Nucleoside/nucleobase transport and metabolism by microvascular endothelial cells isolated from ENT1\(^{-/-}\) mice

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Bone DB, Choi DS, Coe IR, Hammond JR. Nucleoside/nucleobase transport and metabolism by microvascular endothelial cells isolated from ENT1\(^{-/-}\) mice. Am J Physiol Heart Circ Physiol 299: H847–H856, 2010. First published June 11, 2010; doi:10.1152/ajpheart.00018.2010.—Nucleoside and nucleobase uptake is integral to mammalian cell function, and its disruption has significant effects on the cardiovasculature. The predominant transporters in this regard are the equilibrative nucleoside transporter subtypes 1 (ENT1) and 2 (ENT2). To examine the role of ENT1 in more detail, we have assessed the mechanisms by which microvascular endothelial cells (MVECs) from ENT1\(^{-/-}\) mice transport and metabolize nucleosides and nucleobases. Wild-type murine MVECs express mainly the ENT1 subtype with only trace levels of ENT2. These cells also have a Na\(^{+}\)-independent equilibrative nucleobase transport mechanism for hypoxanthine (ENBT1). In the ENT1\(^{-/-}\) cells, there is no change in EN2T or ENBT1, resulting in a very low level of nucleoside uptake in these cells, but a high capacity for nucleobase accumulation. Whereas there were no significant changes in nucleoside transporter subtype expression, there was a dramatic increase in adenosine deaminase and adenosine A\(_2\) receptors (both transcript and protein) in the ENT1\(^{-/-}\) tissues compared with WT. These changes in adenosine deaminase and A\(_2\) receptors likely reflect adaptive cellular mechanisms in response to reduced adenosine flux across the membranes of ENT1\(^{-/-}\) cells. Our study also revealed that mouse MVECs have a nucleoside/nucleobase transport profile that is more similar to human MVECs than to rat MVECs. Thus mouse MVECs from transgenic animals may prove to be a useful preclinical model for studies of the effects of purine metabolite modifiers on vascular function.

Adenosine; microvasculature; heart; transgenic; equilibrative nucleoside transporter

All mammalian cells express at least one form of equilibrative transporter responsible for the cellular uptake and release of endogenous nucleosides such as adenosine. The predominant transporters are equilibrative nucleoside transporter subtypes 1 (ENT1; SLC29A1) and 2 (ENT2; SLC29A2) (3). The inhibition of ENTs has been shown to enhance the cardioprotective (21) and neuroprotective (13) actions of adenosine. These transporters are also responsible for the cellular uptake of cytotoxic nucleoside analogs used to treat cancer and viral infections (25).

An ENT1-null (ENT1\(^{-/-}\)) mouse model has been generated by Choi and colleagues (10). The ENT1\(^{-/-}\) mice are phenotypically normal with the only difference from wild-type (WT) littermates being a slight (<10%) decrease in body weight and some differences in their behavioral characteristics. The ENT1-null mice are less sensitive to the intoxicating effects of ethanol compared with their WT littermates (10), and they also display less anxiety-like behavior (9). It has been noted that there is less tonic A\(_1\) adenosine receptor signaling in the brains of ENT1\(^{-/-}\) mice (10), reflecting either reduced extracellular adenosine levels or a decreased sensitivity of the receptor to adenosine, suggesting that ENT1 plays an important role in modulating the interaction of adenosine with its concomitant receptors in the central nervous system.

In addition to its regulatory activities in the nervous system, adenosine is well established as an endogenous cardioprotective agent via interactions with cardiomyocytes and is also a potent vasodilator through actions on vascular smooth muscle and endothelial cells (18). The availability of ENT1\(^{-/-}\) mice will now allow an in-depth examination of the role of ENT1 (and the effects of its absence) in adenosine metabolism and bioactivity in the various cell types that constitute the cardiovascular system under both normal and pathophysiological conditions. We have recently published the results of a study based on cardiomyocytes isolated from WT and ENT1\(^{-/-}\) mice (22). The present study extends this work to an examination of the impact of ENT1 loss on the nucleoside flux and metabolism in microvascular endothelial cells (MVECs).

Previous studies from our laboratory have established that rat MVECs express high levels of both ENT1 and ENT2 (2), whereas human MVECs express predominantly the ENT1 subtype of nucleoside transporter (6). MVECs are considered to be the major site of accumulation and metabolism of the adenosine released into the interstitial space by myocytes during periods of high energy demand in heart and skeletal muscle (11); therefore, in the absence of biological compensation, one might expect the loss of ENT1 to have dramatic effects on the vascular actions of adenosine.

In this communication, we describe the nucleoside and nucleobase transport profile of MVECs derived from skeletal muscle of ENT1\(^{-/-}\) mice and their WT littermate controls.

Methods

Materials. 8-[\(^{3}\)H]2-chloroadenosine (4–7 Ci/mmole), 2,8-[\(^{3}\)H]hypoxanthine (24–35 Ci/mmmole), \[^{3}\]H]nitrobenzylmercapturine riboside (NBMPR) (5.5 Ci/mmmole), and \[^{3}\]H]water (1 mCi/g) were purchased from Moravek Biochemicals (Brea, CA). Nonradiolabeled 2-chloroadenosine, hypoxanthine, NBMPR, nitrobenzylthioguanine riboside, dipryridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine), collagenase, trypsin, and BSA were obtained from Sigma-Aldrich (St. Louis, MO). All cell culture media, fetal bovine serum (FBS), culture grade Dulbecco’s PBS, trypsin-EDTA, antibiotic/antimycotic (penicillin, streptomycin, and amphotericin B), and heparin were purchased from Gibco/BRL (Burlington, ON, Canada). Endothelial cell growth supplement (ECGS) was

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supplied by Beckton Dickinson (Oakville, ON, Canada). Rabbit polyclonal anti-adenosine deaminase (ADA), goat polyclonal anti-ENT3, rabbit polyclonal anti-adenosine receptor A3, and donkey anti-goat horseradish peroxidase-conjugated antibodies were all from Santa Cruz Biotechnology (Santa Cruz, CA). Mouse monoclonal anti-adenosine receptor A2a and rabbit-anti mouse horseradish peroxidase-conjugated and mouse anti-rabbit horseradish peroxidase-conjugated antibodies were from Abcam (Cambridge, MA).

**ENT1+/− mouse model.** ENT1+/− mice were generated in the laboratory of Dr. Doo-Sup Choi as described previously (10); ENT1+/− mice were backcrossed with outbred WT C57BL/6 mice to generate ENT1+/− mice. Only ENT1+/− mice were crossed (producing ENT1+/−, ENT1+/-, and ENT1−/− offspring) to establish a breeding colony at the University of Western Ontario. Genotyping was done using isolated genomic DNA and standard PCR as previously described (9). All aspects of this study were conducted in accordance with the policies and guidelines set forth by the Canadian Council on Animal Care and were approved by the Animal Use Committee of the University of Western Ontario.

**MVEC isolation/culture.** MVECs were isolated as described previously for rats (2), based on the affinity of their α-α (+)-galactosyl residues for isolate B2 from Bandeiraea simplicifolia (BSI-B4; Sigma) bound to magnetic microbeads (Dynabeads, M-450 Epoxy, BSA in 50 ml Krebs-Ringer solution containing (in mM) 127 NaCl, 6.3 NaH2PO4, 2.7 KCl, 1.5 KH2PO4, 1.5 MgCl2, 2H2O, and 0.5 M MgCl2·6H2O (pH 7.4) or a modified Na+-free PBS containing N-methyl-D-glucamine (NMG) buffer consisting of (in mM) 140 NMG, 10 HEPES, 5 KCl, 4.2 KH2PO4, 0.44 KH2PO4, 0.36 K2HPO4, 1.3 CaCl2·2H2O, and 0.5 MgCl2·6H2O (pH 7.4) as appropriate to the assay and then resuspended in the same buffer for immediate use as described in [3H]NBMPR binding and [3H]substrate uptake. In some cases, the cells were depleted of ATP by sequential incubation at 37°C with rotenone and 2-deoxyglucose as described previously (2).

[3H]NBMPR binding. MVECs (~300,000 cells assay−1, in PBS; pH 7.4) were incubated with [3H]NBMPR (± inhibitors) in borosilicate glass tubes for 45 min at room temperature (~22°C) to attain steady-state binding (1 ml final vol). The 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). The filters were then assessed for radioactive content by standard liquid scintillation counting. Nonspecific binding of [3H]NBMPR was defined as that which remained cell associated in the presence of 10 µM nitrobenzylthioguanosine. Specific binding was defined as the total minus nonspecific binding. Kd and maximum binding capacity (Bmax) values for [3H]NBMPR binding were calculated from nonlinear (hyperbolic) curves fitted (GraphPad Prism 5) to nontransformed specific binding data plotted against the free [3H]NBMPR concentrations at steady state.

[3H]substrate uptake. The uptake of [3H]formycin B, [3H]-2-chloroadenosine, and [3H]hypoxanthine by MVECs was measured at room temperature (~22°C) in Na+-free NMG buffer in the presence and absence of maximally inhibiting concentrations (10 µM) of the ENT1 inhibitor NBMPR and the ENT1/ENT2 inhibitor diprydamole. Total ENT- mediated [3H]substrate uptake was defined as the difference between the total uptake and that observed in the presence of diprydamole. ENT1-mediated uptake was defined as that inhibited by 50 nM NBMPR. ENT2-mediated uptake was calculated as the difference between the uptake in the presence of 50 nM NBMPR and that seen in the presence of 10 µM NBMPR/diprydamole. In some cases, the cells were incubated with both adenine and diprydamole to test for the diprydamole-insensitive transporter-mediated uptake of [3H]hypoxanthine. Uptake assays were initiated by the addition of 250 µl of cell suspension (~750,000 cells assay−1) to 250 µl of [3H]substrate layered over a 200 µl cushion of silicone/mineral oil (21:4 vol/vol) in 1.5-ml microcentrifuge tubes. Assays were terminated after defined time intervals (~5–600 s) by centrifugation (12,000 g) of the cells through the oil layer. Aqueous supernatant and oil were removed, and the cell pellets were digested in 1 M sodium hydroxide for ~16 h at room temperature. The digest was analyzed for [3H] content using standard liquid scintillation counting techniques.

Uptake data are presented as intracellular [3H]substrate concentrations (in pmol/µl intracellular volume; µM) after the correction for the amount of [3H] label present in the extracellular space of the cell pellet. Total water volumes of the cell pellets were determined by incubating cells with [3H]water for 3 min and then processing the samples as described above. An estimate of the extracellular water volume was obtained from extrapolation of the linear time courses of nonmedicated uptake (in the presence of supermaximal concentrations of uptake blockers) back to zero time. In our hands, this method of assessing extracellular water space was more reliable than using membrane-impermeable tracers such as [125I]Julin. Initial rates (V) of flux were calculated as the uptake at 1 s determined by an extrapolation of hyperbolic curves fitted (GraphPad Prism 5) to transporter-mediated time course data. Kin and Vmax values were calculated from nonlinear curves fitted to plots of [3H]substrate concentration versus V.
digested with 7% perchloric acid for ~16 h. The acid soluble extract was removed and neutralized with an equal volume of 1 M potassium hydroxide and then centrifuged (10,000 g for 10 min) to remove insoluble precipitate. Extracts were stored at 20°C until required.

The separation of purine nucleobases, nucleosides, and nucleotides was performed using two one-dimensional thin-layer chromatography methods modified from Metz et al. (19) (6 min 0.5 M LiCl, 10 min 1.0 M LiCl, and remainder in 1.5 M LiCl, all at pH 4.0 and included 0.1 M EDTA; condition 1) and Shimizu et al. (24) (n-butanol:ethyl acetate:methanol:ammonium hydroxide; 7:4:3:4, vol:vol; condition 2). Plastic-backed PEI-cellulose F TLC plates (EMD Chemicals or Merck) were loaded with 10-μl aliquots of neutralized extract and developed using the two solvent conditions in parallel. Purine metabolite locations were determined under UV light, and the Rf values were calculated and compared with standards for identification. The plates were then cut into 5-mm strips for liquid scintillation analysis of [3H] content using Beckman Ready Protein® scintillation fluid.

Neither solvent system on its own was capable of separating all the purine metabolites of interest. The organic solvent (condition 2) was able to retard all nucleotides at the loading point on the plate and could clearly isolate adenosine and xanthine. However, the hypoxanthine and inosine spots overlapped. The LiCl gradient (condition 1), on the other hand, could isolate inosine effectively but produced a blended hypoxanthine-adenosine spot. Thus the amount of hypoxanthine was determined from the equation: dpm hypoxanthine = dpm hypoxanthine/adenosine (condition 1) – dpm adenosine (condition 2). Since xanthine oxidase removes the tritium at the two position of 2,8-[3H]hypoxanthine to give 8-[3H]xanthine, the dpm values associated with xanthine were doubled to compensate for this.

Table 1. RT-PCR primers

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer 5’-3’</th>
<th>Product Size, bp</th>
<th>No. of Cycles</th>
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<tr>
<td>ENT1</td>
<td>CAAGTATTACAAACGCGCTGGAC</td>
<td>196</td>
<td>25</td>
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<tr>
<td>ENT2</td>
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<td>288</td>
<td>35</td>
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<td>ENT3</td>
<td>GCCCTTCTGCTGCTTCTGCTG</td>
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<td>ENT4</td>
<td>ATGTGGCCGCTGCTGCTGCTA</td>
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<tr>
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<td>35</td>
</tr>
<tr>
<td>CNT2</td>
<td>ATTTTGTGGCTGCTGCTGCTG</td>
<td>169</td>
<td>35</td>
</tr>
<tr>
<td>CNT3</td>
<td>GGCTCTCAGTGATGCTCATA</td>
<td>243</td>
<td>35</td>
</tr>
<tr>
<td>Adenosine kinase</td>
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<td>Adenosine deaminase</td>
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<td>Xanthine oxidase</td>
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<td>β-Actin</td>
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Primers used for the amplification of each of the indicated genes are shown along with the expected product size and the number of cycles used in the PCR reactions. The cycle number was selected based on the lowest number that revealed a product up to a maximum of 35 cycles in the qualitative reverse-transcriptase PCR reactions. ENT, equilibrative nucleoside transporter; CNT, concentrative nucleoside transporter.

Fig. 1. Microvascular endothelial cells (MVECs) isolated from either wild-type (WT) or equilibrative nucleoside transporter subtype-1 (ENT1) knockout (KO) (ENT1−/−) littermates were exposed to a range of concentrations of [3H]nitrobenzylmercaptopurine riboside (NBMPR) (abscissa) in the absence (total binding) or presence (nonspecific binding) of 10 μM nitrobenzylthioguanine riboside. Specific binding (ordinate) was calculated as the difference between total and nonspecific binding. Each point is the mean ± SE of 5 experiments performed in duplicate.
Fig. 2. Analysis of purine transporter/metabolic enzyme gene expression in MVECs: A: qualitative reverse-transcriptase PCR: PCR reactions were conducted using the primers shown in Table 1, and the products were resolved on 2% agarose gels. The same PCR conditions were used for each primer pair in both the WT and ENT1−/− samples, and all amplifications shown on each gel set were done in parallel using the same WT and KO cDNA preparations. The first lane on each gel set represents the DNA ladder (bp sizes as labeled). Set 1: ENT1, ENT2, ENT3, and β-actin (the vertical bar indicates where a blank section of the gel image was removed between the ENT3 and β-actin lanes); set 2: adenosine A2a receptor, adenosine A2b receptor, A3 receptor, and β-actin; and set 3: adenosine kinase (ADK), adenosine deaminase (ADA), adenine phosphoribosyltransferase (ARPT), hypoxanthine-guanine phosphoribosyltransferase (HGPRT), purine nucleoside phosphorylase (PNP), xanthine oxidase (XO), and β-actin. All products were of the expected size. B: quantitative real-time PCR: apparent differences in gene expression between the WT and ENT1−/− MVECs, as noted in the studies shown in A, were confirmed by real-time PCR. Crossing point (CP) values, obtained from SYBR green I fluorescence signals, were used to compute the concentrations of ENT3, ADA, and adenosine A2a and A3 receptor transcripts, relative to β-actin. β-Actin CP values were 19.3 ± 0.6 and 18.8 ± 0.7 for the WT and ENT1−/− samples, respectively. The gene-to-β-actin ratios derived from each independent experiment were then averaged to obtain the data shown. Each bar is the mean ± SE of 3 separate amplification runs from 2 independent mRNA isolations (N = 6). *P < 0.05, significant difference in expression between the WT and ENT1−/− cells (Student’s t-test). C: data from B plotted as the relative change between the WT and ENT1−/− mice.
Reverse-transcriptase polymerase chain reaction. Total RNA was isolated from the WT and ENT1−/− MVECs using the phenol-chloroform extraction. First-strand DNA template was generated using 5 μg of DNaseI (Invitrogen, Carlsbad, CA)-treated total RNA and the Superscript First Strand Synthesis System for RT-PCR using oligo d(T) primers (Invitrogen). RT-PCR amplifications were performed using Platinum Taq DNA polymerase (Invitrogen) in a Thermocycler PE 480 (Perkin Elmer, Norwalk, CT) using an oil-overlaid 50-μl reaction mixture in 500-μl thin-walled reaction tubes. Reaction conditions included a 2-min 94°C initial activation, followed by 25, 30, or 35 cycles of 30 s at 94°C, 30 s at 55°C, and 30 s at 72°C. Preliminary experiments determined the number of cycles of PCR that were required to obtain a visible product on agarose gels for each of the transcripts while remaining on the log-linear portion of the cycle number versus product formation curves. PCR products were resolved by electrophoresis on 2% agarose gels against the O’GeneRuler 50-bp DNA ladder mix (Fermentas, Burlington, ON, Canada). Based on qualitative PCR, ENT1, ENT3, ADA, and the adenosine receptor A2a and A3 genes were chosen for quantitative real-time PCR analysis using a Roche LightCycler (Hoffmann-La Roche, Mississauga, ON, Canada). β-Actin was chosen as a reference control. All primers used were the same as those for qualitative PCR. Standard curves for all transcripts were generated using the same cDNA preparation at concentrations ranging from 10−7 to 10−12 M. The PCR was conducted using 1 μl of WT or knockout (KO) cDNA and SYBR Green Jumpstart Taq ReadyMix capillary formulation (Sigma). PCR conditions were as follows: initial denaturing at 94°C for 30 s, followed by 40 cycles of 94°C for 0 s, 55°C for 5 s, 72°C for 17 s, and 78°C for 1 s. The melting curve analysis of products at the end of the reaction revealed single products from each primer pair. At least three individual reactions were performed using two different cDNA preparations.

Primer sequences and expected sizes are summarized in Table 1 for ENT1; ENT2; ENT3; concentrative nucleoside transporter subtypes 1 (CNT1), 2 (CNT2), and 3 (CNT3); adenosine kinase; ADA; adenosine phosphoribosyltransferase; hypoxanthine-guanine phosphoribosyltransferase; purine nucleoside phosphorylase; xanthine oxidase; A1, A2a, A3b, and A3 adenosine receptors; and β-actin.

Immunoblots. Extensor digitorum longus muscles were isolated from age- and sex-matched mice and quickly placed in ice-cold lysis buffer containing 20 mM Tris·HCl, 150 mM NaCl, 1 mM Na2EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na3VO4, and 1 μg/ml leupeptin (pH 7.5), supplemented with 1 mM phenylmethylsulfonyl fluoride just before use (Cell Signaling Technology, Pickering, ON, Canada). Total protein from tissue lysates (100 μg for ADA and ENT3; and 200 μg for A3) were separated by electrophoresis and immunoblotted, in parallel, with antibodies to GAPDH (37 kDa), ADA (50 kDa), adenosine A2a receptor (45 kDa), adenosine A3 receptor (43 kDa), and ENT3 (52 kDa). Representative immunoblots are shown in A. The results (means ± SE) of densitometry analysis of 3 independent experiments with adenosine receptor A2a and ADA are shown in B as a ratio relative to GAPDH. *P < 0.05, significant difference in expression between the WT and ENT1−/− muscle (Student’s t-test).

Fig. 3. Proteins extracted from the extensor digitorum longus muscles of WT and ENT1−/− mice were separated by electrophoresis and immunoblotted, in parallel, with antibodies to GAPDH (37 kDa), ADA (50 kDa), adenosine A2a receptor (45 kDa), adenosine A3 receptor (43 kDa), and ENT3 (52 kDa). Representative immunoblots are shown in A. The results (means ± SE) of densitometry analysis of 3 independent experiments with adenosine receptor A2a and ADA are shown in B as a ratio relative to GAPDH. *P < 0.05, significant difference in expression between the WT and ENT1−/− muscle (Student’s t-test).

Fig. 4. MVECs isolated from either WT (A) or ENT1−/− (B) mice were incubated with 10 μM [3H]formycin B (FB) in the absence (Total, □) or presence of 50 nM NBMPR (+NBMPR; selective inhibition of ENT1-mediated uptake, □) or 10 μM dipyridamole/NBMPR (+DY; complete inhibition of transporter-mediated uptake, ●) for the times indicated. Uptake is represented as picomoles of [3H]FB accumulated per microliters of intracellular water (μM). Each point is the mean ± SE from 4 experiments.
The adenosine receptor A2a and A3 was subjected to SDS-PAGE and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight (≥12 h) at 4°C with one of the following primary antibodies: ADA, 1:1,000; ENT3, 1:400; adenosine receptor A2a, 1:1,000; or adenosine receptor A3, 1:200. Secondary antibodies were used at dilutions of 1:10,000, 1:1,000, 1:4,000, and 1:1,000 for ADA, ENT3, A2a, and A3, respectively, and incubated with membranes for 1 h at room temperature. Detection was performed using LumiGLO chemiluminescent substrate (Cell Signaling Technology) and the VersaDoc Imaging System (Bio-Rad). After exposure, the membranes were stripped (200 mM glycine, 1% Tween-20, and 0.1% SDS; pH 2.2) and then probed for GAPDH using a 1:1,000 dilution of primary antibody for 2 h at room temperature. The secondary antibody was used at dilutions of 1:2,000 for ADA and ENT3 and 1:4,000 for adenosine receptor A2a to account for the double amount of protein used. Densitometry was performed using ImageJ software (http://rsbweb.nih.gov/ij/index.html). In all cases, the major protein detected by each antibody reflected the expected size of the product based on manufacturers’ specifications and previously published data using these antibodies (8, 20).

RESULTS AND DISCUSSION

The gross phenotype of the ENT1−/− mice was similar to that reported previously by Choi and colleagues (10). The ENT1−/− mice had a consistently lower body weight. They were also notably easier to handle than WT mice (less distress because of handling), likely reflecting the anxiolytic phenotype reported previously (9). Quantitative RT-PCR confirmed the loss of the full-length ENT1 transcript in the ENT1−/− mice.

MVECs were successfully isolated from the skeletal muscle tissue of WT and ENT1−/− mice. MVECs from the ENT1−/− mice had no detectable high-affinity binding sites for the ENT1-selective radioligand probe [3H]NBMPR (Fig. 1), confirming the specificity of this radioligand for the ENT1 protein. WT mouse MVECs had 150,000 ± 27,000 [3H]NBMPR sites/cell with an affinity of 0.09 ± 0.02 nM, which is typical of NBMPR binding to ENT1. We found no evidence for Na+/H+ -dependent CNT1, CNT2, and CNT3, and we confirmed that MVECs do not express the A1 adenosine receptor subtype (14). No changes were observed in the expression of the ENT2 subtype of plasma membrane-located nucleoside transporters in ENT1−/− mice compared with littermate controls (Fig. 2A), nor was the ENT4 transcript detected in either the WT or ENT1−/− cells. Furthermore, no significant differences were noted in the expression of adenosine kinase, xanthine oxidase, HGPRT, adenine, hypoxanthine-guanine phosphoribosyltransferase, purine nucleoside phosphorylase, or A2a adenosine receptors in the ENT1−/− MVECs compared with WT controls (Fig. 2A). However, MVECs from ENT1−/− mice showed significant increases of 15 ± 3- and 11 ± 2-fold, respectively, in the expression of mRNA transcripts for ADA and A2a adenosine receptors, relative to WT littermate controls (Fig. 2B). There was a trend toward a decrease in adenosine A3 receptor transcript in the ENT1−/− mice, but this did not reach statistical significance and there was no corresponding change in A3 receptor protein levels (Fig. 3). Likewise, there was a small but significant increase in ENT3 transcript (Fig. 2B), which was also not paralleled by an increase in ENT3 protein in skeletal muscle (Fig. 3). This lack of correlation between changes in mRNA versus protein levels for these relatively low abundance transcripts may reflect posttranscriptional regulation of translation efficiency and protein processing. Attempts to measure ADA and A2a adenosine receptor protein levels in the MVECs proved to be problematic. However, significant increases in ADA (2.1-fold) and adenosine A2a receptor (2.4-fold) protein was evident by immunoblotting using the skeletal muscle tissue of ENT1−/− mice from which the MVECs were derived, relative to WT mice (Fig. 3).

These adaptive changes can be rationalized, from a cell homeostasis perspective, if one assumes that the loss of ENT1 is leading to a reduced efflux of adenosine (arising from ATP metabolism) from the MVECs. This would result in an increased cytoplasmic adenosine and a decreased extracellular adenosine in the ENT1−/− compared with WT mice. The increase in ADA would lead to more adenosine metabolism to inosine, which may then be converted to either hypoxanthine or inosine monophosphate (1), thereby reducing intracellular adenosine. Adenosine has well-established vasodilatory (4) and anti-inflammatory activities (16), mediated in part via extracellular adenosine A2a receptors on vascular endothelial cells. Thus the increase in adenosine A2a receptor expression

Fig. 5. MVECs isolated from either WT (A) or ENT1−/− (B) mice were incubated for 5 or 15 s, respectively, with the indicated concentrations of [3H]2-chloroadenosine. Parallel assays were conducted in the absence (total influx) and presence of either 50 nM NBMPR (NBMPR-resistant influx) or 10 μM DYM/NBMPR (nonmediated uptake) as described in Fig. 4 for [3H]FB uptake. The total transporter-mediated uptake of substrate was calculated as the total influx minus the nonmediated component. The ENT2-mediated uptake was calculated as the NBMPR-resistant uptake minus the nonmediated component, and the ENT1-mediated uptake was estimated as the difference between the total uptake and that seen in the presence of NBMPR. Results are plotted as picomoles of [3H]2-chloroadenosine accumulated per microliter of cell water per second (Vv, ordinate) against the concentration of [3H]2-chloroadenosine used (abscissa). Each point is the mean ± SE from 5 experiments. The transporter kinetic constants derived from these data are shown in Table 2.

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may be compensating for the lower levels of adenosine-mediated signaling in the ENT1−/− mice as a consequence of reduced extracellular adenosine concentrations. A previous study has also invoked lower extracellular adenosine levels as an explanation for the reduced A1 adenosine receptor signaling observed in the brains of ENT1−/− mice (10). In contrast to our findings in MVECs, there does not appear to be a compensatory upregulation of adenosine A2a receptors in cardiomyocytes isolated from the ENT1−/− mice (22), suggesting that different cell types respond differently to changes in adenosine bioavailability. With the consideration that adenosine receptor expression varies between different tissues and therefore adenosine has differing physiological effects in each tissue, the compensatory changes that would result from altered adenosine levels might be expected to differ in different tissues and cell types.

In terms of the ability to accumulate [3H]substrates associated with ENT activity, the WT MVECs accumulated both [3H]formycin B (Fig. 4) and [3H]2-chloroadenosine (Fig. 5) via two distinct Na+-independent dipyridamole-sensitive processes (Table 2). The majority (~90%) of the substrate uptake in WT MVECs was inhibited by 50 nM NBMPR and hence was mediated by ENT1. The remainder of the uptake was inhibited by dipyridamole, but not NBMPR, and hence was mediated by ENT2. It is noteworthy that mouse MVECs are very different from rat MVECs where ENT2 mediates ~50% of the total nucleoside uptake (2). Thus one would expect an ENT1-selective inhibitor such as NBMPR to have a much greater effect on nucleoside levels in the cardiovasculature of mouse compared with its effects in rat.

The uptake of [3H]formycin B (Fig. 4) and [3H]2-chloroadenosine (Fig. 5) by MVECs was reduced dramatically in the cells derived from the ENT1−/− mice (Table 2). The initial rate of uptake of 10 μM [3H]formycin B in the ENT1−/− MVECs was 0.07 ± 0.01 pmol·μl−1·s−1, which is not significantly different from the rate of ENT2-mediated formycin B uptake by WT mMVECs (0.06 ± 0.01 pmol·μl−1·s−1) (Fig. 4). Similar time courses were observed for [3H]2-chloroadenosine uptake by the ENT1−/− and WT MVECs (data not shown). These functional data are compatible with the lack of any change in the transcript levels of ENT2 in the ENT1−/− mice (Fig. 2). The Vmax of 2-chloroadenosine uptake in the ENT1−/− mice (0.19 ± 0.07 pmol·μl−1·s−1) was actually significantly lower than the Vmax of ENT2-mediated 2-chloro-

Adenosine uptake in the WT mice (1.2 ± 0.5 pmol·μl−1·s−1) (Fig. 5).

All of the above studies were done in Na+-free media to eliminate the potential influence of Na+-dependent uptake of nucleosides (17). MVECs do not appear to express any of the known CNTs. However, to determine whether there are any other Na+-dependent mechanisms for the uptake of nucleosides operating in these cells, the rate of uptake of 10 μM formycin B was compared in normal PBS and Na+-free NMG buffer. These experiments indicated that the mouse MVECs had a small amount of Na+-dependent formycin B uptake, but

### Table 2. Kinetic parameters for the transporter-mediated uptake of 2-chloroadenosine and hypoxanthine by MVECs isolated from WT and ENT1−/− mice

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th></th>
<th></th>
<th>ENT1−/−</th>
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</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total</td>
<td>ENT1</td>
<td>ENT2</td>
<td>Total</td>
<td>ENT1</td>
<td>ENT2</td>
</tr>
<tr>
<td>2-Chloroadenosine</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
</tr>
<tr>
<td>Vmax, pmol·μl−1·s−1</td>
<td>18 ± 3</td>
<td>24 ± 5</td>
<td>1.2 ± 0.5</td>
<td>—</td>
<td>ND*</td>
<td>0.19 ± 0.06*</td>
</tr>
<tr>
<td>Km, μM</td>
<td>84 ± 17</td>
<td>147 ± 44</td>
<td>9 ± 3</td>
<td>—</td>
<td>ND*</td>
<td>13 ± 6</td>
</tr>
<tr>
<td>Hypoxanthine</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vmax, pmol·μl−1·s−1</td>
<td>12 ± 2</td>
<td>17 ± 4</td>
<td>ND</td>
<td>10 ± 2</td>
<td>12 ± 2</td>
<td>ND</td>
</tr>
<tr>
<td>Km, μM</td>
<td>84 ± 20</td>
<td>126 ± 29</td>
<td>ND</td>
<td>96 ± 29</td>
<td>131 ± 32</td>
<td>ND</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 5 WT and 5 ENT1−/− mice. MVECs, microvascular endothelial cells; WT, wild-type; ENT1−/−, ENT knockout; DY, dipyridamole; ND, no transport activity detected. *P < 0.05, significant difference between WT and ENT1−/− (Student’s t-test).

Fig. 6. MVECs isolated from either WT (A) or ENT1−/− (B) mice were incubated with 5 μM [3H]hypoxanthine in the absence (Total) or presence of 10 μM DY (inhibition of ENT2-mediated uptake) or 1 mM adenine for the times indicated. Uptake is represented as picomoles of [3H]hypoxanthine accumulated per microliter of intracellular water (in μM). Each point is the mean ± SE from 4 experiments.
adenine with or without the inclusion of 10^(-6)M hypoxanthine with nonmediated uptake component, the WT mouse MVECs accumulated hypoxanthine via an ENT-independent transport process. When adenine (1 mM) is used to define the initial time course studies using 10^(-6)M hypoxanthine as substrates in addition to nucleobases, such as hypoxanthine, as substrates in addition to the adenine-insensitive component. Each point is the mean ± SE from 5 experiments. The transporter kinetic constants derived from these data are shown in Table 2.

Fig. 7. MVECs isolated from either WT (A) or ENT1−/− (B) mice were incubated for 10 s with the indicated concentrations of [3H]hypoxanthine (abscissa). Parallel assays were conducted in the absence and presence of 1 mM cumulated hypoxanthine with or without the inclusion of 10 μM DMY as described in Fig. 6. Results are plotted as the transporter-mediated uptake of substrate (in pmol per μl of cell water accumulated per s; Vi, ordinate) after subtraction of the adenine-insensitive component. Each point is the mean ± SE from 5 separate experiments. The transporter kinetic constants derived from these data are shown in Table 2.

Fig. 8. MVECs isolated from WT and ENT1−/− mice were depleted of ATP and incubated with 100 μM [3H]hypoxanthine in the absence and presence of 10 μM DMY for 20 min. Acid extracts of cell pellets were neutralized with 1 M KOH, and aliquots were subjected to thin-layer chromatography as described in the text. An ANOVA test was performed within each metabolite group. *P < 0.05 compared with WT−ATP condition; †P < 0.05 compared with WT−ATP + DMY condition.

Interestingly, the apparent rate of [3H]hypoxanthine influx tended to increase when cells from WT mice were incubated with the ENT blocker dipyridamole before the uptake measurements, particularly at higher (>100 μM) concentrations of hypoxanthine (Fig. 7). In contrast, dipyridamole had no effect on hypoxanthine uptake by ENT1−/− MVECs. Given that part of the hypoxanthine uptake in the MVECs would be due to the activity of ENT2, one should actually expect to see a small decrease in hypoxanthine uptake in the presence of dipyridamole. One interpretation of these data is that dipyridamole was blocking the efflux of a metabolite of hypoxanthine via ENT1. In normal cell metabolism, hypoxanthine is typically formed as a metabolite of inosine and is efficiently metabolized by xanthine oxidase to xanthine or by HGPRT to inosine monophosphate thus there is little free hypoxanthine in cells. However, under the experimental conditions used in this study, where cells are exposed to high concentrations of hypoxanthine, it would be produced in the endothelial cells under conditions of high adenosine release from surrounding cells, such as would occur during tissue ischemia (1, 11, 12). This hypoxanthine is metabolized intracellularly by xanthine oxidase with the consequent production of reactive oxygen species. Reactive oxygen species are believed to play a role in mediating the endothelial dysfunction observed in various pathological conditions including diabetes and ischemia-reperfusion injury (5, 15, 23). Therefore, understanding how MVECs handle hypoxanthine is important in the overall evaluation of purine metabolism in the vasculature. It appears that both mouse and human MVECs, which have low or no ENT2, respectively, rely heavily on this nucleobase transporter for the transfer of nucleobases across the cell membrane. Rat MVECs, on the other hand, which express high levels of ENT2 (which can transport nucleobases), do not appear to express ENBT1. Further studies on this novel nucleobase transport system are needed.

It it represented only about 3% of the total uptake, and there was no difference in this component between the WT and ENT1−/− mice (data not shown).

To further assess the ENT2-mediated component in the MVECs, the uptake of [3H]hypoxanthine was examined after depleting the cells of ATP to reduce trapping of [3H]hypoxanthine metabolites. ENT2 differs from ENT1 in that it accepts nucleobases, such as hypoxanthine, as substrates in addition to purine and pyrimidine nucleosides. It was apparent from the initial time course studies using 10 μM hypoxanthine that mouse MVECs readily accumulated hypoxanthine via a saturable system that was relatively insensitive to dipyridamole but that was inhibited by adenine (Fig. 6). These data suggest that hypoxanthine was being accumulated via an ENT-independent transport process. When adenine (1 mM) is used to define the nonmediated uptake component, the WT mouse MVECs accumulated hypoxanthine with Km and Vmax estimates of 125 ± 29 μM and 16.5 ± 3.8 pmol·μl⁻¹·s⁻¹, respectively, while ENT1−/− cells had similar Km and Vmax values of 123 ± 28 μM and 11.6 ± 2.1 pmol·μl⁻¹·s⁻¹, respectively. A dipyridamole-insensitive equilibrative nucleobase transporter subtype 1 (ENBT1) with similar characteristics as that described herein has recently been characterized by us in human MVECs (6). Hypoxanthine is a metabolite of adenosine metabolism and
is possible that the [3H]hypoxanthine is being metabolized to [3H]inosine via purine nucleoside phosphorylase (7). As noted above, inosine is a substrate for ENT2 and ENT1, both of which are blocked by dipyridamole. The WT MVECs, with their high level of expression of ENT1, would release the [3H]inosine formed in this way much more rapidly than would the ENT1-deficient cells and would thus be more sensitive to the effects of dipyridamole. To test the above hypothesis, we examined the [3H]metabolite profile of WT and ENT1−/− MVECs after exposure to [3H]hypoxanthine under the conditions that we used for the uptake assays (Fig. 8). As predicted, ENT1−/− MVECs had significantly higher intracellular levels of [3H]hypoxanthine than did the WT MVECs after exposure to 100 μM [3H]hypoxanthine for 20 min. [3H]inosine levels showed a trend toward being increased in ENT1−/− MVECs; however, this difference was not significant unless dipyridamole was present to block the remaining ENT2-mediated efflux. The difference between the WT and ENT1−/− MVECs was also attenuated in the presence of the ENT blocker dipyridamole. These data indicate that [3H]hypoxanthine is being metabolized to [3H]inosine by nucleoside phosphorylase under these assay conditions and that this [3H]inosine is not released from ENT1−/− cells as effectively as it is from WT MVECs. These data also show that the ATP-depletion protocol used led to the expected reduction in the conversion of hypoxanthine to its phosphorylated metabolites (Fig. 8).

In summary, the loss of ENT1 in the ENT1−/− mice leads to a dramatic reduction in the ability of MVECs to accumulate and release nucleosides such as adenosine. Despite this loss of nucleoside transport capacity, the ENT1−/− mice appear phenotypically normal under baseline conditions. There did not appear to be any compensatory upregulation of the other major plasma membrane nucleoside transporter ENT2 in the ENT1−/− mice, but there were increases in both ADA and A2a adenosine receptor expression in MVECs and the skeletal muscle tissue from which the cells were isolated. The consequent changes in adenosine metabolism and adenosine-mediated signaling via the A2a receptor likely compensate for decreased extracellular adenosine levels in the ENT1−/− mice and allow them to function normally under baseline conditions. However, given the widely acknowledged role of adenosine as a cardioprotectant in conditions of cardiovascular stress, it might be anticipated that the ENT1−/− mice will be less able to respond to cardiovascular stresses associated with conditions such as diabetes and ischemia-reperfusion injury. Alternatively, a reduced uptake of extracellular adenosine might potentiate adenosine-receptor activation and subsequent cardioprotection, suggesting that ENT1−/− mice might be permanently preconditioned (17, 18) and thus protected from ischemic or hypoxic challenge. Investigations into the relative roles and contributions of ENT1 and ENT2 in purine-dependent cardiovascular physiology are in progress in our laboratory. In addition, this study also highlights significant differences in the way MVECs from rats and mice handle nucleosides and nucleobases. Indeed, human MVECs are similar to mouse MVECs in that they express ENT1, but very little ENT2, and display a large component of the adenine-sensitive hypoxanthine transporter ENBT1. Thus mouse MVECs cultured from transgenic animals may prove to be a useful preclinical model for use in studies of the effects of purine transport/metabolism modifiers on vascular function.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

REFERENCES


