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Glutamine Stimulates Amino Acid Transport during Ischemia-Reperfusion in Human Intestinal Epithelial Cells[1]

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Background. **The potential mechanism of intestinal ischemia-reperfusion (I/R) injury includes oxygen-derived toxic free radicals. We tested the hypothesis that glutamine increases intracellular glutathione, a protective substrate against oxidative stress, by stimulating membrane amino acid transport during I/R using human intestinal epithelial cell line Caco-2.**

Methods. **Ischemic conditions were obtained by combining both hypoxic (1%O_2-5%CO_2-94% N_2) and nutrient-deprived (Phosphate-Buffered Saline; PBS) conditions. After 2 h of ischemia, re-oxygenation (5%CO_2-95% air) was initiated and the culture medium was changed to PBS, PBS supplemented with amino acids (A.A.), and PBS supplemented with 2 mM glutamine plus amino acids (Gln) (reperfusion). After 4 h of reperfusion, the transport of ^{3}H-glutamine, ^{3}H-glutamate, and ^{3}H-leucine was assayed and intracellular glutathione was measured. ^{3}H-thymidine incorporation was measured for the determination of DNA synthesis. Data (mean ± SD) were analyzed by ANOVA.**

Results. **Ischemia decreased Na^+-dependent glutamine, Na^+-dependent glutamate, and Na^+-independent leucine transport compared with control ($P < 0.01$). After reperfusion, glutamine and glutamate transport in the PBS and A.A. groups decreased significantly compared with control ($P < 0.01$), whereas glutamine supplementation increased glutamine transport to the levels in control ($P < 0.01$) and partially increased glutamate transport ($P < 0.01$). Leucine transport significantly increased in the A.A. and Gln groups compared with the PBS group. Glutamine significantly increased intracellular glutathione and DNA synthesis compared with the PBS and A.A. groups ($P < 0.01$).**

Conclusions. **This study demonstrated that glutamine up-regulates amino acid transport during I/R in human intestinal epithelial cells, possibly resulting in increased intracellular glutathione and DNA synthesis.**

Key Words: **intestine; ischemia-reperfusion; Caco-2 cell; glutamine; amino acid transport; glutathione.**

INTRODUCTION

Intestinal injury resulting from ischemia and reperfusion (I/R) is common in a variety of clinical conditions, such as small bowel transplantation [1], heart or aortic surgery [2], and septic shock [3]. Intestinal I/R injury is characterized by interstitial edema and disruption of the structural and functional mucosa [4], which promote bacterial translocation [5, 6]. These clinical features play a role in the pathogenesis of systemic inflammation, respiratory failure, and multiple organ failure [7]. Progressive intestinal necrosis may continue despite restoration of blood flow, and further bowel resection may be required, with the consequent development of short gut syndrome. Therefore, drugs that prevent a reduction in intestinal viability may be useful in the treatment of intestinal I/R injury.

The potential mechanisms for intestinal I/R injury include the production of oxygen-derived toxic free radicals, which are generated by activation of the xanthine oxidase system [8]. Oxygen free radicals target cell membrane constituents, causing iron-dependent lipid peroxidation, and consequent membrane disintegration and increased microvascular permeability [9]. Normally, reactive oxygen metabolites are reduced to less-toxic substances by glutathione [10]. Glutathione

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levels are of critical importance to enterocytes, affecting their ability to withstand oxidative attack.

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 [11]. Enterocytes rely heavily on glutamine as an essential metabolic precursor in nucleotide, glucose and amino sugar, and protein synthesis [12]. Although classified as "non-essential," glutamine appears essential for the viability and growth of intestinal cells. We have shown that alanylglutamine protects against morphological and functional mucosal injury after intestinal I/R in rats [13]. Glutamine maintains intestinal glutathione levels and improves survival after intestinal I/R [14]. However, the precise mechanism for glutamine to protect the intestinal damage in intestinal I/R models has not yet been elucidated.

Amino acid transport across the plasma membrane is essential for supplying enterocytes with amino acids for cellular metabolism [15]. We have shown that intestinal ischemia down-regulates amino acid transport and intracellular glutathione [16]. We put forward the hypothesis that glutamine maintains intracellular glutathione by stimulating amino acid transport during intestinal I/R. To investigate our hypothesis, we examined the effect of glutamine on membrane amino acid transport, intracellular glutathione, and DNA synthesis during intestinal I/R using human intestinal cell line Caco-2.

MATERIALS AND METHODS

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, Inc. (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).

Cell Culture

Caco-2 cells were cultured in 100-mm tissue culture dishes in DMEM supplemented with 10% heat-inactivated FBS, 1% non-essential amino acids, 1,000 units/ml penicillin, and 1,000 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.

Ischemia and Reperfusion

Ischemic conditions were obtained by combining both hypoxic and nutrient-deprived conditions [17]. 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, Italy) of culture media. The PO_2 of the culture media was found to be 30 ± 4% of normal levels (150 ± 6 mmHg) at 30 min after changing to the hypoxic conditions. Nutrient-deprived conditions were obtained by changing the culture medium to Dulbecco's Phosphate-Buffered Saline (PBS). Reperfusion was obtained by re-oxygenation (5% CO_2-95% air) and changing the culture medium to PBS, PBS supplemented with amino acids (A.A.), and PBS supplemented with 2 mM glutamine plus amino acids (Gln). MEM amino acids solution (GIBCO/BRL Life Technologies, Inc.) was used for amino acids solution, which mainly contain essential amino acids; arginine, cystine, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, threonine, tryptophan, tyrosine, and valine. Amino acid concentrations of both solutions were equal (1022 mg/L) and 2 mM glutamine was produced by replacing 29% of the total amino acid with glutamine. The number of viable cells was determined by the trypan blue dye exclusion test after 4 h of reperfusion. Cell viability was more than 95% in these conditions.

Amino Acid Transport

Cells were seeded into 24-well tissue culture plates (0.5 ml/well). After getting 90 to 100% cell confluence, cells were incubated in the ischemic conditions. After 2 h of ischemia, reperfusion was started by the method described earlier. The transport of L-glutamine, L-glutamate, and L-leucine was measured after 2 h of ischemia and 4 h of reperfusion. The transport of radiolabeled amino acids by cell monolayers was assayed by the cluster tray method of Gazzola *et al.* [18]. 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. There were 100 μl of the cell extract neutralized with 10 μl of 2 N HCl and subjected to liquid scintillation counter. Protein content was measured by the bicinchoninic acid method [19] using the BCA Protein Assay Reagent (Pierce Chemical, Rockford, IL). 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 min.

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 for 2 h of ischemia and 4 h of reperfusion. After reperfusion, cells were rinsed twice with PBS and were detached from the plate with trypsin. They were centrifuged at 500 × *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. 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 BCA method.