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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

coincubated with NEM to block system y^+/CATs contribution was regarded as system y^+L mediated [1,10,11].

2.7. Kinetics of L-arginine transport

Overall 0–20 μmol/L or 0–1000 μmol/L L-arginine transport (for system y^+L and system y^+/CATs, respectively) was measured in CS as above. Overall transport of L-arginine was defined as the sum of a saturable component plus a non-saturable, linear component of transport in the ranges of L-arginine concentrations used in this study (hereafter referred as a K_D value defined by $m \cdot [Arg]$, where m corresponds to slopes of lineal phases of transport at a given L-arginine concentration [Arg]) [26]. Cell monolayers were rinsed with ice-cold CS to terminate tracer uptake.

The initial rate of transport (i.e., linear uptake up to 10 s) was derived from the slope of the linear phases of L-arginine transport. Values for transport were adjusted to the one phase exponential association equation considering the least squares fit:

$$v_i = V_m \cdot (1 - e^{-(k \cdot t)})$$

where v_i is initial velocity, V_m is mayor velocity at a given time (t) and L-arginine concentration, and e and k are constants. Overall L-arginine transport at initial rates was adjusted to the Michaelis-Menten hyperbola plus a nonsaturable, linear component (K_D) as described [26]. The saturable transport of L-arginine was derived by subtracting the $m \cdot [Arg]$ components from overall transport, and the transport kinetic parameters maximal velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) of transport were calculated [26].

The relative contribution of system y^+L and system y^+/CATs ($^{y+L/y+}F$) to total transport (i.e., y^+L plus y^+/CATs mediated transport) in cells non-treated ($-NH_4$) or treated ($+NH_4$) with NH_4Cl was estimated from V_{max}/K_m values by:

$$^{y+L/y+}F_{-NH4} = \frac{^{y+L}V_{max} \cdot {}^{y+}K_m}{^{y+L}K_m \cdot {}^{y+}V_{max}}$$

or

$$^{y+L/y+}F_{+NH4} = \frac{^{y+L}V_{max} \cdot {}^{y+}K_m}{^{y+L}K_m \cdot {}^{y+}V_{max}}$$

where $^{y+L}V_{max}$ and $^{y+L}K_m$ are kinetic parameters for system y^+L-saturable transport, and $^{y+}V_{max}$ and $^{y+}K_m$ for system y^+/CATs saturable transport.

The relative effect of NH_4Cl on transport activity via system y^+L ($1/^{-NH4/+NH4}F_{y+L}$) or system y^+/CATs ($1/^{-NH4/+NH4}F_{y+}$) was estimated by:

$$\frac{1}{^{-NH4/+NH4}F_{y+L}} = \frac{^{+NH4}V_{max} \cdot {}^{-NH4}K_m}{^{-NH4}V_{max} \cdot {}^{+NH4}K_m}$$

or

$$\frac{1}{^{-NH4/+NH4}F_{y+}} = \frac{^{+NH4}V_{max} \cdot {}^{-NH4}K_m}{^{-NH4}V_{max} \cdot {}^{+NH4}K_m}$$

where $^{-NH4}V_{max}$ and $^{-NH4}K_m$, or $^{+NH4}V_{max}$ and $^{+NH4}K_m$ are kinetic parameters for transport in cells non-treated or treated with NH_4Cl, respectively [6].

The efficiency of the effect of a change in the pHi (E_{pHi}) on the uptake at a fixed concentration ($^{U}E_{pHi}$) or the maximal transport capacity ($^{Vmax/Km}E_{pHi}$) for a range of concentrations of L-arginine via system y^+/CATs and system y^+L was estimated by:

$$^{U}E_{pHi} = \frac{^{-NH4}U - {}^{+NH4}U}{^{-NH4}pHi - {}^{+NH4}pHi}$$

or

$$^{Vmax/Km}E_{pHi} = \frac{^{-NH4}V_{max}/K_m - {}^{+NH4}V_{max}/K_m}{^{-NH4}pHi - {}^{+NH4}pHi}$$

where uptake (U) at a given concentration of L-arginine (2 or 100 μmol/L in this study) was measured in the absence ($-NH_4$) or presence ($+NH_4$) of NH_4Cl at basal pHi (^{-NH4}pHi) or pHi in the presence of NH_4Cl (^{+NH4}pHi). For a range of concentrations of L-arginine (0–20 or 0–1000 μmol/L in this study), the values for V_{max}/K_m were used. Each transport assay was run in duplicate with transport activity expressed as pmol/μg protein/min. Values for $^{U}E_{pHi}$ and $^{Vmax/Km}E_{pHi}$ are expressed as a change in the pmol/μg protein/min relative to 1. Radioactivity in 0.5 N KCl cell digests was determined by liquid scintillation counting, and uptake was corrected for D-[^{3}H]mannitol disintegrations per minute (d.p.m.) in the extracellular space [26].

2.8. NOS activity

NOS activity was assayed by quantification of the intracellular content of L-citrulline by high-performance liquid chromatography in confluent HUVECs in the absence or presence of 100 μmol/L L-NAME, as reported [15,26].

2.9. Western blot for eNOS

Total protein was obtained from confluent cells washed twice with ice-cold PBS and harvested in 100 μL of lysis buffer composed by 63.7 mmol/L Tris/HCl (pH 6.8), 10% glycerol, 2% sodium dodecylsulphate, 1 mmol/L sodium orthovanadate, 50 mg/mL leupeptin, and 5% 2-mercaptoethanol, as described [26]. Cells were sonicated (6 cycles, 5 s, 100 W, 4 °C), and total protein was separated by centrifugation (14,000g, 15 min, 4 °C). Proteins (60 μg) were separated by polyacrylamide gel (10%) electrophoresis and transferred onto Immobilon-P polyvinylidene difluoride membranes. The proteins were then probed against total eNOS (1:500 dilution, 12 h, 4 °C), eNOS phosphorylated at serine^{1177} (P∼Ser^{1177}-eNOS, 1:1000 dilution, 12 h, 4 °C), eNOS phosphorylated at threonine^{495} (P∼Thr^{495}-eNOS, 1:1000 dilution, 12 h, 4 °C), and β-actin (1:3000, 1 h, room temperature). Membranes were rinsed in Tris buffer saline-Tween (TBS-T) and incubated (1 h) in TBS-T/0.2% BSA containing secondary horseradish peroxidase-conjugated antibodies. Proteins were detected by enhanced chemiluminescence (film exposure time was 1 min) in a ChemiDoc-It 510 Imagen System (UVP, LCC Upland, CA, USA) and quantified by densitometry [26].

2.10. Statistical analysis

The sample size was estimated considering a power of 80% to detect a difference between groups (by a two-sided alpha level of 0.05). Values for clinical parameters are given as mean ± S.D. For in vitro assays the values were mean ± S.E.M., where n indicates the number of different biological samples and corresponding cell cultures with 3–4 replicates per experiment. Comparisons between two groups were performed using Student's unpaired t-test and between more than two groups by analysis of variance (ANOVA, two-ways). If the ANOVA demonstrated a significant interaction between variables, post hoc analyses were performed by the multiple-comparison Bonferroni test. The statistical software GraphPad InStat 3.1 and GraphPad Prism 7.0d (GraphPad Software Inc., San Diego, CA, USA) was used for data analysis. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Study group

Pregnant women included in this study were with normal pregnancy, normotensive, normal fasting glycaemia at delivery, singleton, and of similar age and height (Table 1). Weight and body mass index

Table 1
Clinical variables in pregnant women and newborns.

Mother	
Age (years)	31.4 ± 3.9 (26.2–37.5)
Height (cm)	161.7 ± 3.9 (154.1–163.2)
Weight (kg)	
9–16 weeks of gestation	56.2 ± 3.4 (54.6–59.5)
Delivery	68.1 ± 3.9 (64.2–69.1)*
BMI (kg/m^2)	
9–16 weeks of gestation	21.5 ± 1.7 (20.1–22.3)
Delivery	26.1 ± 0.9 (25.1–29.3)*
Mean arterial pressure (mm Hg)	
9–16 weeks of gestation	77.1 ± 3.5 (75.5–79.2)
Delivery	81.7 ± 6.1 (79.8–89.2)
Glycemia fasting (mg/dL)	83.1 ± 6.9 (74.9–89.5)
OGTT (mg/dL)	
Glycemia basal	82.7 ± 3.2 (78.2–85.3)
Glycemia 2 h after glucose	83.3 ± 4.1 (82.1–89.0)
Newborn	
Sex (female/male)	13/10
Gestational age (weeks)	38.3 ± 0.7 (38.0–38.9)
Birth weight (g)	3182 ± 210 (3051–3346)
Height (cm)	49.7 ± 1.2 (48.1–52.2)
Ponderal index (g/cm^3 × 100)	2.59 ± 0.12 (2.12–2.63)

Women that coursed with normal pregnancies (n = 23) were included in this study. Weight, body mass index (BMI), and blood pressure were determined at the first interview with the obstetrician (9–16 weeks of pregnancy) and at delivery. BMI was calculated by weight in kilograms divided by the square of the height in meters. Ponderal index was calculated by weight in grams divided by the cube of height in centimeters multiplied by 100. Oral glucose tolerance test (OGTT) was measured at the 1st trimester of pregnancy in all women with a normal glycaemia (see Material and methods section). Values are mean ± S.D. plus range in brackets.

* $P < 0.05$ versus corresponding values at 9–16 weeks of gestation.

(BMI) at delivery were higher compared with the first determination early in pregnancy (9–16 weeks of gestation) where women were normoweight (BMI < 25 kg/m^2). The total gestational weight gain between early in pregnancy and delivery was 11.9 ± 0.7 kg with a BMI variation of 0.6 kg/m^2 reaching BMI values that were > 25–29.9 kg/m^2.

3.2. Basal pHi

Exposure of cells to 20 mmol/L NH_4Cl increased the pHi value, and NH_4Cl removal caused rapid acidification (~1 s, pHi = 6.51 ± 0.04) lasting for ~25 s before a significant pHi recovery started reaching initial pHi value in ~6 min (Fig. 1A). The basal pHi value (7.19 ± 0.03) was reduced by NH_4Cl in a concentration-dependent manner (half-maximal effective concentration (EC_{50}) = 1.29 ± 0.03 mmol/L NH_4Cl, equivalent to pHi = 6.75 ± 0.02) (Fig. 1B), without altering the cell survival (97–99% alive cells between 0.1 and 20 mmol/L NH_4Cl) (not shown), confirming previous observations in this cell type [6].

3.3. System y^+/CATs and system y^+L mediated uptake of L-arginine

In the absence of NH_4Cl (i.e., at basal pHi), the overall uptake of 100 μmol/L L-arginine was inhibited mainly by NEM (69 ± 6%), with a minor inhibition caused by L-leucine (25 ± 3%) but blocked in cells coincubated with NEM + L-leucine (Fig. 1C). In cells exposed to 20 mmol/L NH_4Cl and washed with CS solution (i.e., pHi ~6.5), the overall uptake was reduced compared with cells in the absence of NH_4Cl in a proportion (0.14 ± 0.02 pmol/μg protein/min) that was similar in cells incubated with L-leucine in the absence of NH_4Cl (0.15 ± 0.06 pmol/μg protein/min). In the presence of NH_4Cl, the uptake of L-arginine was inhibited by NEM or NEM + L-leucine in a similar proportion (0.42 ± 0.02 and 0.44 ± 0.02 pmol/μg protein/min, respectively), but NH_4Cl did not alter the uptake of L-arginine inhibited by L-leucine in the absence of this salt. Uptake of L-arginine mediated by system y^+/CATs predominates over a minor contribution of system y^+L at 100 μmol/L L-arginine (Fig. 1D).

The overall uptake at 2 μmol/L L-arginine (0.17 ± 0.05 pmol/μg protein/min) in the absence of NH_4Cl was lower (67 ± 3%) compared with 100 μmol/L L-arginine (Fig. 1E). The uptake detected at this concentration of L-arginine was similar to the fraction of uptake inhibited by L-leucine in 100 μmol/L L-arginine (0.15 ± 0.06 pmol/μg protein/min). Overall uptake was unaltered by NEM but blocked by L-leucine or NEM + L-leucine. In the presence of NH_4Cl the 2 μmol/L L-arginine uptake was abolished in all experimental conditions. Uptake of L-arginine mediated by system y^+L accounted for 2 μmol/L L-arginine in HUVECs (Fig. 1F). NH_4Cl did not alter 100 or 2 μmol/L L-arginine uptake via system y^+/CATs. However, the system y^+L activity in the presence of NH_4Cl at these two concentrations of L-arginine was abolished.

3.4. pHi-dependent uptake of L-arginine via system y^+L/CATs and system y^+L

Overall uptake of 100 μmol/L L-arginine was unaltered by 0.1 or 1 mmol/L NH_4Cl in the absence of NEM and by 0.1 mmol/L NH_4Cl in the presence of NEM, but reduced by 20 mmol/L NH_4Cl in the absence or presence of NEM (Fig. 2A). Incubation of cells with NEM resulted in a NH_4Cl concentration-dependent inhibition of uptake (EC_{50} = 0.32 ± 0.04 mmol/L NH_4Cl). Overall uptake of 2 μmol/L L-arginine in the presence of NEM was inhibited by NH_4Cl (EC_{50} = 0.31 ± 0.0.3 mmol/L NH_4Cl), but uptake was unaltered in cells incubated with NEM + L-leucine (Fig. 2B). Uptake mediated by system y^+/CATs was not significantly altered by NH_4Cl (Fig. 2C) and independent of the resulting pHi (Fig. 2D). However, uptake mediated by system y^+L was reduced in a concentration-dependent manner by NH_4Cl (EC_{50} = 0.29 ± 0.03 mmol/L NH_4Cl) and the resulting acidic pHi (EC_{50} = 6.89 ± 0.11 pHi).

The efficiency of inhibition of a change in pHi (ΔpHi) on 2 μmol/L L-arginine uptake ($^{U}E_{pHi}$) mediated via system y^+L was higher at the smaller variation of pHi (ΔpHi 0.06 in this study) and less pronounced but reaching comparable values at higher variations of pHi (ΔpHi 0.38 and 0.69 in this study) from the basal pHi value in HUVECs (Fig. 2E). However, the $^{U}E_{pHi}$ for uptake via system y^+/CATs was unaltered by the pHi. The $^{U}E_{pHi}$ for system y^+L compared with system y^+/CATs mediated uptake was higher at pHi 7.19 compared with uptake at pHi 6.81 or 6.5 (Fig. 2F).

3.5. pHi-dependent system y^+/CATs and system y^+L transport kinetics

In the absence of NH_4Cl, the overall transport of L-arginine in the range of 0–1000 μmol/L was semi-saturable, inhibited mainly by NEM but marginally inhibited by L-leucine, and blocked by NEM + L-leucine (not shown) as previously reported [10]. The derived transport obtained after subtracting the linear, non-saturable component from overall transport (i.e., K_D), was saturable in all experimental conditions and adjusted to a first order linear regression in Eadie-Hofstee plots as reported [10,15]. Incubation of cells with increasing concentrations of NH_4Cl did not alter the K_D for overall transport and the V_{max}, K_m, or V_{max}/K_m for saturable transport in this range of L-arginine concentrations (Table 2).

Overall transport of L-arginine in the range of 0–20 μmol/L L-arginine was semi-saturable, unaltered by NEM but abolished by L-leucine or NEM + L-leucine (not shown) as previously reported [10]. The derived saturable L-arginine transport was unaffected by NEM but blocked by L-leucine and NEM + L-leucine (Fig. 3A). A first-degree regression line well fitted saturable transport at different pHi values in Eadie-Hofstee plots (Fig. 3B). Increasing concentrations of NH_4Cl did not alter the K_D for overall transport but reduced the V_{max} (Table 2) and V_{max}/K_m (Fig. 3C) without changing the K_m for saturable transport.

The efficiency of inhibition of a given ΔpHi in 0–20 μmol/L L-arginine transport kinetic parameters ($^{Vmax/Km}E_{pHi}$) for system y^+L was also

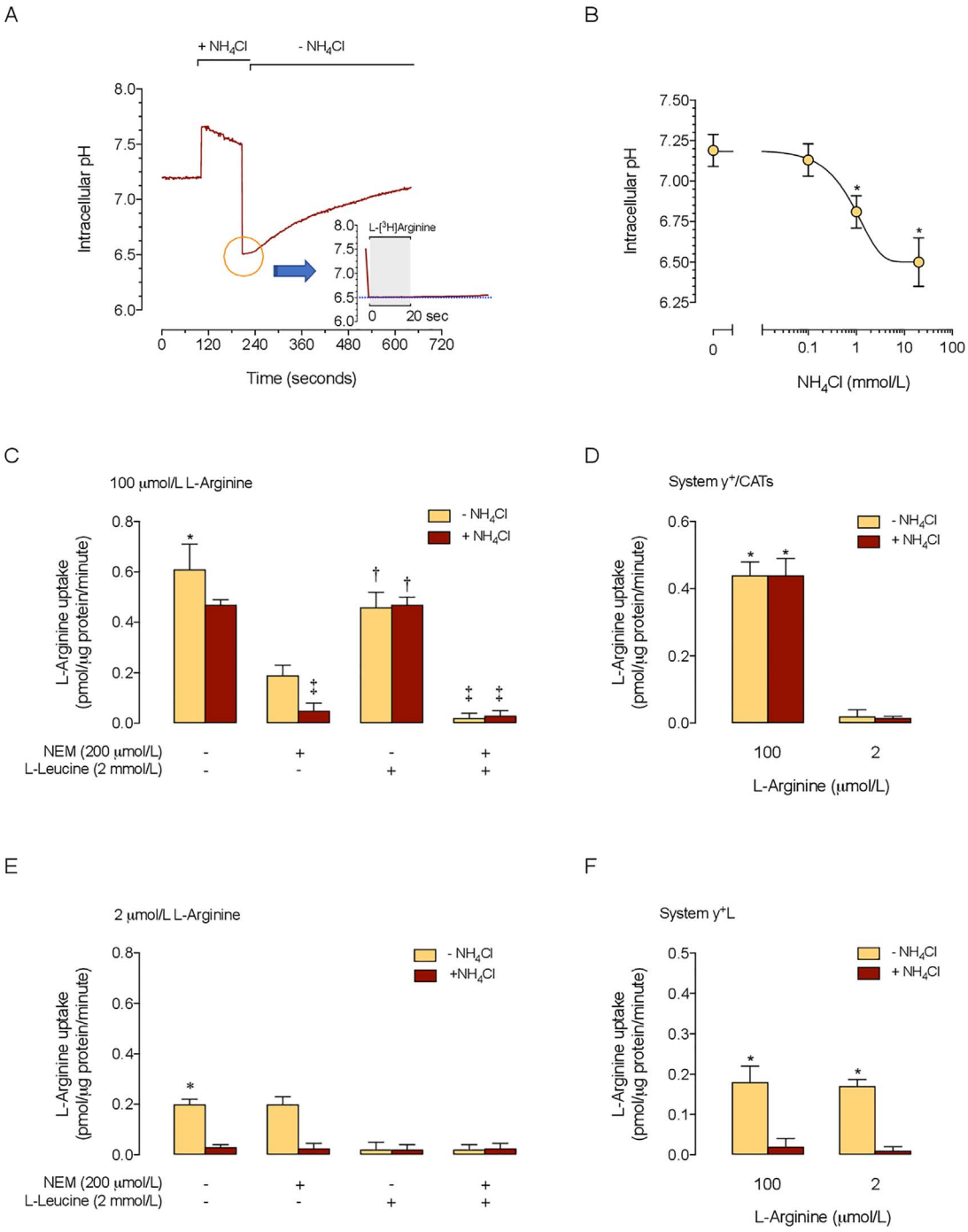

Fig. 1. Effect of cell pHi on L-arginine uptake in HUVECs. A. Cells were preloaded with BCECF-AM and transferred into a spectrofluorometer. After basal pHi stabilisation, the cells were exposed (2 min) to a control solution containing 20 mmol/L NH_4Cl (+NH_4Cl). Cells were then rinsed with a NH_4Cl-free solution (−NH_4Cl) and left in this medium for pHi recovery (see Material and methods section). A typical record is shown. The *insert* indicates the data for the first 55 s after removal of NH_4Cl. The grey area indicates the time (10 s) used for overall uptake of 2 or 100 µmol/L L-arginine (6 µCi/mL L-[^{3}H]arginine, 37 °C). B. pHi values for cells exposed to a Na^+-free solution without (0) or with NH_4Cl. C. Overall 100 µmol/L L-arginine uptake in +NH_4Cl or −NH_4Cl solution in the absence (−) or presence (+) of *N*-ethylmaleimide (NEM) or L-leucine. D. L-Arginine uptake mediated via system y^+/CATs derived from data in C. E. Overall 2 µmol/L L-arginine uptake as in C. F. L-Arginine uptake mediated via system y^+L derived from data in E. In B, $^*P < 0.05$ versus without or with 0.1 mmol/L NH_4Cl. In C, $^*P < 0.05$ versus all other values, †$P < 0.05$ versus corresponding values in the presence of NEM or NEM + L-leucine. ‡$P < 0.05$ versus values in −NH_4Cl in the presence of NEM. In D, $^*P < 0.03$ versus corresponding values in 2 µmol/L L-arginine. In E, $^*P < 0.03$ versus all other values except for −NH_4Cl in the presence of NEM. In F, $^*P < 0.03$ versus corresponding values in +NH_4Cl. Values are mean ± S.E.M. ($n = 18$).

higher at the smaller variation of pHi (ΔpHi 0.06) and less pronounced but reaching comparable values at higher variations of pHi (ΔpHi 0.38 and 0.69) from the basal pHi (Fig. 3D). The pHi unaltered the $^{Vmax/Km}E_{pHi}$ for transport via system y^+/CATs. The $^{Vmax/Km}E_{pHi}$ for system y + L compared with system y^+/CATs mediated transport was higher at all pHi used in this study (Fig. 3E).

3.6. NOS activity

In the absence of NH_4Cl, total synthesis of L-citrulline in cells incubated with 100 µmol/L L-arginine was partially reduced by NEM and L-leucine but abolished by NEM + L-leucine (Fig. 4A). NH_4Cl similarly inhibited L-citrulline synthesis in the absence or presence of L-leucine

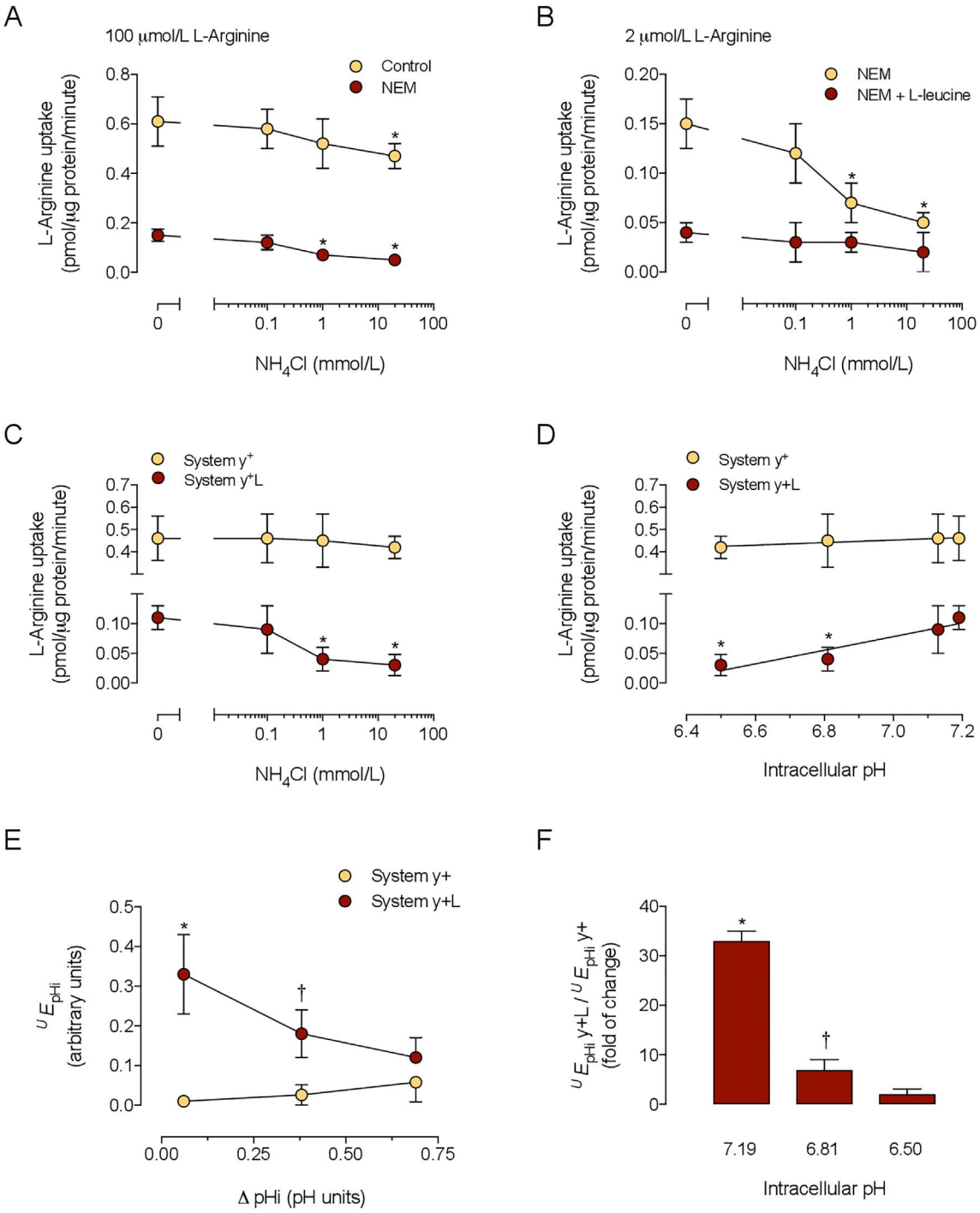

Fig. 2. Effect of pHi on L-arginine uptake in HUVECs. A. L-Arginine (100 μmol/L) uptake (6 μCi/mL L-[^{3}H]arginine, 10 s, 37 °C) in primary cultures of HUVECs non-treated (0) or treated with increasing concentrations of NH_4Cl as described in Materials and methods. Cells were in the absence (Control) or presence of 200 μmol/L *N*-ethylmaleimide (NEM). B, L-Arginine (2 μmol/L) uptake as in A in the presence of 200 μmol/L NEM (i.e., Control for this concentration of L-arginine) or NEM plus 2 mmol/L L-leucine (NEM + L-leucine). C. L-Arginine uptake via system y^+/CATs and system y^+L derived from data in A and B, respectively, in the absence of presence of NH_4Cl. D. L-Arginine uptake against pHi values in cells as in C. E. Efficiency of a change in the pHi (ΔpHi) on the uptake of L-arginine ($^UE_{pHi}$) via system y^+/CAT and system y^+L from data in D. Values for $^UE_{pHi}$ are expressed as a change in the pmol/μg protein/min relative to 1 (see Material and methods section). F. Relative $^UE_{pHi}$ for system y^+/CAT and system y^+L from data in E. In A, B, and C, $^*P < 0.05$ versus corresponding values without NH_4Cl. In D, $^*P < 0.05$ versus corresponding values at pHi 7.13 and pHi 7.19. In E, $^*P < 0.03$ versus all other corresponding values, †$P < 0.05$ versus corresponding value at ΔpHi = 0.69 pHi units. In F, $^*P < 0.03$ versus all other values, †$P < 0.05$ versus values at pHi = 6.5. Values are mean ± S.E.M. ($n = 19$).

but blocked by NEM and NEM + L-leucine. Incubation of cells with L-NAME blocked L-citrulline synthesis in all experimental conditions. The NOS-dependent fraction of synthesis of L-citrulline was reduced by NEM or L-leucine but abolished by NEM + L-leucine (Fig. 4C). In the presence of NH_4Cl, NOS-dependent L-citrulline synthesis was decreased partially reaching similar values to those in the presence of L-leucine. However, it was abolished in the presence of NEM or NEM + L-leucine.

In the absence of NH_4Cl, total synthesis of L-citrulline in cells incubated with 2 μmol/L L-arginine was unaltered by NEM but abolished by L-leucine and NEM + L-leucine (Fig. 4B). NH_4Cl and L-NAME also abolished L-citrulline synthesis. The NOS-dependent synthesis of L-citrulline in the absence of NH_4Cl was unaltered by NEM but blocked by

Table 2
Effect of intracellular pH on the kinetic parameters for L-arginine transport in HUVECs.

		Saturable transport			Overall transport	
		V_{max} (pmol/μg protein/min)	K_m (μmol/L)	V_{max}/K_m (pmol/μg protein/min/(μmol/L))	K_D (pmol/μg protein/min/(μmol/L))	v_i (pmol/μg protein/0.5 s)
System y^+/CATs						
Without NH_4Cl	pHi 7.19	0.60 ± 0.18	79 ± 49	0.008 ± 0.004	0.0025 ± 0.0002	0.00279 ± 0.00022
With NH_4Cl (mmol/L)						
0.1	pHi 7.13	0.61 ± 0.11	97 ± 29	0.006 ± 0.002	0.0022 ± 0.0002	0.00258 ± 0.00022
1	pHi 6.89	0.58 ± 0.12	73 ± 31	0.008 ± 0.003	0.0022 ± 0.0002	0.00279 ± 0.00018
20	pHi 6.50	0.53 ± 0.08	84 ± 32	0.006 ± 0.002	0.0023 ± 0.0003	0.00240 ± 0.00021
System y^+L						
Without NH_4Cl	pHi 7.19	0.38 ± 0.11	1.98 ± 1.06	0.192 ± 0.079	0.0492 ± 0.0048	0.00159 ± 0.00019
With NH_4Cl (mmol/L)						
0.1	pHi 7.13	0.33 ± 0.08	2.01 ± 0.91	0.164 ± 0.057	0.0511 ± 0.0041	0.00137 ± 0.00019
1	pHi 6.89	0.21 ± 0.09*	2.12 ± 0.99	0.099 ± 0.044*	0.0488 ± 0.0052	0.00084 ± 0.00011*
20	pHi 6.50	0.04 ± 0.02	1.99 ± 0.12	0.020 ± 0.003	0.0479 ± 0.0048	0.00017 ± 0.00008

Transport of L-arginine (20 s, 37 °C) was measured in HUVECs from normal pregnancies. Transport assays were done in cells not treated (*Without NH_4Cl*) or treated (*With NH_4Cl*) in a NH_4Cl-acid pulse as described in Materials and methods. The resulting intracellular pH (pHi) values are indicated. Maximal velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) of saturable transport in the range of 0–20 μmol/L (for system y^+L) or 0–1000 (for system y^+/CATs) L-arginine were calculated assuming a single Michaelis-Menten hyperbola. V_{max}/K_m represents maximal L-arginine transport capacity. The lineal phase of overall transport of L-arginine (K_D) was obtained from transport data fitted to a Michaelis-Menten equation increased in a lineal component. Initial velocity (v_i) was calculated for 0.5 s with 100 or 2 μmol/L L-arginine transport. All values for V_{max}, K_m, and v_i for system y^+L are lower ($P < 0.05$) and values for V_{max}/K_m and K_D were higher ($P < 0.05$) than corresponding values for system y^+/CATs. *$P < 0.05$ versus all other corresponding values for System y^+L. Values are mean ± S.E.M. ($n = 19$).

L-leucine and NEM + L-leucine (Fig. 4D). NH_4Cl reduced, but NEM, L-leucine, and NEM + L-leucine blocked the NOS-dependent synthesis of L-citrulline.

3.7. eNOS expression and activation

Total eNOS protein abundance was unaltered by an acidic pHi (Fig. 5A,B). Phosphorylation of eNOS at Ser^{1177} was reduced in a pHi-dependent manner (EC_{50} = 0.77 ± 0.03 pHi) (Fig. 5C); however, eNOS phosphorylation at Thr^{495} was unaltered by acidic pHi (Fig. 5D).

4. Discussion

This study shows that pHi is a factor that modulates the L-arginine transport in primary cultured HUVECs from normal pregnancies. Intracellular acidification causes a reduction in the L-arginine transport via system y^+L but not via system y^+/CATs, and in the activity of eNOS due to lower activator phosphorylation in Ser^{1177} at this enzyme. Since

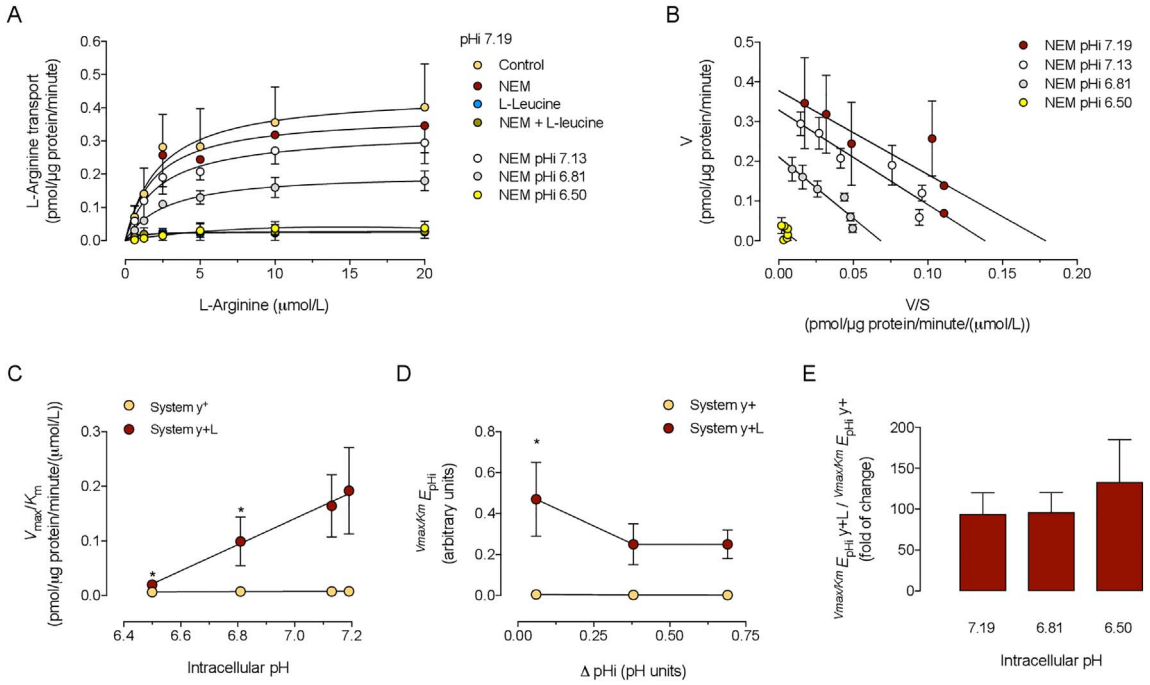

Fig. 3. Effect of NH_4Cl on L-arginine saturable transport in HUVECs. A. Saturable L-arginine transport (6 μCi/mL L-[^{3}H]arginine, 10 s, 37 °C) was measured in primary cultures of HUVECs non-treated (Control, i.e., pHi 7.19) or treated with 0.1, 1, or 20 mmol/L NH_4Cl reaching pHi 7.13, 6.81, or 6.5, respectively (see Material and methods section). Cells at pHi 7.19 were in the absence or presence of 200 μmol/L *N*-ethylmaleimide (NEM) or NEM plus 2 mmol/L L-leucine (NEM + L-leucine). Cells in pHi 7.13, 6.81, or 6.5 were exposed to NEM. B. Eadie-Hofstee plots for transport data in cells in the presence of NEM as in A. C. Maximal transport capacity (V_{max}/K_m) for systems y^+/CATs and system y^+L from data in A (see also Table 2). D. The efficiency of a change in the pHi (ΔpHi) on maximal transport capacity for L-arginine ($^{Vmax/Km}E_{pHi}$) via system y^+/CATs and system y^+L from data in A. Values for $^{Vmax/Km}E_{pHi}$ are expressed as a change in the pmol/μg protein/min relative to 1 (see Material and methods section). E. Relative $^{Vmax/Km}E_{pHi}$ for system y^+/CAT and system y^+L from data in D. In C, *$P < 0.05$ versus corresponding values at pHi 7.13 and 7.19. In D, *$P < 0.05$ versus all other values. Values are mean ± S.E.M. ($n = 19$).

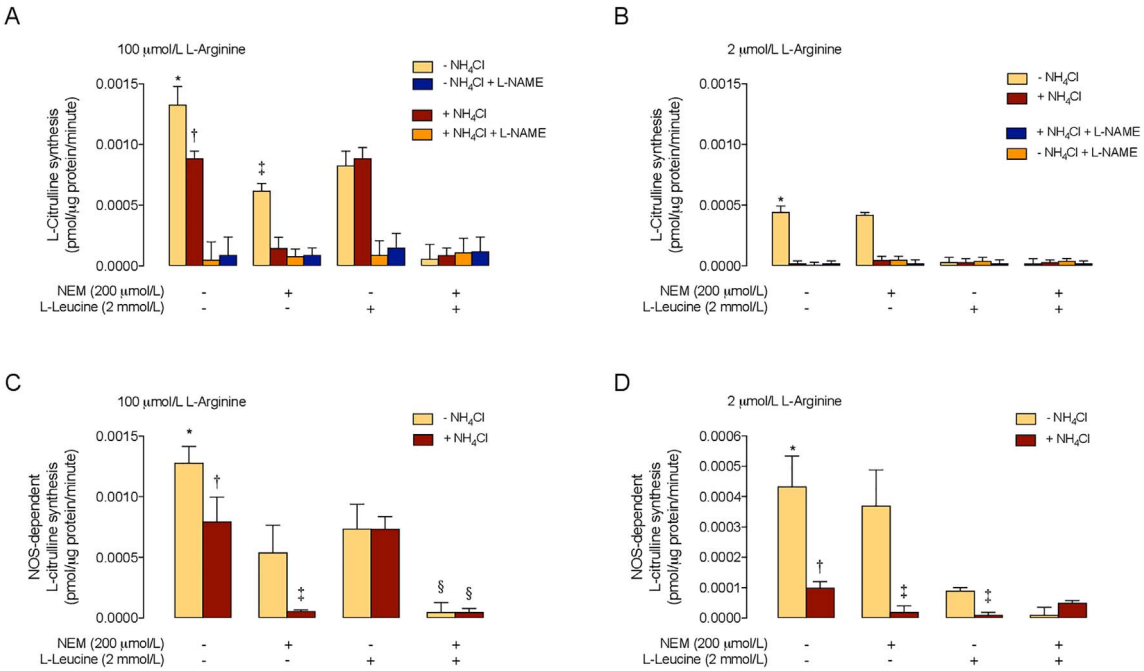

Fig. 4. pHi dependency of NOS activity in HUVECs. A. Intracellular L-citrulline level was determined by H.P.L.C. in primary cultures of HUVECs not treated ($-NH_4Cl$) or treated ($+NH_4Cl$) with 20 mmol/L NH_4Cl in the absence or presence of 100 µmol/L N^G-nitro- L-arginine methyl ester (L-NAME) as described in Material and Methods section. Assays were in cells in 100 µmol/L L-arginine in the absence or presence of 200 µmol/L *N*-ethylmaleimide (NEM) or NEM plus 2 mmol/L L-leucine (NEM + L-leucine). B. The intracellular L-citrulline level in HUVECs in 2 µmol/L L-arginine as in A. C. Nitric oxide synthase (NOS) activity-dependent L-citrulline synthesis derived from data in A. D. Nitric oxide synthase (NOS) activity-dependent L-citrulline synthesis derived from data in B. In A, $^*P < 0.05$ versus all other values, $\dagger P < 0.05$ versus corresponding values except in the presence of L-leucine. $\ddagger P < 0.05$ versus all other corresponding values. All values in the presence of L-NAME are significantly different ($P < 0.03$) from values in the absence of this inhibitor. In B, $^*P < 0.03$ versus all other values. In C, $^*P < 0.05$ versus all other values, $\dagger P < 0.05$ versus all other corresponding values except in the presence of L-leucine, $\ddagger P < 0.05$ versus all other corresponding values except in the presence of NEM + L-leucine, $\S P < 0.03$ versus corresponding values in the presence of L-leucine. In D, $^*P < 0.05$ versus all other values except for $-NH_4Cl$ in the presence of NEM, $\dagger P < 0.05$ versus all other corresponding values, $\ddagger P < 0.05$ versus corresponding values in $-NH_4Cl$. Values are mean ± S.E.M. ($n = 19$).

NO is involved in a broader range of biological effects other than regulation of vascular tone, intracellular acidification may have significant implications in diseases associated with endothelial dysfunction, such as gestational diabetes mellitus and cancer, where the pHo and pHi are altered [6,27–29].

HUVECs show a pHi ~ 7.19 as previously reported (pHi ~ 7.21) [6,30,31], and is close to the pH reported in the human umbilical vein blood (pH ~ 7.35) [25,32–34]. Intracellular acidification down-regulates the transport activity of the human equilibrative nucleoside transporters 1 and 2 in HUVECs [6], and Na^+/H^+ exchanger 1 in human lymphoblasts [34] and the human colonic carcinoma T_{84} cell line [35]. Since overall L-arginine transport was reduced as the pHi changed to acidic in HUVECs, L-arginine transport mechanisms are responsive to a change in the pHi in this cell type. Inhibition by the acidic pHi was partial and similar to that induced by L-leucine at basal or acidic pHi in the presence of 100 µmol/L L-arginine. Considering the apparent K_m for L-arginine uptake via hCAT-1 (K_m ~ 120 µmol/L) and hCAT-2B (K_m ~ 250 µmol/L) in this cell type [1,2,36] it is likely that these isoforms were involved in this phenomenon. L-Arginine transport via system y^+/CATs is independent of pHo in mammalian cells [2,11,37]. However, there are no studies addressing modulation of L-arginine transport by pHi in endothelium or other cell types [2,11,34,37]. Our results suggest that system y^+/CATs activity (likely hCAT-1 and hCAT-2B) is independent of intracellular acidification up to ΔpHi ~ 0.69 from the basal pHi since the effect of the ΔpHi on 100 µmol/L L-arginine uptake ($^UE_{pHi}$ 0.022 ± 0.012, range 0.01–0.06) or the V_{max}/K_m for transport ($^{Vmax/Km}E_{pHi}$ 0.0037 ± 0.0011, range 0.002–0.005) of this amino acid was unaltered.

Uptake of L-arginine is also mediated by system y^+L in HUVECs [9,10]. Uptake of 2 µmol/L L-arginine was almost exclusively mediated via system y^+L meanwhile at higher levels (100 µmol/L) transport was via system y^+/CATs and system y^+L. The results show that system y^+L transport activity was sensitive to acidic pHi in HUVECs [34,38], an effect that was higher at smaller changes from the basal pHi. Thus, system y^+L seems more efficiently modulated by a discrete change in pHi (ΔpHi 0.06 in this study) from the physiological pHi in HUVECs. Similar changes were seen with 2 µmol/L or a broader concentration of L-arginine ($^UE_{pHi}/^{Vmax/Km}E_{pHi}$ ~ 0.8). Thus, pHi modulation of system y^+L activity results from changing the V_{max}/K_m for system y^+L, an effect that results from reduced V_{max}. Several possibilities may explain this finding, i.e., (i) reduced number of transporters available at the plasma membrane due to lower expression or recycling with no change in their transport capacity, (ii) decreased transport capacity of a fixed number of membrane transporters, or (iii) both phenomena. Since pHi effect on transport was assayed for 15 s and system y^+L half-life is most likely unaltered at this incubation time, the reduced L-arginine transport may result from a lower activity rather than expression of system y^+L in HUVECs.

An increase in the activity of system y^+L associated with a higher synthesis of NO in HUVECs [1,10], human platelets [16,17], and rat cortical astrocytes [39]. Also, since system y^+L may be located close to eNOS in the plasma membrane [2], the pHi-decreased system y^+L transport activity may result in lower eNOS activity in HUVECs. Our results show that L-NAME–inhibited L-citrulline formation from L-arginine (index of NOS activity) [8] was lower at acidic pHi. This phenomenon was associated with a reduced activity of system y^+L, but not system y^+/CATs and abolished at 2 µmol/L but partially reduced at 100 µmol/L L-arginine. Interestingly, eNOS activity seems linked to system y^+/CATs (particularly hCAT-1 and hCAT-2) in HUVECs [2,40–42]. However, an intracellular L-arginine pool not fed from this amino acid extracellular content is also a supplying source for eNOS in this cell type [40,42]. Thus, CATs transport activity could be unaltered, up or downregulated and these changes will not necessarily lead to parallel changes in NOS activity. Indeed, HUVECs from late-onset

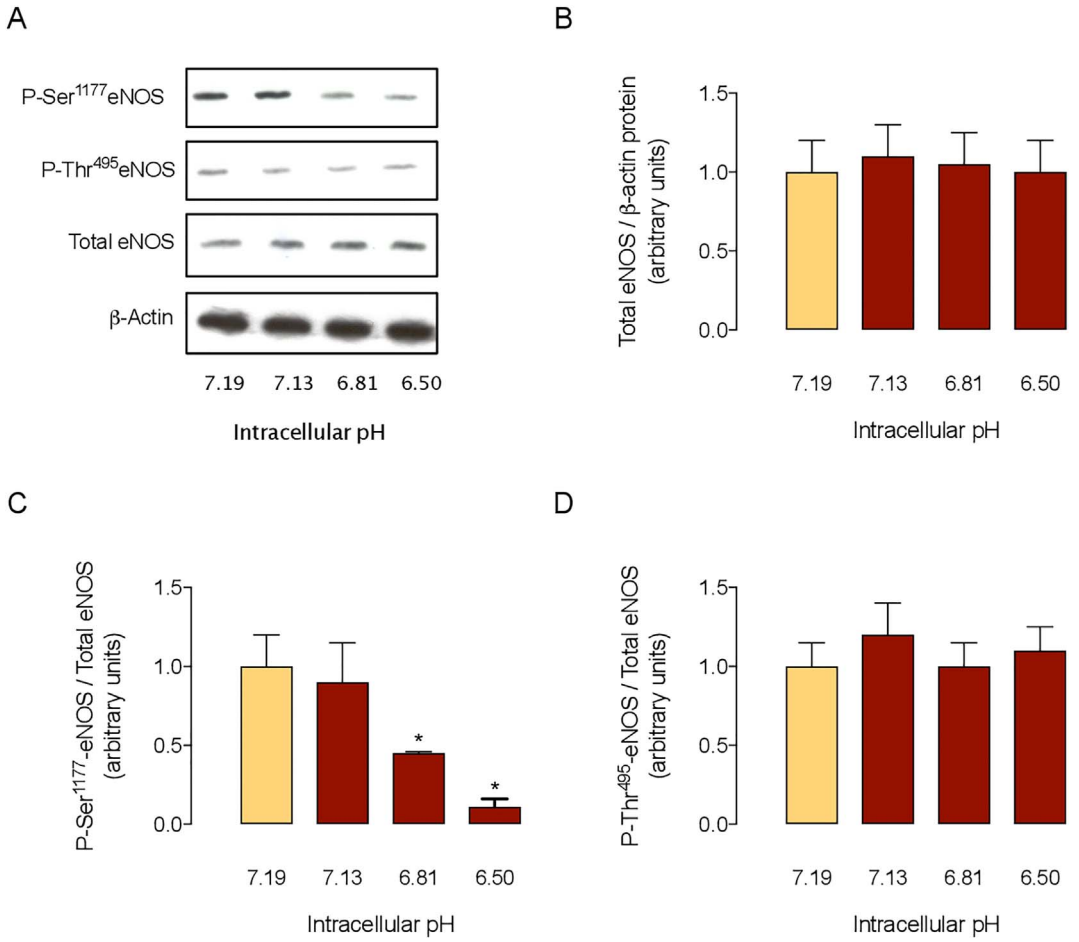

Fig. 5. pHi dependency of eNOS expression and phosphorylation in HUVECs. A. Western blot for total (Total eNOS) or phosphorylated at Serine^{1177} (P-Ser^{1177}eNOS) or Threonine^{495} (P-Thr^{495}eNOS) eNOS protein in primary cultures of HUVECs non-treated (Control, i.e., pHi = 7.19) or treated with 0.1, 1, or 20 mmol/L NH_4Cl reaching pHi 7.13, 6.81, or 6.5, respectively (see Material and methods section). β-Actin is the loading control. Total eNOS/β-actin (B), P-Ser^{1177}eNOS/Total eNOS (C) or P-Thr^{495}eNOS/Total eNOS (C) protein ratios from cells as in A. *$P < 0.05$ versus values at pHi 7.19 and 7.13. Values are mean ± S.E.M. ($n = 19$).

preeclampsia show increased hCAT-1–mediated L-arginine transport but reduced eNOS activity [43,44]. Potential explanations for this phenomenon include the possibility that system y^+/CATs activity may deliver L-arginine for NOS activity and NO generation, arginase activity for the synthesis of polyamines, or for protein synthesis [43,44].

The reduced NOS activity seen in HUVECs in an acidic pHi was likely due to lower eNOS activation since its lower activator phosphorylation at serine^{1177} [8,18,26] instead of an increased inhibitory phosphorylation of threonine^{497} [8,18,26]. Since intracellular alkalization activates eNOS in HUVECs [18], and other endothelium including human pulmonary aortic [19] and rat aortic [20] endothelial cells, intracellular acidification may result in reduced NO synthesis in HUVECs. Interestingly, a change in pHi from 7.5 to ~6.5 resulted in a more significant reduction of NOS activity compared with a pHi shift from 6.5 to 5.5 [18], thus complementing similar findings for system y^+L activity in HUVECs. Thus, a change in pHi causing intracellular acidification is a phenomenon involved in downregulation of the system y^+L/eNOS activity in HUVECs. The possibility that intracellular acidification inhibited NOS was not related to L-arginine uptake is unlikely since incubation of cells with NEM + L-leucine abolished L-arginine uptake and NO synthesis. This proposal is supported by studies in rat astrocytes knockdown for system y^+/LAT-2 expression where system y^+L activity and NO generation was reduced [39].

In summary, intracellular acidification results in reduced membrane transport of L-arginine mediated via system y^+L but not via system y^+/CATs in HUVECs (see Fig. 6). Diminished transport resulted from lower maximal transport capacity due to reduced V_{max} without significant alterations in the apparent K_m for transport. Therefore, an acidic pHi seems not to alter the intrinsic properties of system y^+L but the activity of membrane transporters in HUVECs. Interestingly, the pHi sensitivity of L-arginine transport was higher as smaller the change in the pHi from the basal pHi in this cell type. Additionally, intracellular acidification also reduced the synthesis of NO and activator phosphorylation of eNOS, which seems to result from reduced system y^+L activity and lower activation of eNOS. Interestingly, preliminary results show that basal pHi is alkaline in HUVECs exposed to an A_{2A} adenosine receptors antagonist (L Sobrevia, unpublished), suggesting that basal pHi is potentially maintained by activation of this type of adenosine receptors in HUVECs. We hypothesise that changing the pHi into an acidic intracellular environment is a phenomenon likely involved in the lower adenosine-mediated relaxation of foetoplacental vasculature via reducing the endothelial system y^+L/eNOS activity as seen in diseases of pregnancy such as preeclampsia [44,45], obesity [46,47], or gestational diabetes mellitus [21,27,28].

Conflict of interest

There is no conflict of interest.

Transparency document

The http://dx.doi.org/10.1016/j.bbadis.2018.01.032 associated with this article can be found in online version.

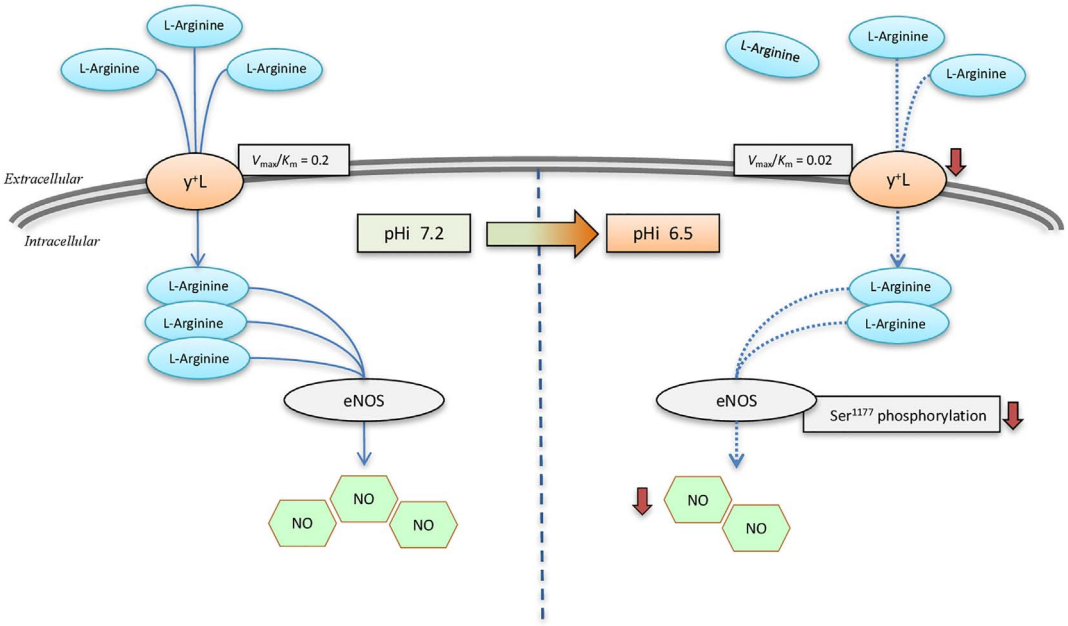

Fig. 6. Modulation of L-arginine/NO signalling pathway by intracellular pH in the human foetoplacental endothelium. The physiological intracellular pH (pHi) value in human umbilical vein endothelial cells (HUVECs) from normal pregnancies (pHi 7.2) maintain the L-arginine transport via the very high affinity transport system y^+L with a maximal transport capacity (V_{max}/K_m) of ~0.2 pmol/μg protein/min/(μmol/L). L-Arginine is metabolised by the endothelial nitric oxide synthase (eNOS) into L-citrulline and nitric oxide (NO). When pHi value is acidic (pHi 6.5), the V_{max}/K_m is reduced (⇩) to ~0.02 pmol/μg protein/min/(μmol/L) resulting in lower uptake of L-arginine (dotted lines). The reduced uptake in L-arginine transport and its subsequent lower bioavailability to eNOS leads to minor NO generation likely due to lower activator phosphorylation at serine 1177 (Ser^{1177}) residue at eNOS.

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