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Absence of equilibrative nucleoside transporter 1 in ENT1 knockout mice leads to altered nucleoside levels following hypoxic challenge

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ABSTRACT

Aims: Equilibrative nucleoside transporters (ENT) modulate the flux of adenosine. The ENT1-null (KO) mouse heart is endogenously cardioprotected but the cellular basis of this phenotype is unknown. Therefore, we investigated the cellular mechanisms underlying ENT1-mediated cardioprotection.
Main methods: Circulating adenosine levels were measured in WT and KO mice. Cellular levels of nucleosides and nucleotides were investigated in isolated adult cardiomyocytes from WT and KO mice using HPLC following hypoxic challenge (30 min, 2% O_2). Changes in hypoxic gene expression were analyzed by PCR arrays and cAMP levels were measured to investigate contributions from adenosine receptors.
Key findings: Circulating adenosine levels were significantly higher in KO (416 ± 42 nmol/l, $n = 12$) compared to WT animals (208 ± 21, $n = 13$, $p < 0.001$). Absence of ENT1 led to an elevated expression of genes involved in cardioprotective pathways compared to WT cardiomyocytes. Following hypoxic challenge, extracellular adenosine levels were significantly elevated in KO (4360 ± 1840 pmol/mg protein) versus WT cardiomyocytes (3035 ± 730 pmol/mg protein, $n \geq 12$, $p < 0.05$). This effect was enhanced in the presence of dipyridamole (30 μM), which inhibits ENT1 and ENT2. Enhanced extracellular adenosine levels in ENT1-null cardiomyocytes appeared to come from a pool of extracellular nucleotides including IMP, AMP and ADP. Adenosine receptor (AR) activation mimicked increases in cAMP levels due to hypoxic challenge suggesting that ENT1 modulates AR-dependent signaling.
Significance: ENT1 contributes to modulation of extracellular adenosine levels and subsequent purinergic signaling via ARs. ENT1-null mice possess elevated circulating adenosine levels and reduced cellular uptake resulting in a perpetually cardioprotected phenotype.

Introduction

The purine nucleoside adenosine has long been studied for its role in cardioprotection (Eltzschig, 2009). Extracellular adenosine is produced from precursors ATP and AMP via ecto-apyrase (CD39) and 5′-ecto-nucleotidase (CD73), respectively and can also be released by multiple cell types, such as inflammatory cells, platelets, endothelia and epithelia (Eltzschig, 2009). Extracellular adenosine signals through cell surface adenosine receptors (AR), A_1, A_{2a}, A_{2b} and A_3 and AR signal activation has been associated with cellular protection in various pathophysiological conditions in a variety of tissue types (Vassort, 2001; Eckle et al., 2007; Schepp and Reutershan, 2008).

Adenosine receptor activation is terminated by breakdown of adenosine or by re-uptake of adenosine into the cell via nucleoside transporters (Young et al., 2008). In cardiomyocytes, the equilibrative nucleoside transporter 1 (ENT1) and 2 (ENT2) are primarily responsible for adenosine flux (Chaudary et al., 2002). Since ENTs are bidirectional transporters, facilitating movement of nucleosides down their endogenous gradient, they also have the capacity to release adenosine to the extracellular milieu and contribute to extracellular purinergic signaling (Zamzow et al., 2008). Therefore, the presence and activity of NTs significantly influences extracellular levels of adenosine and thus NTs modulate adenosine signaling (Eltzschig et al., 2005; Löffler et al., 2007). However the role of NTs in modulating the effects of adenosine in cardiomyocytes is poorly understood and the relative contribution of ENT1 and ENT2 is not clear. Recent findings have highlighted the need to examine the role of ENT1 with respect to the activation of adenosine receptors, since ENT1 mediates the transport, and therefore the effects of adenosine at the cellular level. Moreover, the clinical use of adenosine, adenosine

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analogs and ENT inhibitors to treat various cardiovascular conditions (Pelleg et al., 2002), highlights the importance of understanding the role and contribution of ENTs to purinergic signaling.

Recently, a global murine knockout of ENT1 was described and has proved useful in establishing the role of ENT1 in the central nervous system and neurotransmission (Chen et al., 2007; Choi et al., 2004). In addition, various characteristics (transporter, receptor, enzyme profiles) of both the cardiomyocyte and the microvascular endothelial cells of the ENT1-null mouse have been described (Bone et al., 2010; Rose et al., 2010) and we have determined that these animals show decreased heart damage in response to induced ischemia and hypoxia (Rose et al., 2010). These data suggest that the ENT1-null mouse heart is inherently protected from damage due to myocardial infarction suggesting that ENT1 plays a major role in overall modulation of adenosine-mediated cardioprotection. Here, we describe the underlying mechanisms responsible for ENT1-mediated cardioprotection and demonstrate the role of ENT1 in modulating levels of extracellular adenosine and thus adenosine receptor signaling which leads to the protected phenotype.

Materials and methods

Animals

Genotyped female ENT1-null and ENT1 wild-type pups (Choi et al., 2004) were shipped after weaning from Mayo Clinic (Rochester, MN) to York University (Toronto, ON). Primary cardiomyocytes were isolated from a different mouse for each experiment. 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 (CCAC) guidelines. Littermates were housed in standard cages with chow and water available ad libitum. The animal room was maintained on a 12 hour light/dark cycle.

Isolation of primary adult mouse cardiomyocytes

Primary adult cardiomyocyte isolation was performed on mice two to four months of age as previously described (Rose et al., 2010). Briefly, mice were injected with 0.5 ml of heparin diluted in PBS to a final concentration of 100 IU/ml. After 20 min, an anesthetic mixture of ketamine (1.5 mg/ml) and xylazine (0.1 mg/ml) was injected at a volume of 1% of total mouse weight. Surgery for cardiomyocyte isolation commenced 20 min post anesthetic injection in accordance to the guidelines set by the Animal Care Committee of York University and CCAC. 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 incubated at 2% CO2 for 2 h to allow attachment of the rod-shaped cells and were used for experiments immediately. The plating medium was aspirated, plates were washed twice with 1 ml of HBSS and 1.25 ml of HBSS was added to each plate before start of the experiment. At this step all of the nonattached, floating dead cells were removed. Final cell yields of cardiomyocytes were equivalent to previously published reports and number of rod shaped cells did not change after 30 min of hypoxia showing no increase in cell death. Prior to all assays we confirmed that cell death did not contribute to nucleoside and nucleotide levels (data not shown).

Hypoxic treatment of isolated primary adult mouse cardiomyocytes and collection of cellular extracts

Cardiomyocytes were plated as previously described (Rose et al., 2010). Nitrobenzylthioinosine (NBTI, 100 nM, 10 min) was used to inhibit ENT1 and dipyridamole (DIPY, 30 μM, 10 min) was used to inhibit ENT1 and ENT2. Hypoxic challenge was achieved by using 1.25 ml de-gassed HBSS (± inhibitors) and transfer of cardiomyocytes to a vented Plexiglas GasPak hypoxia chamber (VWR Canlab, Mississauga, ON) for 30 min at 37 °C with a humidified hypoxic atmosphere of 2% O_2, 5%CO_2 and 93% N_2. Cardiomyocytes were plated in 60 mm plates (coated with 5 μg/ml laminin) and incubated at 2% CO_2 in minimum essential medium (MEM) containing Hank's Balanced Salt Solution (HBSS, GIBCO®, Invitrogen, Carlsbad, CA), supplemented with 2.5% (w/v) bovine calf serum (BCS) and 10 mM 2,3-butanedione monoxime (BDM) as previously described (Rose et al., 2010). Typically, 1×10^6 rod-shaped cardiomyocytes are obtained per adult mouse heart and 120,000–150,000 cardiomyocytes are plated at a density of approximately 3500 rod-shaped cells/cm^2 on 60 mm plates. All experiments are conducted the same day as isolation. Cells were then rinsed twice with HBSS and incubated in 1.25 ml HBSS (in the presence or absence of NBTI and DIPY) for 30 min at 37 °C, 2% CO_2. Normoxic cardiomyocytes were kept at 37 °C with 2% CO_2. After hypoxic challenge, HBSS media was collected and immediately frozen at −80 °C prior to HPLC analysis. Cardiomyocytes were lysed with ice cold perchloric acid (PCA, 0.5 ml, 0.4 M) and scraped to collect. Cells were flash frozen in liquid N_2 and thawed at room temperature prior to syringing on ice. Centrifugation (10 min at 16000×*g*, 4 °C) allowed for separation of intracellular solution from protein pellet of lysed cells, which were dissolved in NaOH (300 μl, 0.5 M) and analyzed for protein content (Bio-Rad Protein Assay, Bio-Rad Laboratories (Canada) Ltd., Mississauga, ON). Supernatants were neutralized with 10 μl ice-cold 4 M K_2CO_3 per 100 μl volume, incubated on ice for 7 min and centrifuged for 10 min (16000×*g* at 4 °C). Supernatants, containing intracellular components, were then frozen at −80 °C prior to HPLC analysis.

High-performance liquid chromatography for intracellular and extracellular nucleosides and nucleotides

HPLC was conducted using a Beckman-Coulter System Gold ® 126NM Solvent Module with a System Gold® 166NM Detector and a 50 μl injection loop (Beckman Coulter Inc., Mississauga, ON). The C18 column for chromatographic separation was 250 mm × 4.6 mm, 4 μm particle size, Synergi Fusion-RP, preceded by an analytical guard cartridge system (Phenomenex Inc., Torrance, CA). A 1% (v/v) methanol (HPLC grade, Sigma-Aldrich, St. Louis, MO) buffer (1 M KH_2PO_4, pH 6.9) was used for nucleotide chromatographic separation and a 6% (v/v) acetonitrile (HPLC grade, Sigma-Aldrich, St. Louis, MO) buffer (1 M KH_2PO_4, pH 6.5) was used for nucleoside chromatographic separation of cellular extracts (Fig. 3A, B). The system was operated at room temperature with a flow rate of 1 ml/min, an operating pressure of approximately 2 kpsi and a wavelength of 254 nm for detection and quantification. Detector output of peak was measured and integrated by 32 Karat 8.0 system software (Beckman Coulter Inc., Mississauga, ON) and analyzed using GraphPad Prism version 4.0 for Windows (GraphPad Software, San Diego, CA). In order to identify and quantify

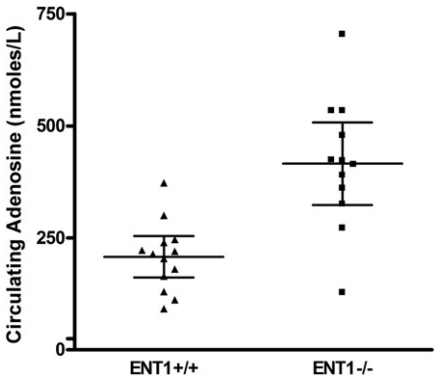

Fig. 1. Circulating adenosine levels are elevated in ENT1 null mice. Circulating adenosine levels were significantly higher in ENT1-null (−/−) (416 ± 42 nmol/l, n = 12) compared to wild-type (ENT1+/+) (208 ± 21, n = 13, $p<0.001$). Data expressed as mean ± 95% confidence intervals.