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Enteral Nutrients Alter Enterocyte Function Within an *In Vitro* Model Similar to an Acute *In Vivo* Rat Model During Hypoxia

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ABSTRACT. *Background:* Early enteral nutrition in patients following traumatic injury is an important intervention. However, after shock-resuscitation, intestinal hypoperfusion persists despite adequate systemic resuscitation. Our previous *in vivo* rat studies indicate that hypoperfusion impairs mucosal function in the small intestine. Therefore, the current study sought to improve previous *in vitro* models by the following means: (1) We used Caco-2 monolayers stably transfected with the brush-border sodium-glucose co-transporter (SGLT-1); and (2) we created an environment that mimicked the physiologic enterocyte environment. We hypothesized that hypoxic alterations of epithelial function in an *in vitro* model are comparable to those of an *in vivo* rat model. *Methods:* After 21 days, monolayers were randomized to receive 24 hours of incubation in a normoxic or hypoxic environment. Cells were further randomized to receive 1 of 4 nutrient treatments: mannitol (an osmotic control), glucose (uses SGLT-1 and is metabolized), 3-O-methylglucose (3-O-mg; uses SGLT-1 and is not metabolized), or fructose (does not use SGLT-1 but can be metabolized). *Results:* Transepithelial resistance ($p = .007$) and short-circuit current ($p = .05$) were lower in hypoxic groups. When compared with normoxic groups, hypoxic groups had significantly impaired glucose ($p < .001$) but not glutamine transport, irrespective of nutrient treatment. Additionally, adenosine triphosphate/adenosine diphosphate ratio was reduced ($p = .01$) and lactate concentration was increased ($p < .001$) during hypoxia. *Conclusions:* In summary, results from this *in vitro* study using Caco-2BBe cells stably transfected with SGLT-1 correspond to results obtained in the *in vivo* rat model. Therefore, this is an appropriate *in vitro* model in which to study cellular alterations caused by the hypoxic small intestine, with the goal of ensuring safe early enteral nutrition following traumatic injury. (*Journal of Parenteral and Enteral Nutrition* **26:**71–76, 2002)

Traumatic injury is the fifth leading cause of death in the United States, killing approximately 100,000 people each year. There is an emerging consensus that early enteral nutrition benefits the high-risk trauma patient.[1] Over the past 2 decades, the timing of nutrition intervention and the route of nutrient administration have been debated in many clinical studies.[2–5] After traumatic injury, the metabolic stress response peaks at 3 to 4 days and subsides in 7 to 10 days.[2] This hypermetabolism typically increases energy and protein requirements, sometimes resulting in protein malnutrition. Skeletal muscle contributes to the protein needs of the body; however, once stores are depleted, visceral stores and circulating protein are used.[2] As a result of this protein malnutrition, the cardiac, pulmonary, hepatic, gastrointestinal (GI), and immunologic systems are disturbed. Based on this knowledge, intensive care units have made the provision of early enteral nutrition a priority. Compared with total parenteral nutrition (TPN), total enteral nutrition (TEN) decreases the risk of disease, is safer, and is more economical and more convenient. In addition, TEN prevents mucosal atrophy, attenuates the stress response, maintains immunity, and preserves the GI microbiota better than TPN.[6–8] Furthermore, complications related to sepsis are reduced with TEN compared with TPN.[2,4,6] This evidence has led to the commonly used phrase, "If the gut works, use it!" and the advent of early enteral nutrition for trauma patients.

The danger of early enteral nutrition in the critically ill patient is related to hypoperfusion of the intestine. In healthy individuals, the presence of food in the intestine increases local blood flow. After hypovolemic shock, however, there is persistent GI hypoperfusion, despite adequate systemic resuscitation. Therefore, the presence of food in the intestine may increase oxygen demand beyond available delivery of blood flow, potentially leading to intestinal ischemia.[9–11] Although very few prospective, controlled trials are described in the literature, there are many case reports of nonocclusive small bowel necrosis, suggesting that early enteral nutrition may be harmful in patients.[2,12–18] In the vast majority of reported cases, small bowel necrosis resulted in patient death, with a survival rate of only 23%. The causative mechanism remains unknown, but the consistent association with early enteral nutrition indicates that the inappropriate

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administration of specific nutrients into a poorly perfused small bowel plays a pathogenic role. Further investigation is necessary to elucidate the physiologic response after the provision of early enteral nutrition into the poorly perfused small intestine during hypoxia. Our previous *in vivo* rat studies indicate that hypoperfusion: (1) impairs SGLT-1 but not glutamine transport; (2) decreases transmural resistance; (3) increases lactate concentration; and (4) increases adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio during glucose perfusion.[19] The objective of the current study was to establish a physiologically relevant cell culture model that mimics the previously reported *in vivo* model's responses during hypoxia. The following criteria were set: (1) decreased brush-border sodium/glucose co-transporter (SGLT-1) activity; (2) impaired epithelial barrier function; (3) increased lactate concentration; and (4) reduced ATP concentration.

CLINICAL RELEVANCY

Early enteral nutrition following traumatic injury is an important intervention in patients who have undergone shock-resuscitation. However, following shock-resuscitation, intestinal hypoperfusion persists, despite adequate systemic resuscitation. The dangers of early enteral nutrition may be related to poor intestinal perfusion. Hypoperfusion may increase oxidative demand beyond what is available, resulting in altered nutrient transport. Additional information is necessary to elucidate the appropriate nutrients for enteral formulas in traumatically injured patients. Therefore, an *in vitro* model is critical for investigating the cellular and molecular mechanisms involved. Information obtained by such studies is necessary to ensure that early enteral nutrition following traumatic injury is appropriate.

METHODS

Experimental Model

Caco-2BBe monolayers, stably transfected with SGLT-1[20] were grown and maintained on polyester, collagen-coated, 6-mm diameter, .4-micron pore size, 1.13 cm^2 Transwell Snapwell inserts (Corning Costar, Costar, NY). The Transwell Snapwell inserts allow isolation of the brush-border and serosal medium (Fig. 1). Cells were maintained in an incubator (Jouan, Model IG650, Winchester, VA) at 37°C with 5% CO_2 and 95% atmosphere for 21 days, with renewal of medium (DMEM medium, 10% fetal bovine serum, 15 mmol/L HEPES, and 0.25 mg/mL Geneticin) every 3 to 4 days (Gibco Scientific, Grand Island, NY). Twenty-ones days postsubculture, cells were randomized to receive 1 of 4 brush-border nutrient treatments: (1) mannitol (an osmotic control); (2) glucose (undergoes active transport across the brush-border membrane via SGLT-1 and is metabolized); (3) 3-o-methylglucose (3-O-mg; uses SGLT-1 but is not metabolized); or (4) fructose (does not use SGLT-1 but can be metabolized). All of the sterile, filtered (Corning Costar, 0.4-micron pore size) nutrient treatments were provided at a concentration of 120 mmol/L in a modified Kreb's solution (140 mmol/L Na, 119.8 mmol/L Cl, 25 mmol/L HCO_3, 12 mmol/L Mg, 1.2 mmol/L Ca, 4.8 mmol/L K, 2.4 mmol/L HPO_4, and 0.4 mmol/L H_2PO_4, pH 7.4) to the brush-border for 24 hours. After addition of the brush-border nutrient treatment, monolayers were further randomized to receive incubation in a normoxic or hypoxic atmospheric culturing (n = 5 per nutrient treatment). The hypoxic environment was created using a modular incubator chamber (Billups-Rothenberg, Del Mar, CA) with infusion of 5% CO_2 and 95% N_2 at 10 L/min for 2 minutes. Cells were maintained in a hypoxic environment during the entire experiment and therefore did not undergo reperfusion. Normoxic cells were maintained under control atmospheric conditions (5% CO_2 and 95% atmosphere). Both normoxic and hypoxic cells were maintained at 37°C for the 24-hour treatment period. The cells were disrupted with 500 μL 2% trichloroacetic and 2 mmol/L EDTA, sonicated for 30 seconds at setting 3 (Branson Sonifier, Model 450; Branson, Danbury, CT) and centrifuged for 5 minutes at 10,000 × *g*. The supernatant was stored at −80°C for further analysis of lactate, pyruvate, and ATP and ADP.

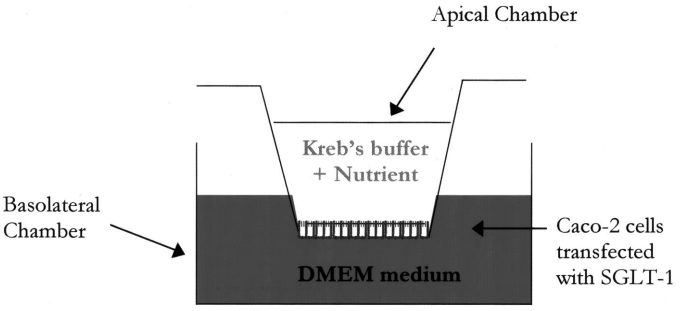

FIG. 1. Transwell Snapwell insert model. Caco-2 monolayers, stably transfected with SGLT-1, were grown on collagen-coated, polyester, permeable, 6-mm diameter membranes. Twenty-one days postconfluency, the nutrient treatment was added for 24 hours to the brush-border chamber of the Transwell Snapwell insert and standard DMEM medium was added to the basolateral chamber of the monolayer.

Ion Transport Measurements in Modified Ussing Chambers

Techniques that assess GI function by measuring ion flux in modified Ussing chambers have been previously described.[19] Briefly, 1.13 cm^2 Transwell Snapwell inserts were placed in modified Ussing chambers (Physiologic Instruments, Inc, San Diego, CA) and bathed in 8 mL of modified Kreb's solution maintained at 37°C with a circulating water bath (Fischer Scientific, Itasca, IL). Normoxic monolayers were oxygenated (95% O_2/5% CO_2). Basal transepithelial short-circuit current, resistance, and potential difference were measured after a 20- to 30-minute equilibration period. Sodium-dependent nutrient transport was determined by measuring changes in short-circuit current induced by the addition of either 10 mmol/L glucose or glutamine to the medium in the brush-border reservoir. The modified Ussing chambers were connected to dual channel voltage/current clamps (VCC MC2; Physiologic Instruments, San Diego, CA) with a

computer interface, allowing for real-time data acquisition and analysis (Acquire & Analyze software; Physiologic Instruments).

Lactate Concentration

As a marker of anaerobic metabolism, cellular lactate concentration was measured in each monolayer (n = 3 per treatment). Measurements were made using the Sigma Diagnostics lactate kit (Procedure No. 735) at 540 nm using an El_x800 plate reader (Bio-Tek Inc, Winooski, VT). Sample lactate concentrations were determined using a lactate standard curve (Sigma Chemical, St. Louis, MO). To normalize for differences in available glycolytic substrate between treatment groups, data were normalized for pyruvate concentration (μmol lactate/μmol pyruvate) per milligrams protein. Cellular pyruvate concentration was quantified on a fluorometer (F-2000, Hitachi Instruments Inc, Chicago, IL) based on a pyruvate standard curve. Briefly, 10 μL of each sample was placed in 1 mL of NADH buffer (0.95 μmol/L, 0.5 mol/L NaH_2PO_4, 0.5 mol/L K_2HPO_4) and fluorescence was recorded after excitation at 340 nm and emission at 460 nm. The sample was then reduced after addition of 5 U/L of lactate dehydrogenase (LDH) and fluorescence was requantified using the same parameters.[21–22] The differences in samples emission values (initial value minus value after LDH addition) were determined and compared with a pyruvate standard curve (Sigma Chemical).

Epithelium ATP Concentration

Epithelial ATP and ADP concentrations were measured to discern the impact of hypoxia and the nutrient treatments on tissue energy reserves after 24 hours (n = 3 per treatment). Epithelium ATP levels were determined using the luciferin/luciferase method for detection.[23] Briefly, 100 μL (10 mg/mL) of luciferan/luciferase (Sigma Chemical) was added to 100 μL of the monolayer cytosol fraction (diluted 50-fold) and counted for 30 seconds on a scintillation counter (LS 6500; Beckman, Fullerton, CA) using chemiluminescence parameters.[23–24] Sample ATP concentrations were then calculated based on an ATP standard curve generated using a scintillation counter. ADP concentration was measured on a fluorometer (F-2000; Hitachi Instruments Inc) by measurement of the reduction of NADH recorded after excitation at 340 nm and emission at 460 nm. Briefly, samples and standards were placed in a buffer of NADH (20 μmol/L); phosphoenolpyruvate (40 μmol/L) and ADP reduction occurred after addition of pyruvate kinase (0.30 U/mL).[21] The sample ADP concentration was calculated based on an ADP standard curve (Sigma Chemical).

Statistical Analysis

The effects of hypoxia and nutrient treatment on the dependent variables were determined using a 2-way analysis of variance. The sources of variation were hypoxia (h = 2), nutrient treatment (n = 4), and hypoxia interacting with nutrient treatment. Comparisons within the hypoxia-treated cells by nutrient treatment interaction were completed using Tukey's posthoc analysis. Statistical significance was defined as $p \leq .05$. Computations were performed using SAS (Version 8.0; SAS Institute Inc, Cary, NC).

TABLE I
Transepithelial Ion Transport

Nutrient treatment	Short circuit current $\mu A/cm^2$	Resistance $\Omega \cdot cm^2$	Potential difference mV
	NORMOXIA		
Mannitol	1.7 ± 1.5	154 ± 11	.02 ± .21
Glucose	3.4 ± 1.4	154 ± 10	.21 ± .19
3-O-mg	0.8 ± 1.7	167 ± 12	.07 ± .23
Fructose	5.1 ± 1.5	174 ± 11	.21 ± .21
	HYPOXIA		
Mannitol	0.1 ± 1.5	153 ± 11	.28 ± .21
Glucose	1.5 ± 1.4	130 ± 10	.34 ± .19
3-O-mg	0.5 ± 1.7	147 ± 12	.59 ± .23
Fructose	1.2 ± 1.5	146 ± 11	.67 ± .21
Hypoxia	$p = .05$	$p = .007$	$p = .02$
Nutrient	NS	NS	NS

NS, not significant.

RESULTS

Transepithelial Ion Transport Measurements

Transepithelial short-circuit current, resistance, and potential difference were measured in epithelial monolayers to provide a direct assessment of epithelial ion transport (Table I) Transepithelial short-circuit current, a measure of active ion transport, was lower ($p = .05$) in the hypoxia groups compared with normoxia controls. Transepithelial resistance, a measure of passive ion transport, was significantly lower ($p = .007$) in the hypoxia groups, indicating increased epithelial permeability. Transepithelial potential difference, a measure of total ion transport, was higher ($p = .02$) in the hypoxia groups than in normoxia controls. Transepithelial short-circuit current, resistance, and potential difference were not altered by any of the brush-border nutrient treatments.

Sodium-Dependent Nutrient Transport

Sodium-dependent glucose and glutamine transport were measured to assess the effects of hypoxia and nutrient treatment on epithelial nutrient transport capacity. When compared with normoxia controls, glucose transport was significantly impaired ($p < .001$) in the hypoxia groups (Fig. 2). However, glucose transport activity was not altered as a result of nutrient treatment (Fig. 2). Glutamine transport activity was not altered by either hypoxia or nutrient treatment (Fig. 3).

Lactate Concentration

The degree of anaerobic metabolism assessed by the cellular lactate concentration [(μmol lactate/μmol

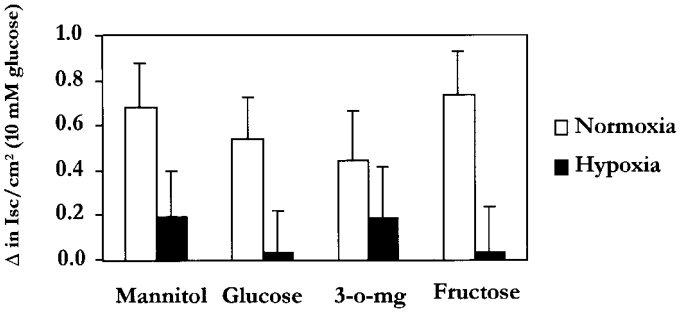

FIG. 2. Sodium-dependent glucose transport was significantly lower ($p < .001$) in the hypoxia groups than in the normoxia controls, irrespective of nutrient treatment. Data reported as means ± SEM (n = 5/treatment).

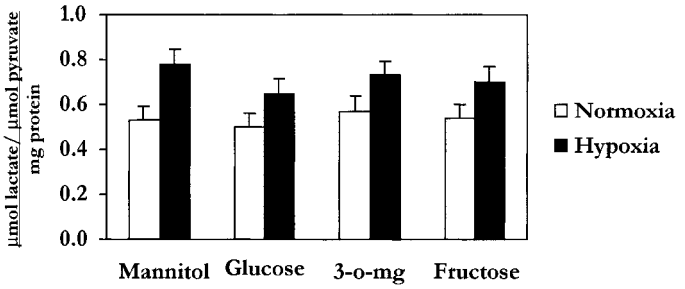

FIG. 4. Cellular lactate concentration was measured as a marker of anaerobic metabolism (n = 3 per treatment). Lactate concentration (μmol lactate/μmol pyruvate)/mg protein was significantly higher ($p < .001$) in hypoxia groups than in normoxic controls irrespective of brush-border nutrient treatment. Data reported as means ± SEM (n = 5/treatment).

pyruvate)/mg protein] was significantly higher ($p < .001$) in the hypoxic groups, irrespective of nutrient treatment (Fig. 4).

Enterocyte ATP/ADP Ratio

ATP/ADP was measured to discern the impact of hypoxia and nutrient treatment on tissue energy reserves. Epithelial ATP/ADP ratio was lower during hypoxia ($p = .01$) compared with normoxic controls (Fig. 5).

DISCUSSION

Early enteral nutrition has become a widely used treatment in patients with traumatic injury. However, relatively little data exist regarding the provision of enteral nutrients in the hypoperfused bowel; a situation known to occur in patients having undergone shock-resuscitation.[25–26] Preliminary *in vivo* studies demonstrate that various nutrients produce different effects on the hypoperfused small intestine, and therefore, establishment of valid experimental models is necessary so that further investigations can be conducted.[19] Although *in vivo* animal models serve a useful purpose for testing preclinical hypotheses, other more focused hypotheses regarding the cellular mechanisms that are fundamental to physiologic responses are explored using highly reproducible and validated cell culture models. The Caco-2 cell line originated from a human colon carcinoma and, on differentiation, exhibits small intestinal characteristics (ATCC, Manassas, VA). It is the best described, most highly used small intestinal epithelial monolayer for assessment of intestinal digestive and absorptive function.[27] Because of these characteristics, the Caco-2 cell line is an ideal cell type for initial use in developing an *in vitro* nutrition support model.

A limitation of the Caco-2 cell line is that it does not demonstrate functional activity of SGLT-1.[27] As this protein is the route of entry for luminal glucose and galactose into the enterocyte, we deemed it necessary to use Caco-2 cells that had been stably transfected with SGLT-1. The Caco-2 cells stably transfected with SGLT-1 were particularly important because the enteral nutrient treatments and results that we compared with *in vivo* treatment results were indeed hexoses. Other investigations focused on examining issues of nutrition support should be cautious to ensure that this major route of hexose entry into the enterocyte is undeniably present and functional.

After the most appropriate cell line was selected, a suitable structural foundation was necessary to allow for treatment exposure to either the brush-border or basolateral chamber of the monolayer. Transwell Snapwell inserts were used because this unique system allows for culturing with different medium on either chamber of the cell. In the current study, this system allowed for provision of isolated nutrient treatments to the brush border and maintenance of the serosal environment with complete cell culture medium, which simulated the composition of the blood.

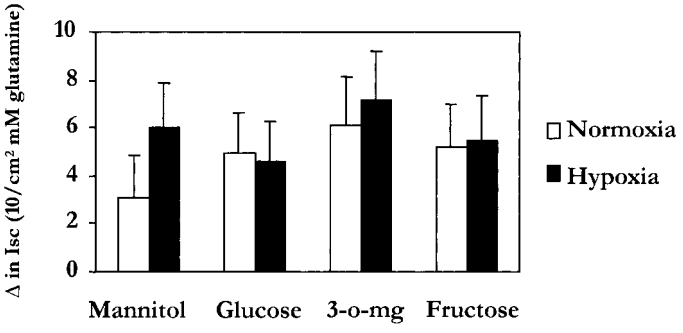

FIG. 3. Sodium-dependent glutamine activity was not altered by hypoxia or nutrient treatment. Data reported as means ± SEM (n = 5 per treatment).

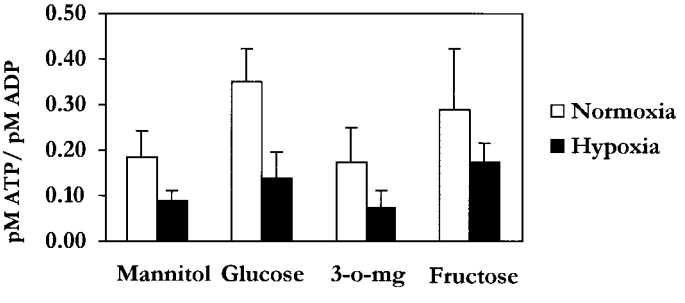

FIG. 5. Adenosine triphosphate/adenosine diphosphate (ATP/ADP) ratio (pM ATP/pM ADP) was significantly lower ($p = .02$) in hypoxic groups than in normoxic groups (n = 3 per treatment). Data reported as means ± SEM.

Although the Transwell Snapwell inserts have been used in many studies for subsequent experimental applications,[23,24,28,29] the use of this system to simulate scenarios related to route of nutrient administration is a relatively novel concept.

The construction of an appropriate *in vitro* system designed to simulate *in vivo* responses is inadequate without further validation to ensure that observed physiologic responses occur in parallel in both models. We therefore sought to validate the described *in vitro* model with a previously reported hypoperfused intestine rodent model.[19] To ensure that the *in vitro* model mimicked the established *in vivo* physiologic responses to intestinal hypoperfusion, we established certain validation criteria. After 24 hours in the hypoxic environment, monolayers must exhibit the following parameters: (1) decreased SGLT-1 activity; (2) impaired barrier function; (3) increased lactate concentration; and (4) reduced ATP concentration.

SGLT-1 activity was significantly impaired after hypoxia treatment compared with normoxic controls. This result indicates a parallel response between the *in vivo* and *in vitro* studies. Interestingly, hypoxia does not seem to affect sodium-dependent glutamine transport in either model, and further investigations are warranted to examine the differential regulation of these active nutrient transporters. The preservation of glutamine transport indicates that the cell is still capable of active transport processes and that the provision of certain classes of nutrients may be more appropriate for patients having undergone shock-resuscitation. Glutamine transport may be maintained because it is a preferential substrate of the small intestine[30] and is hypothesized to be a conditionally essential amino acid during critical illness.[31] Additional data are necessary to distinguish the factors that determine the differential regulation of nutrient processing during hypoxia in the jejunum.

In addition to being an appropriate model for digestive and absorptive function, Caco-2 monolayers are often used for measurement of barrier function.[25,32] In the present study, barrier function decreased during hypoxia, which correlated with results in the *in vivo* rat model. Decreased barrier function results in a leakier intestine and increased risk for translocation of bacteria and sepsis in traumatically injured patients.[33–34] The potential for specific enteral nutrients to regulate mucosal barrier function during hypoxia is an issue of significant clinical interest and, therefore, is worthy of further experimental investigation.

Hypoxic lactate concentration and ATP/ADP ratio in the *in vitro* study meet our final validation criteria. Hypoxia did increase lactate concentration, an observation consistent with that observed in septic patients.[7] In our previous *in vivo* hypoperfusion rat study, we reported that metabolizable substrates can be detrimental to the hypoperfused intestine. This was based on the finding that lactate production increased during hypoperfusion after the enteral administration of glucose and fructose.[18] However, the *in vitro* results obtained in the current study indicate that providing these metabolizable nutrients does not increase cellular lactate concentrations. Therefore, the current *in vitro* model did not adequately assess the effects of metabolizable substrate in the hypoxic small intestine, however it did meet the validation criteria by demonstrating increased lactate concentration during hypoxia. In addition, cellular ATP/ADP ratio was significantly decreased during hypoxia when compared with normoxic controls, thereby satisfying the final validation criteria. In the previous *in vivo* rat model, brush-border normoxic glucose increased in ATP/ADP ratio, but this effect of glucose during normoxia on ATP/ADP was not observed in the current study. Perhaps further investigation is necessary to determine hexose use in Caco-2 cells transfected with SGLT-1. Hexoses can access the enterocyte by both SGLT-1 and GLUT2 (from the basolateral membrane); however, the literature contains several reports of low-level GLUT2 expression in Caco-2 cells.[35–36] The limited transport of hexoses across the basolateral membrane, in combination with the impaired SGLT-1 activity described herein, may result in limited lactate and ATP/ADP production and could be responsible for the modest discrepancy observed between the *in vitro* and *in vivo* models.

In conclusion, the *in vitro* nutrition support model appears to be an ideal model for investigating issues related to nutrient transport. When coupled with atmospheric hypoxia, nutrient transport data mimic *in vivo* responses observed during intestinal hypoperfusion. Therefore, the *in vitro* nutrition support model provides a highly reproducible and validated system in which to test focused hypotheses regarding cellular mechanisms underlying physiologic responses during hypoxia. In combination with investigations conducted in *in vivo* animal models, these models should provide important information regarding the safe provision of early enteral nutrition after traumatic injury.

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