Sodium-dependent Neutral Amino Acid Transport by Human Liver Plasma Membrane Vesicles

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The activities of several selected Na⁺-dependent amino acid transporters were identified in human liver plasma membrane vesicles by testing for Na⁺-dependent uptake of several naturally occurring neutral amino acids or their analogs. Alanine, 2-(methylamino)isobutyric acid, and 2-aminoisobutyric acid were shown to be almost exclusively transported by the same carrier, system A. Kinetic analysis of 2-(methylamino)isobutyric acid uptake by the human hepatic system A transporter revealed an apparent $K_m$ of 0.15 mM and a $V_{max}$ of 540 pmol·mg⁻¹·protein⁻¹·min⁻¹. Human hepatic system A accepts a broad range of neutral amino acids including cysteine, glutamine, and histidine, which have been shown in other species to be transported mainly by disparate carriers. Inhibition analysis of Na⁺-dependent cysteine transport revealed that the portion of uptake not mediated by system A included at least two saturable carriers, system ASC and one other that has yet to be characterized. Most of the glutamine and histidine uptake was Na⁺-dependent, and the component not mediated by system A constituted system N. The largest portion of glycine transport was mediated through system A and the remainder by system ASC with no evidence for system Gly activity. Our examination of Na⁺-dependent amino acid transport documents the presence of several transport systems analogous to those described previously but with some notable differences in their functional activity. Most importantly, the results demonstrate that liver plasma membrane vesicles are a valuable resource for transport analysis of human tissue.

The liver is a major site of amino acid metabolism in the body. Hepatocytes require amino acids for a myriad of physiologically essential processes, they serve as metabolic precursors, protein synthesis substrates, and regulators of protein degradation. Alanine transporters exhibit affinity for a number of amino acids, including the branched chain and aromatic amino acids excluded by system A. Given its constitutive level of expression and the lack of any evidence for regulation, system ASC may represent the primary mode for much of the active amino acid transport in mammalian cells. In addition to these Na⁺-dependent carriers with rather broad specificity, many cells have been shown to contain a glycine-specific system A transporter. This limited specificity of this carrier appears to be the exception in mammalian cells, with most transporters exhibiting affinity for a number of amino acids.

Hepatic amino acid transport activity has been well characterized in rat liver tissue, and a few observations have been made regarding the uptake of selected amino acids and thereby provide evidence for specific amino acid transporters. For example, the plasma membrane transport of alanine, an important substrate for gluconeogenesis, is rate-limiting for hepatic alanine metabolism. Likewise, the influence of membrane transport may alter basic cellular processes such as protein synthesis and degradation by changing amino acid availability.

Although substrate selectivity and regulation of several amino acid transport systems have been described in certain species and tissues, much remains to be understood in terms of the contribution of individual amino acid transporters to the biology and pathophysiology of the cell. Illustrative of this point is the belief that amino acid transport systems play an important role in cellular growth and development. System A, a plasma membrane activity responsible for the transport of neutral amino acids having unbranched side chains, is hormonally regulated, elevated in transformed cells, and responsive to substrate deprivation. Further, the hepatocellular regeneration that occurs following partial hepatectomy of rats has been shown to coincide with a marked increase in system A activity. System N, an amino acid transport system originally described in rat hepatocytes, is specific for glutamine and histidine and, to a lesser extent, asparagine. Although it is induced by substrate deprivation and is subject to hormonal control, it is not regulated to the same extent as system A. Another well characterized Na⁺-dependent transporter is system ASC, which transports nearly all of the neutral amino acids to some extent, including the branched chain and aromatic amino acids excluded by system A. Given its constitutive level of expression and the lack of any evidence for regulation, system ASC may represent the primary mode for much of the active amino acid transport in mammalian cells. In addition to these Na⁺-dependent carriers with rather broad specificity, many cells have been shown to contain a glycine-specific system A. The limited specificity of this carrier appears to be the exception in mammalian cells, with most transporters exhibiting affinity for a number of amino acids.

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vesicles has been described recently (24). Thus, plasma membrane vesicles freshly isolated from normal human liver were examined for the presence of specific amino acid transport activities.

**EXPERIMENTAL PROCEDURES**

**Tissue Procurement**—Following approval from the University of Florida College of Medicine Institutional Review Board, adult human liver tissue was obtained from patients undergoing hepatic surgery, typically partial hepatectomy for benign or isolated malignant hepatic disease. Tissue was also obtained from a multiorgan donor from whom the liver was not transplanted for technical reasons. Liver tissue was obtained intraoperatively and immediately placed on ice and perfused. A sample of the liver specimen was removed prior to perfusion for pathological study. The data described in this report are derived from patients with normal biochemical tests of liver function and from liver specimens found to be histologically representative of normal human tissue.

**Plasma Membrane Vesicle Isolation**—Liver tissue was perfused with an ice-cold buffer containing 250 mM sucrose, 10 mM Hepes,1 and 1 mM EGTA, pH 7.5 (SEB). Plasma membrane vesicles were prepared immediately, or perfused liver was stored at -70 °C for later analysis. Plasma membrane-enriched vesicles were prepared by a modification of the method described by Prpic et al. (25). Briefly, liver was minced with scissors and mechanically disrupted with a Polytron homogenizer (Brinkmann Instruments). The homogenate (diluted to 6% [w/v] with SEB) was centrifuged at 100,000 × g for 2 min. The pellet was discarded, and the supernatant was centrifuged at 1,500 × g for 10 min at 4 °C in a Sorvall SS-34 rotor. The supernatant was discarded and the pellet resuspended in SEB in a glass Dounce homogenizer. Percoll was added to create a 12% (v/v) solution that was thoroughly mixed at 4 °C. The Percoll/SEB mixture was added in equal volumes to Corex tubes and centrifuged at 34,500 × g for 1 h in a Sorvall SS-34 rotor. The band of unbroken tissue just below the top lipid layer was carefully aspirated and washed free of Percoll with a 1:6 dilution of 250 mM sucrose, 1 mM MgCl2, 10 mM Hepes, pH 7.5 (SMB), through centrifugation at 34,500 × g for 30 min in a Sorvall SS-34 rotor. The final pellet was resuspended in SMB and stored in small aliquots at -70 °C. Each aliquot was thawed only once, just prior to use.

Protein concentration was determined by a modified Lowry method (26). Plasma membrane enrichment relative to homogenate was determined through assessment of the activity of Na+,K+-ATPase (27) for basolateral membrane and alkaline phosphodiesterase or 5' nucleotidase for canalicular surface (28). Marker enzyme studies revealed a 10-33-fold enrichment in the plasma membrane fraction relative to the homogenate. Electron micrographs verified the vesicular nature of our preparation and the lack of significant contamination by nuclei or mitochondria. Measurement of the accumulation at equilibrium of 5-O-methyl-D-[14C]glucose by the plasma membrane vesicles (37 °C) revealed an intravesicular volume of 0.2-0.3 μl/mg of protein (29).

**Vesicle Transport Assays**—Immediately prior to uptake assays, membrane vesicles were thawed on ice and diluted with SMB to a concentration of 2.5-3.5 mg of protein per ml. Amino acid uptake was initiated after rapidly warming 20 μl of vesicle suspension (50-60 μg) to 37 °C in a water bath and then adding it to 20 μl of 200 mM NaSCN or KSCN, 1 mM MgCl2, 10 mM Hepes, pH 7.5, including 100 μM radiolabeled amino acid. Transport was terminated by the addition of 1 ml of ice-cold stop buffer (150 mM NaCl, 10 mM sodium phosphate, pH 7.4). The mixture was rapidly passed over a 0.45-μm nitrocellulose filter (type GN-6, Gelman Sciences, Ann Arbor, MI). The filter was washed with two 5-ml aliquots of stop buffer and then placed in 4 ml of scintillation mixture (Ready Protein, Beckman Instruments) prior to analysis in a liquid scintillation counter. Non-specific binding (no vesicles present) of the radiolabeled amino acid was measured for every experiment and subtracted from all corresponding assays.

**Data Analysis**—Transport data were derived from two to four plasma membrane isolations from a minimum of two different normal human liver specimens/experiment. Each individual assay was performed in triplicate or quadruplicate. Kinetic transport parameters were determined with the assistance of a FORTRAN computer program designed to describe uptake by one saturable and one nonsaturable component (30). Nonsaturable uptake was subtracted from the total uptake prior to the calculation of the kinetic constants. Student's t test was used to determine the statistical significance of differences between means.

**Chemicals**—The radiolabeled amino acids used were 2-(methylamino)[1,4C]isobutyric acid (Du Pont-New England Nuclear), 2-amino-[3H]isobutyric acid, l-[2-3H]alanine, l-[2,3-3H]serine (ICN Biomedicals, Costa Mesa, CA), l-[3-3H]glutamine, l-[2,5-3H]histidine, l-[3,3,4-3H]cysteine, and l-[3,3-3H]cystine obtained from Amersham Corp. l-[5-3H]Cysteine was obtained from l-[3,3-3H]cystine by the addition of dithiothreitol. Sigma was the source of the unlabeled amino acids and other reagents.

**RESULTS**

Complete characterization of amino acid transport by human liver will require extensive analysis of neutral, cationic, and anionic amino acid uptake. This report focuses only on the transport of selected amino acids chosen because their Na+-dependent accumulation in rat liver is indicative of specific carrier activities. Experiments were designed to test for similarities and differences between the human transport systems and those described previously for rat tissue.

**MeAIB and AIB**—Fig. 1 shows the time course of the Na+-dependent uptake of 2-(methylamino)isobutyric acid (MeAIB) into adult human liver plasma membrane vesicles. The accumulation of intravesicular MeAIB was rapid, with maximal uptake occurring by 30 s with a peak distribution ratio (vesicular amino acid concentration versus medium concentration) of greater than 4:1.

An overshoot indicating Na+-dependent secondary active transport (cotransport) by a membrane vesicle transport protein was detected (31). The Na+-dependent overshoot observed during amino acid uptake time course measurements reflects the energy-dependent nature of their Na+-dependent amino acid uptake (31). Typical of secondary active transport, the peak distribution ratio occurred during the overshoot at a velocity far exceeding that at equilibrium when the electrochemical gradient was dissipated (32). The time course of the Na+-dependent uptake of 2-aminoisobutyric acid (AIB) was similar to that of MeAIB, with a maximum distribution ratio of over 4:1 at 30 s. The inhibition of Na+-dependent AIB uptake by increasing concentrations of the system A-specific substrate MeAIB is shown in Fig. 2. MeAIB was a potent inhibitor and essentially abolished Na+-dependent AIB uptake at a concentration of 2.5 mM. Analysis of the MeAIB inhibition of AIB uptake by a Dixon plot yielded an

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1 The abbreviations used are: Hepes, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid; MeAIB, 2-(methylamino)isobutyric acid; AIB, 2-aminoisobutyric acid; EGTA, [ethylenebis(oxyethyl-1,2cyclentriitol)]tetraacetic acid.

**Fig. 1.** Time course of MeAIB uptake by human liver plasma membrane vesicles. The uptake of 50 μM MeAIB was measured at 37 °C in the presence of either 100 mM NaSCN (●) or KSCN (○) and the Na+-dependent portion (●) determined as described under "Experimental Procedures." Each point represents an average ± S.D. of assays in quadruplicate. Where not shown, the standard deviation bars are contained within the symbol.
data estimated an apparent $K_i$ of 0.12 M for MeAIB. The uptake of 50 mM AIB was assayed for 10 s at 37 °C as described under "Experimental Procedures." Computer-assisted analysis of the data estimated an apparent $K_i$ of 0.12 mM for MeAIB. The results are reported as an average ± S.D. of assays in triplicate, and where not shown, the standard deviation bars are contained within the symbol.

approximate $K_i$ of 0.12 mM. In a similar manner, alanine completely inhibited the Na+-dependent uptake of either AIB or MeAIB at concentrations of alanine greater than 5 mM (data not shown).

The kinetics of Na+-dependent MeAIB transport into human liver plasma membrane vesicles were analyzed to estimate kinetic constants (Fig. 3). Linear transformation (Eadie-Hofstee plot) of the MeAIB velocity versus concentration allowed the estimation of an apparent $K_m$ of 0.15 mM and a $V_{max}$ of 540 pmol-mg⁻¹ protein⁻¹ min⁻¹. Rat hepatic system A has been shown to be inactivated by increasing the H⁺ concentration of the uptake assay (13, 33). The Na+-dependent transport of 50 mM MeAIB by human liver vesicles was pH sensitive; the transport values at an assay pH of 6.5 or 8.0 were 26 and 12%, respectively, of the rate at pH 7.5 (50.1 ± 5.3 pmol of MeAIB-mg⁻¹ protein⁻¹ min⁻¹).

Alanine—The time course of Na+-dependent alanine uptake exhibited a Na+-dependent overshoot similar to that of MeAIB but had a markedly greater distribution ratio of 7:1 at 10 s (Fig. 4A). Approximately 75% of the Na+-dependent alanine uptake was inhibited by 2 mM MeAIB, and 20 mM MeAIB decreased alanine uptake by 85% (Fig. 4B). With regard to the remaining 15%, the addition of 20 mM unlabeled alanine abolished this MeAIB-insensitive alanine transport, pointing to the possible existence of an additional Na+-dependent carrier for alanine (Fig. 4B). When [3H]alanine uptake was tested in the presence of 20 mM unlabeled alanine, 96% of alanine uptake was found to be inhibitable.

The substrate specificity of the MeAIB-insensitive portion of alanine uptake was evaluated through testing the degree of inhibition by several amino acids. At an inhibitor concentration of 10 mM, this component of Na+-dependent alanine uptake was reduced with the following order of effectiveness: cysteine > serine > threonine > glutamine > leucine > histidine. Although cysteine and serine were the most potent inhibitors (<95%), even the weakest inhibitor tested, histidine, decreased this portion of Na+-dependent alanine uptake by 66.7%.

Cysteine—Cysteine transport into human liver plasma membrane vesicles was examined because of its strong inhibition of the MeAIB-insensitive alanine uptake and its known specificity to system ASC in rat liver (34, 35). Virtually all of the total uptake of 50 mM cysteine was found to be Na+-dependent. Unlike alanine, Na+-dependent cysteine uptake was only inhibited by 25% in the presence of 10 mM MeAIB (Fig. 5). Although the addition of either 10 mM alanine, serine, or threonine as inhibitors along with 10 mM MeAIB decreased cysteine uptake further, a significant amount of Na+-dependent transport remained in each case (Fig. 5). The combination...
Fig. 5. Inhibition analysis of Na⁺-dependent cysteine uptake by human liver plasma membrane vesicles. The Na⁺-dependent uptake of 50 μM cysteine was assayed for 10 s at 37 °C in the presence of 10 mM MeAIB alone or 10 mM MeAIB plus 10 mM alanine, serine, threonine, or cysteine. The results are reported as the percent of control (84 ± 10 pmol of cysteine·mg⁻¹ protein·10 s⁻¹) ± S.D. of assays in triplicate.

FIG. 6. Time course of the uptake of histidine or glutamine by human liver plasma membrane vesicles. The uptake of 50 μM histidine (A) or glutamine (B) was measured at 37 °C in either 100 mM NaSCN (●) or KSCN (○), and the Na⁺-dependent portion (●) was calculated as described under "Experimental Procedures." The results are reported as the average ± S.D. of assays in triplicate, and where not shown, the standard deviation bars are within the symbol.

Glutamine and Histidine—The time course of Na⁺-dependent glutamine or histidine uptake, similar to that of alanine, demonstrated a rapid transport rate with a distribution ratio greater than 6:1 (Fig. 6). The Na⁺-independent and Na⁺-dependent rates were similar for both amino acids. Uptake of glutamine in the presence of increasing concentrations of MeAIB, or MeAIB and glutamine, the results are reported as an average ± S.D. of assays in triplicate, and the standard deviation bars, where not shown, are contained within the symbol. B, the Na⁺-dependent glutamine (50 μM) uptake by human liver plasma membranes was assayed for 10 s at 37 °C in the presence of 20 mM MeAIB, or 20 mM MeAIB plus 5 mM glutamine, histidine, asparagine, alanine, serine, or cysteine as shown. The results are reported as percent of control (125 ± 17 pmol of glutamine·mg⁻¹ protein·10 s⁻¹) ± S.D. of assays in triplicate.

inhibition far greater than that for any of the other amino acids tested (Fig. 7B). Complementary inhibition analysis of histidine transport revealed that glutamine diminished the MeAIB-insensitive Na⁺-dependent histidine uptake by 90%; but alanine, serine, or cysteine had little or no effect.

Glycine Uptake—Glycine transport by the human liver vesicles was mediated primarily through an Na⁺-dependent mechanism. The maximal distribution ratio of 8:1 occurred at 15 s. Analysis of glycine transport through inhibition by increasing concentrations of MeAIB revealed an inverse hyperbola that reached a minimum at 20.0 mM MeAIB, yielding an apparent Kᵢ of 0.29 mM. Only about 15% of the Na⁺-dependent glycine transport persists following maximal MeAIB inhibition, and the combined addition of serine and MeAIB completely abolished Na⁺-dependent glycine transport.

DISCUSSION

Although the entire complement of the amino acid transporters is probably not known, examination of the function in different cell types of those identified thus far has revealed notable variations in their characteristics (10, 34, 36). This study of human liver transport activity demonstrates additional evidence of the complexity of amino acid transport systems with overlapping substrate specificities and also reveals important differences when compared with data described previously for rat liver. These findings underscore the
importance of further investigation into human hepatic transport processes.

Inhibition analyses and transport kinetics of alanine establish that its uptake into human liver plasma membrane vesicles occurs predominately by one carrier. Nearly all (90%) of the Na⁺-dependent uptake of AIB was blocked by an excess of the system A-specific analog MeAIB. The fact that the apparent $K_m$ for MeAIB transport equals its $K_i$ for inhibition of AIB uptake argues that the transport of these two alanine analogs is mediated by the same carrier, namely system A (10). The restrictive nature of AIB and MeAIB will allow their use in conjunction with human liver tissue to monitor alterations in system A activity in response to metabolic perturbations or disease states. The properties considered for this carrier included Na⁺ dependence, H⁺ sensitivity, and substrate specificity. Characterization of alanine uptake in the presence of MeAIB by the human vesicles also suggests mediation primarily (>85%) by the system A transporter. Given the high degree of regulation associated with system A in rat liver and the metabolic importance of alanine to hepatic amino acid and carbohydrate metabolism, the nearly complete restriction of alanine uptake to system A warrants further study.

The adult human liver system A transporter differs from that described in rat hepatocytes by accommodating a broader spectrum of substrates which includes amino acids shown previously to be transported to a significant degree by other carriers, in particular, systems ASC and N. For example, in rat hepatocytes, Na⁺-dependent cysteine uptake is almost entirely restricted to system ASC (34, 35), but inhibition analysis in human liver plasma membrane vesicles revealed that MeAIB suppressed nearly half of the cysteine transport, indicating a significant role for system A in cysteine uptake. Furthermore, combining typical system ASC substrates (alanine, serine, or threonine) along with MeAIB as inhibitors did not completely abolish Na⁺-dependent cysteine uptake although adding an excess of unlabeled cysteine demonstrated the saturability of the process. Therefore, it appears that Na⁺-dependent cysteine transport proceeds through at least three saturable carriers: A, ASC, and one other yet to be characterized fully. The additional carrier may be a distinct transport system for cysteine, similar to one that exists in human red blood cells (37).

Our analysis of glutamine and histidine transport by human liver tissue also revealed differences from that described for rat hepatocytes (10). Human liver system N does not mediate the majority of the transport for these 2 amino acids as in the rat hepatocyte, yet measurable system N activity is present. As illustrated by MeAIB inhibition of glutamine uptake, system A more readily accepts glutamine as a substrate in the human vesicles than in rat tissue (13). The Na⁺-dependent component of glutamine uptake not attributable to system A is not mediated by system ASC, as shown by the lack of strong inhibition by typical system ASC substrates. Only glutamine, histidine, and to lesser extent asparagine block this portion of glutamine uptake, in analogy to the rat liver system N activity (13).

In isolated rat hepatocytes and cultured rat hepatoma cells, glycine exhibits a demonstrable portion of its Na⁺-dependent transport through a saturable carrier that is neither system A nor ASC (15). This carrier, termed system Gly, was shown to be specific for glycine. Our study did not provide evidence for the presence of measurable system Gly activity in human liver. The largest portion of glycine transport by human hepatocytes is mediated by system A, and the remainder appears to be taken up through system ASC.

In conclusion, our examination of Na⁺-dependent amino acid uptake by human liver plasma membrane vesicles documents the presence of several amino acid transport systems analogous to those described previously in rat liver, yet with significant variance in their properties. Preliminary evidence for a cysteine-specific carrier similar to one reported in human red blood cells (37) demonstrates that there may also be important differences in the spectrum of transporters expressed between rat and human liver tissue. Further experimentation will be necessary to test for and characterize additional transporters that may be present in human tissue. Quantitative extrapolation of our results using selected human liver samples to the species as a whole is limited by variation in absolute transport rates from specimen to specimen due to the known influences of hormonal or adaptive regulation which are uncontrollable when obtaining human tissue. Although it is not possible to provide broad generalizations about the specific contribution of the various transporters because of this variability in transport velocity, we have clearly demonstrated that distinct amino acid transport activities are measurable in normal human liver samples and that human liver plasma membrane vesicles are a valuable resource for transport analysis of a given individual. Such analysis will be valuable in assessing a variety of hepatic disease states that involve metabolic or membrane abnormalities.

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