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Intracellular acidification reduces L-arginine transport via system y^+L but not via system y^+/CATs and nitric oxide synthase activity in human umbilical vein endothelial cells

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ABSTRACT

L-Arginine is taken up via the cationic amino acid transporters (system y^+/CATs) and system y^+L in human umbilical vein endothelial cells (HUVECs). L-Arginine is the substrate for endothelial NO synthase (eNOS) which is activated by intracellular alkalization, but nothing is known regarding modulation of system y^+/CATs and system y^+L activity, and eNOS activity by the pHi in HUVECs. We studied whether an acidic pHi modulates L-arginine transport and eNOS activity in HUVECs. Cells loaded with a pH-sensitive probe were subjected to 0.1–20 mmol/L NH_4Cl pulse assay to generate pHi 7.13–6.55. Before pHi started to recover, L-arginine transport (0–20 or 0–1000 µmol/L, 10 s, 37 °C) in the absence or presence of 200 µmol/L *N*-ethylmaleimide (NEM) (system y^+/CATs inhibitor) or 2 mmol/L L-leucine (system y^+L substrate) was measured. Protein abundance for eNOS and serine^{1177} or threonine^{495} phosphorylated eNOS was determined. The results show that intracellular acidification reduced system y^+L but not system y^+/CATs mediated L-arginine maximal transport capacity due to reduced maximal velocity. Acidic pHi reduced NO synthesis and eNOS serine^{1177} phosphorylation. Thus, system y^+L activity is downregulated by an acidic pHi, a phenomenon that may result in reduced NO synthesis in HUVECs.

1. Introduction

A variety of membrane transport systems removing metabolic substrates from the extracellular medium are expressed in the foetoplacental endothelium [1–4]. The activity of some of these transport systems is modulated by changes in the extracellular (pHo) and intracellular (pHi) pH [5,6]. The cationic amino acid L-arginine, the substrate for the synthesis of nitric oxide (NO) via the endothelial NO synthase (eNOS) [7,8], is taken up mainly by the cationic amino acid transporters (CATs, also referred as system y^+ or system y^+/CATs) family [4] and system y^+L in human umbilical vein endothelial cells (HUVECs) [2,9,10]. System y^+/CATs corresponds to a family of five proteins of which mainly the high affinity ($K_m \sim 100–250$ µmol/L) hCAT-1 and hCAT-2B isoforms are expressed in HUVECs [4,11]. System y^+L activity results from heterodimers formed by the interaction of the heavy chain of the cell surface antigen 4F2 (4F2hc) with the light

Abbreviations: pHo, extracellular pH; pHi, intracellular pH; 4F2hc, heavy chain of the cell surface antigen 4F2; HUVECs, human umbilical vein endothelial cells; HPAEC, human pulmonary artery endothelial cells; RAEC, rat aorta endothelial cells; hENT1/2, human equilibrative nucleoside transporters 1 and 2; GDM, gestational diabetes mellitus; hCATs, human cationic amino transporters; NO, nitric oxide; NOS, nitric oxide synthase; eNOS, endothelial nitric oxide synthase; NH_4Cl, ammonium chloride; DAF-FM, 4-amino-5-methylamino-2′,7′-difluorofluorescein; L-NAME, N^G-nitro-L-arginine methyl ester; BCECF-AM, bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester; NEM, *N*-ethylmaleimide

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chains 4F2-lc2 (or y^+LAT-1) or 4F2-lc3 (or y^+LAT-2) [1,11–14]. System y^+L activity accounts for L-arginine transport with a very high affinity (K_m ~ 1–20 μmol/L) and small and large neutral amino acids, such as L-leucine, requiring extracellular sodium in HUVECs [4,9]. System y^+/CATs and system y^+L activity are reported as independent of a change in pHo in mammalian cells [11,14]. However, there are no reports addressing whether the activity of these membrane transport systems is modulated by the pHi.

Increased L-arginine transport mediated by system y^+/CAT-1 [15] and system y^+L [10] results in higher eNOS activity in HUVECs and other cell types [16,17]. Interestingly, intracellular alkalization activates eNOS in HUVECs [18], human pulmonary arterial endothelial cells (HPAECs) [19], and rat aorta endothelial cells (RAECs) [20]. However, it is unknown whether eNOS activation in response to a change in the pHi leading to an alkaline or acidic intracellular environment associated with system y^+/CATs and system y^+L transport activity in human endothelial cells. Intracellular alkalization due to lower NHE1 activity reduced the transport of the endogenous nucleoside adenosine in HUVECs [6]. Since adenosine is a vasodilator in most vascular beds including the foetoplacental circulation [21] via increasing the L-arginine transport and NO synthesis in HUVECs [22], and dysfunction of the foetoplacental vasculature is addressed as the cause of altered umbilical vein blood flow in growth restricted foetus [23,24], it is likely that changes in the pHi in HUVECs alters the dynamics of NO-dependent dilation mechanisms of the umbilical vein therefore limiting the delivery of nutrients to the foetus [25]. This study aimed to characterise the role of a change in pHi on L-arginine transport mediated via system y^+/CATs and system y^+L and on NO synthesis in HUVECs.

2. Material and methods

2.1. Antibodies and materials

Primary monoclonal mouse *anti*-eNOS phosphorylated at serine^{1177}, *anti*-eNOS phosphorylated at threonine^{495}, and *anti*-β-actin were from Sigma Aldrich (St Louis, MO, USA). Primary monoclonal mouse *anti*-total eNOS antibody and secondary horseradish peroxidase-conjugated goat *anti*-mouse antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). For isolation of HUVECs from umbilical cords, Collagenase Type II from *Clostridium histolyticum* (Boehringer, Mannheim, FRG) was used. Medium M199, newborn (NBCS) and foetal calf (FCS) sera, L-glutamine, and penicillin-streptomycin were from Gibco Life Technologies (Carlsbad, CA, USA). L-[^{3}H]Arginine and D-[^{3}H]mannitol were from NEN (Dreieich, FRG). N^G-Nitro-L-arginine methyl ester (L-NAME) was from Sigma Aldrich, Immobilon-P polyvinylidene difluoride membranes from BioRad Laboratories (Hertfordshire, UK), and the fluorescent dye 4-amino-5-methylamino-2′,7′-difluorofluorescein (DAF-FM) from Molecular Probes (Leiden, The Netherlands).

2.2. Study group

This study included samples collected from 23 full-term normal pregnancies from the Hospital Clínico UC-CHRISTUS (HCUC-C) in Santiago de Chile and Clínica de la Mujer (CLM) in Antofagasta (Chile). Pregnant women included in this study did not smoke or consume drugs or alcohol and had no intrauterine infection or any other medical or obstetrical complications. The ethnicity of patients involved in this study was Hispanic. The investigation conforms to the principles outlined in the Declaration of Helsinki. Ethics Committee approvals from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and CLM and informed written consent of patients were obtained.

2.3. Human placenta and umbilical cords

Placentas were collected at delivery on ice and transferred to the laboratory until use 15–30 min later. Middle sections of umbilical cords (100–120 mm length) were dissected into 200 mL phosphate-buffered saline (PBS) solution (mmol/L: 130 NaCl, 2.7 KCl, 0.8 Na_2HPO_4, 1.4 KH_2PO_4, pH 7.4, 4 °C) until use 6–12 h later for isolation of endothelial cells [6,26].

2.4. Cell culture

This study was done in primary cultures of HUVECs from normal pregnancies. The reason why selecting this type of cells is because (i) they are from the umbilical vein which carries feotal blood after crossing the placenta circulatory bed towards the foetus body with the umbilical vein blood being rich in oxygen and nutrients and unloaded of toxins and waste from the foetus circulation, (ii) umbilical vein blood carries signalling molecules that are transferred from the mother through the placenta into the foetal circulation, (iii) molecules synthesised and released within the placenta tissue are available at the umbilical vein blood thus transferring regulatory signals from the placenta to this vessel by changing, for example, the offering of nutrients to the growing foetus, and (iv) HUVECs release extracellular vesicles, including exosomes, that could potentially alter the downstream vasculature (i.e., the foetal circulation) altering or changing the function or phenotype of the endothelium in the foetal vascular bed [27,28]. HUVECs were isolated by collagenase digestion (0.25 mg/mL collagenase) from umbilical cords obtained at delivery from normal pregnancies and cultured (37 °C, 5% CO_2) in 1% gelatin-coated Petri dishes (100 mm diameter) up to passage 3 in primary culture medium (PCM; M199 containing 5 mmol/L D-glucose, 10% NBCS, 10% FCS, 3.2 mmol/L L-glutamine and 100 U/mL penicillin-streptomycin) as reported [6,26]. Sixteen hours prior experiments the incubation medium was changed to M199 medium containing 0.25% NBCS and 0.25% FCS. Experiments were in the absence or presence of N^G-nitro-L-arginine methyl ester (L-NAME, 100 μmol/L, NOS inhibitor) and cell viability was assayed using 3-(4,5-dimethylthiazol-2-*yl*)-2,5-diphenyltetra-zolium bromide assay (Sigma-Aldrich) as reported [6].

2.5. pHi measurement and recovery

Cells were loaded (10 min, 37 °C) with the fluorescent pH-sensitive probe 2,7-bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, 12 μmol/L) as described [6]. Probe excess was removed rinsing (×3) with control solution (CS) (mmol/L: NaCl 145, KCl 5, NaH_2PO_4 1, Na_2SO_4 1, $CaCl_2$ 1.8, $MgCl_2$ 1, HEPES 30, D-glucose 5, pH 7.4, 37 °C). Fluorescence ratios were registered every 0.5-seconds interval. The pHi was estimated using standard calibration curves with 10 μmol/L nigericin and high-K^+ in a calibrating solution (pH 6.2, 7.2, 8.2) as described [6]. The pH_i recovery was examined by the NH_4Cl pulse technique [6]. After the basal pHi was stabilized (~3 min) cells were exposed (2 min) to CS with 0.1, 1, or 20 mmol/L NH_4Cl (NH_4Cl/CS solution). Cells were then rinsed with NH_4Cl-free CS, and cell viability assayed as above.

2.6. Uptake of L-arginine

Since pHi recovery started after 25 s of removal of NH_4Cl/CS, transport assays in CS were performed at 20 s (37 °C). To identify the involvement of system y^+/CATs and system y^+L on L-arginine transport the cells were incubated with CS with or without 200 μmol/L *N*-ethylmaleimide (NEM) (a general inhibitor of system y^+/CATs) [1,11], 2 mmol/L L-leucine (a neutral amino acid that competes with L-arginine for system y^+L) [1,11], or NEM + L-leucine as previously described [10]. Overall uptake at 2 and 100 μmol/L L-arginine (6 μCi/mL L-[^{3}H] arginine, 20 s, 37 °C) for system y^+L and system y^+/CATs, respectively, was measured in confluent cells in CS as described [10,26]. The fraction of uptake inhibited by NEM was considered as system y^+/CATs mediated, and the portion of uptake inhibited by L-leucine in cells