Original Article

Influence of Intracellular Glutamine Depletion on Regulation of Amino Acid Transport System A in Placental (BeWo) Cells

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Abstract

Glutamine is known to modulate the expression of various genes and uptake activity of nutrient transporters in mammalian cells. The purpose of the study was to investigate the influence of intracellular glutamine status on amino acid transport system A in placental choriocarcinoma cells (BeWo). Intracellular glutamine was depleted by culturing the cells in regular medium without glutamine and by treating the cells for 16 hours with methionine sulfoximine (MSX), an inhibitor of glutamine synthetase. When cultured in the presence of glutamine, treatment with MSX had no effect on system A activity as monitored by MeAIB uptake. However, when cultured in the absence of glutamine, treatment with MSX (2 mM) reduced system A activity by 70%. The decrease in system A activity induced by MSX treatment was blocked by co-treatment with glutamine but not with glutathione, or diazooxonorleucine (a glutamine analog). The decrease in system A activity caused by MSX treatment was associated with a decrease in the maximal velocity of the transport system but without any significant change in substrate affinity. Northern analyses indicated that there was no change in steady-state levels of ATA1 and ATA2 mRNA, suggesting that the regulation was not at the level of transcription. These data show that intracellular glutamine is obligatory for maintenance of optimal expression/activity of system A in BeWo cells.

Keywords: amino acid transport system A, gene regulation, glutamine, placental cell

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บทคัดย่อ

อิทธิพลของการลดกลูทามีนภายในเซลล์ต่อการควบคุมการขนส่งกรดอะมิโนระบบเอในเซลล์รก

บุญฤทธิ์ ทองทรง

เป็นที่ทราบกันโดยทั่วไปว่ากรดอะมิโนกลูทามีนในเซลล์ของสัตว์เลี้ยงลูกด้วยนมสามารถควบคุมการแสดงออกของยีนที่หลากหลาย รวมทั้งบทบาทของตัวขนส่งสารอาหารต่างๆ วัตถุประสงค์ของการศึกษาเพื่อทราบถึงอิทธิพลของสภาวะกลูทามีนภายในเซลล์ที่มีต่อการขนส่งกรดอะมิโนระบบเอในเซลล์รก โดยทำการลดระดับ กลูทามีนภายในเซลล์ดังกล่าวที่ถูกเพาะเลี้ยงด้วยอาหารเลี้ยงเซลล์ตามปกติแต่ไม่มีกลูทามีน และเติมสารเมทไธโอนีนซัลโฟซิมีนซึ่งทำหน้าที่ยับยั้งเอนไซม์กลูทามีนซินทีเทส เป็นระยะเวลานาน 16 ชั่วโมง ผลพบว่าเมื่อเลี้ยงเซลล์ในสภาวะที่มีกลูทามีนตามปกติ การใส่สารเมทไธโอนีนซัลโฟซิมีน ไม่เห็นผลต่อการนำเมทธิลอะมิโนไอโซบิวทีริคแอซิดเข้าเซลล์ ซึ่งเป็นตัวบ่งชี้การขนส่งกรดอะมิโนระบบเอ แต่ในทางตรงกันข้ามเมื่อเลี้ยงเซลล์ในสภาวะที่ไม่มีกลูทามีนและเติมสารเมทไธโอนีนซัลโฟซิมีนพบว่า การขนส่งกรดอะมิโนระบบเอลดลงร้อยละ 70 และเมื่อทำการยับยั้งผลดังกล่าวข้างต้นโดยการเติมกลูตามีนพบว่าให้ผลได้ดี แต่พบว่าไม่ได้ผลเมื่อเติมกลูต้าไธโอนหรือสารไดอะโซอ๊อกโซนอร์ลิวซีน (สารเสมือนกลูทามีน) การนำกรดอะมิโนเข้าสู่เซลล์รกที่ลดลงโดยตัวขนส่งกรดอะมิโนระบบเอที่เป็นผลมาจากเมทไธโอนีนซัลโฟซิมีนมีผลสัมพันธ์ต่อการลดจำนวนของตัวขนส่งระบบดังกล่าว แต่ไม่เปลี่ยนแปลงค่าความชอบต่อสับสเตรต เมื่อทำการวิเคราะห์การแสดงออกของยีนตัวแทนในระบบดังกล่าว ได้แก่ เอทีเอ1 และ เอทีเอ2 ไม่พบว่าเกิดการเปลี่ยนแปลงจากระดับคงที่ของทั้งสองยีนตัวขนส่งดังกล่าว จึงเสนอแนะว่า การควบคุมที่เกิดขึ้นไม่ได้เกิดในระดับที่เป็นการลอกรหัสหรือสร้างอาร์เอ็นเอ ข้อมูลทั้งหมดที่ได้จากการศึกษาแสดงให้เห็นว่า ระดับกลูทามีนภายในเซลล์เป็นสิ่งจำเป็นต่อการคงอยู่ของบทบาทหน้าที่ที่เหมาะสมสำหรับการแสดงออกหรือนำกรดอะมิโนในระบบเอผ่านเข้าสู่เซลล์รก

คำสำคัญ: ขนส่งกรดอะมิโนระบบเอ การควบคุมยีน กลูทามีน เซลล์รก

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Introduction

Glutamine is by far the most abundant free amino acid in circulation and is an important molecule that not only serves as a building block for protein synthesis but also performs a variety of additional biological functions (Karinch et al., 2001). The intracellular concentration of glutamine is very high where it plays a central role as a ready source of nitrogen, carbon, and energy in various metabolic processes. Some findings showed that it was also a regulator of gene expression, controlling gene expression at the level of transcription (Bellon et al., 1995; Brasse-Lagnel et al., 2003). Glutamine is taken up into mammalian cells from extracellular medium by several active and passive amino acid transport systems (Ganapathy et al., 2003). In addition, glutamine is also synthesized by the amidation of glutamate. This ATP-dependent reaction is catalyzed by glutamine synthetase, an enzyme inhibitable by the glutamine analog methionine sulfoximine (MSX).

Gene expression is controlled by complex interaction of various intracellular and extracellular factors. Nutrients play a critical role in this phenomenon. Previous studies had shown that amino acids had marked ability to control gene expression and other cellular processes (Jousse et al., 2000; Averous et al., 2003; Jefferson and Kimball, 2003; Kadowaki and Kanazawa, 2003). Several genes have been identified to possess *cis*- and *trans*- acting elements that respond to the nutritional status of the cells with respect to specific amino acids (Guerrini et al., 1993). With the identification of these amino acid-responsive elements in genes, the molecular events involved in the control of gene expression by amino acids are beginning to be unraveled (Barbosa-Tessmann et al., 2000; Fafournoux et al., 2000; Kilberg and Barbosa-Tessmann, 2002).

Mammalian cells express a number of amino acid transport systems that mediate the uptake into and release of amino acids from the cells (Ganapathy et al., 2003). These transporters are thus vital for the homeostasis of amino acids in cells. The role of intracellular glutamine in the regulation of some important amino acid transporters has not been investigated. The purpose of the present study was to investigate the influence of depletion of intracellular glutamine on the activity and gene expression of the amino acid transport system A. These studies were carried out using the BeWo cells, a human placental choriocarcinoma cell line. These cells express several

amino acid transport systems, of which system A is of special importance. System A, which accepts glutamine and other short-chain neutral amino acids as substrates (Ganapathy et al., 2003), is robustly expressed in BeWo cells. These cells are similar to normal placental trophoblasts that express two of the three known isoforms of system A, namely ATA1 and ATA2 in the brush border membrane (Jones et al., 2002). The activity of system A in the placental brush border membrane has been shown to directly correlate with the birth weight of babies under various physiological and pathological conditions (Kuruvilla et al., 1994; Glazier et al., 1997; Sibley et al., 1997; Harrington et al., 1999). Therefore, information on the identity of factors that regulate the activity of system A in these cells is highly relevant to the understanding of fetal growth and development.

Materials and Methods

Cell culture and chemicals: The BeWo choriocarcinoma cell line, cell culture media, fetal bovine serum, MACS mRNA isolation kit, hybond N^+ nylon membrane, ready-to-go oligolabeling kit, unlabeled and radiolabeled amino acid of [^{14}C]α-(methylamino) isobutyric acid (MeAIB) (55 mCi/mmol) were provided by Professor Dr. Vadivel Ganapathy and Professor Dr. Puttur D. Prasad.

***Cell culture and treatment*:** BeWo cells were cultured in 12-well culture plates for uptake assays or in 150 cm^2 flasks for RNA isolation in DMEM/F-12 (50:50) medium containing 2.5 mM glutamine and supplemented with 10% fetal bovine serum, 100 U/ml penicillin and 100 µg/ml streptomycin. The culture condition was as mentioned in the previous paper (Thongsong. 2012). Confluent cultures were treated with MSX in a glutamine-free culture medium. These culture conditions effectively lead to the depletion of intracellular glutamine because of the lack of glutamine in the medium and also because of the inhibition of endogenous synthesis of glutamine by MSX. After treatment of the cells with or without MSX for 16 hours, the cells were used for the amino acid uptake measurements. Cells cultured in glutamine-free medium but not treated with MSX were used as control.

MeAIB uptake measurement: Uptake measurement was carried out at 37^{0}C. The medium was aspirated and the cell monolayer was washed once with the uptake buffer. Uptake was then initiated by the addition of 500 µl of uptake buffer containing 0.5 µCi of radiolabeled MeAIB. The incubation was continued for a desired length of time, following which the uptake was terminated by aspirating the uptake medium. Initial time course studies showed that the uptake was linear up to 40 min, and therefore, all subsequent measurements were done within this linear phase of uptake. After the termination of the uptake, the cells were washed two times with 1.5 ml of ice-cold uptake buffer. The cells were then solubilized with 0.5 ml of 1% SDS/0.2 N NaOH and transferred to scintillation vials for the determination of the radioactivity associated with the cells. The composition of the uptake buffer was 25 mM Hepes/Tris (pH 7.5), 140 mM NaCl, 5.4 mM KCl, 1.8 mM $CaCl_2$, 0.8 mM $MgSO_4$, and 5 mM glucose.

RNA isolation and Northern analyses: Poly(A)$^+$ RNA was isolated from control and MSX-treated cells using the MACS mRNA isolation kit. RNA (5 µg/lane) was size-fractionated on a denaturing formaldehyde-agarose gel, transferred onto hybond-N^+ membrane and cross-linked by UV irradiation. The blot was then sequentially probed with [α-^{32}P]-labeled cDNA probes specific for ATA1 and ATA2 as described previously (Jones et al., 2002). Hybridization signals were detected and quantified using the storm 840 system. The blot was also probed with [α-^{32}P]-labeled β-actin cDNA probe as an internal control to normalize differences in RNA loading between the lanes.

Statistical analysis: Experiments were made in duplicate or triplicate and each experiment was repeated three times. The results were given as means±SEM. Kinetic analyses were done by both nonlinear and linear regression analyses using the commercially available computer program Sigma Plot.

Results

Influence of intracellular glutamine depletion on system A activity in BeWo cells: The study was investigated the effect of extracellular and intracellular glutamine levels on the regulation of system A activity in BeWo cells. Since system A, which can mediate glutamine transport, is robustly expressed in BeWo cells, the activity of system A in cells grown in medium with and without glutamine in the absence and presence of MSX was determined. Confluent cells were treated with or without MSX (2 mM) for 16 hours in the presence or absence of glutamine (2.5 mM). Uptake of MeAIB (4 µM) was then measured in NaCl-containing buffer. The data obtained show that treatment with MSX did not have any effects on system A activity if the cells were treated with the inhibitor in the presence of glutamine (203±6.0 pmol/mg protein/20 min vs 178±5 pmol/mg protein/20 min) (Fig 1). But, when the treatment with

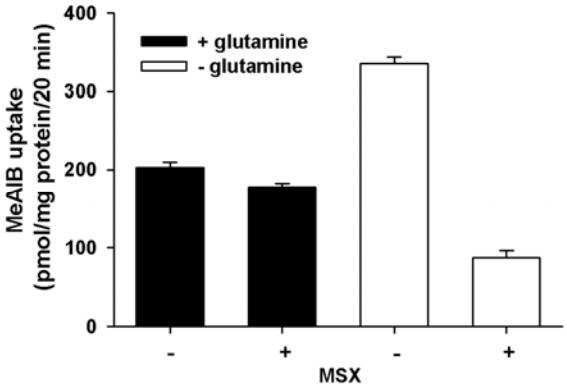

Figure 1 Influence of extracellular glutamine on MSX-dependent regulation of system A activity. BeWo cells were treated with or without glutamine (2.5 mM) in the presence or absence of MSX (2 mM) for 16 hours. The activity of system A was then measured using MeAIB (4 µM) as the substrate.

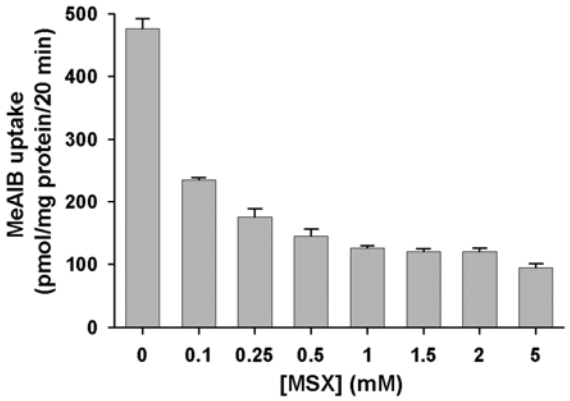

Figure 2 Dose-responsed relationship for the effect of MSX on system A activity. BeWo cells were treated with increasing concentrations of MSX in the absence of extracellular glutamine for 16 hours. The activity of system A was then measured using MeAIB (4 μM) as the substrate.

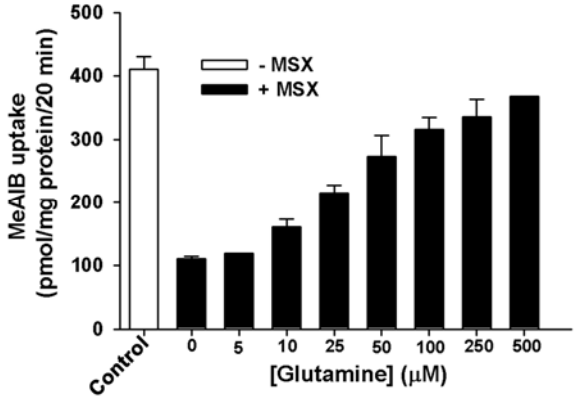

Figure 3 Blockade of MSX effect on system A activity by glutamine. BeWo cells were treated with MSX for 16 hours with increasing concentration of glutamine in the extracellular medium. Cells treated in the absence of both MSX and glutamine served as control. The activity of system A was measured using MeAIB (4 μM) as the substrate.

the inhibitor was done in the absence of glutamine, MSX caused ~4-fold decrease in system A activity (335±9 pmol/mg protein/20 min vs 87±9 pmol/mg protein/20 min). In addition, cells cultured in the absence of glutamine showed significantly higher system A activity than the cells cultured in the presence of glutamine (203±6.0 pmol/mg protein/20 min vs 335±9 pmol/mg protein/20 min).

Consequently, the dose-responsed relationship for the effect of MSX on system A activity was looked at. Confluent cells were treated with increasing concentrations of MSX in the absence of extracellular glutamine for 16 hours. The data show that the activity of system A decreased by nearly 50% when the concentration of MSX is as low as 0.1 mM (476±16 pmol/mg protein/20 min vs 235±4 pmol/mg protein/20 min) (Fig. 2). The effect increased as the concentration of MSX increased. At 5 mM MSX, the highest concentration tested, the uptake value obtained was 95±6 pmol/mg protein/20 min, which was less than 20% of the control value.

The influence of extracellular glutamine on MSX-induced decrease in system A activity was then determined. Confluent cells were treated for 16 hours with 2 mM MSX in the presence of increasing concentrations of glutamine in the extracellular medium. Cells treated in the absence of MSX as well as glutamine served as control. As seen in Fig 3, the data obtained show that MSX treatment decreased the activity of system A by ~75%, from 410±20 pmol/mg protein/20 min to 110±4 pmol/mg protein/20 min. This effect could, however, be blocked to a substantial extent if the treatment was done in the presence of glutamine, indicating that the decrease in system A activity was indeed due to the decrease in cellular glutamine levels. Only very little glutamine in the extracellular medium was needed to block the influence of MSX on system A activity. At extracellular glutamine concentration as low as 10 μM, the inhibition dropped to ~60%. The concentration of glutamine that caused 50% blockade of the MSX-induced system A inhibition was 49±6 μM.

Intracellular glutamine is known to influence the cellular levels of glutathione (GSH) (Oehler and Roth, 2003). To determine whether the effect of MSX on system A activity is due to decreasing cellular levels of GSH, the ability of extracellular GSH to block the effect of MSX was assessed. In addition, the ability of a glutamine analog to block the effect of MSX was also investigated. The experiment was used 6-diazo-5-oxo-L-norleucine (DON), a structural analog of glutamine which acts as a glutamine antagonist in various biological reactions that utilize glutamine as a substrate. Confluent cells were treated with 2 mM MSX in the absence of extracellular glutamine but in the absence or presence of 2 mM GSH or DON. Cells treated in the absence of MSX as well as extracellular glutamine served as control. The data showed that treatment with MSX was able to cause about 70% inhibition of system A activity in the absence or presence of GSH or DON (Fig 4). Thus, neither GSH nor DON was able to block the effect of MSX on system A activity.

The influence of MSX on the kinetic parameters of system A activity was then studied. Confluent cells were treated in the absence of extracellular glutamine with or without MSX (0.25 mM) for 16 hours. Uptake of MeAIB was measured over the concentration range of 0.1-2.5 mM. Linear and nonlinear regression analyses of the data indicated that the decrease in the activity of system A caused by MSX was associated with a decrease in the maximal velocity (V_{max}) of the transport system (control, 99.5±1.1 nmol/mg protein per 30 min; MSX-treated, 38.9±2.9 nmol/mg protein per 30 min) (Fig. 5). The affinity of the transport system for MeAIB remained essentially unaltered. The values for K_t in control and MSX-treated cells were 0.75±0.13 and 0.70±0.09 mM, respectively. These results suggested that treatment with MSX most likely resulted in a decrease in the surface density of functional system A proteins in the plasma membrane of the cells.

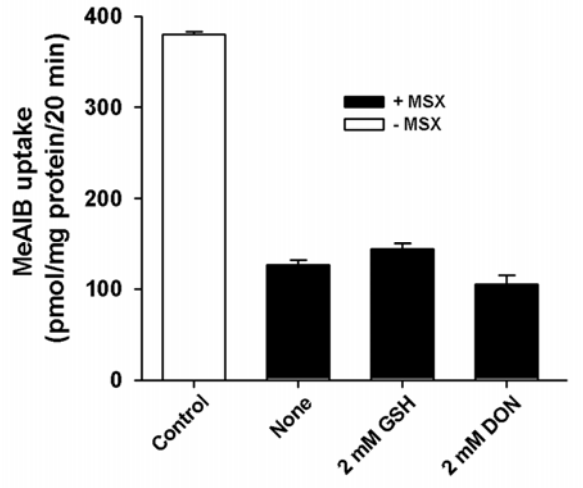

Figure 4 Effect of glutathione (GSH) and diazooxonorleucine (DON) on MSX-induced inhibition of system A activity. BeWo cells were treated with 2 mM MSX in the absence of extracellular glutamine for 16 hours but in the absence or presence of 2mM GSH or DON. Cells treated in the absence of MSX as well as extracellular glutamine served as control. The activity of system A was measured using MeAIB (4 μM) as the substrate.

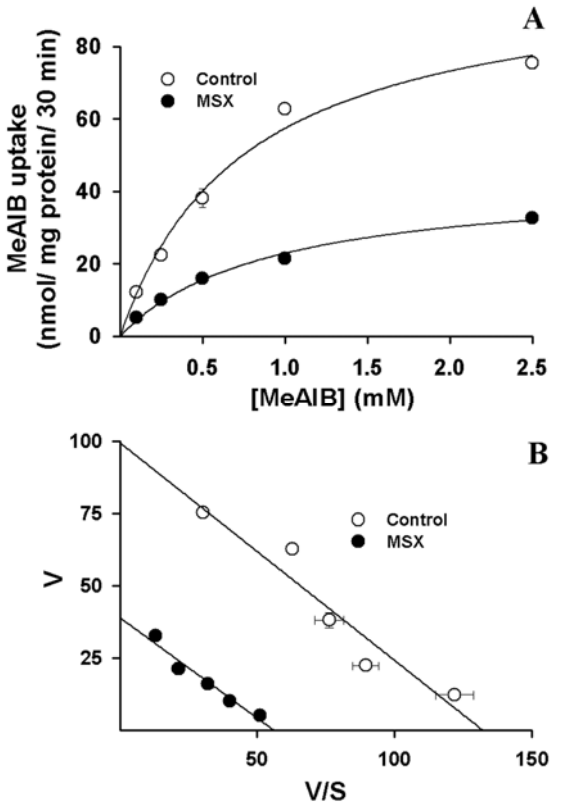

Figure 5 Influence of MSX on the kinetic parameters of system A activity. BeWo cells were treated with or without MSX (0.25 mM) for 16 hours in the absence of extracellular glutamine. Transport of MeAIB was measured over the concentration range of 0.1-2.5 mM. The amount of radiolabeled MeAIB was kept constant at 4 μM. (A) Uptake rate versus MeAIB concentration. (B) Eadie-Hofstee transformation of the same data. V, uptake in nmol/mg protein/30 min; S, MeAIB concentration in mM.

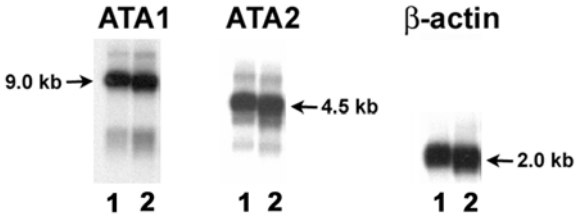

Figure 6 Influence of MSX treatment on ATA1 and ATA2 mRNA levels. BeWo cells were treated with or without MSX (2 mM) in the absence of extracellular glutamine for 16 hours. Poly(A)$^+$ RNA was isolated from these cells and subjected to sequential Northern blot hybridization with [^{32}P]-labeled cDNA probes specific for ATA1, ATA2, and β-actin. Lane 1: absence of MSX, Lane 2: presence of MSX.

To determine if the observed decrease in the system A transport activity is at the transcriptional level and is associated with a decrease in steady-state levels of system A transcripts, northern analyses using RNA isolated from control and MSX-treated cells were performed. The blot was sequentially probed with cDNA probes specific for ATA1 and ATA2 (Fig 6). The data showed that there was no change in steady-state levels of either ATA1 or ATA2 mRNA levels in MSX-treated cells compared to control cells. This showed that the decrease in system A activity associated with MSX treatment occurred without changes in steady-state levels of mRNA specific for system A. The intensity of the hybridization signal obtained with β-actin cDNA probe was comparable in lanes containing RNA isolated from control and MSX-treated cells.

Discussion

This study described the effect of intracellular glutamine depletion on the uptake activity and gene expression of amino acid transport system A in BeWo cells. Depletion of intracellular glutamine had dual effects on system A activity. Removal of extracellular glutamine without affecting the endogenous synthesis of glutamine stimulated the activity of system A. But, removal of extracellular glutamine and simultaneous inhibition of endogenous synthesis of glutamine decreased the activity of system A. Thus, extracellular and intracellular glutamine appeared to have a differential effect on the regulation of system A activity. The effect of glutamine depletion on amino acid transport was not limited to system A. Transport of various nutrients that are not substrates for system A (e.g. taurine and carnitine) were also decreased significantly in MSX-treated cells (Thongsong, 2012).

The decrease in system A activity induced by MSX treatment was associated with a decrease in the maximal velocity of the transport system with no alterations in substrate affinity. This suggests that the transporter density in the plasma membrane of the cells decreased as a result of MSX treatment. However, there was no change in the steady-state levels of mRNA transcripts for ATA1 and ATA2, two

isoforms of system A that are expressed in BeWo cells. These findings ruled out the possibility that MSX treatment interferes with the expression of genes for ATA1 and ATA2 at the level of transcription. Two other potential modes of action remain as a possible mechanism. First, intracellular glutamine levels may be directly affecting the expression of the transporter genes. Putative amino acid-responsive elements (AARE) have been identified in the regulatory region of certain genes which are subjected to regulation based on the amino acid status of the cell (Guerrini et al., 1993; Bruhat et al., 1999; Claeyssens et al., 2003). Recently, an intronic element in the ATA2 gene has been shown to mediate the amino acid availability-dependent transcriptional control of the ATA2 isoform of system A. However, this was unlikely in MSX-mediated regulation of system A activity since the decrease in system A activity was not associated with changes in steady-state levels of ATA1 or ATA2 mRNA (Palii et al., 2004). An alternate possibility is that MSX treatment may interfere with protein expression at the level of translation. Amino acid depletion has also been shown to inhibit cap-dependent translation and increase cap-independent translation of the mRNA for cationic amino acid transporter 1 (Cat-1) (Fernandez et al., 2001). Cat-1 transcripts have internal ribosomal entry site (IRES) which mediate cap-independent translation (Fernandez et al., 2002). It is, therefore, possible that intracellular glutamine depletion by MSX treatment may inhibit cap-dependent translation of ATA1 and ATA2 transcripts, resulting in decreased functional protein on the cell surface. Secondly, MSX treatment and the resultant intracellular glutamine depletion may interfere with the trafficking of ATA1 and ATA2 (Ling et al., 2001). Intracellular glutamine is also an important determinant of GSH levels in cells (Oehler and Roth, 2003). Changes in GSH status of the cells and the resultant redox state of the cell might affect system A expression and activity. Such a mechanism seems unlikely, however, because extracellular GSH is unable to block the effect of MSX on system A. Entry of extracellular GSH into mammalian cells requires the presence of GSH transport systems in the plasma membrane. Whether such transport systems are present in BeWo cells is not known. Further studies, are therefore, needed to understand why extracellular GSH has no effect on MSX-induced decrease in system A activity.

This study and my previous report showed that intracellular glutamine status might influence not only the activity of system A but also that of other transport systems such as the taurine transporter. These findings may have important physiological implications. Since system A is one of the transporters that mediate glutamine entry into normal placental syncytiotrophoblast at the brush border membrane (Novak and Beveridge, 1997), system A activity may modulate intracellular glutamine status in these cells. System A is one of the few amino acid transport systems that are subjected to hormonal regulation. Some previous studies reported the inhibition of amino acid transport system A by ethanol (Jones et al., 2002) and interleukin-1beta in trophoblasts (Thongsong et al., 2005). Therefore, the activity of system A in the placental brush border membrane may be subjected to alterations under various physiological and pathological conditions. Such changes may influence intracellular glutamine status which might in turn affect the expression and activity of not only system A but also other amino acid transport systems. Further studies are, therefore, needed to understand the explanation of the effect.

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