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Equilibrative nucleoside transporter 1 plays an essential role in cardioprotection

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Rose JB, Naydenova Z, Bang A, Eguchi M, Sweeney G, Choi DS, Hammond JR, Coe IR. Equilibrative nucleoside transporter 1 plays an essential role in cardioprotection. *Am J Physiol Heart Circ Physiol* 298: H771–H777, 2010. First published December 24, 2009; doi:10.1152/ajpheart.00711.2009.—To better understand the role of equilibrative nucleoside transporters (ENT) in purine nucleoside-dependent physiology of the cardiovascular system, we investigated whether the ENT1-null mouse heart was cardioprotected in response to ischemia (coronary occlusion for 30 min followed by reperfusion for 2 h). We observed that ENT1-null mouse hearts showed significantly less myocardial infarction compared with wild-type littermates. We confirmed that isolated wild-type adult mouse cardiomyocytes express predominantly ENT1, which is primarily responsible for purine nucleoside uptake in these cells. However, ENT1-null cardiomyocytes exhibit severely impaired nucleoside transport and lack ENT1 transcript and protein expression. Adenosine receptor expression profiles and expression levels of ENT2, ENT3, and ENT4 were similar in cardiomyocytes isolated from ENT1-null adult mice compared with cardiomyocytes isolated from wild-type littermates. Moreover, small interfering RNA knockdown of ENT1 in the cardiomyocyte cell line, HL-1, mimics findings in ENT1-null cardiomyocytes. Taken together, our data demonstrate that ENT1 plays an essential role in cardioprotection, most likely due to its effects in modulating purine nucleoside-dependent signaling and that the ENT1-null mouse is a powerful model system for the study of the role of ENTs in the physiology of the cardiomyocyte.

cardiovascular; hypoxia; adenosine; cardiomyocytes

THE PURINE NUCLEOSIDE ADENOSINE is an important signaling molecule in cardiovascular physiology. Adenosine acts as an antistress molecule and as a regulator of energy use in the cell (35). Adenosine is also required for ATP synthesis and nucleic acid formation, and initiates a cardioprotective response when there is an imbalance between oxygen demand and supply (22, 35, 36, 39). Adenosine and adenosine analogs have been used clinically for their antiarrhythmic effects for nearly 20 years (39) and, more recently, as preconditioning mimetic agents during open-heart surgery (23, 32). Extracellular adenosine can activate adenosine receptors (A_1, A_{2A}, A_{2B}, A_3), located on the cell surface of cardiomyocytes, to produce a variety of physiological responses (30), and levels of adenosine both inside and outside of cells are regulated by the presence and activity of nucleoside transporters (NTs) located in the cell membrane. To date, several mammalian NTs have been characterized and shown to be responsible for nucleoside and nucleobase transport across the cell membrane (19, 24, 33, 42, 50). Flux of adenosine across the cardiomyocyte cell membrane is mediated primarily via the equilibrative nucleoside transporter 1 (ENT1; SLC29A1), which is expressed at higher levels in heart tissue than the other three ENT isoforms (31, 40).

Despite the importance of adenosine in cardioprotection, very little is known about the role of ENTs in purine-dependent physiology in cardiomyocytes. We have previously used the murine cardiomyocyte cell line, HL-1, to demonstrate the importance and contribution of ENT1 and ENT2 to purine nucleoside-dependent responses in hypoxia, preconditioning, and cardioprotection (6, 9, 37). Both hypoxic and pharmacological preconditioning in HL-1 cells were found to involve ENT1, with contributions from ENT2, plus adenosine receptor activation (37, 44). Although HL-1 cells are a useful model for these types of studies (3, 27, 47), the role of ENT1 in cardioprotection in the whole animal has not been confirmed. Therefore, we were interested in extrapolating our findings from the isolated cultured cell model to a whole animal model to better understand the role of ENTs in the cardiovascular system.

Recently, a global ENT1 knockout mouse (ENT1 null) has been developed, which provides a valuable model to study the role of ENT1 in adenosine-dependent activities in the central nervous system (CNS) (7, 8) and in drug transport in erythrocytes (17). The ENT1-null mouse is phenotypically normal, with only a slight decrease (<10%) in body weight compared with that of wild-type littermates ($ENT1^{+/+}$). Thus the ENT1-null mouse model has furthered our understanding of the role ENT1 in adenosine physiology in the CNS and in drug transport. Therefore, based on our previous findings, we hypothesized that lack of ENT1 in this animal will lead to a cardioprotected phenotype in the heart. Consequently, we confirmed the ENT and adenosine receptor expression profiles plus the purine nucleoside transport characteristics of primary adult cardiomyocytes isolated from ENT1-null mice and wild-type littermates. We investigated whether ischemic challenge led to decreased damage in the ENT1-null heart compared with wild-type littermates and compared these findings with our previous observations in HL-1 cells. We also used small interfering RNA (siRNA) to knock down ENT1 in HL-1 cells to mimic the null transgenic mouse model. Taken together, our data clearly demonstrate that ENT1 is a key player in cardioprotection, most likely due to its role in mediating the effects of purine nucleoside-dependent signaling cardioprotection and that the ENT1-null mouse model is a powerful model system for the study of the role of ENTs in the physiology of the cardiomyocyte.

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METHODS

Animals. Genotyped female ENT1-null and ENT1 wild-type pups (8) were shipped after weaning from Mayo Clinic (Rochester, MN) to York University. All animal handling procedures were approved by the Animal Care Committee of York University, and animals were kept according to the Canadian Council on Animal Care guidelines. Littermates were housed in standard cages with chow and water available ad libitum. The animal room was maintained on a 12-h:12-h light/dark cycle. For all experiments, mice were between 2 and 4 mo of age. Primary cardiomyocytes were isolated from a different mouse for each experiment.

Isolation of primary adult mouse cardiomyocytes. Primary adult cardiomyocyte isolation was performed, and cells were used, on the same day since they cannot be maintained beyond 24 h (43) and do not proliferate. Briefly, anesthesia was administered, the murine aorta was cannulated, and the heart was perfused with isolation buffer containing (in mM) 120.4 NaCl, 4.7 KCl, 1.2 $MgSO_4$, 0.6 Na_2PO_4, 0.6 KH_2PO_4, 4.6 $NaHCO_3$, 5.6 glucose, 10 HEPES, 10 2,3-butanedione monoxime (BDM), and 32 taurine (pH 6.9), for 2.5 min, followed by a 10- to 12-min perfusion with an enzyme digestion buffer containing 12.5 μM $CaCl_2$ and 1 mg/ml Collagenase Type II (Worthington Biochemical Corporation, Lakewood, NJ). The heart was cut from the cannula just below the atria and transferred into stop buffer [isolation buffer containing 12.5 μM $CaCl_2$ and 5% bovine calf serum (BCS); Wisent], and the ventricles were gently teased into small pieces with forceps. Pipettes of decreasing size (1 to 0.5 mm diameter) were used to bring the tissue to a suspension. The Cellector Tissue Sieve filter system (Bellco Biotechnology, Vineland, NJ) removed any undigested tissue, and the filtrate was brought to a final volume of 10 ml in stop buffer. In a nonstick 100-mm plate the calcium concentration was adjusted using increasing volumes of 10 mM $CaCl_2$ to bring the final calcium concentration from 12.5 μM to 1 mM. Cells were transferred into a 15-ml tube and allowed to settle for 8 min. The supernatant was spun for 6 min (at 100 *g*), and both pellets were resuspended in MEM/Hanks' medium (GIBCO, Invitrogen, Carlsbad, CA) and supplemented with 2.5% BCS and 10 mM BDM. Cells were counted in suspension and optimally plated for 120,000–150,000 rod shaped cells per 60-mm tissue culture plates coated with 5 μg/ml laminin. Cells were then incubated at 2% CO_2 for 2 h to allow attachment of the rod-shaped cells. Typically, 1×10^6 cells are obtained per adult murine heart.

Coronary artery ligation and infarct size assessment. Myocardial ischemia-reperfusion was performed as previously described (46, 48). Briefly, the coronary artery was ligated using a curved needle and size 7-0 silk thread. Myocardial ischemia was induced by tightening the thread around a polyethylene (PE-10) tube and the coronary artery. Once ischemia (30 min) via coronary artery occlusion (confirmed by change in the color of the heart) was completed, the tube was removed to allow reperfusion of the heart. At 2 h postreperfusion, the animal was reanesthetized and the heart was immediately excised, washed in PBS, weighed, and sliced into four segments parallel to the atrioventricular groove (atria were removed and not stained). To assess infarct size, slices were incubated in 1.5% 2,3,5-triphenyltetrazolium chloride (TTC) PBS solution (37°C, 20 min). Tissue slices were immediately photographed (Nikon SLR D5000; Nikon Canada), and infarct size was measured using image analysis software (ImageJ, version 1.41; National Institutes of Health, Bethesda, MD). Infarct area was expressed as a percentage of total heart mass. Analysis of infarct size was conducted by an observer who was blinded to the genetic background of the mouse.

[^{3}H]nitrobenzylthioinosine binding. Nitrobenzylthioinosine (NBTI) is a high affinity (nM), tight-binding specific inhibitor of ENT1 (4) and can be used as an indicator of ENT1 protein at the plasma membrane as previously described (6). Briefly, cardiomyocytes (~100,000 cells/assay) in (in mM) 10 Tris·HCl, 100 KCl, 0.1 $MgCl_2 \cdot 6H_2O$, and 0.1 $CaCl_2 \cdot 2H_2O$ (pH 7.4) were incubated in the presence or absence of 10 μM NBTI, with [^{3}H]NBTI (Moravek, CA) ranging in concentration from 0.2 to 7.5 nM, in borosilicate glass tubes for 50 min at room temperature to attain steady-state binding (1 ml final volume). Reactions were terminated by filtration through Whatman GF-B filters under vacuum and washed twice with ~5 ml of ice-cold Tris · HCl buffer (10 mM; pH 7.4). Filters were then assessed for radioactive content by standard liquid scintillation counting. Nonspecific binding of [^{3}H]NBTI was defined as that which remained cell associated in the presence of 10 μM nonradioactive NBTI. Specific binding was defined as total binding minus nonspecific binding. K_d and B_{max} values for [^{3}H]NBTI binding were calculated using nonlinear regression analysis (GraphPad PRISM version 4.00 for Windows Software; San Diego, CA).

[^{3}H] substrate uptake. [^{3}H]-2-chloroadenosine and [^{3}H]-inosine uptake was measured according to previously described methods (11). [^{3}H]-2-chloroadenosine is used instead of adenosine because it results in less nonspecific binding to plasticware compared with adenosine. Briefly, cardiomyocytes were plated in six-well plates and incubated in sodium-free transport buffer containing (in mM) 20 Tris · HCl, 3 K_2HPO_4, 1 $MgCl_2$, 2 $CaCl_2$, 5 $C_6H_{12}O_6$, and 130 $C_7H_{17}NO_5$ (pH 7.4) containing permeant (10 μM 2-chloroadenosine or inosine) and radiolabeled nucleosides, [^{3}H]-2-chloroadenosine or [^{3}H]-inosine (Moravek Biochemicals, Brea, CA), in the absence or presence of NBTI (100 nM; 15 min). At room temperature, permeant was added and then rapidly aspirated at 10 s [previously determined to be within the linear phase of uptake for cultured murine cardiomyocytes (10, 11)]. Cells were washed five times in ice-cold sodium-free transport buffer and solubilized in 2 M NaOH for 48 h at 4°C. Aliquots were taken to measure protein content (Lowry protein assay; Bio-Rad) and analyzed for [^{3}H] content using standard liquid scintillation analysis.

RNA isolation and cDNA synthesis from adult mouse cardiomyocytes. Total RNA was isolated from the ENT1 wild-type and ENT1-null cardiomyocytes using a phenol-chloroform extraction method (TRIzol; Invitrogen, Carlsbad, CA). RNA concentration was quantified, and cDNA synthesis was performed using Superscript reverse transcriptase (Invitrogen).

Quantitative RT-PCR. Quantitative RT-PCR (qRT-PCR) amplifications were performed using Finnzymes Dynamo SYBR Green qRT-PCR Kit (New England Biolabs, Ipswich, MA). Primer sequences for A_1, A_{2a}, A_{2b}, and A_3 adenosine receptors were the sense and antisense primers 5′-ATCCCTCTCCGGTACAAGACAGT-3′ and 5′-ACTCAGGTTGTTCCAGCCAAAC-3′, 5′-CCGAATTCCACTCCGGTACA-3′ and 5′-CAGTTGTTCCAGCCCAGCAT-3′, 5′-TCTTCCTCGCCTGCTTCGT-3′and 5′-CCAGTGACCAAACCTTTATACCTGA-3′, and 5′-ACTTCTATGCCTGCCTTTTCATGT-3′ and 5′-AACCGTTCTATATCTGACTGTCAGCTT-3′, respectively. Primers for mENT1, mENT2, mENT3, and mENT4 were previously described (15). GAPDH was used as an internal control (Ambion, an applied biosystems business; Austin, TX). Reaction conditions for amplification of mENTs were: 15 min at 95°C, 40 cycles of 1 min at 94°C, 30 s at 58°C, and 1 min at 72°C. Reaction conditions for amplification of adenosine receptors were: 15 min at 95°C, 40 cycles of 1 min at 94°C, and 30 s at 55°C. Dissociation curves revealed sharp peaks at the melting temperature of each amplicon, and PCR primer efficiency was between 95% and 100% for the primer sets. Data analysis was performed using relative quantification ($2^{-\Delta\Delta Ct}$) and expressed as fold change versus lowest expressed transcript level (one gene set to 1).

siRNA knockdown of ENT1 in HL-1 cardiomyocytes. siRNA specific for ENT1 were designed and chemically synthesized by Ambion (Ambion Silencer Select predesigned siRNAs; Applied Biosystems, Foster City, CA). Two functional siRNA to the same target were used independently to ensure any biological effect observed was due to silencing of the target gene and not due to an off-target effect. Briefly, HL-1 cardiomyocytes were maintained in culture as previously described (6, 9) and seeded in six-well plates 24 h before transfection to allow for 80% cell density. siRNA (60 nmol, ~21 base pairs in length) was mixed with DMEM (250 μl) and, separately, Lipo-

fectAMINE 2000 (15 μl; Invitrogen) was added to DMEM (250 μl). After a brief incubation (4 min), the two vials were mixed and incubated at room temperature (20 min) and then pipetted dropwise onto cells, bringing the final volume to 2.5 ml per well. Transfected cells were incubated for 24 h, at which point media was changed to Claycomb media (JRH Biosciences, Lenexa, KS), and assays were conducted the following day (48 h post-siRNA transfection). The negative control was a scrambled siRNA sequence, which does not target ENT1, allowing validation that the biological effects exhibited were due to knockdown of ENT1 mRNA only. The positive control was siRNA targeted to GAPDH, which allowed for evaluation of efficient siRNA delivery into the cell (Ambion Silencer Select "verified to silence" siRNA; Applied Biosystems).

Data analysis. Data were compared by a one-way ANOVA with a Newman-Keuls or Dunnett post test or a Student's *t*-test or Mann-Whitney nonparametric test, where appropriate. Values are expressed as means ± SE with a $P < 0.05$ considered to be statistically significant. All statistical analyses were performed using GraphPad Prism version 4.00 for Windows (GraphPad Software, San Diego, CA).

RESULTS

The ENT1-null heart is cardioprotected following ischemia-reperfusion. The ENT1-null mouse heart showed significantly smaller myocardial infarct size compared with wild-type littermates following ischemia and reperfusion (Fig. 1*A*; $n = 5$, 3 wild-type, 2 ENT1-null mice; $P < 0.0001$). Myocardial cross sections showed white infarcted tissue in wild-type mouse hearts and minimal damage in ENT1-null littermate hearts (Fig. 1*B*).

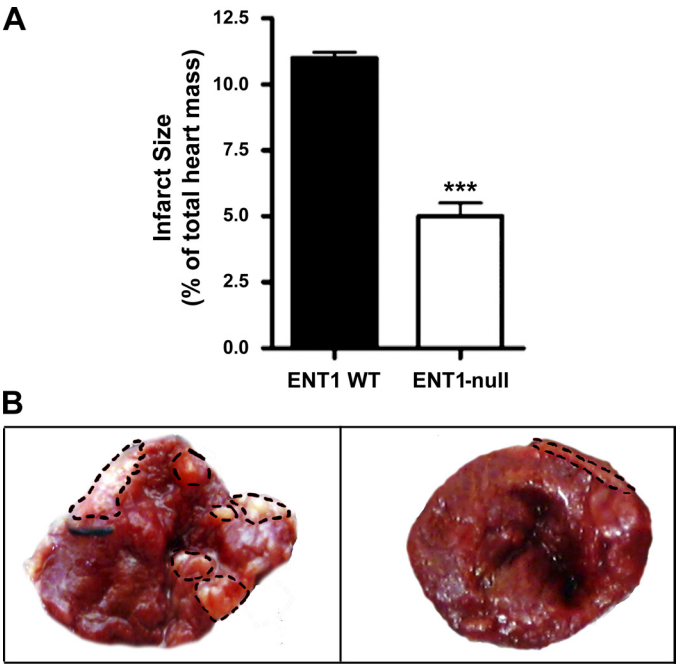

Fig. 1. Equilibrative nucleoside transporter 1 (ENT1)-null mouse heart is cardioprotected following ischemia-reperfusion. *A*: ENT1-null mouse heart showed significantly smaller (***$P < 0.0001$) myocardial infarct size compared with wild-type (WT) littermates upon 30 min of ischemia. Data are expressed as percent infarct size of total heart mass, weighed immediately upon excision, 2 h postreperfusion (means ± SD; $n = 5$: 3 wild-type, 2 ENT1-null mice). *B*: representative images of myocardial cross sections (parallel to atrioventricular groove) for ENT1 wild-type and null littermates (white, infarcted tissue).

ENT1-null mice show no compensatory response in other ENTs or adenosine receptors. Since the ENT1-null mouse heart is cardioprotected, it was essential to investigate whether this was due to a possible upregulation of other ENTs to compensate for the lack of ENT1 or changes in other components of the cardioprotective pathways at the cellular level in cardiomyocytes. Therefore, primary cardiomyocytes were successfully isolated from ENT1-null and wild-type littermate adult mouse hearts and maintained in culture for 24 h. ENT1 was found to be the predominant ENT isoform expressed in adult cardiomyocytes compared with ENT2, ENT3, or ENT4 (Fig. 2*A*) based on quantitative real-time RT-PCR in wild-type littermate controls. Analysis of ENT isoform profiles in ENT1-null adult mouse hearts showed a complete absence of ENT1 transcript compared with littermate controls ($n = 11$ ENT-null and $n = 9$ wild-type littermates; $P < 0.001$) and confirmed no compensatory change in expression of ENT2, ENT3, or ENT4 (Fig. 2*A*). Since reliable antibodies against mENT1 are not available, we confirmed the absence of ENT1 protein by conducting NBTI-binding assays. In ENT1 wild-type cardiomyocytes, over a million high affinity NBTI binding sites per cell were determined to be present (1,110,000 ± 309,000 sites; K_d 0.07 ± 0.02 nM and B_{max} 0.17 ± 0.01 pmol/mg protein), whereas there was no evidence for specific, high-affinity NBTI-binding in ENT1-null cardiomyocytes (Fig. 2*B*; $n = 3$; $P < 0.0001$; K_d 2.47 ± 1.3 and B_{max} 0.05 ± 0.01 pmol/mg protein). Low levels of NBTI-binding in the ENT1-null cardiomyocytes reflect typical residual-specific NBTI binding. The high number of NBTI binding sites per cardiomyocyte likely reflects the large surface area of these cells (12) relative to other cell types (21, 41).

Measurement of transcript levels of adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 showed no statistically significant difference in the expression of any adenosine receptor subtype in ENT1-null cardiomyocytes compared with control littermates ($n = 6$ ENT1-null and $n = 7$ wild-type littermates), suggesting no compensatory effects are present (Fig. 2*C*) in the ENT1-null mouse heart. A slight reduction in the levels of A_{2A} receptor transcript was noted but this was not statistically significant.

ENT1-null cardiomyocytes show severely impaired nucleoside uptake compared with wild-type littermates. Quantitative RT-PCR and NBTI-binding analyses confirmed a lack of ENT1 and an absence of compensatory responses to account for the cardioprotection evident in the ENT1-null mouse heart. Therefore, we next examined whether lack of ENT1 influenced nucleoside uptake by measuring transport in ENT1-null cardiomyocytes and comparing it with uptake by wild-type cardiomyocytes. Absence of ENT1 in ENT1-null cardiomyocytes results in significantly reduced, but not abolished, uptake of 2-chloroadenosine ($P < 0.01$) and inosine ($P < 0.001$; Fig. 3). This reduced 2-chloroadenosine uptake resembled uptake in ENT1 wild-type cardiomyocytes in the presence of NBTI (which inhibits ENT1), demonstrating that 2-chloroadenosine is transported predominantly by ENT1 and confirming the complete lack of ENT1-dependent uptake in ENT1-null cells ($n = 3$ wild-type and $n = 3$ ENT1-null mice; $P < 0.01$; Fig. 3*A*). In addition, as expected, there was no statistically significant effect of NBTI on 2-chloroadenosine uptake in ENT1-null cardiomyocytes (Fig. 3*A*), demonstrating the specificity of NBTI for ENT1 and suggesting that any remaining uptake of 2-chloroadenosine is mediated by ENT2 transport. In addition

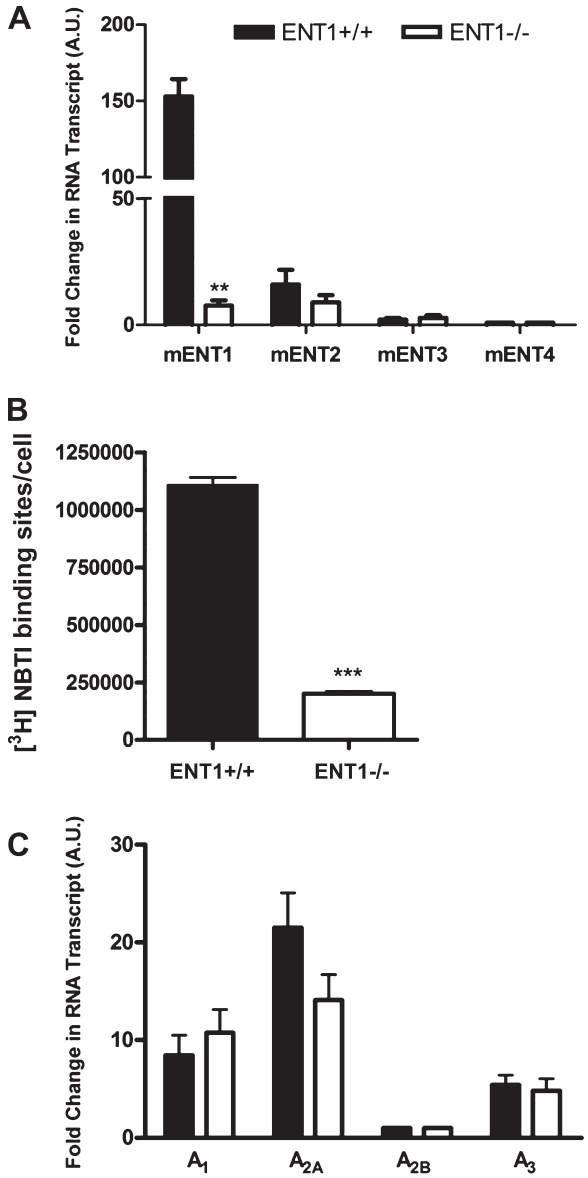

Fig. 2. Lack of ENT1 is not correlated with changes in levels of other ENTs or adenosine receptors. *A*: absence of ENT1 does not lead to compensation in transcript levels of other ENTs. Quantitative RT-PCR (qRT-PCR) of ENTs in cardiomyocytes shows significantly reduced ENT1 transcript levels in ENT1-null mice (**$P < 0.001$) compared with control wild-type littermates, but similar ENT2, ENT3, and ENT4 profiles in both. Data were calculated relative to GAPDH and expressed as fold change versus lowest transcript expressed (wild-type ENT4) ± SE ($n = 11$ ENT-null and $n = 9$ wild-type littermates pairs of mice; 3 replicates per experiment). *B*: lack of nitrobenzylthioinosine (NBTI) binding confirms absence of ENT1 protein in ENT1-null cardiomyocytes. Low levels of NBTI-binding seen in ENT1-null cardiomyocytes (mean bound [^{3}H]NBTI ± SD binding sites per cell; $n = 3$ ENT1-null and $n = 3$ wild-type littermates) were equivalent to residual-specific NBTI binding and significantly lower (***$P < 0.0001$) than NBTI binding seen in wild-type littermates. *C*: absence of ENT1 does not result in changes in adenosine receptor profiles. qRT-PCR of adenosine receptors A_1, A_{2A}, A_{2B}, and A_3 in cardiomyocytes shows similar transcript levels in ENT1-null mice compared with control wild-type littermates. Data were calculated relative to GAPDH and expressed as fold change versus lowest transcript expressed (wild-type A_{2B}) ± SE ($n = 6$ ENT1-null and $n = 7$ wild-type littermates; 4 replicates per experiment). AU, arbitrary units.

to 2-chloroadenosine, we measured the uptake of the endogenous substrate inosine, which we have previously reported to be transported by both ENT1 and ENT2 in HL-1 cells (37). Results showed that inosine uptake was significantly reduced in ENT1-null cardiomyocytes compared with control littermates ($n = 3$ wild-type and $n = 3$ ENT1-null mice; $P < 0.001$; Fig. 3*B*). Inosine uptake by wild-type and ENT1-null cardiomyocytes in the presence of NBTI was reduced slightly but not significantly from that seen in the absence of NBTI, confirming that ENT2 is the transporter primarily responsible for inosine uptake (Fig. 3*B*). ENT1-null cardiomyocytes also exhibited less overall inosine uptake compared with wild-type NBTI-treated cardiomyocytes ($P < 0.01$; Fig. 3*B*), suggesting that uptake via ENT2 alone is limited in the absence of ENT1.

siRNA knockdown of ENT1 in HL-1 cardiomyocytes mimics lack of nucleoside transport in ENT1-null mouse cardiomyocytes. Previous studies in HL-1 cells have suggested that ENT1 is the main transporter responsible for flux of adenosine and inosine (6, 37). To examine the contribution of ENT1-mediated transport,

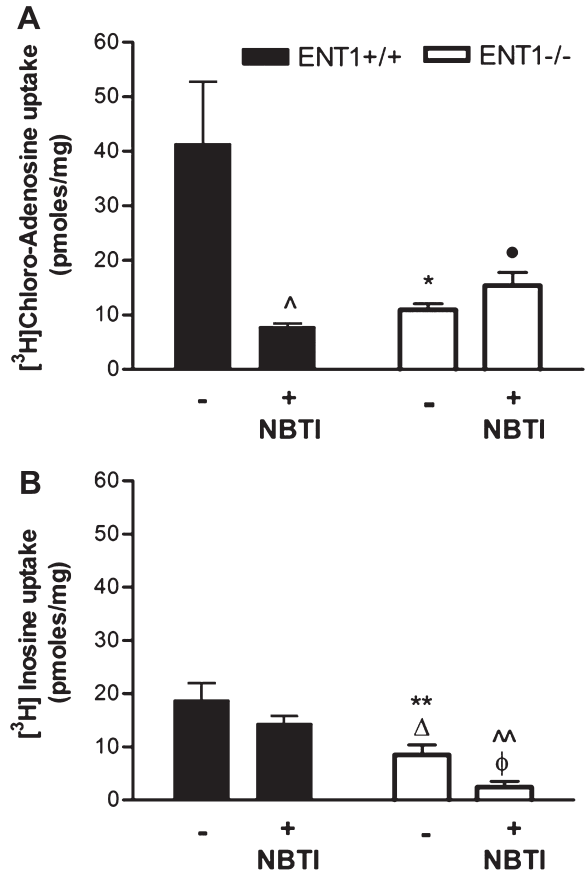

Fig. 3. ENT1-null cardiomyocytes show impaired uptake of nucleosides and confirm ENT1 as the predominant ENT responsible for adenosine uptake in cardiomyocytes. *A*: ENT1-null cardiomyocytes exhibited significantly reduced (*$P < 0.01$) [^{3}H]-2-chloroadenosine (10 μM) uptake compared with control wild-type littermates. Uptake in ENT1-null cardiomyocytes resembles that seen in wild-type cardiomyocytes in the presence of NBTI (100 nM), confirming that 2-chloroadenosine is transported via ENT1 and that functional ENT1-mediated transport is missing in ENT1-null cardiomyocytes (^$P < 0.01$ for wild-type vs. wild-type + NBTI; · $P < 0.01$ for wild-type vs. ENT1-null + NBTI). *B*: ENT1-null cells also exhibited impaired [^{3}H]-inosine uptake (10 μM) compared with wild-type littermates (**$P < 0.001$) and compared with wild-type NBTI-treated cardiomyocytes (Δ$P < 0.01$), suggesting the limited activity of ENT2 to transport both adenosine and inosine in mice lacking ENT1 (φ$P < 0.01$ wild-type + NBTI vs. ENT1-null + NBTI; ^^$P < 0.001$ wild-type vs. ENT1-null + NBTI). Data are expressed as means ± SD and $n = 3$ ENT1-null and $n = 3$ wild-type littermate mice (6 separate cardiomyocyte isolations); each experiment was conducted in sextuplicate.

and to confirm our transport findings in ENT1-null cardiomyocytes, siRNA targeted to ENT1 was used to knockdown ENT1 in HL-1 cells. Significantly reduced ENT1 transcript levels were confirmed in both siRNA targeted to ENT1 ($P < 0.05$; Fig. 4*A*) compared with a negative control (scrambled siRNA) and positive control (siRNA targeted to GAPDH). Similar ENT2, ENT3, and ENT4 transcript profiles were present in all treatments demonstrating efficient ENT1 knockdown at the transcript level. To confirm ENT1 knockdown at the protein level, siRNA-treated and negative control HL-1 cells were exposed to a range of concentrations of [^{3}H]NBTI. Both ENT1-specific siRNA targets showed decreased NBTI binding compared with control (Fig. 4*B*), indicating a lack of ENT1 protein at the plasma membrane. Following these findings, nucleoside uptake by HL-1 cells treated with siRNA was measured, and data showed a statistically significant impairment in both [^{3}H]-2-chloroadenosine ($P < 0.001$) and [^{3}H]-inosine ($P < 0.01$) uptake compared with scrambled siRNA control (Fig. 4, *C* and *D*), confirming our findings in ENT1-null cardiomyocytes.

DISCUSSION

Purinergic cardioprotection in the cardiovascular system has been widely investigated and is generally accepted to involve the purine nucleoside, adenosine, as well as adenosine receptors and their concomitant signaling pathways, metabolic enzymes, and purine nucleoside (adenosine) transporters. The relationships between the various components of the purinergic signaling pathways have been investigated using a variety of approaches including transgenic animals in which receptors, metabolic enzymes, and/or signaling components have been knocked out (14, 20, 25, 34). Studies using whole transgenic animal approaches have proved vital in advancing our understanding of phenomena such as preconditioning (14). However, although nucleoside transporters are routinely implicated in modulating the effects of adenosine in the cardiovasculature and are the targets of an important class of anti-ischemia drugs, very little is known about the role of NTs in cardiovascular physiology, partly due to a lack of appropriate tools, including transgenic models.

Here we present, for the first time, the use of a mouse adenosine (nucleoside) transporter knockout model to demonstrate the essential role of ENT1 in adenosine-mediated cardioprotection. This murine model has already been used to demonstrate the importance of ENT1 in purinergic signaling in the CNS (7, 8). We have extended these studies and here fully describe the transporter and receptor characteristics of cardiomyocytes isolated from ENT1-null animals. Furthermore, we show that the absence of ENT1 is sufficient to provide whole heart protection against ischemia in null mutants compared with wild-type littermates and demonstrate that this effect was not due to

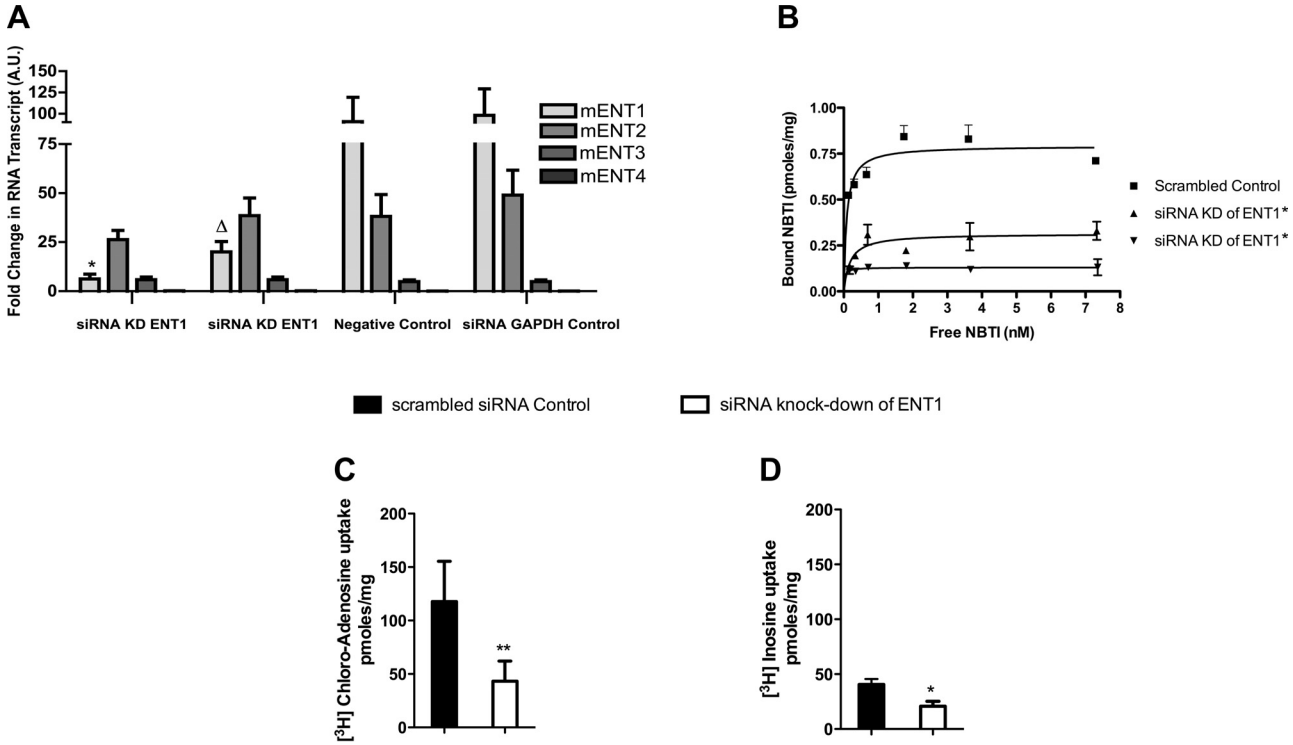

Fig. 4. Small interfering RNA (siRNA) knockdown (KD) of ENT1 in HL-1 cardiomyocytes confirms functional role of ENT1 in purine nucleoside uptake and resembles primary adult ENT1-null cardiomyocyte profile. siRNA KD was first confirmed (*A* and *B*) through the use of 2 independent targets to KD ENT1. *A*: qRT-PCR of ENTs shows significantly reduced ENT1 transcript levels with both siRNA targeted to KD ENT1 compared with a negative control (scrambled siRNA $*P < 0.05$; $\Delta P < 0.05$) and compared with siRNA targeted to GAPDH ($*P < 0.05$; $\Delta P < 0.05$). Similar ENT2, ENT3, and ENT4 profiles were evident. Data were calculated relative to GAPDH and expressed as fold change versus lowest transcript expressed (wild-type ENT4) ± SE ($n \geq 6$; each experiment performed in triplicate). *B*: siRNA-treated HL-1 cells showed significantly less NBTI binding compared with control (bound [^{3}H]NBTI ± SD; $n = 3$; $*P < 0.01$). *C*: siRNA KD of ENT1 in HL-1 cardiomyocytes shows a statistically significant decrease in both [^{3}H]-2-chloroadenosine ($**P < 0.001$) and [^{3}H]-inosine (*D*; $*P < 0.01$) uptake compared with control HL-1 cells treated with scrambled siRNA ($n \geq 4$; data expressed as means ± SD; each experiment performed in sextuplicate) confirming transport findings in ENT1-null cardiomyocytes in which ENT1 plays a predominant role in adenosine uptake and a role in inosine uptake.

compensatory responses of other ENTs. Since ENT1 is hypothesized to be part of an adenosine-dependent signaling network, we also examined expression levels of all four adenosine receptor subtypes and noted no significant difference in receptor expression between wild-type and ENT1-null cardiomyocytes. Adenosine receptors are known to be implicated in various aspects of cardioprotection and cellular homeostasis (18, 28, 34), and all four subtypes were expressed in adult mouse cardiomyocytes, although at differing levels ($A_{2A}<A_1<A_3<A_{2B}$). These data are consistent with previous reports, which show all four adenosine receptors present in the whole heart, with A_1 and A_{2A} receptor mRNA present at significantly higher levels compared with the other subtypes (49). These data demonstrate that ENT1-null mice do not show compensatory effects in the levels of the transcripts encoding nucleoside transporters or adenosine receptors, although we cannot rule out the possibility of altered transporter or receptor function (since expression levels are not necessarily indicative of function). In previous studies using this model (7, 8, 17), a lack of compensatory upregulation of receptors and transporters was confirmed using pharmacological and electrophysiological approaches. Here we have used a molecular approach based on quantitative real-time RT-PCR, and our findings correlate with previous studies (7, 8, 17). Moreover, description of an ENT1 overexpressing transgenic model reported no compensatory effects in the expression of adenosine receptors, other nucleoside transporter isoforms, or purine metabolizing enzymes (38).

Although ENTs have been widely implicated in modulating flux of purines in the cardiovascular system (2, 6, 29, 37), there are discrepancies as to which ENT is primarily responsible. Previous data from humans and rat tissue (2) suggest that ENT4 may play an important role, whereas studies in the rat cardiomyocyte cell line H9c2 (29) suggest that ENT2 may be involved. This study demonstrates that ENT1 is the predominantly expressed ENT in murine cardiomyocytes, supporting previous findings in mice (6, 26) and human tissue (40, Marvi et al. unpublished observations). Species and tissue-specific differences in rat and mouse transporter expression profiles have been previously described (31), but these data, taken together with other studies, suggest a similar pattern of transporter expression exists for mice and humans, supporting the use of a mouse model in gaining a greater understanding of the role of ENTs in the cardiovascular pathophysiology.

Previously, ENT2 has been shown to contribute to purine nucleoside flux in murine cultured cells (37); therefore, we cannot rule out a contribution of this transporter to purinergic physiology in the cardiovasculature. Interestingly, ENT2, similar to the other transporters, was not upregulated in response to the absence of ENT1 in these mice. Inosine uptake is significantly reduced overall in ENT1-null cardiomyocytes, whereas we have shown that it can be effectively transported by ENT2 in both HL-1 cardiomyocytes (37) and in primary wild-type cardiomyocytes when ENT1 is inhibited by NBTI. The explanation for this reduced ENT2 uptake in ENT1-null cardiomyocytes is not clear since there is no difference in the ENT2 expression levels between wild-type and ENT1-null cardiomyocytes but may reflect a complex interplay between the ENTs within the cellular context that remains to be elucidated.

Although the precise mechanism of cardioprotection in the ENT1-null mouse remains to be determined, the importance of ENT1 in response to hypoxic challenge has been demonstrated in previous studies in both mice (5) and humans (16). In these studies hypoxia results in transcriptional downregulation of the ENT1 gene, which presumably leads to elevated extracellular adenosine levels, which activate purinergic signaling pathways that promote cell survival (5, 16). By extension, removal of the ENT1 gene altogether presumably results in a permanently enhanced level of extracellular adenosine and a perpetually cardioprotected phenotype, as we see in this study. Previous research has implicated both CNT2 (1) and/or ENT1 (13) in modulation of adenosine uptake-dependent activation of AMP-dependent protein kinase (AMPK), a key player in cardioprotective responses. However, in contrast, we did not find any evidence for a role of ENT1 in the modulation of AMPK signaling via exogenously added adenosine (50 μM for 5, 15, and 30 min) in ENT1-null and wild-type cardiomyocytes compared with nontreated controls (data not shown), suggesting that the mechanism of cardioprotection in ENT1-null hearts may involve other signaling pathways. In summary, cardioprotection due to the absence of ENT1 clearly highlights the essential role ENT1 plays in mediating adenosine-dependent processes and confirms the potential of this model system as a research tool. This transgenic model, in combination with use of cultured cell models, such as HL-1, and manipulations such as siRNA knockdown, will provide a clinically relevant approach to study the role of ENT1 in purine-nucleoside physiology of the cardiovascular system.

GRANTS

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DISCLOSURES

No conflicts of interest are declared by the author(s).

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