2, using a fluorescent probe, the 5-(and-6)-chloromethyl-2′,7′-dichlorodihydrofluorescein diacetate acetyl ester (DCF-DA, Molecular Probe). In our model, DCF-DA is specific for H_2O_2 [7, 8]. Fluorescence intensities (λ_{Ex} 492–495 nm) were measured at 520 nm with an IonOptix Acquire system (IonOptix, Milton, MA, USA). Before each experiment, basal fluorescence intensity was recorded. The increase in fluorescence associated with ACh stimulation was calculated as the difference between the intensity of DCF-DA fluorescence in the presence of ACh and the intensity of DCF-DA fluorescence in the presence of PE.

Western blots

Whole brain vessels were isolated as previously described [22]. Briefly, two brains from each group were pooled, homogenized with a loosely fitting Dounce tissue grinder in ice-cold phosphate buffer saline (PBS, pH 7.4), and centrifuged at 3,500 rpm for 5 min at 4°C. The pellet was then suspended in PBS, gently layered over 15% dextran solution (molecular mass, 43 kDa), and finally centrifuged at 5,000 rpm for 30 min at 4°C. Pellets containing blood vessels were collected over a 50-μm nylon mesh. Proteins were isolated from the vessels following 30-min incubation in a lysis buffer of the following composition (mM): Tris–HCl 50, β-glycerophosphate 20, NaF 20, EDTA 5, EGTA 10, Na_3VO_4 1, benzamidin 10, dithiothreitol 5, PMSF 0.5, leupeptin 0.02, microcystin LR 1, and Triton X-100 1% (v/v). For Western blot analysis, proteins (50 μg/lane) were subjected to SDS–PAGE, using a discontinuous buffer system (Laemmli), in 10–20% gradient gels and transferred to nitrocellulose. Blots were incubated with the primary antibodies [rabbit polyclonal anti-endothelial NOS (eNOS), 1:200, BD Transduction Laboratories, Franklin Lakes, NJ, USA; rabbit polyclonal anti-inducible NOS (iNOS), 1:1,000, Santa Cruz Biotechnology, Santa Cruz, CA, USA; rabbit polyclonal anti-COX-1, 1:1,000, Cayman Chemical, Arbor, MI, USA; rabbit polyclonal anti-COX-2, 1:1,000, Cayman Chemical; goat polyclonal anti-PGI_2 synthase, 1:200, Santa Cruz Biotechnology; and rabbit polyclonal anti-TXA_2 synthase, 1:1,000, Abcam, Cambridge, MA, USA]. Equal protein loading was established with GAPDH (mouse monoclonal anti-GAPDH, 1:100,000, Ambion, Streetville, ON, Canada) or with α smooth muscle actin (mouse monoclonal anti-α smooth muscle actin, α SMA, 1:100,000, Sigma-Aldrich Canada).

Statistics

"*n*" refers to the number of animals used in each protocol. Continuous variables are expressed as mean± standard error (SEM). One-way ANOVA followed by Tukey's test as post hoc test was used to compare each parameter among the different groups of mice. Unpaired *t* test was performed to study the effects of L-NNA, FUR, APO, and INDO on ACh-induced dilations, MT, and H_2O_2-fluorescence increase. The results were considered to be statistically significant when the *p* value was <0.05.

Results

Heart rate and blood pressure

Heart rate was not affected by age in WT but it tended to decrease (p=0.06) in 12-m/o ATX mice (Table 1). Arterial blood pressure increased with age in both WT and ATX mice. At 3 months, however, ATX mice were already hypertensive compared to WT mice (Table 1). Catechin maintained blood pressure to values measured in 3-m/o WT without affecting heart rate at 12 months (Table 1). Arterial blood pressure was routinely measured and compared in animals under isoflurane anesthesia (Table 1) and also in non-anesthetized animals by the tail-cuff technique (6-m/o ATX mice were hypertensive when compared to WT mice, systolic blood pressure=128±2 versus 146±3 mmHg, WT versus ATX mice, n=8, p<0.05). In 6-m/o conscious mice, HR was not affected by age or dyslipidemia (666±10 versus 682±12 bpm, WT versus ATX mice, n=8, p>0.05). It is apparent that isoflurane anesthesia significantly depressed HR and blood pressure.

Plasma parameters

ATX mice were severely dyslipidemic at both 3 and 12 months of age compared to WT mice (Table 1); as expected, catechin did not modify cholesterol and triglycerides levels (Table 1). Of interest, cholesterol rose with age in WT mice, essentially due to a rise in LDL cholesterol (Table 1). Plasma thromboxane B_2 (the stable metabolite of TXA_2) levels were unaffected by age or dyslipidemia (Table 1). Following catechin treatment, however, TXB_2 levels were reduced by 80% in the plasma of ATX mice (Table 1).

Plaque area and oxidative stress

WT mice did not develop atherosclerotic plaque with age, in contrast to ATX mice (Supplementary Fig. S1). Catechin significantly reduced plaque formation by 35% (Fig. S1). Atherosclerotic lesions were also present in the carotid arteries from middle-aged ATX mice. In contrast, lesions or fatty deposits were observed only occasionally in cerebral arteries from ATX mice (data not shown). Global vascular oxidative stress, assessed by aortic DHE staining, was significantly higher in 12-m/o compared to 3-m/o WT mice (Fig. 1a). DHE staining was already greater at 3 months in ATX than in WT mice but did not increase further with age. Catechin normalized oxidative stress in 12-m/o ATX mice to 3-m/o WT values (Fig. 1a). In cerebral arteries, DHE staining was similar between 3-m/o WT and ATX mice (Fig. 1b), as previously reported [6]. In contrast to aortic-DHE staining, superoxide staining in cerebral arteries decreased in 12-m/o ATX mice and was not affected by catechin (Fig. 1b).

Cerebral myogenic tone: basal eNOS dysfunction and up-regulation of TXA_2 with atherosclerosis

The physiological response of resistance arteries to a rise in intra-luminal pressure is an increase in MT, i.e., a reduction in diameter. The MT of cerebral arteries induced by a pressure of 60 mmHg was greater in ATX than in WT mice at both ages, while aging alone had no effect (Fig. 2a). L-NNA increased MT in cerebral arteries isolated from WT mice only, suggesting an impaired basal eNOS activity in ATX mice (Fig. 2a). The increased MT

in arteries isolated from ATX mice was eliminated by INDO or FUR, while these agents had no effect on MT in WT mice, suggesting a role for TXA_2 in the elevated MT in ATX mice. After 9 months of treatment with catechin, MT of vessels isolated from ATX mice was normalized to WT values, sensitive to L-NNA and unaffected by INDO or FUR (Fig. 2a), demonstrating a reversal of the eNOS dysfunction and a reduction in TXA_2-dependent regulation of cerebrovascular MT.

Decline in cerebral eNOS function and up-regulation of TXA_2 with age and atherosclerosis

In cerebral arteries isolated from 3-m/o WT mice, ACh induced endothelium-dependent dilations (pD_2 of 7.0±0.2 and E_{max} of 48±4%; Table 2) and we previously reported that eNOS-derived H_2O_2 production is a key endothelium-derived relaxing factor in young and healthy mouse cerebral arteries [8]. We therefore investigated eNOS function by simultaneous recording ACh-mediated dilations and H_2O_2 production (Figs. 2b–c, 3, and 4). With age, efficacy to ACh decreased by ≈50% in arteries from WT mice and dilations were almost abolished in middle-aged ATX mice (Fig. 2b, Table 2). Accordingly, H_2O_2 production was reduced in 12-m/o WT mice and in ATX mice when compared to 3-m/o WT mice (Fig. 2c). Chronic catechin treatment prevented the accelerated decline in endothelium-dependent dilations with age in ATX mice and improved eNOS–H_2O_2 production (Fig. 2b–c, Table 2).

In 3-m/o WT mice, ACh-induced dilations were equally reduced in the presence of L-NNA or INDO, while FUR or APO had no effect (Fig. 3a–b, Table 2), suggesting a dilatory role for eNOS and prostacyclin. Accordingly, eNOS–H_2O_2 production was abolished by L-NNA but unaffected by INDO, FUR, or APO (Fig. 3c). In 12-m/o WT mice, the inhibitory effect of L-NNA was lower when compared to 3-m/o WT mice, suggesting the development of eNOS dysfunction with age (Fig. 3). In addition, both INDO and FUR increased ACh dilations and H_2O_2-associated fluorescence in arteries from 12-m/o WT mice (Fig. 3, Table 2), suggesting an up-regulation of TXA_2 with age, counteracting the dilatory effect of eNOS.

Premature eNOS dysfunction in cerebral arteries from 3-m/o ATX mice was illustrated by the lower inhibitory effect of L-NNA on both dilation and H_2O_2 production when compared to 3-m/o WT mice (Fig. 4, Table 2). This was associated with an early deleterious role of TXA_2, as shown by the improvement of ACh dilations in the presence of INDO or FUR (Fig. 4a–b, Table 2). Interestingly, INDO and FUR also improved eNOS–H_2O_2 production (Fig. 4c), suggesting that TXA_2 inhibits eNOS function.

In 12-m/o ATX mice, eNOS function was abolished since ACh dilations and H_2O_2-associated fluorescence were insensitive to L-NNA (Fig. 4, Table 2). ACh dilations in 12-m/o ATX mice were increased by FUR or INDO, although to a lesser extent than in 3-m/o ATX mice (Fig. 4a–b, Table 2). Neither INDO alone nor its combination with L-NNA altered H_2O_2 production (Fig. 4c). In addition, the fluorescent signal measured in the combined presence of L-NNA and INDO was associated with a significant vasodilation (Fig. 4b), suggesting the implication of an EDHF [8]. Thus, the rise in H_2O_2-associated fluorescence induced by ACh was eNOS independent; it was, however, abolished by APO, a non-specific NADP(H) oxidase inhibitor, which had no effect in the other groups (Fig. 4c). This is in accordance with the effects of APO on ACh-induced dilations, which only

improved dilations in arteries isolated from 12-m/o ATX mice (Fig. 4, Table 2), suggesting that NADP(H) oxidase may contribute to endothelial dysfunction in these middle-aged atherosclerotic mice.

Following the chronic treatment with catechin, eNOS function was preserved: in cerebral arteries from 12-m/o ATX mice, both ACh dilations and H_2O_2-associated fluorescence became sensitive to L-NNA and were affected neither by INDO, nor FUR or APO (Fig. 4), suggesting eNOS preservation and TXA_2 down-regulation. eNOS dysfunction and TXA_2 up-regulation observed with age and atherosclerosis are summarized in Fig. 5.

Protein expression: decline in PGI_2 synthase and rise in TXA_2 synthase with atherosclerosis

PGI_2 synthase protein expression was high in cerebral vessels isolated from 3-m/o WT mice only (Fig. 6a), supporting the role of PGI_2 as an EDRF in these mice only. In ATX mice, the expression of the enzyme was significantly lower at 3 months of age compared to WT and was not further reduced with aging (Fig. 6a). Catechin treatment did not reverse PGI_2 synthase protein level, in agreement with the functional data reported in Fig. 5, where INDO had no inhibitory effect on the endothelium-dependent dilations. In contrast, TXA_2 synthase increased dramatically in middle-aged ATX mice (Fig. 6b), an effect that was fully prevented by 9 months of treatment with catechin. This is in accordance with the functional data (Fig. 4) where catechin reversed the effects of FUR on eNOS function. Similarly, COX-1 and COX-2 expression rose with age in WT mice, and further in ATX mice (Supplementary Fig. S2); catechin prevented the rise in COX-1 expression and normalized the rise in COX-2 expression to 3-m/o ATX level (Supplementary Fig. S2). Aging, atherosclerosis, or catechin affected the expression of neither eNOS nor iNOS protein in cerebral vessels (Supplementary Fig. S3). Total expression of eNOS may, however, not be truly representative of eNOS function, unlike eNOS dimer/monomer ratio. We indeed previously showed that in mouse cerebral arteries, monomeric eNOS is the preferred state for the production of H_2O_2, although addition of BH_4 did not significantly increase the dimerization of eNOS, while it strongly increased NO production [8].

Discussion

In the present study, we demonstrate for the first time that the age-related cerebral endothelial dysfunction in WT mice occurs prematurely in ATX mice. This endothelial dysfunction is associated with an up-regulation of TXA_2, counter-acting eNOS activity and the cerebrovascular dilation. This is accompanied by hypertension and severe peripheral conductance artery atheroma. Our data demonstrate that chronic preventive catechin treatment, a dietary cardio-protective polyphenol with antioxidant properties, reduces plaque size, normalizes blood pressure, and, most importantly, prevents the rise in TXA_2 synthase activity maintaining cerebral artery eNOS function.

In mouse cerebral arteries, we reported that the normal physiological function of eNOS is to generate dilatory H_2O_2—and not NO—in response to ACh [8] or to increases in intra-luminal flow [7]. In cerebral arteries of 12-m/o WT mice, endothelial dysfunction developed and eNOS-associated dilation and H_2O_2 release were both reduced by ≈50%. PGI_2 synthase

protein levels were decreased (Fig. 6a), but INDO and FUR restored the normal dilatation. Therefore, in the cerebral arteries of middle-aged WT mice, H_2O_2 and prostacyclin production decline due to a rise in TXA_2 synthase activity, as illustrated in Fig. 5. This contrasts with the report that impaired cerebral dilations in aged rats are insensitive to INDO and thus not related to production of COX-derived constrictor metabolites [30]. In cerebral vessels isolated from 3-m/o ATX, the amplitude of the dilation associated with eNOS-derived H_2O_2 was also reduced by ≈50%; both INDO and FUR restored the dilation and eNOS activity, while PGI_2 synthase expression was very low. Therefore, a similar deleterious profile is measured in young ATX and in middle-aged WT mice.

The early and reversible cerebral endothelial dysfunction observed in 3-m/o ATX mice is in sharp contrast with the almost complete loss of ACh-induced dilations in arteries isolated from 12-m/o ATX mice. In the latter, INDO and FUR only slightly improved dilations to ACh, but the production of H_2O_2 was insensitive to L-NNA; this demonstrates that combined with severe dyslipidemia, aging leads to irreversible damages. Interestingly, in the presence of ACh, the rise in H_2O_2-associated fluorescence—but not the dilatory response—was abolished by apocynin, suggesting that in cerebral arteries isolated from 12-m/o ATX mice (unlike in any other groups), the NADP(H) oxidase is sensitive to muscarinic receptor activation. It has been shown that NADP(H) oxidase was partly responsible for flow-mediated dilation in rat brain arteries in vivo [39], a mechanism absent in isolated mouse cerebral arteries [7]. Further studies including the measurement of NAD(P)H oxidase subunits expression are needed to clarify the exact role of this enzyme in the endothelial dysfunction observed in 12-m/o ATX mice.

The rise in TXA_2 synthase and the potentially augmented NADP(H) oxidase activity in middle-aged ATX mice must contribute to the change in the *redox* environment. The increase in cerebrovascular NADP(H) oxidase activity in animal models of hypercholesterolemia was recently reported [20, 32]. Furthermore, it has been shown that aging limits the ability of antioxidant enzymes to up-regulate in response to an acute and short-term pro-atherogenic challenge [3]. In our experimental conditions, however, this imbalance towards pro-oxidation was likely the result of a long-term pro-atherogenic stimulus leading to chronic damage [53]. This was indeed completely reversed by a chronic and long-term preventive treatment with catechin, a scavenger of superoxide anions [41, 50]. Catechin is, however, a non-specific antioxidant that displays large cardio-protective properties [45], including anti-inflammatory properties [35, 52]. To achieve significant beneficial effects against cardiovascular diseases, frequent and habitual consumption of catechins present in green tea is required [35, 38, 54]. In the present study, beneficial functional effects of long-term chronic catechin treatment were associated with reduced aortic plaque formation and aortic oxidative stress (but not cerebral oxidative stress), as we previously reported [12], and normalization of blood pressure in ATX mice. The catechin treatment preserved ACh-induced endothelium-dependent dilations to the level of arteries isolated from age-matched WT mice. This was associated with a lack of TXA_2 production or a potential contribution of NADP(H) oxidase during ACh stimulation, leading to the prevention of eNOS dysfunction. Catechins have been shown to modulate NOS activities, i.e., to inhibit iNOS and to stimulate eNOS [35, 50, 51]. There are numerous reports showing that antioxidants in general preserve or improve eNOS function either directly or by

increasing NO bioavailability, and reduce the development of atherosclerosis and inflammation in mice models of dyslipidemia [4, 16, 58]. The beneficial role of antioxidants on the cerebral circulation, particularly in the context of atherosclerosis, is, however, less studied [20, 31, 32].

Although previous studies suggested an association between TXA_2 and stroke or cerebral ischemia [2, 21, 43, 55], the role of TXA_2 in cerebrovascular dysfunction associated with severe dyslipidemia and atherosclerosis is less clear [10]. Previous studies suggested that TXA_2 not only competes with but also lowers eNOS activity via an increase in ROS following receptor activation in renal arteries [13], in cultured endothelial cells [57], and in cerebral arteries [36]. In the present study, FUR increased endothelium-dependent dilations and reduced MT, suggesting a major role for TXA_2 in cerebral arteries from 12-m/o ATX mice.

In ATX mice, the reduction in TXA_2 synthase expression by catechin was accompanied by a strong reduction in plasma TXB_2 levels. Accordingly, TXA_2 production in platelets was reported to be inhibited by polyphenols [29]. The phenolic antioxidant AGI-1067 also decreased TXB_2 plasma levels without affecting PGI_2 production in patients with multiple risk factors for cardiovascular diseases [47]. It has also been previously reported that green tea extract was neuroprotective in rats exposed to ischemia/reperfusion by lowering eicosanoids brain concentration, including TXA_2 [19].

Our study has some limitations: catechin and apocynin have potential non-specific effects that should also be considered. Catechin, used for 9 months, likely displays effects other than antioxidative properties [35]. Apocynin is a non-specific inhibitor of the NADP(H) oxidase, which may also act as a ROS scavenger [17, 46]. In addition, although used acutely in the present study, furegrelate, a specific TXA_2 synthase inhibitor, may affect the level of other prostaglandins besides TXA_2. Finally, oxidative stress was assessed only by DHE staining, but the quantification of isoprostane levels could have been a more reliable index.

In conclusion, in our murine model of severe human dyslipidemia, atherosclerosis led to premature endothelial aging via eNOS dysfunction, an increase in TXA_2 production and oxidative stress (see Fig. 7 for summary). The impairment of eNOS function by oxidative stress could promote TXA_2 production, which would further contribute to the rise in ROS and endothelial dysfunction. Catechin, used in prevention, slowed the progression of atherosclerosis and the endothelial dysfunction via a reduction of the role of the TXA_2 pathway and thus the protection of the cerebrovascular eNOS function.

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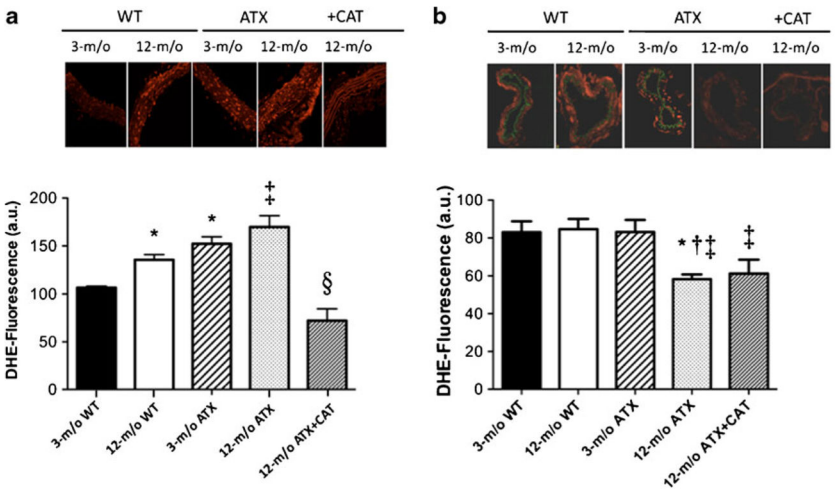

Fig. 1.

Catechin treatment reduces aortic oxidative stress. Effect of 9-month catechin treatment on **a** aortic and **b** cerebrovascular superoxide production quantified by DHE staining (n=4, in triplicate). Data are mean±SEM. $^{*}p<0.05$ versus 3-m/o WT, $^{\dagger}p<0.05$ versus 3-m/o ATX, $^{\ddagger}p<0.05$ versus 12-m/o WT, $^{\S}p<0.05$ versus 12-m/o ATX

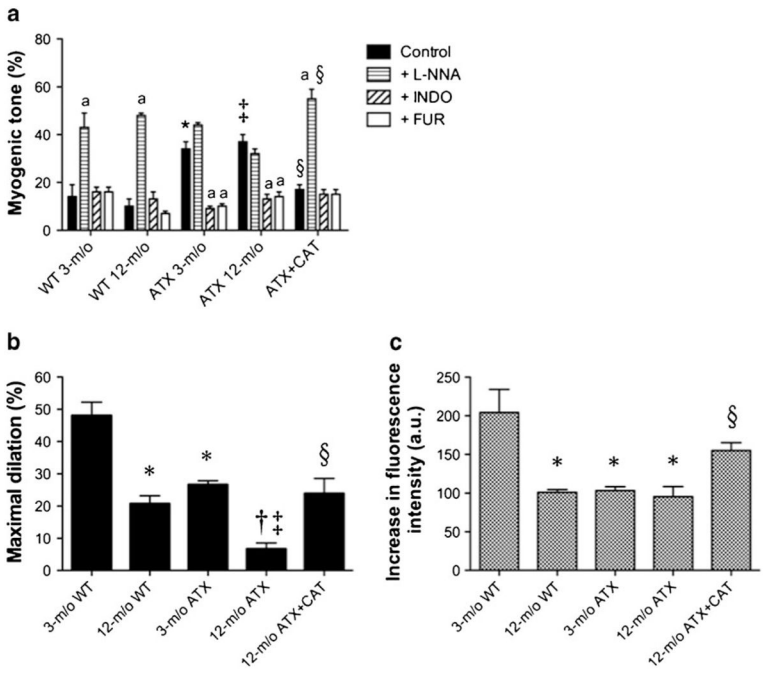

Fig. 2.

Cerebral eNOS dysfunction with aging and atherosclerosis. **a** Effect of eNOS, COX, and TXA_2 synthase inhibition with L-NNA, indomethacin (*INDO*), and furegrelate (*FUR*), respectively, on cerebral myogenic tone (%) in 3- and 12-m/o WT and ATX mice treated or not with catechin (*CAT*). Effect of aging, atherosclerosis (*ATX*), and catechin treatment for 9 months (*ATX+CAT*) on **b** maximal endothelial-dependent dilations to ACh (10 μM) and **c** the associated eNOS–H_2O_2 production in cerebral arteries isolated from 3- and 12-m/o WT and ATX mice. Data are mean±SEM (n=8). $^{*}p< 0.05$ versus 3-m/o WT, $^{\dagger}p<0.05$ versus 12-m/o WT, $^{\ddagger}p<0.05$ versus 12-m/o ATX, $^{\S}p<0.05$ versus MT

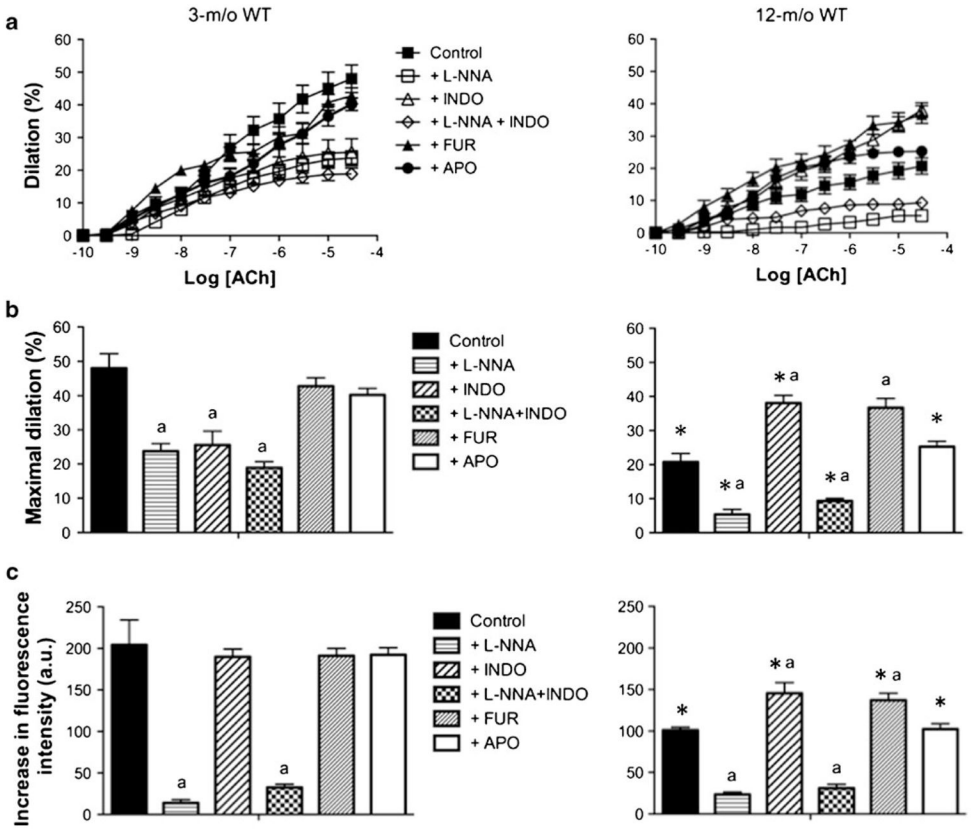

Fig. 3.

Aging is associated with cerebral endothelial dysfunction. Effect of *N*-nitro-L-arginine (*L-NNA*; 10 μmol/l), indomethacin (*INDO*; 10 μmol/l), L-NNA combined with INDO, furegrelate (*FUR*; 10 μmol/l), and apocynin (*APO*; 10 μmol/l) on **a** dose–response curve to ACh, **b** maximal endothelium-dependent dilations to ACh, and **c** the associated eNOS-derived H_2O_2 production in cerebral arteries isolated from 3- and 12-m/o WT mice (n=6). Data are mean±SEM. [a]p<0.05 versus control, *p<0.05 versus 3-m/o WT

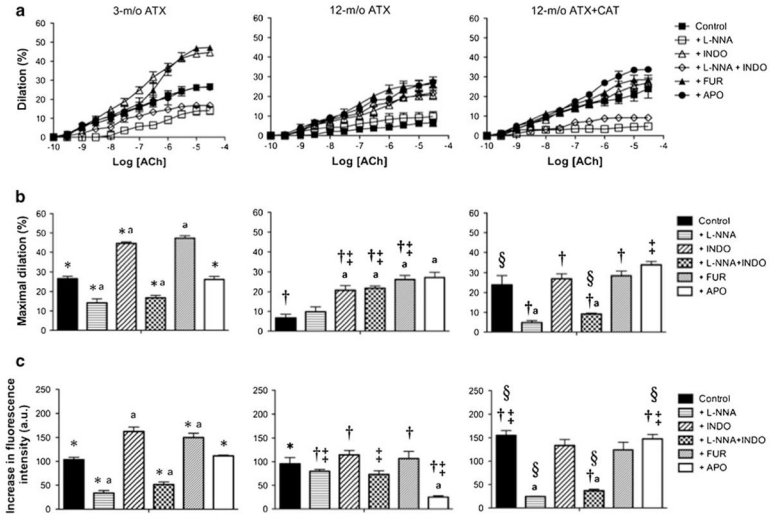

Fig. 4.

Catechin treatment prevents cerebral endothelial dysfunction associated with aging and dyslipidemia. Effect of *N*-nitro-L-arginine (*L-NNA*; 10 μmol/l), indomethacin (*INDO*; 10 μmol/l), L-NNA combined with INDO, furegrelate (*FUR*; 10 μmol/l), apocynin (*APO*; 10 μmol/l), and catechin (*CAT*) treatment on **a** dose–response curve to ACh, **b** maximal endothelium-dependent dilations to ACh, and **c** the associated eNOS-derived H_2O_2 production in cerebral arteries isolated from 3- and 12-m/o ATX mice treated or not with CAT. Data are mean±SEM (n=6). $^{a}p<0.05$ versus control, $^{*}p<0.05$ versus 3-m/o WT, $^{\dagger}p<0.05$ versus 3-m/o ATX, $^{\ddagger}p<0.05$ versus 12-m/o WT, $^{\S}p<0.05$ versus 12-m/o ATX

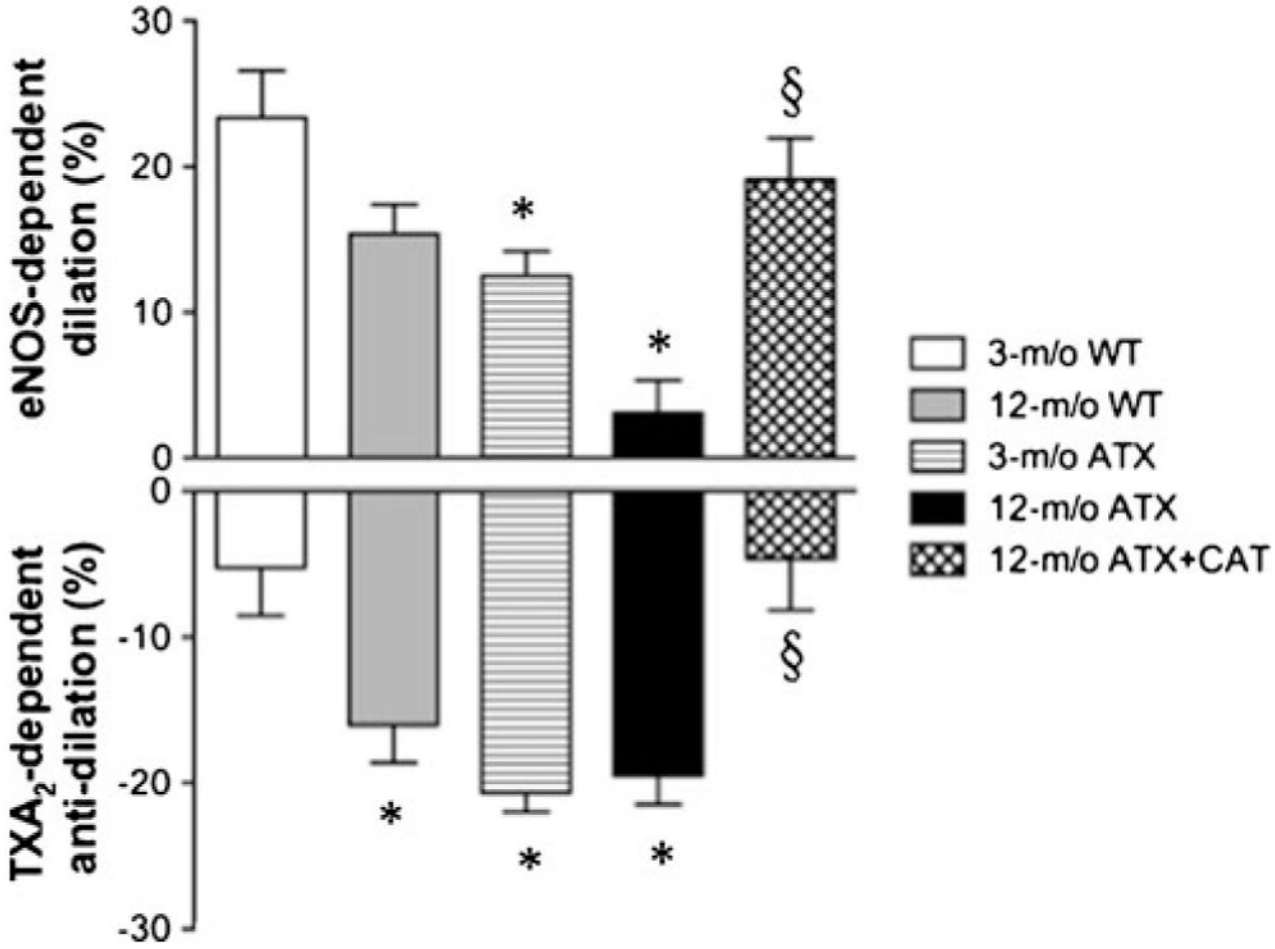

Fig. 5.

Evolution of the contribution of eNOS–H_2O_2 as a dilatory factor and TXA_2 as an anti-dilatory factor with age and severe dyslipidemia. The contribution of eNOS activity to the dilation induced by ACh (10 μM) is reported as the amplitude of the control dilation (%) *minus* the amplitude of the L-NNA-sensitive dilation (%); the inhibitory effect of TXA_2 on the dilatory response induced by ACh is reported as the amplitude of the control dilation (%) *minus* the amplitude of the FUR-sensitive dilation (%). Data are mean±SEM (n=6). $^{*}p<0.05$ versus 3-m/o WT, $^{\S}p<0.05$ versus 12-m/o ATX

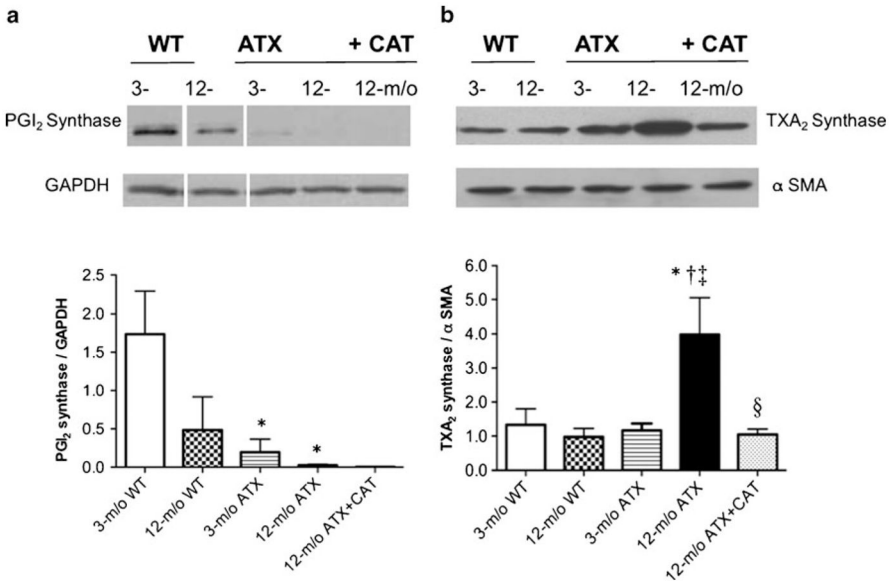

Fig. 6.

Catechin treatment prevents cerebral up-regulation of TXA_2 synthase. Effect of aging, atherosclerosis, and catechin (*CAT*) treatment on whole brain vessels protein expression of **a** PGI_2 synthase and **b** TXA_2 synthase of wild-type (*WT*) and atherosclerotic (*ATX*) mice. Protein expressions are normalized to GAPDH protein expression (PGI_2 synthase) or to α smooth muscle actin protein expression (TXA_2 synthase) (n=5). Data are mean±SEM. $^{*}p<0.05$ versus 3-m/o WT, $^{\dagger}p<0.05$ versus 12-m/o WT, $^{\ddagger}p<0.05$ versus 3-m/o ATX, $^{\S}p<0.05$ versus 12-m/o ATX

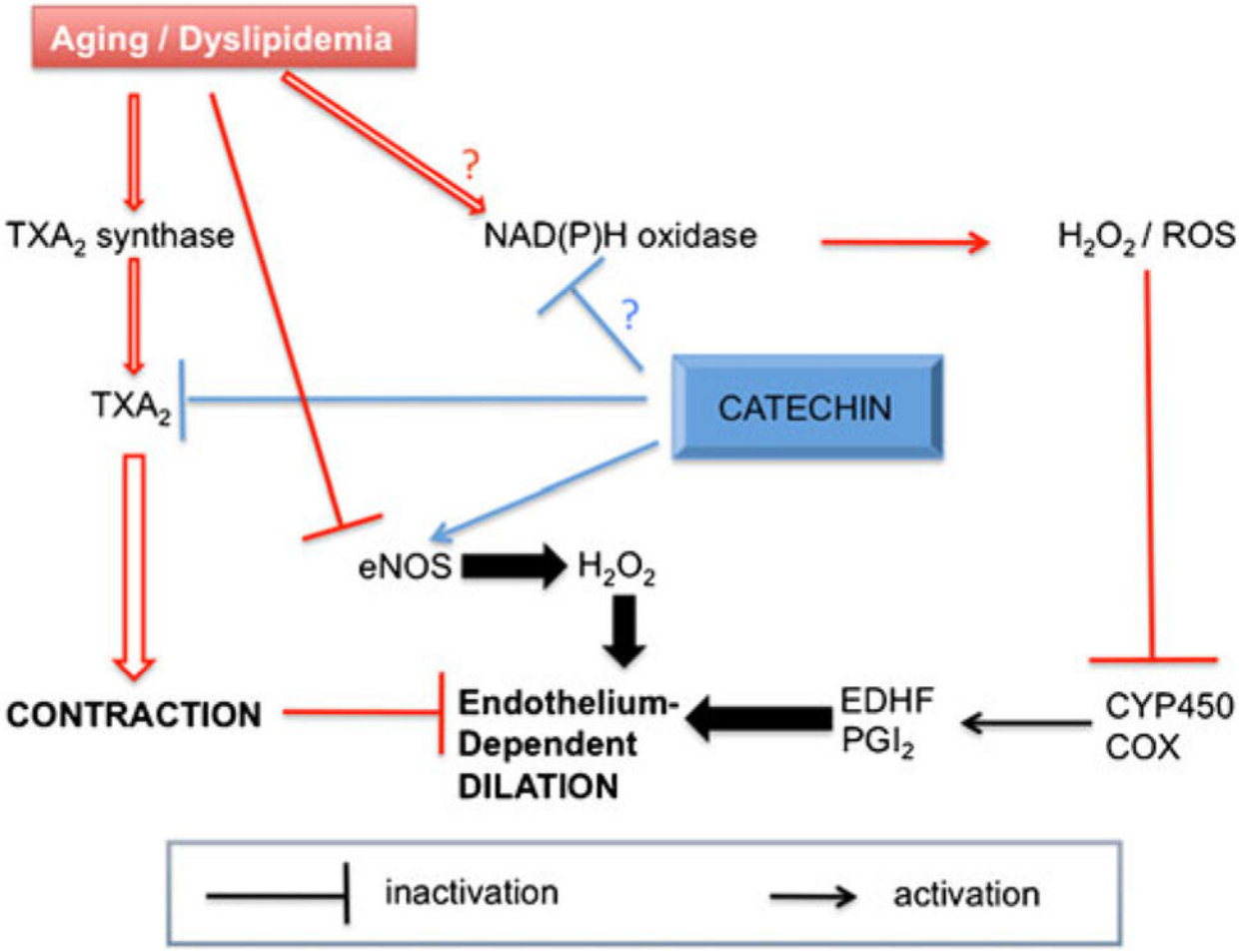

Fig. 7.

Schematic representation of the effects of age and severe dyslipidemia on the regulation of the cerebrovascular function and the preventive effects of catechin. Both aging and dyslipidemia favor the contribution of contractile TXA_2 which compromises endothelium-dependent cerebral dilation mediated through eNOS-derived H_2O_2. Aging and dyslipidemia are also associated with a rise in ROS, potentially originating from NADP(H) oxidase, which further impairs endothelial-dependent dilation via inhibition of the non-NO and non-PGI_2 component of the dilation, likely cytochrome P450-derived EDHF. Chronic treatment with the cardio-protective polyphenol catechin preserves eNOS–H_2O_2 pathway, limits the implications of TXA_2, and potentially that of NADP(H) oxidase

Table 1

Effect of aging, atherosclerosis, and catechin treatment on hemodynamic parameters and plasma levels of cholesterol, triglycerides, and thromboxane B_2 (TXB_2)

	WT 3 m/o	WT 12 m/o	ATX 3 m/o	ATX 12 m/o	ATX+CAT 12 m/o
Hemodynamic parameters					
Heart rate (bpm)	377±19	375±29	371±22	304±18	300±17
DAP (mmHg)	59±3	73±1 *	73±2 *	81±2 **, ***	64±6 ****
SAP (mmHg)	94±2	111±3 *	106±3 *	122±3 **, ***	99±6 ****
MAP (mmHg)	71±3	86±2 *	84±3 *	95±2 **, ***	76±5 ****
Plasma levels					
Cholesterol (mmol/l)	2.1±0.1	4.5±0.2 *	16.6±2.0 *	23.3±3.4 ***	23.5±3.2
LDL (mmol/l)	0.5±0.1	2.5±0.2 *	10.3±1.6 *	14.2±2.1 ***	15.1±2.2
HDL (mmol/l)	1.3±0.1	1.8±0.1	1.7±0.1	2.1±0.2	1.9±0.1
Triglycerides (mmol/l)	0.7±0.1	0.4±0.1	6.2±0.5 *	9.5±0.9 **, ***	9.7±1.3
TXB_2 (pg/ml)	19±2	20±3	22±9	20±2	4±1 ****

Data are mean±SEM of n=8 mice in each group

WT wild-type mice, *ATX* atherosclerotic mice, *bpm* beats per minute, *DAP* diastolic arterial pressure, *SAP* systolic arterial pressure, *MAP* mean arterial pressure, *LDL* low-density lipoprotein, *HDL* high-density lipoprotein, *TXB2* 11-dehydro-thromboxane B_2, the stable metabolite of TXA_2

* $p<0.05$ versus 3-m/o WT,

** $p<0.05$ versus 3-m/o ATX,

*** $p<0.05$ versus 12-m/o WT,

**** $p<0.05$ versus 12-m/o ATX

Table 2

Effect of L-NNA, indomethacin, furegrelate, and apocynin, used acutely to inhibit eNOS, COX, TXA_2 synthase, and NADP(H) oxidase, respectively, on the sensitivity (pD_2) and the efficacy (E_{max}) to ACh in cerebral arteries

	Control	L-NNA	Indomethacin	Furegrelate	Apocynin
3-m/o WT					
pD_2	7.0±0.2	7.4±0.2	7.8±0.1 *	7.5±0.1	6.8±0.2
E_{max} (%)	48.0±4.2	23.7±2.2 *	25.5±4.1 *	42.8±2.4	40.2±1.9
12-m/o WT					
pD_2	7.2±0.3	ND	6.9±0.2 **	7.4±0.2	7.9±0.2 **
E_{max} (%)	20.8±2.5 **	5.3±1.5 *, **	38.1±2.2 *, **	36.7±2.7 *	25.3±1.6 **
3-m/o ATX					
pD_2	7.5±0.2	6.4±0.3 *, **	7.2±0.1	6.7±0.2 **	7.4±0.3
E_{max} (%)	26.6±1.3 **	14.1±2.1 *, **, ****	44.6±0.8 *, **	47.3±1.3 *, ****	26.2±1.6 **
12-m/o ATX					
pD_2	ND	ND	6.9±0.3 **	7.4±0.2 ***	7.2±0.4
E_{max} (%)	6.7±1.9 **, ***	9.8±2.5 **	20.8±2.3 *, ***, ****	26.2±2.1 *, **, ***, ****	27.2±2.7 *, **
12-m/o ATX+CAT					
pD_2	7.5±0.3	ND	7.2±0.3	7.4±0.2	7.0±0.1
E_{max} (%)	23.9±4.7 **, *****	4.8±1.0 *, **, ***	27.0±2.5 ***	28.5±2.3 **, ***	33.8±1.7 ****

Data are mean±SEM of n=8 mice in each group

WT wild-type mice, *ATX* atherosclerotic mice, *ATX+CAT* ATX mice treated with catechin for 9 months, *ND* not determined (values too low)

* p<0.05 versus control,

** p<0.05 versus 3-m/o WT,

*** p<0.05 versus 3-m/o ATX,

**** p<0.05 versus 12-m/o WT,

***** p<0.05 versus 12-m/o ATX