Differential Regulation of Homocysteine Transport in Vascular Endothelial and Smooth Muscle Cells

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Objective—We previously reported that homocysteine (Hcy) inhibits endothelial cell (EC) growth and promotes vascular smooth muscle cell (VSMC) proliferation. This study characterized and directly compared Hcy transport in cultured human aortic ECs (HAECs) and smooth muscle cells (HASMCs).

Methods and Results—Hcy (10 μmol/L) was transported into both cell types in a time-dependent fashion but was approximately 4-fold greater in HASMCs, and is nonstereoenantiomer specific. Hcy transport in HAECs had a Michaelis-Menten constant (Km) of 39 μmol/L and a maximal transport velocity (Vmax) of 873 pmol/mg protein/min. In contrast, Hcy transport in HASMCs had a lower affinity (Km=106 μmol/L) but a higher transport capacity (Vmax=4192 pmol/mg protein/min). Competition studies revealed that the small neutral amino acids tyrosine, cysteine, glycine, serine, alanine, methionine, and leucine inhibited Hcy uptake in both cell types, but the inhibition was greater for tyrosine, serine, glycine, and alanine in HAECs. Sodium-depletion reduced Hcy transport to 16% in HAECs and 56% in HASMCs. Increases in pH from 6.5 to 8.2 or lysosomal inhibitors blocked Hcy uptake only in HAECs. In addition, Hcy shares carrier systems with cysteine, in a preferable order of alanine-serine-cysteine (ASC)>aspartate and glutamate (XASC)=large branched-chain neutral amino acids (L) transporter systems in HAECs and ASC>L>XAG in HASMCs. The sodium-dependent system ASC plays a predominant role for Hcy transport in vascular cells.


Key Words: Homocysteine transport ■ vascular cells

Hyperhomocysteinemia (HHcy) contributes to the etiology of myocardial infarction and stroke,1-3 yet the mechanisms by which homocysteine (Hcy) promotes cardiovascular disease (CVD) are not clear. It was suggested that Hcy promotes CVD via mechanisms involving endothelial injury,4 VSMC proliferation,5 HDL inhibition,6 the conversion to Hcy-thiolactone and induction of an auto-immune response,7 and thrombogenesis.8

During atherogenesis, VSMCs switch to a proliferative phenotype while endothelial cell (EC) growth is suppressed. We were the first to report that Hcy has this distinct cell-type specific proatherogenic effects on vascular cells: it inhibits EC growth and promotes VSMC proliferation.4-9 We demonstrated that Hcy inhibits cyclin A transcription in ECs but activates it in VSMCs.5,8,10 This study is designed to identify the fundamental upstream biochemical mechanisms responsible for the distinct biological and molecular effects of Hcy on vascular cells.

Hcy transport into cells is an essential step of Hcy signaling that leads to its downstream biological effects. Hcy has been reported to enter cultured primary vascular and neuronal cells, and transformed hepatic cells.11-15 In cultured rat embryonic cerebrocortical cells, Hcy causes a neurotoxic response in excitatory amino acid exposure via activation of the N-methyl-D-aspartate subtype of the glutamate receptor.14 Studies with human umbilical vein endothelial cells (HUVECs) found that 2 transporter systems, alanine-serine-cysteine (ASC) and large branched-chain neutral amino acids (L), mediate Hcy uptake (ASC>L), and that the neutral amino acids cysteine and serine inhibit Hcy transport.12 Subsequently, it has been suggested that Hcy uptake is mediated by the sodium-dependent cysteine transport systems in a preferable order of aspartate and glutamate (XASC)≥L>ASC≥alanine (A) in cultured human aortic ECs (HAECs).11

In this study, we hypothesized that Hcy transport may be differentially regulated in vascular cells and characterized the

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transport of a physiologically relevant concentration of Hcy in cultured HAECs and HASMCS.

### Materials and Methods

#### Vascular Cell Culture and Chemicals

HAECs and HASMCS (Cambrex Corporation, East Rutherford, NJ) were cultured as previously described and used between passages 5 to 7. Each experiment was performed with at least 2 different cell preparations from both male and female donors between 29 to 43 years of age (HAEC lot#14841, 2F0572, & 5F1500; HASMC lot# 9F2203 & 6F3533). HEACs were grown on 0.2% gelatin-coated tissue culture flask or plates. All chemicals if not specified below were purchased from Sigma-Aldrich.

#### Preparation of D.L-[35S]Hcy, L-Hcy, and D-Hcy

D.L-[35S]Hcy-thiolactone was prepared by hydrobromic acid digestion of L-[35S]methionine (Met) and converted to D.L-[35S]Hcy as previously described. The hydriodic acid digestion of L-Met is known to afford racemic D.L-Hcy-thiolactone. Using bleomycin hydrolase, which hydrolyzes L-Hcy-thiolactone to L-Hcy, but does not hydrolyze D-Hcy-thiolactone, we confirmed that the [35S]Hcy-thiolactone prepared from L-[35S]Met is indeed an equimolar mixture of L- and D-stereoisomers, D.L-[35S]Hcy-thiolactone. D.L-[35S]Hcy thiolactone (1 mmol/L, 40,000 Ci/mol) was hydrolyzed with 0.1 mol/L NaOH at room temperature for 10 minutes, and the product was treated with 50 mmol/L dithiothreitol (room temperature, 1 minute) to afford D.L-[35S]Hcy (1 mmol/L, 40,000 Ci/mol), which was then neutralized with 0.1 mol/L HCl.

L-Hcy and D-Hcy were obtained by the reduction of L-homocystine and D-homocystine by a 2-fold molar excess of dithiothreitol for 30 minutes at 37°C and pH 8.0. Fresh D.L-[35S]Hcy, L-Hcy or D-Hcy was prepared for each experiment.

#### Hcy Transport Assay

HAECs or HASMCS were grown on 24-well plates to confluence. Transport of Hcy was determined by measuring the influx of radiolabeled D.L-[35S]Hcy as described. For the time course studies, cells were washed with HEPES buffer (140 mmol/L sodium chloride, 5.6 mmol/L D-glucose, 5.0 mmol/L KCl, 1.0 mmol/L MgCl2, 0.9 mmol/L CaCl2, and 25 mmol/L HEPES, pH 7.4) and incubated in HEPES buffer containing D.L-[35S]Hcy (2 μCi, 0.1 μmol/L) and unlabeled L-Hcy (10 μmol/L) for 30 sec and 1, 2, 5, 10, 15, and 30 minutes at 37°C. For all subsequent studies, cells were incubated with Hcy for 10 minutes. Cells were washed with ice-cold HEPES buffer and lysed with 0.2% SDS in 0.2 N NaOH. Cell-associated radioactivity was counted by liquid scintillation spectrometry and normalized by DNA concentration. Hcy transport activity was expressed as pmol intracellular Hcy per μg DNA per min. To correct for nonspecific uptake, at each time point, cells were incubated in parallel with HEPES buffer containing 1 mmol/L unlabeled L-Hcy and the fraction of the radioactivity of the cells was determined and subtracted from each data point. Hcy uptake efficiency was calculated by dividing cell-associated radioactivity by total radioactivity in the culture medium and cells.

#### Kinetic Studies

To study the kinetics of Hcy transport, HAECs or HASMCS were incubated with increasing concentrations of L-Hcy (5, 10, 25, 50, 100, or 250 μmol/L) and D.L-[35S]Hcy (2 μCi, 0.1 μmol/L) for 10 minutes and the rate of [35S]Hcy uptake (V) determined. A nonlinear regression analysis was performed using SigmaPlot 9.0 (Systat software). Kinetic parameters were calculated from the Eadie-Hofstee (V versus V/Hcy) plots as described. Transport velocity was plotted as a function of velocity/Hcy concentration (μmol/L).

#### Amino Acid Competition and pH and Sodium Dependency on Hcy Transport

The competition of other amino acids with Hcy transport was examined by incubating cells with D.L-[35S]Hcy (2 μCi, 0.1 μmol/L) and L-Hcy (10 μmol/L) in the presence of 100-fold excess of a tested amino acids (1 mmol/L). To explore sodium dependency, Hcy transport assays were performed by replacing the sodium chloride with equal molar choline chloride, lithium chloride, or N-methyl-D-glucamine (NMDG) in HEPES buffer to assess the effect of pH. Hcy transport, assays were carried out in HEPES buffer of pH 6.5, 7.0, 7.4, 7.8, or 8.2 (adjusted with 1 M KOH).

#### Effects of Golgi, Lysosomal, and Cysteine Transport Inhibitors on Hcy Transport

To test the relationship between Hcy transport and other intracellular transport systems, cells were preincubated for 1 hour with brefeldin A (BFA; 10 μmol/L), which blocks translocation of proteins from the endoplasmic reticulum (ER), monensin (Mon; 10 μmol/L), which causes lysosomal acidification and degradation, or concanamycin A (Con A; 2 μmol/L), which inhibits vacuolar H+-ATPase and induces lysosomal dysfunction. The role of cysteine (Cys) transport systems in Hcy transport was tested by incubating cells with D.L-[35S]Hcy (2 μCi, 0.1 μmol/L) and L-Hcy (10 μmol/L) in the presence of inhibitors (1 mmol/L for 10 minutes) of the various Cys transport systems, including L-aspartic acid β-hydroxamate (ABH) for sodium-dependent system XAG, Serine (Ser) for system ASC, L-[35S]methionine (Met) and converted to D,L-[35S]Hcy as previously described. The hydriodic acid digestion of L-[35S]Met is indeed an equimolar mixture of L- and D-stereoisomers, D.L-[35S]Hcy thiolactone. D,L-[35S]Hcy thiolactone (1 mmol/L, 40,000 Ci/mol) was hydrolyzed with 0.1 mol/L NaOH at room temperature for 10 minutes, and the product was treated with 50 mmol/L dithiothreitol (room temperature, 1 minute) to afford D.L-[35S]Hcy (1 mmol/L, 40,000 Ci/mol), which was then neutralized with 0.1 mol/L HCl.

L-Hcy and D-Hcy were obtained by the reduction of L-homocystine and D-homocystine by a 2-fold molar excess of dithiothreitol for 30 minutes at 37°C and pH 8.0. Fresh D.L-[35S]Hcy, L-Hcy or D-Hcy was prepared for each experiment.

#### Measurement of Cytosolic Ca2+

Concentration [Ca2+]i

Assays of Cytosolic [Ca2+]i, in HAECs were performed as previously described. Fresh D.L-[35S]Hcy, L-Hcy or D-Hcy was prepared for each experiment.

#### Lysosome Examination by Confocal Microscopy

Lysosomes were localized using Lysotracker Red (LysoT), an acidotropic fluorescent dye that is concentrated in mammalian lysosomes, (Molecular Probes, 1 μmol/L). After a 1-hour pretreatment with vehicle DMSO (0.1% v/v), Con A (2 μmol/L), or Mon (10 μmol/L), fluorescence was examined using a confocal scanning laser microscope (Leica TCS SL) with excitation/emission wavelengths set at 543/620 nm, respectively.

#### Statistical Analysis

Each experiment was repeated 3 times, and every experimental point was measured in triplicate (n=9). Results are expressed as the mean±SD. Statistical comparison of single parameters between 2 groups were performed by paired Student t test. Kruskal-Wallis 1-way ANOVA was used to compare the means of multiple groups, followed by Dunn’s test. A probability value ≤0.05 was considered significant.

#### Results

Hcy Transport Activity Was Lower in HAECs Than in HASMCS, and not Stereorenamtiomer Specific

Specific transport of Hcy was time-dependent in HAECs and HASMCS (Figure 1A). In both cell types, Hcy transport activity progressively increased over the first 15 minutes reaching a plateau at 30 minutes. The uptake of [35S]Hcy was
about 4.85-fold greater in HASMCs than in HAECs after a 30 minutes (202.8 pmol/H9262 g DNA/min in HASMCs and 41.8 pmol/H9262 g DNA/min in HAECs, _P_/H11021 0.05). Hcy, at a physiological relevant concentration (10.1 mol/L), was transported more efficiently in HASMCs (10.8%) than in HAECs (2.6%) after 30 minutes incubation (Figure 1B). Hcy uptake after 10 minutes was in the exponential phase uptake in both cell types (121.9 pmol/H9262 g DNA/min in HASMCs and 29.5 pmol/H9262 g DNA/min in HAECs), and reached 60% and 71% of maximum Hcy transport activity in HASMCs and HAECs, respectively. In all subsequent experiments, Hcy transport was measured over a 10-minute time period. The unlabeled Hcy stereoisomers, L-Hcy and D-Hcy, are similarly effective in competing with D,L-[35S]Hcy transport. Hcy transport was lowered to 5% and 9% by 100-fold excess of L-Hcy, to 10% and 12% by 100-fold excess of D-Hcy, and to 7% and 9% by 100-fold excess of D,L-Hcy in HAECs and HASMCs, respectively (Figure 1C and 1D).

**Figure 1.** Hcy transport activity and uptake efficiency. HAECs and HASMCs were incubated for indicated times with D,L-[35S]Hcy (0.1 µmol/L, 2 µCi) and L-Hcy (10 µmol/L). A, Hcy transport activity was determined by cell-associated radioactivity which was corrected for nonspecific uptake and normalized by DNA concentration. Hcy Transport activity at 15 minutes in each cell type was defined as 100%. B, Hcy uptake efficiency was calculated by dividing cell-associated radioactivity by total radioactivity in the culture medium and cells. Total radioactivity of D,L-[35S]Hcy in each reaction was defined as 100%. C & D, HAECs and HASMCs were incubated with D,L-[35S]Hcy (0.1 µmol/L, 2 µCi) in the presence of 1 mmol/L of L-Hcy, D-Hcy, or D,L-Hcy for 10 minutes. Values represent the mean±SD from 3 separate experiments (n=9).

**Hcy Transport Had Higher Affinity and Lower Transport Capacity in HAECs Than in HASMCs**

Kinetic studies revealed that Hcy transport in HAECs had a Michaelis-Menten constant (Km) of 39 µmol/L and a maximum transport velocity (Vmax) of 23 pmol/µg DNA/min. In contrast, Hcy transport in HASMCs had a lower affinity (Km=106 µmol/L) but a higher transport capacity (Vmax=279 pmol/µg DNA/min; supplemental Figure I, available online at http://atvb.ahajournals.org). Thus, Hcy transport is characterized by a higher affinity and lower transport capacity in HAECs than that in HASMCs.

**Neutral Amino Acids Inhibited Hcy Transport in HAECs and HASMCs**

For our competition studies, we used a concentration of competing amino acids that would saturate the transport system. According to the law of mass action, a useful approximation for this is to use a concentration of the competing amino acid that is 100-times the Km of the transport system. Thus, under our experimental conditions, the competing amino acids would block almost all the transport of Hcy if it was occurring through a similar transport system(s). Small neutral amino acids Cys, Ser, Tyr, Gln, Ala, Leu, and Met, at a 100-fold higher molar concentration (1 mmol/L) significantly inhibited the transport of Hcy (10.1 µmol/L; Figure 2). Cys was the strongest competitor with similar competitive potency in both cell types. Cys reduced Hcy transport to 8% in HAECs and 5% in HASMCs. The neutral nonpolar amino acid Ala and neutral polar amino acids Gln, Ser, Tyr blocked Hcy transport in both cell types but was more efficacious in HAECs. These neutral amino
acids reduced Hcy transport to 23%, 13%, 13%, and 5%, respectively, in HAECs compared with 32%, 26%, 37%, and 54%, respectively, in HASMCs. In contrast, Leu and Met inhibited Hcy transport more efficiently in HASMCs. Leu and Met reduced Hcy uptake to 39% and 37%, respectively, in HAECs and to 23% and 27%, respectively, in HASMCs. Among the basic and acidic amino acids tested, minimal inhibitory effects were observed; however, aspartic acid had a modest stimulatory effect, especially in HASMCs.

Hcy Transport Was More Sensitive to Sodium-Depletion in HAECs

Substitution of sodium in the uptake buffer with choline, lithium, or NMDG at an equimolar concentration significantly decreased Hcy transport. Hcy transport was reduced to 34% in HAECs and to 63% in HASMCs by choline substitution, to 55% and 78% by lithium, and to 16% and 56% by NMDG, a more specific sodium substitute (Figure 3A and 3B). These findings indicate that Hcy is transported by both basic and acidic amino acids tested, minimal inhibitory effects were observed; however, aspartic acid had a modest stimulatory effect, especially in HASMCs.

Figure 2. Amino acids competition with Hcy transport. HAECs and HASMCs were incubated with D,L[S]Hcy (0.1 μmol/L, 2 μCi) and L-Hcy (10 μmol/L) in the presence of 1 mmol/L of indicated competing amino acids for 10 minutes. Hcy transport activity was determined by cell-associated radioactivity which was corrected for nonspecific uptake and normalized by DNA concentration. Values represent the mean±SD from 3 separate experiments (n=9). Hcy Transport activity of the control group (without competing AA) was defined as 100%. *P< 0.05 vs control group. Note that small neutral amino acids cysteine (Cys), glutamine (Gln), serine (Ser), tyrosine (Tyr), alanine (Ala), leucine (Leu), and methionine (Met) inhibited Hcy transport in both cell types but the inhibition was greater in HAECs.

Figure 3. Sodium-dependency and pH sensitivity of Hcy transport. HAECs and HASMCs were incubated with D,L[S]Hcy (0.1 μmol/L, 2 μCi) and L-Hcy (10 μmol/L) in sodium-containing or sodium-free (replaced by choline, lithium or NMDG) HEPES (A & B), or in HEPES of indicated pH (C & D) for 10 minutes. Values represent the mean±SD from 3 separate experiments (n=9). Hcy transport activity of the sodium group was defined as 100% in A & B. Hcy transport activity of the PH 7.4 group was defined as 100% in C & D.*P< 0.05 vs the sodium group. #P< 0.05 vs the PH 7.4 group.
sodium-dependent and sodium-independent carrier systems in both cell types, but that Hcy uptake is predominantly mediated by sodium-dependent carrier systems in HAECS.

**Hcy Transport Activity Was Reduced in Response to Increases in pH in HAECS**

Because pH sensitivity is an important feature of some amino acid transport systems, we examined the effect of pH (6.5 to 8.2) on Hcy transport. We found that Hcy transport was highly sensitive to pH in HAECS and was reduced to 40% when pH was increased from 7.8 to 8.2, a condition which induces lysosomal lysis (Figure 3C and 3D). In contrast, changes in pH minimally affected Hcy transport in HASMCs.

**Hcy Transport Was Partially Inhibited by Lysosomal Inhibitors in HAECS**

To test the role of lysosomes on Hcy transport, we pretreated cells with lysosomal inhibitors for 30 minutes before the transport assay. The lysosome inhibitor Mon significantly inhibited Hcy transport in HAECS, but not in HASMCs. At the concentration of 10 μmol/L, Mon inhibited Hcy transport in HAECS to 76%. Similarly, another lysosomal inhibitor Con A (2 μmol/L) selectively inhibited Hcy transport in HAECS but not in HASMCs. In contrast, the Golgi inhibitor BFA had no effect on Hcy transport in HAECS, but increased it in HASMCs (Figure 4A and 4B).

**Lysosomal Disruption Does Not Alter ATP-Induced [Ca^{2+}]_i Elevation in HAECS**

To confirm that Mon and Con A affect lysosome structure in HAECS, we examined lysosomes with the lysosomal specific fluorescent dye (LysoT). Lysosomes were detected as red fluorescent granules in the cytoplasm of the cells (Figure 4C1). Pretreatment with Con A (2 μmol/L, 1 hour) and Mon (10 μmol/L, 1 hour) largely diminished lysosomal-specific staining in HAECS (Figure 4C2 and 4C3). However, ATP-induced calcium release, a standard indicator of cell viability and reactivity, was not changed by the pretreatment with Con A or Mon (Figure 4D2 and 4D3). Perfusion of ATP (50 μmol/L) elevated cytoplasmic Ca^{2+} ([Ca^{2+}]_i) in HAECS cells by 837±8.6 nM (Figure 4D1). In cells pretreated for 1 hour with Con A or Mon, ATP (50 μmol/L) induced an increase in ([Ca^{2+}]_i) by 814±9.3 nmol/L and 807±8.4 nmol/L, respectively. However, higher concentrations of Con A (2.5 μmol/L) and Mon (25 μmol/L) resulted in a significant inhibition in ATP-induced [Ca^{2+}]_i (data not shown) and were not used.

**Transport System ASC Is Responsible for 90% of Hcy Uptake in HAECS and Is Lysosomal-Dependent**

The classical ASC system inhibitor serine reduced Hcy transport in HAECS to 11% and to 35% in HASMCs, suggesting ASC system mediates about 90% of Hcy uptake in HAECS and about 65% of that in HASMCs (Figure 5A and 5B). A mixture of inhibitors of the 4 Cys transport systems (a competitive substrate inhibitor serine for system ASC, a competitive inhibitor BCH of the system L, an analogue inhibitor ABH for system X_{agi}, and a competitive substrate inhibitor MeAIB for system A, each at 1 mmol/L), reduced Hcy transport to 5% in HAECS and to 13% in HASMCs. Lysosomal inhibitors Con A (2 μmol/L) or Mon (10 μmol/L) inhibited Hcy uptake only in HAECS. Inhibitory effect of serine on Hcy uptake was not further enhanced by the presence of Con A and Mon. ABH and BCH lowered Hcy uptake to 57% and 65%, respectively, in HAECS, and 73% and 48%, respectively, in HASMCs. This inhibitory effect was further increased in both cell types when it was combined with Con A and Mon (Figure 5C and 5D). Finally, the system A inhibitor MeAIB had no effect on Hcy transport in either cell type.

**Discussion**

The present study demonstrates that Hcy transport by vascular cells is mediated by at least 3 different transport systems. Although both ECs and VSMCs use similar transport systems, we found that Hcy is taken up by different biochemical pathways in the 2 cell types. Compared with VSMCs, ECs have a lower transport capacity and a higher affinity for Hcy. In addition, Hcy transport in ECs exhibits a greater sodium dependency than in VSMCs and, unlike in VSMCs, is inhibited at alkaline pH. System ASC plays a predominant role for Hcy transport and is lysosomal-dependent in ECs, but not in VSMCs. Hcy transport was assessed in a physiologically relevant concentration of Hcy with 99% unlabeled L-Hcy (10 μmol/L) and 1% tracer D,L-[35S]Hcy (0.1 μmol/L) in this study. Because both D- and L-stereoisomers of Hcy are equally effective in blocking the uptake of radiolabeled D,L-[35S]Hcy by vascular cells, our data most likely reflect the activity of L-Hcy transport, and D-[35S]Hcy contributes only about 0.5% of Hcy transport activity reported in this study.

Maximal Hcy uptake is 4.15-fold higher in VSMCs than that in ECs (10.8% versus 2.6%). This can probably be explained by a 4.8-fold higher transport velocity in SMCs ($V_{\text{max}}=279$ pmol/μg DNA/min versus 23 pmol/μg DNA/min in HAECS), which suggests an increase in the number of transporters for Hcy in VSMCs than in ECs. Even though Hcy transport in HAECS had a higher affinity ($K_m=39$ μmol/L in ECs versus 106 μmol/L in VSMCs), this did not compensate for its lower transport velocity. These results indicate that Hcy-induced EC injury cannot be attributed to higher Hcy uptake in these cells. It is very likely that other aspects of biochemical mechanisms involving Hcy, independent of total Hcy uptake, are responsible for the selective cytotoxic effects of Hcy in ECs.

Unlike the uptake of amino acids, Hcy transport is not stereoenantiomer specific. Both L-Hcy and D-Hcy equally and significantly compete with D,L-[35S]Hcy for uptake (Figure 1C and 1D).

Hcy shares transporters with other small amino acids Cys, Ala, Gln, Ser, Tyr, Leu, and Met, and that cell-specific mechanisms may contribute to more potent competition by Ala, Gln, Ser, Tyr in ECs and Leu and Met in VSMCs (Figure 2). It was reported that ECs use Cys transport systems to import Hcy, Cys circulates (200 to 300 μmol/L) at a 30-fold higher concentration than Hcy (5 to 10 μmol/L), is not an established risk factor for CVD, and does not inhibit EC growth. Therefore, it is unlikely that the competition between Cys...
and Hcy result from oxidative modifications because Hcy differs from Cys in only having an additional methylene group. The equally potent competition by Cys with Hcy transport in both ECs and SMCs supports the notion stated above that Hcy transport activity does not contribute to the selective cytopathic effects of Hcy in ECs.

Studies with human ECs suggest that Hcy uptake is mediated by the sodium-dependent transport systems XAG, Figure 4.

**Figure 4.** A & B, Effect of Golgi and lysosome inhibitors on Hcy transport. HAECs and HASMCs were pretreated with the Golgi inhibitor brefeldin A (BFA, 10 μmol/L), lysosome inhibitor concanamycin A (Con A, 2 μmol/L), and lysosomal disruptor monensin (Mon, 10 μmol/L) for 1 hour, and then incubated with D,L[35S]Hcy (0.1 μmol/L, 2 μCi) and L-Hcy (10 μmol/L) for 10 minutes. Values represent the mean±SD from 3 separate experiments (n=9). Hcy transport activity of the control group without any inhibitors was defined as 100%. *P<0.05 vs control group. Note that lysosomal inhibitors blocked Hcy transport activity only in HAECs. C&D, Lysosomal confocal imaging and cell viability (ATP-induced [Ca²⁺]). Lysosomes were detected in HAECs using Lysotracker Red as red fluorescent granules by confocal microscopy (C1). Disruption of lysosomes by pretreatment with Con A, 2 μmol/L, 1 hour or Mon, 10 μmol/L, 1 hour produced a marked decrease in fluorescence (C2 and C3). ATP (50 μmol/L) induced an elevation of [Ca²⁺] by 837±8.6 nmol/L. A representative trace is shown in D1. ATP-induced [Ca²⁺] increase was unaffected by pretreatment with Con A or Mon; actual Ca²⁺ traces are shown in D2 and D3, respectively; in all cases n=10. Note that lysosomal disruption does not alter ATP-induced [Ca²⁺] elevation in HAECs.
Sodium-dependent amino acid transporters use free energy stored as the $\text{Na}^+$/H$^+$ electrochemical potential gradient across plasma membranes and is able to transport amino acids against the concentration gradient. Sodium-independent transport system L preferentially transports large neutral amino acids. It appears that Hcy transport is largely dependent on the presence of sodium and reduced to 16% by a highly selective sodium substitute NMDG in ECs and to 56% in VSMCs. These data suggest that ECs have higher capacity to maintain a higher intracellular concentration of Hcy against the plasma concentration gradient. Interestingly, Hcy transport is inhibited at basic pH suggesting that endothelial Hcy transport is retarded in an alkaline environment, a condition which induces lysosomal lysis.

We have also examined the role of intracellular organelles in regulating Hcy transport in vascular cells. The involvement of lysosomes was examined by disrupting lysosome structure with Con A and monensin. We found that lysosome inhibitors specifically block Hcy transport in ECs but not in VSMCs. These data suggest that ECs have higher capacity to maintain a higher intracellular concentration of Hcy against the plasma concentration gradient. Interestingly, Hcy transport is inhibited at basic pH suggesting that endothelial Hcy transport is retarded in an alkaline environment, a condition which induces lysosomal lysis.

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Hcy transport appears to occur mostly via the system ASC in both vascular cells, but HAECs use ASC system for about 90% of Hcy uptake. This is consistent with a previous study in HUVECs, indicating that the system ASC predominantly mediates Hcy transport in human vein and arterial ECs. However, our data do not support a recently proposed model that Hcy transport occurs in the order of XAG/L/ASC in HAECs. We find that system A is not involved in Hcy transport in either type of vascular cells, and that Hcy shares transport systems with Cys, in the order of ASC/L/XAG in VSMCs.

The ASC, XAG, and L systems are ubiquitously expressed in many cell types. The $\text{Na}^+$-dependent ASC system prefers small neutral amino acids without bulky or branched side chains and transports Ala, Ser, Cys, Gln, and Thr. Alternatively, the $\text{Na}^+$-independent L system prefers large neutral amino acids with bulky side chains and transports Leu, Phe, Met, Cys, and Gln. In contrast, the $\text{Na}^+$-dependent system XAG prefers the acidic amino acids Asp, Glu, and Cystine. We found that the lysosomal inhibitors Con A and Mon additively enhanced ABH and BCH (inhibitors of XAG and L, respectively)–mediated inhibition of Hcy transport, but did not increase serine-mediated inhibition. These findings suggest that lysosomal inhibitors target transport system different from that of ABH and BCH, and share the same target of serine. It appears that the system ASC is uniquely lysosome-dependent and plays predominant role for Hcy transport in ECs. The biological significance of Hcy transport systems...
can only be finally confirmed in the future when the structure of these multisubunit protein complexes are characterized to allow genetic or molecular approaches to delete the transport system.

To our knowledge, our study is the first to directly compare the biochemical features of Hcy transport in different vascular cells and to link this atherogenic amino acid with lysosome function. In conclusion, we have fully characterized the transport of Hcy by vascular cells. Significantly, we identified important differences in the uptake properties for this atherogenic molecule between human ECs and VSMCs. In particular, we identified a greater predominant role for the lysosomal pathway in Hcy-mediated pathologies would provide an important insight into understanding the disease process and may suggest novel therapeutic strategies.

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**Disclosures**

None.

**References**

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