Oral Glutamine Attenuates Surgical Manipulation-Induced Alterations in the Intestinal Brush Border Membrane

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**Background.** Our earlier work has shown that surgical manipulation of the intestine results in oxidative stress and mucosal damage along with alterations in the brush border membrane (BBM). Glutamine feeding is known to offer protection against damage to mucosa under various conditions and this study looked at the effect of oral supplementation of glutamine or glutamic acid in the intestinal BBM alterations after surgical manipulation.

**Materials and methods.** Control and rats pretreated for 7 days with 2% glutamine or glutamic acid or isonitrogenous amino acids, glycine, or alanine were subjected to surgical manipulation of the intestine. BBMs were isolated from the intestine and functional and structural alterations to these membranes were assessed and compared.

**Results.** Surgical manipulation resulted in oxidative stress in the enterocyte BBM and these changes included a decrease in alkaline phosphatase activity and α-tocopherol content along with an increase in lipid peroxidation parameters. A decrease in glucose transport by the isolated BBM vesicles suggested functional impairment. Surgical manipulation also resulted in phospholipid degradation possibly mediated by PLA2 and membrane protease activation. Glutamine or glutamic acid supplementation prevented these changes but not by glycine or alanine.

**Conclusion.** This study suggests that oral glutamine or glutamic acid supplementation prior to surgery can offer protection to the intestine and this might prevent postsurgical complications. © 2003 Elsevier Inc. All rights reserved.

**Key Words:** glutamine; glutamic acid; brush border membrane; oxidative stress; phospholipids.

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**INTRODUCTION**

The microvillus (brush border) membrane (BBM) of the intestine plays an important role in digestion and absorption of nutrients as well as exclusion of foreign substances. It also acts as a barrier to prevent the entry of luminal bacteria and their products into the body. This barrier can be adversely affected in a number of pathological conditions, including burn trauma, hemorrhagic shock, or surgical stress [1]. Recent studies suggest that intestine plays an important role in the development of postoperative complications, such as sepsis, systemic inflammatory response syndrome, and multiple organ failure. Our earlier work has shown that surgical manipulation of the intestine can result in widening of intercellular spaces, increased intestinal permeability, and oxidative stress in enterocytes [2]. Surgical manipulation also leads to structural and functional alterations in the intestinal BBM, resulting in altered lipid composition and sugar transport [3].

Glutamine is the most abundant free amino acid in the circulation. It also has important and unique metabolic functions as a vehicle for the transfer of carbon between tissues, as a fuel for rapidly dividing cells, and as a precursor for many biologically active molecules. The gastrointestinal tract is the principal organ of glutamine use with most of the uptake occurring in the small intestinal epithelial cells that line the villi [4, 5]. The small intestine consumes large amount of glutamine and it can take up glutamine either from the lumen or from the blood stream via basolateral surface. The gut mucosal cells have high glutaminase activity.
consistent with their avid rate of uptake and metabolism [6]. Studies in patients with severe abdominal infection and in endotoxin-treated rats indicate that intestinal consumption of circulating glutamine is markedly diminished under these conditions [7].

Catabolic states, such as major surgery, sepsis, and cancer, are characterized by alterations in the interorgan exchange of amino acids, net skeletal muscle breakdown, and negative nitrogen balance [8]. In good health, glutamine is the most abundant amino acid in plasma and skeletal muscle, but circulating and tissue concentration fall precipitously after injury, surgery, or infection [9, 10]. Extensive research in animal models of illness/injury demonstrate that glutamine supplementation improves survival and immune function [11]. It has been shown to be beneficial in the prevention of infectious morbidity and mortality in seriously ill patients [12]. Specifically, glutamine administration decreased clinical infections and shortened hospital stay in a group of bone marrow transplant patients [13], decreased mortality in critically ill adults [12], and reduced risk of sepsis [14]. The objective of this study was to examine the protective effect of glutamine and other amino acids on structural and functional alterations in the small intestinal BBMs against oxidative stress-induced by surgical manipulation.

MATERIALS AND METHODS

Tris (hydroxy methyl) aminomethane (Tris), N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid] (HEPES), bovine serum albumin, p-nitrophenyl phosphate, thioarbituric acid, guanidine hydrochloride, di-nitro phenyl hydrazine (DNPH), and lipid standards were obtained from Sigma chemical Co. (St. Louis, Mo). Rabbit polyclonal antibody against cPLA2 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and affinity purified alkaline phosphatase-conjugated goat anti rabbit IgG was obtained from Banyan Biotech, Pvt. Ltd., Bangalore, India. Tris (hydroxy methyl) aminomethane (Tris), N-[2-hydroxyethyl] piperazine-N’-[2-ethanesulfonic acid] (HEPES), bovine serum albumin, p-nitrophenyl phosphate, thioarbituric acid, guanidine hydrochloride, di-nitro phenyl hydrazine (DNPH), and lipid standards were obtained from Sigma chemical Co. (St. Louis, Mo). Rabbit polyclonal antibody against cPLA2 was from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA), and affinity purified alkaline phosphatase-conjugated goat anti rabbit IgG was obtained from Banyan Biotech, Pvt. Ltd., Bangalore, India.

Animals

Adult Wistar rats of both sexes (200–250 g), exposed to a daily 12-h light–dark cycle and fed water and rat chow ad libitum, were used for this study. The rats were randomly divided in to six groups (each group consists of four animals): group 1, sham control (laparotomy without intestinal handling); group 2, surgical manipulation (laparotomy with intestinal handling); group 3, glutamine treatment (2% glutamine mixed with diet); group 4, glutamic acid treatment (laparotomy with intestinal handling); group 5, cPLA2 treatment; and group 6, saline treatment. All these treatments (groups 3–6) were given for 7 days before surgical manipulation. This study was approved by the Animal Experimentation Ethics Committee of the Institute.

Surgical Manipulation of the Small Intestine

Both control and amino acid-treated animals were subjected to surgical manipulation as described [2]. Briefly, overnight fasted rats were anaesthetized by ketamine injection. The abdominal wall was opened by a vertical incision of approximately 4 cm. The intestine was gently moved and the ileoceleal junction identified. The intestine was handled along its entire length from the ileoceleal junction proximally, simulating the “inspection” that occurs in a clinical setting. The intestine was then replaced back in the abdominal cavity and the whole process was completed within 1 to 2 min. After this, the abdominal wall was sutured and the animals were killed by decapitation 60 min after the surgical procedure. Our earlier work has shown that maximum alteration in the intestine occur 60 min after surgical manipulation [2].

Isolation of BBMs

BBMs were isolated from the small intestine of control and surgically manipulated rats by the PEG precipitation method [15]. Briefly, luminal contents were washed thoroughly with ice-cold saline and the mucosa scraped using a glass slide. Approximately 3% homogenate of the mucosa was prepared in 2 mM Tris-HCl containing 50 mM mannitol pH 7.1 using a Porter–Elvehjem homogenizer for 2–3 min at full speed. This was allowed to remain at 4°C for 15 min and filtered using nylon cloth. To this, a 50% PEG solution was added to make a final concentration to 10% PEG, stirred for 15 min, and centrifuged at 7500g for 15 min. The pellet was discarded and the supernatant was spun at 27,000g for 40 min. To the pellet, 30 mL of suspension buffer (10 mM Tris HCl and 300 mM mannitol, pH 7.1) was added and centrifuged at 27,000g for 40 min. The pellet was washed twice with the same suspension buffer and finally suspended in 1 mL of the same buffer using a syringe fitted with a 26-gauge needle. Purity of the isolated BBMs was checked by enrichment of the marker enzyme alkaline phosphatase (ALP), activity of ALP was assayed as described [16] and specific activity is expressed as units/mg protein (unit is expressed as µmole/min/mg protein). Protein was estimated using bovine serum albumin as standard [17].

Oxidative Stress Parameters

Various peroxidation parameters were measured in the isolated BBMs. Malonalddehyde (MDA) was measured using thiorbarbituric acid [18]. The amount of MDA formed was calculated from the standard curve prepared using 1,1’-3,3’ tetramethoxy propane and value expressed as nmole/mg protein. For conjugated diene measurement, total lipids from BBMs were extracted as described [19], dissolved in 1 mL of heptane, and used for lipid analysis. Neutral lipids were separated on silica gel G plates using the solvent system hexane: diethyl ether: acetic acid (80:20:1, v/v). Spots corresponding to the standard were identified by iodine exposure and eluted. Individual phospholipids were separated on silica gel H plate using the solvent system chloroform:methanol:acetic acid:water (25:14:4:2, v/v) and quantitated by phosphate estimation after acid hydrolysis [24].
Measurement of D-Glucose Uptake

Isolated BBMs were assessed for their ability to transport glucose by uptake measurements carried out by rapid filtration technique, at room temperature as described [25]. Briefly 50 μL of BBM corresponding to 100 μg of protein was incubated with 150 μM D-glucose, 0.8 μCi (14C) D-glucose, and 10 mM HEPES (pH 7.5) at varying time intervals. At the end of incubation, the mixture was diluted with 2 mL of ice-cold stop buffer (150 mM NaCl, 10 mM HEPES, pH 7.5) and immediately filtered using 0.45-μm pore size Millipore membrane under constant vacuum. The filter was washed three times with 5 mL of stop buffer and transferred to counting vials. The radioactivity retained in the filter was counted using LKB Rack-Beta scintillation counter.

Immunoblotting

Translocation of cPLA2 on to the BBMs was detected by immunoblotting [26]. Protein corresponding to 75 μg of BBMs prepared from six different group of animals were resolved on sodium dodecyl sulfate–polyacrylamide gels (7.5%) using electrophoresis over 1 h at a constant voltage of 100 V. The samples were then electrophoretically transferred to a nitrocellulose membrane (type NC, 0.45-μm pore size) using a blotting apparatus. Nonspecific binding sites were blocked overnight with wash buffer containing Tween-20 (10 mM Tris, pH 7.4; 150 mM NaCl; 5 mM sodium azide; and 20% Tween-20) containing 5% w/v fat-free dry milk powder at 4°C. The membranes were incubated for 2 h at room temperature with 1:200 diluted rabbit polyclonal antibody against cPLA2. After 10- to 15-min washes in wash buffer-Tween 20, the membranes were incubated for 2 h at room temperature with 1:1000 diluted rabbit anti-goat IgG against cPLA2 conjugated to alkaline phosphatase. The membranes underwent 10- to 15-minute washings in wash buffer-Tween 20 before detection of the alkaline phosphatase activity using substrate, bromochloroindolyl phosphate.

Gelatin Zymography

Zymogram of protease activity was performed as described [27] with slight modification. Polyacrylamide gel (12%) containing 0.1% gelatin was cast and 10 μg of BBM protein was applied on the gel in standard sodium dodecyl sulfate loading buffer containing 0.1% sodium dodecyl sulfate but lacking 2-mercaptoethanol. It was not boiled before loading. The gel was run at 100 volts for 2 h and then soaked in 2% Triton X-100 and incubated at 20°C on a shaker for 90 min with three changes. After this, the gel was soaked in reaction buffer (0.1 M Tris-HCl, pH 7.5) overnight at 37°C and then stained with coomassie blue.

Glutathione Estimation by High-Performance Liquid Chromatography (HPLC)

Intestinal homogenate proteins were precipitated with trichloroacetic acid and glutathione in the acid supernatant was quantitated using HPLC after derivatization. The derivatization was based on the initial conversion of S-carboxyl methyl derivatives of free thiols with iodoacetate followed by the conversion of free amino groups to 2,4-dinitrophenyl derivatives. Dinitrophenyl derivatives were separated by HPLC on an Ultrasil amino column (Shimadzu, Kyoto, Japan) with a gradient of methanol and sodium acetate and detected at 365 nm [28] and expressed as nmole/mg protein.

Statistical Analysis

Data are expressed as means ± SD. The Student’s t test was used for independent comparisons and Bonferroni correction was applied for multiple t test where necessary. Statistical calculations were performed using SPSS for windows (version 9.0) software.

RESULTS

Figure 1 shows the activity of BBM marker enzyme ALP, which was found to be decreased after surgical manipulation, and this was prevented by either glutamine or glutamic acid supplementation prior to surgical manipulation. This protective effect was not seen with two other isonitrogenous aminoacids, glycine and alanine, suggesting the specific effect by glutamine and...
glutamic acid. Activity of ALP and other parameters studied between glutamine fed sham control and normal control were found to be identical and hence data are shown only with normal control. Surgical manipulation resulted in an increase in oxidative stress parameters, such as MDA, conjugated diene, and protein carbonyl content and a decrease in \( \alpha \)-tocopherol; these changes were prevented by oral feeding of glutamine or glutamic acid prior to surgical manipulation (Fig. 2). This protective effect on oxidative stress was not seen either with glycine or alanine feeding. Functional integrity of isolated BBMs was compared between sham control and after surgical manipulation with or without different amino acid feeding by measuring the glucose transport ability of membrane vesicles. There was a decrease in glucose transport by membranes isolated

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**FIG. 2.** Oxidative stress parameters of BBM in sham control, after surgical manipulation, and various amino acid treatment prior to surgical manipulation. A, MDA; B, conjugated diene; C, protein carbonyl content; D, \( \alpha \)-tocopherol. (Each value represents mean ± SD of four separate estimations, \( n = 4 \). *\( P < 0.05 \) when compared with sham control; \( \#P < 0.05 \) when compared with surgical manipulation.)
after surgical manipulation as compared with controls and this was again prevented by feeding the animal either with glutamine or glutamic acid but not with glycine or alanine prior to surgical manipulation (Fig. 3A). Peak glucose uptake occurred at 20 s and this uptake after various treatments is shown in Fig. 3B. Oxidative stress-induced alteration in the BBM after surgical manipulation may be associated with membrane structural alterations and because lipids are important constituents of membranes, lipid composition was analyzed in different group of animals. Figure 4 shows the level of certain phospholipids in the BBM after surgical manipulation. A decrease in phosphatidylcholine (PC) and phosphatidylethanolamine (PE) along with an increase in lyso PC and lyso PE were seen after surgical manipulation (Fig. 4). These lipid alterations were prevented by prior feeding of animals with either glutamine or glutamic acid but not glycine or alanine. There was no significant change in neutral or other phospholipids in the BBM after surgical manipulation (data not shown). We have earlier shown that surgical manipulation results in the appearance of cytosolic PLA2 (cPLA2) in the BBM as evidenced by immunoblot and as shown in Fig. 5, cPLA2 was not present in BBM isolated from control animals or those treated with glutamine or glutamic acid prior to surgi-

FIG. 3. D-glucose transport by intestinal BBM in sham control, after surgical manipulation, and various amino acid treatment prior to surgical manipulation. A, 14C D-Glucose uptake after various time period. B, 14C D-Glucose uptake at 20 s. (Each value represents mean ± SD of four separate estimations, n = 4. *P < 0.05 when compared with control; #P < 0.05 when compared with surgical manipulation.)
Cal manipulation whereas cPLA₂ was present in membranes isolated after surgical manipulation or glycine or alanine feeding, prior to surgical manipulation. Oxidative stress is known to activate proteases and BBM-associated proteases were studied by gelatin zymography after various treatments. As can be seen in Figure 6, control membranes showed predominantly three proteases of mol.wt 105, 88, and 22 kDa and these proteases were activated by surgical manipulation. This protease activation was prevented by glutamine or glutamic acid feeding prior to surgical manipulation but not by glycine or alanine. It was observed that glutamine or glutamic acid feeding alone increased the GSH level in the intestine by three as compared to control fold (8.86 ± 0.226 vs. 2.86 ± 0.22 nmoles/mg protein). Surgical manipulation resulted in a decrease in the GSH level (0.847 ± 0.08 nmoles/mg protein) and prior treatment with glutamine or glutamic acid maintained the GSH level similar to glutamine or glutamic acid control.

**DISCUSSION**

Intestinal mucosal alterations occur after surgical manipulation and oxidative stress has been implicated in the pathophysiology of postoperative complications.
We have earlier shown that after surgical manipulation of the intestine, activation of xanthine oxidase (XO) [2] and mitochondrial dysfunction [29] occur, which results in generation of oxygen free radicals, and this in turn leads to structural and functional alterations in BBM, including degradation of phospholipids and generation of arachidonic acid [3]. It was also shown that these mucosal changes could be prevented by pretreating the animals with XO inhibitor, allopurinol, suggesting a role for XO-derived superoxide in the mucosal damage after surgical manipulation. It was also shown that heat preconditioning offers protection from damage to the intestinal BBM after surgical manipulation [30].

In the present study, glutamine supplementation to the animal prior to surgical manipulation prevented BBM alterations, which was shown by various parameters of peroxidation as well as structural and functional alterations. Similar protection was seen with glutamic acid supplementation but not with isonitrogenous compounds alanine or glycine. Glucose uptake was decreased after surgical manipulation, which was prevented by glutamine or glutamic acid supplementation but not by alanine or glycine. We have earlier shown that in vitro exposure of BBM to oxidative stress decreased glucose and amino acid transport [31] and lipid peroxidation of BBM decreases sodium-dependent glucose transport [32]. Structural alterations seen in the BBM after surgical manipulation include changes in BBM phospholipids, especially degradation of PC and PE and generation of lysophospholipids, which suggested a possible role for PLA2 in BBM lipid alterations. These phospholipid alterations were prevented by prior feeding with glutamine or glutamic acid but not with glycine or alanine. It is known that oxygen free radicals can activate PLA2 [33], and increased lyso PC and lyso PE were evident after lipid peroxidation of PLA2 containing liposomes and microsomes [34]. We have earlier shown that oxygen free radicals generated during surgical manipulation result in the translocation of cytosolic PLA2 on to the BBM, which is responsible for phospholipid degradation [30]. Immunoblot analysis showed that cPLA2 was absent in the control BBMs but appeared in the BBMs isolated after surgi-
cal manipulation. Moreover, glutamine or glutamic acid feeding prior to surgery prevented the appearance of cPLA₂ on to the BBM. It is known that cPLA₂, preferably hydrolyses phospholipids containing arachidonic acid and responds to physiological increments of Ca²⁺ with translocation on to the membrane [35]. It has been shown that effects of membrane-bound proteases are of great importance in some biological regulations and oxidative stress can activate proteases. We have shown earlier that surgical stress results in activation of proteases in the small intestinal epithelial cells, both in mitochondria and in cytosol [36]. BBM proteases were also activated by surgical manipulation, which was prevented by glutamine or glutamic acid feeding. Proteases have been implicated in the pathophysiology of various disease states, and potent inhibitors of these enzymes have the potential to be developed as new therapeutic agents.

Glutamine plays an important role in the metabolism of rapidly proliferating cells such as enterocytes and this amino acid is a preferred fuel source to these cells. In addition it can also act as a precursor of the antioxidant, glutathione. Studies have shown that glutamine-enriched nutrition can reduce the mucosal damage after radiation injury, or chemotherapy [37, 38]. In the present study, it was observed that both glutamine and glutamic acid provided similar protection against surgical manipulation-induced changes in the BBM, suggesting that glutamine protection is mediated possibly through conversion to glutamic acid. It is known that enterocytes are rich in glutaminase, which can convert glutamine to glutamic acid [6]. The fact that surgical manipulation-induced generation of free radicals are responsible for mucosal alterations and both glutamine or glutamic acid can provide protection suggests that increasing the antioxidant glutathione level may be an important factor offering protection. Because isonitrogenous compounds, glycine, or alanine were not able to offer protection suggest that these effects are specific to glutamic acid. Recently, it has shown that Caco-2 cells supplemented with glutamine can prevent cytokine-induced bacterial translocation and this was caused by glutamine acting as an energy supplement rather than through other mechanisms [39]. Glutamine has also been shown to induce heat shock proteins [40], and this has been suggested as a mechanism by which protection is offered under stress condition. Our attempt to identify the induction of HSP-70 or HSP-30 by immunoblot after feeding these amino acids was not successful (data not shown). Currently, we are pursuing to identify the exact mechanism by which glutamine and glutamic acid offer protection against surgical manipulation-induced alterations in the intestine.

In conclusion, this study has shown that during surgical manipulation of the intestine structural and functional alterations occur in the BBMs, including lipid peroxidation, protease activation, altered transport, and lipid composition. These biochemical events in the intestinal mucosal cell can be prevented by oral feeding of either glutamine or glutamic acid and suggest that supplementation of these amino acids might offer protection from post surgical complications.

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