Glutamine Deprivation Causes Hydrogen Peroxide-induced Interleukin-8 Expression via Jak1/Stat3 Activation in Gastric Epithelial AGS Cells

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Background: The Janus kinase (Jak)/Signal transducers of activated transcription (Stat) pathway is an upstream signaling pathway for NF-κB activation in Helicobacter pylori-induced interleukin (IL)-8 production in gastric epithelial AGS cells. H. pylori activates NADPH oxidase and produces hydrogen peroxide, which activates Jak1/Stat3 in AGS cells. Therefore, hydrogen peroxide may be critical for IL-8 production via Jak/Stat activation in gastric epithelial cells. Glutamine is depleted during severe injury and stress and contributes to the formation of glutathione (GSH), which is involved in conversion of hydrogen peroxide into water as a cofactor for GSH peroxidase.

Methods: We investigated whether glutamine deprivation induces hydrogen peroxide-mediated IL-8 production and whether hydrogen peroxide activates Jak1/Stat3 to induce IL-8 in AGS cells. Cells were cultured in the presence or absence of glutamine or hydrogen peroxide, with or without GSH or a Jak/Stat specific inhibitor AG490.

Results: Glutamine deprivation decreased GSH levels, but increased levels of hydrogen peroxide and IL-8, an effect that was inhibited by treatment with GSH. Hydrogen peroxide induced the activation of Jak1/Stat3 time-dependently. AG490 suppressed hydrogen peroxide-induced activation of Jak1/Stat3 and IL-8 expression in AGS cells, but did not affect levels of reactive oxygen species in AGS cells.

Conclusions: In gastric epithelial AGS cells, glutamine deprivation increases hydrogen peroxide levels and IL-8 expression, which may be mediated by hydrogen peroxide-mediated Jak1/Stat3 activation and therefore, reducing IL-8 production. Scavenging hydrogen peroxide or targeting Jak1/Stat3 may also prevent oxidant-mediated gastric inflammation.

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Key Words: Glutamine, Hydrogen peroxide, Janus kinase, Interleukin-8, Signal transducers of activated transcription

INTRODUCTION

Interleukin (IL)-8 is the major activator of neutrophil extravasation into the gastric mucosa, which is an important aspect of gastric inflammation. Elevated levels of IL-8 are found in gastric tissues from the patients with Helicobacter pylori infection. Previously, we showed that Janus kinase (Jak)/Signal transducers of activated transcription (Stat) signaling is a prerequisite for NF-κB activation, leading to IL-8 production in H. pylori infected gastric epithelial AGS cells. H. pylori infection activates NADPH oxidase and produces reactive oxygen species (ROS) such as hydrogen peroxide (H₂O₂) that activate Jak/Stat signaling in AGS cells. H₂O₂ activates the oxidant-sensitive transcription factor NF-κB and induces the expression of IL-8 in gastric epithelial cells. H₂O₂, not superoxide or nitric oxide, activates Jak2, Stat1, and Stat3 in various types of cells, including fibroblasts and the A431 adenocarcinoma cell lines. Therefore, by activating Jak/Stat H₂O₂ may have a critical role in IL-8 expression. IL-8 increases in the gastric tissues of cancer patients. IL-8 is also reported to be a promoter of angiogenesis and acts as an autocrine growth factors for colon carcinoma cells. Therefore, IL-8 may be an important mediator of gastric inflammation and carcinogenesis.

Glutathione (GSH) is composed of three amino acids, cysteine,
glycine, and glutamate. After cellular uptake, glutamine is converted to glutamic acid, which is a precursor of GSH. Previously, we demonstrated that glutamine deprivation increases ROS levels and induces IL-8 expression by activating NF-kB in ataxia telangiectasia fibroblasts. In Caco cells, glutamine deprivation increases IL-8 production after treatment with lipopolysaccharide. In human intestinal cells, glutamine supplementation reduces IL-6 levels by suppressing NF-κB activation. Glutamine supplementation also reduces inflammation and foveolar hyperplasia in H. pylori-infected mice. The same study found that body weight gain, food consumption, H. pylori colonization, and serum immunoglobulin G did not differ in H. pylori-infected mice fed supplemental glutamine compared with mice fed a control diet. Therefore, glutamine deprivation may induce IL-8 expression mediated by H2O2-stimulated inflammatory signaling in gastric epithelial cells.

In the present study, we investigated whether glutamine deprivation induces IL-8 production by increasing H2O2 levels and whether H2O2 directly activates Jak1/Stat3 to induce IL-8 expression in gastric epithelial AGS cells.

**MATERIALS AND METHODS**

1. **Cell culture**

   The human gastric epithelial AGS cell line (American Type Culture Collection, Manassas, VA, USA) was cultured in RPMI 1640 medium (GIBCO, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (GIBCO), 100 U/mL penicillin, and 100 μg/mL streptomycin, with or without 2 mM glutamine, (Sigma, St. Louis, MO, USA) at 37°C in a humidified atmosphere of 5% CO2 and 95% air.

2. **Experimental protocol**

   In the first set of experiments, cells were cultured in the absence or presence of glutamine (2 mM) with or without GSH (10 mM) for 12 hours (for measurement of IL-8 mRNA levels) or 24 hours (for measurement of the levels of GSH, H2O2, and IL-8 in the medium). In the second set of experiments, cells were cultured in the presence or absence of H2O2 (100 μM) with or without a Jak/Stat specific inhibitor AG490 (40 μM) for 30 minutes (for measurement of ROS levels), 60 minutes (for measurement of Jak1/Stat3 activation), 4 hours (for measurement of IL-8 mRNA levels), and 12 hours (for measurement of IL-8 levels in the medium).

3. **Determination of intracellular glutathione and hydrogen peroxide levels in the medium**

   Cells were washed with ice-cold PBS, harvested by scraping into PBS, and homogenized in 300 μL of extraction buffer (0.1% Triton X-100 and 0.6% sulfosalicylic acid in KPE [0.1 M potassium phosphate buffer with 5 mM EDTA disodium salt, pH 7.5]). GSH in the acid-soluble supernatant was analyzed with an enzyme assay using a microplate reader. GSH content was expressed as nmole/mg protein, determined by the Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA) at excitation and emission wavelengths of 485 and 520 nm, respectively.

4. **Determination of intracellular reactive oxygen species levels**

   Cells were loaded with 10 μg/mL of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA; Invitrogen, Carlsbad, CA, USA) and incubated in 5% CO2 and 95% air at 37°C for 30 minutes. DCF fluorescence was measured using a VICTOR X5 multilabel plate reader (PerkinElmer, Boston, MA, USA) at excitation and emission wavelengths of 485 and 520 nm, respectively.

5. **Western blot analysis for Jak1, p-Jak1, Stat3 and p-Stat3**

   Cells were trypsinized, washed, and then homogenized in Tris-HCl (pH 7.4) buffer containing 1% NP-40 and protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN, USA). The protein concentration of each sample was determined by Bradford assay (Bio-Rad Laboratories). Total cell extracts (50 μg) isolated from the cells were loaded per lane, separated by 6% SDS polyacrylamide gel electrophoresis under reducing conditions and transferred onto nitrocellulose membranes (Amersham Inc., Arlington Heights, IL, USA) by electroblotting. After blocking using 3% nonfat dried milk in TBS-T for 2 hours, the membrane was incubated with polyclonal antibodies for Jak1 (1:500 dilution, cat. no. 3332; Cell Signaling, Beverly, MA, USA), Stat3 (1:500 dilution, cat. No. 06-596; Upstate Biotechnology, Lake Placid, NY, USA), phospho-Jak1 (1:500 dilution, cat. no. sc-16773; Santa Cruz Biotechnology, Santa Cruz, CA, USA) and phospho- Stat3 (1:500 dilution, cat. no. 9131; Cell Signaling) diluted in TBS-T containing 3% nonfat dried milk at 4°C overnight. After washing with TBS-T, the immunoreactive proteins were visualized by using goat anti-rabbit secondary antibodies (1:2,000 dilution, cat. no. sc-2004; Santa Cruz Biotechnology) conjugated to horseradish peroxidase, which was followed by enhanced chemilumine-
scence (Santa Cruz Biotechnology).

6. Real-time PCR analysis of interleukin-8

Total RNA in cells was isolated by TRI reagent (Molecular Research Center Inc., Cincinnati, OH, USA). Total RNA was converted to cDNA by reverse transcription process using a random hexamer and virus reverse transcriptase (Promega, Madison, WI, USA) using the following conditions: 23°C for 10 minutes, 37°C for 60 minutes, and 95°C for 5 minutes. cDNA was used for real-time PCR with human specific primers for IL-8 and β-actin. The sequences of the IL-8 primers were 5′-ATGACTTCCAAGCTGGCGTGCT-3′ (forward primer) and 5′-TCTCAGGCTCTTCAAATACTTCT-3′ (reverse primer), which gave a 297 bp PCR product. For β-actin, the forward primer was 5′-ACCAAATGGGACGACATGGAG-3′ and the reverse primer was 5′-GTGAGGATGAGGATGTC-3′, which gave a 349 bp PCR product. Real-time PCR for quantifying IL-8 gene expression was conducted using a Light Cycler (Roche Applied Sciences, Indianapolis, IN, USA). cDNA was added to SYBR Green real-time PCR Master Mix (Toyobo Co., Osaka, Japan) containing 10 pg/mL of forward and reverse primers for IL-8 and was amplified using a Light Cycler. For PCR amplification, the cDNA was amplified using 40 cycles, with denaturation at 95°C for 15 seconds, annealing at 60°C for 15 seconds, and extension at 72°C for 45 seconds. The β-actin gene was amplified in the same reaction to serve as the reference gene.

7. ELISA for interleukin-8 level

IL-8 levels in the medium were determined by ELISA kits (Invitrogen) according to the manufacturer’s instructions.

Figure 1. Levels of glutathione (GSH), H2O2, and interleukin (IL)-8 expression in AGS cells cultured in the absence or presence of glutamine, with or without GSH. The cells were cultured in the absence or presence of glutamine (2 mM) with or without GSH (10 mM) for 12 hours (for measurement of IL-8 mRNA levels; C) or 24 hours (for measurement of levels of GSH, H2O2, and IL-8 in the medium; A, B, and D). Values are expressed as mean ± SE of four different experiments. Gln+, cells cultured in the presence of glutamine; Gln−, cells cultured in the absence of glutamine. *P < 0.05 vs. Gln+; **P < 0.05 vs. Gln− control.
8. Statistical analysis

Statistically significant differences were determined using one-way ANOVA and Newman-Keul's test. All values are expressed as mean ± SE of four different experiments. A value of $P < 0.05$ was considered statistically significant.

RESULTS

As shown in Figure 1A and 1B, glutamine deprivation decreased intracellular GSH levels and increased hydrogen peroxide levels in the medium, an effect that was inhibited by treatment with GSH. mRNA and protein levels of IL-8 were higher in the cells cultured in the absence of glutamine than in cells cultured in the presence of glutamine. Therefore, GSH inhibited the glutamine deprivation-induced increase in IL-8 levels in AGS cells (Fig. 1C and 1D).

To evaluate whether $H_2O_2$ induces the activation of Jak1/Stat3 and IL-8 expression, cells were cultured in the absence or presence of $H_2O_2$ with or without AG490. Prior to the experiment, phospho-specific and total forms of Jak1/Stat3 were determined during 90 minutes-culture. As shown in Figure 2A, $H_2O_2$ induced phosphorylation of Jak1 and Stat3 time-dependently. Total forms of Jak1 and Stat3 were not changed by $H_2O_2$ treatment. Figure 2B shows that $H_2O_2$-induced activation of Jak1/Stat3 was inhibited by AG490 treatment at 60 minutes-culture. $H_2O_2$-induced mRNA and protein expression of IL-8 was lower in AG490-treated cells than in non-treated cells (Fig. 2D and 2E). However, the $H_2O_2$-induced increase in ROS levels was not affected by AG490 treatment (Fig. 2C).

![Figure 2.](image-url)
DISCUSSION

In the present study, we found that glutamine deprivation induces IL-8 production by increasing H$_2$O$_2$ levels, and that H$_2$O$_2$ activates Jak1/Stat3 to induce IL-8 expression in gastric epithelial AGS cells. These results suggest that glutamine supplementation suppresses oxidative stress-mediated gastric inflammation and carcinogenesis through maintenance of GSH levels in the cells and thus, suppressing H$_2$O$_2$-mediated activation of Jak/Stat and IL-8 expression.

H$_2$O$_2$ has been reported to cause activation of Jak/Stat in astrocytes and periodontal ligament cells. Following activation, Stat3 dimmers translocate into the nucleus where they selectively bind to $\gamma$-interferon activation sequence (GAS) element in the IL-8 promoter and induce IL-8 transcription. In H. pylori-infected human gastric mucosal cells, the expression of IL-8 was shown to be mediated by ROS, including H$_2$O$_2$. Therefore, reducing H$_2$O$_2$ levels by treatment with GSH or glutamine supplementation may inhibit gastric inflammation.

Glutamine is a conditionally essential nutrient since it is depleted from muscle stores during severe injury, illness, or stress. In cells, glutamine is broken down into glutamate and used in the synthesis of the antioxidant GSH. Therefore, glutamine supplementation can be expected to increase GSH levels, an effect has been confirmed in rats with breast cancer. In human intestinal mucosa, glutamine deprivation increases ROS levels and reduces the production of IL-6 by inhibiting NF-kB activation. The anti-inflammatory activity of glutamine is suggested to be attributable to its inhibition of prostaglandin synthesis, but the use of glutamine in the synthesis of GSH, which reduces H$_2$O$_2$ levels, may also be important for preventing oxidant-mediated inflammation. In relation to carcinogenesis, glutamine administration has been shown to markedly alleviate oxidative/nitrosative stress, normalize SOD activity, increase levels of total GSH and block NO overproduction, but it does not reduce angiogenesis induced by hypertension in gastric tissues. In contrast, glutamine promotes ovarian cancer cell proliferation, by increasing the activity of glutaminase and glutamate dehydrogenase through modulation of the mTOR/S6 and MAPK pathways, leading to cell proliferation. A recent clinical study has shown that supplements of glutamine, eicosapentaenoic acid, and branched-chain amino acids can help maintain nutrition status, decrease the complications and improve compliance of esophageal cancer patients receiving concurrent chemo-radiotherapy and gastric cancer patients receiving postoperative adjuvant chemotherapy.

In myc-expressing human renal cell carcinoma cells, cells use glutamine more efficiently than glucose. Therefore, pharmacologic inhibition of glutamine metabolism is suggested as a potential therapeutic approach for the treatment of renal cell carcinoma. Glutamine also influences the signaling pathways involving the oncogenes myc and k-ras and the tumor suppressors p53 and sirt 4. Since glutamine could stimulate proliferation of some cancer cells, more studies should be performed to fully understand these effects before glutamine is used for cancer therapy.

In conclusion, we show that reducing H$_2$O$_2$ levels by glutamine supplementation inhibits the expression of the inflammatory cytokine IL-8 through the suppression of Jak1/Stat3 activation in gastric epithelial cells. Thus, glutamine may prevent gastric inflammation as well as oxidant-mediated carcinogenesis. The inhibition of H$_2$O$_2$-induced activation of Jak1/Stat3 and IL-8 expression by glutamine suggests that it could be used as a chemo-preventive nutrient. In addition, preventing Jak1/Stat3 activation or scavenging excess H$_2$O$_2$ may be beneficial for preventing oxidant-mediated gastric inflammation.

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CONFLICTS OF INTEREST

No potential conflicts of interest were disclosed.

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