The American Journal of Pathology, Vol. 181, No. 1, July 2012

http://dx.doi.org/10.1016/j.ajpath.2012.03.024

Vascular Biology, Atherosclerosis, and Endothelium Biology

Ectonucleotide Triphosphate Diphosphohydrolase-1 (CD39) Mediates Resistance to Occlusive Arterial Thrombus Formation after Vascular Injury in Mice

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Modulation of purinergic signaling, which is critical for vascular homeostasis and the response to vascular injury, is regulated by hydrolysis of proinflammatory ATP and/or ADP by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD-1; CD39) to AMP, which then is hydrolyzed by ecto-5′-nucleotidase (CD73) to adenosine. We report here that compared with littermate controls (wild type), transgenic mice expressing human ENTPDase-1 were resistant to the formation of an occlusive thrombus after $FeCl_3$-induced carotid artery injury. Treatment of mice with the nonhydrolyzable ADP analog, adenosine-5′-0-(2-thiodiphosphate) trilithium salt, Ado-5′-PP[S], negated the protection from thrombosis, consistent with a role for ADP in platelet recruitment and thrombus formation. ENTPD-1 expression decreased whole-blood aggregation after stimulation by ADP, an effect negated by adenosine-5′-0-(2-thiodiphosphate) trilithium salt, Ado-5′-PP[S] stimulation, and limited the ability to maintain the platelet fibrinogen receptor, glycoprotein α_{IIb}/β_3, in a fully activated state, which is critical for thrombus formation. *In vivo* treatment with a CD73 antagonist, a nonselective adenosine-receptor antagonist, or a selective A_{2A} or A_{2B} adenosine-receptor antagonist, negated the resistance to thrombosis in transgenic mice expressing human ENTPD-1, suggesting a role for adenosine generation and engagement of adenosine receptors in conferring *in vivo* resistance to occlusive thrombosis in this model. In summary, our findings identify ENTPDase-1 modulation of purinergic signaling as a key determinant of the formation of an occlusive thrombus after vascular injury. *(Am J Pathol 2012, 181:322–333; http://dx.doi.org/10.1016/j.ajpath.2012.03.024)*

Arterial thrombosis secondary to rupture of an atherosclerotic plaque is the underlying event in the majority of acute myocardial infarctions and the leading cause of death in the westernized world.[1] Atherosclerotic plaque rupture exposes the subendothelial matrix, which leads to platelet activation secondary to the convergence of numerous signaling cascades that release platelet-dense granules, increasing local concentrations of the purinergic mediators, ATP and ADP.[2,3] Engagement of specific receptors on platelets results in further activation and recruitment of platelets into the growing thrombus.[2,3] After thrombotic coronary arterial occlusion, myocardial ischemia and subsequent necrosis ensues, resulting in myocardial damage and dysfunction if blood flow is not restored promptly.

An extracellular purinergic regulatory pathway is positioned uniquely to modulate thrombosis, inflammation, and myocardial ischemia-reperfusion injury.[4] A key com-

Supported by NIH, National Heart, Lung, and Blood Institute grants HL094703 and HL096038 (R.J.G.), an American Heart Association-Great Rivers Affiliate Student Undergraduate Research Fellowship grant 10UFEL4180090 (Z.M.H.), and the Davis Heart and Lung Research Institute and Ross Academic Advisory Committee (R.J.G.).

Accepted for publication March 8, 2012.

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Presented in part at the American Heart Association Scientific Session, November 12–16, 2011, Orlando, FL.

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ponent of this pathway is ectonucleoside triphosphate diphosphohydrolase-1 (ENTPD-1; CD39), a 70- to 100-kDa transmembrane protein expressed on platelets, endothelium, and leukocytes that hydrolyzes the proinflammatory prothrombotic molecules ATP and ADP to AMP.[5,6] AMP subsequently is converted by ecto-5′-nucleotidase (CD73) to the anti-inflammatory, anti-thrombotic, and cardiac protective compound, adenosine,[7] with multiple receptors existing for ATP, ADP, and adenosine.[8,9] Our laboratory recently showed that ENTPD-1 expression reduces myocardial infarct size after ischemia-reperfusion injury in both mouse and pig.[10,11] Furthermore, decreased ENTPD-1 activity has been shown in atherectomy specimens from patients with acute coronary syndromes, suggesting a pivotal role for ENTPD-1 in modulating the balance between a prothrombotic and antithrombotic milieu.[12]

Given the complex regulation of the purinergic system in thrombosis, we hypothesized that overexpression of ENTPDase-1 may modulate *in vivo* large-conduit vessel arterial thrombosis by affecting both platelet reactivity and tissue factor expression levels. Here, we show that overexpression of human ENTPDase-1 in mice modulates purinergic signaling, which attenuates activation of the platelet fibrinogen receptor, glycoprotein α_{IIb}/β_3 (GP IIb/IIIa; CD41/CD61), and conveys resistance to *in vivo* thrombosis not only via hydrolysis of ADP but also through specific adenosine-receptor engagement.

Materials and Methods

Transgenic Mice

The generation of the human ENTPDase-1–expressing mice has been described previously.[13] The human ENTPD-1 (CD39) transgene is expressed from the mouse H-2K^b promoter, resulting in global expression of ENTPD-1 in these mice. Transgenic mice expressing human (ENTPD-1-Tg) were back-crossed for more than 10 generations onto the BALB/c background and compared with littermate controls. The investigations described conform to the Guidelines for the Care and Use of Laboratory Animals of the National Institutes of Health and were approved by The Ohio State University Institutional Animal Care and Use Committee.

Chemicals

The following inhibitor of CD73 activity was used in these studies: α-β-methylene-ADP (APCP; Sigma-Aldrich, St. Louis, MO).[14] The following chemical antagonists of adenosine receptors were used in this study[15,16]: 8-(p-sulfophenyl) theophylline (8-SPT), a nonselective antagonist for adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 (Sigma-Aldrich); 4-(2-[7-amino-2-(2-furyl)[1,2,4]triazolo[2,3-*a*][1,3,5]triazin-5-ylamino]ethyl) phenol (ZM 241385), a highly selective A_{2A} adenosine antagonist (Tocris, Ellisville, MI); N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide (MRS 1754), a selective A_{2B}-receptor antagonist (Sigma-Aldrich). The following ADP mimetic, adenosine-5′-0-(2-thiodiphosphate) trilithium salt, Ado-5′-PP[S] (ADP-β-S; Sigma-Aldrich) was used.[15]

In Vivo *Carotid Thrombosis*

Ferric chloride ($FeCl_3$)-induced carotid artery thrombosis was used.[17] Wild-type (WT) or ENTPD-1-Tg mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Atropine (0.05 mg s.c.) was administered to reduce airway secretions. Animals were intubated and ventilated with room air (tidal volume, 250 μL; 150 breaths/minute) with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37°C by a thermo-regulated heating pad. The left common carotid artery was dissected gently, a flow probe was placed on the artery (MA0.5PSB; Transonic Systems; Ithaca, NY), and blood flow was measured with a pulsed Doppler flow system. After obtaining baseline flow recordings, carotid artery injury was induced by application of filter paper saturated with 10% $FeCl_3$ solution on the adventitial surface proximal to the flow probe for 3 minutes. The flow as a percentage of baseline and the time to thrombotic occlusion (blood flow, 0 mL/minute) was measured from the placement of the $FeCl_3$-saturated filter paper. The surgeon was blinded to the animal genotype and any drug treatment during all experiments. Animals were reanesthetized as needed and at each hour under anesthesia the animals were administered normal saline intraperitoneally. For those animals treated with pharmacologic antagonists, the antagonists were dissolved in a final concentration of 0.5% dimethyl sulfoxide and administered at the designated dose as a single intraperitoneal injection 15 to 30 minutes before the application of $FeCl_3$.

For visualization of thrombus formation, mice were injected intravenously with 5 μL of a 100-μmol/L solution/gram of body weight of the cationic lipophilic dye 3,3′-dihexyloxacarbocyanine iodide 10 minutes before application of $FeCl_3$ to induce vessel injury.[18] The carotid artery was video recorded continuously using a Leica M165 FC fluorescent stereomicroscope (Leica, Wetzlar, Germany) equipped with a Hamamatsu ORCA-R^2 Digital CCD camera (Hamamatsu, Hamamatsu City, Japan) for 30 minutes.

Carotid Histology

At 15 minutes after the application of $FeCl_3$ animals were perfusion-fixed with 4% buffered paraformaldehyde and the injured and noninjured carotid arteries from WT or ENTPD-1-Tg mice were isolated. The samples were embedded in paraffin, sectioned at 3 μm, and stained with H&E.

Whole-Blood Aggregation

Blood was collected from the inferior vena cava of anesthetized WT or ENTPD-1-Tg mice into 70 U/mL of sodium heparin and then diluted 1:2 into physiological saline and whole-blood aggregometry was performed on these diluted samples.[19] One milliliter of diluted blood was

placed into 2-mL cuvettes and incubated for 6 minutes at 37°C in the Chrono-Log Whole Blood Lumi-Ionized Calcium Aggregometer (Chrono-Log Corp., Havertown, PA). The cuvettes then were placed into the test chamber and incubated with the impedance electrode at 37°C while stirring at 900 rpm. After 2 minutes of stabilization, ADP (20 μmol/L final concentration; Chrono-Log Corp., Havertown, PA) or ADP-β-S (20 μmol/L final concentration; Sigma-Alrich) was added. Aggregation, measured as ohms of electrical impedance, was recorded for up to 6 minutes. All whole-blood aggregometry tests were performed within 2 hours of blood acquisition.

Flow Cytometric Analysis

Whole blood was obtained from the inferior vena cava of anesthetized WT or ENTPD-1-Tg–expressing mice. All antibodies were obtained from EMFRET Analytics GmbH & Co. KG (Eibelstadt, Germany). Whole-blood fluorescence-activated cell sorting analysis for the levels of the total fibrinogen receptor, glycoprotein α_{IIb}/β_3 complex expression (clone Leo.H4; Rat IgG_{2b}), was examined at baseline to ensure that there was no difference in the level of these critical platelet membrane proteins involved in platelet activation and thrombosis. Platelet activation was analyzed by activation of diluted whole blood with 20 μmol/L ADP and activated GP α_{IIb}/β_3 complex (clone JON/A; Rat IgG_{2b})[20] expression was examined by flow cytometric analysis at specified times to examine the level of platelet activation (BD LSR II Flow Cytometer; Becton Dickinson, Franklin Lakes, NJ). Nonspecific staining was determined with isotype control antibodies.

ENTPDase Activity Assay

Total platelet lysates were assayed for ENTPDase activity as previously described.[13] One unit of ATP diphosphohydrolase activity corresponds to the release of 1 μmol inorganic phosphate/minute at 37°C.[21]

Quantification of Vascular Tissue Factor Expression and Platelet $P2Y_1$- and $P2Y_{12}$-Receptor Levels

Vascular tissue factor (TF) was measured semiquantitatively using Western blot analysis as published previously.[22] Aortas were removed, cleaned of adventitia, placed in ice-cold phosphate-buffered saline, and then homogenized using an Omni tissue homogenizer (model TH-794; Omni International, Marietta, GA) in lysis buffer consisting of 30 mmol/L 3-[(3-cholamidopropyl)dimethylammonio]propanesulfonic acid, 10 μmol/L E64, 1 mmol/L phenylmethylsulfonyl fluoride, and 10 mmol/L EDTA in PBS (pH 7.4). For platelet homogenates, whole blood was obtained from the inferior vena cava of anesthetized WT or ENTPD-1-Tg–expressing mice, diluted 1:1 in physiological saline, and layered onto platelet Fico/Lite (Atlanta Biologicals, Lawrenceville, GA). Platelet-rich plasma was generated by centrifugation at 350 $\times$ *g* for 15 minutes. Platelets then were washed with physiological saline and pelleted. Samples from 3 mice were pooled and homogenized in lysis buffer consisting of 50 mmol/L Tris (pH 7.4), 150 μmol/L NaCl, 0.5% Nonidet P-40, 1 mmol/L sodium pyrophosphate, 5 mmol/L sodium vanadate, 1 mmol/L benzamidine, and 1 mmol/L sodium fluoride with protease inhibitor cocktail (Sigma-Aldrich).

The crude homogenates were centrifuged at 10,000 $\times$ *g*, and supernatants were transferred and stored at −80°C until analyzed. Protein content of supernatants was measured using the D_C protein assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (12 μg for TF, 15 μg for P2Y1 and P2Y12) were diluted 1:2 in Laemmli sample buffer (Bio-Rad) and placed in a boiling water bath for 5 minutes. Samples then were run on 12.5% Criterion ready gels (Bio-Rad) using SDS-PAGE, proteins were transferred to polyvinylidene difluoride membranes, and blocked overnight in Tris-buffered saline (pH 7.4) with 0.05% Tween 20 (Sigma-Aldrich) and 5% powdered milk. Membranes were incubated with primary antibodies to mouse TF (1:500, 12 hours; American Diagnostica, Stamford, CT) or mouse $P2Y_{12}$ receptor (1:1000, 12 hours; AnaSpec, Freemont, CA), mouse $P2Y_1$ (1:400, 12 hours; Alomone, Jerusalem, Israel), or glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:15,000, 12 h; Abcam, Inc, Cambridge, MA), and after washing were incubated for 45 minutes with horseradish-peroxidase–conjugated secondary antibody. Immunoblots were developed using Supersignal (Pierce, New Haven, CT) and quantified by densitometry (ChemiDoc; Bio-Rad). TF, $P2Y_1$, and $P2Y_{12}$ were normalized to GAPDH and data are presented as the relative quantification of TF $P2Y_1$ and $P2Y_{12}$ (relative to each individual sample GAPDH).

Statistical Analysis

The results of experiments were analyzed by several statistical methods using standard software (eg, GraphPad Prism, version 4.0, San Diego, CA). Results were expressed as mean ± standard error of the mean. For comparison between 2 groups, significance was determined by an unpaired Student's *t*-test. For comparison of multiple groups, multifactorial analysis of variance with *post hoc* comparison of the means with Bonferroni correction was used to determine statistical significance. For all evaluations, probability values less than 0.05 were considered significant.

Results

Overexpression of ENTPDase-1 Markedly Delays Formation of Occlusive Thrombus Formation in Vivo

Analysis of platelets from human ENTPDase-1–expressing mice revealed a twofold increase in ENTPDase activity and increased surface level expression of ENTPD-1 compared with WT control platelets consistent with a prior study showing increased expression on the platelets, endothelium, and leukocytes of the transgenic mice

Table 1. Complete Blood Count from WT and ENTPD-1-Tg Mice

Parameter	WT (n = 6)	ENTPD-1-Tg (n = 6)
WBC (K/μL)	5.7 ± 0.8	7.5 ± 1
NE (%)	26.9 ± 1.6	25.6 ± 1.6
LY (%)	60.7 ± 2.1	61.7 ± 1.7
MO (%)	9.2 ± 1.2	9.6 ± 1.2
EO (%)	2.4 ± 0.3	2.4 ± 0.6
BA (%)	0.8 ± 0.2	0.6 ± 0.1
HCT (%)	51.9 ± 5.3	54.9 ± 5.4
RBC (K/μL)	10.1 ± 1.0	10.8 ± 1.2
Hb (g/dL)	17.1 ± 0.8	16.2 ± 0.8
MCV (fL)	51.4 ± 0.4	51.2 ± 0.8
MCHC (g/dL)	34.2 ± 2.6	30.9 ± 3.0
RDW (%)	15.4 ± 0.2	15.6 ± 0.3
Retics (%)	1.3 ± 0.2	1.1 ± 0.4
PLT (K/μL)	1295.7 ± 52.6	1128.7 ± 98.5
MPV (fL)	5.3 ± 0.1	5.1 ± 0.2

None of the data were found to be statistically significantly different.
BA, basophills; EO, eosinophils; Hb, hemoglobin; HCT, hematocrit; LY, lymphocytes; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; MO, monocytes; MPV, mean platelet volume; NE, neutrophils; PLT, platelets; RBC, red blood cells; RDW, red cell differential width; Retics, reticulocyte; WBC, white blood cells.

on a different genetic background (C57BL/6) (data not shown).[13] No differences in the complete blood count were observed between WT and ENTPD-1-Tg mice (Table 1). To determine whether ENTPDase-1 overexpression translated to a resistance to *in vivo* conduit artery thrombosis, WT or ENTPD-1-Tg mice were evaluated in a model of $FeCl_3$-induced carotid artery thrombosis.[17] In WT animals there was an initial increase in carotid flow followed by a precipitous decrease in flow to zero that correlated *in vivo* with the formation of an acute occlusive thrombus. However, in ENTPD-1–expressing animals, although there was no increase in flow observed after ferric chloride application and there was a gradual decrease in carotid flow to approximately 70% of the baseline flow, no occlusive thrombus was formed within the injured vessel (Figure 1, A and B). These findings were confirmed by histologic assessment of the carotid arteries after $FeCl_3$ treatment that revealed occlusive thrombus present in the injured WT animals by 15 minutes, but not in the injured carotid arteries of ENTPD-1-Tg animals (data not shown).

To determine the absolute extension of the time to thrombosis conveyed by ENTPD-1 expression, a separate group of animals was monitored until occlusion was observed. ENTPD-1 expression profoundly delayed the time to occlusion (WT: 13.7 ± 0.88 minutes, N = 14 versus ENTPD-1-Tg: 281.5 ± 57.31 minutes, N = 8; $P <$ 0.001; Figure 1C). Given the role of TF in ferric chloride–induced carotid thrombosis,[23–26] as well as prior studies showing an increase in TF levels in ENTPD-1 knockout mice[27] and decreased expression of TF on endothelial cell cultures treated with adenosine,[28] the level of TF in the vasculature was measured and found not to differ between WT and ENTPD-1-Tg aortae at baseline (relative TF/GAPDH densitometric ratios were as follows: WT, 0.20 ± 0.044; ENTPD-1-Tg, 0.21 ± 0.033; $P > 0.05$; N = 3 per group; Figure 1D). Furthermore, strain variations with regard to coagulation and thrombosis parameters did not account for the resistance to occlusive thrombus formation because C57BL/6 background mice overexpressing human ENTPD-1 showed a comparable prolongation in the time to thrombosis (data not shown). Thus, overexpression of ENTPD-1 conveys a marked protection against the generation of an occlusive arterial thrombus *in vivo*.

ADP-Receptor Engagement in ENTPDase-1–Mediated Resistance to Thrombosis

As stated earlier, ENTPDase-1 sequentially converts extracellular ATP and ADP to AMP. ADP is a potent platelet agonist required for the continued expression of activated glycoprotein α_{IIb}/β_3-complex. To determine whether the ENTPDase-1–mediated *in vivo* resistance to thrombosis involved ADP hydrolysis, animals were treated with the nonhydrolyzable ADP analog (ADP-β-S). Treatment with 6.25 mg/kg ADP-β-S, administered intravenously, 10 minutes before the application of ferric chloride, resulted in abrogation of the antithrombotic efficacy observed in ENTPD-1-Tg mice (ADP-β-S–treated mice: WT: 11.3 ± 1.53 minutes versus ENTPD-1-Tg: 17.1 ± 2.59 minutes; $P > 0.05$; N = 3 per group; Figure 1C), consistent with the interpretation that hydrolysis of ADP is the critical step in the protection from *in vivo* thrombosis in ENTPD-1-Tg mice.

ENTPDase-1 Modulates ex Vivo *Platelet Aggregation and Activation*

To examine the effect of ENTPD-1 overexpression in an *ex vivo* model, whole-blood aggregation was performed. In response to ADP stimulation (20 μmol/L), both WT and ENTPD-1-Tg whole blood displayed a rapid initiation of aggregation that persisted in WT blood. In contrast, although an initial aggregatory response was observed after ADP stimulation, there was a rapid and near-complete disaggregation observed in ENTPD-1-Tg whole blood (Figure 2A). This resulted in a decrease in the total area under the curve in response to 20 μmol/L ADP stimulation in ENTPD-1-Tg versus WT (area under the curve was as follows: WT: 20,900 ± 746.2 $\Omega \cdot$ seconds; ENTPD-1-Tg: 5586 ± 1544 $\Omega \cdot$ seconds; N = 4 per group; P = 0.0001) and a decrease in the total aggregation at 6 minutes (Figure 2B; aggregation at 6 minutes: WT: 69.8 ± 6.35 Ω; N = 4 per group: ENTPD-1-Tg: 4.5 ± 1.55 Ω; N = 4 per group; $P < 0.001$). These results differ slightly from what was reported previously for the transgenic mice on a different genetic background using whole blood anticoagulated with citrate.[13] Heparin, an *in vivo* anticoagulant frequently used in the treatment of acute coronary syndromes, may increase platelet responsiveness to agonists.[29] However, regardless of the anticoagulant used, the data are consistent with the interpretation that ENTPD-1 expression inhibits platelet reactivity in whole blood. Next, to determine whether the differences in whole-blood aggregation are caused by ADP hydrolysis, whole blood from ENTPD-1-Tg mice was stimulated with ADP-β-S (20 μmol/L), which resulted in a persistent aggregatory response in ENTPD-1-Tg whole blood compared with ADP stimulation with a correspond-

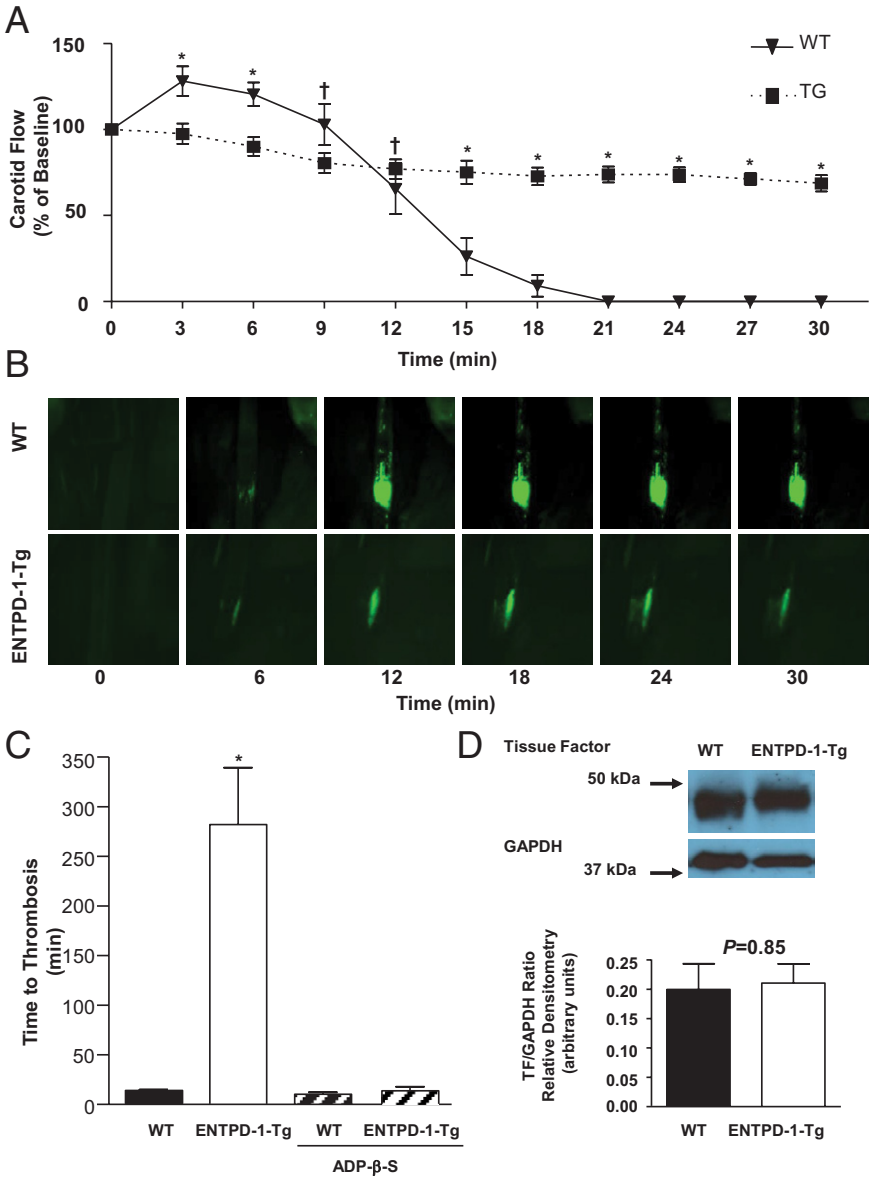

Figure 1. ENTPD-1 conveys resistance to *in vivo* thrombosis. **A:** Carotid flow after $FeCl_3$ application is expressed as a percentage of baseline flow in WT and ENTPD-1-Tg animals. **B:** *In vivo* real-time fluorescence imaging of thrombus formation in WT and ENTPD-1-Tg animals after $FeCl_3$ application. **C:** Time to thrombosis after $FeCl_3$ application in WT and ENTPD-1-Tg mice (WT: 13.7 ± 0.9 minutes; ENTPD-1-Tg: 281.5 ± 57.3 minutes; $N = 8$ per group) and the effect of ADP-β-S treatment on the time to thrombosis after $FeCl_3$ application in WT and ENTPD-1-Tg mice (ADP- β-S: WT: 11.3 ± 1.53 minutes versus ENTPD-1-Tg: 17.1 ± 2.59 minutes; $N = 3$ per group). **D:** Tissue factor expression on aortae from WT and ENTPD-1 mice. Representative immunoblot and normalized densitometry (relative TF/GAPDH densitometric ratios: WT: 0.20 ± 0.044; ENTPD-1-Tg: 0.21 ± 0.033; $P = 0.85$; $N = 3$ per group). Values are mean ± standard error of the mean. $^*P < 0.001$; $^\dagger P < 0.05$; TG, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

ing increase in the total area under the curve (Figure 2C; ADP: 5358 ± 443.7 Ω · second; ADP-β-S: 15,380 ± 680.2 Ω · second; $N = 3$ per group; $P = 0.0002$) and an increase in the total aggregation at 6 minutes (Figure 2D; ADP: 1.5 ± 0.75 Ω; $N = 3$ per group; ADP-β-S: 55.8 ± 3.38 Ω; $N = 3$ per group; $P < 0.0001$), consistent with ENTPDase-1–mediated hydrolysis of ADP inhibiting the whole-blood aggregation.

To investigate the effects of overexpression of ENTPD-1 further, platelet activation in whole blood from either WT or ENTPD-1-Tg mice treated with ADP was compared. Prior studies have shown a time-dependent increase in activated platelet fibrinogen receptor, the integrin glycoprotein (GP) α_{IIb}/β_3 (CD41/CD61) with ADP stimulation.[20] Basal expression levels of GPIX, GPVI (not shown), and the integrin glycoprotein α_{IIb}/β_3 did not differ between WT and ENTPD-1-Tg platelets (Figure 3, A and B). Although ADP stimulation resulted in an increase in surface expression of activated glycoprotein α_{IIb}/β_3 (Figure 3C) in a time-dependent manner in WT platelets, the level of activated GP α_{IIb}/β_3 on platelets from ENTPD-1-Tg mice was reduced significantly at all time points. However, stimulation of WT or ENTPD-1 blood with ADP-β-S showed comparable expression of activated GP α_{IIb}/β_3 on platelets at 6 minutes after stimulation (mean fluorescence intensity was as follows: WT: 207.3 ± 22.41 versus ENTPD-1: 229.0 ± 22.94; $P > 0.05$), consistent with intact expression and signaling mechanism via $P2Y_1$ and $P2Y_{12}$ receptors in ENTPD-1 platelets. Furthermore, to determine whether differences in the level of $P2Y_1$- and $P2Y_{12}$-receptor expression on platelets from ENTPD-1-Tg mice contributed to the observed effects, immunoblot analysis was conducted. No differences in either $P2Y_1$ (relative P2Y1/GAPDH densitometric ratios were as follows: WT: 0.80 ± 0.158; ENTPD-1-Tg: 0.92 ± 0.116; $P > 0.05$; $N = 3$ per group; Figure 3D) or $P2Y_{12}$-receptor levels (relative P2Y12/GAPDH densitometric ratios were as follows: WT: 1.83 ± 0.122; ENTPD-1-Tg: 2.03 ± 0.069; $P > 0.05$; $N =$

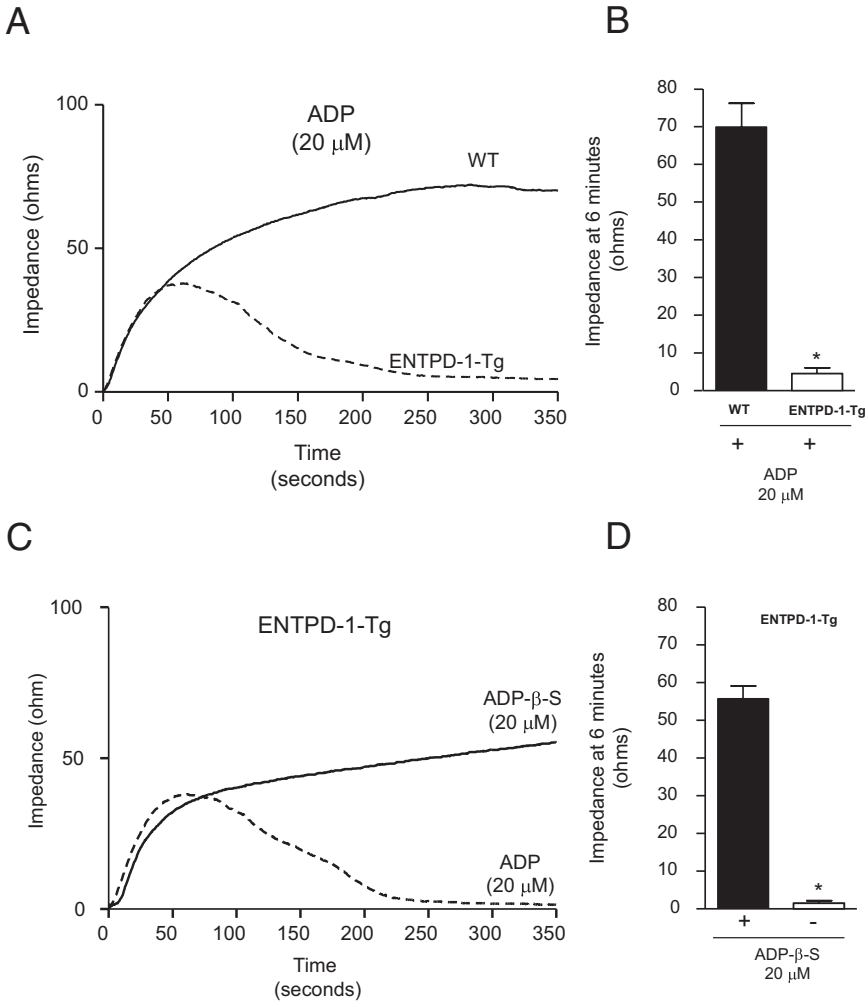

Figure 2. ENTPDase-1 attenuates whole-blood aggregation and ADP-β-S treatment restores whole-blood aggregation in ENTPDase-1 mice. **A:** Whole-blood aggregation curve in response to 20 μmol/L ADP in WT and ENTPD-1-Tg blood. Area under the curve for whole-blood aggregation in WT and ENTPD-1-Tg blood in response to 20 μmol/L ADP (WT: 20,900 ± 746.2 Ω · second; ENTPD-1-Tg: 5586 ± 1544 Ω · second; $N = 4$ per group; $P = 0.0001$). **B:** Aggregation at 6 minutes in WT and ENTPD-1-Tg blood in response to 20 μmol/L ADP (WT: 69.9 ± 6.35 Ω; ENTPD-1-Tg: 4.5 ± 1.55 Ω; $N = 4$ per group). ADP-β-S restores whole-blood aggregation in ENTPDase-1 mice. **C:** Whole-blood aggregation in ENTPD-1-Tg blood in response to 20 μmol/L ADP or 20 μmol/L ADP-β-S, a non-hydrolyzable ADP analog. Area under the curve for whole-blood aggregation in ENTPD-1-Tg blood in response to 20 μmol/L ADP or 20 μmol/L ADP-β-S (ADP: 5358 ± 443.7 Ω · second; ADP-β-S: 15,380 ± 680.2 Ω · second; $N = 3$ per group; $P = 0.0002$). **D:** Aggregation at 6 minutes in ENTPD-1-Tg blood in response to 20 μmol/L ADP or 20 μmol/L ADP-β-S (ADP: 1.5 ± 0.75 Ω; ADP-β-S: 55.8 ± 3.38 Ω; $N = 3$ per group). Values are mean ± standard error of the mean. $^*P < 0.001$; TG, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

3 per group; Figure 3E) were observed. Together with the *in vivo* data of treatment of mice with ADP-β-S, these data suggest that ENTPD-1 activity mediates antiaggregatory efficacy by hydrolyzing ADP, thus limiting the activation state of platelet glycoprotein α_{IIb}/β_3, preventing the acute generation of an occlusive thrombus *in vivo*.

Adenosine in ENTPDase-1–Mediated Resistance to Occlusive Thrombosis

As outlined earlier, ENTPDase-1 converts extracellular ATP and ADP to AMP and ecto-5′-nucleotidase (CD73) converts extracellular AMP to adenosine, which can regulate platelet reactivity and *in vivo* thrombosis.[30,31] To determine whether the *in vivo* resistance to carotid thrombosis observed in ENTPD-1-Tg animals also involves the generation of adenosine by ecto-5′-nucleotidase, mice were treated with APCP, a specific ecto-5′-nucleotidase antagonist. Treatment with APCP also abrogated the resistance to occlusive thrombus formation mediated by ENTPD-1 overexpression (APCP-treated WT: 11.9 ± 1.01 minutes versus APCP-treated ENTPD-1-Tg: 15.5 ± 1.08 minutes; $N = 4$ per group; $P < 0.05$; Figure 4, A and B), suggesting the possibilities that APCP inhibits ENTPDase-1 activity directly, or that inhibition of ecto-5′-nucleotidase activity inhibits ENTPDase-1 activity via accumulation of AMP, or that ecto-5′-nucleotidase–mediated generation of adenosine is involved in the *in vivo* resistance to thrombosis.

To address if adenosine-receptor–mediated signaling contributes to the resistance to *in vivo* thrombosis observed in ENTPDase-1–expressing mice, mice were treated with the nonselective adenosine-receptor antagonist 8-SPT. Treatment with 8-SPT abrogated the resistance to occlusive thrombus formation observed in mice overexpressing ENTPDase-1, consistent with the interpretation that adenosine receptor engagement is required for the resistance to occlusive thrombus formation mediated by ENTPDase-1 (8-SPT–treated WT: 10.5 ± 1.42 minutes versus 8-SPT–treated ENTPD-1-Tg: 14.3 ± 1.57 minutes; $N = 8$ per group; $P > 0.05$; Figure 4, A and C). Thus, APCP and 8-SPT, both with distinct molecular structures, either inhibit ENTPDase-1 activity directly or these pharmacologic data are consistent with an interpretation that, in the mouse, generation of adenosine and engagement of adenosine receptors contributes to the *in vivo* antithrombotic efficacy conveyed with ENTPD-1 expression. Prior studies have shown that adenosine al-

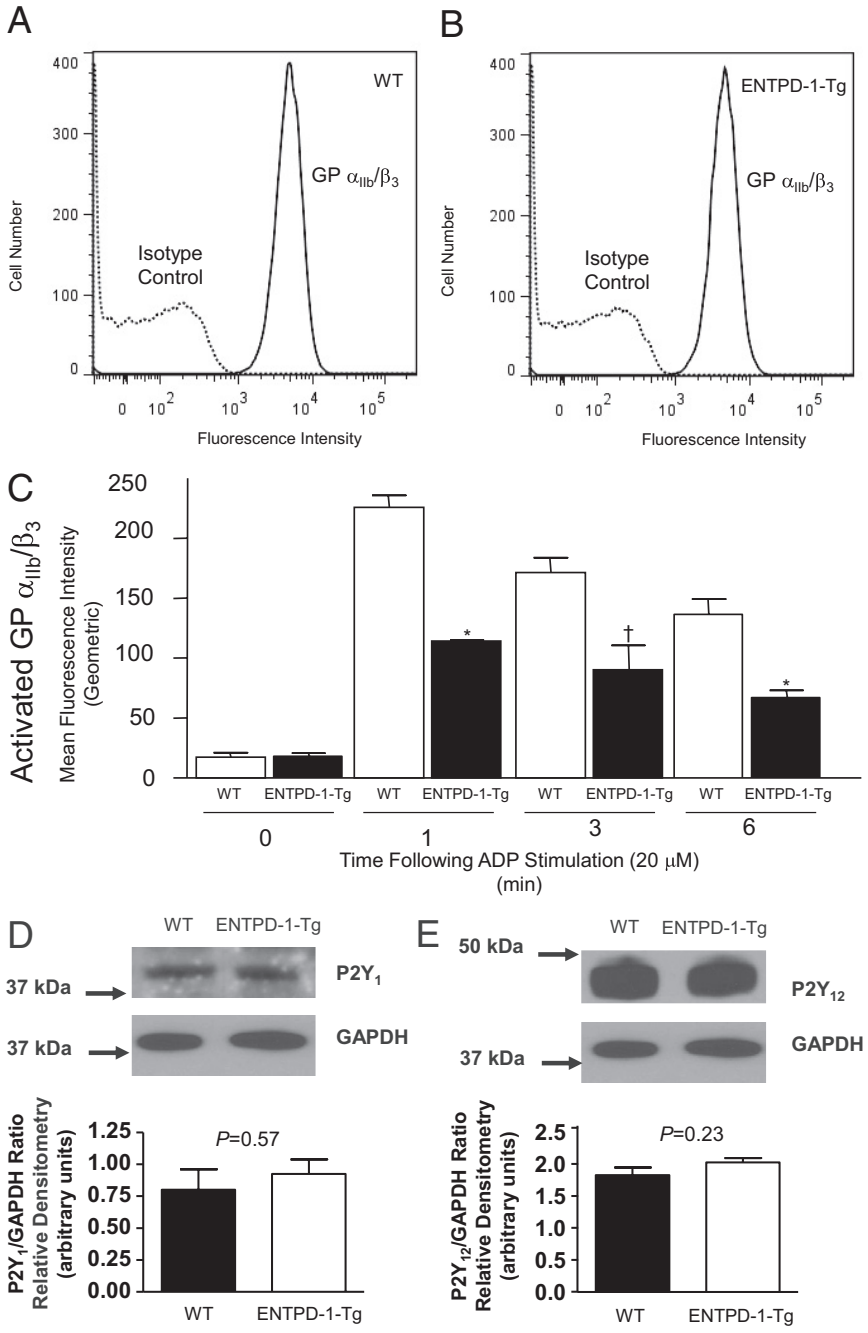

Figure 3. ENTPDase-1 expression attenuates fibrinogen receptor activation. **A:** Representative fluorescence-activated cell sorter analysis of expression of total GP α_{IIb}/β_3 on WT (**A**) and ENTPD-1-Tg (**B**) resting platelets (isotype control: WT: 24.2 ± 1.40; ENTPD-1-Tg: 23.3 ± 0.59; GP α_{IIb}/β_3: WT: 3,215 ± 273; ENTPD-1-Tg: 3,488 ± 203; $N = 4$ per group). **C:** Fluorescence-activated cell sorter analysis of activated GP α_{IIb}/β_3 on WT and ENTPD-1-Tg platelets after stimulation with 20 μmol/L ADP. Geometric mean ± standard error of the mean from 10,000 platelet events are shown: baseline (WT: 17.5 ± 3.40; ENTPD-1-Tg: 18.2 ± 2.62; $N = 3$ per group; $P = 0.8732$); 1 minute (WT: 226.0 ± 9.74; ENTPD-1-Tg: 114.3 ± 0.75; $N = 3$ per group); 3 minutes (WT: 171.4 ± 12.28; ENTPD-1-Tg: 90.4 ± 20.36; $N = 3$ per group), and 6 minutes (WT: 136.3 ± 12.95; ENTPD-1-Tg: 67.05 ± 6.074; $N = 3$ per group). **D:** $P2Y_1$-receptor levels on platelets from WT and ENTPD-1 mice. Representative immunoblot and normalized densitometry (relative P2Y1/GAPDH densitometric ratios: WT: 0.80 ± 0.158; ENTPD-1-Tg: 0.92 ± 0.116; $P = 0.57$; $N = 3$ per group) **E:** $P2Y_{12}$-receptor levels expression on platelets from WT and ENTPD-1 mice. Representative immunoblot and normalized densitometry levels (relative P2Y12/GAPDH densitometric ratios: WT: 1.83 ± 0.122; ENTPD-1-Tg: 2.03 ± 0.069; $P = 0.23$; $N = 3$ per group). $^*P < 0.001$; $^\dagger P < 0.05$; TG, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

ters platelet reactivity via interaction with the A_{2A} receptor. To delineate if occupation of the adenosine A_{2A} receptor is involved in the ENTPDase-1–mediated resistance to thrombosis, the acute effect of ZM 241385, a selective antagonist of the A_{2A}-adenosine receptor, was examined. Acute pretreatment of mice with ZM 241385 completely abrogated the *in vivo* resistance to thrombosis conveyed by ENTPDase-1 overexpression, consistent with an interpretation that the *in vivo* ENTPDase-1–mediated effects require A_{2A}-receptor occupancy (ZM 241385 1 mg/kg: WT: 11.1 ± 0.58 minutes versus ENTPD-1: 14.4 ± 1.31 minutes; $N = 5$ per group; $P > 0.05$; Figure 4, D and E). Given recent data suggesting an inhibitory role of adenosine A_{2B}-receptor activation on platelet reactivity *ex vivo*,[32] the effect of MRS 1754, a selective A_{2B}-receptor antagonist, also was examined. At all doses examined, pretreatment with MRS 1754 (0.1 mg/kg) abrogated the resistance to thrombosis achieved with ENTPD-1 overexpression (0.1 mg/kg MRS 1754–treated: WT: 12.3 ± 1.88 minutes versus ENTPD-1-Tg: 20.5 ± 11.05 minutes; $P > 0.05$; $N = 5$ per group; Figure 4, D and F), consistent with the interpretation that adenosine A_{2B}-receptor occupation also is required for the *in vivo* ENTPDase-1–mediated resistance to thrombosis. Thus, 8-SPT, ZM 241385, or MRS 1754, all structurally distinct molecules, attenuate ENTPDase-1 activity di-

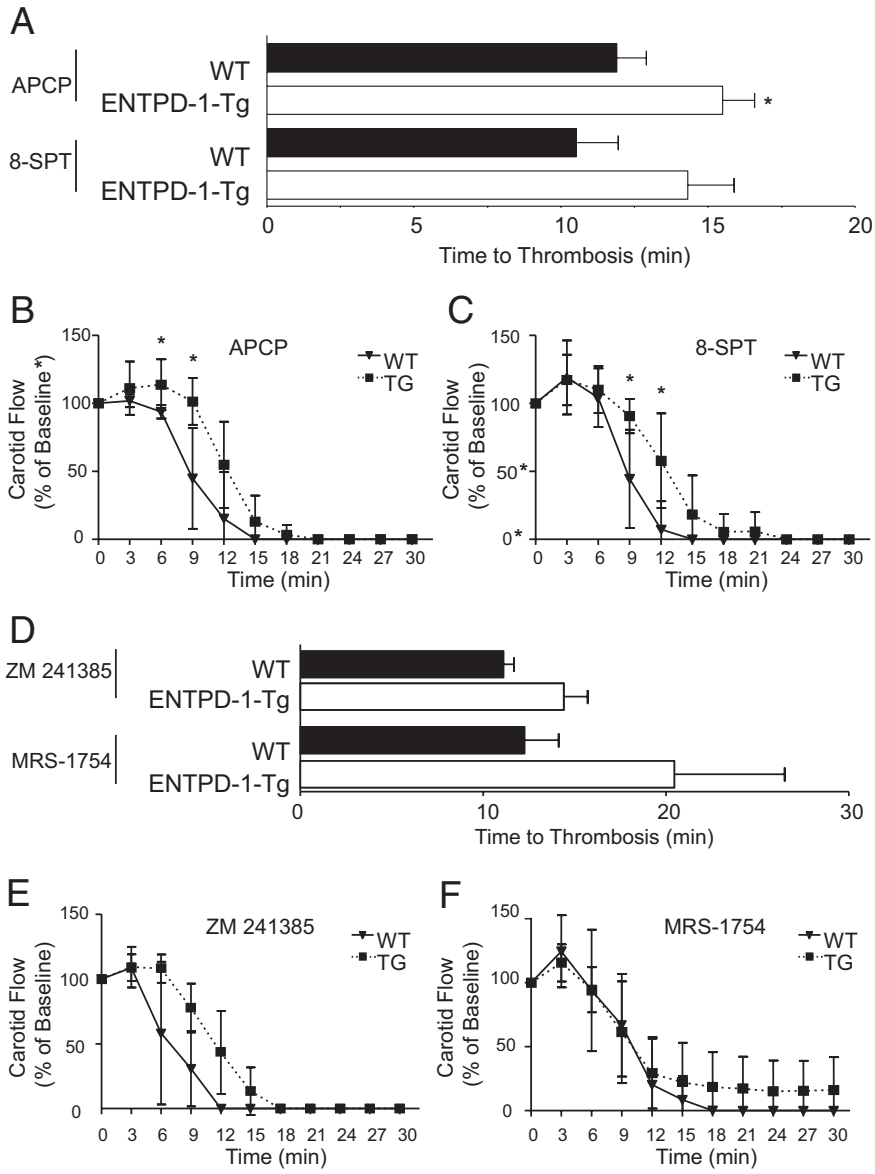

Figure 4. Adenosine in ENTPDase-1–mediated resistance to occlusive thrombosis. **A:** Administration of the ecto-5′-nucleotidase inhibitor APCP or the nonselective adenosine receptor antagonist 8-SPT abrogates ENTPDase-1–mediated resistance to occlusive thrombus formation *in vivo* (APCP-treated WT: 11.9 ± 1.01 minutes; APCP-treated ENTPD-1-Tg: 15.5 ± 1.08 minutes; $N = 4$ per group, $P = 0.045$ versus APCP-treated WT; $P < 0.001$ versus untreated ENTPD-1-Tg; 8-SPT–treated WT: 10.5 ± 1.42 minutes; 8-SPT–treated ENTPD-1-Tg: 14.3 ± 1.57 minutes; $N = 8$ per group, $P > 0.05$ versus 8-SPT–treated WT; $P < 0.001$ versus untreated ENTPD-1-Tg). **B:** Carotid flow after $FeCl_3$ application expressed as a percentage of baseline flow in WT and ENTPD-1-Tg animals treated with APCP. **C:** Carotid flow after $FeCl_3$ application expressed as a percentage of baseline flow in WT and ENTPD-1-Tg animals treated with 8-SPT. **D:** Administration of the adenosine A_{2A}-receptor antagonist ZM 241385 or the adenosine A_{2B}-receptor antagonist MRS-1754 abrogates ENTPDase-1–mediated resistance to occlusive thrombus formation *in vivo* (ZM-treated WT: 11.1 ± 0.58 minutes; ZM 241385-treated ENTPD-1-Tg: 14.4 ± 1.31 minutes; $N = 5$ per group, $P > 0.05$ versus ZM 241385-treated WT; $P < 0.001$ versus untreated ENTPD-1-Tg; MRS-1754–treated WT: 12.3 ± 1.88 minutes; MRS-1754–treated ENTPD-1-Tg: 20.5 ± 11.05 minutes; $N = 5$ per group, $P > 0.05$ versus MRS-1754–treated WT; $P < 0.001$ versus untreated ENTPD-1-Tg). **E:** Carotid flow after $FeCl_3$ application expressed as a percentage of baseline flow in WT and ENTPD-1-Tg animals treated with ZM 241385. **F:** Carotid flow after $FeCl_3$ application expressed as a percentage of baseline flow in WT and ENTPD-1-Tg animals treated with MRS-1754. Values are mean ± standard error of the mean. *$P < 0.05$; TG, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

rectly, or these pharmacologic data are consistent with the interpretation that, in the mouse, engagement of both the A_{2A} and A_{2B} adenosine receptors contribute to the antithrombotic efficacy conveyed with ENTPD-1 expression.

Discussion

Acute arteriosclerotic plaque rupture with subsequent adhesion and aggregation of platelets results in thrombus formation and coronary vessel occlusion, the pathophysiologic basis of acute coronary syndromes.[1] The current studies show that increased ENTPD-1 (CD39) activity can modulate purinergic signaling, thereby attenuating activation of the platelet fibrinogen receptor, the integrin GP α_{IIb}/β_3 (CD41/CD61), resulting in a profound resistance to *in vivo* occlusive thrombus formation. Increased expression of ENTPDase-1 pacifies the response to vascular injury. Thus, overexpression of ENTPD-1 conveys a marked protection against *in vivo* arterial thrombosis and treatments aimed at increasing ENTPD-1 levels might convey vascular protection.

The accumulation of platelets at sites of vascular disruption via engagement of specific platelet receptors to the exposed subendothelial matrix leads to platelet activation. On activation, numerous signaling cascades converge to release of ATP and ADP from platelet-dense granules, which further activate and recruit platelets into the growing thrombus. ATP and ADP also affect vascular reactivity, inducing vasodilation when administered abluminally to intact carotid arteries.[33] ENTPDase-1 appears to control nucleotide-dependent vasoconstriction because knockout of the *ENTPDase-1* gene leads to increased aortic ring constriction in response to UDP or UTP.[34,35] Thus, ATP and ADP mediate prothrombotic and inflammatory signaling through interactions with specific

purinergic receptors on endothelium, vascular smooth muscle, platelets, and inflammatory cells.[34–36] Modulation of platelet function is achieved in part through the action of several purinergic receptors on platelets, $P2Y_1$, $P2Y_{12}$, and $P2X_1$.[36]

The $P2Y_1$ is a $G\alpha_q$ G-protein–coupled receptor critical to ADP-induced shape change, aggregation, thromboxane A2 generation, and thrombus formation under shear conditions.[37–39] Knockout of the $P2Y_1$ receptor results in absence of platelet shape change and reduced aggregation in response to ADP as well as reduced thrombus size and stability.[40,41] Overexpression of the $P2Y_1$ receptor results in increased platelet reactivity both *ex vivo* and *in vivo*.[42] The $P2Y_{12}$ receptor is a $G\alpha_i$-coupled G-protein–coupled receptor that suppresses cAMP formation,[43] thereby potentiating platelet activation by a number of stimuli.[36] Knockout of the $P2Y_{12}$ receptor results in reduced ADP-induced platelet aggregation, an inability to inhibit adenylyl cyclase activity, and prolonged bleeding time, however, platelet shape change and intracellular aggregation are not affected.[44,45] Interestingly, the $P2Y_{12}$ receptor, the target of the clinically approved antiplatelet agents clopidogrel and prasugrel, has a lower affinity for ADP than the $P2Y_1$ receptor. Furthermore, the prodrug form of clopidogrel appears to facilitate platelet aggregation by inhibition of ENTPDase-1.[46] In contrast to the $P2Y_1$ and $P2Y_{12}$ receptors, the $P2X_1$ receptor is stimulated by ATP, resulting in an influx of calcium that does not lead to aggregation but is necessary for full activation of platelets. Knockout of the $P2X_1$ receptor results in resistance to thrombosis of small arteries,[42] whereas overexpression of $P2X_1$ receptors results in increased thrombus formation in response to various agonists.[47] Thus, genetic studies have shown that activation of each of the purinergic receptors is necessary for full platelet activation.

In the current study, increased ENTPDase-1 activity reduced ADP-mediated whole-blood aggregation. Similarly, in response to ADP activation, ENTPDase-1 expression decreased activation of the platelet fibrinogen receptor, GP α_{IIb}/β_3 complex. These data are consistent with prior work showing that persistent ADP-mediated $P2Y_1$- and $P2Y_{12}$-receptor stimulation is required to maintain calcium-mediated activation of the platelet fibrinogen receptor, glycoprotein α_{IIb}/β_3.[48] Thus, ENTPDase-1 not only maintains basal vascular function, but in pathologic states, ENTPDase-1–mediated hydrolysis of ATP and ADP released from damaged tissue regulates the purinergic pathways involved in platelet activation and thrombosis. However, the antithrombotic effects conveyed by ENTPD-1 expression were reversed by stimulation with the nonhydrolyzable ADP analog, ADP-β-S, suggesting that continued ADP stimulation is required to maintain maximal aggregation and platelet activation, a well-known phenomenon,[49–52] and that $P2Y_1$ and $P2Y_{12}$ expression and signaling are intact and capable of mediating platelet activation in ENTPD-1 platelets.

ATP and ADP are metabolized to AMP, which is converted to adenosine by the actions of ecto-5′-nucleotidase (CD73). Recent work has shown that soluble 5′-nucleotidase derived from *Crotalus atrox* venom inhibits *ex vivo* platelet aggregation, suggesting a role for adenosine-mediated signaling in the inhibition of platelet function.[53] However, in our current model, we observed no effect with antagonism of ecto-5′-nucleotidase or of adenosine-receptor blockade in whole-blood aggregation (data not shown). Indeed, prior studies have shown no effect of adenosine on whole-blood aggregation except under conditions in which dipyridamole is added to prevent red blood cell uptake and metabolism of adenosine.[54–56]

The current data suggest that in our model inhibition of whole-blood aggregation is dependent on ADP degradation rather than adenosine generation. However, a more complex situation appears to be involved in the cascade leading to *in vivo* thrombosis in which both ADP degradation and adenosine generation via ecto-5′-nucleotidase (CD73) activity appear capable of modulating occlusive thrombus formation after arterial injury. Indeed, prior studies[31] have demonstrated that endogenous adenosine production appears to inhibit *in vivo* platelet aggregation. It has been proposed that localized effects might explain the observation reported here and in other studies regarding adenosine modulation of platelet activation and thrombus growth *in vivo*; adenosine-receptor effects may be more pronounced in pathologic processes, such as a growing thrombus.[56] In our *in vivo* model, ENTPD-1–mediated resistance to formation of an occlusive thrombus appears dependent on ecto-5′-nucleotidase activity because treatment of ENTPD-1-Tg mice with the CD73 antagonist APCP normalized the time to thrombosis. These data are consistent with prior studies that have shown that knockout of ecto-5′-nucleotidase results in a shortened time to thrombosis in a murine model of $FeCl_3$-induced carotid arterial thrombosis.[57] Future studies examining the effect of genetic ablation of ecto-5′-nucleotidase in mice that overexpress ENTPDase-1 will provide further insight into this complex signaling cascade both *ex vivo* and *in vivo*. Our data suggest that not only ADP hydrolysis but also adenosine generation are necessary for ENTPD-1–mediated resistance to *in vivo* occlusive thrombus formation after vascular injury. Thus, therapies that lead to increased ENTPD-1 activity may have profound vascular protective efficacy.

Platelets possess both A_{2A} adenosine receptors and A_{2B} adenosine receptors,[58,59] and both couple to adenylate cyclase, causing an increase in cAMP levels, which inhibits platelet activation.[60,61] Genetic ablation of either the A_{2A} adenosine receptor[58] or the A_{2B} adenosine receptor[32] results in higher platelet aggregation in response to ADP. The A_{2A} and A_{2B} adenosine receptors also are expressed on circulating neutrophils and monocytes. Indeed, not only platelet-platelet interactions but also platelet-leukocyte adhesion occur with acute arterial injury; circulating platelet-leukocyte aggregates are increased in acute coronary syndromes.[62] Thus, one might hypothesize that ENTPDase-1 expression on leukocytes may modulate platelet/leukocyte interactions and affect thrombosis. Prior studies have shown that in patients with leukocytosis, ENTPDase-1 activity is increased and platelet aggregation is reduced in response to various stimuli.[63]

Recent work has shown that ENTPDase-1 polymorphisms regulate the level of expression of ENTPDase-1 on leukocytes.[64] Furthermore, ENTPDase-1 regulates leukocyte chemotaxis by hydrolyzing released ATP to adenosine, which coordinate to modulate chemotaxis via activation of purinergic nucleotide and adenosine receptors,[65–70] and modulates P2X purinoreceptor 7–dependent function in murine macrophages.[71] The regulation of TF procoagulant activity is complex, with a number of pathways capable of switching TF from a cryptic, nonactive state, to a decrypted, active state.[72] Recent work has shown a critical role for activation of the macrophage P2X purinoreceptor 7 in a protein disulfide isomerase-regulated thiol pathway that controls the release of procoagulant TF–positive microparticles and also TF-dependent thrombosis.[26] Although the level of TF does not differ between WT and ENTPD-1-Tg aortae, further work will be required to determine whether ENTPDase-1 influences TF decryption. Indeed, ongoing work is investigating the influence of the cellular expression of ENTPDase-1 on *in vivo* thrombosis.

In conclusion, these results show a pivotal role of ectonucleoside triphosphate diphosphohydrolase-1 in the modulation of purinergic-mediated platelet activation. ENTPDase-1 expression results in an attenuation of activation of the platelet fibrinogen receptor, glycoprotein α_{IIb}/β_3, which translates to a resistance to *in vivo* occlusive thrombus formation. Our data suggest that not only ADP removal, but also adenosine-receptor engagement, delays *in vivo* thrombosis because antagonism of CD73 or nonselective adenosine-receptor antagonism abrogates the resistance to occlusive thrombus formation conveyed by ENTPDase-1 expression. Supporting our work is recent data showing that in mice subjected to hypothermia, a condition used frequently to treat patients with out-of-hospital arrest, ENTPDase-1 activity was decreased, expression of the platelet activation marker P-selectin was increased, and platelet thrombus formation in $FeCl_3$-injured murine mesenteric arteries was increased.[73] These effects were reversed by administration of recombinant soluble ENTPD-1.[73] We acknowledge that a limitation of the current studies was the specificity and selectivity of the pharmacologic agents used and that the generalizability of the current findings to other models of vascular injury cannot be inferred. However, the ferric chloride model has been used to show the efficacy of agents used clinically to treat acute arterial thrombosis, including tirofiban, eptifibatide, and clopidogrel.[17,74] Continuing investigations using *in vivo* thrombus imaging, alternate methods of arterial vascular injury, and adenosine-receptor knockout animals will help define the interplay between ENTPDase-1 activity and adenosine receptor–mediated signaling on platelet activation and *in vivo* thrombus formation and stability further. The current data show that ENTPDase-1–mediated modulation of purinergic signaling is a key determinant of the formation of an occlusive arterial thrombus after vascular injury and supports the hypothesis that therapy focused on increasing ENTPD-1 expression and activity could have profound *in vivo* antithrombotic efficacy.

Acknowledgments

We thank Dr. Peter J. Newman (Blood Research Institute, Blood Center of Wisconsin) for his critical reading of the manuscript relative to the regulation of platelet activation and thrombosis.

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