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# Glutamine supports recovery from loss of transepithelial resistance and increase of permeability induced by media change in Caco-2 cells[1]

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**Abstract**

Recent evidence suggests that the conditionally essential amino acid glutamine is important for intestinal barrier function. However, the mechanism remains undefined. To determine the effects of glutamine on permeability of intestinal epithelial cell monolayers, Caco-2 cells were grown on membrane filters and exposed to 4 mmol/L sodium butyrate in order to rapidly achieve high levels of alkaline phosphatase and high transepithelial resistance as seen in functionally mature enterocytes. A standard method of medium exchange consisting of removal and replacement resulted in a catastrophic loss of transepithelial resistance and increase of mannitol and dextran fluxes that required 2-4 hrs and protein synthesis to recover. The effect was attributed to exposure of the upper monolayer surface to atmosphere and could be avoided by refeeding by incremental perfusion. Spontaneously-differentiated Caco-2 monolayers were resistant to this stress. This novel stress test was employed as a sensitive assay for the requirement of glutamine for monolayer transepithelial resistance and mannitol permeability. Pre-stress glutamine availability was more important than Gln-availability during the recovery phase. Thus the transepithelial resistance and permeability of butyrate-induced monolayers is dynamically-regulated in response to atmospheric exposure, by a mechanism that depends on threshold levels of glutamine availability. © 2003 Elsevier Inc. All rights reserved.

*Keywords:* Nutrition; Transepithelial resistance; Paracellular permeability; Tight junctions; Stress

## 1. Introduction

A major function of intestinal epithelial cells is to provide a physical barrier between the potentially hostile intestinal lumen and the highly immunoreactive subepithelial tissue. The barrier function of the intestinal mucosa is partially maintained by the tight junction (TJ) complex joining adjacent epithelial cells, a highly regulated structure that confers selectivity to the permeability of the small intestine [1,2]. Permeability properties of the epithelium are dynamically regulated by diverse physiological and pathological stimuli [3]. Disruption of the intestinal epithelial TJ complexes results in a "leaky gut" with an increase in intestinal paracellular permeability [4,5]. A defective intestinal epithelial TJ barrier has been implicated in the paracellular permeation of toxic luminal substances, which leads to intestinal inflammation and mucosal injury [6–8]. Enhanced paracellular permeability across intestinal epithelium occurs in patients with Crohn's disease, and in colonic epithelium from patients with ulcerative colitis suggesting that altered TJ permeability may be a contributing factor in these processes [9,10].

Intestinal epithelial cell function has been studied in cell culture using immortalized cell lines that conserve function. A commonly used method to evaluate intestinal epithelial intercellular junctional integrity is the transepithelial electrical resistance (TER) across a confluent monolayer of cells grown on a permeable membrane in a bicameral system [4,11–14]. The markers [$^{14}$C]mannitol and FITC-dextran were also used for measurement of paracellular permeability and monolayer integrity [5,13,15]. The Caco-2 cell line, derived from a human colon adenocarcinoma, spontaneously differentiates 3 weeks after achieving confluence, exhibiting several morphological and functional characteristics of mature enterocytes [16–19]. The short-chain fatty

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1 Abbreviations used. DMEM, Dulbecco's Modified Eagle Medium; FBS, fetal bovine serum; GS, glutamine synthetase; MEM, Minimum Essential Medium; MS, methionine sulfoximine; BT, sodium butyrate; PBS, Phosphate-Buffered Saline; Gln, glutamine; TER, transepithelial resistance; TJ, tight junction; FITC-dextran, fluorescein isothiocyanate-dextran.

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acid butyric acid (BT) inhibits cell proliferation, induces certain differentiation markers [20–23], and induces a very high TER [18]. This occurs within 2 days, rather than a 3 weeks time interval. Butyric acid is produced in the colonic lumen by bacterial fermentation of carbohydrate and therefore may similarly influence epithelial cells in vivo. It has been proposed that the phenotypic changes in the 3-week Caco-2 cultures model mimics those that occur in normal colonic cells in vivo during their migration from the crypt base to the neck [18]. BT-induced cells more closely model epithelial cells from the crypt neck to villus surface compartment [18].

In cultured monolayer models of the intestinal epithelium, certain toxic substances such as furazolidone, *Clostridium difficile* toxin A, ethanol and cytochalasin B specifically alter TJ permeability of differentiated intestinal cells [12,13,24,25]. These models provide opportunities to help define the intracellular processes that regulate intestinal epithelial TJ permeability in pathological and normal physiological conditions.

Since the work of Windmueller and Spaeth, the importance of glutamine (Gln) for intestinal mucosal function *in vivo* has become generally accepted [26,27]. Recent studies have shown that supplementation of the conditionally essential amino acid glutamine may benefit the gut of individuals who are highly stressed [28–30]. Exogenous Gln also seems to contribute to the maintenance of the intestinal barrier [31–33]. Preliminary studies also have shown that deprivation of dietary Gln in an infant rat artificial feeding model increases bacterial translocation and this effect can be exacerbated by inhibiting intestinal glutamine synthetase by enteral administration of methionine sulfoximine (MS) [34].

The importance of Gln availability for small intestinal epithelial function is supported by cell culture studies. The importance of Gln for cultured cells has been known since the 1950's when it was demonstrated that some cells rely more on Gln for optimal growth than any other amino acid [35,36], even though most cells are capable of synthesizing Gln from glutamate and ammonia [14,37]. Rat intestinal epithelial crypt (IEC-6) cells proliferate in relation to the level of extracellular and endogenously synthesized Gln [37]. In the presence of MS, an inhibitor of glutamine synthetase (GS), supraphysiologic Gln is required to attain maximal proliferation [37]. In cultured Caco-2 cells, addition of MS substantially inhibits the appearance of differentiation markers and junctional integrity even in the presence of physiologic concentrations of extracellular Gln [14]. These studies suggest that endogenously synthesized Gln may play a specific, but as yet uncharacterized role in the intestinal epithelial cell.

In the course of studies on the effects of Gln-deprivation on the junctional integrity of BT-induced Caco-2 monolayers, we observed the paracellular permeability barrier of these cultures are remarkably sensitive to the stress of conventional media change with the apical surface being more sensitive than the basal. The parameters of this response are reported here. In addition, we used media change stress to help define the dependence of butyrate-induced monolayers on Gln-availability and find that the prior history of Gln-availability is more important than its availability during stress recovery.

## 2. Materials and methods

### 2.1. Reagents

Trypsin, Minimum Essential Medium (MEM), Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), Dulbecco's Phosphate-Buffered Saline (PBS) and antibiotic antimycotic solution were from GIBCO BRL (Grand Island, NY). Biocoat® Cell Culture Inserts (Fibrillar Collagen, type I rat tail) and BD MITO+ Serum Extender were from Becton Dickinson Labware (Bedford, MA). D-[$^{14}$C]mannitol was purchased from Amersham Pharmacia Biotech (Piscataway, NJ). Methionine sulfoximine (MS), L-glutamine, sodium butyrate (BT), FITC-dextran, cycloheximide and actinomycin D and all other chemical reagents were from Sigma Chemical (St. Louis, MO).

### 2.2. Cell culture

Caco-2 cells were from the ATCC (Rockville, MD) and grown in a humidified incubator at 37°C with 5% $CO_2$, 95% air. For each experiment, cells (passage 20-30) were collected by dissociation of a confluent stock culture with 0.05% trypsin and 0.53 mmol/L EDTA in HBSS, counted in a hemacytometer, and seeded into 24-well Biocoat® Cell Culture Inserts at 200,000 cells per well. The volume of culture media was 0.5 ml in apical and 1 ml in basal chambers. For the short-term BT-induced cultures, cells were fed with Biocoat® Seeding Basal Medium (DMEM with Gln) with MITO+ ™ Serum Extender (a substitute of serum, including hormones, growth factors and defined metabolites but not Gln) for 48 hr. Cells were washed once with PBS and induced with 4 mmol/L NaBT in Gln-free DMEM with Serum Extender, supplemented with the indicated concentration of Gln. In some trials, the medium contained 4 mmol/L MS, a specific inhibitor of glutamine synthetase [14,37,38]. To inhibit protein synthesis or transcription, BT-induced cultures were incubated in 10 $\mu$mol/L cycloheximide or 0.25 $\mu$g/ml actinomycin D, respectively [11], starting at the time of refeeding. For 3-week spontaneously differentiated cells, cultures were fed 8:2 glutamine free MEM and fetal bovine serum with 4 mmol/L Gln,

changed every other day. Media contained 200 U/ml penicillin, 200 μg/ml streptomycin, and 0.05 μg/ml amphotericin B.

### 2.3. Media change

The standard media replacement method consisted of removal of old media from apical and basal chambers by aspiration, and replacement with either the same amount of fresh, new, pre-warmed media or return of the old media. Care was taken to not disturb the monolayer while aspirating and adding the media. In a modified, 'perfusion' method, 0.5 volumes of old media was removed and the same amount of new media was added back to the same well both apically and basally. This 1:1 dilution procedure was repeated 8 times so that old media was essentially completely replaced by new media.

### 2.4. TER measurement

Electrical resistance across the monolayer was measured using a Millicell Electrical Resistance System with a dual electrode (Millipore Corp., Bedford, MA), by placing separate electrodes in the upper and lower wells according to the manufacturer's instructions. Two readings were taken and averaged. The resistance from each well was subtracted by a blank value from an insert without cells to arrive at the resistance of the monolayer, which was multiplied by the area of the membrane to obtain TER ($\Omega \cdot cm^2$).

### 2.5. Transepithelial mannitol and dextran flux studies

Caco-2 cells on collagen-coated membranes were treated with BT in the absence or presence of the indicated concentration of Gln. D-[$^{14}$C]mannitol (2.15 GBq/mmol; MW: 184) or FITC-dextran (MW: 4,000) were added to 0.2 μCi/ml or 200 μg/ml, respectively, to the apical compartments. Basal medium samples were taken after 2 hrs, 20 μl for liquid scintillation counting and 100 μl for quantitation of fluorescence using a TECAN SPECTRA FLUOR fluorescence spectrophotometer (Tecan, NC) at excitation and emission wavelengths of 492 nm and 515 nm. Apparent permeability coefficients (Papps) were calculated by the following formula [39]: $Papp = P\,(A \times C_o)$ (cm/s), where $P$ is the permeability rate (mol/s), $C_o$ is the initial apical concentration of test substance (mol/ml), and $A$ is the surface area of the monolayer.

### 2.6. Statistical analysis

Results are presented as means ± S.D. Two-way ANOVA was performed to assess the impact of different levels of Gln (factor A) and time following media change (factor B) on TER. $P < 0.05$ was considered statistically significant.

## 3. Results

### 3.1. Stress effect of media change on TER

When Caco-2 cells were seeded at high density on collagen-coated membranes in transwell chambers, they achieved plateau TER values of 150 $\Omega \cdot cm^2$ within 2 days of culture in Seeding Basal Medium with Serum Extender containing 4 mmol/L Gln (data not shown). When shifted to medium containing 4 mmol/L BT in the presence of 0.6 mmol/L Gln, the normal plasma concentration [14], TER values increased by a factor of 6 within 2 days of additional culture (day 4). However, when cultures were refed by media exchange on d 4, TER values were observed to fall to the low value observed before addition of NaBT (Fig. 1A). The standard media change protocol involved aspiration of old medium from both the upper and lower chambers of each well of the multiwell plate followed by serial replacement of new medium, resulting in an average time of exposure of the monolayer to atmosphere of 5 min. To minimize this exposure time, old media was removed and then immediately returned to the well from which it came. Even though exposure to atmosphere was reduced to <10 s, TER values were reduced to <25% of the prefeeding values. A time course analysis showed that the reduction of TER values was temporary (Fig. 1B). TER values of the cultures exposed to atmosphere for <10 s recovered normally within 4 hrs, whereas, cultures exposed for 5 min took 6 hrs. When exposure to atmosphere was increased to 15 min, TER values remained low even after 6 hrs. These findings suggest that media change is a stressor that temporarily decreases the tightness of intercellular junctions.

Since TER recovery required many hours we tested its dependence on new mRNA and protein synthesis (Fig. 1C). Inclusion of 0.25 μg/ml actinomycin D [11] at the time of refeeding had little effect on TER recovery although these cultures suffered a later decline in TER between 8 and 24 hrs confirming an effect of the drug. In contrast, inclusion of 10 μmol/L cycloheximide [11] allowed partial recovery of TER at 2 hrs but no additional recovery after that time. This suggests that the stress of atmospheric exposure is a major insult that requires new protein synthesis for recovery.

To determine whether the stress of media change was mediated at the upper (apical) or lower (basal) surface of the epithelial monolayers, media in the upper and lower chambers were changed separately. Removal of medium from the lower chamber leaving the underside of the membrane substratum exposed to atmosphere for up to 15 mins, followed by replacement with fresh medium, had no effect on the TER values right after media change (Fig. 2A). In contrast, the similar operation on the upper chamber caused the same catastrophic declines in TER seen in Fig. 1. These declines increased in degree as the time of exposure to atmosphere was increased from 10 s to 15 mins. When the exposure time was 10 s to 10 mins, TER values returned to over 90% and 77% of normal by 4 hrs, respectively (Fig. 2B). How-

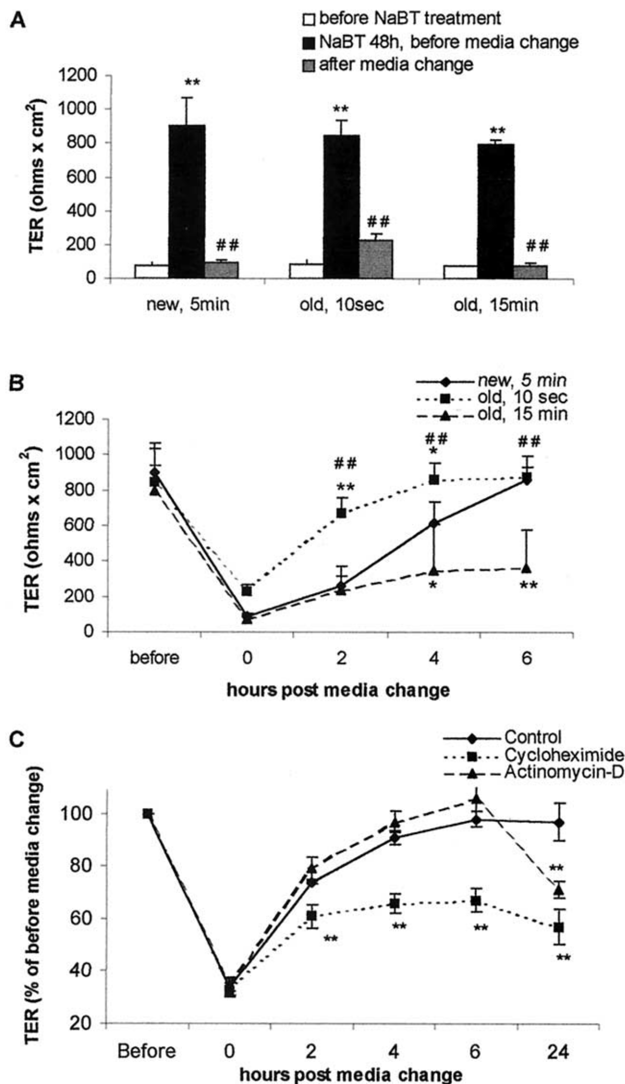


Fig. 1. Effect of media change on BT-induced Caco-2 cell monolayers. A: 2-day Caco-2 monolayers raised in seeding medium were refed and incubated for an additional 2 days in 4 mmol/L NaBT and 0.6 mmol/L Gln. The old medium was removed and either returned to the dish (old) or replaced with fresh medium (new). Time of exposure to atmosphere is given (10 s, 5 min, 15 min). TERs were measured before and after media change. Media change caused a large decrease of TER in Caco-2 cells (**: P <0.01, vs. before NaBT treatment. # #: P <0.01, vs. before media change). B: Reversibility of TER following media change. TER values were measured at 0, 2, 4, and 6 hr after media change. Recovery of TERs was complete except in the group receiving old medium after 15 min exposure to atmosphere (*: P <0.05, **: P <0.01, vs. new media, 5 min. ##: P <0.01, vs. old media, 15 min). C: Effect of cycloheximide and actinomycin D on Caco-2 TER recovery after refeeding. After media change, cells were treated with 10 μmol/L cycloheximide or 0.25 μg/ml actinomycin D. TERs were measured at indicated time points. Cycloheximide consistently reduced the TER recovery following media change (**: P <0.01, vs. control).

ever, TER was only partially restored (42%) during this time interval when the exposure time was increased to 15 mins (Fig. 2B). Thus the stress of medium change appeared to be mediated at the upper surface of the epithelial monolayer.

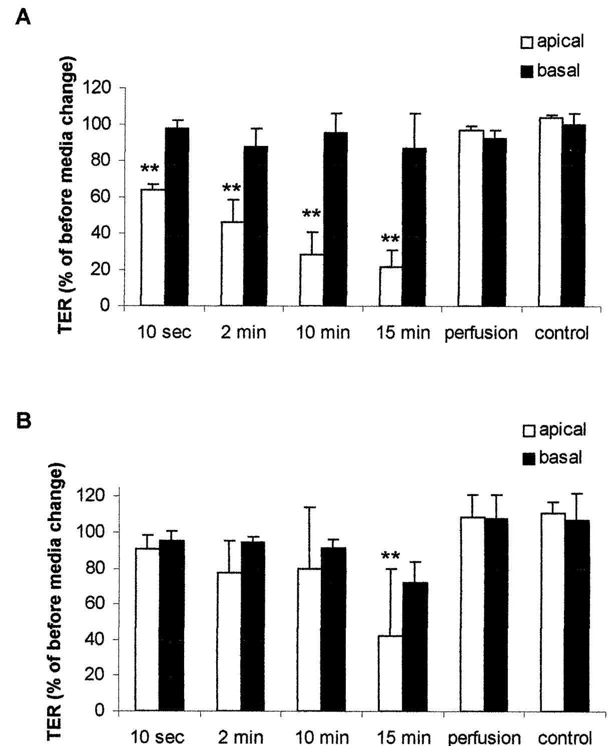


Fig. 2. Effect of changing upper or lower media on TER values. A, B: 2-day Caco-2 cultures were treated with 4 mmol/L NaBT and 0.6 mmol/L Gln for an additional 48 hrs. Media were removed from either the upper or lower wells, and then replaced to their original positions after 10 sec, 2 min, 10 min or 15 min of atmospheric exposure. In the "perfusion" group, old media were 1:1 diluted for 8 times with new media as described in Material and Methods. A: TER measurements taken immediately after refeeding, relative to values prior to refeeding. B: TER measurements taken 4 hr after refeeding, relative to values prior to refeeding. Apical media change caused a significant decrease of TER. There was no change in basal media change group and "perfusion" group. (**: P <0.01, vs. control).

To determine whether the effect of media change was due to exposure of the monolayer to atmosphere or due to the convective disturbance of refeeding, medium in the upper chamber was exchanged by repetitive replacement of 0.5 vols for 8 cycles, a process referred to here as perfusion. This method of medium replacement did not alter the TER (Fig. 2A), suggesting that the ability of monolayers to maintain their TER values was sensitive to exposure to the atmosphere.

### 3.2. Dependence of TER recovery on extracellular Gln

Our previous study revealed variable dependence of TER values of the BT-induced cultures on extracellular Gln, with some trials showing normal TER values in the absence of added free Gln and others showing a high level of dependence (DeMarco V, Li N, West CM, Neu J, unpublished data). Since cultures treated with an inhibitor of GS, an enzyme which provides an intracellular source of Gln [14,37], were absolutely dependent on free Gln, it was

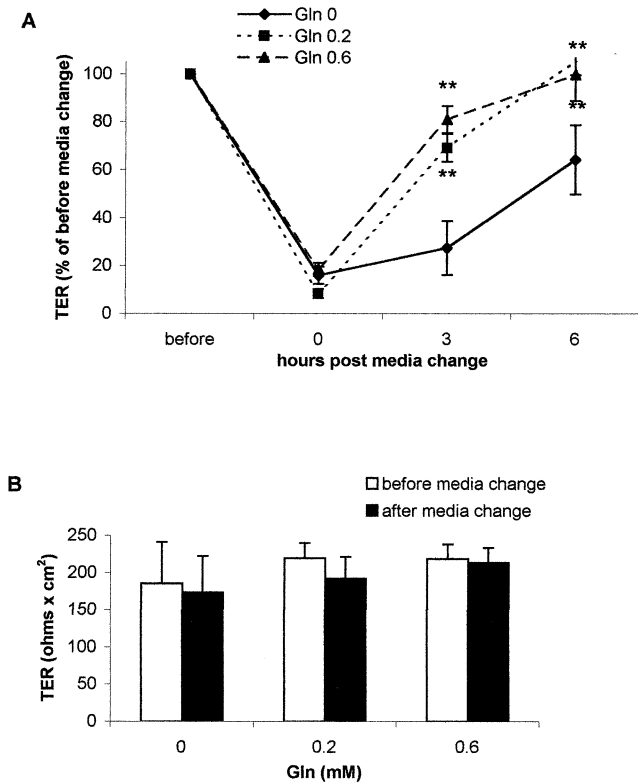


Fig. 3. Effect of pre-refeeding concentration of Gln on TER recovery after media change. After culture in seeding media for 2 days, Caco-2 cells were treated for an additional 2 days with 4 mmol/L NaBT and 0, 0.2 or 0.6 mmol/L Gln. TERs were measured before and after replacement with fresh media of the same composition, using the standard protocol. Cultures maintained with no added Gln showed delayed recovery of TER values to pre-feeding levels (**: P <0.01, vs. Gln 0). TER values before media change ($\Omega \cdot cm^2$): Gln 0 = 602 ± 151, Gln 0.2 = 1160 ± 128, Gln 0.6 = 683 ± 36.5. B: Effect of media change on TER in 21-day, spontaneously differentiated Caco-2 cultures. Caco-2 maintained in MEM containing 20% FBS and 4 mmol/L Gln for 21 days were treated for 3 additional days in 0, 0.2, or 0.6 mmol/L Gln in DMEM with Serum Extender. On d 3 of treatment, TERs were measured before and immediately after media change. TERs of spontaneous differentiated Caco-2 cells were not altered following the media change (P >0.05).

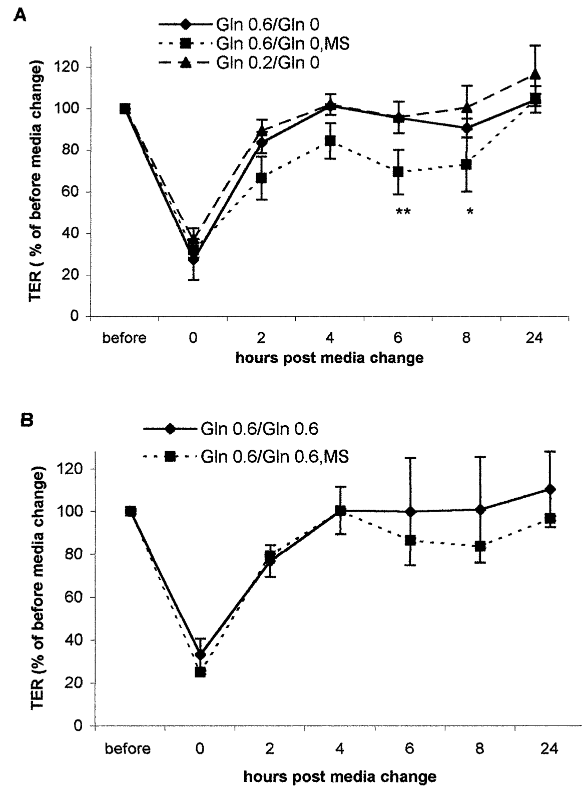


Fig. 4. Effect of post-refeeding concentration of Gln on TER recovery after media change. After culture in seeding media for 2 days, Caco-2 cells were treated for an additional 2 days with 4 mmol/L NaBT and 0.2 or 0.6 mmol/L Gln. Media were then replaced, using the standard protocol, with 0 Gln (A) or 0.6 mmol/L Gln (B), in the absence or presence of 4 mmol/L MS. TERs were measured before and after media change. In the presence of MS, deprivation of exogenous Q delayed the recovery of the TER (*: P <0.05, **: P <0.01, vs. Gln 0.6/Gln 0.6). TER values before media change ($\Omega \cdot cm^2$): Gln 0.6/Gln 0 = 808 ± 404, Gln 0.6/Gln 0, MS = 734 ± 142, Gln 0.2/Gln 0 = 704 ± 149, Gln 0.6/Gln 0.6 = 721 ± 280, Gln 0.6/Gln 0.6, MS = 790 ± 159. Note that the standard deviations for the Gln 0.6/Gln 0 and Gln 0.6/Gln 0.6 initial TER values were <200 in other trials, but the data shown here were all collected from the same group of cell cultures to facilitate comparisons.

suggested that GS provides a level of Gln close to a minimal threshold requirement. The media change stress model provided an opportunity to test this concept.

Fig. 3A shows a comparison of 4-day BT-induced cultures maintained during d 2–4 at 0, 0.2 or 0.6 mmol/L Gln. The figure plots TER, as a percentage of initial pre-feeding value to correct for sample-to-sample variation, at 4 days (time 0) and a 6-hr recovery period. All cultures experienced similar media change stress when subjected to atmosphere for 5 mins, based on comparison with their pre-stress TER values. However, the 0 Gln culture exhibited abnormally slow recovery and lagged significantly behind the 0.2 and 0.6 mmol/L Gln cultures after 6 hrs. Thus although BT-induced cells were able to achieve high TER values in the absence of added Gln, recovery from media change stress required exogenous Gln despite the ability of these cells to synthesize Gln intracellularly via GS.

Similar media change protocols on 21-day spontaneously differentiated Caco-2 cultures revealed that the TER values of these monolayer cultures were not sensitive to exposure to atmosphere for up to 5 mins (Fig. 3B) even when the cultures had been reared for 3 days in the absence of Gln. Since the NaBT-induced 3-day cultures exhibit much higher TER values than the 21-day cultures (800 vs. 200 $\Omega \cdot cm^2$), the mechanism responsible for increased TER of the short term NaBT-induced cultures may be selectively sensitive to the stress effect of atmospheric exposure.

To address whether rapid TER recovery depended on the availability of exogenous Gln after the medium change, glutamine was deleted from the refeeding medium of NaBT-induced, Gln-fed cultures produced as described in Fig. 3A. These cultures recovered at a normal rate, even when the concentration of Gln prior to media change was only 0.2 mmol/L (Fig. 4A). Thus although protein synthesis appeared to be required during recovery (Fig. 1C), neces-

sary Gln could be provided from endogenous sources. To address whether endogenous Gln derived from biosynthesis, production of Gln by glutamine synthetase was blocked by addition of 4 mmol/L MS to the cells in addition to deleting Gln from the medium. Under these conditions, recovery was slightly retarded until the following d (Fig. 4A). Since this delay could be rescued by addition of Gln to the medium (Fig. 4B), the effect of MS appeared to be specific for glutamine synthetase. This suggested that, in the absence of exogenous Gln, biosynthesis of Gln via GS is required to support optimal recovery monolayer integrity, as assessed by TER, after media change stress.

### 3.3. *Effects of media change and Gln on transepithelial fluxes of radiolabeled mannitol and FITC-dextran*

The effect of refeeding on epithelial permeability was also tested by small molecule mannitol (MW: 184) and large molecule dextran (MW: 4,000) flux measurement. [$^{14}$C]Mannitol or FITC-dextran were added to the apical chamber, and after 2 hrs aliquots were taken from the lower chamber for liquid scintillation counting or quantitation of fluorescence. As shown in Figs. 5A and 5B, media change caused significant increases of mannitol and dextran flux in the presence or absence of Gln. In cultures fed with 0.2 or 0.6 mmol/L Gln, permeability coefficients for mannitol returned to pre-feeding levels within 4 hrs (Fig. 5A). However, cultures not provided with Gln only partially recovered. Thus mannitol flux was affected similarly to TER during refeeding in the presence or absence of extracellular Gln. However, permeability coefficients for dextran recovered completely in the presence or absence of extracellular Gln (Fig. 5B). This result suggests that the permeability recovery of large molecules occurs before that of small molecules and does not appear to depend on extracellular Gln.

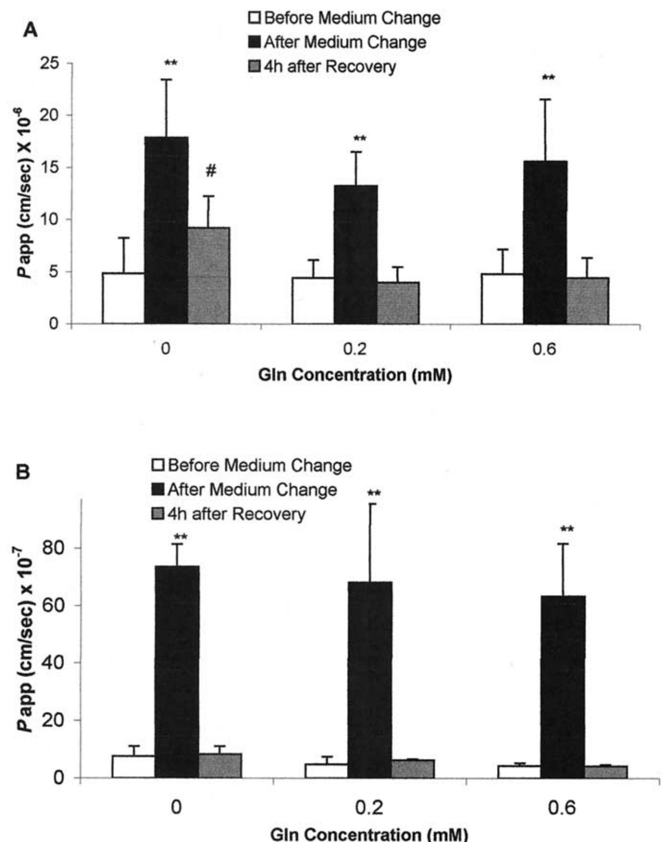


Fig. 5. Effects of media change and Gln on transepithelial fluxes of radiolabeled mannitol and FITC-dextran. Caco-2 cells were treated for 2 days with BT as in Fig. 1 in the presence or absence of Gln as indicated. [$^{14}$C]mannitol or FITC-dextran were added to apical compartment and $^{14}$C accumulated in the basal compartment was analyzed after 2 hrs, for the 'before medium change' sample. For the 'after medium change sample', [$^{14}$C] mannitol or FITC-dextran were included in the fresh medium, and a basal aliquot was withdrawn after 2 hrs. For the 'recovery' sample, [$^{14}$C]mannitol or FITC-dextran were added 4 hrs after medium change, and a basal aliquot was withdrawn after 2 additional hrs. Media change caused significant increases of mannitol and dextran flux in the presence or absence of Gln (**: $P < 0.01$, vs. before media change). Permeability coefficients for mannitol in 0.2 and 0.6 mmol/L Gln groups were completely recovered at 4 hr after media change, but only 48% recovered in the 0 Gln group (#: $P < 0.05$, vs. before media change) (A). Permeability coefficients for dextran recovered completely at 4 hr after media change in the presence or absence of Gln (B).

## 4. Discussion

When cultured on membrane filters, Caco-2 monolayers model many aspects of intestinal epithelial function *in vivo,* including establishment of a permeability barrier to small molecules such as mannitol and phenol red which is inversely correlated to TER values [15]. Exposure of monolayers to BT rapidly induced high TER values in confluent cultures and expression of other markers that correlate with the epithelium of the villus surface [18]. In the course of an investigation to analyze the dependence of monolayer integrity on Gln availability, we observed that TER values of BT-induced cultures were rapidly but transiently reduced dramatically by simple media change and refeeding. The lower TER values appear to reflect an increase in paracellular permeability as increased fluxes of [$^{14}$C]mannitol and FITC-dextran were also observed. This effect was attributed to exposure of the upper (apical) monolayer surface to atmosphere rather than to convective disturbance, and was not associated with refeeding per se. Furthermore, the response to media change was limited to BT-induced cultures, as standard 3-week spontaneously differentiated monolayers were not affected by the media change regime.

The transiently reduced TER following media change stress provides a sensitive, instantaneous, quantitative measure of monolayer integrity that can be used to assess dependence on environmental or intracellular factors. As an example, we examined dependence on the availability of extracellular Gln. Another study (DeMarco VD, Li N, West CM, Neu J, unpublished data) had found that the high TER values of BT-induced monolayers was variably dependent

upon extracellular Gln, as evidenced by high TER values of some cultures induced in the absence of added Gln. However, we find here that these 0 mmol/L-Gln monolayers consistently exhibit slow recovery after media change stress regardless of whether their initial TER values were affected (Fig. 3A). Even when cultures were initially provided a subphysiological level of Gln (0.2 mmol/L), intracellular sources of Gln were sufficient to support rapid recovery of TER and mannitol flux values in cultures refed in the absence of exogenous Gln, consistent with prior evidence that GS is induced by Gln-free media [38]. However, rapid recovery was dependent upon Gln biosynthesis (Fig. 4), consistent with the evidence for dependence on new protein synthesis (Fig. 1C). The fact that cells treated with MS in these experiments eventually did recover suggests that in the short term sufficient GLN can be provided by protein turnover.

The media change stress reduced TER values to the pre-BT levels, suggesting that stress might affect only the increment induced by BT. This is consistent with the absence of an effect on the TER values of spontaneously differentiated, 3-week cultures, which also have relatively low TER values (Fig. 3B). Although the mechanism by which BT or Gln increases TER is not known, villus enterocytes uniquely express claudin-4 [40] which might be the target of Gln-deprivation. The requirement for Gln may be nutritional, or alternatively may comprise a regulatory signal [41]. The physiological significance of exposure of the monolayer to atmosphere is not known. *In vivo,* the apical epithelial surface is covered by a thick mucous layer which is expected to buffer the monolayer from the effects of luminal gas bubbles that may contact the epithelium. However, the results raise the possibility that recovery from other physiological stressors is also selectively dependent on Gln -availability. We can currently only speculate on the nature of the damage induced by media change. It could be secondary to high oxygen potential or mechanical damage. Regardless of the relevance of this stressor *in vivo,* it is a significant response that must be considered in paracellular permeability studies on BT-induced monolayer cultures. Such studies will be critical to test mechanisms related to the hypothesis of whether glutamine-mediated regulation of intestinal barrier function relates to gut origin sepsis and other diseases [6,33,42].

## Acknowledgments

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