Epidermal growth factor receptor expression and signaling are essential in glutamine’s cytoprotective mechanism in heat-stressed intestinal epithelial-6 cells

Stefanie Niederlechner,1 Christine Baird,1 Benjamin Petrie,1 Erhard Wischmeyer,2 and Paul E. Wischmeyer1

1Department of Anesthesiology, University of Colorado, Colorado, Aurora, Colorado; and 2Institute of Physiology, University of Würzburg, Würzburg, Germany

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Niederlechner S, Baird C, Petrie B, Wischmeyer E, Wischmeyer PE. Epidermal growth factor receptor expression and signaling are essential in glutamine’s cytoprotective mechanism in heat-stressed intestinal epithelial-6 cells. Am J Physiol Gastrointest Liver Physiol 304: G543–G552, 2013. First published December 28, 2012; doi:10.1152/ajpgi.00418.2012.—Epidermal growth factor receptor (EGFR) expression and signaling can induce cellular protection after intestinal inflammation. L-Glutamine (GLN) is known to prevent apoptosis after intestinal injury by activating MAPK and phosphatidylinositol 3-kinase (PI3-K)/Akt pathways. However, the role of EGFR expression and signaling in GLN-mediated cellular protection in intestinal epithelial-6 (IEC-6) cells after heat stress (HS) is unknown. To address the role of EGFR in GLN-mediated protection, IEC-6 cells were treated with GLN in the presence or absence of EGFR small interfering RNA, the EGFR tyrosine kinase inhibitor AG1478, the ERK1/2 inhibitor PD98059, the p38MAPK inhibitor SB203580, or the PI3-K/Akt inhibitor LY294002 under basal and HS conditions. GLN-mediated cell survival was measured using 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. Phosphorylated and/or total levels of EGFR, cleaved caspase-3, poly(ADP-ribose) polymerase-1, ERK1/2, p38MAPK, and Akt were assessed by Western blotting. We showed that HS induced a decrease in total, cytoplasmic, and nuclear EGFR levels in IEC-6 cells, which was prevented by GLN supplementation, leading to attenuated apoptosis via EGFR small interfering RNA. Furthermore, the protective effect of GLN was lessened by AG1478, PD98059, and LY294002 but was not affected by SB203580. AG1478 attenuated GLN-mediated increases in ERK1/2 and decreases in p38MAPK phosphorylation. However, AG1478 had no effect on GLN-mediated augmentations in Akt phosphorylation. In summary, EGFR expression was important in the protective mechanism of GLN, as well as GLN-mediated activation of EGFR tyrosine kinase activity. GLN-mediated EGFR signaling activated ERK1/2 and decreased p38MAPK signaling. However, GLN-mediated Akt phosphorylation after HS seems to be independent of EGFR signaling. ERK1/2; p38MAPK; phosphatidylinositol 3-kinase; Akt; apoptosis

THE INTESTINAL MUCOSA is under continuous physiological stress from factors such as osmotic changes, inflammation, and luminal bacteria (45, 46). L-Glutamine (GLN) is known to be a key nutrient in the maintenance of gut homeostasis (11, 30, 43, 48). In addition to its role as the primary fuel for the gut, GLN has been shown to have growth factor-like signaling functions, activating a number of genes involved in promoting intestinal cell survival (5, 42, 57). GLN is able to stimulate MAPK, which is known to be activated by EGF, and is necessary for the optimal growth factor effect in intestinal cells (26, 44). Furthermore, Seth and colleagues (48) showed that GLN induces a rapid increase in tyrosine phosphorylation of the EGFR receptor (EGFR) in Caco-2 cells.

In critically ill patients, GLN levels are decreased, and this decrease in GLN has been associated with increased patient mortality (38). It has been hypothesized that this increased mortality may be due to withdrawal of luminal nutrients and growth factors, leading to atrophy of the epithelial layer and, thus, impairment of vital gut barrier function (51, 52, 54). Therefore, GLN, traditionally termed a nonessential amino acid, is now considered “conditionally essential” for the small intestinal mucosa and other cell types following stress.

During severe illness and malnutrition, GLN-supplemented parenteral and enteral nutrition becomes essential for the gut, because GLN reduces intestinal permeability, decreases bacterial translocation, enhances immune function, protects the gut mucosa against injury, accelerates healing of the small intestine, and improves nitrogen balance in animal models of intestinal injury (20, 27, 50, 60, 61). GLN is an osmotically acting amino acid that is cotransported with sodium into the cell, causing an influx of water and inducing a “cell-swelling” effect (37). Since osmotic changes are a major physical stress that may be experienced by all cells during their lifetime, osmosis-linked cell signaling plays an important role in activation of specific survival genes (22). GLN has been studied extensively; however, its molecular mechanisms of action, especially the initial key steps, remain a mystery.

EGFR, shown to be important in overall intestinal homeostasis, regulates multiple cellular functions such as proliferation, ion transport, cell survival, and cell growth (40, 41, 49, 53, 58, 59). This is probably best demonstrated in studies using transgenic mice with defective EGFR (waved-2 mice), which display an increased susceptibility to colitis induced by administration of dextran sulfate (16). These results suggest a key role for EGFR in protecting against the development of chronic intestinal disease. Furthermore, reduced EGFR signaling has been shown in inflammatory bowel disease patients (2, 14), indicating that impairment of EGFR might contribute to disease etiology. Other reports have supported the role of EGFR in recovery of the small intestine (32, 35). Moreover, it was shown that EGFR becomes internalized to the nucleus; nuclear EGFR is crucial for DNA repair because of its potential role as a transcription factor (13, 31). EGFRs, like integrins, also function as osmosensors (22), which work with integrins as a “duet” with similar downstream signaling pathways (1). More-
over, it has been shown that extracellular matrix proteins such as fibronectin are involved in integrin-mediated EGFR activation (12). How integrins, EGFR, and extracellular matrix proteins are integrated proximal to MAPK signaling is not well understood.

MAPK pathways, such as ERK1/2 and p38MAPK, as well as the phosphatidylinositol 3-kinase (PI3-K) pathway, are important downstream survival signaling cascades from the membrane to the nucleus (8, 24, 30). Activated MAPK regulates the activities of transcription factors or kinases downstream via phosphorylation, control of gene expression, and other key cellular functions (5, 17, 18, 34). It is well established that ERK1/2 is activated by most growth factors for cell proliferation, differentiation, and survival (28, 47). In contrast, p38MAPK is considered to be a stress-related kinase, and its activation often leads to apoptosis. However, p38MAPK activation is highly divergent, and its pro- or antiapoptotic function appears to be dependent on the cell type and cellular content (29, 34). PI3-K is a ubiquitous lipid kinase comprising a large and complex family with multiple subunits and isoforms. Together, these subunits catalyze upstream effectors, which, in turn, phosphorylate Akt kinases (4, 9, 10). The PI3-K/Akt pathway is an intracellular signaling pathway important in apoptosis. PI3-K phosphorylation activates Akt, reducing apoptosis and allowing proliferation (30). Recent studies have demonstrated that Akt1 is responsible for maintaining cell size and survival by increasing nutrient uptake (15).

The molecular mechanisms by which GLN promotes cell survival and prevents apoptosis in the intestine continue to evolve and have not been fully defined. We hypothesize that EGFR may assist in mediating the protective mechanism of GLN, and this protective pathway may be mediated by MAPK and PI3-K/Akt signaling in intestinal epithelial cells after injury.

![Fig. 1. L-Glutamine (GLN) prevents heat stress (HS)-mediated decreases in EGF receptor (EGFR) levels. A and B: Western blots showing total EGFR levels in IEC-6 cells with 0 or 10 mM GLN (15 min before injury) under unstressed and stressed (HS, 43°C) conditions after 0 and 3 h of recovery; β-actin was used as loading control. Densitometric analysis shows total EGFR expression as fold change relative to 0 mM GLN. Values are means ± SE; n = 3 (A) and 4 (B). C: Western blot and densitometric analysis of cytoplasmic and nuclear EGFR levels after 3 h of recovery in cells treated as described in A and B. Values are means ± SE; n = 6.](http://ajpgi.physiology.org/)
MATERIALS AND METHODS

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO), unless otherwise specified.

Cell culture. Intestinal epithelial (IEC-6) cells (American Type Culture Collection, Manassas, VA) were cultured as previously described (21). Briefly, IEC-6 cells were grown in DMEM supplemented with 10% FBS, 2 mM L-GLN, 10 ml/l antibiotic solution containing penicillin G (10,000 U/ml) and streptomycin (10,000 µg/ml; Cellgro Mediatech), and 0.01 mg/ml insulin.

Heat stress injury. The model of heat stress (HS) injury in IEC-6 cells was used to mimic intestinal inflammation and injury, because it represents the most widely accepted method for inducing a “stress protein response” or heat shock protein expression (23, 55, 56). For cell viability, 96-well plates were submerged to a lethal HS in a 44°C water bath (model 260, Precision, Winchester, VI) for 50 min (21) and allowed to recover at 37°C for 24 h. For protein expression experiments, cells were subjected to a nonlethal HS at 43°C for 45 min (21) and allowed to recover for 0 or 3 h.

MTS cell viability assay. IEC-6 cells were seeded in 96-well plates (7,000 cells per well) and allowed to grow for 42 h in full medium until they achieved 80% confluence. Cells were then cultured for 24 h in GLN-free serum containing DMEM. After GLN starvation for 24 h, cells were exposed to 0, 2, and 10 mM GLN for 15 min. AG1478 (20 µM) or SB203580 (10 µM) was used 1 h prior to GLN treatment to inhibit EGFR and p38MAPK signaling. Cells were then subjected to lethal HS (see Heat stress injury). After 24 h, cell viability was evaluated using a soluble tetrazolium salt [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS)] assay (Promega, Madison, WI) according to the manufacturer’s instructions. Briefly, 1 part phenazine methosulfate was added to 20 parts MTS immediately before the solution was diluted 1:5 in phenol red-free DMEM and added to IEC-6 cells. MTS was bioreduced by cells to a colored, soluble formazan product. Absorbance values were read after 3 h at 490 nm by an ELISA plate reader (Thermo Electro, San Jose, CA); references included readings at 650 nm and no-cell blank wells. Higher absorbance values reflect greater cell viability. Every well (n = 4 per group in each experiment) was normalized to its own individual non-HS control to account for possible differences in cell growth.

Small interfering RNA transfection. Small interfering RNA (siRNA) against EGFR (Invitrogen, Carlsbad, CA) was utilized to evaluate the specific role of EGFR in GLN-mediated cellular protection. Cells were seeded in 96-well plates and allowed to grow for 24 h (to 50–60% confluence) in full medium. Medium was changed to DMEM (with 2 mM GLN) + 10% FBS only, and cells were transfected for 48 h using SilencerFect (Bio-Rad, Hercules, CA) with no RNA, EGFR siRNA (40 nM), or control noncoding oligonucleotides (40 nM) with a guanine-cytosine content comparable to that of EGFR siRNA (Invitrogen). Cells were pretreated with DMEM (with 0 mM GLN) + 10% FBS only 24 h before HS (transfection reagents still present). IEC-6 cells were then treated with 0 or 2 mM GLN and subjected to HS, as described above. Cell survival was measured as described above (see MTS cell viability assay).

Protein extraction and Western blot analysis. Cells were seeded in 10-cm dishes and allowed to grow for 3 days in full medium; then the medium was replaced with GLN-free serum containing DMEM for 24 h. Cells were then treated with and without 10 mM GLN for 15 min with or without 1 h of pretreatment with the EGFR kinase activity inhibitor AG1478 (20 µM), the ERK1/2 kinase inhibitor PD98059 (50 µM; Calbiochem, Philadelphia, PA), the p38MAPK inhibitor siRNA (40 nM) and treatment with 0 or 2 mM GLN. Cells were then transfected with no siRNA, NC siRNA (40 nM), or EGFR siRNA (siEGFR, 40 nM) for 48 h. Expression of starved IEC-6 cells transfected with no siRNA, NC siRNA (40 nM), or EGFR siRNA (siEGFR, 40 nM) was determined using MTS assay following lethal HS (44°C) and under basal conditions after transfection of IEC-6 cells with no siRNA, NC siRNA (40 nM), or EGFR siRNA (40 nM) and treatment with 0 or 2 mM GLN. All groups were normalized to their non-HS controls to account for differences in cell growth. Values are means ± SE; assays were carried out in triplicates, and experiments were performed 3 times.
SB203580 (10 μM), or the PI3-K inhibitor LY294002 (25 μM) and subjected to HS. At the end of experimental treatment, medium was removed from the culture, and cells were immediately washed and harvested in ice-cold PBS. For total protein extraction, the cells were lysed at 4°C using 180 μl of M-PER lysis buffer (Pierce, Rockford, IL) with inhibitor protease and phosphatase cocktail (Roche, Indianapolis, IN). For EGFR levels and localization, cells were lysed with the Pierce NE-PER nuclear and cytoplasmic extraction reagent kit (Pierce).

The NE-PER reagents efficiently solubilize and separate cytoplasmic and nuclear proteins into fractions with minimal cross-contamination or interference from genomic DNA and mRNA. Protein was determined using the BCA protein assay (Pierce). After addition of 15 μg of each sample to a 4× treatment buffer (250 mM Tris-Cl, 8% SDS, 27.5% glycerol, 20% 2-mercaptoethanol, and 0.1% bromphenol blue, pH 6.8), the sample was boiled for 3 min and then loaded onto a NuPAGE 4–12% Bis-Tris gel (Invitrogen). After electrophoresis, gels were equilibrated with transfer buffer (1.2 g of Tris-Cl, 7.5 g of glycine, 100 ml of methanol, and 950 ml of distilled H2O). Proteins were electrophoretically separated with a mini-gel system and transferred to polyvinylidine difluoride membranes (Millipore, Billerica, MA) using the Bio-Rad transfer system (Invitrogen). Membranes were blocked with 5% nonfat milk in PBS-Tween or 5% BSA in PBS-Tween for phosphorylated protein antibody for 1.5 h at room temperature. Primary antibodies against EGFR, total ERK1/2, Thr202/Tyr204-phosphorylated ERK1/2, total p38MAPK, Thr180/Tyr182-phosphorylated p38MAPK, total Akt, Ser473-phosphorylated Akt, poly-(ADP-ribose) polymerase (PARP), and caspase-3 (1:1,000 dilution; Cell Signaling, Danvers, MA) were added to antibody buffer (blocking solution), and the samples were incubated overnight at 4°C. After the samples were washed three times with PBS-Tween over 30 min, secondary antibodies, peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG (Pierce), were applied at a 1:3,000 dilution for 1.5 h. Blots were washed three times with PBS-Tween over 30 min, incubated in commercial enhanced chemiluminescence reagents (Pierce), and exposed utilizing a chemiluminescence darkroom system (UVP, Upland, CA). Densitometry was normalized against β-actin (1:50,000 dilution).

**Data analysis and statistics.** All experiments were repeated at least three times with IEC-6 cells at passages 15–20. Statistical analysis was validated with GraphPad Prism analysis software. Conditions were compared using one-way ANOVA followed by Tukey’s post hoc test or Student’s t-test, where appropriate, and are expressed as means ± SE; n = 3.
RESULTS

GLN is protective by preventing decreases in EGFR expression after HS. Total EGFR expression was significantly reduced in IEC-6 cells immediately following HS (43°C) and at 3 h after HS. GLN treatment (10 mM) prevented this decrease in EGFR expression after HS (Fig. 1, A and B). Since recent literature showed that EGFR becomes internalized to the nucleus and that nuclear EGFR is crucial for DNA repair and cell survival (13, 31), we looked at cytoplasmic and nuclear levels of EGFR after HS and GLN treatment to see if GLN accumulates EGFR in the nucleus after HS. However, data showed that GLN supplementation inhibited decreases in EGFR expression caused by HS in the nucleus and cytoplasm (Fig. 1C). To demonstrate that EGFR expression is important in GLN’s protective mechanism of IEC-6 cells, cells were transfected with 40 nM EGFR siRNA. EGFR knockdown, shown in Fig. 2A, did not affect cell viability in non-HS cells (Fig. 2B). Attenuation of EGFR expression via siRNA (40 nM) led to a significant decrease of GLN-mediated protection following HS shown using MTS assay (Fig. 2C). This indicates that GLN-mediated protection against apoptosis after HS is lost when EGFR expression is attenuated.

Involvement of EGFR tyrosine kinase signaling in GLN-mediated cellular protection. Seth and colleagues (48) showed that GLN induces a rapid increase in the tyrosine phosphorylation of EGFR, and GLN’s protective effect was prevented by AG1478, the EGFR tyrosine kinase inhibitor, in Caco-2 cells. Therefore, using MTS assay, we investigated whether AG1478 had the same effect on GLN-mediated cellular protection following lethal HS (44°C) in a nontransformed intestinal epithelial cell line (IEC-6) (Fig. 3B).

AG1478 (0, 5, 10, and 20 μM) was added to non-HS groups to measure any possible toxicity of the inhibitor reagents. As shown in Fig. 3A, AG1478 (0, 5, 10, and 20 μM) alone did not affect cell viability. HS plates (44°C) were normalized to their non-HS control groups (Fig. 3B) to account for differences in cell growth. GLN increased cell survival in HS cells in a dose-dependent manner. Importantly, 0, 5, 10, and 20 μM AG1478 completely attenuated protection by 2 and 10 mM GLN (Fig. 3B). Evaluation of key apoptotic pathway markers confirmed the cell survival results of the MTS assay. HS increased apoptotic markers such as cleaved PARP and cleaved caspase-3 levels. Supplementation with 10 mM GLN decreased markers of apop-

Fig. 4. ERK1/2 activation is involved in GLN’s protective mechanism and attenuates after inhibition of EGFR tyrosine kinase activity. A: Western blot showing inhibitory effect of PD98059 on Thr202/Tyr204-phosphorylated [T(P)202/Y(P)204] and total ERK1/2 levels in IEC-6 cells treated with the ERK1/2 kinase inhibitor PD98059 (50 μM) for 1 h or DMEM without GLN under basal conditions. B: representative Western blots from 3 independent experiments showing procaspase-3 and cleaved caspase-3 levels in IEC-6 cells treated as described in B, but with addition of the inhibitor AG1478 (20 μM). ERK1/2 activation is shown as mean fold change relative to total ERK1/2; results were normalized to 0 mM GLN. Values are means ± SE; n = 3.

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tosis after HS, indicating that 10 mM GLN can improve cell survival and prevent apoptosis during HS (Fig. 3, C and D). However, AG1478 (20 μM) decreased GLN-mediated antiapoptotic effects, as shown by increased cleaved PARP and cleaved caspase-3 levels (Fig. 3, C and D). These data suggest that, in the absence of EGFR signaling, GLN-mediated cellular protection against hyperthermia is lost.

GLN is protective by activating ERK1/2 via EGFR signaling. Since it is well established that ERK1/2 is activated by a number of key growth factors for cell survival (28, 47), we examined the effect of GLN on ERK1/2 activation after hyperthermia and investigated whether EGFR tyrosine kinase activity may play an initial role in GLN’s activation of ERK1/2. In our previous study, we were able to show via MTS assays and use of the ERK1/2 inhibitors PD98059 (50 μM) and U0126 (30 μM) that GLN’s protective effect was attenuated (36). By inhibiting ERK1/2 kinase with 50 μM PD98059 (Fig. 4A), we confirmed in this study via evaluation of cleaved caspase-3 and cleaved PARP that ERK1/2 activation is important in the protective mechanism of GLN (Fig. 4, B and C). GLN supplementation decreased cleaved caspase-3 and cleaved PARP levels after HS. However, PD98059 (50 μM) attenuated GLN-mediated antiapoptotic effects, as demonstrated by increased cleaved caspase-3 and cleaved PARP levels (Fig. 4, B and C). Next, Western blots of the ratio of Thr202/Tyr204-phosphorylated ERK1/2 to total ERK1/2 showed that HS increased phosphorylated ERK1/2 by threefold. GLN treatment further elevated ERK1/2 phosphorylation by sixfold after HS. Importantly, this GLN-mediated increase in ERK1/2 phosphorylation was inhibited after AG1478 (20 μM) treatment (Fig. 4D).

GLN is protective by dephosphorylating p38MAPK via EGFR signaling. In contrast to ERK1/2, p38MAPK is considered to be a stress-related kinase. However, since p38MAPK activation is highly divergent and its pro- or antiapoptotic function appears to be dependent on the cell type and cellular content (29, 34), we examined its role in GLN’s protective mechanism in IEC-6 cells after hyperthermia. We were also interested in whether EGFR signaling was essential in GLN-mediated p38MAPK dephosphorylation. Phosphorylation of p38MAPK was increased by HS (P < 0.05) and significantly attenuated by GLN (P < 0.05; Fig. 5C). MTS assays in IEC-6 cells revealed that 10 mM SB203580 increased cell survival in HS control groups (P < 0.05; Fig. 5A). Furthermore, GLN was...
still protective when IEC-6 cells were treated with GLN + 10 μM SB203580 (Fig. 5B), suggesting that dephosphorylation of p38MAPK regulates cell survival. To see whether EGFR signaling was essential in GLN-mediated p38MAPK dephosphorylation, we added AG1478 to cells exposed to HS and treated with GLN. The decrease in HS-induced p38MAPK phosphorylation caused by AG1478 (20 μM) was not significant. However, in cells treated with HS + GLN + AG1478, phosphorylation of p38MAPK was significant (P < 0.001; Fig. 5C), indicating that EGFR signaling is involved in GLN-mediated dephosphorylation of p38MAPK after HS.

EGFR signaling has no effect on GLN-mediated Akt activation. Another essential cell survival signaling pathway is the PI3-K pathway. PI3-K phosphorylation activates Akt, reducing apoptosis and allowing proliferation (30). Using the PI3-K inhibitor LY294002 (25 μM), we confirmed that PI3-K signaling activates Akt in IEC-6 cells after hyperthermia in control and GLN-treated groups (Fig. 6A). Western blots of cleaved caspase-3 and cleaved PARP expression showed that the PI3-K pathway is essential in GLN’s protective mechanism (Fig. 6, B and C). LY294002 treatment significantly attenuated GLN’s reduction of apoptosis, as measured by cleaved caspase-3 (Fig. 6B) and cleaved PARP (Fig. 6C) levels after hyperthermia. To see if EGFR signaling is involved in GLN-mediated PI3-K signaling, we looked at total Akt protein levels and Akt activation after GLN and AG1478 (20 μM) treatment in HS IEC-6 cells. Investigating total Akt levels, we could show that total Akt is significantly decreased after HS. However, GLN returned Akt protein levels to normal after thermal injury to prevent cell death (Fig. 6D). From the ratio of phosphorylated to total Akt, we could demonstrate that HS increased phosphorylated Akt by twofold and 10 mM GLN supplementation increased phosphorylated Akt by threefold after HS. Addition of AG1478 (20 μM) to the GLN-treated group did not change the GLN-mediated increase in Akt phosphorylation (Fig. 6D).

Fig. 6. Phosphatidylinositol 3-kinase (PI3-K) signaling is involved in GLN’s protective mechanism independently from EGFR signaling. A: representative Western blots of total and Ser473-phosphorylated [S(P)473] Akt in IEC-6 cells treated with 0 or 10 mM GLN with or without 1 h of pretreatment with LY294002 (25 μM) under basal and stressed (43°C) conditions. B: representative Western blot of caspase-3 in IEC-6 cells treated as described in A. Cleaved caspase-3 was analyzed using densitometry and is shown as fold change; results were normalized to 0 mM GLN. Values are means ± SE (n = 4). C: representative Western blot of PARP1 in cells treated as described in A. Cleaved PARP is shown as fold change; results were normalized to 0 mM GLN. Values are means ± SE; n = 4. D: representative Western blots of Ser473-phosphorylated total Akt in cells treated with 0 or 10 mM GLN for 15 min with or without 1 h of pretreatment with AG1478 (20 μM) under basal and stressed (43°C) conditions. Ser473-phosphorylated and total Akt are shown as mean fold change relative to total Akt; results were normalized to 0 mM GLN. Values are means ± SE; n = 4.
DISCUSSION

After intestinal injury, GLN depletion leads to ongoing tissue injury, apoptosis, and failure of cellular repair (25). However, despite multiple experimental and clinical studies demonstrating GLN’s beneficial effects in the intestine, the molecular mechanism of GLN remains unclear. Our findings provide novel mechanistic insight into the antiapoptotic effects of GLN in the intestine after injury. We show that GLN, known to have growth factor-like signaling functions, can protect against intestinal injury by expressing EGFR after HS and activating EGFR signaling (Figs. 1 and 2).

Expression of the EGFR is essential to the maintenance of cellular integrity and to the intestinal epithelial cell’s response to injury (14, 33). Utilizing IEC-6 cells, a small intestinal crypt epithelial cell line, we show that, under nonstress conditions, intestinal epithelial cells produce substantial amounts of EGFR. Our data further show that, 0 and 3 h following thermal injury (43°C), total, cytoplasmic, and nuclear EGFR levels are significantly reduced. This reduction in EGFR is associated with significant apoptosis. GLN supplementation is able to restore epithelial cell EGFR levels following HS to preinjury levels in the cytoplasm and nucleus; GLN did not just accumulate EGFR in the nucleus. The preservation of EGFR levels seems to correlate with reduced apoptosis (Fig. 2). Here, we show, for the first time, that GLN can protect intestinal epithelial cells in vitro from cell death by preserved EGFR expression after HS injury (Fig. 2). Silencing EGFR inhibited GLN’s protection (Fig. 2), suggesting that EGFR expression is essential in GLN’s protective mechanism. Insufficient EGFR expression impairs gastrointestinal function and can lead to worsening of disease in patients with inflammatory bowel disease (14). Our results demonstrate that GLN was able to preserve EGFR content after HS and did not appear to lead to excessive EGFR expression (Fig. 1). Further studies will evaluate whether GLN prevents EGFR degradation or whether GLN induces EGFR expression after HS. Different possible EGFR degradation pathways (lysosomal, proteosomal, and other pathways such as EGFR cleavage by caspases) have been reported (3, 6). Alwan et al. (3) demonstrated that ligand-induced polyubiquitination of EGFR is an essential factor in the downregulation of activated EGFRs. Bae et al. (6) showed that caspases are capable of proteolytic cleavage of EGFRs. The exact mechanism responsible for EGFR degradation following stress, however, remains obscure and is an issue of debate. Thus the EGFR degradation pathway, in conjunction with GLN’s prevention of EGFR degradation after HS, is a key area of research for future studies.

In this study, we show that preservation of EGFR expression is important for GLN-mediated protection and that the activation of EGFR tyrosine kinase signaling via ERK1/2 activation and p38MAPK inactivation (Figs. 1–5) appears to play a key signaling role. EGFR, MAPKs, and PI3-K signaling are important signal transduction pathways stimulated by growth factors affecting survival and apoptosis (30, 58, 59). Therefore, we hypothesized that there may be a relationship between these pathways and GLN’s protection in IEC-6 cells after thermal injury. We have presented evidence that EGFR, ERK1/2, p38MAPK, and PI3-K signaling play essential roles in GLN-mediated cell survival (Figs. 3–6). However, only ERK1/2 and p38MAPK were involved in GLN-mediated EGFR signaling after HS (Figs. 4 and 5). GLN seemed to activate ERK1/2 phosphorylation and to dephosphorylate p38MAPK after hyperthermia to prevent cell death (Figs. 4 and 5). The potential interaction between these pathways will be an interesting area of investigation for future studies.

GLN seemed to activate Akt independently from EGFR signaling after thermal injury (Fig. 6D). Even the high concentration of AG1478 (20 μM) was not able to decrease GLN-mediated Akt phosphorylation (Fig. 6D). These results suggest that GLN is protective via distinct signaling pathways after intestinal injury. It appears that the PI3-K pathway signals upstream of EGFR and has an impact on EGFR signaling or PI3-K signaling is activated by GLN simultaneously and signals in parallel to EGFR. The specific role and molecular mechanism of PI3-K signaling in GLN’s protective mechanism will be key areas of future research. Recent studies have demonstrated that Akt1 is responsible for maintaining cell size and survival by increasing nutrient uptake (15). Since EGFRs are known to be osmosensors, like integrins (22), it will be vital to study whether PI3-K regulates EGFR expression and osmosensing in GLN’s protective mechanism after HS.

As our previous data showed that GLN is protective via fibronectin-integrin signaling, independent of intracellular GLN concentrations (36), and after previously published literature revealed that integrins, EGFR, and extracellular matrix proteins are interacting (1, 12), it may be possible that EGFR expression and signaling are regulated via fibronectin-integrin signaling or vice versa in GLN’s molecular protective mechanism in IEC-6 cells after HS. As specific details of interactions between EGFR, integrins, and the extracellular matrix become clearer, it should be possible to identify GLN’s precise molecular pathway leading to prevention of cell death.

Fig. 7. Proposed working model. GLN is protective in the intestine by preventing EGFR degradation after HS injury, as well as by activating the protective EGFR signaling pathway. GLN phosphorylates ERK1/2 and inactivates p38MAPK via EGFR tyrosine kinase signaling to prevent apoptosis. However, GLN is protective by activating the PI3-K pathway independently from EGFR signaling.
We could also confirm the study of Galadari et al. (19) and show that Akt depletion is a prelude to cell death following HS. GLN was able to preserve total Akt levels at preinjury levels after HS to prevent cell death. We also investigated Akt activation, since Akt activation plays an important role in survival signaling pathways (30). Bang et al. (7) demonstrated that HS phosphorylates Akt, enhances the ability of cells to survive HS, and suppresses apoptosis. We were interested to know whether GLN enhances Akt activation after hyperthermia to overcome cell death. Also, we wanted to investigate if GLN-mediated EGFR signaling regulates increases in Akt activation. We could show that GLN increased Akt activation by threefold (HS by 2-fold); however, even at the highest concentration of AG1478 (20 μM), we could not see any inhibitory effect on GLN-mediated increases in Akt phosphorylation, indicating that GLN-mediated PI3-K/Akt signaling occurs before EGFR signaling or simultaneously.

In summary, EGFR expression and kinase activity are involved in GLN’s protective mechanism in IEC-6 cells after HS. Our current working hypothesis suggests that GLN expresses EGFR after HS and activates EGFR tyrosine activity, which phosphorylates ERK1/2 and deactivates p38MAPK, preventing apoptosis independently from PI3-K signaling (Fig. 7). However, GLN is also protective via activation of PI3-K signaling (Fig. 7). Future studies are needed to determine whether GLN is protective via two distinct simultaneous pathways or whether PI3-K signaling has a unique effect on EGFR signaling. As the mechanistic details of EGFR and apoptosis pathways are further delineated, it should be possible to identify the exact molecular pathways by which GLN acts via these pathways to prevent cell death.

We believe that our study raises a new and clinically important hypothesis. Many conditions of injury and stress, including inflammatory gut injury, lead to rapid GLN deficiency (39). GLN deficiency may lead to a reduction of EGFR expression (Fig. 7). This hypothesis is correct, these data have particular relevance to the clinical care of human disease because of GLN’s potential to optimize EGFR expression and signaling to protect tissues/organs and, ultimately, improve clinical outcome.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.N. and C.B. are responsible for conception and design of the research; S.N. and B.P. performed the experiments; S.N. analyzed the data; S.N., C.B., and P.E.W. interpreted the results of the experiments; S.N. prepared the figures; S.N. drafted the manuscript; S.N., E.W., and P.E.W. edited and revised the manuscript; S.N., E.W., and P.E.W. approved the final version of the manuscript.

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