Bidirectional supply of glutamine maintains enterocyte ATP content in the in vitro Ussing chamber model

Abstract Glutamine is the principal energy source for enterocytes, but it is not known whether parenteral or enteral supplementation is most beneficial to gut integrity. The aim of this study was to evaluate the effects of glutamine in uni- or bidirectional supply on the viability of intestinal mucosa of starved rats during incubation in Ussing chambers. Segments of jejunum from rats starved for 48 h were randomly mounted in Ussing chambers with three nutrient solutions: Krebs buffer without glutamine; 6 mM glutamine added to the mucosal side; 6 mM glutamine added to the mucosal side and 0.6 mM glutamine to the serosal side. A TP content of the mucosa, electrophysiology, and 51Cr-ethylenediaminetetraacetate (EDTA) permeability were studied during 180 min of incubation. The addition of glutamine to both sides of the stripped mucosa improved ATP levels compared to the Krebs solution (P<0.05), and the addition of glutamine resulted in an increase in short circuit current (P<0.05). No significant differences were seen in 51Cr-EDTA permeability or epithelial electrical resistance. Glutamine supplementation to both the luminal and serosal side in the Ussing chamber was more effective than luminal glutamine only in maintaining ATP levels of intestinal mucosa. Bidirectional supplementation of glutamine might improve intestinal energy metabolism and viability in in vitro studies.

Keywords Ussing chamber · Glutamine · Energy metabolism · Intestinal mucosa · Epithelial resistance · Permeability

Introduction

One important feature of small-bowel function is to maintain the barrier function while absorbing nutrients. The function of tight junctions is vital to the barrier and can be measured by studying the permeation of markers that pass paracellularly. In vitro studies are valuable tools for studying this phenomenon in specific parts of the intestine under standardized conditions. In the Ussing chamber, transepithelial potential difference (PDt), short circuit current (Isc), and transepithelial electrical resistance (TER) can be monitored over the membrane for optimal surveillance of the mucosa in different disease states [1, 2, 3]. Stripped mucosal segments can be kept viable in Krebs solution containing glutamate in terms of permeability and electrical properties, but the effects of glutamine, the preferred energy source, have not been fully examined.

Glutamine is considered essential for the maintenance of gut metabolism, structure, and function in stress conditions [4]. It plays an important role as a respiratory substrate for cells in the mucosa of the small intestine [5,6]. Metabolism of this amino acid to α-ketoglutarate with subsequent complete oxidation in the Kreb’s cycle yields 30 mol ATP per mol glutamine [7]. Enterocytes are strongly dependent on an external glutamine supply because of the small size of the cellular glutamine pool in the small intestine [5]. In addition, the glutamine syn-
the activity in the intestinal mucosa is extremely low [8]. The mucosa is dependent on both luminal and plasma glutamine and extracts 20–30% of circulating glutamine [9]. Glutamine is also an important nucleotide precursor for the gastrointestinal tract [10, 11]. Using in vivopolyethylene glycol (PEG) recovery as a measurement of epithelial absorptive and discriminatory function, we found that glutamine supplementation resulted in an increased absorptive area of the small bowel [12]. Using the Ussing chamber, we have found that starvation opens up the paracellular pathway for $^{41}$Cr-ethylenediaminetetraacetate (EDTA). We have previously shown that starvation and surgery increase paracellular permeability and that starvation is the most influential factor in this experimental model [13]. We have also examined the effects of adding glutamine at different concentrations, i.e., 0.6 mM, 3 mM, 6 mM, and 30 mM in Krebs solution without any nutrients, i.e., glutamate, glucose, fumarate, and pyruvate, to the mucosal side of the jejunum of starved rats and found that this unidirectional supply of glutamine increased ion pump activity but did not restore the intracellular ATP level [14]. Whether an optimal glutamine plasma concentration or enteral administration of glutamine is needed to restore gut integrity in a metabolically stressed state is not known.

The purpose of this study was to determine the effects of glutamine supplemented to one or both sides of the mucosa on the viability, energy metabolism, and epithelial permeability of stripped jejunal mucosa from starved rats in the Ussing chamber.

Material and methods

Animals and specimens

Male Wistar rats (B&K Universal AB, Stockholm, Sweden), weighing 240–260 g, were used. The rats were housed two by two in a room maintained at 23°C in a 12-h dark/light cycle throughout all the experiments and were fed a normal diet with no restriction on food or water supply for 2 weeks.

Prior to the Ussing chamber experiments, the animals had no access to food but free access to water for 48 h. Rats fed ordinary chow who were not starved served as controls ($n=6$). Anesthesia with ketamine (Ketalar, 80 mg/kg) and xylazine (Rompun, 8 mg/kg) was administered intraperitoneally. The abdomen was opened by a full-length midline incision, and a 20-cm segment of the proximal jejunum was stripped from the mesentery and immediately put in ice-cold, oxygenated Krebs buffer. While immersed in cold Krebs buffer, the outer muscle layers were removed under a dissection microscope, and the tissue sections were cut into specimens of an appropriate size and mounted in modified Ussing chambers [15] (Precision Instrument Design, Los Altos, CA, USA).

Ussing chamber experiments

After mounting, each half cell was filled with 5 ml preheated Krebs buffer at 37°C, bathing the stripped mucosa specimen on both the mucosal and serosal side. The exposed tissue surface area was 1.78 cm$^2$. The Krebs buffer was continuously oxygenated with $O_2/CO_2$ (95/5%) and stirred by gas flow in the chambers. After a 40-min equilibration period to achieve steady-state conditions regarding PDt, the Krebs buffer in the serosal compartment was replaced and marker solution containing $^{51}$Cr-EDTA with or without glutamine was added to the mucosal compartments; 0.6 mM glutamine corresponds to normal plasma levels of glutamine. The three groups ($n=6$ in each group) were treated as follows: KB group, Krebs buffer solution; 6 KB group, 6 mM glutamine on the mucosal side in the Krebs buffer solution; 0.6+6 KB group, 6 mM glutamine on the mucosal side and 0.6 mM glutamine on the serosal side in Krebs buffer solution.

Samples of 1 ml were taken every 20 min from the serosal compartment for subsequent analysis of $^{51}$Cr-EDTA and were replaced by fresh Krebs buffer. Incubation was performed for 180 min after equilibration, and specimens were then removed for analysis of epithelial ATP.

Electrical measurements

One pair of Ag/AgCl electrodes (Radiometer, Copenhagen, Denmark) with 3 M KCl/2% agar bridges was used for measurements of the transepithelial potential difference, and another pair of Pt electrodes was used for current passage. The electrodes were coupled to an external six-channel electronic unit with a voltage-controlled current source. Data sampling was computer controlled via an A/D–D/A board (Lab NB, National Instruments) by a program developed in Lab View (National Instruments). A linear least square fit was performed on the current ($I$) to voltage ($U$) pair relationship:

$$U = PD + TER \times I$$

The transepithelial resistance (TER) is obtained from the slope of the line, potential difference (PDt) from the intersection of the voltage axis (when $I=0$), and the short circuit current (Isc) determined from the quotient PD/TER. Three representative time points, i.e., at equilibration point (0 min), 15 min (the effect of adding glutamine to the system), and 180 min after equilibration (the long-term effect on cellular metabolism and viability) during incubation in Ussing chambers were chosen for analysis.

Chemicals and measurement

Krebs buffer was prepared for each day of experiment and contained the following: NaCl 110.0 mM, CaCl$_2$ 3.0 mM, KCl 5.5 mM, KH$_2$PO$_4$ 1.4 mM, NaHCO$_3$ 29.0 mM, Na pyruvate 5.7 mM, Na fumarate 7.0 mM, Na glutamate 5.7 mM, and glucose 13.4 mM. It was adjusted to a pH of 7.3 and equilibrated with $O_2/CO_2$ (95/5%) before use. Glutamine was prepared and dissolved in Krebs buffer before the Ussing chamber study. All the glutamine solutions were adjusted to a pH of 7.3, and the addition of glutamine did not significantly affect osmolarity.

$^{51}$Cr-EDTA (Du Pont, Dreieich, Germany), specific activity 126 Ci/mmol, was added to the mucosal compartment at the start of the experiments to a concentration of 44.3 µg/l (0.13 µM). Permeability of $^{51}$Cr-EDTA was assessed by measuring the appearance of the marker on the serosal side during the experiments. The radioactivity in 1-ml samples was counted for 600 s in a γ-counter (1282 Compugamma, LKB, Bromma, Sweden).

Calculations

The apparent permeability coefficient ($P_{app}$) was calculated according to the following equation:

$$P_{app} (cm/s) = \frac{(dC/dt) \times V/(I/Co \times A)}$$
where \( dC/dt \) is the change in concentration on the serosal side per unit time (mol/l per s), \( V \) is the volume of the chamber (cm\(^3\)), \( A \) is the area of exposed intestine (cm\(^2\)), and \( C_0 \) is the initial marker concentration in the mucosal reservoir (mol/l) [16]. \( P_{\text{app}} \) was calculated for the 20–120 min period.

**ATP in jejunal mucosa**

Samples of jejunal mucosa were taken at operation as control value (n=6) and after 180 min of incubation in the Ussing chamber. The samples were frozen in liquid nitrogen, stored at −70°C, and freeze dried. At analysis, mucosa powder was disected free of blood and connective tissue under a microscope and ground to a homogenous powder. ATP was extracted from no less than 2.5 mg freeze-dried mucosa powder using 1 M perchloric acid containing 1 mM EDTA. The extracts were neutralized with 2.2 M KHCO\(_3\) and stored at −70°C until analysis. Analyses were performed with enzymatic fluorometry using methods modified from Harris et al. [17]. The contents of ATP were expressed as micromoles per gram dry mucosa powder.

**Statistics**

Data are presented as mean±standard error of mean (SEM). Comparisons between groups were evaluated using the Kruskal Wallis test and in-group comparisons using the Wilcoxon’s paired signed rank test. Differences were considered significant at \( P<0.05 \).

**Ethics**

The study was approved by the Animal Ethics Committee of the Faculty of Health Science, Linköping University, Sweden.

**Results**

**ATP contents of the stripped jejunal mucosa**

When no glutamine was added (KB group), ATP decreased by 40\% compared to sampling at the start of experiment (3.4±0.5 vs. 5.7±0.5 µmol/g d.w., \( P<0.05 \)). The mucosal ATP level when glutamine was added only on the mucosal side was also significantly lowered (4.0±0.5 µmol/g d.w., \( P<0.05 \)). On addition of glutamine to both the mucosal and the serosal side, the ATP content (5.1±0.6 µmol/g d.w.) was maintained compared to the start of the experiment (Fig. 1).

**Permeability of stripped jejunal mucosa**

The cumulated jejunal permeability of \(^{51}\)Cr-EDTA of starved rats did not differ significantly at 120 min in the three groups (Fig. 1), and \( P_{\text{app}} \) was 5.9±0.7 cm/s in the 0.6+6 KB group, 5.5±0.7 cm/s in the KB group, and 4.9±0.6 cm/s\(^{-1}\) in the 6 KB group (not significant). The \( P_{\text{app}} \) of intestinal mucosa found in previous experiments in rats feeding normally is 3.3±0.3 cm/s [10] (Fig. 2).

**Electrophysiologic parameters**

The short circuit current (Isc) in stripped jejunal mucosa at equilibration was 131±12 µA/cm\(^2\) in the KB group vs. 138±22 µA/cm\(^2\) in the 6 KB group and 127±22 µA/cm\(^2\) in the 0.6+6 KB group (not significant) (Figs. 3, 4). Relative values during the experiment are presented in Fig. 3. The addition of glutamine significantly increased Isc after 15 min in the Ussing chamber(\( P<0.05 \)), and bidirectional glutamine increased Isc more rapidly. Isc was also higher at 180 min in glutamine-containing KB (\( P<0.05 \)).

The TER in stripped jejunal mucosa at equilibration was 26.2±3.2 Ωcm\(^2\) in the KB group vs. 28.5±2.3 Ωcm\(^2\).
in the 6 KB group and 27.6±2.8 Ω cm² in the 0.6+6 KB group (not significant). There was a significant drop in TER after 15 min in the 0.6+6 KB group and in the KB group (Fig. 4). There were no significant differences in TER between the three groups at the end of the experiment.

Discussion

Starvation and surgical stress increase the vulnerability of the intestinal mucosa and might interfere with barrier integrity. Specific nutrients to support mucosal energy restoration and to stimulate proliferation are of clinical interest. However, the optimal route of supply for glutamine is still under debate. To elucidate the specific role of glutamine from the luminal side or both the luminal and serosal side, we used the Ussing chamber model to study the effects on the energy metabolism and permeability of stripped intestinal mucosa. We found that the addition of glutamine supports ATP levels and ion pump activity to a higher degree when glutamine is provided from both sides of the mucosal lining. We suggest that the supply of a physiologic concentration of glutamine to the serosal side is important for maintenance of mucosal viability.

Our hypothesis was that glutamine is beneficial to enterocyte energy metabolism in the stressed situation in the Ussing chamber. We have previously reported beneficial effects of glutamine on proliferation, but these effects are only demonstrated when there is depletion or an increased demand of glutamine compared to the fed state. For this reason, starved animals were studied. Glutamine has been suggested to be particularly efficient when glycolysis is depressed [18]. It has recently been shown that glutamine supplement can increase transmucosal resistance and decrease mannitol flux through the epithelium in a severe acute colitis model in an Ussing chamber [19]. Keurkhubascke et al. [20] found that intestinal membrane perfused with a solution consisting of Dulbecco’s modified Eagle’s medium with 20 mM glutamine maintained the TER for 3 h in an Ussing chamber. In our study, we found that the permeability perturbation resulting from starvation was not attenuated by adding glutamine to the Ussing chamber. This might be due to structural changes rather than to an energy deficit in the epithelial lining. TER is thought to reflect tissue integrity. A low TER value suggests increased permeability to ionic movement or lower tissue integrity. When glutamine was added to both sides, there was a rapid drop in TER, which might be caused by an increase in ionic flux due to activation of Na⁺-coupled absorption of glutamine, since lsc also increased more rapidly in this group at this time. In this short experiment, tight junction permeation might have been initially affected by glutamine, but there were no significant differences in TER or $P_{app}$ at the end of the experiment among the three groups.

Adenine nucleotide levels indicate viability of intestine [21], and ATP levels in enterocytes appears to be important for the maintenance of intestinal barrier function [22]. The importance of ATP for maintaining normal permeability characteristics in epithelial monolayers [23, 24, 25] has been extensively documented. Profound ATP depletion led to loss of both the “gate” and “fence” functions of tight junctions. In both endothelial cells and kidney epithelia, ATP depletion results in a redistribution of cortical membrane-associated filamentous actin [26, 27, 28, 29]. In the study presented here, glutamine on both the luminal and serosal side demonstrated the ability to preserve the energy charge, and ATP content in stripped

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**Fig. 3** Changes in short-circuit current (Isc) in jejunal mucosa of starved rats in a Ussing chamber. White bars, 15 min after equilibration; black bars, 180 min after equilibration. Data are expressed as mean±SEM percentage of the initial value at equilibration time point. *P<0.05 15 min vs. 180 min within group; $P<0.05$ vs. KB group at 15 min

**Fig. 4** Changes in transepithelial resistance (TER) in jejunal mucosa of starved rats in vitro. White bars, 15 min after equilibration; black bars, 180 min after equilibration. Data are expressed as mean±SEM percentage of the initial value at equilibration time point. *P<0.05 15 min vs. 180 min within group; $P<0.05$ vs. KB group at 15 min
mucosa only decreased by 10% of the initial value when glutamine was added to both sides. In contrast, the glutamate and glucose content of the Krebs buffer was unable to maintain adenine nucleotide levels, and ATP content decreased by 40% of the initial value when glutamine was withheld.

The ability to maintain an Isc is a characteristic shared by all transporting epithelia and is dependent on the electrogenic ion pumps in the epithelial function [30]. Thus Isc is equivalent to the sum of all active ion transport processes which require energy production, generally in the form of ATP. Tissue viability in small-intestine tissue can also be evaluated from the basal Isc or from the changes in Isc when resistance is stable [31]. A low Isc suggests that the stripped mucosa has a low metabolic rate or ionic flux. In our study, Isc was higher in all groups when the Krebs buffer was supplemented with glutamine, especially bidirectionally. Adding glutamine might therefore be a way to increase cellular viability and metabolism.

In summary, this study suggests that supply of glutamine from both the luminal and serosal side seems to be important for the maintenance of energy metabolism and mucosal viability in rat intestinal mucosa. The two methods of administration might result in different processing of the supplemented amino acid with regard to transport mechanisms versus oxidation. The possibility of there being a differentiated metabolic cellular response to parenteral and enteral glutamine needs further attention. The addition of glutamine to the mucosal side and a physiologic plasma concentration of glutamine to the serosal side might also be a valuable model to improve intestinal energy metabolism in future in vitro studies.

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