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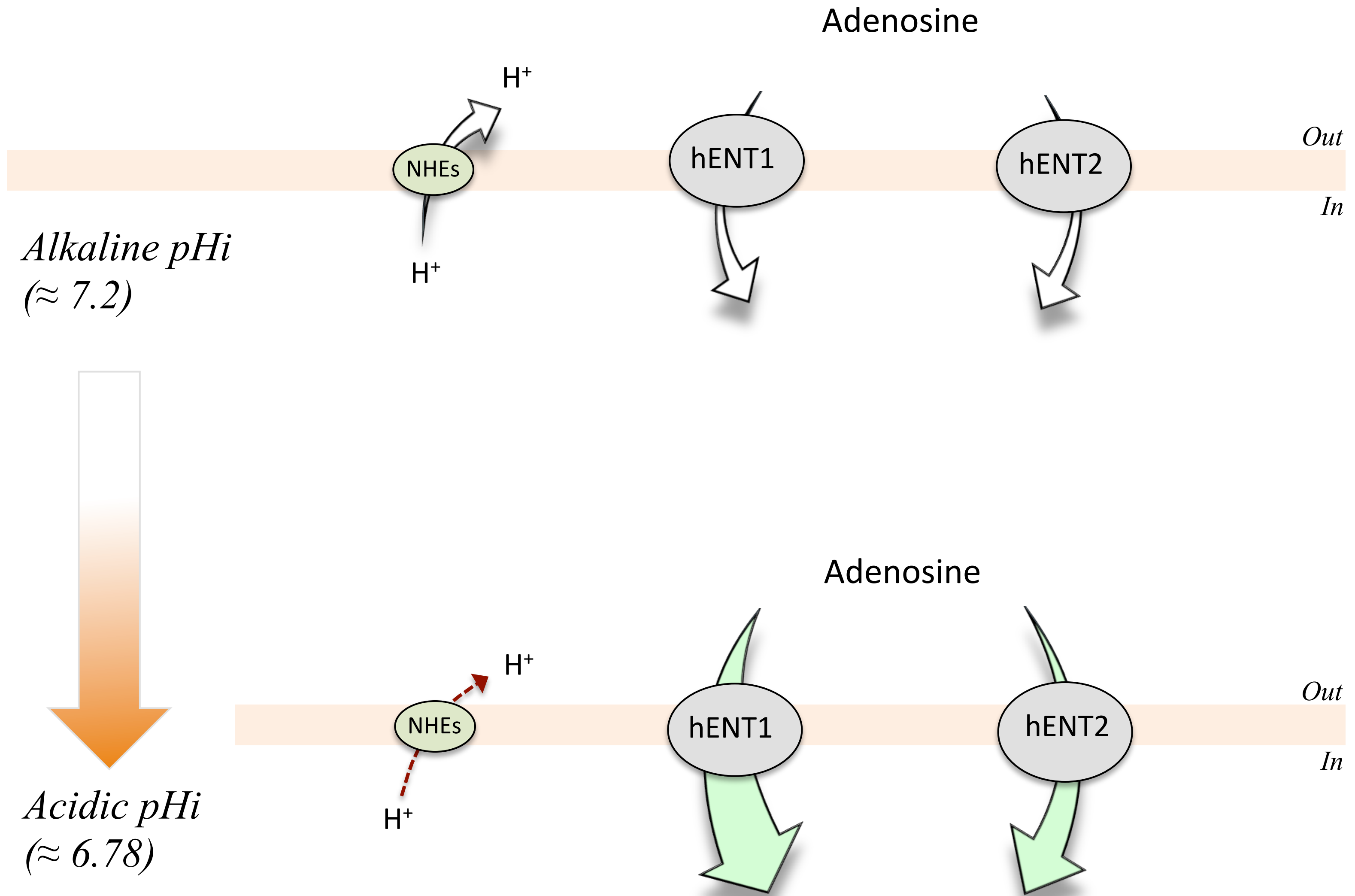
Adenosine
H+
NHEs
H+
hENT1
hENT2
Out
In
Alkaline pHi
(≈ 7.2)
Adenosine
H+
NHEs
H+
hENT1
hENT2
Out
In
Acidic pHi
(≈ 6.78)

Intracellular acidification increases adenosine transport in human umbilical vein endothelial cells

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Running title: Modulation of adenosine transport by intracellular pH

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Abstract

30 *Introduction.* Adenosine is taken up via human equilibrative nucleoside transporters 1 (hENT1) and 2 (hENT2) at a physiological extracellular pH (pHo ~7.4) in human umbilical vein endothelial cells (HUVECs). Acidic pHo increases the uptake of adenosine and 5-hydroxytryptamine (5HT) via hENT4 in this cell type. However, modulation of hENT1 and hENT2 transport activity by the pHi is unknown. We investigated whether hENT1 and
35 hENT2-adenosine transport was regulated by acidic pHi.

Methods. HUVECs loaded with a pH sensitive probe were subjected to 0.1-20 mmol/L NH_4Cl pulse assay to generate 6.9-6.2 pHi. Before pHi started to recover, adenosine transport kinetics (0-500 µmol/L, 37ºC) in the absence or presence 1 or 10 µmol/L *S*-(4-nitrobenzyl)-6-thio-inosine (NBTI), 2 mmol/L hypoxanthine, 2 mmol/L adenine, 100 µmol/L 5HT, or 500
40 µmol/L adenosine, was measured.

Results. Overall adenosine transport (i.e., hENT1+hENT2) was semisaturable and partially inhibited by 1 µmol/L, but abolished by 10 µmol/L NBTI in cells non-treated or treated with NH_4Cl. The initial velocity and non-saturable, lineal component for overall transport were increased after NH_4Cl pulse. hENT1 and hENT2-mediated adenosine transport maximal
45 capacity was increased by acidic pHi. hENT1 activity was more sensitive than hENT2 activity to acidic pHi.

Discussion. hENT1 and hENT2-adenosine transport is differentially regulated by acidic pHi in HUVECs. These findings are important in pathologies associated with pHi alterations such as gestational diabetes mellitus.

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Keywords: adenosine transport; intracellular pH; ENT transporters; endothelium; foetus

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1. Introduction

The maintenance of a physiological intracellular (pHi) and extracellular (pHo) pH is under light modulation by plasma membrane transport mechanisms that remove protons (H^+) to the extracellular space [1-3]. These phenomena include the sodium (Na^+)/H^+ exchanger 1 (NHE1) as the primary regulator of the pHi/pHo ratio. Uptake of metabolic substrates happens via different membrane transport systems in adult and foetoplacental endothelium [4,5]. Some of these transport systems depend on the pHo, such as those mediating adenosine and 5-hydroxytryptamine (5HT) [6]. The endogenous nucleoside adenosine is reported to increase the L-arginine transport and synthesis of nitric oxide (NO) in the human foetoplacental micro and macrovascular endothelium from normal or pathological pregnancies such as gestational diabetes mellitus (GDM) [7-10]. This phenomenon results from activation of adenosine receptors due to the extracellular accumulation of this nucleoside arising from a reduced uptake by the foetoplacental endothelium [8,10,11]. Thus, a proper function of membrane transport mechanisms modulating the extracellular concentration of adenosine is critical to mantaining the physiological cell metabolism in this vascular bed [4,8-10] as in other tissues [5,6].

Adenosine nucleoside is taken up via the human equilibrative nucleoside transporters (hENTs) in the human umbilical vein (HUVECs) [11] and placental microvascular (hPMECs) [12] endothelial cells. hENTs corresponds to a family of at least four proteins, i.e., hENT1, hENT2, hENT3, and hENT4. hENT1 and hENT2 mediate adenosine uptake in HUVECs from normal or pathological pregnancies, including GDM [11]. Despite the proposed activation of hENT4-mediated adenosine and 5HT transport in response to an acidic pHo in HUVECs [6], the role of a change in pHi or pHo regulating hENT1 and hENT2 transport activity in this or other cell type is unknown [3,5]. One study shows that an acidic pHi increases adenosine

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efflux in perfused rat skeletal muscle [13], but not an attempt to address the type(s) of membrane transporters involved in this phenomenon was given. Additionally, pre-gestational diabetes mellitus associated with acidic pH (pH <7.2) in the umbilical vein blood [14]. Since 80 GDM also associates with lower hENT1 and hENT2-mediated adenosine transport in HUVECs [11] and hPMECs [12], we hypothesize that adenosine transport mediated via hENT1 and hENT2 is under regulation by an acidic pHi in HUVECs.

2. Materials and methods

85 See the expanded Methods section in the online Supplementary material.

2.1. Umbilical cords and cell culture

Human placentas were collected after delivery from 31 full-term normal pregnancies from the Hospital Clínico UC-CHRISTUS in Santiago de Chile (investigation conforms to the 90 Declaration of Helsinki, and counts with Ethics Committee approval from the Faculty of Medicine of the Pontificia Universidad Católica de Chile and informed consent of patients). Sections of umbilical cords were transferred 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, 4ºC)) to the laboratory.

95 Human umbilical vein endothelial cells (HUVECs) were isolated by digestion with collagenase from umbilical cord veins and cultured (5% O_2, 5% CO_2, 37°C) in primary culture medium (PCM: medium 199 (M199, Gibco Life Technologies, Carlsbad, CA, USA), 5 mmol/L D-glucose, 10% new born calf serum, 10% foetal calf serum (Gibco), 3.2 mmol/L L-glutamine, 100 U/mL penicillin-streptomycin (Gibco)) as described [10,15]. Experiments were 100 in primary cultured cells in passage 3 in the absence or presence of 1 or 10 μmol/L *S*-(4-

nitrobenzyl)-6-thio-inosine (NBTI) (Sigma, Atlanta, GA, USA), inhibitory concentrations for hENT1 or hENT1+hENT2 transport, respectively, 2 mmol/L hypoxanthine, and 2 mmol/L adenine (nucleobases taken up via ENT2) [11]. Parallel experiments were in the presence of 100 μmol/L 5-hydroxytryptamine (5HT, an amine that is taken up via ENT4 at pHo ~5.5) [6]
105 or 500 μmol/L adenosine. Cell viability was assayed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetra-zolium bromide assay (Sigma-Aldrich) as reported [16].

2.2. pHi measurement and recovery

Cells were loaded (10 minutes, 37°C) with the fluorescent pH sensitive probe 2,7-
110 bicarboxyethyl-5,6-carboxyfluorescein acetoxymethyl ester (BCECF-AM, 12 μmol/L) (Sigma-Aldrich) as described [16-18]. Probe excess was removed rinsing (x3) 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, 37ºC)). Fluorescence ratios were registered every 2 seconds interval (150 seconds). The pHi was estimated using standard calibration curves with 10 μmol/L
115 nigericin in calibrating solution (pH 6.2, 7.2, 8.2) as described [16-18]. The pH_i recovery was examined by the NH_4Cl pulse technique [16-18]. After the basal pHi was stabilized (~3 minutes) cells were exposed (2 minutes) 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 (for media composition see online Supplemental Methods) and cell viability assayed as above.

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2.3. Transport assays

Since pHi recovery started after 15 seconds of removal of NH_4Cl/CS, transport assays in CS were at 10 seconds (37ºC). The difference between total 2,3-[^{3}H]adenosine uptake

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(NEN, Dreieich, FRG) in the absence or presence of 1 µmol/L NBTI was ENT1-mediated
125 adenosine transport. The difference between adenosine uptake in the presence of 1 and 10 µmol/L NBTI, 2 mmol/L hypoxanthine, or 2 mmol/L adenine was hENT2-mediated adenosine transport [11,15]. Overall 100 µmol/L [^{3}H]hypoxanthine (NEN) was also measured as described for adenosine. Initial rate (v_i) for overall transport, maximal velocity (V_{max}) and apparent Michaelis-Menten constant (K_m) of saturable transport was determined as reported
130 [11,12]. The relative contribution of hENT1 and hENT2 ($^{hENT1/2}F$) to total transport in cells non-treated ($-NH4$) or treated ($+NH4$) with NH_4Cl was estimated from V_{max}/K_m, and the relative effect of NH_4Cl on transport activity via hENT1 ($1/^{-NH4/+NH4}F_{hENT1}$) or hENT2 ($1/^{-NH4/+NH4}F_{hENT2}$) was estimated (see Supplemental Methods).

135 *2.4. Western blotting*

Total protein samples separated via polyacrylamide gel electrophoresis were probed with a primary polyclonal goat *anti*-hENT1 (1:1000) or *anti*-hENT2 (1:1000) (Santa Cruz Biotechnology, USA), or monoclonal mouse *anti*-ß-actin (1:3000) (Sigma Aldrich, St Louis, MO, USA) antibody, followed by incubation (1 hour) in Tris buffer saline Tween/0.2% bovine
140 serum albumin containing secondary horseradish peroxidase conjugated goat anti-goat or anti-mouse antibodies (Santa Cruz Biotechnology) as described [11,12]. Proteins were detected by enhanced chemiluminescence in a ChemiDoc-It 510 Imagen System (UVP, LCC Upland, CA, USA) and quantified by densitometry [11,12].

145 *2.5. Statistics*

Values are mean ± SEM, where n indicates the number of different cell cultures (3-4 replicates) from various pregnant women. Comparisons between two and more groups were

performed by Student's unpaired *t*-test and analysis of variance (2-ways ANOVA), respectively. $P<0.05$ was considered statistically significant.

150

3. Results

3.1. Basal pHi

The basal pHi value (7.21 ± 0.04) (Fig. 1A) was lower in cells exposed to NH_4Cl in a concentration-dependent manner, without altering the cell survival (Fig. 1B). NH_4Cl increased
155 the pHi value (Fig. 1C), and NH_4Cl removal caused rapid acidification (~1 second) recovering to initial pHi value in ~7 minutes. Intracellular acidification was for 15 seconds before a significant pHi recovery (Fig. 1D).

3.2. NH_4Cl effect on adenosine uptake

160 Overall adenosine uptake was equally inhibited by adenosine or 1 µmol/L NBTI in the absence of NH_4Cl (Fig. 2A). Hypoxanthine and adenine caused a minor inhibition unaltered by these molecules. Adenosine uptake was also inhibited by 10 µmol/L NBTI in a higher proportion than 1 µmol/L NBTI. The fraction of uptake inhibited by 10 versus 1 µmol/L NBTI (0.051 ± 0.01 pmol/µg protein/second) was similar to the inhibition by hypoxanthine, adenine,
165 or hypoxanthine + adenine. Hypoxanthine + adenine + adenosine caused the larger inhibition (0.187 ± 0.021 pmol/µg protein/second) and was similar to the added individual inhibitions caused by these molecules alone.

The challenge with 1 mmol/L NH_4Cl increased overall adenosine transport (Fig. 2A). A higher inhibition of 1 µmol/L NBTI and adenosine was seen compared with cells in the
170 absence of NH_4Cl. Adenosine uptake was further reduced by 10 µmol/L NBTI. The inhibition caused by 10 versus 1 µmol/L NBTI (0.063 ± 0.008 pmol/µg protein/second) was similar to

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the inhibition by hypoxanthine, adenine, or hypoxanthine + adenine. hENT1-adenosine uptake was higher than hENT2-mediated uptake in cells non-treated or treated with NH_4Cl (Fig. 2B). Incubation with 1 mmol/L NH_4Cl increased hENT1- and hENT2-adenosine transport;
175 however, the increase in hENT1- was higher than hENT2-mediated transport. Incubation of cells with 5HT unaltered adenosine uptake.

Hypoxanthine uptake was inhibited by 10 µmol/L NBTI with or without adenine or 5HT, but increased by NH_4Cl (Fig. 2C). hENT2-hypoxanthine uptake was higher than hENT1-uptake in cells non-treated or treated with NH_4Cl (Fig. 2D). NH_4Cl increased only
180 hENT2-hypoxanthine uptake.

3.3. pHi-dependent adenosine uptake

Overall adenosine uptake was higher in cells incubated with NH_4Cl in a concentration-dependent manner (Fig. 3A). NBTI inhibited uptake was greater as NH_4Cl concentration
185 increased. hENT1- and hENT2-adenosine uptake increased with NH_4Cl (Fig. 3B) with similar EC_{50} values (EC_{50} = 1.2 ± 0.3 and 1.3 ± 0.3 mmol/L NH_4Cl for hENT1 and hENT2, respectively) and comparable to overall transport (EC_{50} = 1.2 ± 0.2 mmol/L NH_4Cl). hENT1- and hENT2-adenosine uptake increased by acidic pHi values (Fig. 3C). Equally, the slope of hENT1 and hENT2-mediated uptake increase as the pHi was lower, and slope of pHi effect
190 was higher for hENT1- compared with hENT2-adenosine uptake (-0.298 ± 0.029 versus -0.081 ± 0.031 pmol/µg protein/second/(pHi unit)). The NH_4Cl-increased slope and adenosine uptake via hENT2 were less pronounced than these parameters for hENT1-mediated transport. Parallel experiments show that protein abundance for hENT1 was higher (2.5 ± 0.2 fold) than hENT2 in HUVECs (Fig. 3D), confirming previous observations in this cell type [11,15].

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3.4. pHi-dependent hENT1 and hENT2 transport kinetics

Overall adenosine transport was semisaturable and partially inhibited by 1, but abolished by 10 µmol/L NBTI (Fig. 4A). The v_i and K_D for overall transport were higher in NH_4Cl treated compared with non-treated cells (Table 1). The K_D was reduced by 1 µmol/L
200 NBTI to similar values in NH_4Cl treated and non-treated cells. Eadie-Hofstee plot for overall transport was biphasic, except in the presence of 10 µmol/L NBTI.

Saturable adenosine transport via hENT1 (Fig. 4B) and hENT2 (Fig. 4C) were saturable, adjusted to a single Michaelis-Menten equation, with linear Eadie-Hofstee plots. NH_4Cl increased the V_{max} and apparent K_m for saturable transport (Table 1). The V_{max}/K_m was
205 higher in NH_4Cl-treated cells for both membrane transporters types. The relative contribution of NH_4Cl to a change in the V_{max}/K_m for adenosine transport was higher for hENT1 compared with hENT2-mediated.

4. Discussion

210 This study shows that ENTs activity in HUVECs is under regulation by the pHi. Intracellular acidification increases adenosine transport with hENT1 being more sensitive to pHi than hENT2-mediated transport. Increase transport shows with higher maximal adenosine transport capacity (V_{max}/K_m) in HUVECs. This phenomenon could be crucial to maintaining the extracellular concentration of adenosine thus regulating its broad biological effects with
215 implications in diseases associated with dysregulation of hENTs activity, such as GDM and cancer, where pHo [11,12] and pHi [17] are altered.

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Primary cultured HUVECs show a pHi ~7.2 when exposed to pHo ~7.4, suggesting adequate pHi modulation under physiological conditions. The pHi in HUVECs was similar [19-22] or slightly lower [23] to previous reports in this cell type, but close to pH in the human
220 umbilical vein blood (pH ~7.35) [14,24,25]. Thus, pHi modulation in foetal endothelium is tunned with pHo changes (see review [3]). Lower pHi value is observed in lymphoblasts incubated with 25 mmol/L D-glucose likely due to activated NHE1 [25]. NHE1 is an isoform of NHE's membrane transporters playing a significant role in the regulation of pHi [1-3]. It was reported that a delay in pHi recovery after intracellular acidosis reduces endothelial cell
225 activation [21] probably due to lower activation of NHE1 and V-type H^+ ATPases [26,27]. Thus, an acidic intracellular medium results in modulation of plasma membrane transporters activity.

hENT1 and hENT2 isoforms of the *SLC29A* family members mediates adenosine uptake under a physiological pHo (pHo ~7.4) in HUVECs [11]. hENT1 protein abundance is
230 higher than hENT2 protein abundance under these conditions, confirming previous results in HUVECs [11,15]. Also, the relative contribution of hENT1 to adenosine transport (${}^{hENT1/2}F_{-NH4}$) was ~5.5 fold than for hENT2 mediated adenosine transport. HUVECs also expresses hENT4, an isoform with nucleoside and 5HT transport activity increased at pHo ~5.0-6.5, but not functional for adenosine uptake at pHo >7.0 [6]. Since changes in pHo results in
235 modulation of pHi, as described in neonatal rat carotid body type-I cells [28], increased hENT4 activity reported in HUVECs could be a response to pHi acidification. However, the latter is unlikely since an extracellular alkalization increased the pHi, but extracellular acidification did not alter pHi in rat aorta endothelial cells [29]. Our results show that overall adenosine uptake in HUVECs is increased by intracellular acidification with a preferential

activation of the maximal transport capacity via hENT1 compared with hENT2 $((1/{}^{-NH4/+NH4}F_{hENT1})/(1/{}^{-NH4/+NH4}F_{hENT2})$ ~1.15). Since EC_{50} values for NH_4Cl-reduced pHi were comparable to NH_4Cl-increased overall and hENT1 or hENT2-transport, a positive correlation between intracellular acidification and increased adenosine transport is likely in HUVECs. The latter was confirmed by results showing higher slope (~3.7 fold) of hENT1 compared with hENT2-adenosine transport. Since NH_4Cl pulse also increased hENT2-, but not hENT1-hypoxanthine uptake, and was unaltered by 5HT, hENT2 is under modulation of acidic pHi in HUVECs.

The acidic pHi-dependent increase in hENT1 transport activity is seen from a reduction in 0.3 pHi units; a phenomenon maintained up to a change in 1 pHi unit. Interestingly, a decrease in ~0.5 pHi units increases adenosine efflux in perfused rat soleus muscle fibers [13]. Since ENTs-adenosine transport is bidirectional down its concentration gradient, similar kinetics properties (i.e., apparent affinity ($1/K_m$), V_{max}, or V_{max}/K_m) for influx and efflux are likely [5]. Additionally, the acidic pHi-dependent increase in ENTs-adenosine transport is not exclusive for HUVECs. The possibility that adenosine uptake at pHi 6.0-6.5 was mediated by activation of hENT4 in HUVECs is unlikely since 5HT, which is taken up by hENT4 [6], did not alter overall, hENT1, and hENT2 transport.

Overall adenosine transport (up to 500 μmol/L adenosine) was mediated by a saturable plus a linear, non-saturable component working in parallel, as reported for adenosine [11,15] and other substrates [17,30] and cell types [30-32]. This phenomenon was reflected in a non-linear, biphasic representation of the Eadie-Hofstee plots of overall data. The latter meaning that two or more membrane transport mechanisms with similar kinetics transport parameters or availability at the plasma membrane [31,32] is likely. Indeed, K_m values for hENT1- and

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hENT2-mediated transport were similar in HUVECs non-treated or treated with NH_4Cl. Inhibition of overall transport with 1 μmol/L NBTI, a concentration that inhibits ENT1 transport activity [5,11], resulted in a straight first order regression line in the Eadie-Hofstee plot suggesting a transport mechanism that was likely mediated by hENT1 and hENT2 acting in parallel. Indeed, 10 μmol/L NBTI, a concentration inhibiting ENT1 and ENT2 activity [5,11], abolished adenosine transport. Since NH_4Cl increased the saturable hENT1- and hENT2-adenosine transport and Eadie-Hofstee plots were linear, it is unlikely that an acidic pHi will induce expression or recruitment of additional membrane transport mechanism(s) in HUVECs. Interestingly, NH_4Cl increased the K_m and V_{max} values for hENT1- and hENT2-mediated transport. Thus, activation caused by acidic pHi likely involves a higher availability of these membrane transporters at the plasma membrane and changes in their affinity or other intrinsic properties. An increase in ENTs activity is suggested by the increase in V_{max}/K_m seen in these cells. Since the relative effect of NH_4Cl on V_{max}/K_m for hENT1-transport was higher ($1/{}^{-NH4/+NH4}F_{hENT1}$ / $1/{}^{-NH4/+NH4}F_{hENT1}$ ~1.2) than for hENT2-transport, confirms that an acidic pHi affects the transport capacity of HUVECs.

In conclusion, HUVECs show adenosine transport mediated by hENT1 and hENT2 whose activity depends on pHi. An acidic pHi increases the activity of these membrane transporters by increasing their V_{max}/K_m, an effect higher in hENT1 compared with hENT2-adenosine transport. As far as we know, this is the first study addressing that adenosine transport via hENT1 and hENT2 is modulated by changes in pHi in HUVECs, and complement those showing modulation of nucleoside transport by pHo in this cell type [6]. Since the broad biological actions of adenosine in the human body [5], including the foetoplacental vasculature [3,5,9,11,12,33-35], changes in the capacity of the endothelium to remove this nucleoside from the extracellular space is crucial. Our results may have

implications in pathological conditions where hENTs-adenosine transport is reduced such as in GDM-derived HUVECs [11] or human placental microvascular endothelial cells [12] (see Fig. 5). GDM associated with lower V_{max}/K_m and hENT1 expression in these cell types, and

290 pregestational diabetes mellitus courses with acidic pH (pH <7.2) in the umbilical vein blood [3,14]. Thus, GDM could potentially associate with acidic pHo resulting from increased H^+ efflux leading to intracellular alkalization as reported in cancer cells [2,3,17]. However, there is not available information regarding changes in pHi and hENT1 and hENT2 activity in GDM or hyperglycaemia [2,3,34]. Therefore, since hENT1 and hENT2-mediated adenosine

295 transport is increased by acidic pHi, looking for a therapeutic approach to restoring a physiological pHi/pHo ratio, perhaps involving restoration of proper H^+ efflux via NHE1 [2,3], could be beneficial to re-establish GDM-reduced adenosine transport in HUVECs. Interestingly, adenosine down-regulates hENT1 expression and activity involving A_{2B} adenosine receptors subtype in hPMECs from normal pregnancies [36]). However, whether

300 adenosine receptors are involved in pHi modulation in the foetoplacental endothelium is not yet reported [2,3,34,35].

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Conflict of interest

There is no conflict of interest.

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Fig. 1. Effect of NH_4Cl pulse on cell pHi and viability. A, Primary cultures of HUVECs were preloaded with BCECF-AM and transferred into a spectrofluorometer. Basal pHi was stabilized and then cells were exposed (~2 minutes) to a Na^+-free solution without (0) or with NH_4Cl. Cells were then rinsed with NH_4Cl-free solution and left in this
435 medium. pHi values were estimated from a calibration curve with nigericin (see Methods). B, Alive cells counted in a haemocytometer under the same conditions as in A. C, Cells were preloaded with BCECF-AM and transferred into a spectrofluorometer. After basal pHi was stabilized the cells were exposed (2 minutes) to a control solution containing 20 mmol/L NH_4Cl (+ NH_4Cl). Cells were then rinsed with NH_4Cl-free
440 solution (– NH_4Cl) and left in this medium. pHi values were estimated as in A. The circle indicates the section of the trace shown in D. D, Data for the first 15 seconds after removal of NH_4Cl as in C. Arrow indicates the time (10 seconds) used for transport assays. In A and B, *$P<0.05$ versus without NH_4Cl. Values are mean ± S.E.M. (n = 22). In C and D, Data is representative of other 27 different cell cultures.

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Fig. 2. Effect of NH_4Cl on adenosine and hypoxanthine uptake. A, Adenosine (10 µmol/L) uptake (10 seconds, 37ºC) was measured in primary cultures of HUVECs non-treated (–NH_4Cl) or treated (+NH_4Cl) with 20 mmol/L NH_4Cl as described in Materials and Methods. Cells were in the absence (–) or presence (+) of *S*-(4-nitrobenzyl)-6-thio-
450 inosine (NBTI), adenosine, hypoxanthine, adenine, and/or 100 µmol/L 5-hydroxytryptamine (+5HT and –5HT). B, Adenosine uptake mediated by hENT1 or hENT2 from data in A. C, Hypoxanthine (100 µmol/L) uptake (10 seconds, 37ºC) was measured as in A in the absence or presence of NBTI, adenine, or 5HT. D, Hypoxanthine uptake mediated by hENT1 or hENT2 from data in C. In A: *$P<0.05$
455 versus all other corresponding values in +5HT or –5HT. †$P<0.05$ versus 1 µmol/L NBTI and all other values. In B: *$P<0.05$ versus values in hENT1 in –NH_4Cl. †$P<0.05$ versus corresponding hENT2 and hENT1 in +NH_4Cl. In C: *$P<0.05$ versus corresponding values in –NH_4Cl. †$P<0.05$ versus corresponding values in the absence or 1 µmol/L NBTI. In D: *$P<0.05$ versus corresponding hENT1. †$P<0.05$ versus
460 corresponding hENT2. Values are mean ± S.E.M. (n = 16-22).

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465 **Fig. 3. pHi dependency of hENT1 and hENT2-mediated adenosine uptake.** A, Overall adenosine (10 μmol/L) uptake (10 seconds, 37ºC) measured in primary cultures of HUVECs non-treated (0) or treated with NH_4Cl as described in Materials and Methods. Uptake was in the absence (Control) or presence of *S*-(4-nitrobenzyl)-6-thio-inosine (NBTI). B, Adenosine uptake mediated by hENT1 or hENT2 from data in A.
470 C, Adenosine uptake mediated by hENT1 or hENT2 from data in B. The pHi was estimated from a calibration curve with nigericine (see Materials and Methods). D, Representative Western blot for hENT1 and hENT2 in HUVECs at basal pHi (β-actin is internal reference). Lanes are two different cell cultures from different umbilical cords representative of other five samples from pregnant women. *Lower panel*:
475 densitometry ratios for hENT1/β-actin or hENT2/β-actin normalized to 1 for hENT1. In A and B: $*P<0.05$ versus corresponding 0 NH_4Cl. In C: $*P<0.05$ versus pHi 7.2. In D: $*P<0.04$ versus hENT1. Values are mean ± S.E.M. (n = 22).

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480 **Fig. 4. Effect of NH_4Cl on adenosine transport kinetics.** A, Overall adenosine uptake (10 seconds, 37ºC) in primary cultures of HUVECs non-treated (Control) or treated with NH_4Cl as described in Materials and Methods. Transport was in the absence or presence of *S*-(4-nitrobenzyl)-6-thio-inosine (NBTI). B, Saturable adenosine transport mediated by hENT1 from data in A. Data was adjusted to a single Michaelis-Menten
485 equation to obtain maximal velocity and apparent Michaelis-Menten constant of adenosine transport at initial rates (lineal uptake up to 10 seconds) (see online Supplemental Methods). C, Saturable adenosine transport mediated by hENT2 from data in A (data analysed as in B). Lower panels show corresponding Eadie-Hofstee plots. Values are mean ± S.E.M. (n = 22).

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Fig. 5. Physiological context for pHi-modulated adenosine transport in the human foetoplacental endothelium in gestational diabetes mellitus. In human umbilical vein endothelial cells from normal pregnancies the level of protons (H^+) within the
495 cells results in a physiological intracellular pH (pHi). The pHi value (pHi ~7.2 in this study) is maintained by the function of sodium/H^+ exchangers (NHEs). Measurements reported in human umbilical vein blood show that extracellular pH (pHo) is slightly alkaline (pHo ~7.4). These intracellular and extracellular environmental conditions result in physiological extracellular adenosine concentration due to its removal by
500 human equilibrative nucleoside transporters 1 (hENT1) and 2 (hENT2). In pathologies of pregnancy where the metabolism of D-glucose is altered, such as gestational diabetes mellitus (GDM), there is a high risk of generating large amounts of H^+, which are removed via NHEs whose activity is higher (large orange arrow) than in normal cells. This phenomenon will result in a potential intracellular alkalization (pHi >7.2)
505 and extracellular acidification (pHo ~7.2) [3,14,25] in GDM pregnancies. The latter could explain the reduced hENT1 expression and activity (dashed orange arrow) reported in HUVECs and human microvascular endothelial cells (hPMECs) from this disease. Composed from data in this study and [3,11,12,14,25].

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Table 1. Kinetic parameters for adenosine transport.

	Overall transport	
	– NH_4Cl	**+ NH_4Cl**
v_i (pmol/µg protein/0.03 seconds)		
Without NBTI	0.052 ± 0.002	0.315 ± 0.007 *
1 µmol/L NBTI inhibited fraction	0.007 ± 0.002	0.012 ± 0.002 *
10 µmol/L NBTI inhibited fraction	*nm*	*nm*
K_D (pmol/µg protein/second/(µmol/L))		
Without NBTI	0.0063 ± 0.002	0.0092 ± 0.003*
1 µmol/L NBTI inhibited fraction	0.0052 ± 0.002	0.0051 ± 0.002
10 µmol/L NBTI inhibited fraction	*nm*	*nm*

	Saturable transport			
	hENT1		**hENT2**	
	– NH_4Cl	**+ NH_4Cl**	**– NH_4Cl**	**+ NH_4Cl**
V_{max} (pmol/µg protein/second)	2.1 ± 0.1	12.9 ± 0.9 *	0.6 ± 0.1 *	2.2 ± 0.1 †
K_m (µmol/L)	32 ± 4	121 ± 22 *	49 ± 9	130 ± 21 *†
V_{max}/K_m (pmol/µg protein/second/(µmol/L))	0.066 ± 0.005	0.107 ± 0.005 *	0.012 ± 0.001 *	0.017 ± 0.002 *†

	Relative contributions
${}^{hENT1/2}F_{-NH4}$	5.50 ± 0.02
${}^{hENT1/2}F_{+NH4}$	6.37 ± 0.03 *
$1/{}^{-NH4/+NH4}F_{hENT1}$	1.62 ± 0.02
$1/{}^{-NH4/+NH4}F_{hENT2}$	1.41 ± 0.01 ‡

Legend for Table 1 in the next page.

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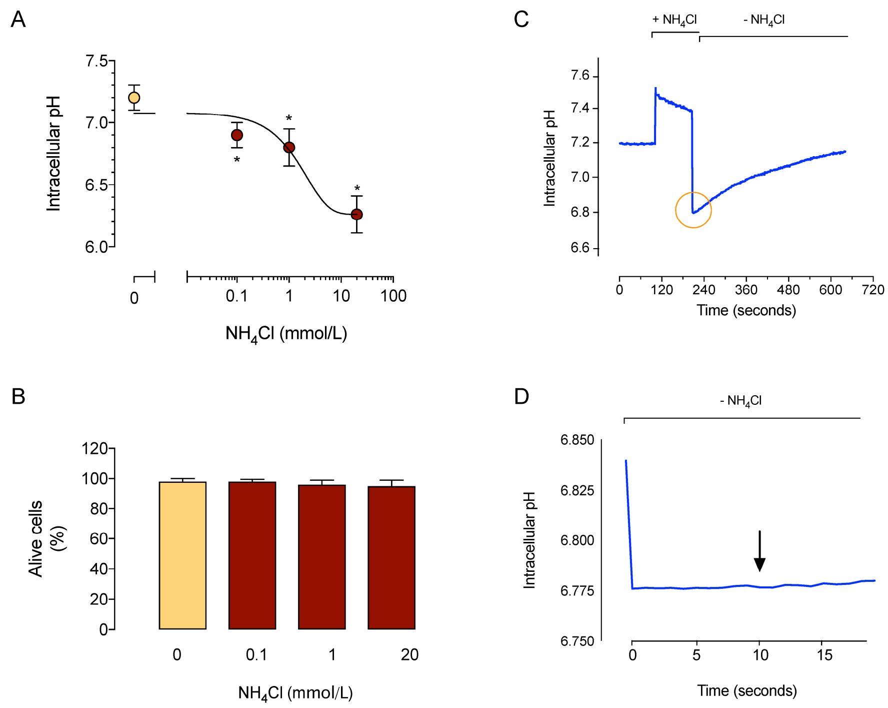
589 *Legend for Table 1.*

590

591 Kinetics for overall (hENT1 + hENT2), hENT1, and hENT2 transport of adenosine (0-500
592 μmol/L adenosine, 10 seconds, 37°C) was measured in primary cultures of HUVECs non-treated
593 (– NH_4Cl) or treated (+ NH_4Cl) with 1 mmol/L NH_4Cl (see Methods). The initial velocity (v_i) for
594 10 μmol/L adenosine was estimated for 0.03 seconds from the slope of lineal phase of uptake
595 adjusted to one phase exponential association equation considering the least squares fit [19]. The
596 lineal, non-saturable component of adenosine transport (K_D) was derived from a Michaelis-
597 Menten hyperbola plus K_D (slope of transport • adenosine concentration) (see Methods). The v_i
598 and K_D were calculated for overall transport (i.e., in the absence of *S*-(4-nitrobenzyl)-6-thio-
599 inosine (NBTI) (Without NBTI)) and for the fraction of adenosine transport inhibited by NBTI.
600 Maximal velocity (V_{max}) and the apparent Michaelis-Menten constant (K_m) were obtained by
601 plotting transport data to a single Michaelis-Menten asymptotic equation having removed the K_D
602 component from overall transport [19]. V_{max}/K_m is maximal transport capacity of adenosine. The
603 relative contribution (*F*) of hENT1 versus hENT2 to adenosine transport in cells non-treated
604 (${}^{hENT1/2}F_{-NH4}$) or treated (${}^{hENT1/2}F_{+NH4}$) with NH_4Cl, and the relative contribution of treating cells
605 with NH_4Cl to hENT1 ($1/{}^{-NH4/+NH4}F_{hENT1}$) or hENT2 ($1/{}^{-NH4/+NH4}F_{hENT2}$) mediated transport were
606 calculated from corresponding V_{max}/K_m values (see Supplementary Methods). *P<0.05 versus
607 corresponding values for hENT1 in cells non-treated with NH_4Cl. †P<0.05 versus corresponding
608 values for hENT2 in cells non-treated with NH_4Cl. ‡P<0.05 versus $1/{}^{-NH4/+NH4}F_{hENT1}$. Values are
609 means ± S.E.M. (n = 23). *nm*, not measurable.



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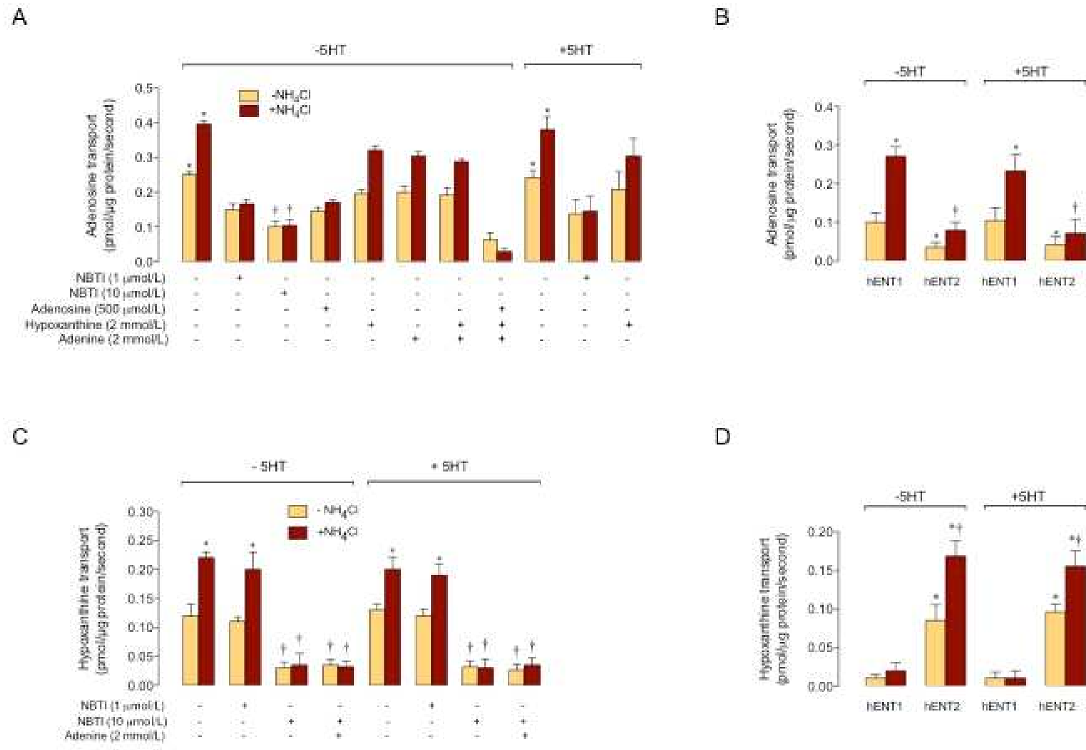

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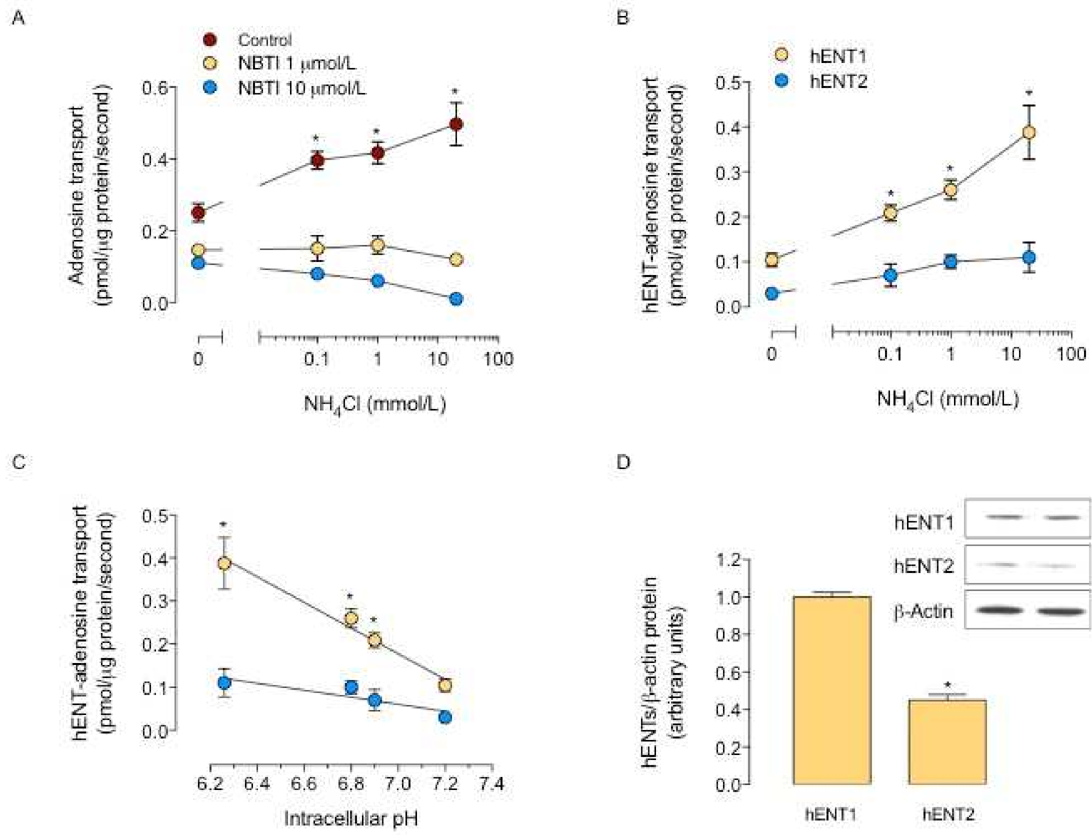

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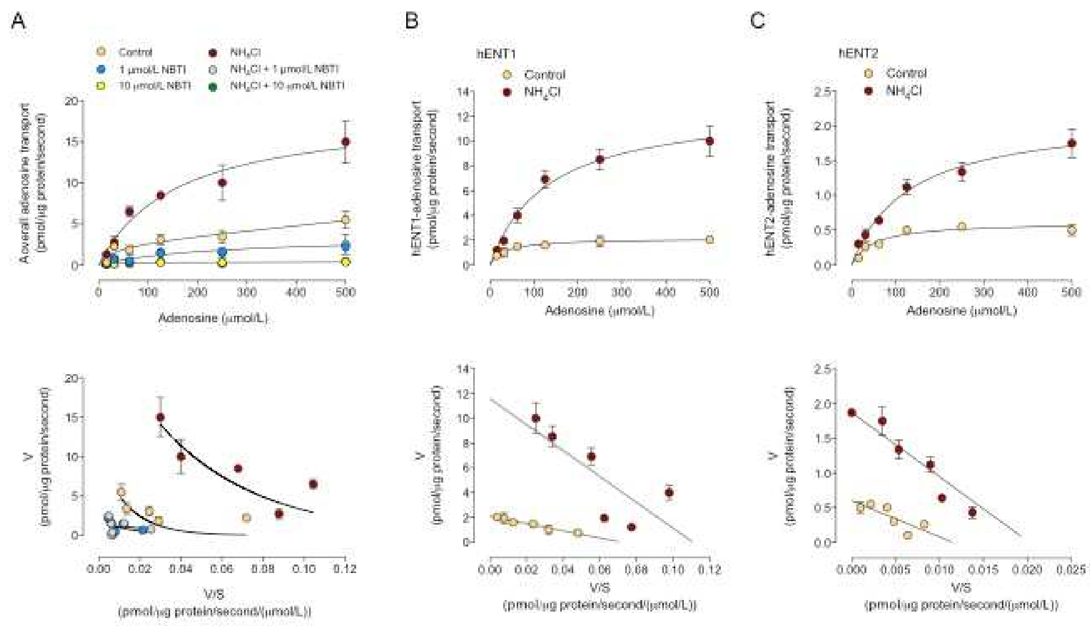

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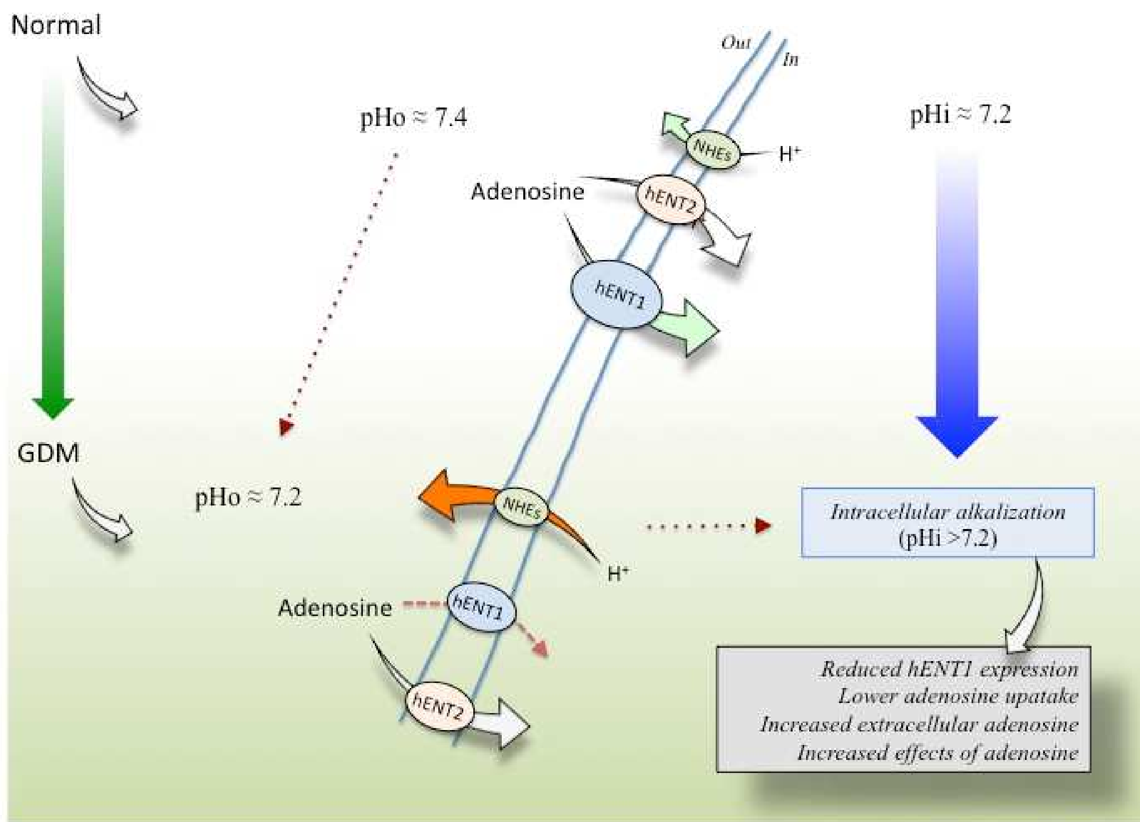

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Highlights

- Acidic pHi increases the overall adenosine transport in foetoplacental endothelium.
- Acidic pHi increases hENT1 and hENT2 maximal transport capacity.
- hENT1 activity is more sensitive than hENT2 to intracellular acidification.
- ENTs activity in human foetoplacental endothelium is regulated by pHi.

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