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ORIGINAL ARTICLE

Loss of vascular expression of nucleoside triphosphate diphosphohydrolase-1/CD39 in hypertension

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Abstract

Ectonucleoside triphosphate diphosphohydrolase-1, the major vascular/immune ectonucleotidase, exerts anti-thrombotic and immunomodulatory actions by hydrolyzing extracellular nucleotides (danger signals). Hypertension is characterized by vascular wall remodeling, endothelial dysfunction, and immune infiltration. Here our aim was to investigate the impact of arterial hypertension on CD39 expression and activity in mice. Arterial expression of CD39 was determined by reverse transcription quantitative real-time PCR in experimental models of hypertension, including angiotensin II (AngII)-treated mice (1 mg/kg/day, 21 days), deoxycorticosterone acetate-salt mice (1% salt and uninephrectomy, 21 days), and spontaneously hypertensive rats. A decrease in CD39 expression occurred in the resistance and conductance arteries of hypertensive animals with no effect on lymphoid organs. In AngII-treated mice, a decrease in CD39 protein levels (Western blot) was corroborated by reduced arterial nucleotidase activity, as evaluated by fluorescent (etheno)-ADP hydrolysis. Moreover, serum-soluble ADPase activity, supported by CD39, was significantly decreased in AngII-treated mice. Experiments were conducted in vitro on vascular cells to determine the elements underlying this downregulation. We found that CD39 transcription was reduced by proinflammatory cytokines interleukin (IL)-1β and tumor necrosis factor alpha on vascular smooth muscle cells and by IL-6 and anti-inflammatory and profibrotic cytokine transforming growth factor beta 1 on endothelial cells. In addition, CD39 expression was downregulated by mechanical stretch on vascular cells. Arterial expression and activity of CD39 were decreased in hypertension as a result of both a proinflammatory environment and mechanical strain exerted on vascular cells. Reduced ectonucleotidase activity may alter the vascular condition, thus enhancing arterial damage, remodeling, or thrombotic events.

Keywords Ectonucleotidase · CD39 · ATP · Angiotensin II · Hypertension

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Abbreviations

ADO	Adenosine
ADP	Adenosine 5′-diphosphate
AMP	Adenosine 5′-monophosphate
ATP	Adenosine 5′-triphosphate
AngII	Angiotensin II
DOCA	Deoxycorticosterone acetate
EC	Endothelial cell
IL	Interleukin
IFN-γ	Interferon gamma
MRA	Mesenteric resistance artery
SHR	Spontaneously hypertensive rat
NTPDase	Nucleoside triphosphate diphosphohydrolase
RT-qPCR	Reverse transcription quantitative real-time PCR
TGF-β1	Transforming growth factor beta 1
TNF-α	Tumor necrosis factor alpha
VSMC	Vascular smooth muscle cell

Introduction

Signaling by extracellular adenine nucleotides and nucleosides (i.e., purinergic signaling) serves as intercellular communication. Extracellular nucleotides and adenosine (ADO) act as cofactors, which amplify biological responses or exert feedback to a primary stimulus through the activation of P2 (P2X and P2Y) and P1 receptors. In the vasculature, extracellular nucleotides are considered "danger signals" because they cause platelet aggregation, inflammation, and vascular permeability [1, 2]. P1 and P2 receptor activation also contributes to the regulation of vasomotricity [3], promoting relaxation or constriction depending on either endothelial or vascular smooth muscle activation. In pathological conditions, nucleotides have been shown to promote atherothrombosis and to contribute to other inflammatory diseases such as sepsis and inflammatory bowel disease [4].

Extracellular nucleotides are metabolized by membrane-bound ectonucleotidases. Among which, the nucleoside triphosphate diphosphohydrolases (NTPDases) play a major role as regulators of purinergic signaling in the cardiovascular system [5]. NTPDase1 (CD39), the major ectonucleotidase expressed in the vascular wall, converts adenosine 5′-triphosphate (ATP) and adenosine 5′-diphosphate (ADP) into adenosine 5′-monophosphate (AMP). AMP is hydrolyzed by a second enzyme, ecto-5′-nucleotidase (CD73), to generate ADO, which is usually considered a vasculoprotective molecule that displays vasodilatory and anti-inflammatory effects [6]. CD39 constitutes the rate-limiting enzyme in the hydrolysis of nucleotides into ADO, consequently modulating danger and protective signals in the vasculature. An increase in the expression of CD39 has been shown to be a key mechanism in preventing vascular permeability, leukocyte extravasation [7], and tissue damage [8] after ischemia. We have shown previously that CD39 controls vasoconstriction and vasodilation, depending on P2 receptor activation [9, 10], thus potentially contributing to the regulation of peripheral arterial resistance and blood pressure. An important role for this enzyme is that, by reducing ATP and ADP concentration and favoring ADO accumulation, it exerts anti-thrombotic and anti-inflammatory effects [6]. Together, these data underline the wide spectrum of potential vascular protection effects exerted by CD39.

Hypertension represents the main risk factor for cardiovascular ischemic and thromboembolic complications. It is associated with arterial functional and structural remodeling and endothelial dysfunction, which is mainly attributed to oxidative stress [11] and elevated arterial tone, consequently increasing peripheral vascular resistance and systemic blood pressure. In the longer term, arterial remodeling involves hypertrophy, fibrosis, and proteolytic alterations of the extracellular matrix [12]. In recent decades, numerous studies have shown that hypertension may be considered a chronic inflammatory disease in which innate immune cell infiltration in the perivascular environment, together with pro- and anti-inflammatory cytokines, plays a central role [13, 14]. Immune cells such as macrophages and T lymphocytes contribute to endothelial dysfunction, arterial remodeling, and elevated blood pressure [14]. Angiotensin II (AngII) plays a pivotal role in these effects not only by promoting vasoconstriction and oxidative stress, leading to endothelial dysfunction, but also by recruiting, activating, and polarizing immune cells [15]. In this context, extracellular nucleotides and their associated regulatory ectonucleotidases are privileged candidates.

To the best of our knowledge, no data are yet available on the impact of systemic hypertension on CD39. With the aim of anticipating the development of vascular purinergic homeostasis, here we investigated the impact of systemic arterial hypertension on NTPDase1/CD39 expression in vivo and in vascular cells in vitro in conditions that mimic the vascular environment of hypertension.

Materials and methods

Complete materials and methods are available in the online-only supplementary material.

Animals

Animals were manipulated in accordance with European Community Standards on the Care and Use of Laboratory Animals (authorization No. 6422). C57Bl6/J male mice (15–20 weeks old) were treated with AngII (1 mg/kg/day) by using an osmotic pump (ALZET model 2004) for 12 or 21 days. Alternatively, mice were treated with deoxycorticosterone acetate (DOCA; Innovative Research of America) and salt (NaCl 1% in drinking water) associated with uninephrectomy

for 21 days. Spontaneously hypertensive rats (SHRs; 16 weeks old) were compared with Wistar Kyoto (WKY) rats.

Cell culture

Mouse vascular smooth muscle cells (VSMCs) were obtained from collagenase-digested mouse aorta. Mouse endothelial cells (ECs) (Mile Sven 1; MS1) were obtained from ATCC. Cells were cultured in 24-well plates and starved 24 h before stimulation. The origin and concentration of the recombinant molecules used are presented in Table S1.

Alternatively, cyclic stretch was imposed on cells by using type I collagen-coated six-well plates (Bioflex, Dunn Labortechnik, GMBH) connected to a Flexercell Strain Unit FX-4000 (Flexcell). CD39 mRNA expression was measured by using reverse transcription quantitative real-time PCR (RT-qPCR).

RT-qPCR analysis

Tissues from AngII-infused mice, DOCA-salt mice, and SHR rats and their respective controls were stored at − 20 °C in RNAlater Stabilization Reagent (Qiagen). RNA extraction was performed with the RNeasy® Micro Kit (Qiagen).

RNA extract was used to synthesize cDNA. RT-qPCR was performed with SYBR® Select Master Mix (Applied Biosystems) by using a LightCycler 480 Real-Time PCR System (Roche). Sequences of primer pairs are represented in Table S2.

Western blot

Arterial CD39 protein expression was assessed in mouse abdominal aorta homogenates by using antibody directed against mouse CD39 (mN1-2c 1/600; ectonucleotidases-ab.com).

Measurement of CD39 activity

Serum (3 μl) or aorta homogenate protein (5–10 μg) was added to the reaction mixture (Hank's Balanced Salt Solution containing HEPES 10 mM, $CaCl_2$, 2 mM, and $MgCl_2$, 1 mM at pH 7.5). The reaction also contained an inhibitor of adenylate kinase (Ap5a; 80 μg/ml) and of alkaline phosphatase (levamisole; 1 mg/ml). The reaction was started at 37 °C by adding etheno-ADP (100 μM) fluorescent derivative as a substrate. Hydrolysis was stopped by precipitation with 10% trichloroacetic acid. A fraction of the supernatant was used for HPLC etheno-ADP and etheno-AMP determination. Etheno-ADP, etheno-AMP, and atheno-ADO were separated by HPLC on a C18 reversed-phase column, and ADPase activity was calculated by measuring fluorescent etheno-ADP hydrolysis.

Data analysis

Multiple groups were compared by using a one-way analysis of variance (ANOVA) or a two-way ANOVA with post-test Bonferroni's correction. Two groups were compared by using an unpaired Student's t test (two-tailed); p values of < 0.05 were considered statistically significant.

Results

Reduced CD39 expression and activity in arteries of hypertensive animals

Hypertension was induced pharmacologically by AngII infusion or DOCA-salt treatment in mice or genetically in SHR rats. The associated increase in systolic blood pressure and heart hypertrophic remodeling was validated in all hypertensive groups compared with suitable controls (Table 1). Among the actors of purinergic signaling, CD39 mRNA was the only one to be systematically downregulated. Indeed, the CD39 arterial transcript was significantly decreased in three different experimental models of hypertension (Fig. 1). This decrease was observed both in mesenteric resistant arteries (MRAs) and in large elastic arteries (thoracic aorta) in the two mouse models (Fig. 1a, b) and in the MRA but not in the aorta in SHR rats. We further evaluated other members of the NTPDase family: NTPDase2, 3, and 8. NTPDase2 (CD39L1) was the only other member expressed in aortas but, in contrast to that of CD39, its expression was not significantly modified by AngII treatment (Fig. S1). To evaluate the potential impact of hypertension on CD39 expression in immune cells, we measured it in lymphoid organs. No changes were found in the spleen (Fig. 1c) and lymph nodes (Fig. 1d) of AngII-treated animals compared with those of controls.

Focusing on AngII-treated mice, we investigated CD39 protein expression by Western blot. The expression of CD39

Table 1 Validation of experimental models of hypertension

	SBP	HW/BW (×1000)
Sham	96 ± 2, n = 4	5.3 ± 0.1, $n = 4$
Angll	138 ± 6, $n = 5$ ***	7.0 ± 0.3, $n = 5$ **
Sham	101 ± 3, $n = 6$	4.4 ± 0.1, $n = 6$
DOCA-salt	123 ± 4, $n = 6$ *	5.0 ± 0.1, $n = 5$ **
WKY	77 ± 1, $n = 6$	3.0 ± 0.1, $n = 6$
SHR	104 ± 3, $n = 6$ ***	4.0 ± 0.1, $n = 6$***

Systolic blood pressure (SBP mmHg) and hypertrophic cardiac remodeling (evaluated by heart weight to body weight ratio, HW/BW) were measured in the three models of hypertension, AngII-infused mice, DOCA-salt mice, and SHR rats

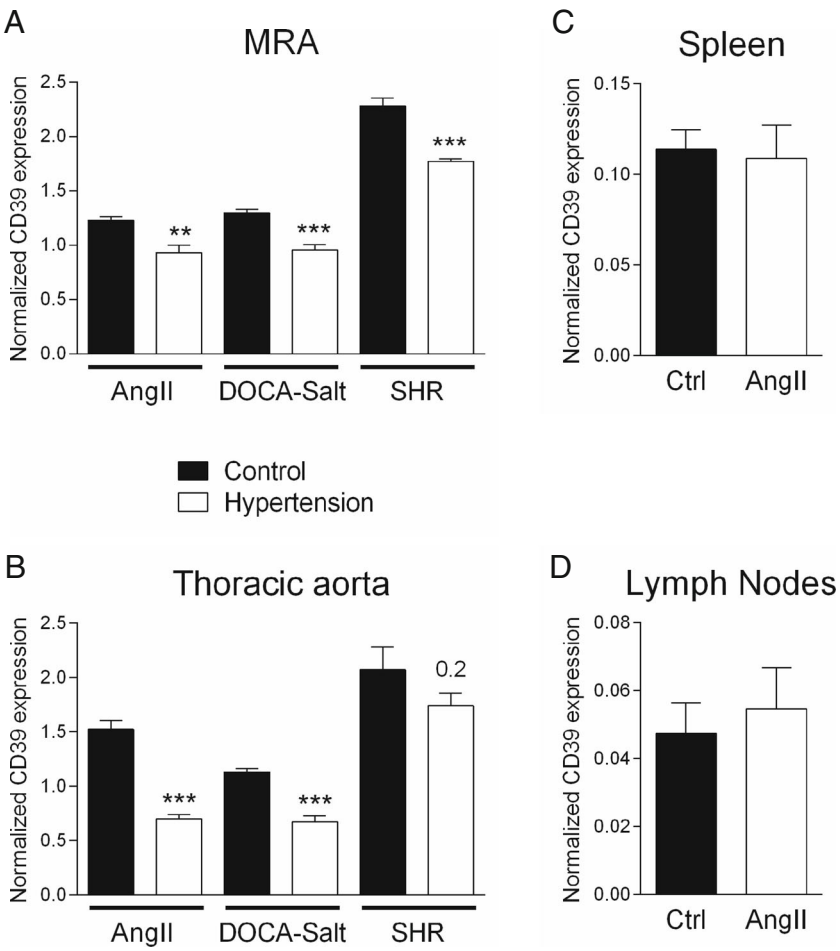

Fig. 1 Alteration of vascular CD39 mRNA expression in hypertensive mice. Vascular CD39 mRNA expression in (**a**) MRA and (**b**) thoracic aorta in three different models of hypertension (AngII-infused mice $n = 7$, DOCA-salt mice $n = 6$, WKY and SHR rats $n = 5$). CD39 expression in spleen (**c**) and lymph nodes (**d**) $n = 4$–7. Each experimental model of hypertension was compared with its control. Data are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (Student's t test) vs. wild-type controls

protein in abdominal aorta was not significantly changed 12 days after the start of AngII treatment (Fig. 2a), but it decreased by ≈ 30% after 21 days of treatment (Fig. 2b), corroborating the RT-qPCR results. These data suggest a slow regulatory process that likely occurs as a consequence of hypertension. To evaluate the vascular activity of CD39, we measured hydrolysis of fluorescent etheno-ADP in aortic homogenates. Compared with wild-type, ADPase activity was reduced by 45% while is virtually disappeared in aortic homogenates of *Entpd1*+/− and *Entpd1*−/− mice, respectively (Fig. S2), suggested the dominant contribution of CD39 in vascular ADP hydrolysis and a gene dosage effect, as previously shown [9, 16]. In homogenates from AngII-treated mice, tissular activity of CD39 was also affected, as shown by the reduced hydrolysis of etheno-ADP compared with that of control mice (Fig. 2c).

An important finding was that in AngII- and DOCA-salt-treated mice, CD39 mRNA expression was significantly negatively correlated to cardiac remodeling, a hallmark of hypertension (Fig. 3a, c). No such correlation was observed in SHR rats (Fig. 3d), which can be explained by the absence of significant CD39 mRNA downregulation in SHR thoracic aorta compared with that in their WKY controls. Finally, in AngII-treated mice, CD39 activity was also negatively correlated to cardiac remodeling, although this result did not reach significance (Fig. 3b).

Decrease in circulating CD39 ADPase activity in the serum of AngII-treated mice

We further measured circulating ADPase activity in the serum of AngII-treated mice. Serum ADPase activity was abolished in *Entpd1*−/− mice, demonstrating that it mostly relies on CD39. In accordance with a delayed impact of hypertension on CD39 protein expression, ADPase activity was not significantly reduced after 12 days of AngII treatment compared with that of non-treated control animals (Fig. 4a). However, corroborating protein expression and tissular activity, a significant decrease in soluble ADPase activity was observed in hypertensive mice after 21 days of treatment with AngII (Fig. 4b).

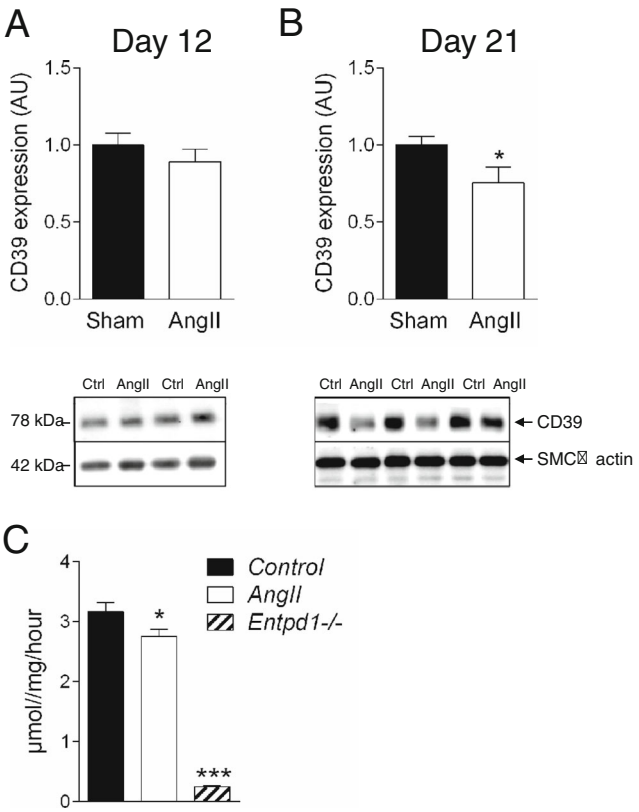

Fig. 2 CD39 protein expression and ADPase activity in hypertensive mice. Quantification of CD39 protein expression from abdominal aorta was assessed by Western blot after 12 days ($n = 5$–6) (**a**) and 21 days ($n = 19$) (**b**) of AngII treatment. Measurement of ADPase activity from the abdominal aorta ($n = 18$ in control mice and $n = 22$ in AngII mice) specific to CD39 was assessed by HPLC using the abdominal aorta of *Entpd1*−/− mice ($n = 6$) as a negative control (**c**). Data are presented as means ± SEM. $^{*}p < 0.05$ and $^{***}p < 0.001$ (Student's *t* test) vs. wild-type controls

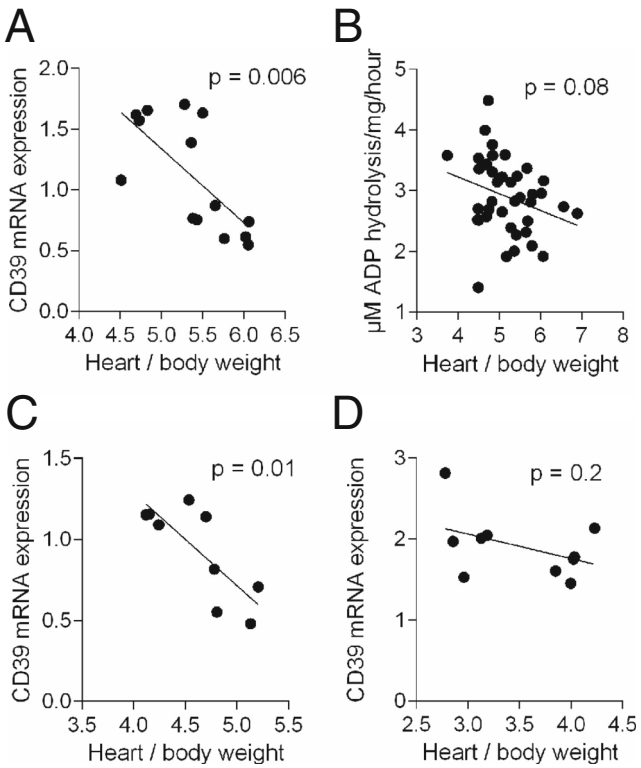

Fig. 3 Link between cardiac remodeling and CD39 downregulation in hypertensive animals. Correlation between cardiac remodeling and CD39 mRNA expression in thoracic aorta ($n = 14$) (**a**) or ADPase activity in abdominal aorta homogenates ($n = 39$) (**b**) in AngII-infused mice. Correlation between cardiac remodeling and CD39 mRNA expression in thoracic aorta in DOCA-salt ($n = 9$) (**c**) and SHR rats ($n = 10$) (**d**). $^{*}p < 0.05$, $^{**}p < 0.01$

Reduction of CD39 mRNA expression in response to proinflammatory cytokines and mechanical strain

In order to identify elements underlying CD39 mRNA downregulation, we conducted in vitro experiments. We stimulated ECs or VSMCs with vasoactive molecules and pro- and anti-inflammatory cytokines that are potentially encountered within the vasculature during hypertension. Results showed a decrease in CD39 mRNA expression in ECs in response to proinflammatory cytokine interleukin 6 (IL-6) and profibrotic cytokine transforming growth factor beta 1 (TGF-β1) and an increase in response to ATP. In VSMCs, a reduction in CD39 expression was measured in response to tumor necrosis factor alpha (TNF-α) and interleukin 1 beta (IL1-β), while it was upregulated by monocyte and macrophage attracting protein 1 (MCP-1 or CCL2; Table 2).

Besides pharmacological stimuli, we wanted to mimic in vitro mechanical strains encountered in hypertension. Orbital shear stress upregulated CD39 expression in mouse ECs compared with static conditions (Fig. S3), corroborating a recent report on CD39 downregulation by turbulent flow compared with laminar flow [16]. In addition, sinusoidal stretch was applied to ECs and VSMCs with the Flexcell method (15% elongation; 0.5 Hz) for 6, 24, and 72 h

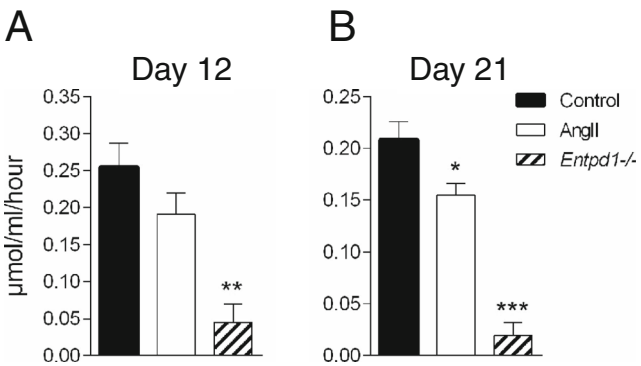

Fig. 4 Soluble ADPase activity in AngII-infused mice. ADP hydrolysis in sera of hypertensive mice 12 days ($n = 3$ in control mice and $n = 5$ in AngII-infused mice) (**a**) and 21 days ($n = 9$ in control mice and $n = 10$ in AngII mice) (**b**) after AngII treatment. This activity is abolished in *Entpd1* −/− mice ($n = 3$ in each experiment). Data are presented as means ± SEM. $^{*}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$ (one-way ANOVA) vs. wild-type controls

Table 2 CD39 expression in vascular cells

	VSMC	EC
	CD39 expression (% of unstimulated)	
TNF-α	**61.3 ± 5.3***	80.7 ± 7.4
IFNγ	141.2 ± 30.5	105.5 ± 7.5
MCP1	**125.0 ± 3.3****	127.3 ± 14.2
IL-6	116.1 ± 6.3	**61.3 ± 6.0***
IL-1β	**47.4 ± 4.4****	82.8 ± 9.9
IL-27	131.2 ± 26.0	100.5 ± 6.0
IL-17a	107.8 ± 14.2	113.1 ± 9.8
IL-12	118.4 ± 23.7	96.4 ± 8.8
IL-10	114.9 ± 14.3	122.2 ± 16.1
TGF-β1	78.5 ± 4.2	**50.9 ± 6.4***
AngII	98.5 ± 20.6	89 ± 12.6
ET-1	93.3 ± 4.3	105.9 ± 12.3
ATP	82.3 ± 5.8	**136.6 ± 6.3***
ADP	79.7 ± 11.4	119.2 ± 14.1
UTP	77.0 ± 10.0	94.7 ± 11.0
UDP	92.8 ± 4.4	85.3 ± 13.7
ADO	107.4 ± 11.3	125.0 ± 12.3

Relative expression of CD39 in ECs and VSMCs stimulated in the presence of various cytokines, endothelin-1 (ET-1), AngII, nucleotides, and nucleosides for 24 h. Data represent mean relative expression ±SEM of three and four to six independent experiment for EC and VSMC respectively. Comparison was done using ratio based t test on raw expression data

*$p < 0.05$,
**$p < 0.01$

(Fig. 5). We validated the effect of stretch by evaluating the upregulation of Mmp2 in VSMCs [17] and Hif1-α in ECs [18] (Table S3). Contrasting with the effect of shear, ECs stretch induced a progressive downregulation in CD39 mRNA which was significant at (72 h). A fast decrease in expression (after 6 h) was observed in VSMC (Fig. 5).

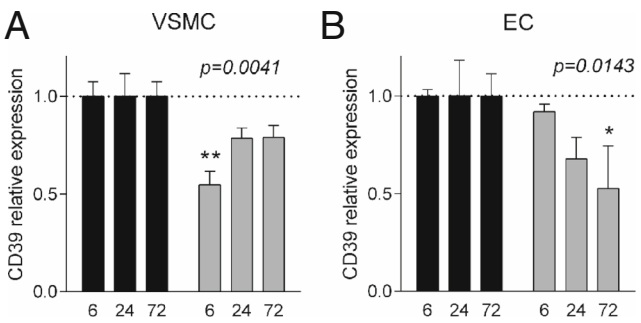

Fig. 5 Regulation of CD39 mRNA expression on vascular cells in vitro by mechanical stretch. CD39 expression on VSMCs (**a**) and ECs (**b**) after mechanical stretch for 6, 24, and 72 h. Data represent mean ± SEM of relative CD39 expression normalized to time-matched controls of five independent VSMC cultures and three (for 72 h) or five (for 6, 24 h) independent EC cultures. *$p < 0.05$, **$p < 0.01$ vs. time-matched control. The p value of stretch effect is shown (two-way ANOVA)

Discussion

In the present work, we showed that a decrease in CD39 occurs in the vasculature of three different experimental models of hypertension. In AngII-treated animals, the CD39 decrease was not measured in the lymphoid organs neither in the kidneys (data not shown and [19]), suggesting a vascular-specific alteration. Moreover, the more pronounced drop in CD39 expression in the thoracic aorta (≈ 50%) compared with that in the MRA (≈ 25%) suggests that large elastic arteries are more affected than resistance arteries. Considering the vasculoprotective effects reported for CD39, we anticipate that this decrease may worsen the vascular prognosis of hypertension.

Reduced ectonucleotidase activity by increasing pericellular nucleotide bioavailability results in enhanced P2 receptor activation. This enhances P2 receptor-dependent vascular effects that are mostly deleterious in the context of hypertension, such as proliferation (hypertrophic remodeling), apoptosis, and fibrosis [20] or excessive constriction [9]. Pathological blood pressure rise has been linked to specific P2 receptor activation. For instance, other groups and ours recently reported that the $P2Y_6$ receptor is involved in microvascular tone, blood pressure rise, and fibrosis caused by AngII infusion [21, 22]. Deficiency of CD39 exacerbates $P2Y_6$-dependent vasoconstriction [9] and this may increase peripheral arterial resistance in vivo and worsen hypertension. The P2X7 receptor was shown by another group to contribute to blood pressure elevation in DOCA-salt mice [23]. Here again, a drop in CD39 might exacerbate deleterious signaling of P2X7 receptors, such as macrophage IL-1β secretion, as discussed earlier. Considering that nucleotides are unequivocal danger signals in vascular conditions, CD39 activity is most likely a protector. In contrast, the presence of ADO, the final product of their hydrolysis, is more ambiguous. Indeed, upregulation of CD73/5′-nucleotidase activity has been shown to promote hypertension through ADO accumulation and endothelin formation in the kidney [19]. Since CD39 constitutes the rate-limiting enzyme of the ATP to ADO hydrolytic chain, a decrease in its activity might be in this case "beneficial" regarding its contribution to renal ADO accumulation.

On the other hand, together with nitric oxide and prostacyclin, endothelial CD39 represents a well-identified anti-thrombotic mechanism through hydrolysis of the key platelet activator ADP [24, 25]. In addition, by contributing to ADO generation and EC sealing, it prevents endothelial permeability [26]. Hence, a reduction in CD39 activity such as that observed in our experimental models of hypertension may increase platelet activation, promote vascular permeability, and favor immune cell infiltration to have an impact on vascular remodeling, as well as thrombogenesis.

Interestingly, other data from the literature suggest that CD39 is affected in many types of vascular dysfunction, such as xenotransplantation-associated endothelial activation [27], atherogenesis [28], and aortic aneurism [29]. What these pathological conditions have in common is that they are associated with thrombo-inflammatory manifestations; as a consequence, we hypothesize that the disappearance of vascular CD39 may contribute to a hypertension-associated thrombo-inflammatory state.

Conversely, we showed that an inflammatory environment affects CD39 expression. Experiments conducted in vitro highlighted the modulation of CD39 by various cytokines. In VSMCs specifically, we report a decrease in CD39 mRNA expression in response to proinflammatory TNF-α and IL1-β. TNF-α has been previously reported to decrease CD39 activity in ECs through oxidative inactivation of the enzyme, resulting in enhanced ADP-dependent platelet recruitment [27, 30]. TNF-α is well-known to mediate endothelial damage in resistant hypertension [31] and is responsible for matrix remodeling and modulation of the VSMC phenotype [32]. We also report for the first time downregulation of CD39 by IL-1β. This cytokine is massively produced by monocytes/macrophages that play a key role in arterial remodeling [33]. The present data suggest that immune infiltration and released cytokines may contribute to arterial remodeling partly through vascular CD39 downregulation. Major determinants of arterial remodeling, i.e., reactive oxygen species, metalloproteinase release, VSMC phenotypic changes, and apoptosis [3], have been shown to depend on P2 receptor signaling. These mechanisms could be overactivated with CD39 downregulation. On the other hand, ATP has been reported to induce IL-1β (and IL-18) secretion by monocytes/macrophages [34, 35]. This effect involves the P2X7 receptor and is largely dependent on CD39 activity [36]. Consequently, reduced ectonucleotidase activity through an increase in ATP concentration could enhance the infiltration of macrophage IL-1β secretion, creating a deleterious amplifying loop. Such a mechanism could participate in the previously reported cross-talk that exists between VSMCs and monocytes throughout IL-1β production [37]. In contrast, CD39 was upregulated in response to MCP-1.

In ECs, we found that CD39 was downregulated by IL-6 and TGF-β1. Mice invalidated for IL-6 are protected against AngII-dependent hypertension [38], underlying the important role of this cytokine in hypertension. An important finding by Thiolat et al. is that the IL-6 receptor blockade enhances the immunosuppressive phenotype in T regulatory cells [39] through CD39 overexpression, which highlights the mechanisms common to immune and non-immune cells. An interaction between CD39 expression and the anti-inflammatory/profibrotic cytokine TGF-β1 has not been reported. ECs exposed to TGF-β1 undergo endothelial-mesenchymal transition, which results in endothelial phenotype loss and the appearance of markers of myofibroblastic differentiation and therefore fibrosis [40, 41]. Several P2 receptors participate in cardiac, vascular, and lung fibrosis [42], and one can hypothesize that the profibrotic action of TGF-β1 partly relies on CD39 downregulation. In contrast, CD39 was upregulated in response to ATP, suggesting a feedback mechanism involving P2 receptors activation. Since we did not observe a direct effect of AngII in vitro, these cytokines (IL-6, IL-1β, TNF-α, TGF-β1) likely represent intermediates between AngII receptor activation, immune cells, and CD39 downregulation. This relies once more on the immune component of AngII-dependent hypertension.

We also found that mechanical forces modulate CD39. Kanthi et al. recently reported that endothelial CD39 is decreased by perturbed flow in the context of atherosclerotic plaque development and clearly demonstrated in an in vitro approach the sensitivity of the enzyme to shear stress [16]. We show here that stretch reduced CD39 transcription in both VSMCs and ECs. Of note, one may hypothesize that the more pronounced stretch imposed on large arteries (≈ 15–20%) compared with small arteries (≈ 5%) during hypertension may correlate with the higher repression of CD39 observed in large arteries. Interestingly, a mechanical stretch applied to cardiomyocytes has been described as inducing secretion of cytokines such as TNF-α, leading to modulation of gene expression and cellular function [43]. In this work, we link CD39 downregulation to TNF-α and mechanical stretch independently, and an overlap of the two pathways cannot be excluded.

Future experiments would be needed to define the mechanisms by which proinflammatory molecules, cell strains, or a combination are responsible for CD39 downregulation.

The protection exerted by CD39 against thrombosis and ischemia-reperfusion injury has drawn particular interest to its therapeutic potential. Recent work suggested a protective effect of soluble forms of nucleotidases to counter thrombosis by scavenging pro-aggregating ADP [44] and reperfusion lesions [45]. In the context of hypertension, few data are available; however, transgenic mice that systemically overexpress CD39 have been shown to be protected against preeclampsia in a model of Th1 lymphocyte injection [46]. More recently, Visovatti et al. demonstrated a protective role of apyrase, a soluble enzyme with CD39-like activity, in pulmonary arterial hypertension in *Entpd1−/−* mice [47]. These results seem to lend credence to the idea that "extra" CD39 activity can exert a protective effect against blood pressure elevation. However, in these studies, the etiology of hypertension (Th1 injection/hypoxia) and the models used (CD39TG vs. apyrase treatment of *Entpd1−/−* animals) do not allow identification of the mechanisms involved in this protection. The beneficial effect of "additional" CD39 activity, however, does not allow anticipation of the potential deficit caused by the 50% loss

in CD39 expression that we report here. In the context of primary hypertension, the potential protection exerted by a soluble CD39 or equivalent ATPDase undoubtedly deserves special attention.

Beside the soluble enzyme, substances that increase endogenous CD39 such as IL-27 [48] and/or statins [49] could be envisaged as potential treatments and/or reinterpreted in light of these new data.

Many soluble nucleotidase and kinase activities exist in blood circulation [50]. Among these, ADPase activity is largely dependent on CD39 (abrogated in *Entpd1−/−* mice; Fig. 2c). Here, we demonstrated a significant decrease in soluble ADPase activity specific to CD39 in the circulation of hypertensive mice. This finding is in agreement with the work of Jalkanen et al., who reported that levels of ADP in the sera of hypertensive patients were significantly increased as a probable consequence of decreased levels of CD39 [51]. Circulating ADPase activity, if not a therapeutic option, may thus serve as a potential biomarker in primary hypertension.

Our data show that the arterial expression and functionality of CD39 is decreased in hypertension. Reduced nucleotidase activity may enhance pathology-associated vascular damage, increasing endothelial permeability and inflammation and increasing the risk of end-organ damage and thrombogenesis.

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Compliance with ethical standards

Conflicts of interest Charlotte Roy declares that she has no conflict of interest.

Julie Tabiasco declares that she has no conflict of interest.
Antoine Caillon declares that he has no conflict of interest.
Yves Delneste declares that he has no conflict of interest.
Jean Merot declares that he has no conflict of interest.
Julie Favre declares that she has no conflict of interest.
Anne Laure Guihot declares that she has no conflict of interest.
Ludovic Martin declares that he has no conflict of interest.
Daniele Nascimento declares that she has no conflict of interest.
Bernhard Ryffel declares that he has no conflict of interest.
Simon C. Robson declares that he has no conflict of interest.
Jean Sévigny declares that he has no conflict of interest.
Daniel Henrion declares that he has no conflict of interest.
Gilles Kauffenstein declares that he has no conflict of interest.

Ethical approval Animals were manipulated in accordance with European Community Standards on the Care and Use of Laboratory Animals (authorization No. 6422).

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