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Oxidative stress modulates nucleobase transport in microvascular endothelial cells

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ABSTRACT

Purine nucleosides and nucleobases play key roles in the physiological response to vascular ischemia/reperfusion events. The intra- and extracellular concentrations of these compounds are controlled, in part, by equilibrative nucleoside transporter subtype 1 (ENT1; SLC29A1) and by equilibrative nucleobase transporter subtype 1 (ENBT1). These transporters are expressed at the membranes of numerous cell types including microvascular endothelial cells. We studied the impact of reactive oxygen species on the function of ENT1 and ENBT1 in primary (CMVEC) and immortalized (HMEC-1) human microvascular endothelial cells. Both cell types displayed similar transporter expression profiles, with the majority (>90%) of 2-chloro[^{3}H]adenosine (nucleoside) uptake mediated by ENT1 and [^{3}H]hypoxanthine (nucleobase) uptake mediated by ENBT1. An in vitro mineral oil-overlay model of ischemia/reperfusion had no effect on ENT1 function, but significantly reduced ENBT1 V_{max} in both cell types. This decrease in transport function was mimicked by the intracellular superoxide generator menadione and could be reversed by the superoxide dismutase mimetic MnTMPyP. In contrast, neither the extracellular peroxide donor TBHP nor the extracellular peroxynitrite donor 3-morpholinosydnonimine (SIN-1) affected ENBT1-mediated [^{3}H]hypoxanthine uptake. SIN-1 did, however, enhance ENT1-mediated 2-chloro[^{3}H]adenosine uptake. Our data establish HMEC-1 as an appropriate model for study of purine transport in CMVEC. Additionally, these data suggest that the generation of intracellular superoxide in ischemia/reperfusion leads to the down-regulation of ENBT1 function. Modification of purine transport by oxidant stress may contribute to ischemia/reperfusion induced vascular damage and should be considered in the development of therapeutic strategies.

Introduction

Microvascular endothelial cells (MVEC) play a critical role in the local regulation of vascular tone via the production and release of vasoactive agents such as nitric oxide and prostacyclin (Tune, 2007). A key factor that stimulates the release of these agents is the endogenous nucleoside adenosine (Nyberg et al., 2010). Adenosine, a 'retaliatory metabolite', is released from cells under biological stress (e.g. ischemia) and stimulates a family of G-protein coupled receptors present in vascular endothelial and smooth muscle cells (Headrick et al., 2011; Ryzhov et al., 2007; Volonte and D'Ambrosi, 2009). Activation of adenosine receptors has been shown to be cardioprotective. Adenosine inhibits platelet aggregation, has anti-inflammatory activities, and is a potent vasodilator. It has also been implicated in the phenomenon of preconditioning, where a short period of ischemia/reperfusion will attenuate the damage caused by a subsequent ischemia/reperfusion event (Headrick and Lasley, 2009; Yang et al., 2010).

Adenosine concentrations are regulated in the vasculature by the concerted activities of a number of enzymes and membrane transporters (Arch and Newsholme, 1978; Deussen, 2000b; Deussen et al., 2006). Under baseline physiological conditions, adenosine produced via 5′-ecto-nucleotidase activity, or arising from ATP metabolism within surrounding tissue, is rapidly taken up into cells by specific plasma membrane transporters (Loffler et al., 2007). In MVEC, adenosine is accumulated and released predominantly via the SLC29A1

Abbreviations: CMVEC, cardiac microvascular endothelial cells (primary); CNT, concentrative nucleoside transporter; EDTA, ethylenediaminetetraacetic acid; ENBT1, equilibrative nucleobase transporter subtype 1; ENT, equilibrative nucleoside transporter; ENT1, equilibrative nucleoside transporter subtype 1, SLC29A1; ENT2, equilibrative nucleoside transporter subtype 2, SLC29A2; FeTTPs, 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron III chloride; HMEC-1, human microvascular endothelial cells type 1 (dermal, immortalized); MnTMPyP, manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin; NBMPR, nitrobenzylmercaptopurine riboside; NBTGR, nitrobenzylthioguanine riboside; NMG, *N*-methyl-glucamine; PBS, phosphate buffered saline; SIN-1, 3-morpholinosydnonimine; TBHP, tert-butyl hydroperoxide.

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transporter (equilibrative nucleoside transporter subtype 1; henceforth referred to as ENT1). Once inside the cells, adenosine is immediately used to maintain the cellular adenine nucleotide pool via the adenosine kinase pathway or, once the kinase pathway is saturated, metabolized by adenosine deaminase to inosine. Hence, intracellular adenosine concentrations are typically very low, maintaining an inward adenosine gradient across the plasma membrane. Intracellular inosine arising from adenosine deaminase activity is converted to hypoxanthine, which is subsequently metabolized by xanthine oxidase to xanthine and uric acid. Xanthine oxidase mediated metabolism produces oxygen free radicals as catalytic by-products that, in turn, contribute to the reactive oxygen stress seen in ischemia/reperfusion injury (Baudry et al., 2008; Lakshmi et al., 2009; Lee et al., 2009). Xanthine oxidase-derived superoxide can also react with nitric oxide produced by endothelial cells to form peroxynitrite, which has also been implicated in the etiology of vascular dysfunction (Forstermann, 2010). The amount of inosine and hypoxanthine available to the xanthine oxidase pathway is dependent not only on the intracellular adenosine levels and adenosine deaminase activities, but also on the capacity of the cells to efflux inosine and hypoxanthine via ENT1 and ENBT1 (equilibrative nucleobase transporter subtype 1), respectively. It is also important to note that cells can replenish nucleotide pools via scavenging of extracellular adenine nucleobases released from surrounding tissue, thus limiting post-ischemic vascular injury; this cytoprotective process would also be sensitive to the activity of ENBT1. We have established previously that primary human (h) MVEC have extensive capacity for purine nucleoside and nucleobase transport via the aforementioned transporters (Bone and Hammond, 2007).

The important roles of ENT1 and ENBT1 in regulating purine levels in the vasculature make them a target for pharmacological manipulation of vascular activity in ischemia/reperfusion pathologies. Recent work by Abd-Elfattah and colleagues showed the effectiveness of blockade of ENT1 in the protection of canine hearts during surgery via both the enhancement of adenosine receptor activity and the maintenance of intracellular adenosine for nucleotide pool replenishment (Abd-Elfattah et al., 2012a,b). Nucleoside transport inhibitors have also been studied as cardioprotectants for cardiac transplant procedures (Chang-Chun et al., 1992; Masuda et al., 1992). Furthermore, dipyridamole, a broad spectrum equilibrative nucleoside transport blocker, is used therapeutically to prevent postoperative thromboembolic complications after cardiac valve replacement and diagnostically to induce vasodilation for myocardial perfusion imaging (Bolognese et al., 1991; Schaper, 2005; Wackers, 1991) . Notwithstanding the therapeutic potential of ENT inhibitors, the development of cardiovascular therapies based on manipulation of adenosine concentrations has been hampered by a lack of information on the cellular regulation of these transporters in the microvasculature, as well as lack of understanding of how these transporters respond to changes in the cellular environment associated with ischemia/reperfusion.

Because the expression of nucleoside transporter subtypes in endothelial cells, as well as their sensitivity to transport inhibitors, is species-related (Archer et al., 2004; Bone and Hammond, 2007; Bone et al., 2010; Hyde et al., 2001), it is imperative to assess the roles of these transporters in human cell/tissue models. However, the isolation and culture of the large amounts of human primary endothelial cells required to perform pharmacological and physiological studies have its disadvantages in terms of cost, biosafety, and limited proliferative capacity in culture. These circumstances suggest the need for an immortalized cell model that retains the endothelial phenotype and responds to regulatory factors in a manner comparable to primary MVEC. In that regard, the HMEC-1 cell line, derived from human dermal MVEC via transfection with the simian virus 40 A gene product (Ades et al., 1992; Bouis et al., 2001; Lidington et al., 1999), appears to have the greatest potential.

The present study focused on the impact that reactive oxygen species, generated during ischemia/reperfusion, have on the function of ENT1 and ENBT1 in human cardiac MVEC (CMVEC; primary cultures) and HMEC-1. Previous data suggest that ENT1 is down-regulated upon exposure to reactive oxygen species (Barankiewicz et al., 1995). However, no similar work has been done for ENBT1. We hypothesize that increased intracellular production of reactive oxygen species by MVEC during ischemic episodes leads to rapid changes in cellular ENT1 and ENBT1 activities which influence the biological activities of adenosine in the vasculature. To test this hypothesis we determined whether the immortalized MVEC cell line HMEC-1 can be used as a model for studying purine transporters in the microvascular endothelium and assessed the impact of simulated ischemia/reperfusion (sI/R) and oxygen free radical generation on nucleoside and nucleobase uptake by human MVEC.

Materials and methods

Materials

2-Chloro[8-^{3}H]adenosine (16 Ci/mmol), [2,8-^{3}H]hypoxanthine (28.7 Ci/mmol), [^{3}H]nitrobenzylmercaptopurine riboside ([^{3}H]NBMPR, 23.8 Ci/mmol), and [^{3}H]water (1 mCi/ml) were purchased from Moravek Biochemicals (Brea, CA). Non-radiolabeled 2-chloroadenosine, hypoxanthine, NBMPR, nitrobenzylthioguanine riboside (NBTGR), dipyridamole (2,6-bis(diethanolamino)-4,8-dipiperidinopyrimido-[5,4-d]pyrimidine), adenine, tert-butyl hydroperoxide (TBHP) and menadione were from Sigma-Aldrich. 3-Morpholinosydnonimine (SIN-1) was from Tocris Bioscience (Minneapolis, MN), and manganese(III) tetrakis(1-methyl-4-pyridyl)porphyrin (MnTMPyP) and 5,10,15,20-tetrakis (4-sulfonatophenyl) porphyrinato iron III chloride (FeTTPs) were from Calbiochem (Mississauga, ON).

Cell culture

Primary human CMVEC were purchased from Lonza (Walkersville, MD) and were cultured in EGM-2 MV medium as supplied by the manufacturer. Cells were provided at *passage* 3 and used between *passages* 4 and 7. No morphological changes occurred during culture up to *passage* 7. Quality assurance from the manufacturer verified the isolation as pure endothelial cells using immunohistologic staining for the presence of acetylated LDL and von Willebrand's (factor VIII) antigen and the absence of smooth muscle α-actin.

HMEC-1 were obtained from the Centers for Disease Control and Prevention (Atlanta, GA) and cultured in MCDB-131 medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, 10 ng/ml epidermal growth factor, and 1 μg/ml hydrocortisone. HMEC-1 were used between *passages* 15 and 25. It was noted in preliminary experiments that after *passage* 30 the amount of hypoxanthine transport by the HMEC-1 increased significantly, suggesting a change in cell characteristics at higher passage numbers.

Cells were maintained in a humidified atmosphere at 37 °C with 5% CO_2 in T175 culture flasks. For experimental assays, cells were trypsinized (0.05% trypsin–0.53 mM EDTA) and then diluted four-fold in 500 μg/ml trypsin inhibitor in phosphate buffered saline (PBS; in mM: 137 NaCl, 6.3 Na_2HPO_4, 2.7 KCl, 1.5 KH_2PO_4, 0.9 $CaCl_2$–$2H_2O$, 0.5 $MgCl_2$–$6H_2O$; pH 7.4). Cells were collected by centrifugation, and pellets were washed in either PBS or a Na^+-free buffer [NMG (in mM): 140 *N*-methyl-D-glucamine, 5 KCl, 4.2 KH_2PO_4, 0.36 K_2HPO_4, 10 HEPES, 1.3 $CaCl_2$–$2H_2O$, and 0.5 $MgCl_2$–$6H_2O$; pH 7.4] and re-suspended in the same buffer as required for immediate use.

For low oxygen exposure, culture flasks were placed in hypoxia chambers (Billups-Rothenberg). Chambers were flushed with 1% O_2 for 5 min and expelled air was measured with an O_2 detector to confirm low oxygen levels. Chambers were then sealed and placed in a 37 °C incubator for 2 h. Following hypoxia, flasks were removed from the chambers and harvested for substrate uptake as described above.