Amino Acids (2001) 20: 335–352

Amino
Acids

Asparagine uptake in rat hepatocytes: Resolution of a paradox and insights into substrate-dependent transporter regulation

T. M. Pawlik, W. W. Souba, and **B. P. Bode**

Surgical Oncology Research Laboratories, Division of Surgical Oncology, Department of Surgery Massachusetts General Hospital and Harvard Medical School, Boston, Massachusetts, U.S.A.

Accepted May 18, 2000

Summary. Extracellular asparagine has previously been shown to markedly stimulate both ornithine decarboxylase and System N-mediated glutamine transport activities in hepatocytes by a transport-dependent mechanism. However, as a weak substrate of its inferred transporter System N, the specific route of asparagine uptake has remained enigmatic. In this study, asparagine transport was studied in detail and shown to be Na^+-dependent, Li^+-tolerant, stereospecific, and inhibited profoundly by glutamine and histidine. Coupled with competitive inhibition by glutamine (K_i = 2.63 ± 1.11 mM), the data indicated that asparagine was indeed slowly transported by System N in rat hepatocytes, albeit at rates an order of magnitude less than for glutamine. The differential substrate transport velocities were shown to be attributable to a low transporter asparagine affinity (K_m = 9.3 − 17.5 mM) compared to glutamine (K_m ~ 1 mM). Consistent with its slow uptake, asparagine accumulated to a fivefold lesser degree than glutamine after 60 min, yet stimulated System N activity to the same extent as glutamine. The transaminase inhibitor aminooxyacetate and starvation of the donor animal each enhanced asparagine uptake twofold and augmented subsequent transporter activation. Conversely, asparagine-dependent System N stimulation was abrogated by hyperosmotic media and blunted 30%–40% by phosphatidylinositol 3-kinase (PI3K) inhibitors wortmannin and LY294002. Collectively, the data suggest that System N-mediated asparagine uptake serves an autostimulatory role, mediated by cellular swelling and in part by a PI3K-dependent signal transduction pathway.

Keywords: Amino acids – Transport – Asparagine – Glutamine – Hepatocytes – Cell volume – Phosphatidylinositol 3-kinase

Abbreviations: PI3K, phosphatidylinositol 3-kinase; ODC, ornithine decarboxylase; RPCD, RPMI 1640-based chemically-defined culture medium; AAFRPCD, amino acid-free RPCD; MeAIB, α-(methylamino)-isobutyric acid; AOA, aminooxyacetic acid; ASN, asparagine; GLN, glutamine.

Introduction

The role of glutamine – the most abundant plasma amino acid – in systemic nitrogen homeostasis and hepatic ammonia metabolism has been well-established and reviewed (Haussinger et al., 1992). In support of this role, mammalian hepatocytes express a Na^+-dependent amino acid transport activity termed System N for its unique narrow substrate specificity described as "glutamine, histidine, *and to a lesser extent asparagine*" – all amino acids bearing nitrogenous side chains (Kilberg et al., 1980; Bode et al., 1990, 1995). This transporter supports and regulates hepatic nitrogen metabolism by governing cytoplasmic glutamine levels, especially during periods of heightened amino acid metabolism (Haussinger et al., 1985a, b; Low et al., 1993). Accordingly, System N activity has been shown to be accelerated during catabolic states such as endotoxemia (Fischer et al., 1996; Inoue et al., 1993), cancer (Dudrick et al., 1993; Easson et al., 1998), diabetes (Handlogten and Kilberg, 1984; Low et al., 1992) and after burn injury (Lohmann et al., 1998). While the gene(s) responsible for this unique transport activity remains to be isolated, its regulatory and functional characteristics have been well-studied (Bode et al., 1990).

The regulation of cellular function and gene expression by amino acids has garnered considerable interest over the past decade (Kilberg et al., 1994). Perhaps the most unique System N regulatory characteristic is its activation by extracellular amino acids (Weissbach and Kilberg, 1984). This novel form of transporter regulation is rapid (evident 5 min after addition of amino acids), Na^+-dependent, and independent of transcription, translation, and metabolism. The stimulated activity is attributable to an increased transporter V_{max} (Weissbach and Kilberg, 1984), but is not retained in plasma membrane vesicles isolated from stimulated hepatocytes (Bode and Kilberg, 1991). This form of regulation is also operative *in vivo* (Haussinger and Lang, 1990; Haussinger et al., 1990) and enhanced by starvation of the animal prior to hepatocyte isolation (Weissbach and Kilberg, 1984; Bode and Kilberg, 1991). Furthermore, the mechanism by which this regulation occurs is linked to concentrative amino acid transport-induced cell swelling in both rat (Bode and Kilberg, 1991; Haussinger and Lang, 1990; Haussinger et al., 1990) and human (Bode et al., 1995) hepatocytes. Although System N may be activated by substrates of other Na^+-dependent transport systems, of all amino acids tested, asparagine was consistently the most effective (Weissbach and Kilberg, 1984; Bode and Kilberg, 1991). Similarly, asparagine has been shown to stimulate hepatocyte polyamine biosynthesis (ornithine decarboxylase (ODC) activity) to a greater extent than other amino acids (Kanamoto et al., 1987, 1991). The stimulation of both System N and ODC activities by extracellular asparagine is concentration- and transport-dependent and linked to cell swelling (Bode and Kilberg, 1991; Law and Fong, 1987; Tohyama et al., 1991).

While its effects are well-studied, asparagine uptake in hepatocytes has never been fully characterized. Paradoxically, it is taken up more slowly than less effective System N activators (Bode and Kilberg, unpublished observations). Given the unique stimulatory properties of this amino acid, the studies

presented here were undertaken to more clearly define the route by which asparagine is taken up into hepatocytes and to provide further insights into the mechanism(s) by which it activates glutamine transporter System N.

Materials and methods

Animals

Adult male Sprague-Dawley rats (150–250 g; Charles River Laboratories, Wilmington, MA) were used for these studies. All experiments were approved by the Committee on Research, Subcommittee on Research Animal Care, Massachusetts General Hospital, and conform to guidelines from the National Institutes of Health and "Guide for the Care and Use of Laboratory Animals". Animals were housed in the animal care facility under standard conditions (i.e., 12-hour light-dark cycle and ad libitum access to standard rat chow and water) and were allowed at least 2 days to acclimate to the surroundings.

Materials

Chemicals, amino acids, and liver perfusion medium (S-MEM) were purchased from Sigma (St. Louis, MO). Tissue culture medium (RPMI 1640) and SelectAmine® kits were from Gibco/BRL (Gaithersburg, MD). Tissue culture medium additives were from Gibco/BRL and Biofluids, Inc. (Rockville, MD), and fatty acid-free bovine serum albumin was from Intergen (Purchase, NY). Radiolabeled L-[[G]-^{3}H] asparagine was from American Radiolabeled Chemicals (St. Louis, MO) and L-[[G]-^{3}H] glutamine was from New England Nuclear Corp (Boston, MA). Collagenase was from Boehringer-Mannheim (Indianapolis, IN), rat tail collagen was from Collaborative Biomedical Products (Bedford, MA), and the 24-well tissue culture trays were obtained from Costar Corp. (Cambridge, MA). Bicinchoninic acid (BCA) protein assay reagent was obtained from Pierce Chemical (Rockford, IL), while the PI3K inhibitors wortmannin and LY294002 were from Biomol (Plymouth Meeting, PA).

Hepatocyte isolation

Unless indicated otherwise, all animals were subjected to an overnight fast prior to surgery and hepatocytes were isolated between 8 and 10 a.m. on the day of the experiment. Animals were weighed and anesthetized with ketamine (75 mg/kg, Ketaset; Fort Dodge Laboratories, Fort Dodge, IA) and xylazine (1.3 mg/kg, Rompun; Mabay, Shawnee, KA). After laparatomy and exposure of the viscera, rat liver parenchymal cells were isolated using a modified two-step collagenase perfusion technique (Berry and Friend, 1969; Kilberg, 1989), described in detail previously (Fischer et al., 1996; Lohmann et al., 1999).

Isolated hepatocytes were resuspended in a modified chemically-defined RPMI 1640-based medium (RPCD) (Woodworth, 1986) supplemented with: 2 mM L-glutamine, 10 mM HEPES (pH 7.4), 3.7 mg/ml bovine serum albumin, 2.2 μg/ml each of linoleic and linolenic acids, 1 μM dexamethasone, 10 nM glucagon, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml selenium, 500 nM phosphorylethanolamine/ethanolamine, trace elements (manganese, silicate, molybdenum, vanadium, nickel and tin salts), 100 units/ml penicillin, and 100 μg/ml streptomycin. The cell suspensions (2.7×10^5 cells/well) were transferred to 24-well culture trays previously coated with rat tail collagen and allowed to attach for two hours at 37°C in a humidified atmosphere of 5% CO_2/95% air prior to experimental treatments and transport studies. In studies addressing the activation of System N by asparagine, hepatocytes were incubated in amino acid-free RPCD (AAFRPCD), made

with RPMI-1640 Selectamine® kits (Gibco), which contained all of the components of RPCD except the amino acids.

Amino acid transport

Transport was measured using the 24-well cluster tray technique (Gazzola, 1981) as described previously (Fischer et al., 1996; Lohmann et al., 1999). Briefly, the hepatocytes were washed twice with 2 ml/well of warm (37°C) cholKRP (Na^+-free Krebs Ringer Phosphate Buffer in which choline chloride and choline phosphate replace the corresponding sodium salts), followed by exposing the cells to the radiolabeled amino acid of interest (either asparagine or glutamine, at 4 μCi/ml) for specific periods of time in either Na^+-containing or Na^+-free buffer. For most experiments, the unlabeled amino acid substrate was present at 50 μM, but for kinetic analyses, it was varied as indicated. Transport assays were terminated after specific times by three rapid washes with 2 ml/well of ice-cold wash buffer (119 mM NaCl, 25 mM Na_2HPO_4 (pH 7.5), 5.9 mM KCl, 0.5 mM $CaCl_2{\cdot}2H_2O$, and 1.2 mM $MgCl_2$).

Intracellular (transported) radiolabeled amino acids were extracted with 0.2 ml/well of 0.2% sodium dodecyl sulfate (SDS) + 0.2 N NaOH; after 1 h, 0.1 ml of the lysate was neutralized with 10 μl of 2 N HCl and subjected to scintillation spectrophotometry in a Packard TopCount™ (Packard Instruments, Meriden, CT). The remaining lysate was utilized for the determination of cellular protein by the bicinchoninic acid (BCA) method. Rates of amino acid transport were calculated from the cpm per sample, the specific activity of the uptake mix (in cpm/nmol) and normalized to cellular protein content in a Microsoft© Excel spreadsheet program. Transport values obtained in the absence of extracellular Na^+ (diffusion and Na^+-independent uptake) were subtracted from those in the presence of Na^+ (total uptake) to yield Na^+-dependent rates which are reported in units of pmol or nmol·mg^{-1} protein·$time^{-1}$. All transport values depicted are the average ± standard deviation of four separate determinations.

Kinetic analysis

Kinetic analysis was performed by varying the final concentration of unlabeled asparagine from 0.05 mM to 50 mM. Analysis of Na^+-dependent transport data was performed by nonlinear regression in Data Desk (Data Description, Ithaca, NY), with the equation for transporters that display Michaelis-Menten kinetics:

$$v = (V_{max} \bullet [S])/(K_m + [S]) \quad (1)$$

where V_{max} represents the maximum velocity (capacity), K_m (Michaelis constant) represents the transporter affinity for the amino acid, and [S] represents the concentration (mM) of asparagine. Calculation of the transport inhibition constant (K_i) was performed using the equation:

$$v = (V_{max} \bullet [S]/K_m((1 + [I] + [S])/K_I))) \quad (2)$$

where [I] represents the concentration of the inhibitory amino acid. All results were depicted graphically using Cricket Graph® (Computer Associates, Islandia, NY).

Statistical analysis

Statistical analyses were performed by paired t-test in Excel, and differences in measured values were considered significant at $p < 0.050$.

Results

Asparagine transport in hepatocytes

The data presented in Figs. 1 and 2 demonstrate the paradoxical nature of asparagine uptake in isolated hepatocytes. When the Na^+-dependent transport of 50 μM L-glutamine was measured in the absence or presence of individual unlabeled System N substrates at 5 mM, histidine and glutamine almost completely inhibited uptake of radiolabeled glutamine (by 95% and 83%, respectively ($p < 0.001$)), whereas asparagine diminished uptake by only 24% ($p < 0.050$). Indeed, this observation has led to the description of the System N substrate profile as "glutamine, histidine, *and to a lesser extent, asparagine*" (Kilberg et al., 1980; Bode et al., 1995). Furthermore, Fig. 2 illustrates that 50 μM L-asparagine is taken up at rates twentyfold slower than 50 μM L-glutamine (e.g. 0.018 ± 0.008 vs. 0.385 ± 0.020 nmol·mg^{-1} protein·30 s^{-1} for asparagine and glutamine, respectively ($p < 0.001$)). The uptake of asparagine is linear over 5 min (Fig. 2 inset) whereas glutamine transport velocities change slope after the first 30 s. Based on these results, all subsequent asparagine transport assays were carried out for 1 min.

The disparity in transport rates coupled with the weak inhibition of glutamine uptake by asparagine collectively raised the possibility that asparagine is taken up by hepatocytes via a different mechanism than System N. To test this possibility, the Na^+-dependent transport of 50 μM L-asparagine was measured in the absence or presence of unlabeled amino acids and amino acid analogues at 5 mM. The results in Fig. 3 indicate that of all test amino acids, glutamine

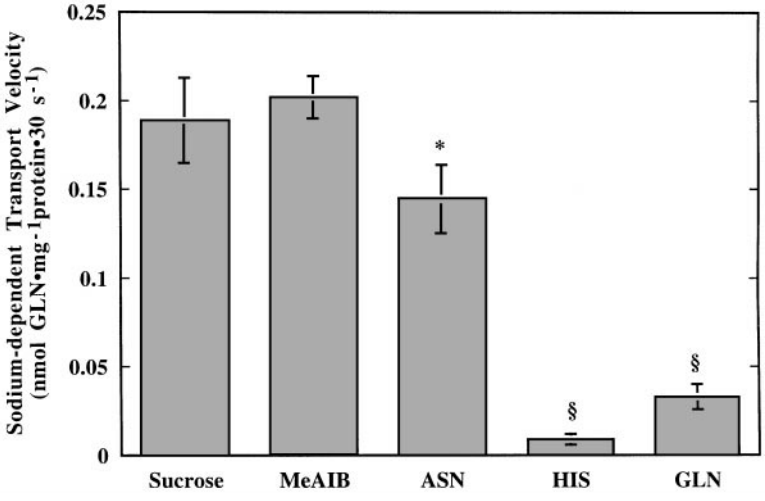

Fig. 1. Inhibition analysis of Na^+-dependent glutamine uptake by System A and N amino acids. The transport of 50 μM L-glutamine was measured in isolated rat hepatocytes as described in the Methods section in the absence and presence of the indicated unlabeled amino acids at 5 *m*M. Sucrose (5 mM) was added to control transport mixes to osmotically compensate for the test amino acids. Transport rates are the average ± SD of four separate determinations. MeAIB is the System A-specific substrate α-(methylamino)-isobutyric acid. *$p < 0.050$ and §$p < 0.010$ vs. control

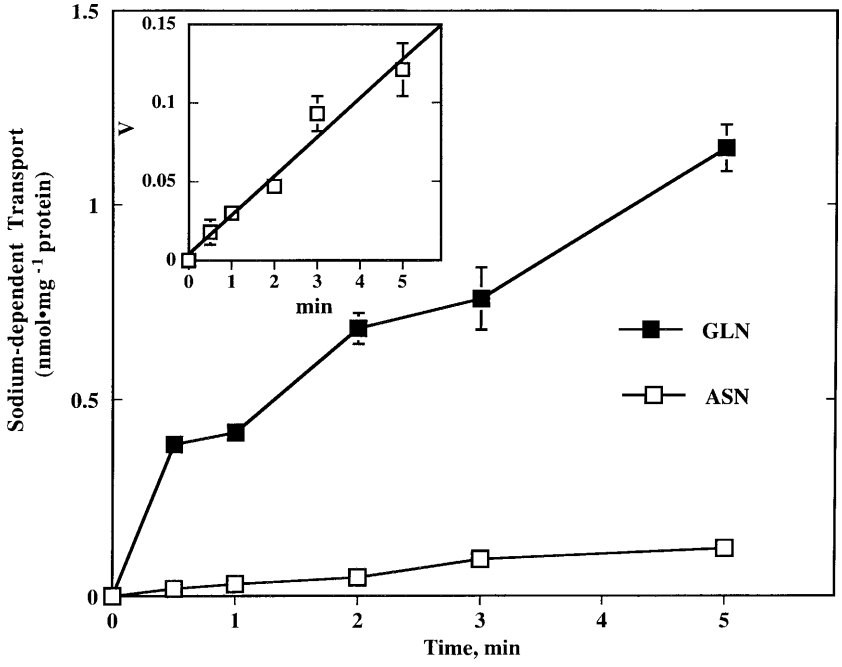

Fig. 2. Time course of 50μM L-glutamine and L-asparagine uptake. The Na^+-dependent transport of asparagine and glutamine was measured in isolated rat hepatocytes as described in the Methods section for the indicated periods of time prior to termination of the assay. Each point is the average ± SD of four separate determinations. Inset shows the asparagine time course on a tenfold lower scale to display the temporal linearity of uptake

(90%), histidine (87%), and asparagine (65%) were the three most inhibitory ($p < 0.010$). However, D-asparagine was ineffective, illustrating the stereospecificity of the carrier. Likewise, the System A substrate MeAIB, the System Gly substrate glycine, the System $X^-_{a,g}$ substrates L- and D-aspartate, and the System A/ASC substrate alanine were all ineffective or weak inhibitors. Thus, the amino acid inhibition profile for asparagine transport was consistent with System N mediation. Surprisingly, the transaminase inhibitor aminooxyacetic acid (AOA) reproducibly "cis-stimulated" asparagine transport rates twofold (13.6 ± 7.7 vs. 28.6 ± 5.4 pmol·mg^{-1} protein·min^{-1} in the absence and presence of 5 mM AOA, respectively ($p < 0.010$)) when present during the transport assay. AOA also stimulated glutamine transport velocities twofold (data not shown). The mechanistic basis for this observation is unclear, but the activation of System N-mediated transport by AOA was further investigated later in the study.

Asparagine uptake at 50μM was also 62% Na^+-dependent (10.6 ± 0.7 vs. 27.3 ± 3.6 pmol·mg^{-1} protein·min^{-1} in choline and Na^+, respectively) compared to 94% Na^+-dependent for glutamine (17.1 ± 2.8 vs. 264.5 ± 11.8 pmol·mg^{-1} protein·30 s^{-1} in choline and Na^+, respectively). The remaining 38% of asparagine uptake (which involved saturable and nonsaturable components) was not further characterized, as the counts were too low to draw definitive conclusions and the focus of the study was to define the Na^+-

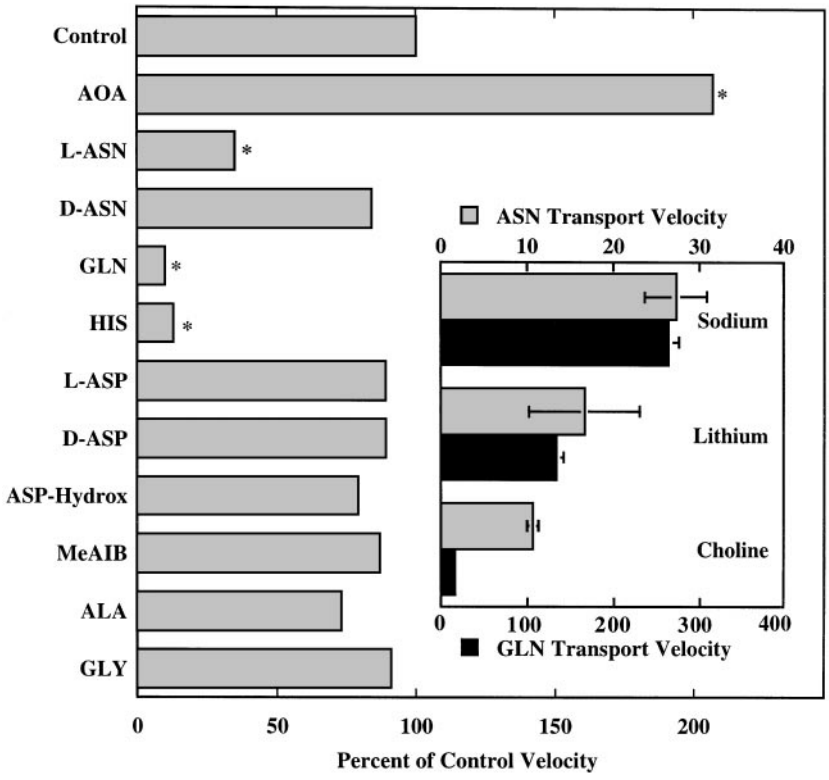

Fig. 3. Amino acid inhibition analysis of Na^+-dependent L-asparagine transport in isolated rat hepatocytes. Hepatocytes from an overnight-fasted rat were subjected to the asparagine transport assay as described in the Methods section. The transport of 50 μM L-asparagine was measured for 1 min at 37°C in the absence and presence of the indicated unlabeled amino acid at 5 mM. *AOA* Aminooxyacetic acid; *ASN* asparagine; *GLN* glutamine; *HIS* histidine; *ASP* aspartic acid; *ASP*-Hydrox L-aspartic acid β-hydroxamate; *MeAIB* α-(methylamino)-isobutyric acid; *ALA* alanine, *GLY* glycine. *$p < 0.050$ vs. control (5 mM sucrose). Inset: Cation tolerance of glutamine and asparagine uptake. The transport of 50 μM L-asparagine was measured at 37°C in Krebs-Ringer Phosphate Buffer containing the indicated chloride salt as the major cation (140 mM). Note that the transport velocities for GLN (in units of pmol·mg^{-1} protein·30 s^{-1}) and ASN (in units of pmol·mg^{-1} protein·min^{-1}) are on two different scales. Values depicted are the averages ± SD of four separate determinations

dependent component through which this amino acid elicits its stimulatory effects. Finally, lending further support to System N mediation was the observation that Li^+ partially substituted (61%) for Na^+ in driving the concentrative uptake of asparagine (Fig. 2 inset), compared to 51% for glutamine. Such "lithium tolerance" is a hallmark feature of System N (Kilberg et al., 1980).

Kinetic analysis

While the substrate and cation tolerance assays suggested that aparagine uptake occurs via System N, the kinetics of this process and nature of the

inhibitory effect of glutamine were assessed to support this hypothesis. First, Na^+-dependent asparagine uptake was measured over a concentration range of 50μM to 50 mM. When analyzed via nonlinear regression analysis according to equation 1, a K_m of 17.5 ± 4.4 mM was obtained with a V_{max} of 5.3 ± 2.5 nmol·mg^{-1} protein·min^{-1}. Indeed, if mediated by System N, the transporter affinity for asparagine was nearly twentyfold less than for glutamine (K_m ~ 1 mM) (Kilberg et al., 1980). This observation could alone explain the disparity in substrate transport rates reported earlier (Fig. 2). Next, the Na^+-dependent transport of 50μM asparagine was measured in the presence of increasing concentrations (0.1 to 50 mM) of unlabeled L-glutamine. The results shown in Fig. 4 reveal that glutamine inhibited asparagine uptake in a concentration-dependent manner. Using equation 2 and the constant values obtained in the nonlinear regression analysis, an inhibitory constant (K_i) of 2.63 ± 1.11 mM was obtained. This value is close to the K_m of rat hepatocyte System N for glutamine (Kilberg et al., 1980). Finally, to determine the nature of glutamine inhibition, Na^+-dependent asparagine transport was measured over the concentration range of 0.05 to 50 mM, in the absence or presence of 2 mM L-glutamine. The resulting double reciprocal (Lineweaver-Burk) plot of the data (Fig. 4 inset) revealed that glutamine competitively inhibits

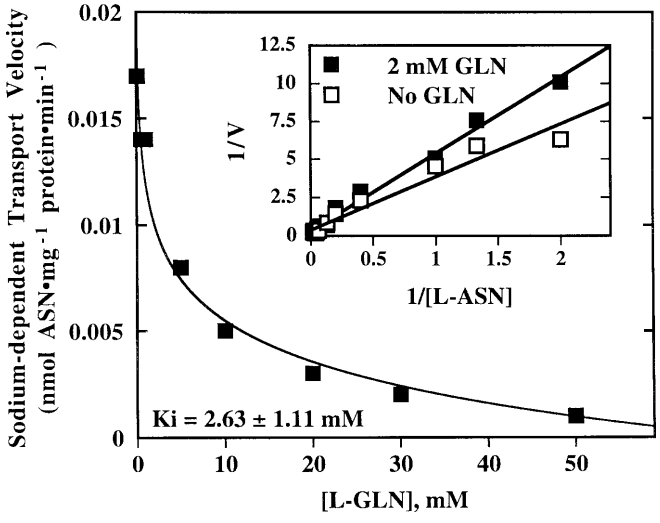

Fig. 4. Inhibition of Na^+-dependent asparagine transport by glutamine in isolated hepatocytes from an overnight-fasted rat. The transport of 50μM L-asparagine was measured as described in the Methods section in the presence of increasing concentrations of unlabeled L-glutamine from 0.1 to 50 mM. Each data point represents the average of four separate determinations. An inhibitory constant (K_i) was derived for glutamine of 2.63 ± 1.11 mM, using the constant values from Equation 2. Inset: Double reciprocal plot of the effect of 2 mM L-glutamine on the Na^+-dependent transport of 0.5 to 50 mM L-asparagine. When analyzed by linear regression, the y-intercept was similar (V_{max} = 2.67 vs. 3.22 pmol·mg^{-1} protein·min^{-1} in the absence and presence of 2 mM glutamine, respectively), but the slopes (K_m/V_{max}) were different (derived K_m = 9.29 vs. 16.30 mM in the absence and presence of 2 mM glutamine, respectively). These data suggest that the effects of glutamine are competitive in nature

asparagine uptake, as the slopes of the lines were altered, but they y-intercept (V_{max}) remained nearly identical for each condition.

Effect of starvation on basal and amino acid-stimulated asparagine transport rates

Amino acid-dependent System N stimulation has been shown to require prior starvation of the donor animal for 24 h or more for functional manifestation, but a precise correlation between the length of starvation and degree of transporter induction has been difficult to establish (Weisbach and Kilberg, 1984; Bode and Kilberg, 1991). To determine if fasting affects hepatic asparagine uptake, hepatocytes were isolated from rats fed ad libitum or fasted for 48 h prior to surgery, followed by the transport assay 2 h later. The results shown in Fig. 5 demonstrate that fasting indeed accelerated Na^+-dependent hepatocyte asparagine transport velocities approximately twofold (23 ± 4 vs. 51 ± 9 pmol·mg^{-1} protein·min^{-1} in hepatocytes from fed and fasted animals, respectively ($p < 0.050$)). This observation is consistent with starvation-dependent enhancement of System N-mediated glutamine uptake reported previously (Fischer et al., 1996). Moreover, after maintenance of these two sets of hepatocytes in amino acid-free RPCD (AAFRPCD) for 1 h, which allows System N activity to decay to "basal" rates (Bode and Kilberg, 1991),

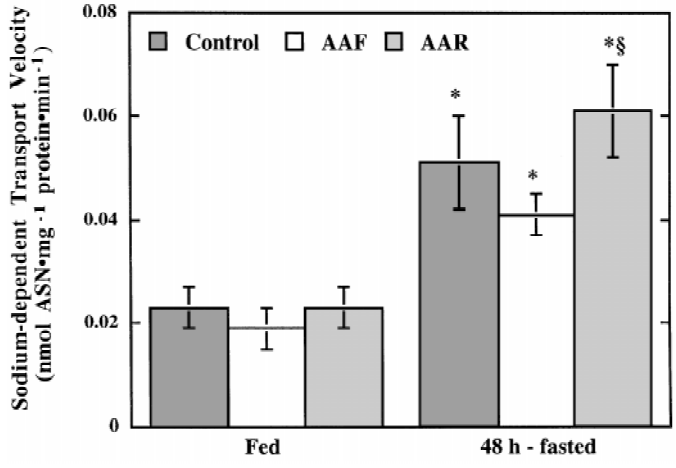

Fig. 5. Effect of starvation on basal and amino acid-dependent asparagine transport rates in isolated rat hepatocytes. Hepatocytes were isolated from rats fed ad libitum and fasted for 48 h prior to surgery and placed in culture in RPCD. After 2 h the Na^+-dependent transport of 50 μM L-asparagine was measured as described in the Methods section (Control). The medium was changed to amino acid-free RPCD, and the cells were incubated for 1 h to allow transport activities to subside to basal levels, followed by another transport measurement (*AAF*). Thereafter, the medium was changed to amino acid-containing RPCD and the cells were incubated for an additional 1 h to allow reactivation of transport rates, followed by a final transport assay (*AAR*). The values shown are the average ± SD of four separate determinations. *$p < 0.050$ vs. values in hepatocytes from fed animals, §$p < 0.050$ vs. 48 h-fasted AAF

the difference in asparagine transport velocities was retained (19 ± 4 vs. 41 ± 4 pmol·mg^{-1} protein·min^{-1} hepatocytes from fed and fasted animals, respectively ($p < 0.050$)). Thereafter, the medium was changed to RPCD with the full complement of RPMI-1640 amino acids for 1 h followed by asparagine transport measurement. As shown in Fig. 5, only hepatocytes from fasted animals displayed amino acid-enhanced asparagine transport rates ($p < 0.050$). Based on these results, all subsequent System N induction studies were performed in hepatocytes from 48 h-fasted animals.

Transporter activation by asparagine

Given that asparagine is transported more slowly than less effective amino acids and that cell swelling is linked to transporter activation, we hypothesized that the accumulation rather than initial-rate velocities may account for its stimulatory properties. To test this possibility, isolated hepatocytes were incubated in AAFRPCD supplemented with either glutamine or asparagine at 5 mM in the presence of 5 μCi/ml of the cognate radiolabeled amino acid (^{3}H L-[G]-glutamine and ^{3}H L-[G]-asparagine) and incubated for 1 h. In parallel trays, each amino acid was tested for the ability to restimulate System N-mediated glutamine uptake. The results indicated that there was no correlation between the extent of amino acid accumulation and the ability to stimulate System N, as asparagine and glutamine each stimulated uptake 1.4-fold (191 ± 14 and 189 ± 25, respectively, vs. 135 ± 6 pmol GLN·mg^{-1} protein·30 s^{-1} in control ($p < 0.050$)), yet asparagine accumulated to a far less extent than glutamine (10,042 ± 949 vs. 45,678 ± 4,740 cpm·mg^{-1} protein, respectively ($p < 0.010$)).

To further investigate the mechanism by which asparagine activates System N, we sought to study the effects of AOA. When present at 5 mM during the transport assay, this transaminase inhibitor accelerates initial-rate asparagine uptake twofold (Fig. 3). The basis for this observation was provided by kinetic assessment (Fig. 6A) which revealed that AOA enhanced the V_{max} of System N-mediated asparagine uptake (9.9 ± 2.1 vs. 20.2 ± 3.0 nmol·mg^{-1} protein·min^{-1} in the absence and presence of AOA, respectively) and increased the carrier affinity for its substrate by 30% (K_m = 12.2 ± 2.6 vs. 8.6 ± 1.3 mM in the absence and presence of AOA, respectively). Based on these results and the prerequisite of transport for asparagine's stimulatory properties, we next assessed the influence of AOA on System N activation. To this end, the media of isolated hepatocytes maintained in AAFRPCD were changed to AAFRPCD ± 5 mM L-ASN, AOA, or both, and System N glutamine transport rates were measured at specific times thereafter. While 5 mM AOA treatment alone failed to stimulate transport activity (160 ± 19 vs. 147 ± 19 pmol GLN·mg^{-1} protein·30 s^{-1} for control and AOA-treated cells, respectively, after 1 h), its presence enhanced the stimulatory effects of asparagine at every time point assayed, though this effect did not reach statistical significance over the first 20 min (Fig. 6B). After 70 min, AOA enhanced the efficacy of asparagine twofold (155 ± 1 vs. 195 ± 23 vs., 244 ± 24 pmol

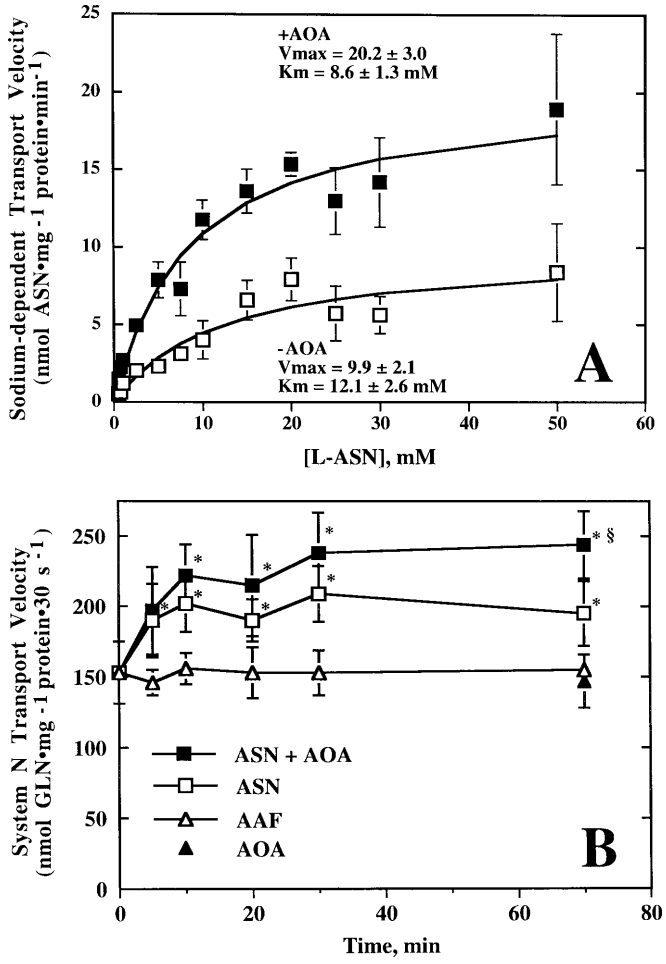

Fig. 6. Kinetic analysis of the effects of AOA on Na^+-dependent asparagine uptake and on asparagine-dependent System N activation. **A**. Kinetic analysis of AOA-stimulated asparagine uptake. Hepatocytes isolated from a 48 h-fasted rat were subjected to kinetic analysis of Na^+-dependent asparagine uptake as described in Fig. 4, in the absence and presence of 5 mM aminooxyacetic acid (*AOA*). Nonlinear regression analysis revealed the kinetic constants listed in the figure, and that AOA increases asparagine uptake by increasing the V_{max} of the transporter. **B**. Effect of AOA-enhanced uptake on the asparagine-dependent System N activation. Hepatocytes isolated from a 48 h-fasted rat were maintained in AAFRPCD for 1 h. At time zero, the medium was changed to AAFRPCD + 5 mM sucrose (*AAF*), 5 mM asparagine (*ASN*) or 5 mM asparagine + 5 mM AOA (*ASN + AOA*), and the Na^+-dependent transport of 50 μM L-glutamine was measured for 30 s at 37°C at the indicated times thereafter. Each point is the average ± SD of four separate determinations. *$p < 0.050$ vs. AAF, §$p < 0.050$ vs. ASN

GLN·mg^{-1} protein·30s^{-1} for control, ASN- and ASN+AOA-treated cells, respectively ($p < 0.050$ between all conditions)). These data indicate that asparagine uptake indeed plays a significant role in stimulating System N activity, as AOA not only accelerates the transport of this amino acid but also enhances its stimulatory effects.

Transport and cell swelling play central roles in the asparagine-dependent activation of hepatic glutamine uptake, but the signal transduction mechanisms that link these events remain poorly defined. Recently, it was shown that phosphatidylinositol 3-kinase (PI3K) mediates cell volume-linked changes in muscle glutamine transport System N^m activity (Low et al., 1997) and glutamine-evoked swelling-dependent alterations in hepatic carbohydrate and fatty acid metabolism (Krause et al., 1996). To determine if PI3K plays a similar role in transducing the stimulatory effects of asparagine, System N activity was temporally monitored after the addition of this amino acid to cells in AAFRPCD in the absence or presence of wortmannin, a specific PI3K inhibitor (Arcaro and Wymann, 1993). A concentration of 0.3 μM was chosen, as this concentration was previously shown to be both efficacious and specific for PI3K in isolated hepatocytes (Krause et al., 1996). Due to its delayed effects, cells were also subjected to a 30 min wortmannin preincubation prior to asparagine addition, as deemed necessary in earlier studies (Krause et al., 1996). Upon switching the culture medium from RPCD to AAFRPCD, the presence of wortmannin for 30 min resulted in a further depression (from 10% to 30%) of basal System N rates beyond that caused by the absence of extracellular amino acids (e.g. 143 ± 24 vs. 95 ± 14 pmol GLN·mg^{-1} protein·30 s^{-1} in AAF + DMSO and AAF + wortmannin, respectively ($p < 0.050$)). Likewise, as shown in Fig. 7, wortmannin inhibited the

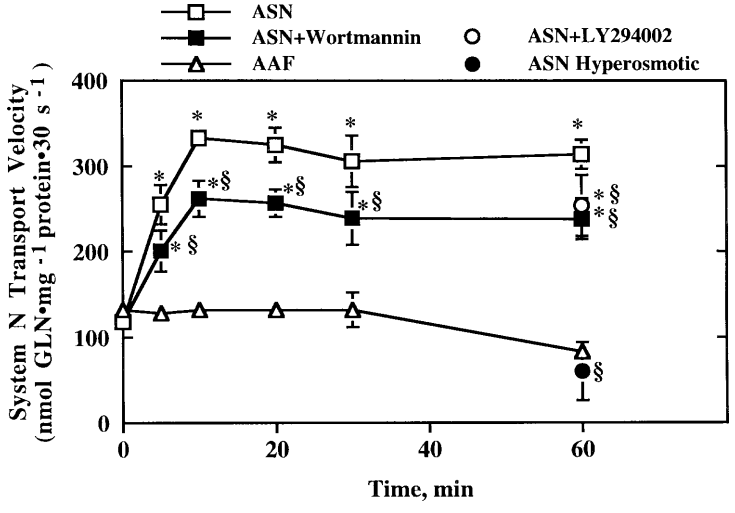

Fig. 7. Attenuation of asparagine-dependent System N activation by phosphatidylinositol 3-kinase (PI3K) inhibitors and hyperosmotic medium. After an initial 2 h attachment period, hepatocytes isolated from a 48 h-fasted rat were incubated for 1 h in AAFRPCD. At time zero, the medium was replaced with AAFRPCD + 5 mM sucrose (*AAF*) or 5 mM asparagine (*ASN*) in the absence or presence of 0.3 μM wortmannin, 50 μM LY294002, or hyperosmotic medium (300 mM sucrose). The medium with ASN alone contained 0.1% DMSO as a vehicle control for the PI3K inhibitors. At the times indicated after media change, the Na^+-dependent transport of 50 μM L-glutamine was measured for 30 s at 37°C. Each point is the average ± SD of four separate determinations. The effects of hyperosmotic medium and LY294002 were measured only at the 60 min time point. *$p < 0.050$ vs. AAF, §$p < 0.050$ vs. ASN

rapid asparagine-dependent stimulation of System N by between 30% and 40% at all times assayed ($p < 0.050$). However, as mentioned above, wortmannin depression of basal System N rates may account for a portion of this inhibition. Similarly, 50μM LY294002, another PI3K-specific inhibitor (Vlahos et al., 1994), attenuated the asparagine activation by 26% ($p < 0.050$). Prevention of asparagine-induced cell swelling by hyperosmotic medium (made by the addition of 300 mM sucrose) completely abolished System N stimulation (($p < 0.010$), Fig. 7). In a separate experiment, activation of System N activity by artificial swelling in hypoosmotic medium (Bode and Kilberg, 1991) was completely inhibited by wortmannin (data not shown).

Discussion

Asparagine stimulates several hepatic processes including glutamine uptake (Weissbach, 1984) and ODC activity (Kanamoto et al., 1987) to a greater extent than other individual amino acids. The paradox that currently exists is that asparagine – whose stimulatory effects on System N and ODC are transport-dependent – is taken up much more slowly than less effective amino acids. The ascription of hepatocyte asparagine uptake to System N has never been examined in detail, resulting in the "glutamine, histidine, *and to a lesser extent asparagine*" qualifier when the description of this transporter's substrate specificity is offered (Kilberg et al., 1980; Bode et al., 1995). This assignment was based upon a significant but relatively weak inhibition of glutamine and histidine uptake by asparagine in isolated rat hepatocytes coupled with a more profound reciprocal inhibition of asparagine transport by glutamine and histidine. Here it is confirmed that asparagine is taken up primarily via System N (Figs. 3 and 4), but the present study provides the first kinetic basis for the observation that asparagine is a seemingly less desirable System N substrate than glutamine and histidine. Partial inhibition of glutamine transport (Fig. 1) and the sluggish uptake of asparagine (Fig. 2) are attributable to marked differences in System N affinity for its three substrates glutamine (K_m ~ 1 mM) (Kilberg et al., 1980), histidine (K_m ~ 0.3 mM) (Weissbach and Kilberg, 1984), and asparagine (K_m ~ 9–17 mM) (Results and Figs. 4 and 6). The order-of-magnitude lower affinity for asparagine relative to the other two substrates clearly accounts for these differences in System N-mediated transport velocities. Such kinetic analysis has not previously been performed in hepatocytes, but there is one report of a derived K_i for asparagine on System N-mediated glutamine uptake in H35 rat hepatoma cells of 7.9 $\pm$ 1.3 mM (Vadgama and Christensen, 1983). This value is close to the 9.3 mM K_m value obtained in Fig. 4. Finally, as shown in Fig. 5, only hepatocytes from fasted animals displayed amino acid-enhanced asparagine transport and transporter activation. This data provides additional evidence that asparagine uptake is indeed mediated by System N, which is uniquely subject to this starvation-dependent form of regulation (Weissbach and Kilberg, 1984; Bode and Kilberg, 1991).

Due to its central role in ammonia and nitrogen metabolism, the liver has been a frequent focus of investigation with respect to amino acid transporters, which have been functionally defined on the basis of amino acid selectivities, kinetic, and regulatory properties (Kilberg, 1982). Among the various transporters, System N activity plays a regulatory role in governing the transmembrane delivery of glutamine for use in hepatic gluconeogenesis (Nurjhan et al., 1995) and ureagenesis (Haussinger, 1986), and constitutes a rate-limiting step in glutamine metabolism when intracellular metabolism is accelerated (Haussinger et al., 1985a,b; Low et al., 1993). Asparagine-stimulated glutamine uptake may serve to maintain adequate cytoplasmic pools of this amino acid when intracellular utilization rates for ureagenesis and gluconeogenesis are enhanced, such as during starvation. In rats, fasting portal asparagine levels are 70μM, but increase to 200–300μM during the prandial period (Ishikawa, 1976). Likewise, portal glutamine levels increase from 400μM in the fasting state to near 700μM after feeding. It should be noted that global hepatic nitrogen metabolism (glutaminase, urea cycle, proteolysis, etc.) is markedly affected by glutamine-induced cell swelling and that these effects are elicited half-maximally at portal levels (0.6mM) of this amino acid (Haussinger et al., 1990). The pharmacological level of asparagine (5mM) used in these studies for transporter induction was previously found to maximally stimulate System N, and is equivalent to the normal levels of all 20 amino acids in this capacity (Bode and Kilberg, 1991). With globally increased portal amino acids after feeding, it is unlikely that asparagine is solely responsible for the activation of this transporter. However, given the disproportionate stimulatory effects elicited by asparagine (Weissbach and Kilberg, 1984; Bode and Kilberg, 1991) in the face of its slow uptake and accumulation (Fig. 2), its fourfold portal increase may significantly contribute to the stimulation of flux through System N and the urea cycle after a meal. The autostimulatory transport mechanism described in this study may therefore help to ensure adequate detoxification of portal amino acid-derived and free ammonia during the prandial and postprandial states, especially after a period of fasting (Fig. 5).

One of the more interesting and novel observations made during the course of these studies was that the transaminase inhibitor aminooxyacetic acid (AOA) reproducibly "cis-stimulated" asparagine transport rates twofold via an increase in the V_{max} when present in the transport assay (Figs. 3 and 6). AOA also enhanced glutamine transport velocities twofold while failing to stimulate other non-System N substrates (data not shown), suggesting that the effect is not specific to asparagine *per se*, but involves the System N transporter. The observation that AOA concomitantly enhances System N activation (Fig. 6B) further underscores the transport-dependent nature of asparagine's stimulatory qualities. It is presently unclear whether this transaminase inhibitor affects the carrier directly or indirectly. One possibility for indirect activation is that AOA prevents the transamination of glutamine- or asparagine-derived metabolites that otherwise would rapidly efflux from the cell during the transport assay. Such intracellular metabolites (e.g. glutamate or aspartate) may in turn allosterically activate the System N transporter, although no precedent for this scenario has been established.

The studies presented here also provide initial insights into signal transduction pathways that link asparagine to System N activation. Although asparagine transport is known to cause cell swelling which is both necessary and sufficient for activation of System N (Bode and Kilberg, 1991 and Fig. 7), it remains unclear why asparagine is such an effective amino acid in the face of its slow uptake. While such avenues of investigation are beyond the scope of the present study, the answer to this question may reside in the subsequent ionic fluxes that are induced by asparagine uptake. Membrane phospholipids (phosphatidylinositol) have recently been shown to occupy a key role in regulating the activity of the ATP-sensitive potassium channel (Shyng and Nichols, 1998). Opening of conductive potassium channels in hepatocytes is an integral part of the regulatory volume decrease (O'Neill, 1999), and potassium efflux has been shown to be involved in System N activation (Bode and Kilberg, 1991). System N^m activation by swelling in the muscle has been shown to be linked to PI3K (Low et al., 1997), as has cell swelling-dependent activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes (Krause et al., 1996). Compelled by these studies, we show here that the rapid asparagine-dependent System N activation in hepatocytes is also mediated, at least in part, by PI3K, based on the 30% to 40% inhibition by wortmannin (Fig. 7). The more specific PI3-kinase inhibitor LY294002 inhibitor exerted similar effects, attenuating the asparagine-dependent activation by 26% (Fig. 7). These observations are consistent with those of Krause et al. who demonstrated that wortmannin inhibits glutamine-dependent intracellular glutamate accumulation by approximately 40%, and more effectively inhibits activation of glycogen synthase by hypotonicity than by glutamine (Krause et al., 1996). When taken together, the results indicate that asparagine, once taken up by System N, induces cell swelling and stimulates a PI3K-dependent signal transduction pathway that partially governs the activity of its transporter.

A model based on the data was synthesized and is schematically depicted in Fig. 8. System N-mediated asparagine uptake (Figs. 1–4), a process augmented by starvation (Fig. 5), results in cellular swelling (Bode and Kilberg, 1991). Increased hepatocellular hydration in turn elicits signal transduction pathway(s), mediated in part by PI3K (Fig. 7), that stimulate System N activity. Partial inhibition of asparagine's effect on the transporter by wortmannin and LY294002, but complete inhibition by hyperosmotic medium (Fig. 7) suggest that swelling is a common but bifurcating pathway for System N stimulation. Such complex physiological responses to altered volume in mammalian cells are well-established and depend upon the stimulus for swelling. Volume regulatory pathways are often incomplete in osmotically altered media, but are fully operative during isoosmotic shrinkage and swelling stimuli such as concentrative amino acid uptake (O'Neill, 1999). It has also been shown that wortmannin blocks swelling-induced signal transduction in hepatocytes without affecting the hydration state of the cells (Krause et al., 1996). While we have defined asparagine uptake in hepatocytes and the role of this process in System N activation, the mechanism of its pronounced swelling and stimulatory potential remains elusive, especially in the face of its

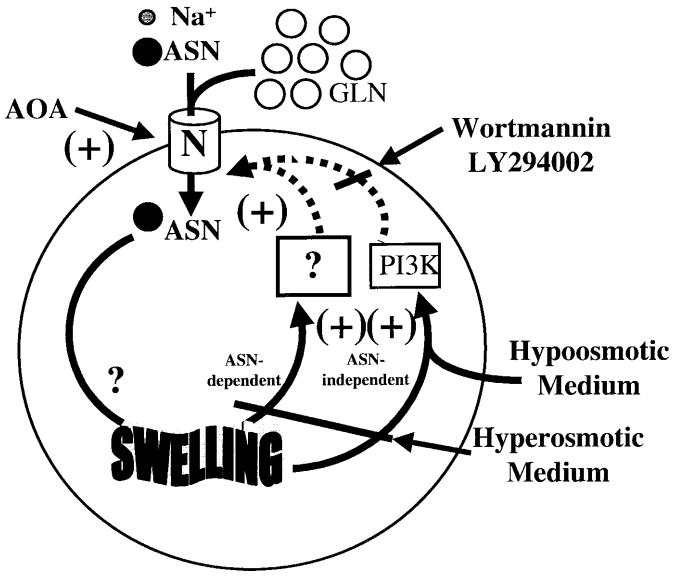

Fig. 8. Proposed model for asparagine-dependent activation of System N in hepatocytes. Asparagine transport into hepatocytes induces cell swelling, but the mechanism of this effect remains unclear in the context of the slow transport and accumulation of this substrate. Cellular swelling is both necessary and sufficient for System N activation, as prevention of swelling by hyperosmotic medium abolishes the effect of asparagine, and hypoosmotic medium stimulates glutamine transport to the same extent as asparagine (Bode and Kilberg, 1991). However, the data suggest that the signal transduction pathway(s) elicited by asparagine-induced swelling bifurcate into asparagine (amino acid) – dependent and – independent pathways. This hypothesis is based on the partial inhibition of asparagine-dependent System N activation by PI3K inhibitors (Fig. 7), and the complete inhibition of osmotically-induced System N activation by these compounds

relatively slow transport rates. As shown in Fig. 8, it is likely that a separate asparagine (amino acid)-dependent mechanism accounts for the wortmannin-insensitive portion of System N activation. This model will serve as the basis for furture investigations, but definitive answers to these questions must await the isolation of the gene(s) responsible for System N activity.

Acknowledgements

This study was supported by a grant from the National Institute of General Medical Sciences (#5P50 GM21700-23).

References

Arcaro A, Wymann M (1993) Wortmannin is a potent phosphatidylinositol 3-kinase inhibitor: the role of phosphatidylinositol 3,4,5-triphosphate in neutrophil responses. Biochem J 296: 297–301

Berry MN, Friend DS (1969) High-yield preparation of isolated rat liver parenchymal cells: a biochemical and fine structural study. J Cell Biol 43: 506–520

Bode BP, Kilberg MS (1991) Amino acid-dependent increase in hepatic system N activity is linked to cell swelling. J Biol Chem 266: 7376–7381
Bode BP, Tamarappoo BK, Mailliard M, Kilberg MS (1990) Characteristics and regulation of hepatic glutamine transport. JPEN: J Parent Enteral Nutr 14: 51S–55S
Bode BP, Kaminski DL, Souba WW, Li AP (1995) Glutamine transport in isolated human hepatocytes and transformed liver cells. Hepatology 21: 5115–5120
Dudrick PS, Inoue Y, Espat NJ, Souba WW (1993) Na^+-dependent glutamine transport in the liver of tumour-bearing rats. Surg Oncol 2: 205–215
Easson AM, Bode BP, Fischer CP, Souba WW (1998) Effects of endotoxin challenge on hepatic amino acid transport during cancer. J Surg Res 77: 29–34
Fischer CP, Bode BP, Souba WW (1996) Starvation and endotoxin act independently and synergistically to coordinate hepatic glutamine transport. J Trauma 40: 688–693
Gazzola GC, Dall'Asta V, Franchi-Gazzola R, White MF (1981) The cluster-tray method for rapid measurement of solute fluxes in adherent cultured cells. Anal Biochem 115: 368–374
Handlogten ME, Kilberg MS (1984) Induction and decay of amino acid transport in the liver. Turnover of transport activity in isolated hepatocytes after stimulation by diabetes or glucagon. J Biol Chem 259: 3519–3525
Haussinger D (1986) Regulation of hepatic ammonia metabolism: the intercellular glutamine cycle. Adv Enzyme Reg 25: 159–180
Haussinger D, Lang F (1990) Exposure of perfused liver to hypotonic conditions modifies cellular nitrogen metabolism. J Cell Biochem 43: 355–361
Haussinger D, Soboll S, Meijer AJ, Gerok W, Tager JM, Sies H (1985a) Role of plasma membrane transport in hepatic glutamine metabolism. Eur J Biochem 152: 597–603
Haussinger D, Stehle T, Gerok W (1985b) Glutamine metabolism in isolated perfused rat liver. The transamination pathway. Biol Chem Hoppe-Seyler 366: 527–536
Haussinger D, Lang F, Bauers K, Gerok W (1990) Interactions between glutamine metabolism and cell-volume regulation in perfused rat liver. Eur J Biochem 188: 689–695
Haussinger D, Lamers WH, Moorman AF (1992) Hepatocyte heterogeneity in the metabolism of amino acids and ammonia. Enzyme 46: 72–93
Haussinger D, Wettstein M, Warkulat U, vom Dahl S, Noe B, Schliess F (1997) Cell volume signaling, osmolytes and liver function. Digestion 58: 21–23
Inoue Y, Pacitti AJ, Souba WW (1993) Endotoxin increases hepatic glutamine transport activity. J Surg Res 54: 393–400
Ishikawa E (1976) The regulation of uptake and output of amino acids by rat tissues. Adv Enzyme Regul 14: 117–136
Kanamoto R, Boyle SM, Oka T, Hayashi S (1987) Molecular mechanisms of the synergistic induction of ornithine decarboxylase by asparagine and glucagon in primary cultured hepatocytes. J Biol Chem 262: 14801–14805
Kanamoto R, Nishiyama M, Matsufuji S, Hayashi S (1991) Translational control mechanism of ornithine decarboxylase by asparagine and putrescine in primary cultured hepatocytes. Arch Biochem Biophys 291: 247–254
Kilberg MS (1982) Amino acid transport in isolated rat hepatocytes. J Memb Biol 69: 1–12
Kilberg MS (1989) Measurement of amino acid transport by hepatocytes in suspension or monolayer culture. Meth Enzymol 173: 564–575
Kilberg MS, Handlogten ME, Christensen HN (1980) Characteristics of an amino acid transport system in rat liver for glutamine, asparagine, histidine, and closely related analogs. J Biol Chem 255: 4011–4019
Kilberg MS, Hutson RG, Laine RO (1994) Amino acid-regulated gene expression in eukaryotic cells. FASEB J 8: 13–19
Krause U, Rider M, Hue L (1996) Protein kinase signaling pathway triggered by cell swelling and involved in the activation of glycogen synthase and acetyl-CoA carboxylase in isolated rat hepatocytes. J Biol Chem 271: 16668–16673

Law CL, Fong WF (1987) Asparagine transport and the induction of ornithine decarboxylase in H-35 rat hepatoma cells. Biochem Int 14: 667–674
Lohmann R, Souba WW, Zakrzewski K, Bode BP (1998) Stimulation of rat hepatic amino acid transport by burn injury. Metabolism 47: 608–616
Lohmann R, Souba WW, Bode BP (1999) Rat liver endothelial cell glutamine transporter and glutaminase expression contrast with parenchymal cells. Am J Physiol 276: G743–G750
Low SY, Taylor PM, Hundal HS, Pogson CI, Rennie MJ (1992) Transport of L-glutamine and L-glutamate across sinusoidal membranes of rat liver. Effects of starvation, diabetes and corticosteroid treatment. Biochem J 284: 333–340
Low SY, Salter M, Knowles RG, Pogson CI, Rennie MJ (1993) A quantitative analysis of the control of glutamine catabolism in rat liver cells. Use of selective inhibitors. Biochem J 295: 617–624
Low SY, Rennie MJ, Taylor PM (1997) Signaling elements involved in amino acid transport responses to altered muscle cell volume. FASEB J 11: 1111–1117
Meijer A, Lamers W, Chamuleau R (1990) Nitrogen metabolism and ornithine cycle function. Physiol Rev 70: 701–748
Nurjhan N, Bucci A, Perriello G, Stumvoll M, Dailey G, Bier D, Toft I, Jenssen TG, Gerich JE (1995) Glutamine: a major gluconeogenic precursor and vehicle for interorgan carbon transport in man. J Clin Invest 95: 272–277
O'Neill CW (1999) Physiological significance of volume-regulatory transporters. Am J Physiol 276: C995–C1011
Shyng SL, Nichols CG (1998) Membrane phospholipid control of nucleotide sensitivity of K_{ATP} channels. Science 282: 1138–1141
Tohyama Y, Kameji T, Hayashi S (1991) Mechanisms of dramatic fluctuations of ornithine decarboxylase activity upon tonicity changes in primary cultured rat hepatocytes. Eur J Biochem 202: 1327–1331
Vadgama JV, Christensen HN (1983) Comparison of System N in fetal hepatocytes and in related cell lines. J Biol Chem 258: 6422–6429
Vlahos C, Matter W, Hui K, Brown R (1994) A specific inhibitor of phosphatidylinositol 3-kinase, 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). J Biol Chem 269: 5241–5248
Weissbach L, Kilberg MS (1984) Amino acid activation of amino acid transport System N early in primary cultures of rat hepatocytes. J Cell Physiol 121: 133–138
Woodworth C, Secot T, Isom H (1986) Transformation of rat hepatocytes by transfection with simian virus 40 DNA to yield proliferating differentiated cells. Cancer Res 46: 4018–4026

Authors' address: Barrie P. Bode, Ph.D., St. Louis University, Department of Biology, 3507 Laclede Ave., St. Louis, MO 63103-2010, U.S.A.,
Fax: (314) 977-3658, E-mail: bodebp@slu.edu

Received January 3, 2000