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Amino acid transport is down-regulated in ischemic human intestinal epithelial cells

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Abstract

Amino acid transport across the plasma membrane is essential for supplying enterocytes with amino acids for cellular metabolism. We studied amino acid transport during ischemic conditions using human intestinal epithelial cell line Caco-2. Cells were incubated under nutrient-deprived (phosphate-buffered saline, PBS), hypoxic, and ischemic (PBS + hypoxia) conditions. Ischemia resulted in a significant decrease in glutamine transport by a mechanism that decreased V_{max} without affecting K_m. The expression of system ATB° (glutamine transporter) mRNA decreased in the ischemic and nutrient-deprived groups, suggesting that the down-regulation of glutamine transport is due to modification of expression of the ATB° gene. The transport of glutamate and leucine, DNA synthesis, and intracellular glutathione also decreased in the ischemic group. These findings throw some light on the mechanism of intestinal epithelial damage during ischemia.

Keywords: Intestinal ischemia; Caco-2 cell; Glutamine; Amino acid transport; System ATB°

1. Introduction

Intestinal ischemic injury plays a role in the pathogenesis of systemic inflammation, respiratory failure, and multiple organ failure [1]. The potential mechanisms include the production of cytokines and oxygen-derived toxic free radicals, which are generated by activation of the xanthine oxidase system [2]. Oxygen free radicals target cell membrane constituents, causing iron-dependent lipid peroxidation, and consequent membrane disintegration and increased microvascular permeability [3]. Normally, reactive oxygen metabolites are reduced to less-toxic substances by glutathione [4].

Glutamine is essential for the synthesis of glutathione, as its metabolism by the intestinal epithelium generates substantial quantities of glutamate, a precursor for glutathione synthesis [5]. Enterocytes rely heavily on glutamine as an essential metabolic precursor in nucleotide, glucose and amino sugar, and protein synthesis [6]. Although classified as ‘‘nonessential’’, glutamine appears essential for the viability and growth of cells maintained in tissue culture.

Amino acid transport across the plasma membrane is essential for supplying enterocytes with amino acids for cellular metabolism [7]. Intestinal epithelial sodium-dependent neutral amino acid transporters are divided into several specific systems, each with its own sensitivity and specificity for various amino acids. System B°, the broad-spectrum neutral amino acid transporter, is thought to belong to the system ASC family of transporters [8]. Recently, specific cDNAs that confer system B° activity in mammalian cell expression systems have been cloned from human and rabbit tissues, and designated ATB° [9]. System ATB° has high affinity for glutamine [10].

A significant correlation has been found between glutamine transport and its disappearance rates in cultured cells, suggesting that glutamine transport is rate-limiting for glutamine utilization [11]. Therefore, demonstrating the ability of intestinal epithelial cells to regulate amino acid transport during ischemic conditions is potentially of great importance in studying the mechanism of intestinal injury in ischemia. However, the regulation of amino acid transport and the molecular mechanism of intestinal ischemia have not yet been elucidated. This study characterized glutamine transport and the molecular nature of its trans-

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porter ATB° under ischemic conditions using human intestinal cell line Caco-2. We also determined the effects of intestinal ischemia on DNA synthesis, intracellular glutathione, and membrane transport of glutamate and leucine, which are transported via the different transport systems from glutamine.

2. Materials and methods

2.1. Chemicals

Radiolabeled amino acid (^{3}H-L-glutamine, ^{3}H-L-glutamate, ^{3}H-L-leucine), and ^{3}H-L-thymidine were purchased from Amersham (Arlington Heights, IL). Dulbecco's modified Eagle medium (DMEM) was from GIBCO/BRL Life Technologies (Grand Island, NY) and fetal bovine serum (FBS) was from JRH Biosciences (Lenexa, KS). Tissue culture plates were obtained from Costar Corp. (Corning, NY). Amino acids and all biochemicals were purchased from Sigma Chemical Inc. (St. Louis, MO). A human intestinal cell line, Caco-2, was obtained from Dainippon Pharmaceutical Company (Osaka, Japan).

2.2. Cell culture

Caco-2 cells were cultured in 10 mm tissue culture dishes in DMEM supplemented with 10% heat-inactivated FBS, 1% nonessential amino acids, 1000 units/ml penicillin, and 1000 units/ml streptomycin at 37 °C under a humidified atmosphere of 5% CO_2/95% air. The culture medium was changed every 2 days until cells were confluent, at which point they were used for experiments. Cells cultured in this condition were used as the control.

2.3. Hypoxic, nutrient-deprived, and ischemic conditions

Hypoxic conditions were obtained by transferring the culture dishes to a modular incubator (personal CO_2 incubator/Multi Gas Incubator, Astec, Fukuoka, Japan) which was flushed with 1% O_2–5% CO_2–94% N_2. Hypoxia was verified by blood gas analysis (IL 1400 BGElectrolyte Analyzer, Instrumentative Laboratory, Milano) of culture media. The PO_2 of the culture media was found to be $30 \pm 4\%$ of normal levels (150 ± 6 mm Hg) at 30 min after changing to the hypoxic conditions, and these oxygen levels were maintained until the end of the experiment. Nutrient-deprived conditions were obtained by changing the culture medium to Dulbecco's phosphate-buffered saline (PBS). Ischemic conditions were obtained by combining both hypoxic and nutrient-deprived conditions [12]. The number of viable cells was determined by the Trypan blue dye exclusion test after 6 h of hypoxia, nutrient-deprivation (PBS), and ischemia (PBS + hypoxia). Cell viability was more than 95% in these conditions.

2.4. Amino acid transport

Cells were seeded into 24-well tissue culture plates (0.5 ml/well). After getting 90–100% cell confluence, cells were incubated in the hypoxic, nutrient-deprived, and ischemic conditions. L-glutamine, L-glutamate, and L-leucine transport was measured at 0, 2, 4, and 6 h. The transport of radiolabeled amino acids by cell monolayers was assayed by the cluster tray method of Gazzola et al. [13]. Before the transport assays, cells were rinsed twice with 37 °C sodium-free Krebs–Ringer phosphate buffer (Chol-KRP, which was made by replacing the corresponding sodium salts with choline chloride and choline phosphate) to remove extracellular sodium and amino acids. After removal of Chol-KRP, the transport assay was initiated by transferring 0.25 ml of the uptake medium to 24-well trays. The transport of radiolabeled amino acid (5 μCi ^{3}H-amino acid/ml) was performed for 1 min at 37 °C at 10 μmol/l unlabeled amino acid in both sodium Krebs–Ringer phosphate (Na-KRP) and Chol-KRP buffer. The assay was terminated by discarding the uptake buffer and ringing the cells with ice-cold Chol-KRP buffer three times. The wells containing the cells were allowed to dry and were solubilized in 200 μl of 0.2 N NaOH/0.2% sodium dodecyl sulfate (SDS) solution. One hundred microliters of the cell extract was neutralized with 10 μl of 2 N HCl and subjected to liquid scintillation counter. Protein content was measured by the bicinchoninic acid protein method [14]. The Na^+-dependent transport values were obtained by subtracting the transport values in Chol-KRP from those in Na-KRP. Saturable sodium-independent transport values were determined in Chol KRP by subtracting the values in the presence of excess (10 mM) unlabeled amino acid from those in its absence. Transport velocities were expressed in picomoles per milligram of protein per minute.

2.5. Expression of system ATB° mRNA

The effect of ischemia on the expression of system ATB° was analyzed by using the reverse transcription-polymerase chain reaction (RT-PCR). Cells were seeded at a density of 1×10^5 cells (2 ml/well) into six-well tissue culture plates. After getting 100% cell confluence, cells were incubated in the hypoxic, nutrient-deprived, and ischemic conditions. After 8 and 16 h, total RNA was extracted from the cells using a RNAce Total Pure Purifications kit (Bioline, London, UK). The amount of extracted RNA was calculated from optical density measurements at 260 nm. RNA (1.0 μg) was used to generate first-strand complementary DNA by using Advantage RT for PCR kit (Clontech, Palo Alto, CA) following the manufacturer's recommended procedures. Then, PCR was performed for system ATB° and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) from the same complementary DNA samples by using a Takara PCR Thermal Cycler. Oligonucleotide primers for system ATB° was: 5' CCGCTGATGATGAAGTGC 3′ (forward),

5′ CCCCCGATAGTGTTTGAG 3′ (reverse). These primers corresponded to human ATB° cDNA. The size of the RT-PCR product is 507 base pairs. Oligonucleotide primers for system GAPDH was: 5′ GAAGGTGAA GGTCGG 3′ (forward), 5′ GAAGAT GGT GAT GGG 3′ (reverse), which correspond to human GAPDH cDNA. The size of the RT-PCR product is 226 base pairs. Amplification was initiated by 2 min at 50 °C, 10 min of denaturation at 95 °C for 1 cycle followed by 28 cycled at 95 °C for 15 s and 60 °C for 60 s. After the last cycle of amplification, the samples were incubated for 10 min at 72 °C. The PCR products were then visualized by UV illumination after electrophoresis through 1.6% agarose gels containing 0.5 μg/ml ethidium bromide.

2.6. DNA synthesis

For the determination of DNA synthesis, we measured the incorporation of ^{3}H-thymidine into acid-insoluble material. When 90–100% cell confluence was reached, cells were incubated in the hypoxic, nutrient-deprived, and ischemic conditions. ^{3}H-thymidine incorporation was measured at 2, 4, and 6 h. At each time point, ^{3}H-thymidine (1 μCi/ml) was added to the culture medium and cells were incubated for 2.5 h at 37 °C. The assay was terminated after 2.5 h, when the cells were washed twice with PBS and fixed by washing three times with ice-cold 10% trichloroacetic acid. Thereafter, the cells were rinsed twice with 70% and 95% ethanol, respectively. They were allowed to dry and solubilized in 200 μl of 0.2 N NaOH/0.2% SDS solution. Radioactivity and protein content were measured by the same procedures described for the amino acid transport measurement.

2.7. Intracellular glutathione

For the determination of intracellular glutathione, equal number of cells (5×10^5 cells) was seeded in each 100 mm culture dishes. After getting cell confluence, cells were incubated in the hypoxic, nutrient-deprived, and ischemic conditions. After 6 h, cells were rinsed twice with PBS and were detached from the plate with trypsin. They were centrifuged at $500 \times g$ for 5 min and the cell pellet was suspended in 0.2 ml of PBS. The suspension was stored at − 80 °C until use. The samples were allowed to thaw and were refrozen once more at − 80 °C before being thawed in the assay (two rounds freeze/thaw). Glutathione levels of the cell lysates were measured colorimetrically using the Bioxytech GSH-420 (OXIS International Inc., Portland, OR). Protein concentrations in lysates were determined by the method described for the amino acid transport measurement.

2.8. Statistical analysis

Data (mean ± standard deviation) were analyzed by one-way analysis of variance with post hoc Turkey–Kramer or Student's *t*-test where appropriate, using a commercial software program (Statview SE+, Graphics, Abacus Concepts, Berkeley, CA). A P value <0.05 was considered statistically significant.

3. Results

3.1. Time-dependent effect of ischemia on glutamine transport

Glutamine uptake was linear for at least 3 min, and the Na^+-dependent component was shown to account for more than 90% of total glutamine uptake. Therefore, 1 min was chosen for the measurement of initial rate Na^+-dependent transport. As shown in Fig. 1A, Na^+-dependent glutamine transport in the PBS and ischemic (PBS + hy-

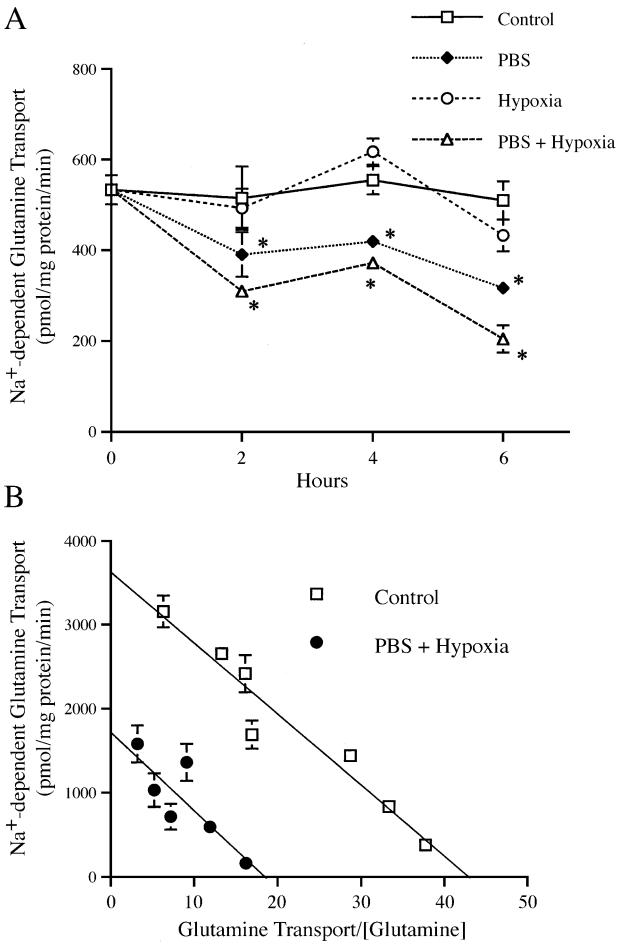

Fig. 1. (A) Time-dependent effect of nutrient-deprivation (PBS), hypoxia, and ischemia (PBS + hypoxia) on Na^+-dependent glutamine transport in a human intestinal epithelial cell line Caco-2. (B) Eadie–Hofstee plot of saturable Na^+-dependent glutamine transport in control and ischemia at 6 h. Transport velocity is plotted as a function of velocity/[glutamine]. Data are presented as mean ± S.D. of quadruplicate determinations. Where not shown, error bars are within the symbol. *$P<0.01$ vs. control.

poxia) groups decreased significantly compared with control at 2, 4, and 6 h ($P<0.01$). There was no significant difference between control and hypoxic groups. To determine the kinetics of the ischemic effect on glutamine transport, the transport of glutamine from 10 μM to 5 mM was determined in both control and ischemic (PBS + hypoxia) cells at 6 h (Fig. 1B). There was a significant decrease in maximum transport velocity in the ischemic group compared with control (V_{max}: control, 3730 ± 316; PBS + hypoxia, 1616 ± 128 pmol/mg protein/min, $P<0.01$), but no significant change was observed in transport affinity (K_m: control, 84 ± 8; PBS + hypoxia, 73 ± 5 μM).

3.2. Effect of ischemia on the expression of system ATB°

Nutrient-deprivation (PBS) and ischemia (PBS + hypoxia) decreased the expression of system ATB° mRNA significantly compared with control at 8 and 16 h, whereas hypoxia did not have any effect on the ATB° expression (Fig. 2).

3.3. Time-dependent effect of ischemia on glutamate and leucine transport

The transport of glutamate and leucine was linear for at least 3 min, and the Na^+-dependent glutamate and Na^+-independent leucine uptake represented at least 90% of the total uptake. Therefore, 1-min assays of Na^+-dependent glutamate and Na^+-independent leucine transport were chosen for subsequent experiments. As shown in Fig. 3, Na^+-dependent glutamate transport in the hypoxic and ischemic (PBS + hypoxia) groups decreased significantly compared with control at 4 and 6 h ($P<0.01$). Na^+-independent leucine transport in the PBS and ischemic (PBS + hypoxia) groups decreased significantly compared with control at 2, 4, and 6 h ($P<0.01$). Nutrient-deprivation (PBS) and hypoxia did not affect glutamate and leucine transport, respectively.

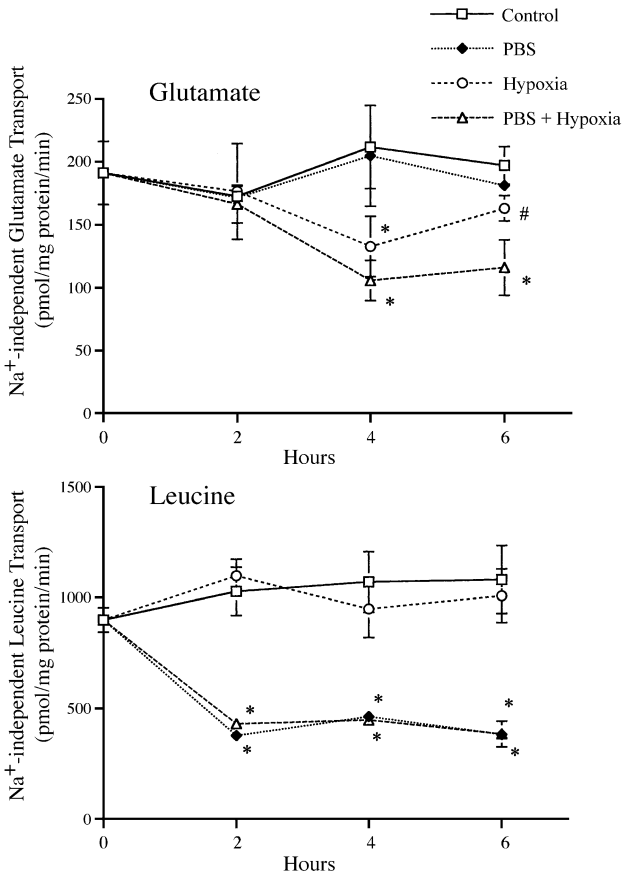

Fig. 3. Time-dependent effect of nutrient-deprivation (PBS), hypoxia, and ischemia (PBS + hypoxia) on Na^+-dependent glutamate and Na^+-independent leucine transport. Data are presented as mean ± S.D. of quadruplicate determinations. $^*P<0.01$, $^{\#}P<0.05$ vs. control.

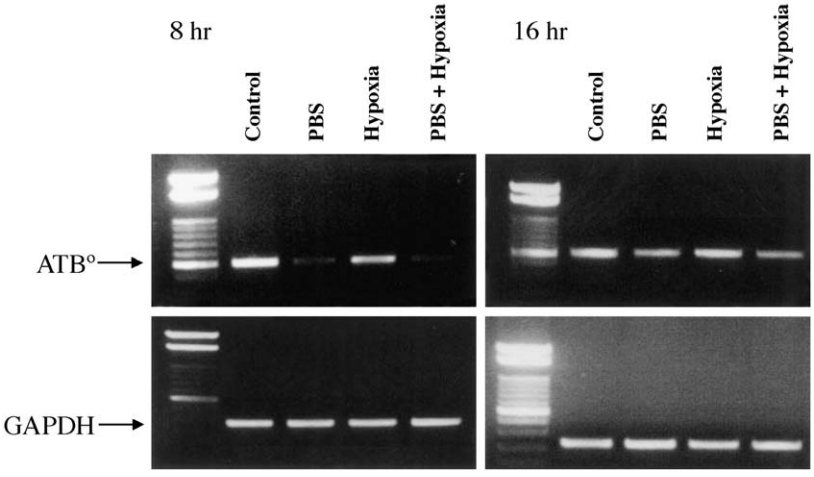

Fig. 2. Effect of nutrient-deprivation (PBS), hypoxia, and ischemia (PBS + hypoxia) on ATB° mRNA expression in a human intestinal epithelial cell line Caco-2. Cells were incubated in each condition for 8 and 16 h. RT-PCR was performed from the same complementary DNA samples with primer pairs specific for human ATB° and GAPDH. The experiment was repeated three times with comparable results.

Fig. 4 illustrates the Eadie–Hofstee plot of the kinetic data obtained from the Na^+-dependent glutamate and Na^+-independent leucine transport at 6 h. In both glutamate and leucine transport, there was a significant decrease in maximum transport velocity in the ischemic group compared with control (glutamate: control, 1107 ± 114; PBS + hypoxia, 714 ± 78 pmol/mg protein/min, $P < 0.01$) (leucine: control, 4549 ± 94; PBS + hypoxia, 2307 ± 181 pmol/mg protein/min, $P < 0.01$), but no significant change was observed in transport affinity (glutamate: control, 61 ± 9; PBS + hypoxia, 67 ± 13 μM) (leucine: control, 58 ± 11; PBS + hypoxia, 50 ± 10 μM).

3.4. Effect of ischemia on DNA synthesis and intracellular glutathione

Fig. 5 shows the effect of ischemia on ^{3}H-thymidine incorporation rates. Ischemia (PBS + hypoxia) decreased ^{3}H-thymidine incorporation significantly compared with control at 2, 4, and 6 h ($P < 0.01$). ^{3}H-thymidine incorporation in the PBS and hypoxic groups decreased significantly compared with control at 6 h and at 4 and 6 h, respectively ($P < 0.05$). At 6 h, ^{3}H-thymidine incorporation decreased significantly in the ischemic group compared with the PBS and hypoxic groups. Intracellular glutathione decreased significantly in the PBS, hypoxic, and ischemic groups compared with control ($P < 0.01$) (Fig. 6). Both nutrient-deprivation (PBS) and ischemia decreased glutathione significantly compared with hypoxia ($P < 0.01$).

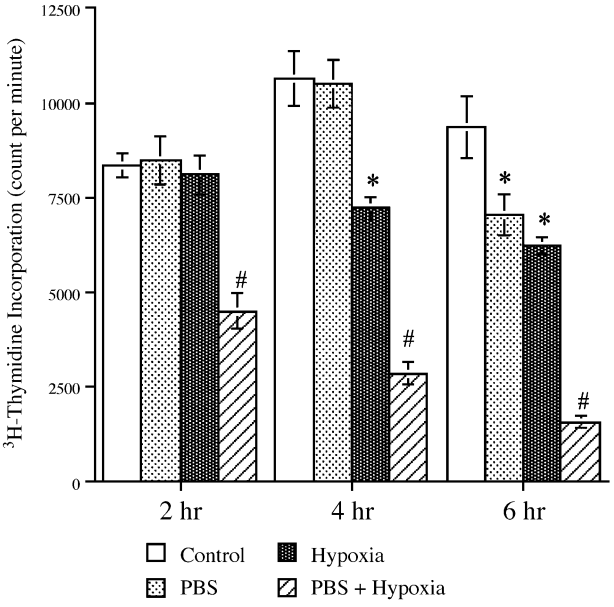

Fig. 5. Effect of nutrient-deprivation (PBS), hypoxia, and ischemia (PBS + hypoxia) on ^{3}H-thymidine incorporation at 2, 4, and 6 h. Data are presented as mean ± S.D. of quadruplicate determinations. *$P < 0.01$ vs. control, #$P < 0.01$ vs. the other three groups.

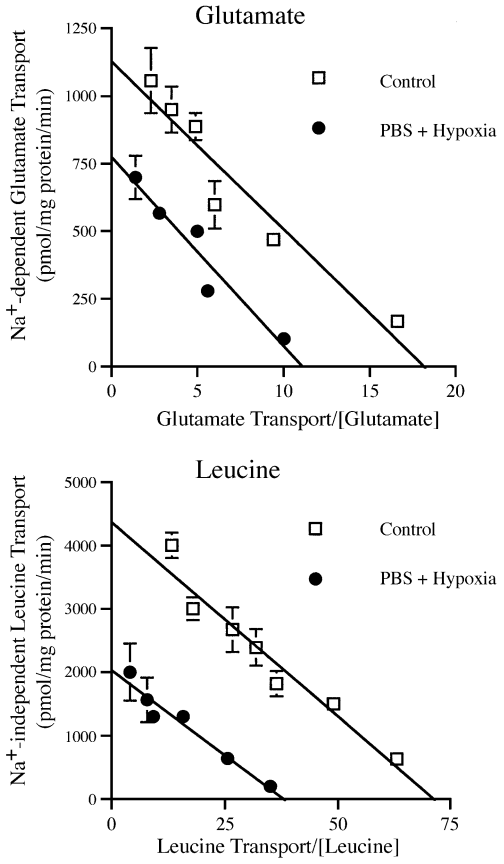

Fig. 4. Eadie–Hofstee plot of saturable Na^+-dependent glutamate and Na^+-independent leucine transport in control and ischemia (PBS + hypoxia) at 6 h. Transport velocity is plotted as a function of velocity/[glutamate] or velocity/[leucine]. Data are presented as mean ± S.D. of quadruplicate determinations. Where not shown, error bars are within the symbol.

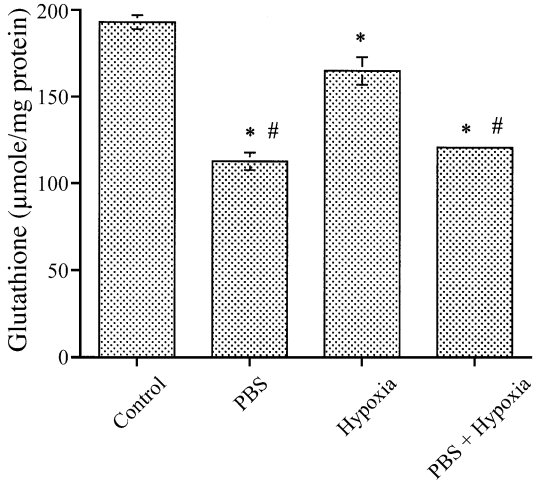

Fig. 6. Effect of nutrient-deprivation (PBS), hypoxia, and ischemia (PBS + hypoxia) on intracellular glutathione at 6 h. Data are presented as mean ± S.D. of quadruplicate determinations. *$P < 0.01$ vs. control, #$P < 0.01$ vs. hypoxia.

4. Discussion

Our study is the first to characterize the details of amino acid transport under ischemic conditions in a human intestinal cell line. Under ischemic conditions, glutamine transport decreased, as a result of a decrease in V_{max} without an affect on K_m. This indicates that ischemic enterocytes decrease the number of active glutamine transporters in the cell membrane without affecting the affinity of the transporter. In Caco-2 cells, glutamine is taken up mainly through system ATB° [10]. In our study, ATB° mRNA expression decreased in the ischemic and nutrient-deprived (PBS) groups, whereas hypoxic conditions did not induce any change in its expression. These results are comparable to those observed for glutamine transport activity. Therefore, we conclude that the down-regulation of glutamine transport induced by ischemia and nutrient-deprivation is due to modification in the expression of the ATB° gene, which may result in a decrease in DNA and protein biosynthesis of the carrier itself.

System ATB°, the broad-spectrum neutral amino acid transporter, is thought to belong to the system ASC family of transporters [15]. Regulation of intestinal ATB° mRNA expression has been reported in in vivo models, such as in intestinal inflammation [16] and massive bowel resection [17]. For example, intestinal epithelial ATB° mRNA levels do not change during chronic intestinal inflammation despite a decrease in alanine uptake rates. Iannoli et al. [18] reported that enterocytes maintain the ability to transport amino acids and glucose across the plasma membrane in a rabbit model of acute intestinal ischemia. The discrepancy between our study and Iannoli's study may be due to the difference of the ischemic models. The in vitro cell culture model that we used in this study has the advantage of testing specific functions of intestinal epithelial cells directly, without being influenced by other potential confounding variables. ATB° takes up glutamine with high affinity, and transports a wide variety of other amino acids, including serine, threonine, alanine, and asparagine, as well as branched-chain amino acids [15]. Down-regulation of ATB° expression induced by ischemia may cause a decrease in system ATB° transport activity, which results in the intracellular deprivation of these amino acids that are essential for cell growth.

The membrane transport of glutamate and leucine decreased under ischemic conditions, as observed with glutamine transport, by a mechanism that decreased V_{max} without affecting K_m. Nutrient-deprivation, but not hypoxia, decreased both leucine and glutamine transport, whereas hypoxia was the principal signal for the decrease in glutamate transport. In Caco-2 cells, Na^+-dependent glutamate uptake occurs mainly through system X^-_{AG} [19]. In our study, Na^+-independent leucine uptake was almost completely inhibited by 2-aminobicyclo-(2, 2, 1)-heptane-2-carboxylic acid, a specific substrate for the Na^+-independent system L, indicating that leucine is transported via system L. Based on these data, one possible explanation for the different transport responses to ischemic damage is that the expression of each transporter gene is regulated by a different signal, such as hypoxia or nutrient-deprivation. In our unpublished data, amino acid transport returned to the control levels when the cells were incubated in the standard culture condition after 6 h of ischemia, suggesting that the responses induced by ischemia are reversible.

^{3}H-thymidine incorporation rates decreased in both hypoxic and nutrient-deprived groups, and the combination of these conditions (ischemia) resulted in remarkable depression of DNA synthetic rates. Both DNA synthesis and Na^+-dependent amino acid transport are energy-dependent processes [20]. Ischemic conditions reduce ATP levels and aspartate transport in cell lines derived from human nervous system [12]. Therefore, in our study, the lower availability of oxygen and key nutrients such as glutamine is likely to depress cellular ATP content, resulting in the decrease of DNA synthesis and Na^+-dependent amino acid transport.

Glutamine utilization can supply two precursors of glutathione, glutamate and cysteine, which serves as a major store of cellular reducing equivalents, and thus provide protection against oxidative stress [11]. Glutamine supplementation maintains gut glutathione levels [21], and absorptive capacity and ATP [22] during intestinal ischemia–reperfusion in rats. This protective action could possibly be through preservation of intracellular amino acid essential for glutathione synthesis and energy production. These results suggest that strategies targeting the maintenance of amino acid transport activity may be of use in the therapy of intestinal ischemic injury.

In conclusion, our present study demonstrates that amino acid transport is down-regulated in ischemic human intestinal epithelial cells, possibly resulting in the decrease of DNA synthesis and intracellular glutathione. These findings help to elucidate the mechanism of intestinal epithelial damage during ischemia.

Acknowledgements

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