NTPDase1 (CD39) controls nucleotide-dependent vasoconstriction in mouse

Gilles Kauffenstein1, Annick Drouin2, Nathalie Thorin-Trescases2, Hélène Bachelard3, Bernard Robaye4, Pedro D’Orléans-Juste5, François Marceau1, Éric Thorin2, and Jean Sévigny1*

1Centre de Recherche en Rhumatologie et Immunologie, Université Laval, 2705 Boulevard Laurier, local T1-49, Québec, QC, Canada G1V 4G2; 2Institut de Cardiologie de Montréal, Université de Montréal, Montréal, QC, Canada; 3Centre de recherche sur les maladies lipidiques, Centre Hospitalier Universitaire de Québec, Université Laval, Québec, QC, Canada; 4Institut de Recherche Interdisciplinaire en Biologie humaine et moléculaire, Université Libre de Bruxelles, Gosselies, Belgium; and 5Département de Pharmacologie, Faculté de Médecine, Université de Sherbrooke, Sherbrooke, QC, Canada

Received 6 August 2008; revised 16 July 2009; accepted 24 July 2009; online publish-ahead-of-print 29 July 2009

Time for primary review: 29 days

Aims
Extracellular nucleotides are vasoactive molecules. The concentrations of these molecules are regulated by ectonucleotidases. In this study, we investigated the role of the blood vessel ectonucleotidase NTPDase1, in the vasoconstrictor effect of nucleotides using Entpd1−/− mice.

Methods and results
Immunofluorescence, enzyme histochemistry, and HPLC analysis were used to evaluate both NTPDase expression and activity in arteries and isolated vascular smooth muscle cells (VSMCs). Vascular reactivity was evaluated in vitro and mean arterial blood pressure was recorded in anesthetized mice after nucleotide i.v. infusion. Expression of nucleotide receptors in VSMCs was determined by RT–PCR. Entpd1−/− mice displayed a dramatic deficit of nucleotidase activity in blood vessel wall in situ and in VSMCs in comparison to control mice. In aortic rings from Entpd1−/− mice, UDP and UTP induced a potent and long-lasting constriction contrasting with the weak response obtained in wild-type rings. This constriction occurred through activation of P2Y6 receptor and was independent of other uracil nucleotide-responding receptors (P2Y2 and P2Y4). UDP infusion in vivo increased blood pressure and this effect was potentiated in Entpd1−/− mice. In addition, pressurized mesenteric arteries from Entpd1−/− mice displayed an enhanced myogenic response, consistent with higher local concentrations of endogenously released nucleotides. This effect was inhibited by the P2 receptor antagonist RB-2.

Conclusion
NTPDase1 is the major enzyme regulating nucleotide metabolism at the surface of VSMCs and thus contributes to the local regulation of vascular tone by nucleotides.

Keywords
NTPDase1 • CD39 • UTP • UDP • P2Y receptor • Vasoconstriction • Myogenic tone • Smooth muscle cell

1. Introduction
Extracellular nucleotides take part in a wide range of physiological and pathological processes including the regulation of vascular tone. In the circulation, sources of extracellular nucleotides are numerous. Red blood cells, endothelial cells (ECs), activated platelets, and neutrophils release adenine nucleotides (ATP and ADP) in a non-lytic manner. This release occurs in response to mechanical constraints, such as shear stress, and also to hypoxia, hyperoxia, or agonist stimulation. In the vessel wall, sympathetic nerve terminals release ATP that is co-stored with noradrenaline. The release of adrenaline nucleotides has been more extensively studied due to accessible methods to measure these nucleotides while uracil nucleotides are more difficult to quantify. Nevertheless, UTP and UDP were shown to be released from ECs and activated platelets, and UTP release has also been reported in kidney microcirculation.

Once released, nucleotides and nucleosides (adenosine) exert their biological effect via the activation of membrane bound P2
and P1 receptors to regulate cell function in an autocrine or paracrine manner. To date, seven ligand-gated P2X (P2X1–7) and eight G protein-coupled P2Y receptors (P2Y1, 2, 4, 6, 11–14) were cloned and characterized in humans. While P2X receptors are all activated by ATP, P2Y receptors respond differently to ATP, ADP, UTP, UDP, or UDP-glucose. Three other receptors, CysLT1 and GPR17 are closely related to the P2Y family and respond to both leukotriens and uracil nucleotides.

Nucleotides participate to local and systemic control of blood flow at different levels. Endothelial P2 receptor activation induces a local vasorelaxation. This effect involves three major dilating factors, nitric oxide, prostacyclin and endothelium-derived hyperpolarizing factor, depending on the species and the vascular territories considered. Several receptors have been implicated in these processes. While P2X1 and P2X4 receptors were shown recently to mediate ATP-induced vasorelaxation in resistance arteries, P2Y1 and P2Y2 are responsible for ADP and UTP/ATP-induced relaxation. In contrast, the activation of P2 receptors from vascular smooth muscle cells (VSMCs) promotes vasoconstriction via P2X3 or pyrimidine-sensitive P2Y receptors that take part in the neurogenic response of resistance arteries.

The concentrations of extracellular nucleotides, and thus P2 receptor activation, are regulated by ectonucleotidases that are membrane-bound enzymes with an extracellularly facing catalytic site. Among these enzymes, the nucleoside triphosphate diphosphohydrolase-1 (NTPDase1) and NTPDase2 are expressed in the vasculature. NTPDase1 is expressed on ECs and VSMCs and NTPDase2 in the adventitia surrounding blood vessels, probably at the surface of fibroblasts. By hydrolysing ADP in the blood stream, a key player in platelets activation, NTPDase1 was first proposed to contribute to the antithrombotic property of ECs before its genetic identification as the lymphocyte marker CD39. The study of NTPDase1 deficient mice confirmed the antithrombogenic role of the enzyme and its implication in the preservation of vascular permeability. To date, the reported functions of vascular NTPDase1 are associated to its endothelial and not muscular expression. We show here that NTPDase1 is the major enzyme hydrolysing nucleotides at the surface of VSMCs and that its absence results in an increase in nucleotide-dependent vasoconstriction.

2. Methods

2.1 Animals

Experiments were carried out in accordance with the guidelines of the Institutional Ethical Committee for Experimental Animals and conform to Guide for the Care and Use of Laboratory Animals (NIH Publication No. 85-23, revised 1996).

2.2 Immunostaining

Cryosections of OCT-embedded tissues were fixed with acetone containing 0.5% phosphate-buffered formalin and immunostained with either mN1-2C (guinea pig anti-mouse NTPDase1 antisera), mN2-36L (rabbit anti-mouse NTPDase2 antisera), anti-α-actin monoclonal antibody (clone 1A4, Sigma, Oakville, ON), or anti-mouse CD31 (clone MEC13.3, BD Pharmingen, San Diego, CA) for endothelium-labelling, in TBS 2% goat serum. Alexa Fluor® 633, 594, and 488-conjugated goat anti-mouse, anti-guinea pig, and F(ab')2 fragment of anti-rabbit were used, respectively, for immunofluorescence. Sections were mounted using antifading mowiol and analysed with an Olympus IX70 microscope. Staining was performed with Vectastain ABC elite kit (Vector Laboratories, Burlingame, CA) with 3,3-diaminobenzidine as chromogen.

2.3 Enzyme histochemistry

Nucleotide hydrolysis in situ was evaluated as previously described.

2.4 NTPDase1 expression and activity in VSMCs

VSMCs primary cultures were obtained as previously described. NTPDase1 expression was evaluated by western blot using mN1-2C. For ectonucleotidase activity, nucleotides were incubated at 37°C with confluent VSMCs and supernatants were analysed by HPLC at indicated time-points.

2.5 Isometric contraction of aortic rings

Vascular tone of thoracic aortas was measured in vertical organ chambers containing Krebs solution.

2.6 Determination of myogenic responses in mesenteric artery

The reactivity of mesenteric arteries (diameter 150 μm) was determined with a wire myograph, and the myogenic tone (MT) with a pressure arteriograph.

2.7 Blood pressure measurement

The carotid artery of anesthetized mice was cannulated with PE-10 polyethylene tubing (Becton Dickinson, Oakville, ON) containing 50 U/mL heparin in saline. A cannula, inserted in the contralateral jugular vein was used for intravenous infusions of agonists. Changes in mean arterial blood pressure (mAP) were detected through the carotid with a pressure transducer connected to a Blood Pressure Analyser-200A (Micro-Med, Tustin, CA).

2.8 Statistical analyses

The raw data were compared using unpaired Student’s t-test. For Figures 5 and 7 measurements were compared by ANOVA followed by a Bonferroni post hoc test for multigroup comparisons. Sources of the different mice strains and a complete detailed method are available in the online supplement.

3. Results

3.1 NTPDase1 is the major ectonucleotidase in VSMCs

In mouse aorta, NTPDase1 is expressed in VSMCs of the media, as indicated by its colocalization with α-actin (Figure 1A). NTPDase2 is expressed in the surrounding adventitia. The aortic endothelium, whose integrity was controlled by uniform PECAM labelling (data not shown), did not display strong immunoreactivity for NTPDase1, contrasting with previous observations in smaller arterioles that showed a strong immunolabelling of NTPDase1 in ECs and weaker staining in VSMCs.

The comparison of nucleotide hydrolysis in situ revealed a broad nucleotidase activity in the media of Entpd1+/+ mice aortic
sections that was not detected in Enptide1−/− tissues (Figure 1B). Such deficit of activity was observed in the Enptide1−/− arterioles of other tissues including heart (Figure 1C), lungs, and liver (data not shown), suggesting that NTPDase1 is the main nucleotidase expressed in VSMCs. The activity of NTPDase2, which is a nucleoside triphosphatase with low diphosphatase activity, was more evident in Enptide1−/− mouse aorta with ATP or UTP as substrates (Figure 1B). In accordance to its expression pattern, NTPDase2 activity was restricted to the adventitia.

NTPDase1 expression in VSMCs primary cultures was confirmed by western blot (Figure 2A), and its biochemical activity by HPLC. While ATP, ADP, UTP, and UDP were rapidly hydrolysed by Enptide1+/+ cells, this hydrolysis was negligible in the presence of Enptide1−/− cells (Figure 2B), highlighting the major contribution of NTPDase1 in nucleotide hydrolysis at the surface of VSMCs.

3.2 Nucleotide-induced vasoconstriction is potentiated in Enptide1−/− mouse aortic rings

The role of NTPDase1 in nucleotide-induced vasoconstriction was assessed by comparing the isometric tension developed by aortic rings from Enptide1+/+ and Enptide1−/− mice. Comparable responses in both genotypes to a depolarizing KCl (Fig 3A), or U46619 (Fig 3G), a thromboxane A2 analogue, confirmed that Enptide1−/− vessels could contract normally. In contrast, nucleotide-induced vasoconstriction was drastically enhanced in Enptide1−/− aortic rings. As illustrated by the effect of UDP, the force and the steadiness of contraction were augmented in the absence of NTPDase1 (Figure 3B, C, H). Similar results were obtained for UTP, ATP, and ADP. Figure 3C–F shows the dose response curve obtained by cumulative concentrations of nucleotides. UDP and UTP induced...
a strong constriction of Entpd1−/− rings (E_max = 0.57 g and 0.54 g, respectively), in comparison to Entpd1+/+ rings (<0.1 g tension at 100 μM, Figure 3C, D), being the second most potent vasoconstrictors after U46619 (E_max = 0.69 g), nearly two times more potent than the α-adrenergic agonist phenylephrine (E_max = 0.33 g). ADP and ATP were less potent vasoconstrictors with EC50 in the high micromolar range (Fig 3E, F). Pharmacological inhibition of NTPDase1 in Entpd1+/+ aortic rings with ARL 67156 concentration-dependently potentiated UDP-induced vasoconstriction (Figure 3H). ARL 67156 had no effect in Entpd1−/− aortic rings confirming that its effect on Entpd1+/+ aortic rings was due to NTPDase1 inhibition.

3.3 Expression of nucleotide receptors in mouse aorta

The potential receptor(s) involved in the contractile response to uracil nucleotides were identified by RT–PCR on both intact thoracic aorta and VSMC cDNAs. Primers for nucleotide-responding, or potentially responding receptors, except P2X receptors that are exclusively activated by ATP, were used to screen for the presence of P2Y, CysLT1,2, GPR17, and two orphan receptors closely related to P2Y receptor family, GPR34 and GPR87.5 The method and primer pairs are available in the online supplement. P2Y1, P2Y2, and P2Y6 receptors were highly expressed in both entire thoracic aorta and VSMCs (Figure 4). P2Y12,13, CysLT1, and GPR34 messengers were amplified in thoracic aorta but not in VSMCs, P2Y4 and P2Y14 were barely detectable, and CysLT2 was absent. The differential expression between aorta and VSMCs may be due to contaminating platelets (P2Y12), red blood cells (P2Y13), and lymphocytes (GPR34) that are impossible to completely eliminate from aortic preparations. Endothelium denudation before thoracic aorta RNA extraction did not modify the expression pattern of these receptors (data not shown). Thus, VSMCs mainly express P2Y1, P2Y2, and P2Y6 receptors.

3.4 Nucleotide-induced vasoconstriction in mouse aorta: evidence for P2Y6 receptor

The pharmacological profile of nucleotide-induced vasoconstriction was evaluated in Entpd1+/− aortic rings. As shown by their EC50 values (Table 1), the rank order of potency revealed a predominant effect of uracil nucleotides with the selective P2Y6 receptor agonist, 3-phenacyl-UDP,25 among the most potent agonists (EC50 = 23 ± 1.2 μM, Table 1). The pA2 of three non-selective P2 receptor antagonists (RB-2, suramin and PPADS) on UDP and UTP-induced contractions were similar, suggesting that a unique receptor population is responsible for the constrictor effect of UDP and UTP (Table 2). Adenine nucleotides displayed a weak effect (EC50 > 200 μM) theoretically ruling out the contribution of P2Y1 (ADP), P2Y12/P2Y4 (UTP and ATP), and P2X receptors (ATP). To definitively determine the receptor(s) involved, we used knockout mice for uracil nucleotide responding P2 receptors, namely P2Y1, P2Y2, P2Y4, and P2Y6. UDP and UTP induced similar contractions in P2y2−/−, P2y4−/− and wild-type aortic rings but was abolished in P2y6−/− aortas (Figure 5). As a control, ATPγS-induced contraction was exclusively diminished in P2y2−/− aortic rings, suggesting that, as reported for the endothelial receptors,26 VSMCs P2Y2 receptor mediates the effect of ATPγS, but not the one of UTP. These results demonstrate that P2Y6 receptor is the mediator of the strong vasoconstrictor property of UDP and UTP in mouse aorta.

3.5 Effect of UDP on blood pressure

Intravenous infusion of UDP (10 μmol/kg) displayed a biphasic effect on blood pressure in mice: a slight transient decrease followed by a marked and sustained increase that reached a maximum between 2 and 3 min after UDP infusion (Figure 6A). The initial slight drop in blood pressure is likely linked to endothelial P2Y6 receptor since its activation has been shown to
Figure 3 Contractile effect of nucleotides is unmasked in Entpd1<sup>−/−</sup> aortas. (A) While the constriction produced by depolarizing KCl (50 mmol/L) was similar in both strain of mice, (B) the vasoconstrictor effect of UDP (100 μmol/L) was greatly enhanced and more stable in Entpd1<sup>−/−</sup> compared with wild-type aortic rings. (C–F) Nucleotides induced a sigmoid shaped dose-dependent contraction in Entpd1<sup>−/−</sup> aortic rings (open circles) but only a weak and progressive tension in Entpd1<sup>+/+</sup> rings (filled circles). (G) The dose–response curve induced by U46619 was similar in both genotypes. (H) Pharmacological inhibition of NTPDase1 with ARL 67156 potentiated the contraction induced by UDP in a dose-dependent manner in Entpd1<sup>+/+</sup> but not in Entpd1<sup>−/−</sup> aortic rings. Each dose-response curve was built from the experiment performed on rings from four to seven aortas from different mice.
produce relaxation in vitro. The pressor effect was significantly more important in Entpd1−/− than in Entpd1+/+ mice upon 1 and 10 μmol/kg UDP infusions (Figure 6B). Angiotensin II induced a similar increase in blood pressure in both strains of mice suggesting that the increased hypertensive effect in the absence of NTPDase1 was specific to UDP (Figure 6B). These data fit with the exacerbated constrictor effect of UDP obtained in Entpd1−/− arteries in vitro and show that this phenomenon can influence blood pressure in vivo.

**Table 1** EC50 values of nucleotide-induced vasoconstriction of Entpd1−/− mice thoracic aorta

<table>
<thead>
<tr>
<th>Nucleotide (n)</th>
<th>EC50 (μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UTP (5)</td>
<td>1.06 ± 0.64</td>
</tr>
<tr>
<td>UDP (7)</td>
<td>1.76 ± 0.94</td>
</tr>
<tr>
<td>UTPA (3)</td>
<td>3.43 ± 0.11</td>
</tr>
<tr>
<td>3 phenacyl-UDP (2)</td>
<td>22.8 ± 12.4</td>
</tr>
<tr>
<td>TDP (4)</td>
<td>24.4 ± 4.9</td>
</tr>
<tr>
<td>TTP (3)</td>
<td>27.7 ± 9.2</td>
</tr>
<tr>
<td>ATPβS (3)</td>
<td>130 ± 86</td>
</tr>
<tr>
<td>ADPβS (1)</td>
<td>53.6</td>
</tr>
<tr>
<td>ITP (3)</td>
<td>109 ± 20</td>
</tr>
<tr>
<td>IDP (3)</td>
<td>120 ± 60</td>
</tr>
<tr>
<td>GTP (3)</td>
<td>190 ± 59</td>
</tr>
<tr>
<td>GDP (3)</td>
<td>323 ± 65</td>
</tr>
<tr>
<td>ATP (5)</td>
<td>&gt;200</td>
</tr>
<tr>
<td>ADP (2)</td>
<td>&gt;200</td>
</tr>
</tbody>
</table>

3.6 Enhanced MT in Entpd1−/− arteries

MT, the reduction in diameter in response to an increase in intraluminal pressure, is the hallmark of resistance arteries. This response is entirely dependent on smooth muscle contraction, although it is regulated by mechanisms including local changes in flow, endothelial function, nerve activation, and autacoids. Stretch, among other cellular stresses, induces the release of nucleotides. Since we observed an enhanced contractile effect of exogenous nucleotides in Entpd1−/− aorta (Figure 3), mesenteric resistance arteries of these mice were used to assess the potential contribution of endogenously released nucleotides in MT. Pharmacological reactivity of mesenteric arteries was investigated with a wire myograph. In agreement with the results obtained with aortas, the constrictor effect of uracil nucleotides was facilitated in Entpd1−/− mesenteric arteries (Figure 7A) while U46619-induced contraction was equivalent in both genotypes (data not shown). EC50 of UDP and UTP-induced contraction were 3.5 and 4.5 μM in Entpd1+/+ arteries, and 0.28 and 0.27 μM in Entpd1−/− arteries. The greater difference obtained in the contractile effect of uracil nucleotides between Entpd1+/+ and Entpd1−/− aortas is likely linked to its thicker SMC layer that slow nucleotides diffusion favouring their exposition to NTPDase1. The P2 receptor antagonist RB-2 potently inhibited those responses (Figure 7A). MT was measured with a pressure arteriograph. Mesenteric arteries developed MT between 25 and 150 mmHg intraluminal pressure which was significantly enhanced in Entpd1−/− arteries, denuded (Figure 7B) or not (data not shown) of their endothelium. The passive dilatation of arteries (absence of extracellular calcium) was equivalent in both strains of mice (Figure 7C). In the same setting, the pharmacological response to phenylephrine was similar: 67 ± 8 vs. 71 ± 7% of contraction for wild-type and Entpd1−/− arteries, respectively. RB-2 prevented the increase in MT observed in Entpd1−/− arteries but did not have a significant effect in wild-type arteries (Figure 7B). These results suggest that nucleotides released by stretching the vessel wall contribute to enhance the MT that can be easily measured in Entpd1−/− arteries.

**Table 2** pA2 values of P2 receptor antagonists on UDP and UTP-induced vasoconstriction of Entpd1−/− mice thoracic aorta

<table>
<thead>
<tr>
<th></th>
<th>UDP</th>
<th>UTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>PPADS</td>
<td>4.25 ± 0.19</td>
<td>4.13 ± 0.11</td>
</tr>
<tr>
<td>Reactive blue 2</td>
<td>4.31 ± 0.16</td>
<td>4.16 ± 0.15</td>
</tr>
<tr>
<td>Suramin</td>
<td>4.08 ± 0.14</td>
<td>4.05 ± 0.15</td>
</tr>
</tbody>
</table>

4. Discussion

To date, the reported vascular functions of NTPDase1 (thromboregulation, vascular permeability) were linked to its endothelial expression. We show here that NTPDase1 is the major ectonucleotidase expressed in VSMCs. Its expression correlates with the high hydrolytic activity previously reported on these cells.
and is absent in the media layer of Entpd1²/² arteries in situ and at the surface of Entpd1²/² VSMCs primary cultures (Figures 1 and 2). Importantly, the absence of NTPDase1 unmasks a strong constrictor effect of UDP and UTP in aortas and mesenteric arteries (Figures 3 and 7).

In agreement with an important nucleotidase activity at the VSMCs surface, hydrolysis-resistant analogues UDP₃S and UTP₅S are 1000-fold more potent as vasoconstrictor agents than UDP and UTP.¹⁰ Based on our pharmacological and molecular data, we found that P2Y₆ receptor is involved in the uracil nucleotide-dependent vasoconstriction in mouse vessels. Using knockout mice, we definitely excluded P2Y₂ and P2Y₄ receptors (Figure 5). It was not really surprising for P2Y₄ receptor since its transcript was barely detected in mouse aorta (Figure 4), and absent in mesenteric artery,²⁰ contrasting with rat vasculature where P2Y₄ receptor was detected in both VSMCs and intact aorta.³¹ The result was not expected for P2Y₂ since this receptor is largely expressed in aorta and VSMCs (Figure 4), and was proposed as the main receptor involved in UTP- and ATP-dependent inositol polyphosphate formation in rat VSMCs.³² Indeed, we found that ATP₅S-dependent contraction was partly dependent on P2Y₂ receptor (Figure 5), but not this of UTP. As P2Y₂ is required for VSMCs migration and proliferation in response to UTP,³³ it appears that distinct P2Y receptors are involved in different functions of VSMCs.

The fact that UTP and UDP were found equipotent vasoconstrictors apparently did not fit with the P2Y₆ as the only receptor type involved. Indeed, UDP was reported to be 100-fold more potent than UTP at the human recombinant P2Y₆ receptor.³⁴ Such pharmacological profile (UDP and UTP equipotent) was previously reported in the vasculature of mouse and rat,³⁵ and was attributed to a P2Y₆-like receptor. Interestingly, UDP was more potent than UTP in wild-type aorta, while the two nucleotides were equipotent in Entpd1⁻/⁻ aortas. As UTP is a much better substrate than UDP for NTPDase1,³³ the enzyme may influence the apparent pharmacology of P2Y₆, affecting the real potency of

Figure 5 P2Y₆ receptor mediates uracil nucleotide-induced vasoconstriction in mouse aorta. Constriction experiments were performed in the presence of the NTPDase1 inhibitor ARL 67156 (100 μmol/L). UTP and UDP-induced an equivalent concentration-dependent contraction in P2y₂⁻/⁻ (filled triangle), P2y₄⁻/⁻ (filled square), and C57Bl/6 control (filled circle) aortic rings which was absent in P2y₆⁻/⁻ (filled diamond) rings. ATP₅S-dependent contraction was partially diminished in P2y₂⁻/⁻ aortic rings. Results are expressed as the percentage of KCl (50 mmol/L)-induced contraction and represent the mean ± SEM of five independent experiments for each group of mice tested except for P2y₆⁻/⁻ that were carried out with the aortic sections from three individuals. *P < 0.05, **P < 0.01, ***P < 0.001.

Figure 6 In vivo effect of UDP. (A) The intravenous infusion of UDP (10 μmol/kg) induced an initial fast hypotensive response followed by a prolonged increase in blood pressure that reached a maximum between 2 and 3 min following infusion that was significantly higher in Entpd1⁻/⁻ mice. (B) The maximal increase in blood pressure was significantly higher in Entpd1⁻/⁻ for 1 and 10 μmol/kg UDP infusions. As a control, similar increase pressor effect was observed in response to angiotensin II (AgII, 100 pmol/kg). Data represent the mean ± SEM of four experiments performed on different mice. *P < 0.05, **P < 0.01.
Further investigations are required to validate this hypothesis that may conciliate the pharmacological differences between P2Y6 and P2Y6-like receptor. The fact that NTPDase1 deficiency was associated with a gain of vessel contractility is interesting as, so far, studies involving Entpd1−/− mice showed that the absence of NTPDase1 was associated with P2 receptor desensitization.17,36–38 Thus, NTPDase1 plays different roles: considering easily desensitized receptors such as P2Y1, NTPDase1, by scavenging extracellular nucleotides, prevents their desensitization,17 in contrast, for poorly desensitizing receptors such as P2Y6, NTPDase1 limits their activation and its absence/inhibition facilitates it. It is likely that NTPDase1 will be essential in view to terminate P2Y6 receptor activation, this being especially true considering the poverty of uracil metabolizing enzymes apart from the NTPDases.39

The absence of NTPDase1 unmasks the contractile effect of UDP also in vivo since intravenous infusion of UDP led to an enhanced pressor effect in Entpd1−/− compared with control mice (Figure 6). This was due to an increase in peripheral resistance, since no modification in the heart rate occurred (data not shown). UTP did not display the same effect being mostly hypotensive (data not shown) contrasting with in vitro experiments in which UTP and UDP were vasoconstrictors (Figure 3). This discrepancy may be due to the involvement of endothelial P2 receptors, such as P2Y2 which may functionally antagonize constrictor effect on VSMCs. It is noteworthy that Entpd1−/− mice do not display elevated blood pressure (data not shown). This may be linked to the fact that the absence of NTPDase1 also facilitates the activation of endothelial P2 receptors, resulting in a facilitated vasodilation that balances the enhanced VSMCs P2 receptor activation (Kauffenstein et al., submitted for publication).

MT controls local blood flow in resistance arteries and depends on the intrinsic property of VSMCs to contract in response to intraluminal pressure increase. Mechanisms underlying MT development are largely unknown but autacoids released by VSMCs such as 20-HETE40 or sphingosine1-phosphate41 have been proposed to participate in this adaptive vascular response. We found that MT was significantly more pronounced in Entpd1−/−
when compared with Entpd1−/− arteries (Figure 7B). This may be attributable to the release of nucleotides upon cell stretching. In agreement with these data, uracil nucleotides exert a potent vasoconstriction in mouse mesenteric arteries (Figure 7A and Vial and Evans36). The enhanced MT observed in Entpd1−/− arteries was strongly inhibited by RB-2, in agreement with the hypothesis that released nucleotides reinforce MT. Several arguments play in favour of uracil nucleotides as autocrine stimulators of SMC. First, uracil nucleotides are released following cellular mechanical stress.32,33 VSMCs themselves could well be a source of uracil nucleotides since vascular smooth muscles have been reported to differ from skeletal muscles and other tissues by a particularly low intracellular ATP/UTP ratio. It has also been reported that pyrimidines are released by rat perfused hind limb in response to constrictor agents (NA, vasopressin, AgII).44 Thus, mechanical distension of SMC could well release extracellular uracil nucleotides. Recently Nishida et al.45 reported that an autocrine loop involving uracil nucleotides release and P2Y6 receptor activation occurs in cardiomyocytes and participates to the development of cardiac fibrosis. This autocrine loop is similar to the one that, as suggested in the present study, participates in MT amplification. Further investigations will be required to identify the nucleotides released and P2 receptors taking part to this process. Nonetheless, P2Y6 receptors seem to be valid candidates since antagonists of this receptor type inhibit MT in mouse mesenteric arteries.46

Altogether, our results suggest that modulation of the expression level or activity of NTPDase1 in VSMCs influence the constrictor effect of nucleotides. As a consequence, in inflammatory or oxidative environment, where NTPDase1 activity is diminished,47 nucleotide receptor activation and the resulting vasoconstriction may be facilitated. On the opposite, an increase in NTPDase1 activity should reduce the constrictor effect of nucleotides. This situation may occur following hypoxic conditions where the expression of the enzyme is dramatically increased.19

Besides high affinity transporters, ectoenzymes constitute an efficient mechanism for extracellular mediators’ clearance. The local production of nucleotides from their precursors is tightly controlled by transporters, in particular the high affinity nucleoside transporter ENT1. Nevertheless, the expression of the enzyme is dramatically increased.19 Uracil nucleotides are released by rat perfused hind limb in response to hypoxic conditions.47 Nucleotide receptor activation and the resulting vasoconstriction may be facilitated. On the opposite, an increase in NTPDase1 activity should reduce the constrictor effect of nucleotides. This situation may occur following hypoxic conditions where the expression of the enzyme is dramatically increased.19

Supplementary material
Supplementary material is available at Cardiovascular Research online.

Acknowledgements
The authors thank S.C. Robson and K. Enyoji for providing Entpd1−/− mice, I. Brochu and M. Pitre for technical assistance, and O. Popa-Nita for reviewing the manuscript.

Conflict of interest: none declared.

Funding
This work was supported by grants from the Canadian Institutes of Health Research (CIHR) to J.S. and E.T., and by the Fonds de la Recherche en Santé du Québec (FRSQ) to J.S. G.K. received a fellowship from the Institut National de la Santé et de la Recherche Médicale (INSERM) in partnership with the FRSQ, that was followed by a second award, this one from the Heart and Stroke Foundation of Canada in partnership with the CIHR and the Canadian Stroke Network. A.D. was the recipient of the Frederick Banting and Charles Best Canada Graduate Scholarships-Doctoral Award in association with CIHR, and J.S. of a new investigator award from the CIHR.

References
29. Gordon EL, Pearson JD, Dickinson ES, Moreau D, Slakey LL. The hydrolysis of

28. Henrion D. Pressure and flow-dependent tone in resistance arteries. Role of


31. Erlinge D, Hou M, Webb TE, Barnard EA, Moller S. Phenotype changes of the

32. Kumari R, Goh G, Ng LL, Boarder MR. ATP and UTP responses of cultured rat

33. Yu N, Erb L, Shivaji R, Weisman GA, Seye CI. Binding of the P2Y2 nucleotide


disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis

effects of cd39/ecto-nucleoside triphosphate diphosphohydrolase-1 in murine


26. Henrion D. Pressure and flow-dependent tone in resistance arteries. Role of

effects of cd39/ecto-nucleoside triphosphate diphosphohydrolase-1 in murine

28. Henrion D. Pressure and flow-dependent tone in resistance arteries. Role of

29. Gordon EL, Pearson JD, Dickinson ES, Moreau D, Slakey LL. The hydrolysis of


31. Erlinge D, Hou M, Webb TE, Barnard EA, Moller S. Phenotype changes of the vascular smooth muscle cell regulate P2 receptor expression as measured by

32. Kumari R, Goh G, Ng LL, Boarder MR. ATP and UTP responses of cultured rat

33. Yu N, Erb L, Shivaji R, Weisman GA, Seye CI. Binding of the P2Y2 nucleotide


disruption of cd39/ATP diphosphohydrolase results in disordered hemostasis

effects of cd39/ecto-nucleoside triphosphate diphosphohydrolase-1 in murine


26. Henrion D. Pressure and flow-dependent tone in resistance arteries. Role of

effects of cd39/ecto-nucleoside triphosphate diphosphohydrolase-1 in murine