Glutamine regulates Caco-2 cell tight junction proteins

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Li, Nan, Patricia Lewis, Don Samuelson, Kellym Liboni, and Josef Neu. Glutamine regulates Caco-2 cell tight junction proteins. *Am J Physiol Gastrointest Liver Physiol* 287: G726–G733, 2004. First published May 6, 2004; 10.1152/ajpgi.00012.2004.—Intestinal epithelial tight junction (TJ) barrier dysfunction may lead to inflammation and mucosal injury. Glutamine (GLN) plays a role in maintenance of intestinal barrier function in various animal models and critically ill humans. Recent evidence from intestinal cell monolayers indicates that GLN maintains transepithelial resistance and decreases permeability. The mechanisms of these effects remain undefined. We hypothesized that GLN affects proteins involved in the intercellular junctional complex. GLN availability was controlled in Caco-2 monolayers by addition to the medium and treatment with methionine sulfoximine (MSO) to inhibit glutamine synthetase (GS). Expression of TJ proteins, claudin-1, occludin, and zonula occluden (ZO)-1 was measured by immunoblotting. Localization of TJ proteins was evaluated by immunofluorescence light microscopy. Structure of TJ was determined by transmission electron microscopy (TEM). Deprivation of GLN decreased claudin-1, occludin, and ZO-1 protein expression and caused a disappearance of perijunctional claudin-1 and a reduction of occludin but had no effect on ZO-1. TEM revealed that MSO-treated cells in the absence of GLN formed irregular junctional complexes between the apical lateral margins of adjoining cells. These findings indicate that TJ protein expression and cellular localization in Caco-2 cell monolayers rely on GLN. This mechanism may similarly relate to GLN-mediated modulation of intestinal barrier function in stressed animals and humans.

nutrition; intercellular junctions; intestinal epithelia cells

IN ADDITION TO BEING THE ORGAN responsible for digestion and absorption of nutrients, the intestine serves a barrier function that is a critical component of the innate immune system (2, 3, 8, 12). Only a single layer of epithelial cells separates the luminal contents from effector immune cells in the lamina propria and the internal milieu of the body. Breaching this single layer of epithelium can lead to pathological exposure of the highly immunoreactive subepithelium to the vast number of microbes and antigens in the lumen. Breakdown of the barrier is implicated in bacterial translocation, leading to sepsis, and in the pathogenesis of acute illnesses such as multiple organ system failure (22). Increased permeability early in life has been implicated in the pathogenesis of several diseases that manifest in later life, including atopy, food allergies, celiac enteropathy, type 1 diabetes, inflammatory bowel disease (2, 8), and autism (39). Studies in rodents show that stresses such as physical restraint can lead to increased ileal permeability (27), and early separation of the mother from the infant is a stress that is associated with intestinal permeability disorders later in life (35).

The mechanisms of stress-related epithelial breakdown are unclear, but regulation of paracellular pathways, especially via the interepithelial tight junction (TJ) proteins, and subsequent stimulation of highly immunoreactive submucosal cells are likely to play a significant role. The TJ represents the major barrier within the paracellular pathway between intestinal epithelial cells (14). These are dynamic structures that readily adapt to a variety of developmental (23, 34), physiological (24), and pathological (28, 29) circumstances. These adaptive mechanisms are still incompletely understood. Multiple proteins that make up the TJ have been identified: occludin (10) and members of the claudin family (9), a group of at least 20 tissue-specific proteins, are the major sealing proteins (36). Studies of occludin "knockout" mice have demonstrated that they retain normal intestinal permeability and, despite demonstrating poor growth and other phenotypic abnormalities, remain viable (33). Recent studies suggest that claudin-1, the intestine-associated family member (17), may directly associate with occludin laterally in the membrane within the same cell but not intercellularly (11), and the combination of these two proteins functioning together performs the major "gatekeeper" function of the TJ (14). These sealing proteins, both transmembrane proteins, interact with cytoplasmic plaques that consist of different types of cytosolic proteins (including the zonula occludens proteins ZO-1, ZO-2, and ZO-3).

Very little is known about specific nutrients and how they affect intestinal epithelial junctions. Evidence suggests that glutamine (GLN) helps maintain intestinal mucosal integrity, especially during stresses, such as radiation therapy (4), chemotherapy (1), and total parenteral nutrition (20), and blunts increased gut permeability associated with experimental sepsis (7).

Of the in vitro models, Caco-2 cells are one of the most widely used to study the assembly of intestinal intercellular junctions and the development of apical-basolateral polarity during differentiation. Caco-2 cells grow to confluence and spontaneously differentiate in a process requiring 21 days (25). Depriving Caco-2 cell monolayers of GLN leads to increased bacterial translocation (31). Previous studies from our laboratory have demonstrated that deprivation of GLN from cell culture medium and inhibition of glutamine synthetase (GS) using methionine sulfoximine (MSO) lead to significant decreases in transepithelial resistance of Caco-2 cell monolayers and increased permeability (6, 38). In the absence of added GLN, sufficient GLN may be provided de novo by GS. Similar results have been found in a cell culture stress model (19). Electron microscopy of the intestine in a GLN-deprived infant rat model also demonstrates intestinal intercellular junction

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breakdown (32). The barrier function breakdown in Caco-2 cells is reversible, suggesting that it is not the result of permanent cell damage (6). These studies establish a role for GLN, either from the medium or via endogenous synthesis, in supporting epithelial cell barrier function of the monolayers.

A relationship between a single nutrient, cell junction biophysical properties, paracellular permeability, and TJ proteins has not been established previously. To test the hypothesis that Caco-2 intercellular junction TJ proteins are involved in the transepithelial resistance and permeability seen with GLN deprivation, immunoblotting, immunofluorescence light microscopy, and transmission electron microscopy were performed.

MATERIALS AND METHODS

Reagents. Vendor-prepared solutions of Trypsin/EDTA, MEM, FBS, and antibiotic antimycotic solution were from GIBCO-BRL (Grand Island, NY). Biocoat Cell Culture Inserts (Fibrillar Collagen, type I rat tail) and MITO$^+$ Serum Extender were from Collaborative Biomedical Products (Bedford, MA). MSO [L-S-(3-amino-3-carboxypropyl)-S-methylsulfoximine], L-GLN, and all other chemical reagents were from Sigma Chemical (St. Louis, MO). Antibodies (anti-claudin-1, occludin, and ZO-1) were obtained from Zymed Laboratories (San Francisco, CA).

Cell culture. Caco-2 cells were purchased from ATCC (Rockville, MD) and grown in a humidified incubator at 37°C with 5% CO$_2$ and 95% air. Cells between passage 20 and 30 were used for all experiments. For each experiment, cells were collected by dissociation of a confluent stock culture with 0.05% trypsin and 0.53 mM EDTA, counted using a hemacytometer. Cells were seeded in 24-well Biocoat Cell Culture Inserts (Collaborative Biomedical Products) at 200,000 cells/well for electron microscopy, Nunc flasks (Nalge Nunc International, Naperville, IL) for immunoblotting, or Lab-Tek II Chamber Slide (Nalge Nunc International) for immunofluorescence microscopy. Culture media consisted of 8:2 GLN-free MEM and FBS. Cells were cultured with 4 mM GLN for 21 days before treatment. Next, cells were fed with GLN-free DMEM with MITO+ Serum Extender (Collaborative Biomedical Products), a substitute of serum, including hormones, growth factors, and defined metabolites, but not GLN. The medium was supplemented with the indicated concentration of GLN for various time points. Media was changed every other day. Media also contained penicillin, streptomycin, and amphotericin B at concentrations of 200 U/ml, 200 μg/ml, and 0.05 μg/ml, respectively.

Preparation of detergent-soluble and -insoluble protein fractions. Caco-2 cells were washed three times with ice-cold PBS, immediately incubated in Nonidet P-40 extraction buffer (25 mM HEPES, pH 7.4, 150 mM NaCl, 4 mM EDTA, 1% Nonidet P-40, 25 mM NaF, 1 mM Na$_3$VO$_4$, 10 mM sodium pyrophosphate, and protease inhibitors) on ice for 30 min, and centrifuged at 20,000 g for 30 min at 4°C. The detergent-soluble fraction was transferred to a microcentrifuge tube. The insoluble fraction was collected in SDS extraction buffer (25 mM HEPES, pH 7.4, 4 mM EDTA, 1% SDS, 25 mM NaF, 1 mM Na$_3$VO$_4$, and 10 mM sodium pyrophosphate) and sonicated. Supernatants (cytoskeleton-associated fractions) were then obtained after centrifugation (20,000 g for 30 min) and diluted with an equal volume of Nonidet P-40 extraction buffer to reduce SDS concentration in the samples. Protein concentrations were measured using a Bio-Rad *Dc* Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA) according to the instructions.

Western blotting. Equal amounts of proteins (20 μg) for each sample were separated on SDS-PAGE (12.5% polyacrylamide gel) and transferred to Immobilon Transfer polyvinylidene difluoride membranes (Millpore, Bedford, MA). Blots were blocked with 5% nonfat milk, incubated sequentially with primary antibody (1:2,000 for claudin-1, 1:1,000 for ZO-1 and occludin) and horseradish perox-

idase-conjugated secondary antibody (1:2,000), and detected with ECL plus (Amersham Pharmacia Biotech, Piscataway, NJ). Protein bands were quantified by densitometry using Adobe Photoshop software.

Immunofluorecence microscopy. Cells (4 × 10^5 cells/cm^2) were grown in the Lab-Tek II chamber slide system (Nalge Nunc International) with various treatments. After being washed with PBS (without Ca^{2+} and Mg^{2+}), cells were fixed with 3% paraformaldehyde. Primary antibody claudin-1, occludin, or ZO-1 (Zymed Laboratories, San Francisco, CA) was diluted 1:50 and incubated for 1 h. Next, cells were incubated with a secondary antibody [fluorescein isothiocyanate (FITC)-conjugated goat anti-rabbit IgG antibody; Sigma] for 30 min at 1:100 dilutions. After being mounted with FITC-compatible media, TJ protein expression was visualized under a fluorescent light microscope (Leitz).

Transmission electron microscopy. After dehydration and embedding in Araldite epoxy resin, 1-μm-thick sections were made using glass knives on a Reichert Ultracut S ultramicrotome and stained with 1% toluidine blue in 1% sodium borate. Areas selected for ultrastructural observations and ultrathin sections (~80 nm in thickness) were cut using a diamond knife and stained with saturated uranyl acetate and Reynold's lead citrate. Sections were viewed on a Hitachi H7000 transmission electron microscope, and images were captured digitally. Ultrastructure observations were made from multiple sites (>10) of junctional complexes that were clearly identified. At least three images from each treatment group were analyzed by three people in a blinded fashion.

Statistical analysis. Sigmastat statistical software (SPSS, Chicago, IL) was used to analyze densitometry results of TJ protein relative expression levels for Western blots. All data were reported as means ± SD from three independent experiments. A one-way ANOVA was used to determine if a significant difference was present among all treatment groups. Additionally, Bonferroni t-tests were performed for unpairwise comparisons when the ANOVA was significant at $P < 0.05$.

RESULTS

Effects of GLN on claudin-1, occludin, and ZO-1 protein expression. To further determine if TJ-associated proteins are involved in the GLN-mediated effects on Caco-2 cell intercellular junctions, Caco-2 cells were cultured for 21 days and treated with or without GLN for 5 days. Detergent-soluble and -insoluble protein fractions were isolated from the cells. Western blots were done to evaluate the expression levels of TJ proteins.

Using an antibody specific for claudin-1, no differences were seen in the detergent-soluble proteins among the different doses of GLN, whereas deprivation of GLN decreased the claudin-1 content and GLN restored claudin-1 expression in a dose-dependent manner when examined in the detergent-insoluble fractions ($P < 0.05$ vs. GLN 0 group; Fig. 1A).

The higher molecular form, a phosphorylated form (85 kDa) of occludin, is associated with the assembling of TJ complexes. In contrast, the low molecular form, a nonphosphorylated form (65 kDa), is involved in the dissembling of TJs (13). As shown in Fig. 1B, both 85- and 65-kDa forms were seen when detergent-insoluble protein extracts were examined. However, the 65-kDa form was predominant in the detergent-soluble fraction. GLN did not affect 65-kDa occludin expression in either detergent-soluble or -insoluble fractions (Fig. 1B). Likewise, Fig. 1C shows no change of ZO-1 expression in either detergent-soluble or -insoluble protein with different doses of GLN. These results suggest that GLN provided in the culture

A

GLN (mM)   0   0.1   0.6   4

S   Claudin-1

I   Claudin-1

B

GLN (mM)   0   0.1   0.6   4

S   Occludin

I   Occludin

C

GLN (mM)   0   0.1   0.6   4
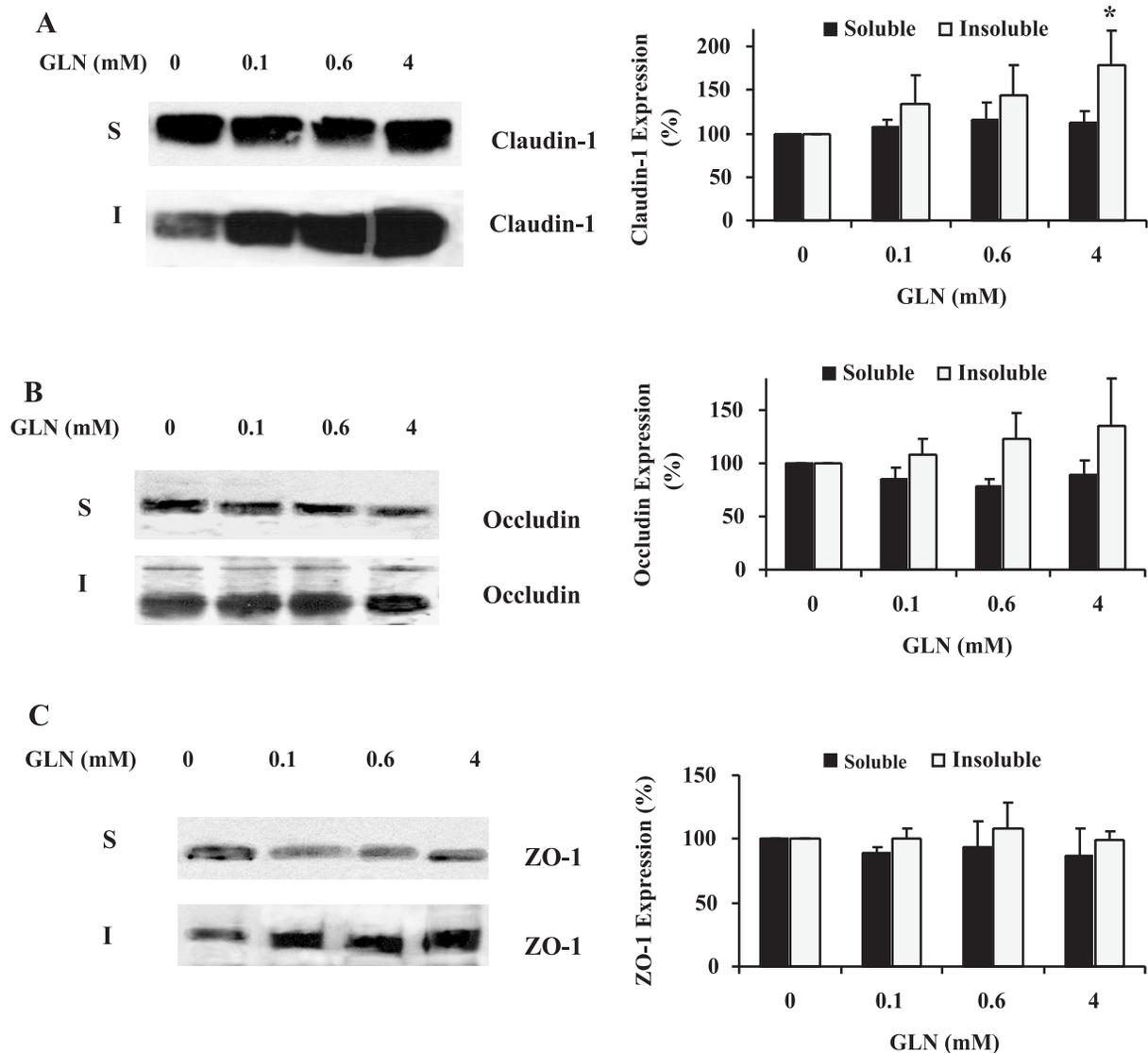
S   ZO-1

I   ZO-1

Fig. 1. Effects exogenous glutamine (GLN) on tight junction proteins. Caco-2 cells were cultured for 3 wk and treated with indicated concentration of GLN for 5 days. Detergent-soluble (S) and -insoluble (I) protein fractions were isolated from the cells. Protein samples were subjected to SDS-PAGE. Western blots were probed with anti-claudin-1 (*A*), anti-occludin (*B*), and anti-zonula occluden (ZO)-1 (*C*) antibodies. Densitometry results were shown in the corresponding bar graphs for relative expression level of claudin-1, occludin, and ZO-1. The protein expression level in GLN-free group was 100%. All data were reported as means ± SD from 3 independent experiments. *$P < 0.05$ vs. GLN-free group.

medium (exogenous GLN) regulates claudin-1 protein level in the detergent-insoluble form, but not occludin or ZO-1.

To investigate the role of GLN synthesized intracellularly via GS (endogenous GLN) in regulating TJ protein, Caco-2 cells were cultured as above and treated with or without GLN in the absence or presence of 4 mM MSO, a specific inhibitor of GS. As shown in Fig. 2, claudin-1 expression level was the lowest when there was no GLN in the medium, and GS was also inhibited by MSO, especially in the detergent-insoluble fraction ($P < 0.01$ vs. GLN 0/MSO 0 group; Fig. 2*A*). GLN supplementation rescued the decrease of claudin-1 caused by GLN deprivation in a dose-dependent manner (Fig. 2, *A* and *D*). Endogenous (synthesized de novo via GS) and exogenous (supplied in the media) GLN deprivation caused a decrease of occludin expression in both detergent-soluble and -insoluble fractions ($P < 0.05$ vs. GLN 0/MSO 0 group; Fig. 2*B*).

Meanwhile, GLN supplementation restored the occludin content even at a very low dose (0.1 mM) of GLN (Fig. 2*B*). Similarly, ZO-1 was reduced by GLN deprivation (Fig. 2*C*). GLN (4 mM) increased the ZO-1, especially in detergent-insoluble fractions ($P < 0.05$ vs. GLN 0/MSO 0 group; Fig 2*C*). These results demonstrate that endogenous GLN also plays an important role in regulation of TJ proteins.

Figure 3 shows the time course of the effects of GLN deprivation on TJ protein expression. Caco-2 cells were treated with GLN-free medium in the presence of MSO at the indicated time points. Cells were treated with 0.6 mM GLN in the presence of MSO for 48 h as a control. In the detergent-insoluble fraction, GLN deprivation downregulated claudin-1, and the maximum effect was at 108 h ($P < 0.05$; Fig. 3*A*). In the detergent-soluble fraction, there appeared to be a trend toward decrease of claudin-1 from 48 h with a maximum effect
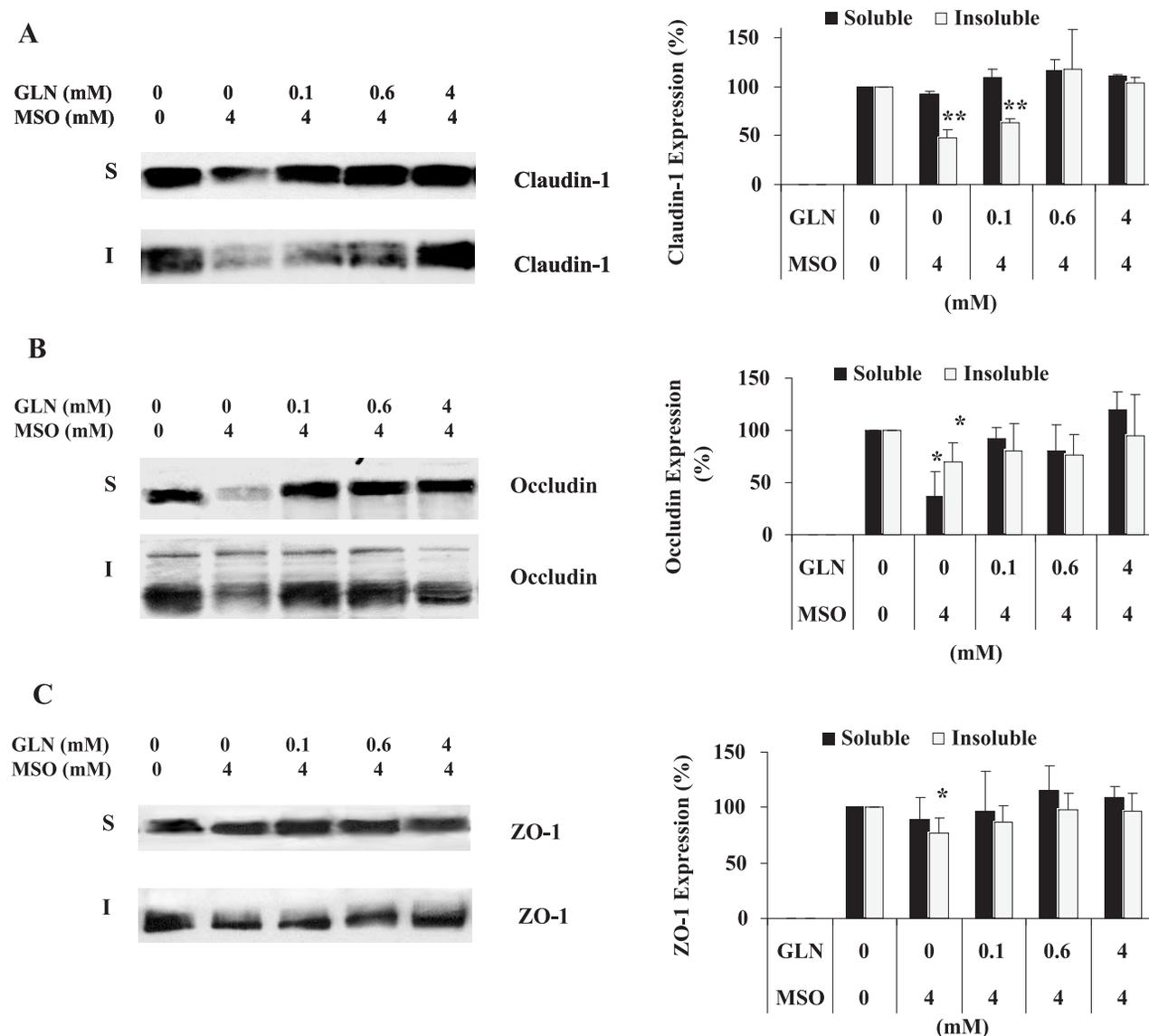
Fig. 2. Effect of endogenous GLN on tight junction proteins. Caco-2 cells were treated as above and in the absence or presence of 4 mM methione sulfoximine (MSO). Detergent-soluble and -insoluble protein fractions were isolated from the cells. Protein samples were subjected to SDS-PAGE. Western blots were probed with anti-claudin-1 (*A*), anti-occludin (*B*), and anti-ZO-1 (*C*) antibodies. Densitometry results were shown in the corresponding bar graphs for relative expression level of claudin-1, occludin, and ZO-1. The protein expression level in GLN 0/MSO 0 group was 100%. All data were reported as means ± SD from 3 independent experiments. *$P < 0.05$ vs. GLN 0/MSO 0 group. **$P < 0.01$ vs. GLN 0/MSO 0 group.

at 108 h, but there is no statistical significance ($P > 0.05$; Fig. 3*A*). GLN deprivation caused a decrease in occludin in the detergent-soluble fraction from 48 to 108 h and in insoluble fractions at 108 h ($P < 0.05$; Fig. 3*B*) and had a similar effect on ZO-1 in insoluble fractions at 108 h ($P < 0.05$; Fig. 3*C*).

Effects of GLN on TJ protein localization (fluorescence microscopy). To test if GLN affects the localization of TJ proteins, cells were treated as above. Immunofluorescence was done by using fluorescent-labeled antibodies to occludin, claudin-1, and ZO-1. Cell morphology among the different treatment groups appeared to differ, with the GLN 0/MSO 4 mM cells appearing slightly larger than the others (Fig. 4, *A-D*). The greatest GLN depletion (no GLN in the medium and endogenous GLN synthesis inhibited with MSO) indicated that claudin-1 (Fig. 4*G*) and occludin (Fig. 4*K*) were markedly de-

creased (intercellular junctions of cells lose bright green, well-defined outline). GLN deprivation did not obliterate junctions in the ZO-1 immunolabeled cells (Fig. 4, *M* and *O*) as it did with occludin and claudin-1, suggesting a different effect of GLN on the individual TJ proteins.

Effects of GLN on ultrastructure of TJ. Ultrastructure of Caco-2 cells treated as previously described was shown by transmission electron microcopy (Fig. 5). The presence of electron-dense material in the space between cells near the brush border reflects the TJ. In cells with 0.6 mM GLN (Fig. 5*B*), the TJ and desmosome displayed an intact structure. In cells without GLN and in the absence of MSO in the medium, the TJ and desmosome appear preserved (Fig. 5*A*). When the cells are deprived of exogenous and de novo synthesized GLN (with 4 mM MSO; Fig. 5*C*), the TJ complex appeared reduced
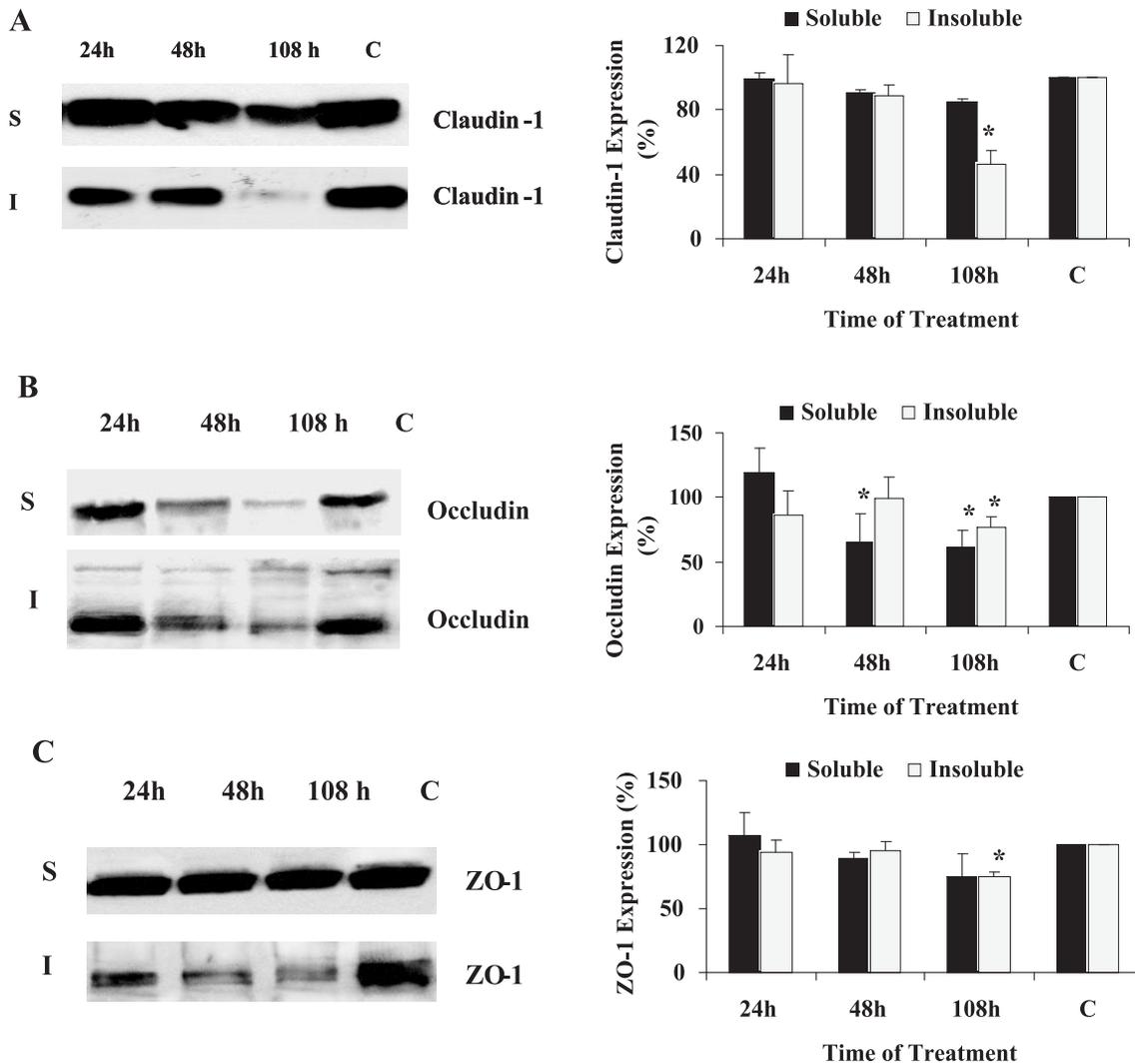
Fig. 3. Time course of tight junction expression. Caco-2 cells were treated with GLN-free medium with 4 mM MSO, a glutathione synthase (GS) inhibitor. Cells were harvested at the indicated time points. Western blotting was done to detect the expression levels of claudin-1 (*A*), occludin (*B*), and ZO-1 (*C*) in Caco-2 cells. Cells were treated with 0.6 mM GLN in the presence of 4 mM MSO as a control. Densitometry results were shown in the corresponding bar graphs for relative expression level of claudin-1, occludin, and ZO-1. The protein expression level in GLN 0.6 mM/MSO 4 mM 48-h group (control) was 100%. All data were reported as means ± SD from 3 independent experiments. *$P < 0.05$ vs. control group.

and contained less electron-dense material, whereas the desmosomes were still evident. With 0.6 mM GLN, this effect was rescued (Fig. 5*D*). These results demonstrated that GLN deprivation resulted in distortion of normal TJ morphology.

DISCUSSION

GLN helps maintain intestinal mucosal integrity, especially during stresses, such as radiation therapy (4), chemotherapy (1), and total parenteral nutrition (20), and blunts increased gut permeability associated with experimental sepsis (7). Previous studies from our laboratory demonstrate that, when endogenous GS is inhibited by MSO in addition to nutritional deprivation of GLN, Caco-2 culture systems exhibit significant breakdown in barrier function (6). Caco-2 cell models subjected to media change stress (19) or "luminal starvation" (18) wherein inhibition of GS was not required for breakdown in barrier function also responded to GLN by decreasing permeability. It has also been shown that GLN deprivation caused a

breakdown of the epithelial junctions in a gastrostomy-fed infant rat model (32).

The Western immunoblots demonstrate that deprivation decreases the insoluble fraction of claudin-1 and occludin and that this effect is partially reversible with GLN supplementation. This is supported by the immunofluorescence studies showing that both occludin and claudin-1 are affected by GLN depletion. To test if GLN 0/MSO 4 mM could cause cell death, lactate dehydrogenase (LDH) assays were done to evaluate cell viability. Cells were treated as above. There was no difference in percentage of LDH release among various GLN/MSO treatment groups (data not shown). This result demonstrated that GLN deprivation does not cause the cell death, and GLN deprivation has a specific effect on TJ protein. The fact that ZO-1 protein was affected on the protein immunoblot but not demonstrated in the immunofluorescent microscopy is of interest. It is notable that the cells in the GLN-depleted group where ZO-1 was examined appeared larger, but we do not
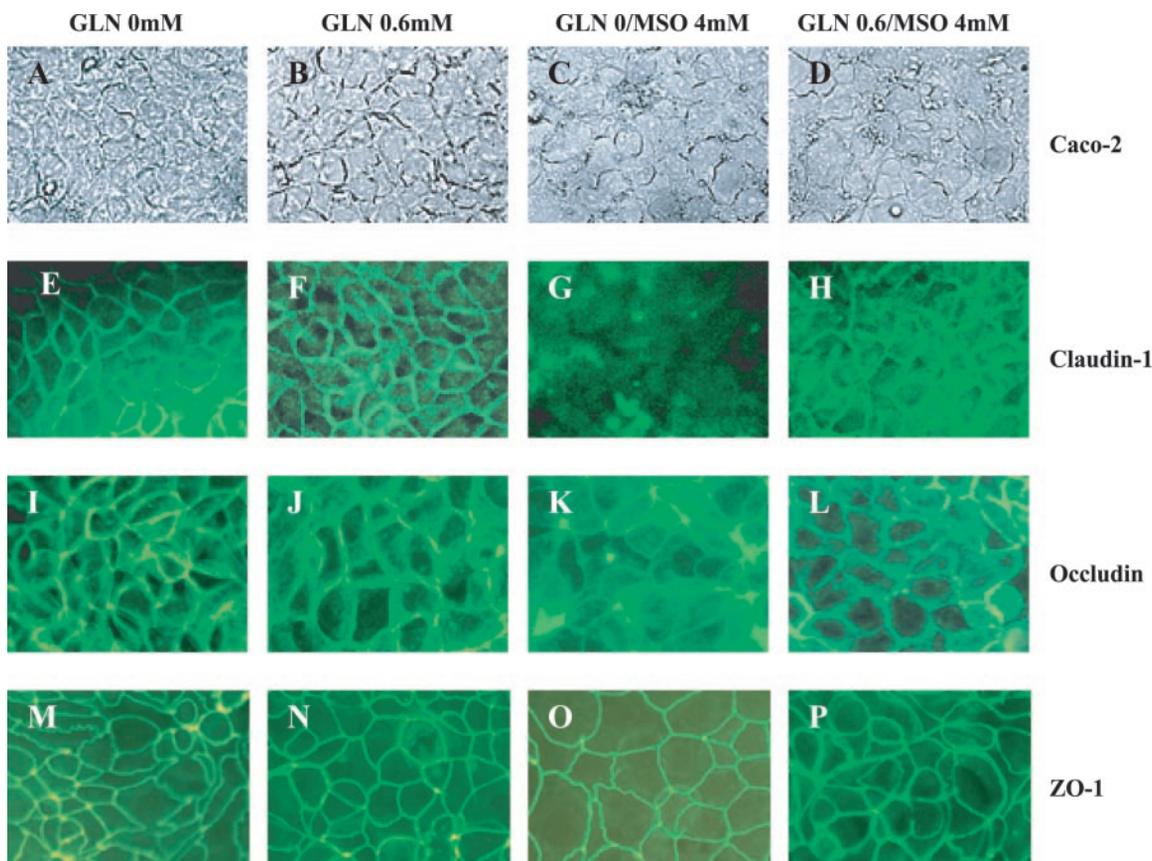
Fig. 4. Effects of GLN on tight junction protein localization using immunofluorescence. Caco-2 cells were treated with (*B*, *D*, *F*, *H*, *J*, *L*, *N*, and *P*) or without (*A*, *C*, *E*, *G*, *I*, *K*, *M*, and *O*) 0.6 mM GLN in the absence (*A*, *B*, *E*, *F*, *I*, *J*, *M*, and *N*) or presence (*C*, *D*, *G*, *H*, *K*, *L*, *O*, and *P*) of MSO for 5 days. Cells were fixed and observed under light microscope (*A*–*D*). Immunofluorescence was done by using specific antibodies for claudin-1 (*E*–*H*), occludin (*I*–*L*), and ZO-1 (*M*–*P*) and observed under a fluorescence microscope. Magnification was ×400.
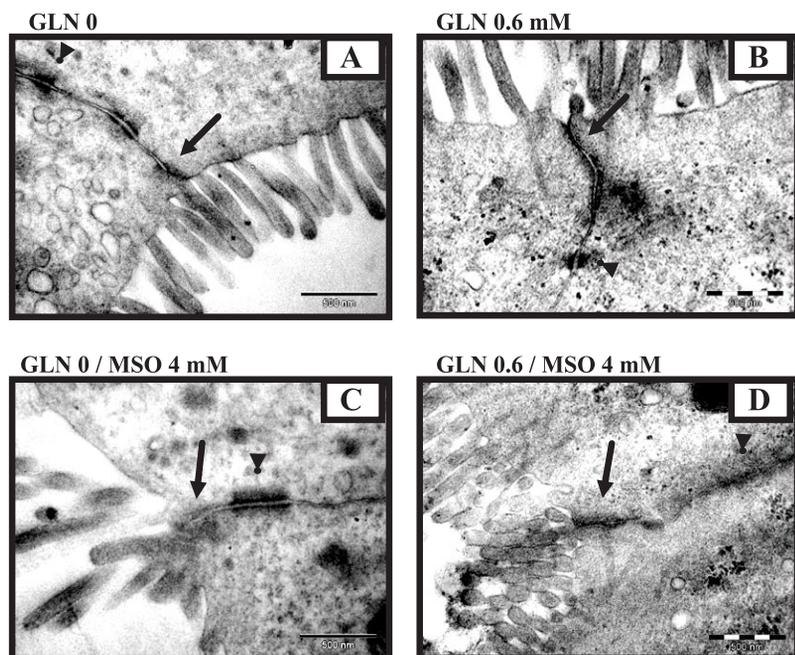

Fig. 5. Effects of GLN on ultrastructure of tight junctions. Caco-2 cells were treated with (*B* and *D*) or without (*A* and *C*) 0.6 mM GLN in the absence (*A* and *B*) or presence (*C* and *D*) of MSO for 5 days. Cells were processed for transmission electron microscopy (TEM) as described in MATERIALS AND METHODS. The junction structures between the adjoining cells were observed under TEM. Arrows, tight junctions; arrowheads, desmosomes. Bars = 500 nM.

know how this might relate to the discrepancy between decreased ZO-1 protein in the immunoblots and normal discrete junctions in the immunofluorescence studies. The electron microscopy supports the protein immunoblot data by demonstrating a poorly visualized TJ in the GLN-deprived group compared with the GLN-supplemented groups or the group that was allowed to synthesize GLN via GS (Fig. 5).

Several stressors have been found to have profound effects on intestinal TJ. These include bacterial toxins (30), commonly used drugs such as indomethacin (30), and allergens including gliadin (5). To our knowledge, the current studies are the first to establish a relationship between intestinal cell GLN status and intercellular junction proteins. The fact that claudin-1 and occludin were affected by GLN is of interest. The claudin family consists of ~15 closely related proteins, several of which have been found in distinct tissues and defects of which have been implicated in several disease processes involving paracellular permeability (16, 37). Claudin-1 was chosen for evaluation in this study primarily because of its previous association with intestinal epithelial cells (41). Claudin and occludin appear to work in concert and appear to be necessary for normal paracellular function (14, 15).

Although these studies demonstrate a relationship between TJ proteins and paracellular biophysical, physiological properties, and intestinal epithelial GLN status, the mechanisms of these effects remain unclear. Although the possibility exists that the decrease in TJ proteins is simply the result of altered protein synthesis, the overall TJ protein concentrations did not differ on the Western blots, whereas the greatest effect was seen in the detergent-insoluble fractions, which suggests a more direct effect on TJ protein localization. This conclusion was validated by both light and electron microscopy in these studies.

There currently exist several candidate pathways that might be related to nutrition and stress-related signaling mechanisms of TJ assembly (26). Several lines of evidence have implicated phosphatidylinositol 3-kinase (PI3K) in regulating TJ assembly. Previous investigators have found that prostaglandins stimulate recovery of paracellular resistance via a mechanism involving transepithelial osmotic gradients and PI3K-dependent restoration of TJ protein distribution (21). These findings suggest that the PI3K, phosphatidylinositols, and filamentous actin rearrangements, in combination, play an important role in the modulation of the junction integrity.

In addition to PI3K, two Rho family GTPases, Rho and Rac, have also emerged as key regulators acting antagonistically to regulate endothelial barrier function: Rho increases actin-myosin contractility, which facilitates breakdown of intercellular junctions, whereas Rac stabilizes endothelial junctions and counteracts the effects of Rho (40). Similar mechanisms (Rho and Rac) are implicated in the regulation of intestinal epithelial junction integrity. Whether GLN affects either PI3K or the Rho/Rac pathways remains speculative.

In summary, GLN status affects TJ proteins in a manner that is commensurate to its effects on electrical resistance and permeability. Further evaluation of nutritional agents affecting these proteins and their mechanism of action will be critical because they have major implications in the pathogenesis of several disease entities.

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