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Glutamine Therapy Improves Outcome of In Vitro and In Vivo Experimental Colitis Models

Hongyu Xue, MD, PhD; Alexandra J. D. Sufit; and Paul E. Wischmeyer, MD

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Background: Pharmacologic doses of glutamine (GLN) can improve clinical outcome following acute illness and injury. Recent studies indicate enhanced heat shock protein (HSP) expression is a key mechanism underlying GLN's protection. However, such a link has not yet been tested in chronic inflammatory states, such as experimental inflammatory bowel disease (IBD). *Methods:* Experimental colitis was induced in Sprague-Dawley rats via oral 5% dextran sulfate sodium (DSS) for 7 days. GLN (0.75 g/kg/d) or sham was administered to rats by oral gavage during 7-day DSS treatment. In vitro inflammatory injury was studied using YAMC colonic epithelial cells treated with varying concentrations of GLN and cytokines (tumor necrosis factor–α/interferon-γ). *Results:* Pharmacologic dose, bolus GLN attenuated DSS-induced colitis in vivo with decreased area under curve for bleeding (8.06 ± 0.87 vs 10.38 ± 0.79, $P < .05$) and diarrhea (6.97 ± 0.46 vs 8.53 ± 0.39, $P < .05$). This was associated with enhanced HSP25 and HSP70 in colonic mucosa. In vitro, GLN enhanced cell survival and reduced proapoptotic caspase3 and poly(ADP-ribose) polymerase cleavage postcytokine injury. Cytokine-induced inducible nitric oxide synthase expression and nuclear translocation of nuclear factor-κB p65 subunit were markedly attenuated at GLN concentrations above 0.5 mmol/L. GLN increased cellular HSP25 and HSP70 in a dose-dependent manner. *Conclusions:* These data demonstrate the therapeutic potential of GLN as a "pharmacologically acting nutrient" in the setting of experimental IBD. GLN sufficiency is crucial for the colonic epithelium to mount a cell-protective, antiapoptotic, and anti-inflammatory response against inflammatory injury. The enhanced HSP expression observed following GLN treatment may be responsible for this protective effect. (*JPEN J Parenter Enteral Nutr*. 2011;35:188-197)

Keywords: glutamine; inflammatory bowel disease; colitis; heat shock protein; NF-κB

Clinical Relevancy Statement

Interventions aiming to enhance heat shock response could furnish a key approach to improve outcomes of inflammatory bowel disease (IBD). The findings of this study suggest that glutamine (GLN) has the potential to be developed as the first clinically relevant enhancer of heat shock response to improve outcomes in IBD. Further, this study demonstrates that the use of a pharmacologically administered bolus dose GLN that has been validated as clinically efficacious in other states of inflammatory injury[42,43] may be utilized in the setting of IBD.

From the Department of Anesthesiology, University of Colorado Denver, Anschutz Medical Campus, Aurora, Colorado.

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Address correspondence to: Hongyu Xue, MD, PhD, University of Colorado Denver, Anschutz Medical Campus, Research Complex II, Room 7490, 12700 East 19th Ave, Aurora, CO 80045; e-mail: Hongyu.Xue@ucdenver.edu.

Introduction

The pathogenesis of inflammatory bowel disease (IBD) largely depends on the sophisticated interplays between injurious factors present in the gut environment and the cytoprotective response employed by the host.[1] Nutrition factors in the diet have frequently been hypothesized to modify key mechanisms involved in the host's protection and repair response following injury.[2-4]

Glutamine (GLN), a preferred respiratory fuel for the gut epithelium, has long been studied as a promising agent to preserve intestinal functional/structural integrity and promote intestinal reconstruction/recovery during various injurious or stress conditions.[5-11] Accumulating evidence has shown that GLN is a key "pharmacologically acting nutrient." High doses of GLN given via bolus administration demonstrate therapeutic benefits in the gut, extraintestinal tissues, and the whole organism during acute lethal and sublethal conditions.[12-17] In these studies, a high-dose bolus paradigm of GLN treatment at or approaching 0.5 g/kg/d is more in a pharmacological mode rather than nutrition mode of administration, which may exceed the physiologic requirements, even in

GLN-deficiency–prone conditions.[18] Recent studies by our group have shown that bolus GLN in pharmacologic doses can safely enhance the key protective heat shock protein (HSP) response in experimental models of inflammatory and infectious injury.[12-15] GLN-mediated induction of the HSP response appears to be vital for GLN's protection against injury.[19,20]

Induction of HSPs is an important innate mechanism to protect cells against stress/injury, and adequate expression of HSPs (eg, HSP25, HSP70, HO-1) is essential to maintain intestinal epithelial resistance to inflammation-related stress in experimental IBD.[21-23] One key mechanism underlying HSPs' protective role is through down regulating the activation of proinflammatory transcription factor nuclear factor (NF)–κB.[24-26] Overactivated NF-κB can lead to a disrupted balance between anti-inflammatory and proinflammatory mediator production by inducing transcription of genes participating in the inflammatory cascade, such as inducible nitric oxide synthase (iNOS).[27] A recent key finding by Hu et al[28,29] demonstrated HSP expression is depressed in human IBD lesions. This impaired HSP expression may contribute to the dysregulated proinflammatory response seen in IBD patients. As a result, promoting HSP expression to attenuate the dysregulated inflammatory response may be a promising therapeutic target for the treatment of IBD. GLN is the only clinically validated enhancer of human HSP expression[30] and has an extensive history of clinical safety in a wide variety of patient populations. Thus, GLN has the potential to be the first clinically relevant enhancer of heat shock response to improve outcomes in IBD. However, the use of pharmacologic bolus doses of GLN has not been tested in the context of IBD, and the role of GLN-mediated enhancement of HSP expression in the treatment of IBD is currently unstudied. In this study, we tested the therapeutic potential of GLN as a pharmaconutrient in the in vivo and in vitro experimental colitis models. Furthermore, we explored the hypothesis that GLN's effects on clinical disease activity, cell survival, and NF-κB signaling are related to enhanced HSP expression.

Materials and Methods

Animal Study

Animal use was approved by the Institutional Animal Care and Use Committee of the University of Colorado. Male Sprague-Dawley rats initially weighing between 250 and 300 g were obtained from Charles River (Wilmington, MA). Rats were housed individually at a constant room temperature, with 12-hour light and dark cycles, and fed standard rodent chow (Purina 5001, Purina Mills, Richmond, IN) and water ad libitum. Following a 7-day acclimation period in individual housing, colitis was induced in all the rats by replacing normal drinking water with distilled water containing 5% dextran sulfate sodium (DSS; w/v; molecular weight 36,000–50,000; MP Biomedicals, Aurora, OH). DSS water was provided ad libitum for 7 days. Concurrent GLN treatment started immediately before DSS treatment initiated and was continued throughout the whole 7-day DSS colitis induction. GLN (0.75 g/kg/d) or sham (isovolemic sterile water) was administered to animals by oral gavage. GLN treatment delivered ~1.2% extra energy intake and ~4.8% extra N intake as compared with sham treatment. GLN (Sigma-Aldrich, St Louis, MO) was made as a 3% (w/v) solution immediately before use and filtered with a 0.45-μm filter. The time when DSS treatment was initiated was designated as day 0. The animals were killed by CO_2 asphyxiation after 7 days (day 7) of DSS ± GLN treatment. A total of 13 rats were used for GLN or sham-treated group.

The disease activity was evaluated by assigning well-established and validated scores for parameters that are analogous to the clinical presentation of human IBD. A modified (17) scoring system of Cooper et al[31] was used: (a) weight loss (0 points—none, 1 point—1%–5% weight loss, 2 points—5%–10% weight loss, 3 points—10%–15% weight loss, and 4 points—more than 15% weight loss); (b) stool consistency/diarrhea (0 points—normal, 2 points—loose stools, 4 points—watery diarrhea); and (c) bleeding (0 points—no bleeding, 2 points—slight bleeding, 4 points—gross bleeding). The disease activity index (DAI) was calculated as the total of these scores: the sum of weight loss, diarrhea, and bleeding. The above clinical measures were scored daily throughout the study period. Data are presented as area under curve (AUC) of bleeding and diarrhea score. AUC of bleeding/diarrhea score was calculated from the diarrhea/bleeding score time graph of each individual animal between days 3 and 7, when diarrhea developed to its full severity.[14,15] All disease activity assessments were conducted by one person who was blinded to experimental treatments.

When rats were killed on day 7, the full length of the colon was washed and longitudinally cut into halves, one of which was fixed in 10% (v:v) neutral buffered formalin for hematoxylin/eosin staining, and the mucosa was gently scraped off the other half and homogenized for protein extraction.

Cell Culture

Conditionally immortalized young adult mouse colonic epithelial cells (YAMC) were provided by Dr Robert Whitehead, Vanderbilt University (Nashville, TN). YAMCs were grown at 33°C, as previously described.[32] Before use, all cells were cultured in interferon (IFN)–γ-free media

under nonpermissive conditions of 37°C for 24 hours to induce colonocyte differentiation. During this time, SV40 large T antigen is no longer produced, and any remaining protein misfolds because of a temperature-sensitive mutation at amino acid 58 (tsA). After the initial 24-hour differentiation induction period, the GLN-containing media (2 mmol/L L-GLN) were replaced by GLN-free media for 24 hours of washout period to induce GLN deprivation. After this period, cells were exposed to media containing 0, 0.25, 0.5, 2, or 4 mmol/L GLN. Concomitantly, a combination of murine IFN-γ (200 U/mL) and tumor necrosis factor (TNF)–α (100 ng/mL) was added to media with varied GLN concentrations to induce injury. A separate set of cells treated with 0 mmol/L GLN media did not receive cytokine treatment, serving as a non–cytokine injury control. Eight hours after the treatment of cytokine and GLN, cell survival was determined via MTS assay, or cells were lysed for protein extraction. Each experiment repeated 4 times.

MTS Cell Proliferation Assay

Cell viability was assayed with 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)/ phenazine methosulfate (PMS) mixture (Promega, Madison, WI). For cell viability preparations, 7,000/well cells were seeded in 96-well plates. According to the manufacturer's recommendations, 1 part PMS was added to 20 parts MTS immediately before the solution was diluted 1:5 in phenol red-free media and added to phosphate-buffered saline (PBS) washed cells. MTS was bioreduced by cells into a colored, soluble formazan product. Absorbance values were read after 2.5 hours at 490 nm using an enzyme-linked immunosorbent assay (ELISA) plate reader (Thermo Electro Corporation, San Jose, CA); references included readings at 650 nm and no-cell blank wells. Higher absorbance values reflect greater cell proliferation/viability. The values for the same 8 wells were averaged. Data are expressed as percent survival of cytokine-treated cells as compared with their corresponding non–cytokine-treated controls at a certain GLN concentration.

Western Blots

Rat mucosal tissue was homogenized or YAMCs were lysed in a solution containing 10 mM Tris (pH 7.2-8), 5 mM $MgSO_4$, DNAse, and RNAse, per manufacturer's instructions (New England Biolabs, Ipswich, MA), plus protease inhibitors (Roche, Indianapolis, IN). To investigate the intracellular localization of HSP25 and HSP70, cells were lysed with the Pierce Nuclear/Cytoplasmic fractionation kit (Pierce, Rockford, IL). Protein was determined with bicinchoninic acid (BCA) protein assay (Pierce). Then, 20 mcg of each sample was added to a 1× treatment buffer (0.125 M Tris [pH 6.8], 4% sodium dodecyl sulfate, 20% glycerol, and 10% β-mercaptoethanol), boiled for 3 minutes, and then loaded into a NuPAGE 4%-12% Bis-TrisGel (Invitrogen, Carlsbad, CA). Proteins were electrophoretically separated with a mini-gel system and transferred to polyvinylidene fluoride (PVDF) membranes (Millipore, Billerica, MA), using the semi-dry transfer system (Invitrogen). Membranes were blocked with 5% nonfat milk in PBS-Tween for 2 hours at room temperature. Primary antibodies against HSP25 (1:3,000 dilution; StessGen, Victoria, BC, Canada), HSP70 (1:25,000 dilution; StressGen), Caspase3 (1:1,000; Cell Signaling, Danvers, MA), poly(ADP ribose) polymerase (PARP; 1:1,000; Cell Signaling), iNOS (1:1,000; Cell Signaling), NF-κB p65 protein (1:2000; Santa Cruz Biotechnology), or β-actin (1:10,000; Sigma-Aldrich) were added to the blocking solution and incubated overnight at 4°C. After washing 3 times with PBS-Tween over 30 minutes, secondary antibodies, peroxidase-conjugated goat antimouse (1:10,000; Pierce) or donkey-antirabbit (1:5,000; Santa Cruz Biotechnology) immunoglobulinG, were incubated at room temperature for 2 hours. Blots were washed 3 times with PBS-Tween over 30 minutes, incubated in commercial-enhanced chemiluminescence reagents (Pierce), and exposed by using a UVP chemiluminescent darkroom system (UVP, Upland, CA). Quantification of images was done by scanning densitometry using LabWorks 4.0 Image Acquisition and Analysis Software (UVP).

Statistical Analysis

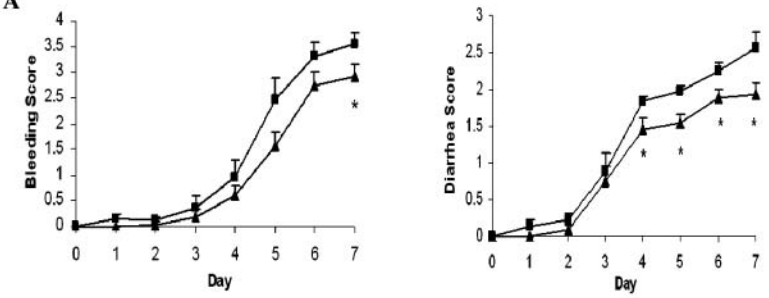
Data are expressed as mean ± standard error of the mean. For animal study, treatment differences in development of bleeding or diarrhea was tested using 2-way analysis of variance (ANOVA) followed by Bonferroni posttest (SPSS 12.0; SPSS, Inc., Chicago, IL); for AUC, DAI, or colon length measures, the effects of GLN were analyzed using the unpaired 2-tailed Student *t* test for independent samples. For cell culture studies, treatment differences in cell survival or densitometry were analyzed using 1-way ANOVA followed by Bonferroni posttest (SPSS 12.0). A P value 0.05 was accepted as being statistically significant.

Results

Bolus GLN Attenuates DSS-induced Colitis In Vivo and Enhances HSP Expression

The time course of bleeding and diarrhea associated with the development of colitis is shown in Figure 1A. DAI reached its maximal level at day 7 for both groups. Bolus GLN treatment significantly improved the IBD-related symptomatology by decreasing the DAI and area under the curve of bleeding and diarrhea scores (Figure 1B).

A



B

Treatment group	†N	DAI at Day 7	AUC of bleeding	AUC of diarrhea	AUC of bleeding+ diarrhea score	‡Colon length (cm/100g body weight)
Sham	13	6.98±0.43	10.30±0.60	8.64±0.26	18.93±0.73	7.53±0.18
GLN	13	5.56±0.54*	7.81±0.64**	6.83±0.50***	14.63±1.06**	8.11±0.22*

Figure 1. Glutamine (GLN) treatment attenuates dextran sulfate sodium (DSS)–induced colitis. Rats received DSS plus GLN or sham treatment for 7 consecutive days (days 0–7); bleeding and diarrhea were scored once per day from days 0-7. (A) Effect of GLN on the time course of bleeding (left) and diarrhea (right); (■) sham, (▲)GLN. *Different from sham at the indicated time point, $P < .05$, via Bonferroni posttests. (B) Effect of GLN on disease activity. †N, total animal number of each treatment group. ‡Colon length was normalized to the animal body weight at sacrifice. +Area under curve (AUC) of bleeding/diarrhea score was calculated from the score time graph of each individual animal between day 3 and day 7. *$P < .05$, **$P < .01$, or ***$P < .005$ compared with sham by Student *t* test. All data are presented as mean ± standard error of the mean.

Colon length has been reported to be negatively correlated with colitis severity and, therefore, is a useful inflammation indicator for colitis.[33,34] GLN treatment significantly increased colon length compared with sham treatment (Figure 1B). Since feed intake was identical between groups (data not shown), its effects on the examined endpoints could therefore be excluded.

Associated with the improvements of the IBD-related disease activity, enhanced expression of stress-inducible HSPs (HSP70 and HSP25) was observed in the colonic mucosa of rats receiving bolus GLN treatment. HSP25 and HSP70 abundance was increased by 152% and 70%, respectively, as compared to sham treatment (Figure 2). No difference in β-actin expression was observed between the 2 groups.

GLN Enhances YAMC Cell Survival and Decreases Apoptosis Following Cytokine Injury

Eight hours after the concurrent exposure to cytokine injury and GLN, only 61.2% ± 5.2% YAMCs treated with 0 mmol/L GLN survived compared with its non–cytokine-treated controls at 0 mmol/L GLN, whereas 0.25 mmol/L GLN increased the percent survival to 79.4 ± 1.5 ($P < .05$ vs 0 mmol/L GLN; Bonferroni posttest), and 0.5 mmol/L GLN further increased the percent survival to 85.8 ± 0.9 ($P < .01$ vs 0.25 mmol/L GLN; Bonferroni posttest; Figure 3A). The protective capacity of GLN seems to approach its maximum at 0.5 mmol/L in this model, as survival rates at higher concentration at 2 and 4 mmol/L (87.9% ± 2.1% and 89.4% ± 1.8%, respectively) are comparable to survival at 0.5 mmol/L ($P = .4$ and .2 vs .5 mmol/L, respectively).

We further related GLN's effect on overall cell viability following cytokine injury to the activation of apoptosis. Cleavage and activation of 2 apoptotic enzymes were evaluated (Figure 3B). Caspase3, an asparagine protease, is considered a primary mediator of the apoptotic pathway that initiates numerous apoptotic signals, including poly (ADP-ribose) polymerase (PARP) cleavage, a key marker for apoptosis.[35,36] Cytokine treatment led to a marked activation of caspase3 and PARP in cells treated with 0 mmol/L GLN. However, such an effect was blunted with increased GLN supply in the media. No cleaved caspase3 was detected when GLN was 0.25 mmol/L or higher. Cleaved PARP was undetectable, and

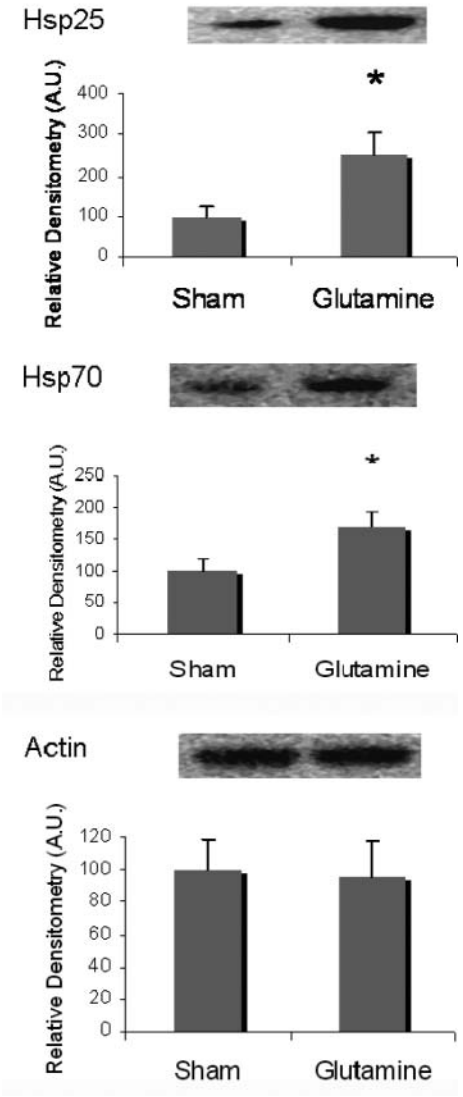


Figure 2. Glutamine (GLN) treatment upregulates colonic mucosal HSP25 and HSP70 protein in rats with colitis. Rats received dextran sulfate sodium (DSS) plus GLN or sham treatment for 7 consecutive days (days 0-7), and accumulation of stress-inducible HSP (HSP25 and HSP70) in colonic mucosa was examined by Western blot at day 7. Relative densitometry is expressed as mean ± standard error of the mean, n = 13/treatment group. *$P < .05$ compared with sham by Student *t* test.

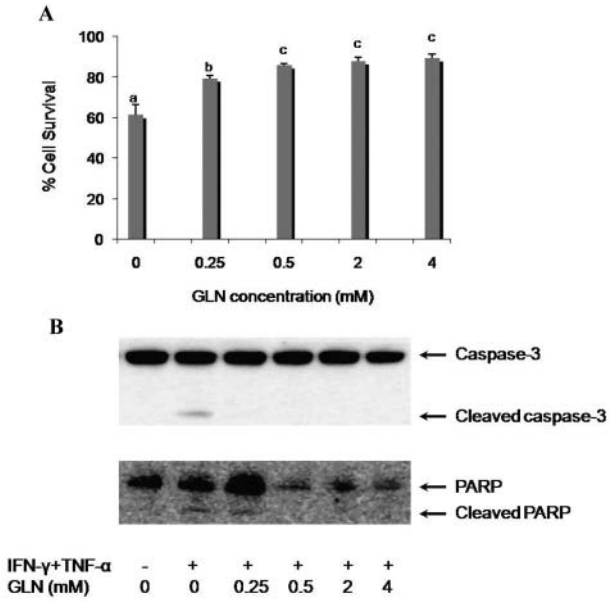


Figure 3. Glutamine (GLN) enhances young adult mouse colonic epithelial (YAMC) cell survival and decreases apoptosis following cytokine injury. YAMC cells were treated concomitantly with 0, 0.25, 0.5, 2, or 4 mmol/L GLN and cytomix (tumor necrosis factor [TNF]–α [100 ng/mL] and interferon [IFN]–γ [200 U/mL]) for 8 hours before harvest. (A) Effect of GLN on cell viability determined via MTS assay. *Y*-axis represents percent survival of cytomix-treated cells as compared with their corresponding non-cytomix-treated controls at a certain GLN concentration. Means that do not share a common symbol are significantly different ($P < .05$) via Bonferroni posttests. All data are presented as mean ± standard error of the mean. (B) Effect of GLN on cleavage of poly(ADP ribose) polymerase (PARP) and caspase-3 examined by Western blot. Cells cultured at 0 mM GLN that did not receive cytokine treatment were used as control. Image shown representative of 4 individual experiments.

total PARP expression was markedly reduced when GLN was 0.5 mmol/L or higher.

GLN Downregulates NF-κB Proinflammatory Pathway Activation in YAMC Cells Following Cytokine Injury

Activation of the NF-κB pathway has been identified as a key factor leading to perpetuation of mucosal inflammation associated with IBD.[37] Upon activation, NF-κB proteins such as p65 are translocated into the nucleus, where they mediate the transcriptional activation of numerous target genes. One of the target proinflammatory genes is iNOS, and excessive NO production via iNOS is believed to play a critical role in initiation and maintenance of IBD.[38] At 0 mmol/L GLN, cytokine treatment induced a 2.91-fold increase in the nuclear appearance of NF-κB p65 ($P < .05$). Consistent with this, a striking cytokine-induced iNOS expression was observed at 0 mmol/L GLN. As shown in Figure 4, nuclear translocation of NF-κB p65 protein was suppressed with increased GLN in media in a dose-dependent manner. Cytokine-induced expression of iNOS was markedly blunted when GLN concentration was 0.5 mmol/L or higher.

GLN Upregulates HSP25 and HSP70 in YAMC Cells Following Cytokine Injury

Associated with enhanced cell viability and suppressed NF-κB activation following cytokine injury, GLN treatment

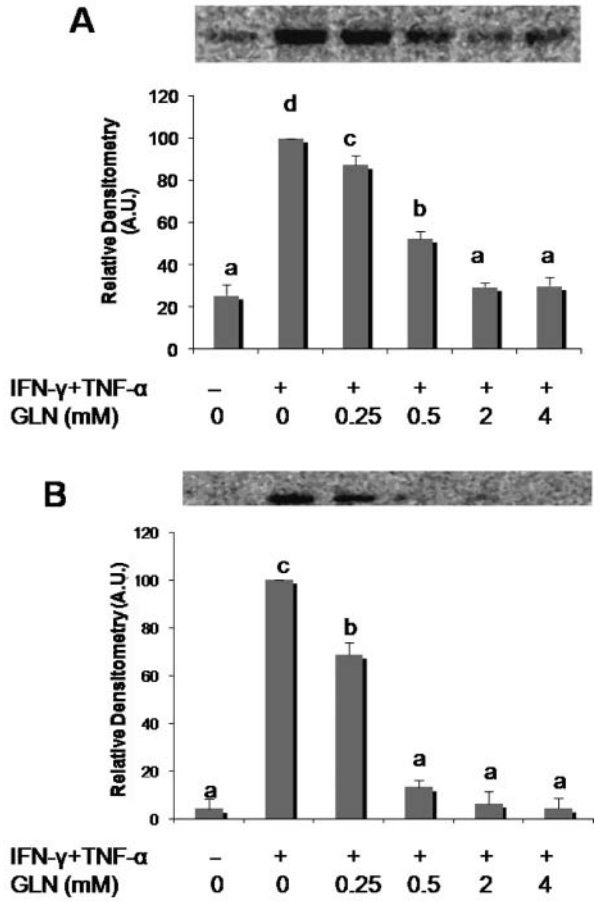


Figure 4. Glutamine (GLN) downregulates nuclear factor (NF) κB proinflammatory pathway activation in young adult mouse colonic epithelial (YAMC) cells following cytokine injury. (A) GLN attenuates NF-κB p65 subunit nuclear translocation following cytokine treatment. NF-κB p65 protein was analyzed by Western blot in the nuclear fraction of cell extracts. (B) GLN attenuates cytokine-induced nitric oxide synthase (iNOS) protein expression as determined by Western blot. Cells cultured at 0 mM GLN that did not receive cytokine treatment were used as control. Image shown representative of 4 individual experiments. Results of relative densitometry are expressed as mean ± standard error of the mean, n = 4. Means that do not share a common symbol are significantly different ($P < .05$) via Bonferroni posttests.

enhanced the total cellular abundance of HSP25 and HSP70 in a dose-dependent manner (Figure 5A) after cytokine challenge. For both HSP25 and HSP70, the maximal enhancement of expression was achieved when GLN was supplied at 2 mmol/L or higher. As shown in Figure 5B, a dose-dependent enhanced nuclear appearance of HSP70 was also observed with increased GLN concentration in the media. When GLN was supplied at 2 mmol/L or higher, nuclear accumulation of HSP25 could also be observed.

Discussion

High-Bolus GLN Conferred Benefits in Colitis-Related Outcomes

There have been a relatively small number of studies directed toward the therapeutic use of GLN in the setting of IBD. Although limited, preclinical data are consistently supportive of a positive role of GLN as a therapeutic in IBD. In models of chemical-induced colitis, enteral or topical GLN treatment was shown to improve a variety of clinical, pathohistological, cellular, and biochemical endpoints of colitis, including DAI,[39] macroscopic and microscopic damage,[39-41] increased gut luminal water content,[42] enhanced epithelial apoptosis,[43] impaired gut barrier function,[44] reduced tissue antioxidant defense/enhanced oxidative stress,[40,41,43,45] and elevated activity or expression of proinflammatory mediators (eg, IL-8, TNF-α, NF-κB, iNOS, and COX-2).[39-41,45]

Nevertheless, results from clinical trials using GLN as a therapeutic in IBD are considerably mixed. A pilot study compared effects of topical use of GLN and butyrate on pouchitis in patients receiving ileal pouch-anal anastomosis. During the 21-day treatment, 6 of the 10 patients who received GLN suppository had no recurrence of symptoms, whereas only 3 of the 9 patients who received butyrate suppository had no recurrence.[46] In long-term follow-up, some of the GLN-treated patients remained in remission for up to 6 months (S. Phillips, MD, personal communication,). Nonetheless, oral GLN treatment at 8.5 g/d via GLN-enriched diet[47] or 7 g 3 times daily in single GLN form[48] or GLN supplied in parental nutrition (PN) at 0.2 g/kg/d[49] was reported to offer no benefits in clinical outcomes in patients with IBD. Markedly insufficient (or nonpharmacologic) doses of GLN used in these trials may potentially confound the interpretation of these observations. In settings of critical illness or cancer chemotherapy, studies with positive results consistently have used higher "pharmacological" GLN doses that exceed the usual physiological requirements.[30,50-52] This is particularly true in critical illness, where systemic inflammation (systemic inflammatory response syndrome [SIRS]) plays a major role in outcome. Data have shown that doses of less than 0.2 g/kg/d are consistently ineffective at inducing improvements in clinical outcomes such as infection rate or mortality. Doses at or approaching 0.5 g/kg/d have consistently shown statistically significant clinical treatment effects on infection and mortality in the critically ill patient (www.criticalcare-nutrition.com).[53,54] As compared with the topical route,[46] the small doses given by oral or parental route used in these IBD trials[47-49] are unlikely to lead to an acute and/or substantial accumulation of GLN in the distal bowel and further are unlikely to induce stress-protective pathways and anti-inflammatory effects.[18]

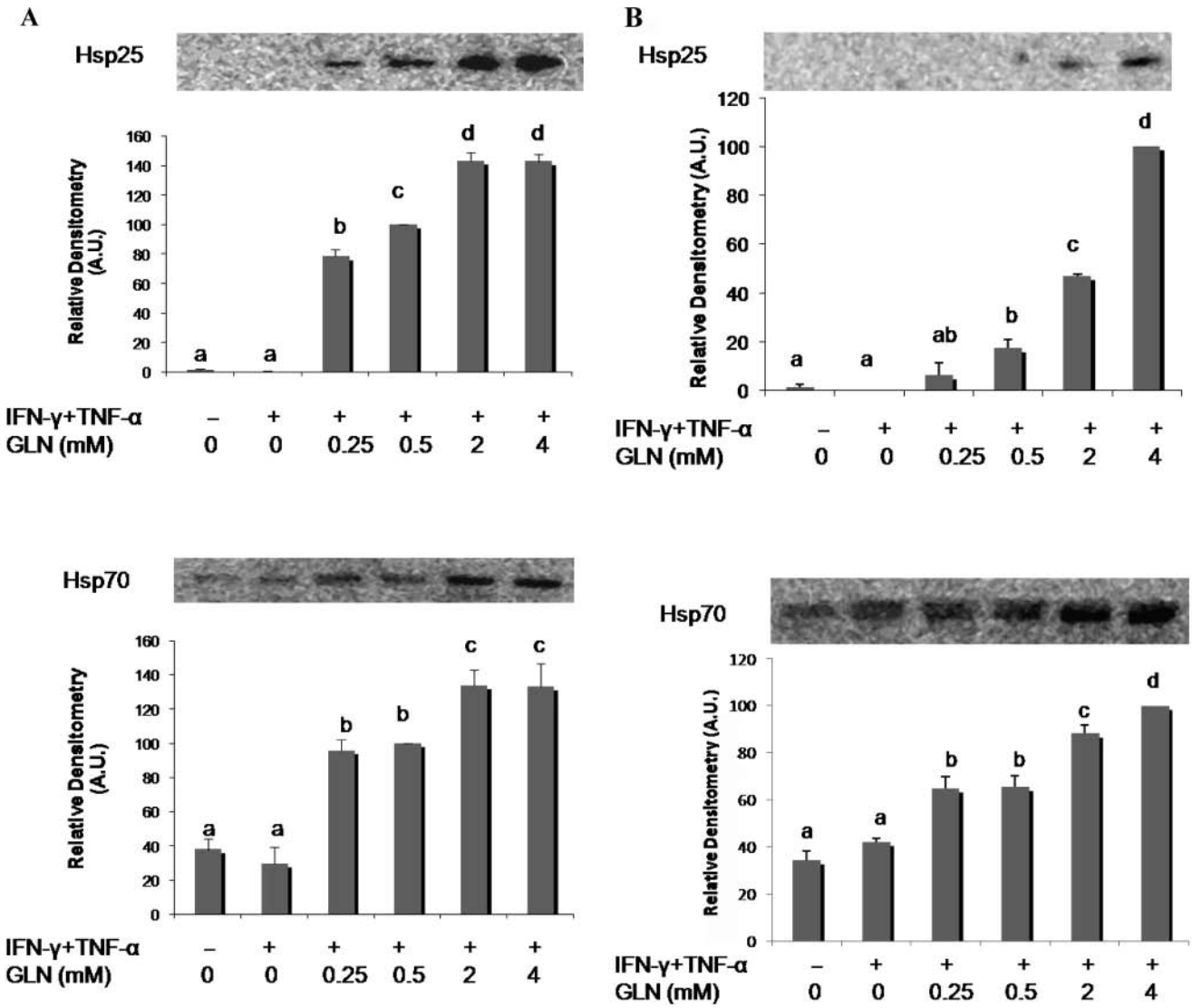


Figure 5. Glutamine (GLN) upregulates heat shock protein (HSP) 25 and HSP70 protein in young adult mouse colonic epithelial (YAMC) cells following cytokine injury. (A) GLN enhances total cellular HSP25 and HSP70 protein expression determined by Western blot. (B) GLN enhances HSP25 and HSP70 nuclear translocation. HSP25 (upper) and HSP70 (lower) proteins were analyzed by Western blot in the nuclear fraction of cell extracts. Cells cultured at 0 mM GLN that did not receive cytokine treatment were used as control. Image shown representative of 4 individual experiments. Results of relative densitometry are expressed as means ± standard error of the mean, n = 4. Means that do not share a common symbol are significantly different ($P < .05$) via Bonferroni posttests.

In this study, we used the pharmacologic dose, bolus GLN administration paradigm, which had shown efficacy in critical illness conditions, including endotoxin shock, ischemia–reperfusion injury, sepsis, hyperthermia, and dose-intensive chemotherapy.[12-17] Compared with the incorporation of amino acids into the diet, a conventional paradigm used in experimental nutrition studies, such a high-bolus paradigm, actually blurs the lines between pharmacologic and nutrition modes of administration. Using this dosing paradigm, we were able to improve clinical outcomes in our colitis model.

Considerable GLN deficiency has been consistently reported in active Crohn's disease and ulcerative colitis lesions of human biopsies.[55,56] In our in vitro inflammatory injury model, insufficient GLN supply at concentrations less than 0.5 mmol/L in the media, which mimics tissue GLN deficiency in vivo, renders the cells more sensitive to cytokine-induced cell death. Cells treated with 0 or 0.25 mmol/L GLN had less viability and increased apoptotic cell death, as shown by the increased caspase-3 and PARP cleavage. Thus, GLN sufficiency is essential for colonic epithelial cells to mount resistance

to inflammation-induced cell death. In light of this, high-bolus GLN supply in vivo may pharmacokinetically lead to an adequate and sustained elevation of tissue GLN level, which is likely essential for correcting GLN deficiency and overcoming the threshold for eliciting benefits of exogenous GLN.

GLN Upregulating Heat Shock Response, an Inherent Cytoprotective Machinery

This set of studies demonstrates for the first time that GLN enhances colonic epithelial expression of inducible HSPs in both in vivo and in vitro experimental IBD-like colitis, suggesting this is a key mechanism underlying GLN's action in this setting. Consistent with our findings in experimental colitis, a marked induction of HSPs was also reported with bolus GLN administration in a variety of other settings such, as hyperthermia, sepsis, and cancer chemotherapy.[12-15,17] In our in vivo study, GLN treatment per se brought in only less than 1/80 and 1/20 extra energy and nitrogen intake, respectively, and nonspecific effects rendered by such a small energy/nitrogen intake increase could be negligible, if not completely ruled out. We have previously shown that isonitrogenous amino acid mixtures at the same pharmacologic doses as GLN produce no effects on heat shock response during stress, suggesting GLN's effect on HSP modulation is rather irreplaceable.[13,30] Still, use of an isonitrogenous and isocaloric control would be an asset for a rigorous comparison to exclude energy/nitrogen-related effects.

The induction of HSP is an important innate mechanism employed by cells to protect themselves against stressors.[57] The intestinal epithelial HSPs (eg, HSP70, HSP25, and HO-1) play a pivotal role in protecting mucosal integrity and function and could be a potential target for boosting the cytoprotective machinery during IBD.[1,58] Inducible HSP, such as HSP70 and HSP25 probably through promoting the refolding of critical, cell-essential proteins,[59] mitigate the injurious actions related to inflammatory insults. HSPs also preserve intestinal barrier function upon stressor insults by maintaining integrity of intestinal epithelial cells' tight junction,[21] preventing permeability changes,[21,60] or preserving cytoskeletal integrity.[61] Furthermore, we also observed enhanced nuclear appearance of HSPs with increased GLN supply in the media. Nuclear translocation of HSPs such as HSP25 and HSP70 has been observed following stress such as heat shock.[62-64] Nuclear appearance of these proteins is proposed to play an antiapoptotic role, such as through protecting nuclear lamina and thus preventing nuclear breakdown during stress.[64]

Accumulating evidence reveals the anti-inflammatory role of HSP70 and HSP25 is largely derived from their downregulation of the NF-κB pathway.[24-26] Transcription factor NF-κB has been established as one key mediator leading to dysregulated anti-inflammatory/proinflammatory balance implicated in IBD pathogenesis.[65-67] Intestinal epithelial cells and macrophages in active lesions of IBD patients have upregulated expression and activation of NF-κB,[66,68] and NF-κB activation is correlated with the severity of gut inflammation.[68] Once activated, transcriptionally active NF-κB p50/p65 protein is translocated into the nucleus, where it initiates the transcription of a multitude of inflammatory genes, including iNOS.[27] Upregulation of iNOS and the overproduction of NO have been consistently reported in both experimental and human IBD,[69-71] and iNOS-derived NO has been shown to directly or indirectly contribute to the initiation and/or progression of IBD.[72-74] In our in vitro model, enhanced HSP expression by GLN was associated with attenuated cytokine-induced p65 nuclear translocation and iNOS expression. This suggests that enhancing epithelial HSP expression may represent a promising therapeutic target for treating IBD via downregulating NF-κB and its downstream proinflammatory mediator(s).

Hu et al[28,29] show that HSP expression is depressed in human patient IBD lesions, which could exacerbate the impaired anti-inflammatory/proinflammatory balance. Thus, promoting inducible HSP expression in intestinal epithelia may be an obvious and effective intervention in treating IBD. Experimental methods using hyperthermia,[75] chemicals (eg, geranylgeranylacetone,[76] zinc L-carnosine[77]), or HSP70 transgenic technology[78] to enhance HSP expression have been shown to protect against chemical-induced colitis by downregulating NF-κB or reducing inflammatory mediator production. Nonetheless, these laboratory approaches are not practical for clinical application due to their inherent toxicities.[79,80] Given the longstanding safety profile of clinical use, GLN has the potential to be developed as the first clinically relevant enhancer of heat shock response in IBD, and this may be key to improving outcomes in IBD patients.[30] Understanding the pharmacokinetics of exogenously supplied GLN in relation to tissue GLN content, epithelial HSP expression, and further control of inflammation/injury will be key in optimizing GLN's efficacy.

Acknowledgment

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