Regulation of glutamate transport and transport proteins in a placental cell line

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Novak, Donald, Forrest Quiggle, Carlos Artime, and Mark Beveridge. Regulation of glutamate transport and transport proteins in a placental cell line. Am J Physiol Cell Physiol 281: C1014–C1022, 2001.—We utilized HRP.1 cells derived from midgestation rat placental labyrinth to determine that the primary pathway for glutamate uptake is via system XAG, a Na+-dependent transport system. Kinetic parameters of system XAG activity were similar to those previously determined in rat and human placental membrane vesicle preparations. Amino acid depletion caused a significant upregulation of system XAG activity at 6, 24, and 48 h. This increase was reversed by the addition of glutamate and aspartate but not by the addition of α-(methylamino)isobutyric acid. Immunoblot analysis of the three transport proteins previously associated with system XAG composed, presumably in combination, system XAG activity at 6, 24, and 48 h. Inhibition analysis suggested key roles for EAAC1 and GLAST1 in basal anionic amino acid transfer, with an enhanced role for GLT1 under conditions of amino acid depletion. In summary, amino acid availability as well as intracellular metabolism regulate anionic amino acid uptake into this placental cell line.

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quent incorporation of metabolic products into the TCA cycle (4). As such, a variety of important energetic intermediates, including NADPH, NADH, FADH, and GTP, are produced. In addition to cell entry via transport, glutamate can be both synthesized and degraded within cells through the action of several enzymes. Although glutaminase activity within the rat placenta is thought to be minimal, phosphate-dependent glutaminase activity has been demonstrated in human placental mitochondria (7) (J. Neu, personal communication) (24). Glutamate can also be derived from α-ketoglutarate through the action of glutamate dehydrogenase or an aminotransferase. These activities have been demonstrated in the placenta (3, 39).

The availability of placental cell type-specific cell lines greatly facilitates studies of the relationships among glutamate transport, transport regulation, and transporter expression (6). The rat placenta is composed of different anatomic and functional regions, foremost of which is the junctional zone, composed largely of spongiosotropic cells, important in placental hormone production, and the labyrinth zone, thought to be the area in which the bulk of maternal-fetal nutrient transfer occurs. Several rat placental cell lines have been derived from midgestation rat placenta and are extensively characterized by Hunt et al. (16, 17). The HRP.1 line, derived from explants of days 11–12 placental labyrinth, exhibits selective characteristics of labyrinth cells. Specifically, it expresses transferrin receptor and alkaline phosphatase, characteristics of labyrinth cells. HRP.1 cells also secrete placental lactogen 2 (consistent with labyrinthine giant cells) but do not form syncytia (16, 17, 41). Because of its rodent origins, derivation from, and similarity to labyrinth cells, we have chosen the HRP.1 cell line for our studies of anionic amino acid transport regulation within a placental cell line.

METHODS

Chemicals. [3H]Glutamic acid was obtained from American Radiolabelled Chemicals (St. Louis, MO). All other chemicals were of reagent grade or of the highest grade commercially available. The EAA1 antibody was made as previously described (28). GLT1 and GLAST1 antibodies were the kind gifts of Jeffrey Rothstein (Johns Hopkins University). The modified 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) assay was obtained from Sigma (St. Louis, MO). 4-[3-(4-Iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1-benzenedisulfonate (WST-1) assay kits were obtained from Boehringer Mannheim.

Cell culture. HRP.1 cells were the kind gift of M. J. Soares. They were grown as described by Hunt et al. (16, 17) except that NCTC-135 media and 10% fetal bovine serum were used. Amino acid depletion experiments were performed using Selectame media with the addition of the designated amino acid in a concentration of 1 mM. Dialyzed serum was used for all studies. Studies described were performed with cells at ~80% confluence unless otherwise noted. Transport was performed as described below after plating cells into 24-well trays.

Enzyme assays. Transaminase activity was assayed with a kit obtained from Sigma. Glutamate dehydrogenase and glutaminase assays were performed according to previously published protocols (7, 29, 37).

Transport. Cells were seeded into 24-well dishes as described earlier, and whole cell transport was performed as previously described (20). Transport was initiated by replacing depletion (amino acid free) buffer (2 × 15 min) with Na+-containing Krebs-Ringer phosphate or Na+-free (choline KRP) buffers that contained the appropriate amount of radiolabeled (10 μCi/ml) amino acids, and, when indicated, inhibitors. After the appropriate time interval, which was typically <60 s, uptake was terminated by four rinses of 4°C choline KRP (2 ml/well). After air drying, cellular protein was precipitated with 10% TCA, and the supernatant radioactivity was analyzed by liquid scintillation counting. Proteins were solubilized in 0.2 N NaOH/0.2% SDS and analyzed for total cellular protein. Uptake velocities (uptake·mg protein−1 min−1) were reported as means ± SE unless otherwise noted. Derived (i.e., sodium-dependent, starvation-induced) velocities were obtained by subtracting uptakes in the absence of Na+/presence of inhibitor from that in the presence of Na+ or absence of inhibitor. SE were then derived in the usual fashion.

Biotinylation. The method utilized for biotinylation of plasma membrane proteins was that of Sims and colleagues (42). Cells were rinsed with PBS containing 0.1 mM Ca2+ and 1.0 mM Mg2+, and then were incubated with biotin solution (sulfo-N-hydroxysuccinimide-biotin, 1 mg/ml in PBS-Ca2+/Mg2+) for 20 min. Biotin solution was aspirated, and the cells were washed two times and then incubated for 30 min with PBS-Ca2+/Mg2+ containing 100 mM glycine. After two additional washes, the cells were incubated with RIPA/lysis buffer (100 mM Tris·HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1% SDS, and 1% sodium deoxycholate) for 30 min at 4°C. Lysates were subsequently centrifuged at 20,000 × g, and supernatants were incubated with avidin-conjugated beads for 1 h at room temperature. After centrifugation at 20,000 × g for 15 min, the pellet was resuspended in RIPA/lysis buffer, washed four times, and then brought up in Laemmli buffer (62.5 mM Tris·HCl, pH 6.8, 2% SDS, 20% glycerol, and 5% 2-mercaptoethanol). After a 30-min incubation, the mixture was centrifuged at 20,000 × g for 5 min, and the supernatant (biotinylated fraction) was used for immunoblotting.

Western analysis. Protein aliquots (50 μg/lane) were electrophoresed on 7.5% SDS-PAGE by the method of Laemmli (21). Proteins were electrotransferred to a 0.45-μm nitrocellulose membrane, and blots were probed with the specified amount/dilution of antibody. Immunoreactive bands were detected with protein A conjugated to horseradish peroxidase (HRP) or HRP-conjugated secondary antibody, as appropriate. Visualization was performed with an enhanced chemiluminescence kit. These antibodies were previously validated and found to be specific through the use of preincubation of primary antibody with the appropriate peptide or fusion protein against which each antibody was made, with subsequent disappearance of visible bands. Densitometry was performed using NIH software.

Data analysis. Kinetic analyses were performed by using computer-assisted least-squares fits of data points with the EnzFitter computer program (Elsevier-Biosoft). Data were examined for the presence of multiple transport systems both graphically and with the aid of a computer. Differences between groups were determined with Student’s two-tailed t-test or by the test of differences of means (in the case of derived values, such as Na+-dependent uptakes). Data were expressed as means ± SE unless otherwise specified.
RESULTS

Glutamate uptake into apical and basal membrane vesicles derived from rat placental labyrinth was previously demonstrated to occur via system $X_{AG}$ (28). Uptake of $0.1\mu M$ glutamate into HRP.1 cells was $Na^+$ dependent and linear through at least 5 min (Fig. 1). The majority of uptake at all time points could be attributed to system $X_{AG}$, defined as $D$-aspartate-inhibitable glutamate uptake in the presence of $Na^+$. Uptake via system $X_{AG}$ has been shown to vary in some cell types with cell density (5). Because of this, and to examine the relationships between cellular protein content, cell number, and confluence in these cells, we determined the impact of cell confluence (estimated at 20, 40, 60, 80, or 100%) on the transport of glutamate expressed not only per milligram of protein but also per cell (measured by counting in parallel dishes) and MTT units, a measure of metabolically active cells. Uptake of glutamate decreased with increasing cell confluences from 60–100%, regardless of whether uptake was measured per unit of protein or per cell (data not shown). At lower confluences, uptake per unit of protein was not concordant with uptake per cell, presumably reflecting diminished cellular protein content at lower confluences. Because our primary interest was in the determination of uptake per cell and because of the relative ease of measurement of uptake per unit of protein, we chose to conduct further experiments at ~80% confluence.

We next examined the kinetics of both $Na^+$-dependent and system $X_{AG}$-mediated glutamate uptake into HRP.1 cells. Initial substrate concentrations were based on our prior work in placental membrane vesicles (28). As shown in Fig. 2A, the majority of glutamate uptake into HRP.1 cells was $Na^+$ dependent. Uptake was saturated by increasing concentrations of substrate. Kinetic parameters were similar but not identical to those delineated for system $X_{AG}$ (Fig. 2B). The Michaelis-Menten constant derived ($15.1 \pm 1.8 \mu M$) was similar to that noted in placental vesicles derived from both human and rat placenta as well as in other cell types (14, 15, 28). There was a small component of $Na^+$-dependent uptake that was not inhibitable by $D$-aspartate, which, therefore, represented uptake not mediated by system $X_{AG}$. This component was previously noted in membrane vesicles derived from...
rat placenta (28). Kinetic characterization was attempted but was not successful because of the low velocities involved.

System $X_{\text{AG}}$ has been shown to be upregulated by substrate deprivation in a renal epithelial cell line (38). We examined glutamate uptake into HRP.1 cells deprived of amino acids for varying lengths of time (Fig. 3). Under these conditions, system $X_{\text{AG}}$ activity was increased two- to threefold at 6, 24, and 48 h. The addition of 1 mM glutamate abolished this increase in activity at each time point. The addition of 1 mM glutamine or leucine to the media resulted in an intermediate increase in activity compared with amino acid complete media, whereas 1 mM $\alpha$-(methylamino)isobutyric acid (MeAIB), a nonmetabolizable amino acid analog substrate of system A, had no effect at any tested time point. Although confluence was visually unaffected by the treatments described above, protein contents were significantly decreased in each treatment group compared with amino acid-replete controls (data not shown), raising the possibility that the results observed were the consequence of diminished confluence rather than substrate deprivation. Conversely, protein contents in the glutamate-, glutamine-, and MeAIB-replete groups were virtually identical in each set (6, 24, and 48 h). Given the large range in uptake shown in these groups, from glutamate replete, which was equivalent to the amino acid-replete control, to MeAIB, which was equivalent to the amino acid-depleted group, it seems unlikely that the observed results were solely attributable to differences in confluence.

Five amino acid transporters capable of glutamate transport consistent with system $X_{\text{AG}}$ activity have been cloned. We previously demonstrated the presence of three of these, EAAC1, GLT1, and GLAST1, in rat placenta. To explore the mechanism by which glutamate uptake was enhanced by amino acid depletion, we used previously characterized antibodies directed against EAAC1, GLT1, and GLAST1, to perform immunoblotting of HRP.1 cell homogenates in the presence/absence of amino acids. As depicted in Fig. 4 and Table 1, GLT1 immunoreactive protein was increased in HRP.1 cell homogenates at 6 h in amino acid-depleted cells compared with controls. This change was not reversed by the addition of glutamate or MeAIB to the media. Addition of glutamine to the media produced an intermediate effect, which did not differ significantly from either the amino acid-depleted or replete cells. Immunoreactive GLAST1 was decreased at 6 h by amino acid depletion compared with control. This effect persisted despite the addition of either glutamate or glutamine to the media. GLAST1 immunoreactive protein was also diminished at 24 h in the amino acid-depleted and glutamine-supplemented groups, whereas GLT1 was not impacted at 24 h under...
any study condition. Levels of immunoreactive EAAC1 were not altered by the deletion of amino acids from the media at either 6 or 24 h. At 48 h, EAAC1 immunoreactive protein was increased approximately fourfold in amino acid-starved compared with amino acid-replete cells; this change did not reach statistical significance. Levels of GLAST1 immunoreactive protein were also increased compared with control in amino acid-starved and glutamate-replete cells; however, as in the case of EAAC1, these differences were not statistically significant given the large variation observed across experiments. GLT1 immunoreactive protein expression differed in that levels were decreased compared with control in the glutamate, glutamine, and MeAIB groups. Expression in the amino acid-starved group was also diminished, but not significantly.

To begin to define the role of individual transport proteins in the transfer of glutamate into HRP.1 cells, we examined glutamate uptake in the presence of varied inhibitors. System X_{AG}^{-} activity was significantly inhibited by micromolar concentrations of both D,L-threo-β-hydroxyaspartate and L-trans-2,4-pyrolidine dicarboxylic acid but not by dihydrokainate (Fig. 5). This inhibition profile suggests that despite its presence within the cell, GLT1 was not an important component of system X_{AG}^{-} in HRP.1 cells. GLAST1, EAAT4, and EAAC1 share similar inhibitor profiles; thus assignment of transport between these entities was not possible utilizing this methodology. We next evaluated the impact of inhibitors on amino acid starvation-induced system X_{AG}^{-} uptake. As shown in Fig. 6, dihydrokainate inhibited approximately two-thirds (67 and 63%) of induced activity at 6 and 24 h, respectively. Inhibition of uptake by dihydrokainate under amino acid-depleted conditions was significant at both time points, whereas inhibition in the presence of amino acids was significantly different at 6 h only (inhibition of 15%; \( P < 0.05 \)). These data suggest that starvation-induced activity might include that which is expressed by GLT1. To begin to reconcile these data with the

![Fig. 5. Inhibition of 1 \( \mu \)M [\(^{3}H\)]glutamate uptake into HRP.1 cells. All uptakes were performed in the presence of a 100 mM inwardly directed Na\(^{+}\) gradient. Each point represents the mean ± SE of 8–24 individual data points from separate experiments. Differences were determined through use of a two-tailed Student’s \( t \)-test. TBHA, D,L-threo-β-hydroxyaspartate; DHK, dihydrokainate; PCDA, L-trans-2,4-pyrolidine dicarboxylic acid.](image)

![Fig. 6. Inhibition of starvation-induced glutamate uptake by dihydrokainate. All uptakes (1 min) were performed with 1 \( \mu \)M [\(^{3}H\)]glutamate in the presence of a 100 mM inwardly directed Na\(^{+}\) gradient with and without 1 mM dihydrokainate in the presence and absence of amino acids. Each point represents the mean ± SE of 12 individual data points from 2–3 separate experiments. Potential differences were determined through use of a two-tailed Student’s \( t \)-test or the test of means of differences (induced uptake). ∗\( P \leq 0.05 \); **\( P \leq 0.07 \).](image)
immunoblot data depicted above, we chose to perform biotinylation studies using a membrane-impermeant biotinylation reagent combined with Western blotting (42). The results of these studies, as depicted in Fig. 7, demonstrate that cell surface expression of all three transport proteins (expressed as arbitrary densitometry units normalized to actin) increased approximately threefold after 24 h of amino acid depletion (8, 42). These data are consistent with the increase in system XAG activity (Fig. 3) demonstrated previously under amino acid-depleted conditions.

We next examined the role of intracellular anionic amino acid metabolism on the regulation of anionic amino acid uptake into HRP.1 cells. Glutaminase, aminotransferase, and glutamate dehydrogenase activities were assessed in HRP.1 cells utilizing previously validated assays [glutamate dehydrogenase (29), aspartate aminotransferase (Sigma kit), and glutaminase (7, 37)]. Both glutaminase and aminotransferase activities were present, and conditions for inhibition of activity with 6-diazoo-5-oxo-l-norleucine (DON) and aminooxyacetic acid (AOA) were established (data not shown). DON is an established inhibitor of phosphate-dependent glutaminase activity, whereas AOA is an established inhibitor of aminotransferases in both human and rat placenta (4, 9, 34, 37). Glutamate dehydrogenase activity could not be demonstrated in HRP.1 cells. Therefore, the impact of DON and AOA on glutamate uptake into HRP.1 cells was examined in the presence/absence of specific amino acids. Neither AOA (10 mM) nor DON (2.5 mM), by themselves, had a significant impact on uptake under any tested condition (data not shown). As shown in Fig. 8, however, the inhibitors in combination had a significant impact on anionic amino acid uptake. In the presence of amino acids, the combination of inhibitors diminished glutamate uptake. This change was not associated with either a decrease in protein or in WST-1 assay (measure of metabolically active cells) results, suggesting that the combination of inhibitors was not associated with a decrease in cell viability. Uptake was also decreased when glutamate, aspartate, or glutamine (not shown) was selectively deleted from the media. In amino acid-free media, inhibitors were associated with a trend toward enhanced glutamate uptake; however, these differences did not reach statistical significance. The presence of inhibitors in addition to either glutamate or glutamine (not shown) elicited a significant increase in uptake. Conversely, in the presence of aspartate, glutamate uptake was diminished compared with that in the absence of inhibitors. Asparagine, in the absence of other amino acids, was insufficient to suppress glutamate uptake to the level noted in the presence of amino acids, glutamate alone, or aspartate alone. In the presence of inhibitors, however, like aspartate and unlike glutamate or glutamine, uptake was diminished compared with that in the absence of inhibitors. Uptake of glutamate into cells whose media lacked only asparagine was also diminished in the presence of inhibitors. Interestingly, this effect was lost if both aspartate and asparagine were depleted. Uptake in this case (presence of inhibitors) was not significantly different from that noted in the absence of amino acids. Together, these data suggest that uptake activity was regulated primarily by the presence of aspartate, rather than glutamate, and that under normal conditions, sufficient intracellular aspartate can be derived from either glutamate or asparagine.
Glutamate is produced by the fetal liver from glutamine and is subsequently taken up by the placenta, wherein it is thought to be an important substrate for placental energy production (31, 45). We have previously demonstrated the presence of system X\textsubscript{AG} transport activity in both basal and apical membranes derived from rat placental labyrinth. This activity has been shown to be decreased in basal membranes derived from rat placenta when a low-protein diet is fed to pregnant dams throughout gestation (25). HRP.1 cells, first obtained from midgestation rat placental labyrinth, have been previously characterized (16, 41). They demonstrate a variety of trophoblast characteristics including the expression of cytokeratin intermediate filaments, transferrin receptor, alkaline phosphatase, and rat PL-II. The current work has demonstrated that these cells exhibit anionic amino acid transport that is qualitatively and kinetically similar to that which we have previously defined in membrane vesicles derived from rat placenta (28) (unpublished data). In addition, they express several transport proteins capable of system X\textsubscript{AG} transport, as does the rat placenta in situ (28). These findings, in addition to those described above, bolster the utility of this cell line for the study of anionic transporter regulation within the placenta.

As an initial step in this process, we demonstrated that system X\textsubscript{AG} activity is regulated by cell confluence. This finding is consistent with those shown by others with regard to the transport of glucose, phosphate, and amino acid transfer mediated by system A (1, 40, 46). Bussolati and coworkers (5) showed that system X\textsubscript{AG} activity increased in quiescent compared with actively proliferating NIH/3T3 cells. These investigators did not directly address the impact of confluence. In addition, quiescence was produced through serum starvation, raising the possibility of growth factor effects. We also demonstrated that system X\textsubscript{AG} activity is regulated in HRP.1 cells in response to amino acid starvation. Similar responses to amino acid depletion have previously been reported for systems A, y\textsuperscript{-}, L, and X\textsubscript{AG} in other tissues (11, 18, 32, 33). To the best of our knowledge, this is the first such report in placental cells. It is of interest that the response to amino acid deprivation in vitro differs from that previously defined in vivo in response to maternal protein deprivation. This discrepancy can, perhaps, be reconciled by the observation that during maternal protein deprivation, maternal serum levels of most amino acids actually rise, while fetal amino acid levels were largely unaffected (25). These data suggest that the downregulation of transport observed during maternal protein deprivation results not from direct effects of substrate deprivation on placental cells but, rather, from the impact of other regulatory substances on transporter function (27). The mechanism by which amino acid starvation regulates amino acid transporter activity/expression has not been delineated. Recently, Franchi-Gazzola and colleagues (12) demonstrated the involvement of the extracellular regulated kinase pathway in the adaptive response of system A. Like system A, the increase in system X\textsubscript{AG} activity is specific for amino acid starvation. Depletion of glucose does not produce a similar upregulation (Novak, unpublished data).

We have previously shown that at least three transporters capable of anionic amino acid transport are present within the rat placental labyrinth. We explored the expression of these transport proteins in response to amino acid depletion. In agreement with prior findings by McGivan and colleagues (30, 35, 38), there was no increase in EAAC1 expression at either 6 or 24 h in amino acid-starved cells. Indeed, expression was actually suppressed by the addition of glutamate and glutamine to the media at 6 h. Expression was increased at 48 h, although this increase reached significance only when glutamate was in the media. GLAST1 expression was qualitatively similar to that of EAAC1. Although GLT1 expression was increased at 6 h, expression decreased with increasing duration of amino acid starvation. These data seemed initially to conflict with our demonstration that a significant portion of anionic amino acid uptake induced by starvation is inhibitable by dihydrokainate, and, therefore, may be attributable to GLT1 expression. The subsequent biotinylation data, conversely, demonstrated that although total GLT1 expression may have fallen with amino acid depletion, the cell surface expression of this protein rises under the same conditions by two- to threefold.

Finally, we explored the impact of intracellular anionic amino acid metabolism on anionic amino acid uptake. Inhibition of glutaminase and aminotransferase activities in the presence of amino acids inhibited glutamate uptake into HRP.1 cells. Uptake continued to be downregulated despite the absence of glutamate, aspartate, or glutamate and aspartate (data not shown), suggesting that either these amino acids do not, of themselves, regulate transporter activity or that intracellular synthesis of these amino acids was not completely inhibited. The increase in uptake associated with the addition of inhibitors in the amino acid-depleted/glutamate-sufficient condition suggests that the products of glutamate metabolism, rather than glutamate per se, were required for the normalization of uptake demonstrated in the absence of inhibitors compared with the amino acid-replete condition. Conversely, the decrease in uptake demonstrated in the amino acid-depleted/aspartate-replete condition in the presence of inhibitors suggests that aspartate, or a metabolite thereof, might participate directly in uptake regulation. Aspartate may be synthesized/degraded via the action of transaminases. It may also participate in the urea cycle, via conversion to arginosuccinate. Finally, asparagine and aspartate may be interconverted by asparaginase or asparagine synthetase activities (44). Our finding that asparagine, like aspartate, sufficiency was associated with inhibition of glutamate uptake in the presence of metabolic inhibitors suggests that this pathway might be of im-
portance in HRP.1 cells. We have, therefore, demonstrated, for what is to our knowledge the first time, that anionic transport activity, at least in HRP.1 cells derived from the rodent placenta, appears to be regulated preferentially by aspartate rather than glutamate. This is perhaps not surprising given the key role of aspartate in cellular metabolic pathways, including the urea cycle, citric acid cycle, and nucleic acid synthesis. We cannot rule out the possibility that other amino acids not tested in this study might have a similar effect.

In summary, HRP.1 cells derived from rat placenta demonstrate system X_{A}G transport activity and express several amino acid transporters known to mediate this activity. Transport activity is regulated by cell density and by substrate availability, particularly that of aspartate. Inhibition analysis suggests that the majority of uptake, in the basal state, is mediated by EAAC1 or GLAST1. GLT1 assumes a more important role under amino acid-depletion conditions. The increase in uptake activity associated with starvation is not, at least within the first 24 h, mediated by an increase in measured whole cell transporter protein expression but, rather, by enhanced cell surface expression.

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