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Homocysteine transport by human aortic endothelial cells: Identification and properties of import systems[☆]

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Abstract

Hyperhomocysteinemia is an independent risk factor for cardiovascular disease. Transport of L-homocysteine into and out of the human vascular endothelium is poorly understood. We hypothesized that cultured human aortic endothelial cells (HAEC) would import L-homocysteine on one or more of the L-cysteine transport systems. Inhibitors of the transporters were used to characterize the uptake of [³⁵S]L-homocysteine, [³⁵S]L-homocystine, and [³⁵S]L-cysteine. We found that L-homocysteine uptake is mediated by the sodium-dependent cysteine transport systems X_{AG}, ASC, and A, and the sodium-independent transport system L. Thus, HAEC utilize multiple cysteine transporters (X_{AG} ≥ L > ASC > A) to import L-homocysteine. Kinetic analysis supported the uptake results. Michaelis–Menten constants (*K_m*) for the four systems yielded values of 19.0, 27.1, 112, and 1000 μM for systems L, X_{AG}, ASC, and A, respectively. The binding and uptake of [³⁵S]L-homocystine, the disulfide homodimer of L-homocysteine, was mediated by systems X_{AG}, L, and ASC but not by system A. In contrast to [³⁵S]L-homocysteine, system x_c was active for [³⁵S]L-homocystine uptake. A similar pattern was observed for [³⁵S]L-cysteine. Thus, L-homocysteine and L-homocystine found in hyperhomocysteinemic subjects can gain entry into the vascular endothelium by way of multiple L-cysteine transporters.

Keywords

Homocysteine; Homocystine; Hyperhomocysteinemia; Homocystinuria; Homocysteine import; Cysteine transport systems; Endothelial cells; Endothelium; Cardiovascular disease

Hyperhomocysteinemia is a modifiable independent risk factor for cardiovascular disease [1], cognitive dysfunction including Alzheimer's disease [2,3], complications of pregnancy [4,5], and osteoporosis [6–8]. The ability of homocysteine to cause endothelial cell dysfunction may be the common feature that links this diverse group of pathologies [9–11]. Cardiovascular cells and tissues are limited in their capacity to metabolize homocysteine in that they do not express cystathionine β-synthase, the first enzyme in the transsulfuration pathway [12,13]. To alleviate intracellular accumulation of homocysteine when the re-methylation pathway is impaired, endothelial cells export homocysteine to the circulation [14]. Because little is known about homocysteine transport in the vascular endothelium, we carried out a detailed

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characterization of homocysteine import by cultured human aortic endothelial cells (HAEC).³ The rationale for this study is that homocystinurics have severe hyperhomocysteinemia with plasma total homocysteine (tHcy) concentrations approaching 500 μ M. Up to 20% of the tHcy in these patients exists as free reduced homocysteine [15], which if imported into the vascular endothelium could have serious adverse effects on endothelial cell function.

Based on the work of Ewadh et al. [16] with HUVEC, we hypothesized that HAEC would utilize one or more of the cysteine transport systems for the binding and uptake of homocysteine. To test this hypothesis, the uptake of [³⁵S]L-homocysteine by HAEC was studied in the presence and absence of cysteine transport inhibitors. Systems X_{AG}, L, ASC, and A are operative in HAEC for homocysteine import, and, on the basis of the kinetic parameters established for each of these systems, X_{AG} and L are likely to be the major entry portals for L-homocysteine in the human aortic vascular endothelium.

Materials and methods

Materials

2-Aminobicyclo(2.2.1)heptane-2-carboxylic acid (BCH), quisqualic acid (Q), L-aspartic acid β -hydroxamate (ABH), serine (Ser), α -(methylamino)isobutyric acid (MeAiB), 1,4-dithioerythritol (DTE), bis-benzimide (Hoechst 33258), 5,5'-dithio-bis-2-nitrobenzoic acid (DTNB), Dulbecco's phosphate-buffered saline (DPBS), endothelial cell growth factor (ECGF), ethylenediaminetetraacetic acid (EDTA), fetal bovine serum (FBS), glucose, heparin sodium salt, L-homocysteine thiolactone, L-homocystine, hydriodic acid, hydroxocobalamin, isopropanol, ninhydrin, *N*-tris[hydroxymethyl]methyl-2-aminoethanesulfonic acid (TES), tris [hydroxymethyl]aminomethane (TRIS), and trypsin-EDTA were obtained from Sigma Chemical (St. Louis, MO). Penicillin, streptomycin, and amphotericin B (antibiotic/antimycotic agents) and culture medium M199 were from Gibco BRL (Gaithersburg, MD). [³⁵S]L-Methionine was obtained from Perkin-Elmer (Wellesley, MA). Cytosint was obtained from ICN Biochemicals (Irvine, CA). Fibronectin was obtained from Boehringer-Mannheim (Indianapolis, IN). All other reagents used were of analytical grade or higher. Diaquocobinamide was prepared from hydroxocobalamin as previously described [17].

Cell culture

Human aortic endothelial cells (HAEC) were isolated from anonymously discarded thoracic aorta from donor hearts used in the Cleveland Clinic Foundation's heart transplant program and subcultured by standard methods [18,19]. The procurement and use of anonymously discarded human thoracic aorta was approved by the Internal Review Board of the Cleveland Clinic Foundation. HAEC were maintained in medium M199 with 90 mg/L heparin, 150 mg/L ECGF, 20% FBS, and antibiotic-antimycotic agents in a humidified-95% air/5% CO₂ incubator at 37 °C. The cells were passaged after trypsinization at a split ratio of 1:3 and grown on fibronectin-coated flasks or 24-well plates. Cells were used between passages 5 and 8.

Correlation between DNA concentration and cell number

HAEC were grown on fibronectin-coated 24-well plates until they attained different degrees of confluency (15–100%). The cells were harvested by trypsinization and counted with a Bright-Line hemacytometer (Reichert, Buffalo, NY). For each well, the cell number was thus

³*Abbreviations used:* HAEC, human aortic endothelial cells; HUVEC, human umbilical vein endothelial cells; BCH, 2-aminobicyclo(2.2.1)heptane-2-carboxylic acid; Q, quisqualic acid; ABH, L-aspartic acid β -hydroxamate; Ser, serine; MeAiB, α -(methylamino)isobutyric acid; DTE, 1,4-dithioerythritol; DTNB, 5,5'-dithio-bis-2-nitrobenzoic acid; DPBS, Dulbecco's phosphate-buffered saline; ECGF, endothelial cell growth factor; EDTA, ethylenediaminetetraacetic acid; FBS, fetal bovine serum; TES, *N*-tris[hydroxymethyl]-methyl-2-aminoethanesulfonic acid; TRIS, tris[hydroxymethyl]amino-methane; tHcy, plasma total homocysteine.

established. DNA content of each well was determined on an aliquot of the trypsinized cells [20]. Cell number was plotted as a function DNA and a value of 1.67×10^5 cells/ μg of DNA was established.

Preparation of [^{35}S]L-homocysteine

[^{35}S]L-Homocysteine was prepared from [^{35}S]L-methionine by modification of the method of Mudd et al. [21]. Briefly, 20.0 μmol L-methionine was mixed with 1 nmol [^{35}S]L-methionine (1.0 mCi), and refluxed with 5 ml hydriodic acid for 18–20 h under argon atmosphere to yield [^{35}S]L-homocysteine thiolactone (specific activity, 50 $\mu\text{Ci}/\mu\text{mol}$). The solution was then evaporated under flowing argon at 45 °C for 24 h to a yellow oil. The latter was dissolved in 0.5 ml of water and [^{35}S]L-homocysteine thiolactone was purified by descending paper chromatography (Whatman 3MM) using a solvent system of isopropyl alcohol, formic acid, and water (70:10:20). Standards of L-methionine and L-homocysteine thiolactone were run in the outer lanes next to the reaction mixture. After running the chromatogram, the central portion containing the resolved reaction components was removed. The positions of the standards were then located with ninhydrin spray. The corresponding [^{35}S]L-homocysteine thiolactone region was cut out, eluted with water, and concentrated in a Speed Vac. The final concentration was determined spectrophotometrically ($\lambda_{\text{max}} = 243 \text{ nm}$; $\epsilon = 2.5 \text{ mM}^{-1}\text{cm}^{-1}$). [^{35}S]L-Homocysteine was prepared from the [^{35}S]L-homocysteine thiolactone by base hydrolysis using the procedure of Duerre and Miller [22]. The pH of the Wnal solution was adjusted to 7.4 with 2 M HCl and 0.05 M TES buVer. The concentration of [^{35}S]L-homocysteine was determined by the procedure of Ellman [23].

Preparation of [^{35}S]L-homocysteine

[^{35}S]L-Homocystine was prepared from [^{35}S]L-homocysteine by catalytic oxidation using a method modified from Büdy et al. [24]. Briefly, an aqueous solution of [^{35}S]L-homocysteine (5.0 mM) was mixed aerobically for approximately 1 h in the presence of 1 μM diaquocobinamide at room temperature. Progress of the reaction was monitored by withdrawing 5 μl aliquots and determining the free thiol concentration [23]. The reaction was terminated when free thiol was undetectable. The reaction product was purified by descending preparative paper chromatography (Whatman 3MM) as described above.

Binding and uptake of [^{35}S]L-homocysteine by HAEC

Saturation-binding studies using [^{35}S]L-homocysteine were carried out on HAEC and Scatchard analysis was used to determine the binding parameters. Binding constants (K_d and B_{max}) were calculated from the data using the non-linear curve-fitting program, Ligand (NIH, Bethesda, MD). The output of Ligand expresses both K_d and B_{max} in micromolar units. To convert the binding capacity, B_{max} , from micromolar units to sites per cell, Avogadro's number, DNA content, sample volume, and the conversion factor of 1.67×10^5 cells/ μg of DNA (determined experimentally) were used.

The binding and uptake of [^{35}S]L-homocysteine was determined in confluent cultures of HAEC grown on fibronectin-coated 24-well plates. After removing the growth medium, the cell layer was washed three times with DPBS–glucose that contained calcium and magnesium to prevent the cells from detaching from the plate (DPBS: 137 mM NaCl, 2.68 mM KCl, 0.90 mM CaCl_2 , 0.50 mM MgCl_2 , 1.50 mM KH_2PO_4 , and 9.58 mM NaH_2PO_4 , pH 7.4) with 0.1% (5.5 mM) D-glucose. The intracellular amino acid pool was depleted by pre-incubation with DPBS–glucose for 90 min at 37 °C. After washing with DPBS–glucose, the cells were incubated at 37 °C for 30 min with increasing concentrations of [^{35}S]L-homocysteine (0–120 μM). The uptake of [^{35}S]L-homocysteine was terminated by removing the incubation medium and washing the cell layer three times with ice-cold PBS. Cells were lysed with 200 μL of 0.1 M NaOH and an aliquot (60 μL) was used for the determination of DNA content [20]. To

determine non-specific cell-associated radioactivity, experiments were performed in the presence of 100-fold molar excess of unlabeled L-homocysteine (specific cell-associated radioactivity = total cell-associated radioactivity – non-specific cell-associated radioactivity). ^{35}S -Radioactivity was determined in a 100 μL aliquot of the NaOH cell lysate by liquid scintillation counting after neutralizing the sample with 2 M HCl. All radioactive measurements were determined by diluting the specified aliquot of sample in 20 ml of Cytoscint scintillation cocktail (MP Biomedicals, Aurora, OH) and counting the sample for 5 min in a Beckman LS6500 liquid scintillation counter (Beckman-Coulter, Fullerton, CA). Lum-Ex correction was selected to correct for chemiluminescence resulting from the lysis buffer. Blanks included the same solvent composition as lysed samples and blank values were subtracted from all sample counts. Binding and uptake of [^{35}S]L-homocystine and [^{35}S]L-cysteine to HAEC were determined similarly.

Analysis of surface-bound versus internalized [^{35}S]L-homocysteine

Total cell-associated L-homocysteine is the sum of surface-bound L-homocysteine plus internalized L-homocysteine. To determine the amount of [^{35}S]L-homocysteine in each compartment HAEC were treated with 50 μM [^{35}S]L-homocysteine in DPBS–glucose for 30 min at 37 °C. The incubation was terminated and the cell layer was washed as described above. However, in this instance, the cells were harvested by trypsinization rather than direct lysis of the cell layer with NaOH to release the surface-bound homocysteine without lysing the cells (200 μl of a trypsin solution containing 500 BAEE units of porcine trypsin and 180 μg $\text{Na}_4\text{EDTA}/\text{ml}$ of DPBS). Total cell-associated [^{35}S]L-homocysteine was determined by counting a 50 μl aliquot of the cell suspension (before centrifugation). After counting the 50 μl aliquot of trypsinized cells, the cells were centrifuged for 10 min at 1000g, and the trypsin supernatant (containing the released surface-bound [^{35}S]L-homocysteine) was collected. The cell pellet was washed once with DPBS and re-centrifuged. The DPBS wash was collected and combined with the trypsin supernatant. Surface-bound [^{35}S]L-homocysteine was determined in an 100 μl aliquot of the combined supernatants. The cell pellet was lysed with 200 μl of 0.1 M NaOH and neutralized with 2 M HCl. Internalized [^{35}S]L-homocysteine was determined by counting an 100 μl aliquot of the neutralized lysate.

Binding and uptake of [^{35}S]L-homocysteine in the presence of cysteine transport inhibitors

Binding of [^{35}S]L-homocysteine was measured in the presence of specific inhibitors of cysteine transport systems, namely BCH, which inhibits system L; Q, which inhibits system x_c ; ABH, which inhibits system X_{AG} ; Ser, which inhibits the sodium-dependent system ASC and the sodium-independent system LAT; MeAiB, which inhibits system A. Initially, a dose-dependency study (0.1–5.0 mM) of each inhibitor was conducted to determine the optimum inhibitor concentrations to be used in the binding and uptake experiments. Based on results of these studies, an inhibitor concentration of 1.0 mM was used in the remainder of the experiments. In those experiments designed to measure the activity of only one transport system, a cocktail of inhibitors was used to block all the other transporters (Table 1). The cell-associated [^{35}S]L-homocysteine was measured as described above. The effect of the inhibitor was calculated as percent of the binding and uptake of [^{35}S]L-homocysteine in the absence of inhibitor(s).

Binding and uptake of [^{35}S]L-homocysteine by HAEC in sodium-free medium

Cysteine transport systems A, ASC, and X_{AG} are sodium-dependent while systems x_c and L (including LAT) are sodium-independent [25]. To explore sodium dependency, cells were incubated in sodium-free medium using the protocol described above. Sodium-free media was prepared by replacing the sodium salts used to prepare DPBS with potassium phosphate and choline chloride. The sodium-free media contains: 100 mM choline chloride, 2.68 mM KCl,

0.90 mM CaCl₂, 0.50 mM MgCl₂, 10 mM KH₂PO₄, and 5.5 mM D-glucose, pH 7.4. Sodium-free [³⁵S]L-homocysteine was prepared by hydrolyzing [³⁵S]L-homocysteine thiolactone with potassium hydroxide as described above.

Kinetic studies on the binding and uptake of [³⁵S]L-homocysteine by HAEC

The initial rates of binding and uptake of [³⁵S]L-homocysteine by HAEC were determined at concentrations ranging from 5 to 32 μM and as a function of time in the absence of cysteine transport inhibitors. The data were normalized to DNA content. Kinetic parameters were obtained from the Lineweaver–Burk plots of the initial rate data.

Statistical analyses

Every experimental point was measured in triplicate and expressed as means ± standard deviation (SD) of the three measurements ($n = 3$). Significance of the data ($p \leq 0.05$) between the means of two groups was determined using an unpaired, two-tailed Student's t test. All experiments were repeated at least three times to confirm reproducibility. Analysis of the binding data, model fitting, and parameter estimation (K_d and B_{max}) were calculated using the least-squares non-linear curve fitting program, Ligand [26] (NIH, Bethesda, MD). The program uses the F ratio test and Hill plots to determine the statistically significant “best fit” model of the data. The kinetic parameters, K_m and V_{max} , were determined using GraphPad Prism (GraphPad, San Diego, CA) to fit the kinetic data.

Results

Binding and uptake of [³⁵S]L-homocysteine and [³⁵S]L-cysteine by HAEC

Initially, the binding studies were carried out at 4 °C to minimize internalization of [³⁵S]L-homocysteine. Because cell detachment occurred at 4 °C, experiments were conducted at 37 °C for 30 min. Approximately 87% of the total cell-associated [³⁵S]L-homocysteine was found to be surface-bound while 13% was internalized. A saturation-binding study for [³⁵S]L-homocysteine is shown in Fig. 1A. Non-specific cell-associated [³⁵S]L-homocysteine was determined in the presence of a 100-fold molar excess of unlabeled L-homocysteine, and non-specific binding was subtracted from total cell-associated [³⁵S]L-homocysteine to obtain specific cell-associated [³⁵S]L-homocysteine. Using Ligand software, a single apparent saturable binding site was observed with $K_d = 130 \pm 15 \mu\text{M}$ and $B_{max} = 1.5 \pm 0.3 \mu\text{M}$ (equivalent to 5700 binding sites per cell). A similar study was carried out for [³⁵S]L-cysteine, however, at the concentrations studied, the binding of [³⁵S]L-cysteine was below saturation (Fig. 1B).

Inhibition of [³⁵S]L-homocysteine binding and uptake by L-cysteine transport inhibitors

To test the hypothesis that L-homocysteine utilizes one or more of the L-cysteine transport systems for import, a pharmacological approach using specific inhibitors of L-cysteine transport systems was conducted. A dose-dependency study (0.1–5.0 mM) on the inhibition of [³⁵S]L-homocysteine binding and uptake by five of the known cysteine transport inhibitors is shown in Fig. 2. Based on these studies, it was concluded that maximal inhibition was achieved with an inhibitor concentration of 1.0 mM for ABH (system X_{AG}), MeAiB (system A), and BCH (system L). However, over the concentration range of 0.1–5.0 mM for inhibitor Q, the binding and uptake of [³⁵S]L-homocysteine was unaffected (Fig. 2D). When serine was used at concentrations greater than 1 mM (2.5 and 5 mM) [³⁵S]L-homocysteine uptake was inhibited an additional 25%, suggesting that serine probably inhibits more than one transporter (Fig. 2E). In Fig. 3, [³⁵S]L-homocysteine binding and uptake was studied in the presence of the individual cysteine transport inhibitors all used at 1.0 mM concentration. The experimental conditions were the same as those outlined above (50 μM [³⁵S]L-homocysteine for 30 min at 37 °C in DPBS–glucose). As shown in Fig. 3A, the binding and uptake of [³⁵S]L-homocysteine

was significantly inhibited by BCH (system L), ABH (system X_{AG}), MeAiB (system A), and Ser (system ASC and possibly LAT), but not by Q (system x_c). These studies suggest that L-homocysteine can be imported by systems X_{AG}, L, A, and ASC but not by system x_c.

Binding and uptake of the homodimer, [³⁵S]L-homocysteine, in the presence of L-cysteine transport inhibitors

In circulation, approximately 10–15% of tHcy exists as L-homocysteine, the oxidized disulfide form of L-homocysteine. To determine if L-homocysteine also utilizes cysteine transporters for import, binding and uptake studies were conducted with 25 μM [³⁵S]L-homocysteine for 30 min at 37 °C in DPBS–glucose in the presence and absence of the inhibitors for cysteine transport. As shown in Fig. 3B, the binding and uptake of [³⁵S]L-homocysteine was significantly inhibited by BCH (system L), Q (system x_c), ABH (system X_{AG}), and Ser (system ASC), but not by MeAiB (system A). These studies suggest that L-homocysteine can be imported by systems ASC, X_{AG}, x_c, and L but not by system A.

Binding and uptake of [³⁵S]L-cysteine in the presence of L-cysteine transport inhibitors

The inhibition of [³⁵S]L-cysteine binding and uptake was determined under the same conditions used for the L-homocysteine studies (50 μM [³⁵S]L-cysteine for 30 min at 37 °C in DPBS–glucose). As shown in Fig. 3C, the binding and uptake of [³⁵S]L-cysteine was significantly inhibited by BCH (system L), Q (system x_c), ABH (system X_{AG}), and Ser (system ASC), but not by MeAiB (system A). These studies suggest that L-cysteine can be imported by systems X_{AG}, L, x_c, and ASC but not by system A.

Binding and uptake of [³⁵S]L-homocysteine in sodium-free medium

Cysteine transport systems X_{AG}, A, and ASC are inactive in the absence of sodium while systems L and x_c are active under sodium-free conditions [25]. Experiments using incubations with 50 μM [³⁵S]L-homocysteine for 30 min at 37 °C were conducted in sodium-free medium in the presence and absence of cysteine transport inhibitors Q (system x_c) and BCH (system L). As shown in Fig. 3D, there was no inhibition of the binding and uptake of [³⁵S]L-homocysteine in the presence of 1.0 mM Q as previously observed in medium containing sodium (Fig. 3A). However, in the presence of 1.0 mM BCH, there was essentially complete inhibition of the binding and uptake of [³⁵S]L-homocysteine suggesting that system L has characteristics of both a Na-dependent and Na-independent transporter of homocysteine (Figs. 3A and D). These observations confirm that in the absence of sodium, systems X_{AG}, A, and ASC are inoperative for the binding and uptake of [³⁵S]L-homocysteine by HAEC, and only system L is functional.

Total inhibition of the binding and uptake of [³⁵S]L-homocysteine by HAEC

A study was conducted to see if the binding and uptake of [³⁵S]L-homocysteine could be completely inhibited by manipulating the contents of the incubation media. As shown in Fig. 4, complete inhibition of the binding and uptake of [³⁵S]L-homocysteine occurred when HAEC were incubated in sodium-free medium containing 1.0 mM BCH to block system L. In sodium-containing medium, 88% of the binding and uptake was inhibited by a cocktail of BCH, ABH, MeAiB, and Ser (all at 1.0 mM final concentration) (Fig. 4). When only two inhibitors were combined, the binding and uptake of [³⁵S]homocysteine was inhibited to a lesser extent (~50%) although still significant. These studies clearly show that the L-cysteine transport systems X_{AG}, A, ASC, and L are also responsible for the import of L-homocysteine into cultured HAEC.

Kinetic studies on the binding and uptake of [³⁵S]L-homocysteine by HAEC

The initial rates of binding and uptake of [³⁵S]L-homocysteine at concentrations ranging from 5 to 32 μM and as a function of time were determined under standard conditions (37 °C in

DPBS–glucose) in the absence of cysteine transport inhibitors. Initial rates were plotted as a function of time (Fig. 5A) and L-homocysteine concentration (Fig. 5B). The data were normalized to the DNA content of each well and rates were expressed as picomole L-homocysteine (Hcy)/ μg DNA versus time. Kinetic parameters were obtained from the Lineweaver–Burk plot using Prism nonlinear least-squares curve fitting (Fig. 5C). The kinetic parameters for global binding and uptake of L-homocysteine were $K_m = 21.3 \pm 0.9 \mu\text{M}$ and $V_{\text{max}} = 13.1 \pm 1.8 \text{ pmol Hcy}/\mu\text{g DNA min}$ (Table 2).

Kinetic studies on the binding and uptake of [^{35}S]L-homocysteine by systems X_{AG} , A, ASC, and L

To obtain kinetic parameters for the rates of binding and uptake of [^{35}S]L-homocysteine by each of the individual L-cysteine transport systems, a cocktail of inhibitors was used in sodium-containing media for systems X_{AG} , A, and ASC (Table 1). System L was studied in sodium-free medium in the absence of inhibitors. The initial-rate data and Lineweaver–Burk plots for systems X_{AG} , A, ASC, and L are shown in Figs. 6A–D, respectively. The kinetic constants of the individual systems and for global binding and uptake are summarized in Table 2. Systems X_{AG} and L with K_m values of 27.1 ± 1.5 and $19.0 \pm 1.3 \mu\text{M}$, respectively, appear to have the most physiological relevance for L-homocysteine transport in HAEC since the plasma tHcy concentrations from hyperhomocysteinemic patients (with either cardiovascular disease, or end-stage renal disease) can range from 15 to $100 \mu\text{M}$. It is likely that these two systems account for most of the L-homocysteine transport in the aortic vascular endothelium.

Discussion

Several lines of evidence suggest that elevated levels of homocysteine disrupt endothelial cell function [27–35]. How homocysteine itself and its oxidized congeners gain entry into vascular endothelial cells is poorly understood. We hypothesized that homocysteine is imported into vascular endothelial cells via one or more of the systems for cysteine transport. Data from the pharmacological studies reported herein strongly support this hypothesis.

The major objective of this study was to identify and characterize the systems that facilitate homocysteine transport into HAEC. We found that homocysteine binding and uptake into HAEC was mediated by four of the known cysteine transport systems. However, we believe that these four transporters may not be the only ones involved in homocysteine uptake and/or efflux. Three of the transporters identified in the present study are sodium-dependent (X_{AG} , A, and ASC) and one is sodium-independent (L). Based upon Scatchard analysis and Michaelis–Menten kinetics, the four transport systems exhibited a wide range of affinities: $K_m = 19.0$, 27.1 , 112 , and $1100 \mu\text{M}$, for systems L, X_{AG} , ASC, and A, respectively. In sodium-containing media, $\approx 50\%$ of the homocysteine uptake is mediated by system X_{AG} and 50% is mediated by system L, while in sodium-free media, 100% is mediated by system L. We did not find any homocysteine binding and uptake mediated by the sodium-independent cystine–glutamate exchanger, system x_c .

In an earlier study, Ewadh et al. [16] examined homocysteine uptake in human umbilical vein endothelial cells (HUVECs) and reported that uptake was mediated by only two systems, namely L ($K_m = 160 \mu\text{M}$) and ASC ($K_m = 73 \mu\text{M}$). Our data support those results; however, in HAEC the K_m values for systems L and ASC were somewhat different ($K_m = 19.0 \mu\text{M}$ and $112.0 \mu\text{M}$, respectively) than those reported in HUVECs. It was also reported by Ewadh et al. [16] that system A was inactive in HUVECs. In contrast, we found that system A was active in HAEC, albeit with very low affinity ($K_m = 1100 \mu\text{M}$). As noted by Ewadh et al. [16] and Cardelli-Cangiano et al. [36], differences in system A activity can be due to the experimental conditions used to isolate endothelial cells. System A transporters appear to be sensitive to the proteases used during cell harvesting from tissues (i.e., collagenase) [16,36]. Also, it must be

noted that HUVEC represent a population of venous endothelial cells while HAEC are derived from arterial cells.

In the present study, we found that system L was responsible for 50% of the homocysteine uptake by HAEC in sodium-containing media, and 100% of the uptake in sodium-free media. Recently, a novel Na-independent system L-transporter, LAT-1, has been cloned from rat glioma cells [37], and this transporter is completely inhibited by BCH and L-cysteine [38] suggesting that it may also be a homocysteine transporter. In those papers, LAT functioned as a transporter for L-leucine [37], and L-serine [38], but the uptake of both amino acids was inhibited by L-cysteine and BCH. Recently LAT-1 expression and transport activity was demonstrated in rat brain capillary endothelial cells and rat retinal vascular endothelial cells [39,40]. In the present study, we used HAEC and found that Hcy uptake was inhibited by BCH by 40% in sodium-containing media and by a further 60% by in sodium-free media (Figs. 3A and D) possibly implicating the system L component, LAT-1/2. In the serine dose-response curve shown in Fig. 2E, there appears to be an inhibition plateau at low concentrations (<0.5 mM) and additional inhibition at higher concentrations (>1 mM) suggesting that serine may inhibit multiple amino acid transporters. If LAT-1/2 is active in Hcy transport in HAEC, one would expect to find additional inhibition by serine in sodium-free media; however, in a small set of experiments we did not find any further inhibition by serine in sodium-free media (data not shown). To really confirm the nature of the BCH and/or serine inhibition (whether it is classical system L and/or additionally LAT-1/2), we would like to study the effect of the LAT-1 antibody on Hcy uptake, or use siRNA to silence LAT-1 expression.

Herein, we report, for the first time, that L-homocysteine binding and uptake is significantly mediated by system X_{AG} ($K_m = 27.1 \mu M$) in human aortic endothelial cells. Using the system X_{AG} inhibitor, ABH, we found that system X_{AG} accounts for 50% of the total cell-associated homocysteine by HAEC. Further, of all the cell-associated L-homocysteine, 87% was surface bound, and only 13% was internalized. The X_{AG} transporters are a family of high affinity glutamate transporters that have been well characterized in the central nervous system (CNS), pulmonary epithelium, and monocyte/macrophages [41–45]. They include the excitatory amino acid transporters, EAAT1–EAAT5, and are sodium and potassium dependent. In the CNS, their primary function is the removal of glutamate from the extra-cellular space in order to terminate its postsynaptic neurotransmitter action and thus protect the cells from neuronal death [41]. However, in addition to glutamate transport, L-cystine is also transported into brain synaptosomes by system X_{AG} , and its transport is inhibited by 75% in the presence of L-homocysteate [46]. In the present study, system X_{AG} slightly favored L-homocystine over L-homocysteine for binding and uptake by HAEC (Fig. 3). In the lung and in macrophages, the primary function of the system X_{AG} transporter is to facilitate the transport of glutamate into the cells for glutathione synthesis, resulting in a protective mechanism against oxidative stress [44,45]. In hyperhomocysteinemia it would be advantageous for endothelial cells to up-regulate the import of glutamate, and thereby increase the production of glutathione to combat the increased oxidative stress imposed by elevated plasma homocysteine levels. Thus, system X_{AG} could have a dual role in HAEC: one involved in homocysteine clearance from the plasma and one involved in up-regulation of glutamate transport into cells to produce glutathione and combat the increased oxidative stress that accompanies hyperhomocysteinemia.

Only a small amount of bound L-homocysteine (13%) was internalized during the 30-min uptake period (Fig. 2). Foreman et al. [47] found similar results with L-homocystine uptake in rat renal cortical tubules. They showed that after 30 min of incubation no detectable levels of homocysteine, homocystine, cystine, or methionine were present in the cells [47]. All of the internalized homocystine had been converted to its metabolites, cystathionine (74%), S-adenosylhomocysteine (18%), and cysteine (8%). However, in HAEC and other cardiovascular cells and tissues, the trans-sulfuration pathway for the conversion of homocysteine to cysteine via

cystathionine is inactive [12,13] because cystathionine β -synthase is not expressed. Thus, the arterial endothelium may be particularly susceptible to the adverse effects of elevated plasma homocysteine.

Without an active transsulfuration pathway to metabolize Hcy, the only routes available for the removal of intra-cellular homocysteine are re-methylation back to methionine or export to the circulation [14]. Homocysteine export in HUVEC [48–50], human hepatoma cells [49], and mouse fibroblasts [51] has been investigated. Existence of a homocysteine carrier for intracellular homocysteine in HUVEC has been suggested [48]. HUVEC exported homocysteine at a constant rate up to 72 h [50]. HAEC exported homocysteine at a higher rate than human hepatoma cells [49]. Methotrexate induced homocysteine export in cultured mouse fibroblasts [51], suggesting that efflux of homocysteine from the cell compensates for the impairment of homocysteine metabolism due to inhibition of dihydrofolate reductase by methotrexate. No difference was observed between the rates of homocysteine export by normal cultured HUVEC and cultured HUVEC obtained from CBS-deficient patients [52]. This finding is not unexpected if one considers that endothelial cells do not express CBS [12,13].

These studies demonstrate for the first time that human aortic endothelial cells can bind and import L-homocysteine via at least four of the known cysteine transport systems, namely X_{AG} , L, ASC, and A, while L-homocysteine is imported through systems X_{AG} , L, ASC, and x_c . In subjects with mild hyperhomocysteinemia, the concentration of tHcy ranges from >12 to 25 μ M. However, 75–80% of the homocysteine in these individuals is disulfide-linked to plasma protein cysteine residues [53–57] and is, therefore, unavailable for transport via the cysteine transport systems. Subjects with intermediate and severe hyperhomocysteinemia have tHcy levels of >25–100 and >100–500 μ M, respectively. In particular, homocystinurics with severe hyperhomocysteinemia often have tHcy concentrations approaching 500 μ M. Up to 20% or higher (≥ 100 μ M) of the tHcy in these patients exists as L-homocysteine and L-homocystine [15], which, if imported into the vascular endothelium would have serious adverse effects on endothelial function. This study has identified and characterized the entry sites for L-homocysteine and L-homocystine in the human vascular endothelium.

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Fig. 1. Binding and uptake of $[^{35}\text{S}]$ L-homocysteine and $[^{35}\text{S}]$ L-cysteine by HAEC. Cells were cultured as described in Materials and methods and then incubated with (A) $[^{35}\text{S}]$ L-homocysteine or (B) $[^{35}\text{S}]$ L-cysteine at the indicated concentrations for 30 min at 37 °C. “Cell associated” is equivalent to “binding and uptake”. Non-specific cell-associated $[^{35}\text{S}]$ L-homocysteine or $[^{35}\text{S}]$ L-cysteine was determined in the presence of 100-fold molar excess of unlabeled L-homocysteine or L-cysteine, respectively. Specific cell-associated = total cell-associated – non-specific cell-associated. Scatchard plots (insets) were generated from the binding data using Ligand (NIH, Bethesda, MD) software. Each data point is the mean $\pm$ SD from three replicates.

Fig. 2.

Binding and uptake of [35 S]L-homocysteine by HAEC in the presence of increasing concentrations of various L-cysteine transport inhibitors. Confluent cultures of HAEC were incubated with 50 μ M [35 S]L-homocysteine for 30 min at 37 °C, in the presence of either 0.1–5.0 mM ABH (inhibitor of system X_{AG}) (A); 0.1–5.0 mM MeAiB (inhibitor of system A) (B); 0.1–5.0 mM BCH (inhibitor of system L) (C); 0.1–5.0 mM Q (inhibitor of system x_c) (D); or 0.1–5.0 mM Ser (inhibitor of system ASC and/or LAT) (E). Non-specific binding (NS) was determined in the presence of 100-fold molar excess of unlabeled L-homocysteine. Results are expressed as percent of the control L-homocysteine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control (C).

Fig. 3.

Binding and uptake of [³⁵S]L-homocysteine, [³⁵S]L-homocystine, and [³⁵S]L-cysteine by HAEC in the presence of L-cysteine transport inhibitors. (A) Inhibition of [³⁵S]L-homocysteine binding and uptake. Confluent cultures of HAEC were incubated with 50 μ M [³⁵S]L-homocysteine for 30 min at 37 °C, and the effect of each cysteine transport inhibitor on [³⁵S]L-homocysteine binding and uptake is shown. All inhibitors: BCH (system L), Q (system X_c), ABH (X_{AG}), MeAiB (system A), and Ser (system ASC) were used at a final concentration of 1.0 mM. Non-specific binding (NS) of [³⁵S]L-homocysteine was determined in the presence of 100-fold molar excess of unlabeled L-homocysteine. Results are expressed as percent of the control L-homocysteine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control (C). (B) Inhibition of [³⁵S]L-homocystine binding and uptake. HAEC were incubated with 25 μ M [³⁵S]L-homocystine for 30 min at 37 °C, and the same concentrations of cysteine transport inhibitors were used as described in (A). Non-specific binding (NS) of [³⁵S]L-homocystine was determined in the presence of 100-fold molar excess of unlabeled L-homocystine. Results are expressed as percent of the control L-homocystine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control (C). (C) Inhibition of [³⁵S]L-cysteine binding and uptake. HAEC were incubated with 50 μ M [³⁵S]L-cysteine for 30 min at 37 °C, and the same concentrations of cysteine transport inhibitors were used as in described in (A). Non-specific binding (NS) of [³⁵S]L-cysteine was determined in the presence of 100-fold molar excess of unlabeled L-cysteine. Results are expressed as percent of the control L-cysteine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control (C). (D) Inhibition of [³⁵S]L-homocysteine binding and uptake in sodium-free medium. Confluent cultures of HAEC were incubated with 50 μ M [³⁵S]L-homocysteine for 30 min at 37 °C in sodium-free medium. The sodium-free media contained: 100 mM choline chloride, 2.68 mM KCl, 0.90 mM CaCl₂, 0.50 mM MgCl₂, 10 mM KH₂PO₄, and 5.5 mM D-

glucose, pH 7.4. No inhibition was observed in the presence of the system x_c inhibitor Q (1.0 mM final concentration), as was also observed in sodium-containing media in (A). Nearly complete inhibition of L-homocysteine binding and uptake was observed in the presence of the system L inhibitor BCH (1.0 mM final concentration). Non-specific binding (NS) of [35 S] L-homocysteine was determined in the presence of 100-fold molar excess of unlabeled L-homocysteine in sodium-free medium. Results are expressed as percent of the control L-homocysteine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control.

Fig. 4.

Total inhibition of binding and uptake of [^{35}S]L-homocysteine by HAEC. Confluent cultures of HAEC were incubated with 50 μM [^{35}S]L-homocysteine for 30 min at 37 $^{\circ}\text{C}$. Complete inhibition of [^{35}S]L-homocysteine binding and uptake was observed in sodium-free medium in the presence of 1.0 mM BCH as previously observed (Fig. 3D). In sodium-containing medium, a cocktail of all four inhibitors (BCH, ABH, MeAiB, and Ser all at 1.0 mM final concentration) was used, and binding and uptake was inhibited 88%. In sodium-containing medium combinations of ABH + BCH, MeAiB + BCH, and Ser + BCH, resulted in inhibitions of 53, 41, and 48%, respectively. Non-specific binding (NS) of [^{35}S]L-homocysteine was determined in the presence of 100-fold molar excess of unlabeled L-homocysteine in sodium-containing medium. Results are expressed as percent of the control L-homocysteine uptake. Each data point is the mean $\pm$ SD from three replicates. * $p \leq 0.05$, significantly different from control.

Fig. 5.

Kinetics of L-homocysteine binding and uptake by HAEC. (A) Confluent cultures of HAEC were incubated with [^{35}S]L-homocysteine in concentrations ranging from 5 to 32 μM . The data were normalized to DNA content in each well and plotted as picomole L-Hcy/ μg DNA. (B) Initial binding and uptake rates were plotted as a function of L-homocysteine concentration. (C) Lineweaver–Burk plot of the data was analyzed using Prism (GraphPad, San Diego, CA) software from which $K_m = 21.3 \pm 0.9 \mu\text{M}$ and $V_{\max} = 13.1 \pm 1.8 \text{ pmol L-Hcy}/\mu\text{g DNA min}$ were derived. Each data point is the mean $\pm$ SD from three replicates.

Fig. 6. Kinetics of L-homocysteine binding and uptake by systems X_{AG}, A, ASC, and L in HAEC. Systems X_{AG}, A, and ASC were studied in sodium-containing medium and a cocktail of inhibitors as described in Table 1. System L was studied in sodium-free medium in the absence of inhibitors. The initial-rate data and Lineweaver–Burk plots (insets) are shown for system X_{AG} (A), system A (B), system ASC (C), and system L (D). The data were analyzed using Prism (GraphPad, San Diego, CA) software to determine the “best-fit” model and to compute the kinetic constants which are summarized in Table 2.

Table 1

Medium cocktails for the study of L-cysteine transporters

System	Media
A	1 mM ABH, 1 mM Ser, 1 mM BCH in DPBS–glucose
ASC	1 mM ABH, 1 mM MeAiB, 1 mM BCH in DPBS–glucose
X _{AG}	1 mM MeAiB, 1 mM Ser, 1 mM BCH in DPBS–glucose
L	Sodium-free medium

Table 2

Kinetic parameters for L-homocysteine global transport and by the individual systems

System	$K_m \pm SD$ (μM)	$V_{max} \pm SD$ (pmol Hcy/ μg DNA min)	<i>n</i>
Global	21.3 $\pm$ 0.9	13.1 $\pm$ 0.2	3
A	1100 $\pm$ 56	84 $\pm$ 5	3
ASC	112 $\pm$ 40	13.4 $\pm$ 3.1	3
X _{AG}	27.1 $\pm$ 1.5	7.9 $\pm$ 3.2	3
L	19.0 $\pm$ 1.3	7.1 $\pm$ 2.5	3