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Transgenic over expression of ectonucleotide triphosphate diphosphohydrolase-1 protects against murine myocardial ischemic injury

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Abstract

Modulation of purinergic signaling is critical to myocardial homeostasis. Ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD-1; CD39), which converts the proinflammatory molecules ATP or ADP to AMP is a key regulator of purinergic modulation. However, the salutary effects of transgenic over expression of ENTPD1 on myocardial response to ischemic injury have not been tested to date. Therefore we hypothesized that ENTPD1 over-expression affords myocardial protection from ischemia-reperfusion injury via specific cell signaling pathways. ENTPD-1 transgenic mice, which over-express human ENTPDase-1, and wild-type (WT) littermates were subjected to either *ex vivo* or *in vivo* ischemia-reperfusion injury. Infarct size, inflammatory cell infiltrate and intracellular signaling molecule activation were evaluated. Infarct size was significantly reduced in ENTPD-1 versus WT hearts in both ex vivo and in vivo studies. Following ischemia-reperfusion injury, ENTPD-1 cardiac tissues demonstrated an increase in the phosphorylation of the cellular signaling molecule extracellular signal-regulated kinases 1/2 (ERK 1/2) and glycogen synthase kinase-3β (GSK-3β). Resistance to myocardial injury was abrogated by treatment with a non-selective adenosine receptor antagonist, 8-SPT or the more selective A_{2B} adenosine receptor antagonist, MRS 1754, but not the A_1 selective antagonists, DPCPX. Additionally, treatment with the ERK 1/2 inhibitor PD98059 or the mitochondrial permeability transition pore opener, atractyloside, abrogated the cardiac protection provided by ENTPDase-1 expression. These results suggest that transgenic ENTPDase-1 expression preferentially conveys myocardial protection from ischemic injury via adenosine A_{2B}

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Disclosures

None

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receptor engagement and associated phosphorylation of the cellular protective signaling molecules, Akt, ERK 1/2 and GSK-β that prevents detrimental opening of the mitochondrial permeability transition pore.

Keywords

ectonucleoside triphosphate diphosphohydrolase 1; CD39; myocardial ischemia; reperfusion; adenosine receptor; ERK 1/2; GSK-3β

1. Introduction

Myocardial infarction (MI), due to acute coronary artery thrombosis causing ischemia and subsequent myocardial cell death, is the leading cause of morbidity and mortality in the Western world [1]. While early restoration of coronary blood flow is imperative to restore oxygen and nutrient delivery to the ischemic tissue, reperfusion contributes to cardiomyocyte injury and a local inflammatory response which leads to further myocardial damage and end organ injury [2].

The extracellular purinergic regulatory pathway is uniquely positioned to modulate myocardial ischemia-reperfusion injury. The significance of this purinergic pathway in cardiovascular disease has been demonstrated by recent human studies demonstrating that mutations in ecto-5'-nucleotidase is associated with the development of symptomatic peripheral vascular disease [3]. Extracellular purinergic modulation is achieved by ectonucleoside triphosphate diphosphohydrolase 1 (ENTPD-1; CD39) mediated hydrolysis of adenosine triphosphate (ATP) or adenosine diphosphate (ADP) released from damaged or activated cells to adenosine monophosphate (AMP) which subsequently is converted by ecto-5'-nucleotidase (CD73) to the anti-inflammatory and cardiac protective molecule, adenosine [4–6].

While innate mechanisms such as ischemic preconditioning and postconditioning exist that modulate cellular susceptibility to ischemia [7], few intrinsic protective pathways have the ability to directly affect both thrombosis and cellular protection. Ectonucleoside triphosphate diphosphohydrolase-1 is one such regulatory mediator that is pivotally positioned to potentially affect both thrombosis and susceptibility to myocardial ischemia-reperfusion injury by promoting the depletion of ADP, a dominant platelet agonist, and the formation of adenosine, a cardiovascular protective molecule [8]. Given several lines of investigation highlighting the role of purinergic pathways in regulating the innate and vascular thrombotic responses to ischemic injury [8–15], we tested the hypothesis that the level of ENTPDase-1 may modulate myocardial susceptibility to ischemia-reperfusion injury. Here we demonstrate that over expression of human ENTPDase-1 (hENTPDase-1) in mice conveys marked myocardial protection from ischemic injury mediated, via adenosine A_{2B} receptor engagement and phosphorylation of the cellular protective signaling molecules, Akt, ERK 1/2 and GSK-3β that prevents detrimental opening of the mitochondrial permeability transition pore.

2. Methods

2.1 Transgenic Mice

The generation of the hENTPDase-1 expressing mice has been described previously [8]. The hENTPD-1 (CD39) transgene in these mice is regulated by the mouse $H\text{-}2K^b$ promoter. hENTPD-1 mice were backcrossed for more than 10 generations onto the C57BL6 background. Transgenic animals and wild-type littermates were compared. The

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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.

2.2 Chemicals

The following antagonists were employed in the studies reported: sodium polyoxotungstate (POM-1) an inhibitor of CD39 activity (3 mg/kg, intravenous, 10 minutes prior to ischemia; Tocris; Ellisville, MI); 8-(p-Sulfophenyl) theophylline (8-SPT; 20 mg/kg intraperitoneal, 15 minutes prior to ischemia) a non-selective antagonist for adenosine receptors A_1, A_{2A}, A_{2B} and A_3 (Sigma-Aldrich; St. Louis, MO); 8-Cyclopentyl-1,3-dipropylxanthine (DPCPX; 1 mg/kg intraperitoneal, 15 minutes prior to ischemia), an A_1 adenosine receptor antagonist; N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide (MRS 1754; 1 mg/kg intraperitoneal, 15 minutes prior to ischemia), an A_{2B} receptor antagonist (Sigma-Aldrich; St. Louis, MO); 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059; 100 micromol/L, 10 minutes prior to ischemia and initial 10 minutes of reperfusion), an ERK 1/2 inhibitor (Sigma-Aldrich; St. Louis, MO). Atractyloside, a mitochondrial permeability transition pore opener (Sigma-Aldrich; St. Louis, MO), Adenosine (HPLC grade #01890; Sigma-Aldrich; St. Louis, MO).

2.3 *In vivo* myocardial ischemia-reperfusion

The *in vivo* myocardial ischemia-reperfusion mouse model was performed using previously described techniques [16]. Mice were anesthetized with ketamine (55 mg/kg) plus xylazine (15 mg/kg). Atropine (0.05 mg SC) was administered to reduce airway secretions. Animals were intubated and ventilated with room air (tidal volume 250 μl, 120 breath/min) with a mouse respirator (Harvard Apparatus, Holliston, MA). Rectal temperatures were maintained at 37°C by a thermo-regulated heating pad. Adequacy of anesthesia was assessed by foot pad stimulation. Following thoracotomy, an 7–0 silk suture was placed around the left coronary artery for ligation. After 60 minutes of ischemia the occlusion was released and reperfusion was confirmed visually. At 24 hours of reperfusion the mice were reanesthetized, intubated and ventilated as outlined above. The chest was reopened along the previous incision line to expose the heart and the left main coronary artery was re-ligated in the same location as before. The heart was excised and the aorta cannulated. Three ml of 10% Phthalo Blue (Heubach Inc.) was slowly injected directly into the aorta to stain the heart for delineation of the ischemic zone from the nonischemic zone. The area of the myocardium that does not stain with Phthalo Blue is defined as the area-at-risk (AAR). Serial, short axis 1-mm-thick sections were cut and incubated in 1.0% 2,3,5-triphenyltetrazolium chloride (TTC) for 15 minutes at 37°C for demarcation of the viable and nonviable myocardium within the AAR (After TTC staining, the area of infarction was pale, whereas the viable myocardium was red). Each of the 1-mm-thick myocardial slices was placed in 10% formalin overnight then weighed and the areas of infarction, AAR, and nonischemic left ventricle assessed. To calculate infarct size/weight, both sides of each of the myocardial slices were photographed with a high resolution digital camera, and contoured with MetaVue software to delineate the borders of the left ventricle, the nonischemic area, and the infarcted area. The sizes of the nonischemic area, AAR, and infarct size area (IS) were calculated as percentages of the total left ventricle (LV) area multiplied by the total weight of that slice.

2.4 *Ex vivo* myocardial ischemia-reperfusion injury

Hearts isolated from ENTPDase-1 expressing mice or wild-type (WT) littermate controls were perfused using a Langendorff system and subjected to global ischemia of 30 minutes and reperfusion of 60 minutes as described previously [16]. At the end of reperfusion, the heart was processed for subsequent analysis of myocardial infarct size as described. *Ex vivo*

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hearts were trimmed of all atria and fat while still cannulated, and perfused with 2 ml 1.5% TTC at 37°C. Hearts were frozen, sectioned in 1mm sections, fixed in 10% formalin, and weighed. To calculate infarct size/weight, both sides of each section were digitally photographed and contoured with MetaVue software to delineate ischemic (red), and infarct (white) tissue. Infarcts were reported as a percentage of total left ventricular (LV) area multiplied by the total weight of that section. For signal molecule assessment, after 30 minutes of ischemia and 15 minutes of reperfusion, the hearts were snap frozen in liquid nitrogen using precooled aluminum blocks then processed as described under "Immunoblot analysis."

2.5 ENTPDase-1 activity assay

Total myocardial lysate was diluted in assay buffer (8 mmol/L $CaCl_2$/5 mmol/L tetramisole/ 50 mmol/L Tris-Base/50 mM imidazole/150 mmol/L NaCl, pH 7.5). After addition of 0.6 mmol/L final concentration of ADP and incubation at 36°C for 20 minutes the reaction was terminated by the addition of 7.5% molybate/malachite green hydrochloride in sulfuric acid/ 11% Tween). After 20 minutes, the absorbance at 620 nm was measured using a SpectraMaxplus plate reader (Molecular Devices, Sunnyvale, CA). One unit of ATPDase activity corresponds to the release of 1 μmol P_i/min at 37°C [8].

2.6 Immunoblot analysis

Hearts were homogenized in buffer containing 50 mmol/L Tris (pH7.4), 150 mmol/L NaCl, 0.5% NP-40, 1 mM sodium pyrophosphate, 5 mmol/L sodium vanadate, 1 mmol/L benzamidine, and 1 mmol/L sodium fluoride with protease inhibitor cocktail (all chemicals from Sigma Alrich, St. Louis, MO) for 10 sec × 3 cycles. After 30 min of protein solubulization, samples were centrifuged at 17,000 × *g* for 10 min and 50% glycerol was added to each supernatant to a final concentration of 10%. Supernatants were vortexed, aliquoted, and frozen at −80°C. All protein processing was carried out at 4°C. Equal amounts of protein, verified with Coomassie staining, were loaded into BioRad SDS-PAGE gels and electrophoresed. After transfer onto nitrocellulose, membranes were washed in 0.05% Tween in Tris buffered saline (TBS; pH 7.5), blocked in 5% milk, and probed with primary antibody (Total Akt; 1:5000, Cell Signaling # 9792 rabbit polyclonal; Akt-P serine 473, 1:1000, Cell Signaling # 4060 rabbit monclonal; p44/42 MAP Kinase (Erk 1/2):1:1000, Cell Signaling #4695 monoclonal rabbit; phosphor-p44/42 MAP Kinase (Thr202/Tyr204) Erk1/2: 1:1000, Cell signaling #9101, polyclonal rabbit; GSK-3β: 1:1000, Cell Signaling #9315 Rabbit monoclonal antibody; phospho-GSK-3β (Ser9): 1:1000, Cell Signaling #9322 Rabbit monoclonal antibody; hCD39: 1: 1000, Ancell #188-020, monoclonal mouse; mCD39 1:1000, eBioscience #24–6392, polyclonal rabbit). Membranes were washed in 0.05% Tween in TBS, incubated in the appropriate secondary antibodies conjugated to horse radish peroxidase (KPL), and Supersignal (Pierce) used to visualize proteins. The blots were imaged and quantified in a BioRad ChemiDoc utilizing Quantity One software.

2.7 Inflammatory cell accumulation

Hearts, trimmed of all atria and fat, were rinsed with PBS and placed in 4% paraformaldehyde in 0.1 M PBS (pH 7.4) overnight. Hearts were transferred to 0.2 M phosphate buffer (pH 7.4) for 2 hours, placed in 30% sucrose for 1 week, embedded in OCT, and sectioned at 5 microns. Frozen sections were dried for 2 hours at 37°C, rinsed with 0.2 M phosphate buffer (pH 7.4), blocked with 5% rabbit serum for 60 min, and incubated with Ly-6G primary antibody overnight at 4 °C in a humid chamber (Rat anti-mouse monoclonal Caltag # RM3000, clone RB6-8C5). After rinsing with 0.5 M Tris Buffer (pH 7.6), slides were incubated with biotinylated rabbit anti-rat secondary (Vector Labs, # BA-4001) for 2 hours at RT in a humid chamber, quenched with 6% hydrogen peroxide in methanol for 15 min, and detected with Elite-ABC Reagent (Vector Labs # PK-6100). After brief

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counterstaining with 0.01% cresyl violet, slides were dehydrated through graded alcohol solutions, cleared with Histoclear (National Diagnostics), and coverslipped with Permount. Leukocytes were quantified within 200 um^2 grids.

2.8 Adenosine measurement

Whole hearts were flash frozen in liquid nitrogen and stored in liquid nitrogen. Whole heart samples are fragmented under liquid nitrogen with a ceramic mortar and pestal in HPLC homogenizing buffer (0.4 M perchloric acid with 1 mM EDTA) then neutralized with 0.4M $K_2H_2PO_4$. An aliquot was removed for subsequent protein content determination using Lowry method. The remaining homogenate was centrifuged at 16,000 × *g* for 5 minutes at 4° C. The supernatant was removed and recentrifuged through in 0.5 micron nylon filter (Millipore) at 16,000 × *g* for 5 minutes at 4° C degrees. The processed sample was loaded into HPLC tubes and separated using a Supelcosil LC-18-T at 15 cm × 4.6 cm ID 3um column and detected with 100mM KH_2PO_4 (Sigma P5379 pH 5.5) for Buffer A and Buffer B consisting of 70 Buffer A:30 HPLC grade methanol (pH 5.5). Both Buffer A and Buffer B were filtered prior to use with 0.22 micron nylon filter and sonicated to degased for 15 minutes. Program gradient ramping from 0% Buffer B to 100% Buffer B. Standard known adenosine samples (Sima-Fluka 01890) were processed in the same manner to generate a standard curve based upon interpolation of the area-under the curve. From this standard curve the concentration of adenosine in each sample was determined then normalized to the sample protein concentration. The values are reported in nmol/mg protein.

2.9 Statistical Analysis

The results of experiments were analyzed by several statistical methods (e.g. paired or unpaired t-tests, analysis of variance, chi-squared analysis, curve fitting functions, etc. using standard software (e.g. GraphPad Prism, version 4.0). Results were expressed as mean ± standard error of the mean. For comparison between 2 groups, significance was determined by unpaired Student *t* test. For comparison of multiple groups, multifactorial ANOVA with post-hoc comparison of the means with Bonferroni correction was used to determine statistical significance. For all evaluations, probability values below 0.05 were considered significant.

3. Results

3.1 hENTPDase-1 expression decreases *in vivo* acute myocardial infarction

Myocardial tissue from hENTPDase-1 expressing animals expressed hENTPD-1 (Figure 1A). To test the hypothesis that hENTPDase-1 expression attenuates acute myocardial injury *in vivo,* myocardial infarct size following 60 minutes of ischemia and 24 hours of reperfusion in WT or hENTPDase-1 expressing mice was assessed. In mice expressing hENTPDase-1 myocardial infarct size was significantly reduced when compared to WT mice (WT: 55.4±3.7% vs. hENTPD-1: 11.5±2.0 %; $P<0.001$; Figure 1C and D). In addition, pretreatment of animals with the ENTPDase-1 inhibitor, sodium polyoxotungstate (POM-1) [17] abrogated the protection conveyed by hENTPDase-1 expression (POM-1; WT: 53.6±5.0% vs. hENTPD-1 expressing: 58.8±5.3%; $P>0.05$) demonstrating that the observed myocardial protection was due to hENTPDase-1 activity and not secondary to compensatory changes. Furthermore, post-infarction leukocyte infiltration was significantly reduced within the infarct border zone in hENTPDase-1 expressing hearts compared to WT hearts at 24 hours of reperfusion (WT: 99.2±4.3 cells per 200 μm^2 vs. hENTPD-1: 28.8±1 cells per 200 μm^2; $P<0.0001$). In total, comparing WT to hENTPDase-1 mice, there was no difference in the age (129.6±6.8 days vs. 140.6±8.61 days; $P>0.05$), body weight (27.2±0.87 grams vs. 29.2±0.92 grams; $P>0.05$), heart weight (69.5±3.93 milligrams vs. 75.5±3.77 milligrams; $P>0.05$), heart weight (mg) to body weight (g) ratio (2.6±0.16 vs. 2.6±0.10; $P>0.05$) or the

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area-at-risk of infarction (AAR) (64.7±1.99 vs. 67.5±1.75; $P>0.05$), heart rate prior during or following ischemia (30 min pre: 236.0±78.1 vs. 242.4±65.1 bpm, $P>0.05$; 30 min ischemia: 246.4±78.8 vs. 228.9±49.3 bpm, $P>0.05$; 30 min reperfusion: 255.6±64.6 vs. 276.2±91.7 bpm, $P>0.05$) or temperature (30 min pre: 36.1±1.18 vs. 36.3 ±1.28, $P>0.05$; 30 min ischemia: 36.4±1.05 vs. 36.8±0.95, $P>0.05$; 30 min reperfusion: 36.0±0.92 vs. 36.8±0.85, $P>0.05$) between groups or treatments. Together, these data support that hENTPDase-1 expression attenuates myocardial injury and reduces the subsequent inflammatory cell infiltration.

3.2 Myocardial expressed ENTPDase-1 mediated cardiac protection

To begin to examine the cellular expression required to mediate cardiac protection in hENTPDase-1 expressing animals, an *ex vivo* model of global myocardial ischemia-reperfusion injury was employed. Comparable to the *in vivo* results, isolated hearts from hENTPDase-1 expressing animals demonstrated a significant reduction in myocardial infarct size following global ischemia-reperfusion injury (WT: 57.5±4.9% vs. hENTPD-1: 21.3±2.3%; $P<0.001$; Figure 2A). These data support that hENTPDase-1 expression in the heart (myocardium, endothelium, vascular smooth muscle) is capable of attenuating ischemia-reperfusion-induced myocardial injury. There was an initial significant recovery in the rate*pressure product (RPP) at 15 minutes of reperfusion with a continued trend toward maintainence of improved ventricular recovery in ENTPD-1 hearts compared to WT hearts (Figure 2B; Baseline: WT:32,469±5,865 *vs*. ENTPD-1: 39,332±8,254 bpm*mmHg; $P=0.83$; 15 min Reperfusion: WT:10,065±3,078 *vs*. 27,086±5,45 bpm*mmHg; $P=0.03$; 30 minutes Reperfusion: WT:15,244.±2,303 *vs*. 31,544±9,969 bpm*mmHg; $P=0.12$; 60 minutes Reperfusion: WT:14,073±1,204 *vs*. ENTPD-1:22,084±6 010 bpm*mmHg; $P=0.19$). Furthermore, the left ventricular end-diastolic pressure was significantly lower in the reperfusion phase in ENTPD-1 hearts compared to WT hearts (Figure 2C; Baseline: 8.95±1.59 *vs*. 7.03±2.0 mmHg: $P>0.05$; 15 min Reperfusion: 29.40±4.18 *vs*. 17.76±2.25 mmHg; $P=0.03$; 30 minutes Reperfusion: 25.74±4.04 *vs*. 14.38±1.71 mmHg; P=0.04; 60 minutes Reperfusion: 25.00±4.18 *vs*. 14.01±1.29 mmHg; $P=0.046$). Analysis ENTPDase-1 activity revealed a 1.6-fold increase in baseline activity in isolated perfused ENTPD-1 hearts compared to WT hearts (WT: 11.15±0.97 vs. ENTP-1:17.55±1.19 mU/mg protein; $P=0.006$). Following 30 minutes of ischemia and 15 minutes of reperfusion, the activityof ENTPDase-1 activity decreased to 45% of the baseline value in WT hearts, while in ENTPD-1 hearts, the activityreduced to 71% of the baseline activity (WT: 4.97±1.33 vs. ENTP-1:12.50±0.99 mU/mg protein; $P=0.008$). Thus, the ENTPD-1 activity is maintained at a higher level in ENTPD-1 hearts following ischemia-reperfusion injury (Figure 2D). Measurement of whole heart adenosine levels demonstrated that while comparable levels of adenosine were observed in WT and hENTPD-1 hearts at baseline (Baseline: WT: 5.91± 1.25 nmol/mg protein vs. hENTPD-1: 6.73±1.20 nmol/mg protein; $P>0.05$; Figure 2E), following myocardial ischemia-reperfusion injury, a significant increase in adenosine levels in ENTPD-1 hearts compared to WT hearts, consistent with the interpretation that adenosine production is involved in facilitating the observed cardiac protection in ENTPD-1 mice (Post-ischemia-reperfusion: WT: 1.99±0.36 nmol/mg protein vs. hENTPD-1: 14.59±3.70 nmol/mg protein $P<0.05$; Figure 2D). Together, these data support that hENTPDase-1 expression attenuates myocardial injury and increase adenosine levels in the post-ischemia-reperfused myocardium.

3.3 hENTPDase-1-mediated attenuation of *in vivo* myocardial injury signals through adenosine receptors

Given the pivotal role of ENTPDase-1 in regulating the purinergic pathway and purinergic signaling we asked whether it is via indirectly driving the generation of adenosine that ENTPDase-1 confers protective efficacy [4–6, 8–15, 17]. Treatment with the non-selective

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adenosine receptor antagonist 8-(p-Sulfophenyl) theophylline (8-SPT) abrogated the decrease in infarct size observed in hENTPDase-1 expressing mice (WT: 48.5±6.1% vs. hENTPD-1: 52.4±5.6%; $P>0.05$; Figure 3), supporting the contention that hENTPDase-1 mediated myocardial protection involves adenosine receptor engagement.

To investigate the role of specific adenosine receptors in hENTPDase-1-mediated infarct size reduction, we initially focused on the effect of the A_{2B} receptor antagonism. Treatment with an A_{2B} receptor antagonist, N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide (MRS 1754), completely abolished the *in vivo* reduction of myocardial infarct size conveyed by hENTPDase-1 expression (WT: 48.4±5.7% vs. hENTPD-1: 54.8±5.1%; $P>0.05$; Figure 3). However, given data suggesting that in mouse, MRS 1754 may also antagonize the adenosine A_1 receptor [18], the A_1 adenosine receptor antagonist 8-cyclopentyl-1,3-dipropylxanthine (DHCPX) also was examined and surprisingly was found to have no effect on the reduction of infarct size conveyed by hENTPDase-1 expression (WT: 55.0±0.04% vs. hENTPD-1: 18.7±0.7%; $P<0.001$; Figure 3). Thus, these data suggest that ENTPDase-1-mediated cardiac protection appears to preferentially involve adenosine A_{2B} receptor occupancy.

3.4 hENTPDase-1-mediates myocardial protection through Akt, ERK 1/2, GSK-3β phosphorylation

To begin to decipher the signaling cascade involved in hENTPDase-1-mediated resistance to ischemic injury, an *ex vivo* model of global ischemia-reperfusion injury was again employed. Analysis of the total level and phosphorylation level of the pivotal reperfusion induced salvage kinase pathway members, Akt, extracellular signal-regulated kinase 1/2 (ERK 1/2) and GSK-3β was conducted. The analysis revealed no difference in the levels of total or phosphorylated Akt at baseline (Figure 4A–C) or of ERK 1/2 (p42 or p44) at baseline (Figure 4D–G), consistent with lack of preactivation of these protective signaling molecules. However, in response to myocardial ischemia-reperfusion injury, ENTPDase-1 expressing hearts demonstrated a significant increase in the level of Akt (Figure 4A–C) and ERK 1/2 phosphorylation (Figure 4D–G). Similar analysis of the total level and phosphorylation levels of GSK-3β revealed no difference in the levels of total or phosphorylated GSK-3β at baseline (Figure 4H–J). However, in response to myocardial ischemia-reperfusion injury, ENTPDase-1 expressing hearts demonstrated a significant increase in the level of GSK-3β phosphorylation (Figure 4H–J). Furthermore, treatment of WT and hENTPDase-1-expressing hearts with the MEK1/2 inhibitor 2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one (PD98059) negated the myocardial protection conveyed by hENTPDase-1 expression (WT: 50.1±4.1% vs. hENTPD-1: 50.9±2.1%; $P>0.05$). These data support that hENTPDase-1 expression conveys cardiac protection via protective signaling through the RISK signaling pathway.

3.5 Mitochondrial permeability transition pore opening negates the protective efficacy of hENTPDase-1

To determine if the signaling pathways activated converged on the mitochondrial permeability transition pore to mediate the protection observed, mice were treated with the mPTP opener, atractyloside (0.1 mg/kg) 15 minutes prior to *in vivo* myocardial ischemia-reperfusion injury. While treatment with atractyloside demonstrated a high mortality (~50%), in the surviving animals, the protection from myocardial injury observed in ENTPD-1 mice was negated (Figure 5)

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4. Discussion

Here we have evaluated the mechanism by which transgenic ENTPDase-1 over expression conveys myocardial protection. The work presented demonstrates the pivotal role of purinergic regulation by ENTPDase-1 as an innate modulator of myocardial susceptibility to ischemia-reperfusion injury. Via modulation of the purinergic axis, ENTPDase-1 expression decreases myocardial injury through adenosine A_{2B} receptor engagement and associated signaling through the RISK pathway.

Extracellular adenosine can signal via any of 4 extracellular adenosine receptors (A_1, A_{2A}, A_{2B}, or A_3) [6, 19]. While all four receptors are expressed in cardiac tissues and have been associated with protection from ischemia-reperfusion injury, the understanding of the contribution of individual receptors to myocardial protection remains complex [6]. Prior data has demonstrated that the A_1 receptor is involved in cardioprotection mediated by IPC or adenosine receptor agonists [20, 21], surprisingly our data reveal that in the presence of increased expression of ENTPDase-1, A_1 antagonism does not inhibit the observed cardiac protection. Indeed, our data support that A_{2B} adenosine receptor antagonism abrogates hENTPDase-1-mediated myocardial protection, suggesting that ENTPDase-1/A_{2B}AR purinergic signaling may represent a "linked" innate cellular protective mechanism. The authors recognize that the use of pharmacologic interrogation of the complexities of adenosine signaling may not be completely selective. Recent cloning and characterization of the A_{2B} adenosine receptors from mouse, rabbit, and dog revealed that significant differences in antagonist pharmacology of the A_{2B}AR exist between species [18]. Furthermore, signaling through the A_{2B}AR is complex; it is reported to involve Gi, G_s and $G_{q/11}$ signaling mechanisms [22–24]. While the A_{2B}AR has a lower affinity for adenosine, modification of the A_{2B}AR receptor by protein kinase C results in the ability of endogenous adenosine levels to activate A_{2B}AR-dependent signaling [25]. Indeed, it has been proposed that activation of protein kinase G (PKG) at reperfusion protects the ischemic-reperfused heart by lowering the threshold for adenosine to initiate signaling from low-affinity A_{2B} receptors during early reperfusion thus allowing endogenous adenosine to activate survival kinases phosphatidylinositol 3-kinase (PI3K) and ERK [26]. In rabbit and rat, treatment with the A_{2B}AR antagonist MRS-1754 blocks ischemic postconditioning (brief ischemic periods following a prolonged ischemic insult) [25, 27, 28]. In wild-type mice, but not A_{2B} adenosine receptor knockout mice, treatment with an A_{2B} adenosine receptor agonist attenuates infarct size following 60 minutes of *in vivo* myocardial ischemia [12]. Similarly, treatment with an A_{2B}AR agonist also demonstrates cardiac protective efficacy in mouse and rabbit models of myocardial ischemia [12, 25]. However, the influence of the A_{2B}AR occupation on ischemic preconditioning (brief episodes of ischemia prior to a prolonged episode of ischemia; IPC) is contradictory. In one study it was concluded that the A_{2B}AR is not required for the acute phase of cardioprotection induced by ischemic preconditioning [29], while another study reported that knockout of the A_{2B}AR prevents IPC-mediated cardiac protection [12]. Stimulation of adenosine A_{2B} receptors appears to block apoptosis in the non-infarcted myocardium even when administered after the onset of infarction [30]. The cellular expression of the A_{2B}AR in the heart is controversial. Several studies have reported mRNA and protein in cardiac tissue and isolated cardiomyocytes [12, 31–34]. More recently, it has been suggested that A_{2B}AR expression in cardiomyocytes localizes in or near mitochondria and A_{2B}AR activation suppresses mitochondrial transition pores in isolated cardiomyocytes [35]. While A_{2B}AR expression on cardiac fibroblasts and endothelial cells has been demonstrated, further confirmation of the expression data on cardiomyocytes is required. Regardless, following an IPC stimulus or exposure to hypoxia, there is selective induction of not only A_{2B} adenosine receptor transcript, but also ENTPDase-1 activity and transcript levels in cardiac tissue [12, 36]. In fact, ENTPDase-1, ecto-5'-nucleotidase, A_1AR and A_{2B}AR also are all found in caveolae [37–42], suggesting that ENTPDase-1/A_{2B}AR

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purinergic signaling may represent a “linked” innate cellular protective mechanism, with all components critical to conveying cardiac protection. Thus, preconditioning stimuli could be used in the clinical arena to induce ENDTPase-1 expression in high risk patients at risk for cardiovascular events.

Several innate myocardial protective mechanisms involve signaling through phosphorylation of RISK pathway components, including Akt, extracellular signal-regulated kinase 1/2 (ERK 1/2) and inactivation of GSK-3β [43–45]. *In vivo* adenosine receptor preconditioning reduces myocardial infarct size via subcellular ERK signaling [43]; blocking ERK phosphorylation abolishes protection from either IPC [27] or A_{2B}AR agonist-mediated cardiac protection [46]. We have demonstrated that increased expression of the innate purinergic pathway modulator ENTPDase-1 leads to increased ERK phosphorylation following ischemia-reperfusion injury that is associated with a reduction in myocardial infarct size when compared to WT hearts. Furthermore, treatment with the MEK 1/2 inhibitor, PD 98059, negates ENTPDase-1-mediated cardiac protection. These data are consistent with ERK 1/2 phosphorylation comprising a key component of the intracellular signaling cascade required for ENTPDase-1-mediated cardiac protection. Indeed, prior work has demonstrated that all adenosine receptors can mediate signaling to the ERK 1/2 [24]. Consistent with our findings is the report that treatment with the A_{2B}AR agonist BAY 60–6583 increases ERK 1/2 phosphorylation and protects the heart from ischemia-reperfusion injury [25]. ERK1/2 phosphorylation is a critical step located upstream from the phosphorylation of GSK-3β by members of the p90-ribosomal S6 kinase (RSK) family (reviewed) [47]. GSK-3β is constitutively active and is regulated by inhibitory phosphorylation. Phosphorylation of GSK-3β has been reported to be involved in IPC [48] and ischemic postconditioning [49], however the requirement for GSK-β inactivation in these innate protective processes has been questioned in more recent studies utilizing genetically altered mice [50]. Work has suggested that inactivation of GSK-3β prevents opening of the mitochondrial permeability transition pore [51] potentially due to action on the voltage-dependent anion chanel (VDAC)[52] and/or the adenine nucleotide translocase (ANT)[53] preventing calcium overload [54]. Indeed, treatment with the mPTP opener atractyloside negated the cardiac protection conveyed by ENTPDase-1 expression. Our data complements recent work demonstrating that A_{2B}AR signaling suppresses mitochondrial transition pore opening [35]. Future studies using both pharmacologic and genetic approaches are required to further delineate the signaling pathways that lead not only to Akt and ERK 1/2 activation, but also GSK-3β inactivation, as well as other upstream and downstream signaling events such as effects on the mPTP and mitochondrial function.

Finally, the authors recognize several limits to the work. The use of pharmacologic interrogation of the complexities of adenosine signaling may not be completely selective. Recent data suggests that DPCPX is also a good antagonist of the A_{2B}AR and the MRS1754 can also bind the A_1AR [18]. Furthermore, there appears to be cross-talk and interdependence in adenosine receptor signaling [55–57], therefore the complexity of the signaling cascade might be understated. Future work employing genetic ablation of the various adenosine receptors will shed further light onto the specificity of this pathway. Additionally, the measurement of adenosine levels provided is supportive of the hypothesis that ENTPDase-1 overexpression leads to increased adenosine production. However, these levels are from whole heart tissue, therefore the contribution of the extent of viable tissue on this measurement can not be ascertained. Future studies utilizing microdialysis techniques will more directly examine the timing and extent of adenosine production in this model.

In summary, the present study demonstrates that regulation of the purinergic system by transgenic ENTPDase-1 over expression conveys myocardial protection from ischemic injury via A_{2B} adenosine receptor engagement and activation of the cellular protective

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signaling molecules, Akt, ERK 1/2 and inactivation of GSK-3β. The protection conveyed by ENTPDase-1 expression is negated by treatment with the mPTP opener atractyloside. Future studies examining the influence of specific cellular expression of ENTPDase-1, as well as pharmacologic and genetic approaches to further decipher the involved signaling cascades, will broaden our understanding of the influence of this purinergic modulating pathway on a number of pathologic processes including thrombosis, ischemia-reperfusion injury and post-infarction inflammation and remodeling.

Highlights

- ENTPDase-1 overexpression protects the myocardium from ischemia-reperfusion injury
- ENTPDase-1 mediated cardiac protection involves signaling through the adenosine A_{2B} receptors.
- ENTPDase-1-mediated cardiac protection involves AKT and ERK1/2 activation.
- ENTPDase-1-mediated cardiac protection involves GSK-3β inactivation.

Abbreviations

ADP	adenosine diphosphate
AMP	adenosine monophophate
ATP	adenosine triphosphate
MI	myocardial infarction
ENTPD-1; CD39	ectonucleoside triphosphate diphosphohydrolase 1
Akt	serine/threonine protein kinase
ERK 1/2	extracellular signal-regulated kinase 1/2
GSK-3β	glycogen synthase kinase-3β
mPTP	Mitochondrial permeability transition pore
CD73	ecto-5'-nucleotidase
WT	wild-type
POM-1	sodium polyoxotungstate
8-SPT	8-(p-Sulfophenyl) theophylline
MRS 1754	N-(4-cyanophenyl)-2-[4-(2,3,6,7-tetrahydro-2,6-dioxo-1,3-dipropyl-1H-purin-8-yl)-phenoxy]acetamide
DPCPX	8-Cyclopentyl-1,3-dipropylxanthine
PD98059	2-(2-Amino-3-methoxyphenyl)-4*H*-1-benzopyran-4-one

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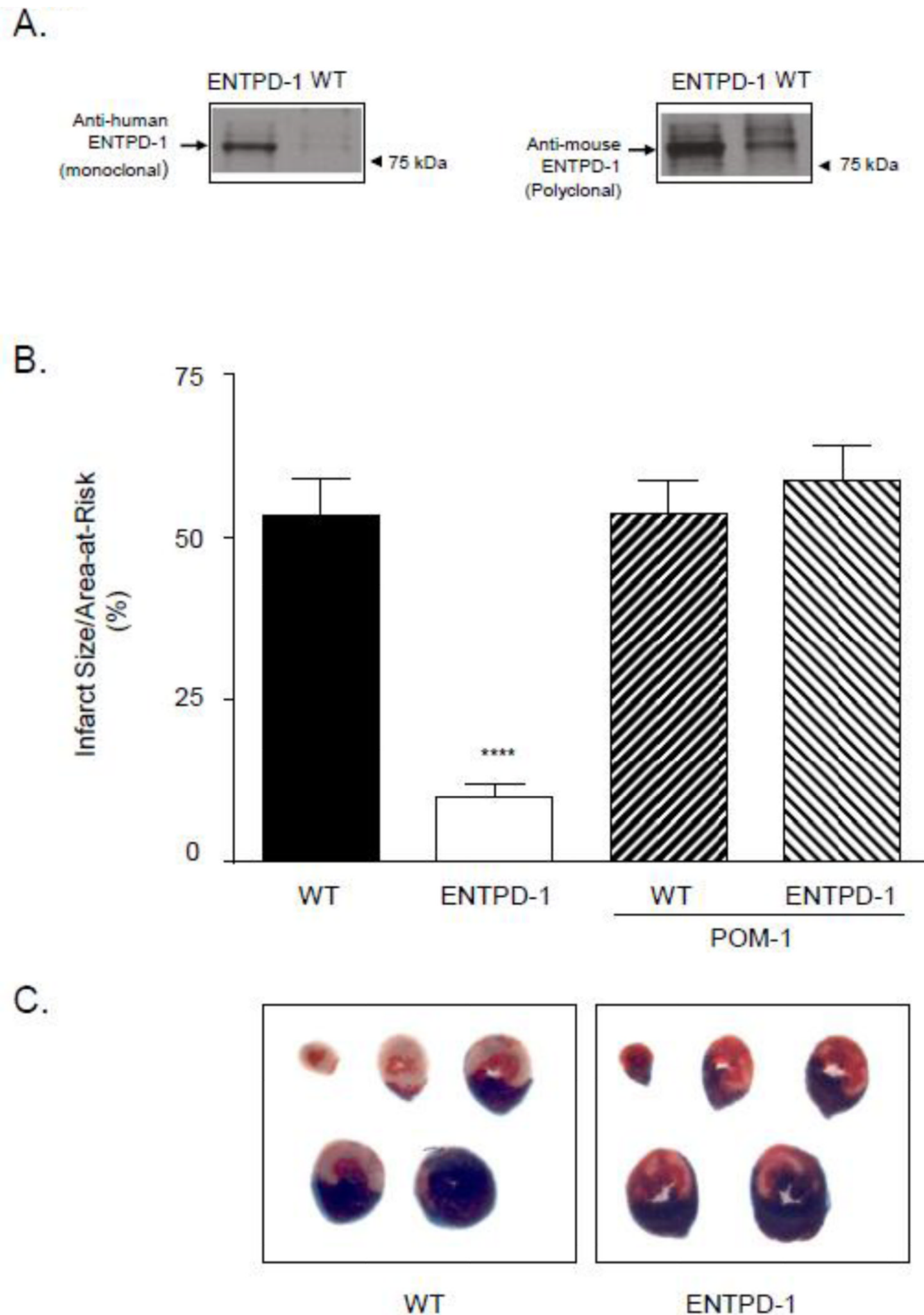

Figure 1.

Ectonucleoside triphosphate diphosphohydrolase-1 expression reduces myocardial infarct size *in vivo*. A, representative images of whole heart homogenates probed with either anti-human ENTPDase-1 monoclonal antibody or anti-mouse ENTPDase-1 polyclonal antibody. Anti-mouse ENTPDase-1 polyclonal antibody cross-reacts with human ENTPDase-1. B, Quantification of infarct size per area-at-risk following 60 minutes of *in vivo* left coronary artery ischemia and 24 hours of reperfusion in WT versus hENTPD-1 mice, untreated versus POM-1 treated (3 mg/kg i.v. 10 minutes prior to ischemia). N= 6 per group; Values are mean±SEM. C, Representative staining for myocardial infarct size following 60 minutes of in vivo left coronary artery ischemia and 24 hours of reperfusion in WT versus hENTPD-1

mice. N= 6 per group; Values are mean ± SEM. **, $P<0.05$; ****, $P<0.001$; WT, wild-type littermate control animals; ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

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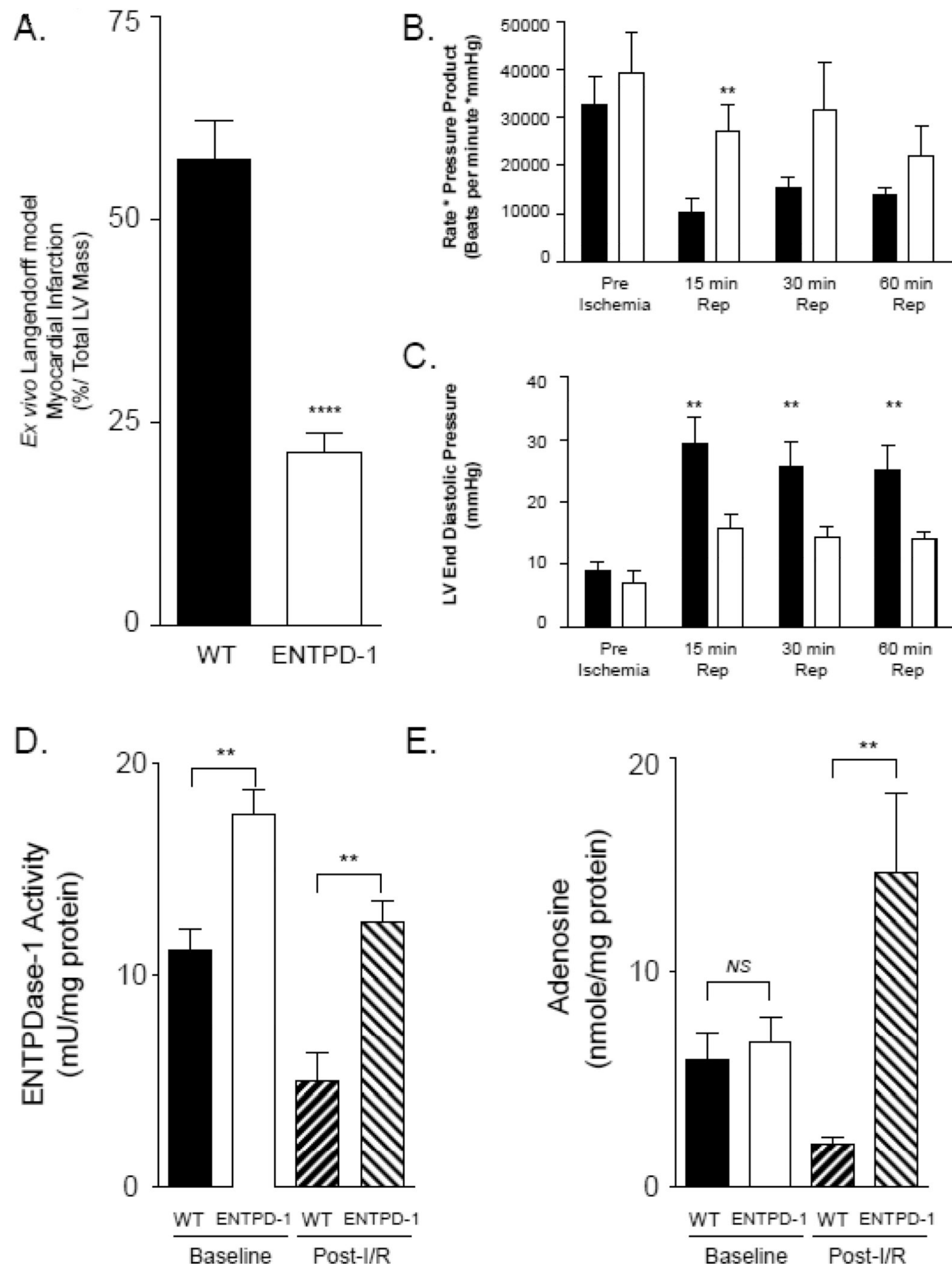

Figure 2.

ENTPDase-1 mediated cardiac protection *ex vivo*. A, Quantification of infarct size from hearts subjected to 30 minutes of *ex vivo* global ischemia and 60 hours of reperfusion WT versus hENTPD-1 mice Infarct size is expressed as a percent of the total left ventricular mass. B, Rate*Pressure product (heart rate * systolic pressure) of WT and ENTPD-1 hearts at baseline (WT:32,469±5,865 *vs*. ENTPD-1: 39,332±8,254 bpm*mmHg; *P*=0.83)and following reperfusion (15 min Reperfusion: WT:10,065±3,078 *vs*. 27,086±5,45 bpm*mmHg; *P*=0.03; 30 minutes reperfusion: WT:15,244.±2,303 *vs*. 31,544±9,969 bpm*mmHg; *P*=0.12; 60 minutes reperfusion: WT:14,073±1,204 *vs*. ENTPD-1:22,084±6 010 bpm*mmHg; *P*=0.19) C, Left ventricular end-diastolic pressure in WT and ENTPD-1

hearts at baseline (8.95±1.59 *vs*. 7.03±2.0: *P*>0.05)and following reperfusion (15 min Reperfusion 29.40±4.18 *vs*. 17.76±2.25; *P*=0.03; 30 minutes Reperfusion: 25.74±4.04 *vs*. 14.38±1.71; P=0.04; 60 minutes Reperfusion: 25.00±4.18 *vs*. 14.01±1.29; *P*=0.046) D, NTPDase activity in WT and ENTPD-1 hearts at baseline (WT: 11.15±0.97 *vs*. ENTP-1:17.55±1.19 mU/mg protein; *P*=0.006) and at 15 minutes following reperfusion (WT: 4.97±1.33 *vs*. ENTP-1:12.50±0.99 mU/mg protein; *P*=0.008). E, Whole heart adenosine levels in WT and ENTPD-1 hearts at baseline (WT: 5.91±1.25 nmol/mg protein *vs*. ENTPD-1: 6.73±1.20 nmol/mg protein; *P*>0.05) and at 15 minutes following reperfusion (WT: 1.99±0.36 nmol/mg protein *vs*. ENTPD-1: 14.59±3.70 nmol/mg protein *P*<0.05). N= 3–4 per group; Values are mean ± SEM. **, *P*<0.05; ****, *P*<0.001; WT, wild-type littermate control animals; ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

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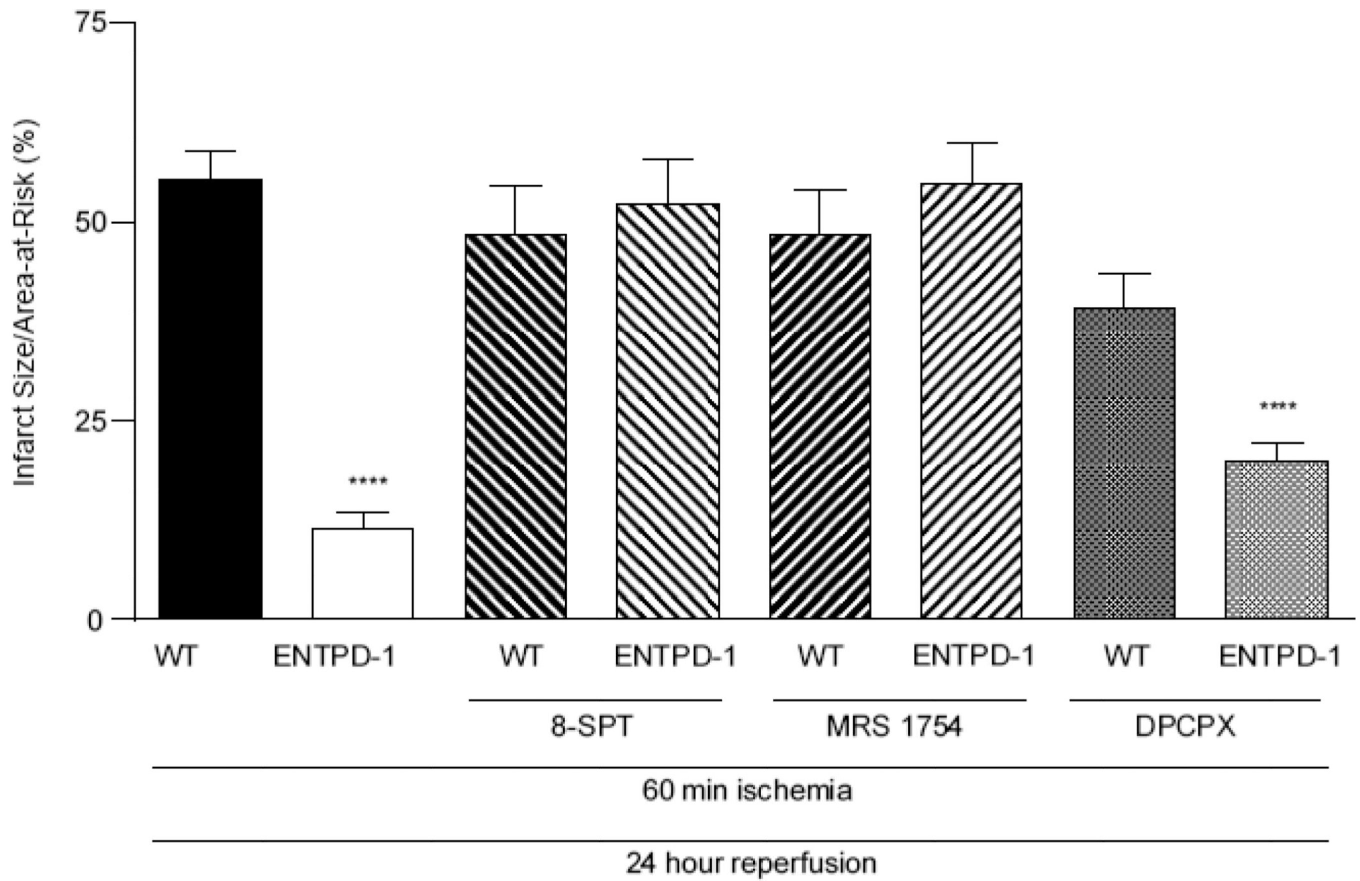

Figure 3.

Adenosine receptor engagement is required for *in vivo* ENTPDase-1-mediated cardiac protection. Quantification of *in vivo* infarct size following 60 minutes of left coronary artery ischemia and 24 hours of reperfusion in vehicle treated WT versus hENTPD-1 mice versus either 8-SPT- treated (20 mg/kg, i.p. 15 minutes prior to ischemia), MRS 1754 (1 mg/kg, i.p. 15 minutes prior to ischemia) or DPCPX (1 mg/kg, i.p. 15 minutes prior to ischemia) treated mice. Values are mean±SEM. ****, P<0.001; N= 3–6 per group; WT, wild-type littermate control animals; ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.

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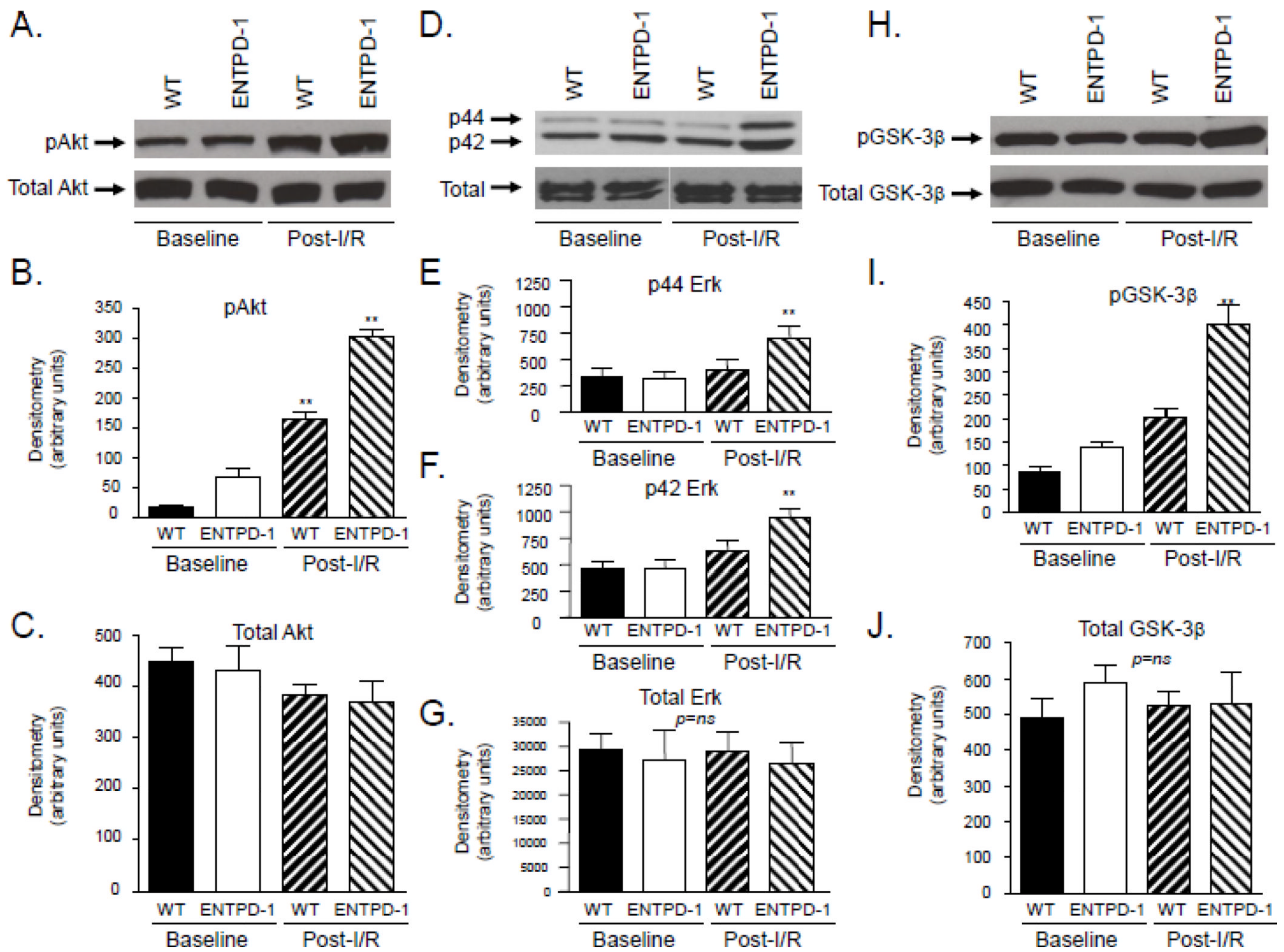

Figure 4.

ENTPDase-1 mediated cardiac protection involves Akt and ERK 1/2 activation and GSK-3β inctivation. Analysis of whole heart lysates from WT and ENTPDase-1 hearts at baseline compared to 30 minutes of *ex vivo* global ischemia and 15 minutes of reperfusion: A, Representative immunoblot analysis for total Akt and phosphorylated Akt. B. Quantification of phosphorylated Akt. C, Quantification of total Akt, D,Representative immunoblot analysis for total ERK 1/2 and phosphorylated ERK1/2. E. Quantification of total phosphorylated p44 Erk. F, Quantification of total phosphorylated p42 Erk. G, Quantification of total ERK 1/2. H, Representative immunoblot analysis for total GSK-3β and phosphorylated GSK-3β. I. Quantification of phosphorylated GSK-3β. J, Quantification of total GSK-3β. N= 4 per group; Values are mean ± SEM. **, $P<0.05$; WT, wild-type littermate control animals; ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals. No difference observed in GAPDH expression between genotype or treatment.

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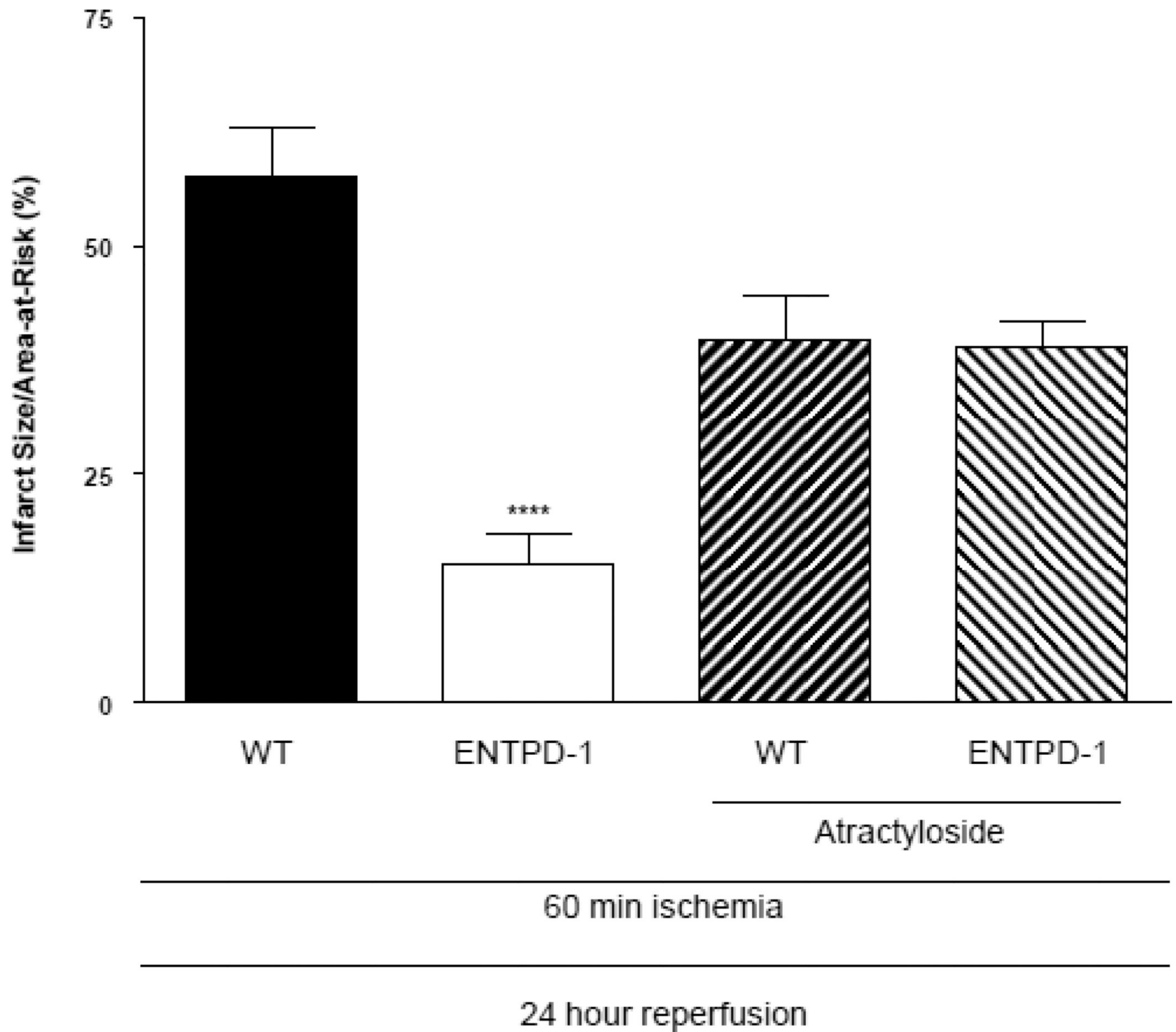

Figure 5.

Mitochodrial permeability transition pore opening negates ectonucleoside triphosphate diphosphohydrolase-1 mediated myocardial protection. Quantification of infarct size per area-at-risk following 60 minutes of *in vivo* left coronary artery ischemia and 24 hours of reperfusion in WT versus hENTPD-1 mice, untreated versus atractyloside treated (0.1 mg/kg i.p. 15 minutes prior to ischemia). N= 3–4 per group; (Untreated: WT: 57.6±5.5% *vs*.hENTPD-1: 15.2±3.4%; $P<0.05$; Atractyloside Treated: WT: 39.8±4.8% *vs*. hENTPD-1: 38.9±2.95; $P>0.05$). Values are mean±SEM. **, $P<0.05$; ****, $P<0.001$; WT, wild-type littermate control animals; ENTPD-1, ectonucleoside triphosphate diphosphohydrolase-1 expressing animals.