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Thiamine transport in human placental brush border membrane vesicles

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Abstract

Pathways for transport of thiamine by the human placental epithelium were investigated using brush border membrane vesicles isolated by divalent cation precipitation. The presence of thiamine transport mechanisms mediating Na^+-thiamine cotransport, proton/thiamine exchange and facilitated diffusion was assessed from [^{3}H]-thiamine tracer flux measurements. The magnitude of intravesicular thiamine accumulation was unaffected by the imposition of an inwardly directed sodium gradient suggesting an absence of a mechanism mediating brush border membrane Na^+-thiamine cotransport. Intravesicular thiamine accumulation was indistinguishable when measured in the presence and absence of conditions favoring the development of an inside-negative, potassium diffusion potential. The observed absence of conductive thiamine uptake suggests the absence of a mechanism mediating facilitated diffusion of thiamine in placental brush border membrane. The imposition of an inside-acid pH gradient was observed to induce concentrative accumulation of thiamine to levels exceeding equilibrium, suggesting the presence of a placental brush border membrane proton/thiamine exchange mechanism. Protonophore- induced dissipation of an imposed inside-acid pH gradient in the absence of membrane potential was observed to abolish concentrative accumulation of thiamine, suggesting a direct chemical coupling of protons and thiamine via a mediated exchange mechanism. Consistent with the functional properties expected for a mechanism mediating thiamine transport by organic cation exchange, the rate and magnitude of intravesicular [^{3}H]-thiamine accumulation was increased when measured in the presence compared to the absence of an outwardly directed thiamine concentration gradient. Substrate specificity studies of the proton/thiamine exchange mechanism suggest that the amine at position four of the pyrimidine ring, but not the hydroxyethyl side chain or an unmodified thiazolium ring, is an important chemical determinant for interaction with the transporter substrate binding site(s). Substrate specificity studies further suggest the possible presence of three separate organic cation exchange mechanisms mediating transport of thiamine, guanidine and MIA across placental brush border membrane.

Keywords: Thiamine; Placenta; Transport; Vesicle

1. Introduction

The human placenta performs an essential function in sustaining normal growth and development of the fetus by mediating net transfer of nutrients from the maternal to the fetal blood supply. Net transplacental transfer of nutrients from the maternal to the fetal circulation occurs at the cellular level as a result of

Abbreviations: FCCP, Carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; HEPES, 4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid; MES, *N*-morpholinoethanesulfonic acid; MIA, Methylisobutylamiloride; NMDG, *N*-methyl-D-glucamine; NMN, *N*-methylnicotinamide; TEA, Tetraethylammonium; TMA, Tetramethylammonium

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the polarized distribution of multiple, substrate-specific transport mechanisms expressed and sorted to function in the apical and basal membrane of syncytiotrophoblast cells. Further supporting an epithelial transport function, the apical or maternal surface of the syncytiotrophoblast is morphologically specialized in the form of a brush border, which greatly expands the absorptive surface of the placenta. Similar to renal and intestinal epithelia, the isolation of the brush border as membrane vesicles has improved our understanding of placental epithelial transport function by permitting the study of nutrient transport pathways present at the maternal surface of the syncytiotrophoblast [1]. Furthermore, for the purpose of identifying and functionally characterizing the presence of individual nutrient transport mechanisms in the apical membrane of human placenta, membrane vesicle studies permit a level of resolution not afforded by the alternative experimental models currently used to investigate placental epithelial transport. Indeed, the presence and nature of nutrient transport pathways in the basal or fetal-facing membrane of human placenta remain largely unknown due to the difficulty of isolating adequately enriched basal membrane vesicle preparations [2,3].

With the aim of further delineating the presence of nutrient transport pathways in human placental epithelia, we have conducted membrane vesicle studies designed to identify and characterize molecular mechanisms mediating organic and inorganic nutrient transport across the maternal-facing membrane of the syncytiotrophoblast [4–8]. More recently, we have extended our study of placental epithelial nutrient transport to include an investigation of the possible presence of an apical membrane transport pathway for the vitamin thiamine. Thiamine is an essential nutrient necessary for normal growth and development of the fetus, and transplacental transfer from the maternal circulation is the sole source of fetal thiamine.

As the diphosphate ester, thiamine is a coenzyme of the mitochondrial α ketoacid dehydrogenase complex that catalyzes the oxidative decarboxylation of α ketoacids to form the corresponding coenzyme A derivative [9]. Thiamine deficiency resulting from chronic alcohol abuse among women who become pregnant may restrict adequate delivery of thiamine to the fetus and possibly contribute to fetal alcohol syndrome [10]. Despite the suggested clinical significance of maternal-to-fetal transfer of thiamine, the placental transport mechanisms mediating uptake at the maternal-facing brush border and efflux at fetal-facing basal membrane remain to be identified and characterized. In studies of thiamine transport by the isolated perfused human placenta, a net transplacental transfer of thiamine towards the fetal space was observed, as well as concentrative thiamine accumulation in placental tissue [10,11]. Thiamine transport was further observed to be saturable, inhibitable by thiamine analogues and to result in higher thiamine concentrations measured at the fetal side of the placental epithelium. These observations suggest the presence of mediated transport pathways for thiamine transfer across the human placenta. Accordingly, we have conducted brush border membrane vesicle studies to identify and functionally characterize the mechanism mediating placental thiamine accumulation from the maternal circulation. To the best of our knowledge, the evidence presented is the first to suggest the presence of a mechanism mediating the coupled exchange of protons for thiamine as a pathway for thiamine transport in the brush border membrane of human placenta.

2. Materials and methods

2.1. Membrane preparations

Brush border membrane vesicles were isolated from human term placenta by divalent cation aggregation and differential centrifugation as described previously [4,6]. Briefly, the villous tissue of placenta obtained within 15 min of elective caesarean section was quickly dissected and minced into small ($\sim$1 cm) fragments at 4°C. The tissue fragments were rinsed three times in 300 mM mannitol, 10 mM HEPES/TMA (OH), pH 7, and gently stirred for approximately 30 min using a motor-driven spatula. The tissue suspension was filtered through cotton gauze, and phenylmethylsulfonyl fluoride was added to a final concentration 0.2 mM. The filtrate was centrifuged at 8100 rpm for 15 min using an SS-34 rotor (Sorvall). The low-speed pellet was discarded, and the supernatant was centrifuged at 19,000 rpm for 40 min. The high-speed pellet was gently resus-

pended, and $MgCl_2$ was added to a final concentration of 12 mM. After incubating for 10 min, the membrane suspension was centrifuged at 5000 rpm for 15 min to pellet the Mg^{2+}-induced membrane aggregates. The low-speed supernatant was centrifuged at 19,000 rpm for 40 min, and the resulting pellet (brush border membrane vesicles) resuspended and washed twice in buffers designated for each experiment. Membranes were stored frozen ($\sim 70°C$) and used within 2 weeks of preparation. The isolated membrane vesicle fraction was typically enriched 25.4 ± 1.3-fold ($n = 7$) in alkaline phosphatase activity [12] compared with homogenates of villous tissue. Typical membrane marker enzyme enrichments for the basal membrane (Na^+/K^+ ATPase), mitochondria (succinic dehydrogenase), and endoplasmic reticulum (NADH dehydrogenase) were 0.68 ± 0.05 ($n = 7$), 0.43 ± 0.02 ($n = 7$), and 0.34 ± 0.03 ($n = 7$), respectively [13–15]. Protein was determined by a sodium dodecyl sulfate-Lowry assay using bovine serum albumin as the standard [16].

2.2. Isotopic flux measurements

Frozen ($\sim 70°C$) aliquots of membrane vesicles were thawed at room temperature, and isosmotic solutions of appropriate ionic composition were added to obtained the desired intravascular solution described for each experiment in the figure and table legends. The membrane suspension was incubated for 120 min at room temperature to attain transmembrane equilibration of the added media. The extravesicular media were similarly prepared, and the final composition for each experiment is given in the figure and table legends. Intravesicular content of [^{3}H] or [^{14}C]-labeled substrate was assayed at least in triplicate at 37°C by a rapid filtration technique described previously [17]. Briefly, a small volume of buffer (40 μl 98 μl) containing radiolabeled substrate was placed at the bottom of a glass test tube followed by positioning a 2.0 μl–10 μl aliquot of membrane suspension (50–200 μg protein) on the test tube wall immediately above the puddle of isotope-containing buffer. Vesicle uptake of radiolabeled substrate was initiated by rapidly mixing the two aliquots using a vortex, and after a predetermined time interval, the uptake reaction was quenched by rapid dilution with isosmotic potassium gluconate, 10 mM HEPES/TMA (OH), pH 7, kept at 4°C. The diluted membrane suspension was passed through a 0.65 μm Millipore filter (DAWP) and washed with an additional 9 ml of quench buffer. The process of quenching, filtration, and washing occurred routinely within a 15-s period. The filters were dissolved in 3 ml of Ready Solv HP (Beckman) and counted by scintillation spectroscopy. The timed uptake values were determined in triplicate, and the mean obtained was corrected by the nonspecific retention of isotope by the filters. Although absolute substrate uptake values expressed per mg of protein varied from membrane preparation to membrane preparation, differences in substrate uptake expressed relative to control substrate uptake values for a given membrane preparation were highly reproducible. Where appropriate, statistical significance has been determined using an unpaired *t*-test for two means, where $p < 0.05$ is taken as the limit determining statistical significance.

2.3. Materials

Valinomycin, FCCP, guanidine, cimetidine, clonidine, amiloride, NMN, TEA, choline, creatinine, serotonin, histamine, dopamine, putriscine, spermidine, spermine, adenine, cytosine, thiamine, pyrthiamine, amprolium, oxythiamine, thiamine monophosphate and cocarboxylase were purchased from Sigma. Imipramine and MIA were purchased from Research Biochemicals. Diethylamiloride was purchased from Molecular Probes. Harmaline was purchased from Aldrich. [^{14}C]-pantothenate, [^{3}H]-MIA and [^{3}H]-choline were obtained from NEN. [^{3}H]-thiamine was obtained from Moravek, and [^{14}C]-guanidine from American Radiolabeled Chemicals. Valinomycin was dissolved in 95% ethanol and was added to the membrane suspension in a 1:100 dilution. Equivalent volumes of ethanol were added to control aliquots of membrane. FCCP dissolved in 95% ethanol or 95% ethanol alone was added to extravesicular solutions in a 1:200 dilution. All solutions were prepared with distilled deionized water and passed through 0.22 μM Millipore filters.

3. Results and discussion

Membrane transport pathways for thiamine at the apical or maternal-facing side of human placental

epithelial cells was first investigated by testing for the possible presence of a mechanism mediating Na-thiamine cotransport. The presence of a brush border membrane Na-thiamine cotransporter was assessed by determining the ability of an imposed, inwardly directed sodium concentration gradient to serve as a driving force for intravesicular thiamine accumulation. As shown in Table 1 the level of thiamine uptake measured in the presence of a sodium gradient was indistinguishable from the levels measured in the presence of lithium or potassium gradients or in the absence of cation gradient. In contrast, and in keeping with the known presence of a placental brush border membrane Na-pantothenate cotransporter [6], pantothenate uptake was observed to be 30- to 40-fold greater when measured in the presence compared to the absence of an inward sodium gradient. The observed inability of an inward sodium gradient to serve as a driving force for intravesicular thiamine accumulation suggests the absence of a mechanism mediating the coupled influx of sodium and thiamine in placental brush border membrane. This evidence would suggest a Na-thiamine cotransport mechanism is not a pathway mediating placental thiamine uptake from the maternal circulation and is further consistent with the reported sodium independence of thiamine transport studied in rat and guinea-pig small intestine brush border membrane [18,19], human red blood cells [20] and culture neuroblastoma cells [21].

Pathways for thiamine uptake across the maternal facing membrane of human placenta was investigated further by testing for the possible presence of a mechanism mediating conductive thiamine uptake. Previously, we identified and functionally characterized a mechanism mediating the facilitated diffusion of choline in placental brush border membrane by determining the ability of an inside-negative voltage difference to serve as a driving force for intravesicular choline accumulation [7]. In these previous studies the presence of excess thiamine was observed to have no effect on conductive choline uptake which suggested thiamine was not a substrate for the mechanism mediating conductive choline uptake and that separate organic cation transport pathways may exist for placental accumulation of choline and thiamine. Accordingly, we assessed the possible presence of a conductive uptake pathway for thiamine by determining the ability of an inside-negative, valinomycin-induced, potassium diffusion potential to serve as a driving force for placental brush border membrane thiamine uptake. As shown in Table 2 in the absence of valinomycin, the imposition of an outward potassium gradient did not result in increased thiamine or choline uptake when compared to uptake measured in the absence of a potassium gradient. However, when measured in the presence of conditions favoring the development of an inside-negative voltage difference, choline uptake, but not thiamine uptake, was markedly increased achieving a level 5-fold greater than observed in the absence of a potassium gradient. The similar level of thiamine uptake measured in the presence and absence of conditions favoring formation of an inside-negative voltage difference indicates the inability of a transmembrane voltage difference to serve as a driving force for thiamine accumulation across placental brush border membrane. Whereas the observed voltage sensitivity of choline uptake further suggests the presence of a mechanism mediating conductive choline uptake, the apparent voltage insensitivity of thiamine uptake would argue against the presence of a similar, but separate mechanism as a pathway for placental thiamine accumulation. Where the voltage sensitivity of thiamine transport has been previously examined in studies of human red blood cells [20] and basolateral membrane vesicles from rat small intestine [22], no evidence for a mechanism mediating conductive thiamine transport was obtained. In contrast, the observed decrease in thiamine accumulation by mouse neuroblastoma cells upon membrane potential depolarization suggests a mecha-

Table 1
Effect of cation concentration gradients on thiamine influx

	NMDG	Na	Li	K
Thiamine	3.49 ± 0.2	2.99 ± 0.15	3.1 ± 0.15	3.34 ± 0.17
Pantothenate	2.65 ± 0.21	117 ± 4.7	4.52 ± 0.02	4.40 ± 0.13

Brush border membrane vesicles were pre-equilibrated with 125 mM NMDG chloride, 42 mM HEPES, 21 mM NMDG, 52 mM MES, pH 6. The 10-s uptake of thiamine (1 μM) or Pantothenate (9 μM) occurred from extravesicular solutions containing 100 mM (NMDG, sodium, lithium, potassium) chloride, 25 mM NMDG chloride, 42 mM HEPES, 21 mM NMDG, 52 mM MES, pH 6. The mean $\pm$ S.E. uptake (pmol/mg protein) was determined from four experiments each performed with a different membrane preparation.

Table 2
Effect of transmembrane voltage difference on thiamine influx

	Ko = Ki + valinomycin	Ko < Ki + valinomycin	Ko < Ki-valinomycin
Thiamine	1.01 ± 0.062	0.983 ± 0.05	0.470 ± 0.03
Choline	6.34 ± 0.57	30.8 ± 1.7	4.1 ± 0.62

Brush border membrane vesicles were pre-equilibrated with 150 mM potassium gluconate, 20 mM MES, 2 mM potassium (hydroxide), pH 5. The 10-s uptake of thiamine (1 μM) and choline (10 μM) occurred from extravesicular solutions containing 20 mM MES, 2 mM potassium (hydroxide), pH 5 and (Ko = Ki) 150 mM potassium gluconate or (Ko < Ki) 135 mM NMDG gluconate, 15 mM potassium gluconate. Where indicated, membrane vesicles were preincubated with 1% (v/v) 25 mg/ml valinomycin or 1% (v/v) ethanol for a minimum of 30 min. The mean ± S.E. thiamine uptake (pmol/mg protein) was determined from four experiments each performed with a different membrane preparation.

nism mediating conductive thiamine uptake may be present in neuronal tissue [21].

Pathways for thiamine uptake across the maternal facing membrane of human placenta was investigated further by testing for the possible presence of a mechanism mediating proton for thiamine exchange. The possible presence of a placental brush border membrane proton/thiamine exchange mechanism was assessed by determining the ability of an imposed proton gradient to serve as a driving force for intravesicular accumulation of thiamine. As shown in Table 3 and consistent with the presence of a previously identified placental brush border membrane proton/organic cation exchange mechanism [23], MIA uptake was approximately 4-fold greater when measured in the presence compared to the absence of an inside-acid pH gradient. Similarly, in the presence of the same inside-acid pH gradient thiamine uptake was observed to be approximately 2–3-fold greater than when measured in the absence of a pH gradient. The pH gradient-induced stimulation of thiamine uptake would not appear to be secondary to an inside-negative, proton gradient-induced diffusion potential because, as shown in Table 2, the presence of a conductive uptake pathway for thiamine was not observed. Consistent with the presence of a placental brush border membrane proton/thiamine exchange mechanism, a direct chemical coupling of proton efflux to thiamine influx is suggested by the observed pH gradient-induced stimulation of thiamine uptake. The nature of proton and thiamine flux coupling was investigated further by determining the ability of an inside-acid pH gradient to serve as a driving force for concentrative accumulation of thiamine in the presence and absence of conditions designed to offset the formation of ion gradient-induced, transmembrane voltage differences. As shown in Fig. 1, the level of thiamine uptake measured in the absence of a pH gradient was low, and slowly approached an equilibrium value where intra- and extravesicular thiamine concentrations are similar. In contrast, when measured in the presence of an inside-acid pH gradient, thiamine uptake was markedly stimulated achieving levels approximately 3-fold greater than observed at equilibrium. Furthermore, although the level of thiamine uptake was somewhat reduced, the observed

Table 3
Effect of proton concentration gradient on thiamine influx

Thiamine		Methylisobutylamiloride	
pHo 6.5/pHi 6.5	pHo 6.5/pHi 5	pHo 6.5/pHi 6.5	pHo 6.5/pHi 5
4.3 ± 0.8	10.1 ± 0.8	53 ± 8.6	207 ± 14

Brush border membrane vesicles were pre-equilibrated with 124 mM potassium chloride, 42 mM MES, 4 mM potassium (hydroxide), and 52 mM mannitol at pH 5 or 25 mM NMDG and 27 mM mannitol at pH 6.5. The 10-s uptake of thiamine (1 μM) or methylisobutylamiloride (1 μM) occurred from extravesicular solutions containing 124 mM potassium chloride, 42 mM MES, 4 mM potassium (hydroxide) and 25 mM NMDG, 27 mM mannitol at pHo 6.5/pHi 6.5 and 27 mM NMDG, 25 mM mannitol at pHo 6.5/pHi 5. The mean ± S.E. uptake (pmol/mg protein) was determined from three experiments each performed with a different membrane preparation.

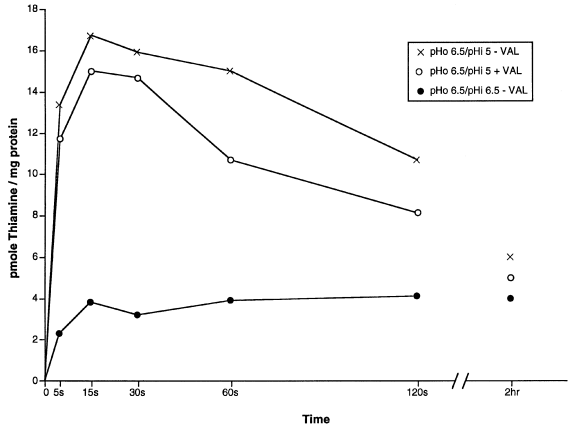

Fig. 1. Effect of proton concentration gradient on thiamine influx. Brush border membrane vesicles were pre-equilibrated with 124 mM potassium chloride, 42 mM MES, 4 mM potassium (hydroxide), 52 mM mannitol at pH 5, or 25 mM NMDG and 27 mM mannitol at pH 6.5. Uptake of [^{3}H] thiamine (1 μM) occurred from extravesicular solutions containing 124 mM potassium chloride, 42 mM MES, 4 mM potassium (hydroxide) and 25 mM NMDG, 27 mM mannitol at pHo 6.5/pHi 6.5 and 27 mM NMDG, 25 mM mannitol at pHo 6.5/pHi 5. Where indicated, membrane vesicles were preincubated with 1% (v/v) 5 mg/ml valinomycin or 1% (v/v) ethanol for a minimum of 30 min. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

pH gradient-induced, concentrative accumulation of thiamine persisted when thiamine uptake was measured in the presumed absence of membrane potential difference. In the presence of charge compensating movements of potassium through valinomycin treated membranes the reduced level of pH gradient-induced thiamine uptake may have resulted from a more rapid dissipation of the imposed pH gradient. The observed concentrative accumulation of thiamine demonstrates the ability of an imposed proton (or hydroxyl) gradient to serve as a driving force for thiamine transport against a chemical potential difference, and is consistent with the presence of a proton/thiamine exchange mechanism (or hydroxyl–thiamine cotransport mechanism) coupling proton efflux (or hydroxyl influx) to thiamine influx. The nature of flux coupling between an imposed pH gradient and the influx of thiamine was investigated further by observing the effect of the protonophore FCCP on pH gradient-driven thiamine uptake. To the extent that pH gradient-induced thiamine uptake occurred by direct chemical coupling through a common ternary intermediate, then a decreased level of thiamine uptake would be expected where dissipation of an imposed pH gradient was enhanced. As shown in Fig. 2, when measured in the presence of FCCP but the absence of valinomycin, the level of pH gradient-induced thiamine uptake was reduced, but remained well above equilibrium and the level measured in the absence of a pH gradient. However, when measured in the presence of both valinomycin and FCCP, the level of pH gradient-driven thiamine uptake did not exceed equilibrium, and was comparable to the level measured in the absence of a pH gradient. These observations suggest that the presence of the protonophore alone is not sufficient to permit a rapid collapse of the imposed pH gradient, and that the presence of an additional charge compensating pathway (valinomycin) is required to shunt the FCCP-induced inside-negative proton diffusion potential. The evidence obtained demonstrating the pH gradient dependence of thiamine uptake further supports the presence of a proton/thiamine exchange mechanism as a pathway for placental thiamine accumulation across the brush border membrane. The possible presence of a placental brush border membrane organic cation exchange mechanism as a pathway for thiamine uptake from the maternal circulation was investigated further by testing for homoexchange diffusion of thiamine.

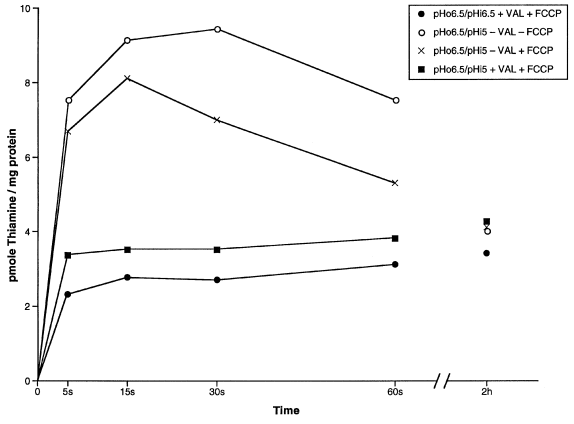

Fig. 2. Effect of FCCP on proton gradient-dependent thiamine influx. Brush border membrane vesicles were pre-equilibrated as described in the legend of Fig. 1. Uptake of [^{3}H] thiamine (1 μM) occurred from extravesicular solutions described in the legend to Fig. 1 and where indicated in the presence or absence of 56 μM FCCP. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

Homo exchange diffusion is a property of exchange mechanisms mediating both proton-coupled cation exchange [24], as well as base equivalent (bicarbonate, carbonate, hydroxyl) coupled anion exchange [25]. Homoexchange diffusion may be demonstrated upon observing the effect of a large outwardly directed gradient of substrate on the rate and magnitude of radiolabelled substrate accumulation. As shown in Fig. 3, [^{3}H] thiamine uptake was markedly stimulated when measured in the presence compared to the absence of an outward thiamine gradient. Consistent with the previous observation of proton gradient-driven thiamine accumulation, the demonstrated homoexchange diffusion of thiamine is evidence further supporting the presence of an organic cation exchange mechanism as a pathway for thiamine accumulation by human placenta. Furthermore, to the best of our knowledge, the identified organic cation exchange mechanism is the first specific example of mediated thiamine transport in human placenta and is distinct from the 'thiamine shuttle' mechanism previously hypothesized [26]. Next, we examined the properties of the identified organic cation exchanger with regard to inhibitor sensitivity and substrate specificity by first determining the effect of thiamine and thiamine analogs on thiamine

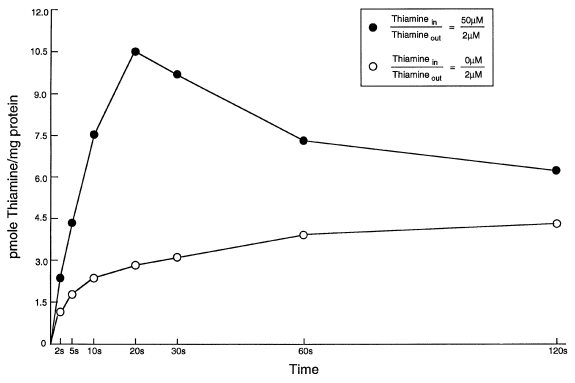

Fig. 3. Effect of intravesicular thiamine on thiamine influx. Brush border membrane vesicles were pre-equilibrated with and without thiamine (50 μM) and with 100 mM potassium chloride, 80 mM MES, 7 mM potassium (hydroxide), pH 5. Uptake of [^{3}H] thiamine (2 μM) occurred from extravesicular solutions containing 100 mM potassium chloride, 80 mM MES, 7 mM potassium (hydroxide), pH 5. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

Table 4
Effect of thiamine and thiamine analogs on thiamine/thiamine exchange

Organic cation	%
Thiamine (25 μM)	43 ± 5.3
Thiamine (100 μM)	15 ± 1.1
Thiamine (500 μM)	6.5 ± 0.9
Pyrthiamine (25 μM)	20 ± 3.0
Pyrthiamine (100 μM)	9.3 ± 1.2
Pyrthiamine (500 μM)	5.6 ± 1.1
Amprolium (25 μM)	26 ± 3.5
Amprolium (100 μM)	12.5 ± 1.3
Amprolium (500 μM)	6.4 ± 1.1
Oxythiamine (25 μM)	96 ± 2.1
Oxythiamine (100 μM)	66 ± 4.8
Oxythiamine (500 μM)	26 ± 1.3
Thiamine monophosphate (25 μM)	68 ± 5.4
Thiamine monophosphate (100 μM)	29 ± 3.7
Thiamine monophosphate (500 μM)	12 ± 0.7
Cocarboxylase (25 μM)	89 ± 2
Cocarboxylase (100 μM)	55 ± 2.2
Cocarboxylase (500 μM)	22 ± 2.8

Brush border membrane vesicles were pre-equilibrated as described in the legend to Fig. 3. The 20-s uptake of thiamine (2 μM or as shown) occurred from an extravesicular solution described in the legend to Fig. 3 and containing the substrates shown below. Thiamine uptake is shown as the percent of thiamine uptake measured in the absence of test substrate (11.7 ± 0.81 pmol/mg). The mean ± S.E. of four experiments each performed with a different membrane preparation is shown.

gradient-driven [^{3}H]-thiamine uptake. As shown in Table 4, a similar decrease in thiamine uptake was measured in the presence of increasing concentrations of thiamine and the thiamine analogs pyrthiamin and amprolium, which suggests the thiazolium ring configuration, and the presence of the hydroxyethyl side chain are not critical determinants for substrate interaction with the transporter. In contrast, [^{3}H]-thiamine uptake was relatively less sensitive to inhibition by oxythiamine, suggesting that substrate interaction with the transporter does require the presence of an amine at position 4 of the pyrimidine ring. Interestingly, phosphorylation of thiamine would not appear to preclude its interaction with the transporter, as both the mono- and diphosphorylated form of thiamine decreased [^{3}H]-thiamine uptake. However, the level of inhibition observed suggests that thiamine phosphorylation does increasingly limit interaction with the substrate binding site(s) within the transporter.

Table 5
Effect of organic cations on thiamine/thiamine exchange

Organic cation	%
Guanidine	105 ± 5.0
Cimetidine	102 ± 5.5
Clonidine	89 ± 5.7
Amiloride	41 ± 0.6
Diethylamiloride	13 ± 1.2
Harmaline	69 ± 2.3
Imipramine	86 ± 3.0
NMN	112 ± 4.6
TEA	110 ± 4.3
Choline	112 ± 6.1
Creatinine	117 ± 6.0
Serotonin	108 ± 4.7
Histamine	114 ± 5.9
Dopamine	113 ± 6.9
Putriscine	119 ± 5.9
Spermidine	123 ± 7.1
Spermine	120 ± 8.2
Adenine	75 ± 1
Cytosine	122 ± 8.6

Brush border membrane vesicles were pre-equilibrated as described in the legend to Fig. 3. The 20-s uptake of thiamine (2 μM) occurred from an extravesicular solution described in the legend to Fig. 3 and containing the substrates (500 μM) shown below. Thiamine uptake is shown as the percent of thiamine uptake measured in the absence of test substrate (10.7 ± 1.4 pmol/mg). The mean ± S.E. of three experiments each performed with a different membrane preparation is shown.

The profile of substrate specificity set forth by the rank order inhibition of [^{3}H]-thiamine uptake shown in Table 4 compares well to the results obtained from similar substrate specificity studies of thiamine transport in the isolated perfused human placenta [27], cultured neuroblastoma cells [21] and rat intestinal brush border membrane vesicles [18].

Previous studies of human placental organic cation transport have resulted in the identification of a proton/organic cation exchange mechanism mediating guanidine [28] and methylisobutylamiloride (MIA) [23] transport in brush border membrane vesicles. Substrate specificity studies of the identified organic cation exchange mechanism(s) did not include an examination of thiamine as a possible substrate, and the role of the identified organic cation exchange mechanisms in human placental epithelial function remains to be defined. To determine whether the same or a different organic cation exchange mechanism mediates thiamine transport, the substrate specificity for thiamine gradient-driven ^{3}H thiamine uptake was determined for substrates shown in Table 5. To the extent that the same organic cation exchanger mediates thiamine and guanidine uptake, then a similar profile of substrate specificity would be expected for thiamine compared to guanidine uptake. As previously described [28], proton gradient-driven guanidine uptake was reduced 56% when measured in the presence compared to the absence of a 67-fold excess of guanidine. However, as shown in Table 5, no effect of guanidine on the level of thiamine uptake was observed when measured in the presence of a 250-fold excess of guanidine. A similar comparison of the concentration-dependent inhibition of proton gradient-driven guanidine uptake [28] and thiamine/thiamine exchange reveals a much greater sensitivity of proton gradient-driven guanidine uptake to amiloride, harmaline and imipramine. The observed differences in substrate specificity, and the apparent inability of guanidine to engage the thiamine transporter suggests that separate organic cation exchange mechanisms mediate placental brush border membrane thiamine and guanidine transport.

To further assess whether a common organic cation exchange mechanism mediates both thiamine and

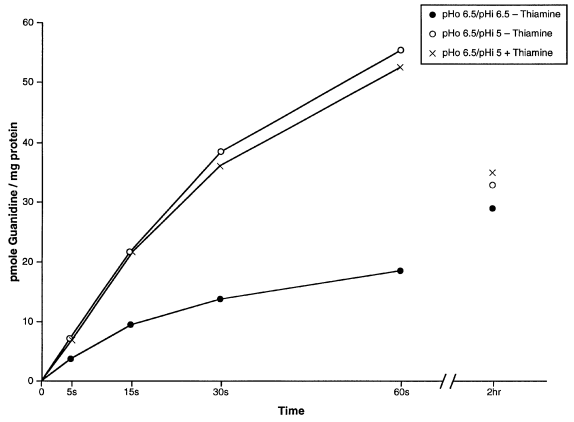

Fig. 4. Effect of thiamine on proton gradient-driven guanidine influx. Brush border membrane vesicles were pre-equilibrated in the absence of valinomycin as described in the legend to Fig. 1. Uptake of [^{14}C] guanidine (10 μM) occurred in the presence and absence of 500 μM thiamine from extravesicular solutions described in the legend to Fig. 1. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

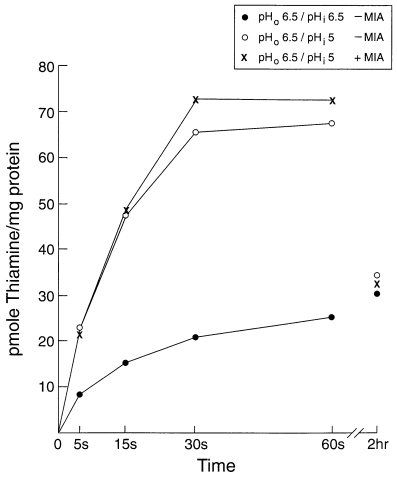

Fig. 5. Effect of MIA on proton gradient-driven thiamine influx. Brush border membrane vesicles were pre-equilibrated in the absence of valinomycin as described in the legend to Fig. 1. Uptake of [^{3}H] thiamine (10 μM) occurred in the presence and absence of 250 μM MIA from extravesicular solutions described in the legend to Fig. 1. A representative experiment of three independent observations each performed with a different membrane preparations is shown.

guanidine transport across the human placental brush border membrane, proton gradient-driven guanidine uptake was measured in the presence and absence of thiamine. To the extent that both thiamine and guanidine are transportable substrates of a common organic cation exchange mechanism then when measured in the presence of a 50-fold excess of thiamine, a reduced level of guanidine uptake would be expected. As shown in Fig. 4, proton gradient-driven guanidine uptake was observed to be virtually identical when measured in the presence and absence of excess thiamine. The observed inability of excess thiamine to decrease proton gradient-driven guanidine uptake further suggests that separate organic cation exchange mechanisms mediate thiamine and guanidine transport across human placental brush border membrane. The mechanism mediating proton/thiamine exchange was investigated further to determine whether a common organic cation exchange mechanism mediates both thiamine and MIA transport across the human placental brush border membrane. Transport of thiamine and MIA by a common organic cation exchange mechanism would be suggested by observing a reduced level of proton gradient-driven thiamine uptake when measured in the presence of excess MIA, and observing a reduced level of proton gradient-driven MIA uptake when measured in the presence of excess thiamine. As shown in Figs. 5 and 6 respectively, the levels of proton gradient-driven thiamine and MIA uptake were essentially indistinguishable when measured in the presence of excess MIA and thiamine. The observed absence of mutual inhibition of thiamine and MIA uptake further suggests MIA is not a substrate of the mechanism mediating proton/thiamine exchange nor is thiamine a substrate of the mechanism mediating proton/MIA exchange. These observations are most consistent with the presence of separate organic cation exchange mechanisms for thiamine and MIA in human placental brush border membrane. The possible presence of multiple organic cation exchange mechanisms mediating human placental brush border membrane thiamine, guanidine and MIA transport was further assessed by determining the effect of excess guanidine on the level of proton gradient-driven MIA uptake. As shown in Fig. 7, a similar level of proton gradient-driven MIA uptake was measured in the

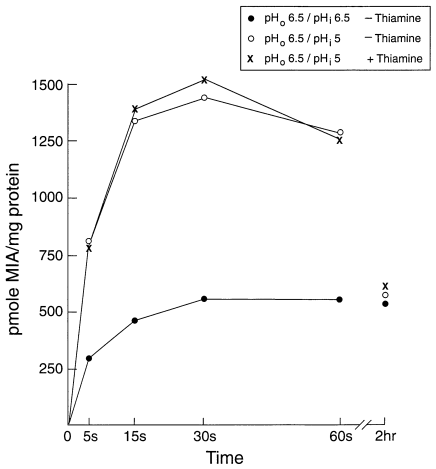

Fig. 6. Effect of thiamine on proton gradient-driven MIA influx. Brush border membrane vesicles were pre-equilibrated in the absence of valinomycin as described in the legend to Fig. 1. Uptake of [^{3}H] MIA (10 μM) occurred in the presence and absence of 500 μM thiamine from extravesicular solutions described in the legend to Fig. 1. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

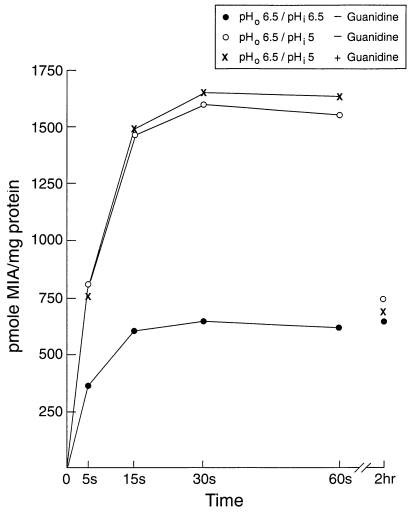

Fig. 7. Effect of guanidine on proton gradient-driven MIA influx. Brush border membrane vesicles were pre-equilibrated in the absence of valinomycin as described in the legend to Fig. 1. Uptake of [^{3}H] MIA (10 μM) occurred in the presence and absence of 500 μM guanidine from extravascular solutions described in the legend to Fig. 1. A representative experiment of three independent observations each performed with a different membrane preparation is shown.

presence and absence of a 50-fold excess of guanidine, suggesting that guanidine does not interact with the substrate binding site of the proton/MIA exchange mechanism. Where the guanidine sensitivity of proton gradient-driven MIA uptake has been previously assessed in human placental brush border membrane vesicles, a 10^6-fold excess concentration of guanidine (1 mM) was observed to have no effect on MIA uptake (1 nM) [23]. The apparent guanidine insensitivity of proton gradient-driven MIA uptake may be considered as evidence suggesting the possible presence of a third placental brush border membrane organic cation exchange mechanism.

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