

L-Glutamine Attenuates Apoptosis in Porcine Enterocytes by Regulating Glutathione-Related Redox Homeostasis

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Abstract

Background: Programmed cell death plays a fundamental role in intestinal development and mucosal homeostasis. Dysregulation of these processes is associated with an impaired intestinal-mucosal barrier, reduced nutrient absorption, and initiation and progression of intestinal diseases. 4-Hydroxy-2-nonenal (4-HNE), a product of lipid peroxidation, is commonly used to induce oxidative stress in cells. L-Glutamine is known to protect cells from apoptosis. However, the underlying mechanisms are largely unknown.

Objective: This study was conducted to test the hypothesis that L-glutamine attenuates 4-HNE-induced apoptosis by modulating glutathione (GSH) and thioredoxin (TXN) antioxidant systems and the expression of genes involved in 4-HNE metabolism in enterocytes.

Methods: Intestinal porcine epithelial cell line 1 (IPEC-1) cells were cultured with or without 4-HNE (30 μ mol/L) in the presence of 0.05 or 0.25 mmol L-glutamine/L (a physiological concentration in the lumen of the small intestine) for indicated time periods. Cell viability, abundances of apoptotic proteins, mitochondrial membrane depolarization, production of reactive oxygen species (ROS) and GSH, and expression of genes involved in the biosynthesis of GSH, thioredoxin, and 4-HNE metabolism were determined.

Results: Compared with basal medium containing 0.05 mmol L-glutamine/L, 4-HNE enhanced apoptosis by 19.6% ($P < 0.05$) in a caspase-3-dependent manner. This effect was accompanied by elevated intracellular ROS production (39.5% and 85.3% for 2- and 4-h treatment, respectively), increased mitochondrial depolarization by 80%, and decreased intracellular GSH concentrations by 17.7%. These effects of 4-HNE were reduced by 0.25 mmol L-glutamine/L. Further study showed that the protective effect of L-glutamine was associated with the enhanced expression of genes involved in GSH production (including *GCLC*, *GCLM*, *GSR*, *CBS*, and *CTH*) by 3.9–14-fold, as well as genes involved in 4-HNE metabolism [e.g., glutathione *S*-transferase A (*GSTA1*) and *GSTA4*] by 1.9–7.2-fold. The mRNA levels for *ADH5*, *AKR1C1*, *AKR1A1*, and *TXNRD1* were enhanced 1.4–8.8-fold by 4-HNE but were not changed in cells co-treated with 4-HNE and L-glutamine.

Conclusion: These findings indicate that L-glutamine attenuates 4-HNE-induced apoptosis by regulating GSH-related redox homeostasis and enhancing *GSTA*-mediated metabolism in enterocytes. *J Nutr* 2018;148:526–534.

Keywords: L-glutamine, 4-hydroxy-2-nonenal, intestinal epithelial cells, apoptosis, reactive oxygen species

Introduction

The intestinal epithelial cells lining the gastrointestinal tract function as a defensive barrier that separates the intestinal luminal contents from the internal environment (1). A striking characteristic of mammalian intestinal epithelial cells is their rapid cell turnover rate (2, 3). The enterocytes of the neonatal small intestine have a lifespan of 2–4 d and are eliminated by cell shedding at the villus tip (4). The intestinal stem cells located at the base of the crypt give rise to multiple cell types

which migrate upward from the crypt to the top of the villi and replace the lost cells (3, 5), thereby maintaining the intestinal-mucosal barrier. It remains unclear whether apoptosis initiates the shedding process and regulates cell turnover (6). However, accumulating evidence indicates that apoptotic cell death plays a fundamental role in the intestinal development and mucosal homeostasis (7). A variety of factors, such as nutrients, growth factors, cytokines, hormones, and gut commensal bacteria, have been reported to regulate apoptotic signaling pathways in intestinal epithelial cells (8, 9). Moreover, excessive

epithelial cell death caused by pathogenic bacteria, metabolic stress, and accumulation of reactive oxygen species (ROS) is associated with increased intestinal permeability, decreased nutrient absorption, and development of intestinal disorders, such as ulcerative colitis, intestinal bowel syndrome, and uncontrolled immune responses in the intestine (10, 11).

Intestinal enterocytes are constantly exposed to lipid peroxidation products, proinflammatory cytokines, luminal toxic molecules, and commensal or pathogenic microorganisms, which are critical factors implicated in ROS generation (12). Under physiologic conditions, ROS levels are maintained at non-toxic levels and have no detrimental effect on enterocytes due to the endogenous antioxidative systems, including glutathione (GSH) and thioredoxin (TXN) (13, 14). Oxidative stress occurs when the antioxidant system is overwhelmed by overproduction of ROS, which, in turn, leads to intestinal dysfunction and the initiation of intestinal diseases (15). 4-Hydroxy-2-nonenal (4-HNE) is one of the most toxic end products of lipid peroxidation, with a concentration estimated to be $\sim 0.05 \mu\text{mol/L}$ in human plasma (16, 17). However, under pathologic conditions, its concentration can reach $100 \mu\text{mol/L}$, which is associated with cellular injury and activation of cell death signaling pathways (15, 18, 19). Although an increase in 4-HNE concentration has been recognized as an indicator of cell damage in the intestine (17, 20), the underlying mechanisms remain largely unknown. In our recent study, we found that 4-HNE can deplete GSH and contribute to apoptosis in intestinal epithelial cells (21). However, nutritional strategies to reduce the cytotoxic effect of 4-HNE are not available.

L-Glutamine is an abundant amino acid in both intracellular and extracellular compartments (22). It provides energy for metabolism, regulates cell proliferation and cellular repair, maintains the acid-base balance and nitrogen balance, modulates gut permeability, and acts as a precursor for the synthesis of peptides, purines, pyrimidines, nucleic acids, and other bioactive molecules in the small intestine (23). Due to the limited synthesis of glutamine by porcine enterocytes, provision of exogenous glutamine is necessary for maintaining intestinal health (24). Thus, glutamine deficiency has been reported to induce villous atrophy, mucosal ulcerations, and increased permeability, which can be ameliorated by glutamine supplementation (23, 25). The protective effect of glutamine on epithelial permeability is partially mediated by modulating the tight junction

proteins, therefore contributing to improving the integrity of the intestinal mucosal barrier (26, 27).

The small intestine of newborn pigs is highly susceptible to oxidative stress (24). This phenomenon may be related to a very low concentration of glutamine (0.05 mM) in the lumen of their small intestine (28). Therefore, sufficient intake of glutamine is essential for optimal health, as well as normal growth and development of the neonatal small intestine (29–33). Using the 4-HNE-induced apoptosis model of intestinal porcine epithelial cells (IPEC-1) (21), we conducted the present study to test the hypothesis that glutamine attenuates 4-HNE-induced apoptosis by modulating GSH and TXN antioxidant systems and the expression of genes involved in 4-HNE metabolism in enterocytes.

Methods

Materials. 4-HNE was procured from Cayman Chemical Co. Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 (F-12), DMEM, and FBS were purchased from Gibco BRL. 2',7'-Dichlorofluorescein diacetate (DCFH-DA) and 1,1',3,3'-tetraethylbenzamidazolocarbocyanin iodide were obtained from Beyotime Institute of Biotechnology. The annexin V-FITC/PI kit was obtained from Jiamay Biotechnology. Antibodies against poly ADP-ribose polymerase (PARP), B cell lymphoma 2 (BCL2), Bcl-2-associated X (BAX), and β -actin were obtained from Santa Cruz Biotechnology. Antibody against cleaved-caspase-3 was a product of Cell Signaling Technology. Peroxidase-conjugated goat anti-rabbit and goat anti-mouse secondary antibodies were purchased from Huaxingbio Biotechnology Co. Unless stated, all other reagents were ordered from Sigma.

Cell culture. IPEC-1, a cell line derived from newborn piglets, was cultured as previously described (34). Cells were cultured in DMEM/F-12 medium supplemented with 5% FBS, insulin ($5 \mu\text{g/mL}$), transferrin ($5 \mu\text{g/mL}$), selenium ($5 \mu\text{g/L}$), epidermal growth factor ($5 \mu\text{g/L}$), and 1% penicillin-streptomycin. For 4-HNE treatment, cells were starved for 6 h in custom-made DMEM medium (35) without FBS, to reduce the intracellular L-glutamine concentrations. The concentration of L-glutamine in the basal custom-made DMEM was 0.05 mmol/L and served as the control. Following the starvation period, the cells were cultured in the basal medium supplemented with or without $0.25 \text{ mmol L-glutamine/L}$ for 6 h, followed by the addition of 4-HNE ($30 \mu\text{mol/L}$) for the indicated time periods. Use of 4-HNE at this dose has been reported to induce apoptosis in IPEC-1 cells (35). The L-glutamine concentration (0.25 mmol/L) was adopted because it was found to be present ($0.25 \pm 0.02 \text{ mmol/L}$, mean \pm SEM, $n = 6$) in the lumen of the jejunum of 12-h-old piglets nursed by sows.

Cell viability assay. IPEC-1 cells ($10,000/\text{well}$) seeded in a 96-well plate were starved for 6 h in basal DMEM media without FBS, and were then incubated with or without $0.25 \text{ mmol L-glutamine/L}$ for 6 h, followed by the addition of 4-HNE ($30 \mu\text{mol/L}$) for 8 h. Cell viability was determined with the use of a cell-counting kit (Zoman Biotech).

Flow cytometry analysis. Cells harvested from the culture dish were stained with annexin V-FITC ($2.5 \mu\text{g/mL}$) for 15 min in the dark. Next, cells were incubated with propidium iodide ($50 \mu\text{g/mL}$) for another 5 min at room temperature. Samples were analyzed by a flow cytometer (Beckman Coulter). Data were assessed using CytExpert software.

Determination of mitochondrial membrane potential by flow cytometry. Flow cytometry was utilized to measure the mitochondrial transmembrane potential, according to the manufacturer's procedures. Briefly, cells were harvested and stained by 1,1',3,3'-tetraethylbenzamidazolocarbocyanin iodide, a permeable cationic potentiometric vital dye. Stained cells were analyzed by a flow

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Supplemental Table 1 and Supplemental Figures 1–3 are available from the "Supplementary data" link in the online posting of the article and from the same link in the online table of contents at <https://academic.oup.com/jn/>.

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Abbreviations used: *ADH5*, alcohol dehydrogenase 5; *AKR1A1*, aldo-keto reductase family 1 member A1; *AKR1C1*, aldo-keto reductase family 1 member C1; *ALDH2*, aldehyde dehydrogenase 2; BAX, B cell lymphoma 2-associated X; BCL2, B cell lymphoma 2; CBS, cystathionine β -synthase; CTH, cystathionine γ -lyase; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DMEM, Dulbecco's Modified Eagle Medium; GCLC, catalytic subunit of glutamate-cysteine ligase; GCLM, glutamate-cysteine ligase modifier subunit; GSH, glutathione; GSR, glutathione reductase; GSS, glutathione synthetase; GSTA, glutathione S-transferase A; IPEC-1, intestinal porcine epithelial cell line 1; PARP, poly ADP-ribose polymerase; ROS, reactive oxygen species; TXN, thioredoxin; TXNRD, thioredoxin reductase; 4-HNE, 4-hydroxy-2-nonenal.

cytometer (Beckman Coulter), with excitation at 488 nm and emission at 530 nm.

Determination of ROS. DCFH-DA, an ROS-sensitive probe, was used to determine intracellular ROS production. Briefly, IPEC-1 cells were treated and incubated with DCFH-DA (10 $\mu\text{mol/L}$) at 37°C for 30 min. ROS were determined by a fluorescence microscope (Zeiss Axiovert) and a flow cytometer (Beckman Coulter).

Determination of GSH. The intracellular GSH concentration was determined using a commercial kit (Beyotime), according to the manufacturer's instructions.

Western blot analysis. Cells were harvested and lysed in RIPA lysis buffer, containing 50 mmol Tris-HCl/L (pH 7.4), 150 mmol NaCl/L, 1% NP-40, 0.1% SDS, 1.0 mmol PMSF/L, 1.0 mmol Na_3VO_4 /L, and 1.0 mmol NaF/L. Protein concentrations were measured by a BCA protein assay kit (Appligen Technologies). Equal amounts of proteins (25 μg) were separated on SDS-PAGE gels, transferred to polyvinylidene difluoride membranes (Millipore), and then blocked in 5% skimmed milk for 1 h at 25°C. The membranes were incubated with a primary antibody overnight at 4°C, followed by incubation with a horseradish peroxidase-conjugated secondary antibody at 25°C for 1 h. The protein bands were developed by an enhanced chemiluminescence kit (Huaxingbio), using the ImageQuant LAS 4000 mini system (GE Healthcare). Band density was quantified by using the Quantity One software (Bio-Rad Laboratories). β -Actin was used as the internal control.

Quantitative real-time PCR. Total RNA was extracted from cells by using the Trizol reagent (CWBio Biotech Co.) and reverse-transcribed into cDNA in a volume of 10 μL , with the use of a high-capacity cDNA archive kit (TaKaRa), according to the manufacturer's protocol. Quantitative real-time PCR was performed with SYBR Green using the ABI 7500 real-time PCR system, according to the manufacturer's instructions. The primer sequences used in this study are shown in Supplemental Table 1. *GAPDH* was used as an internal control for normalization.

Statistical analysis. Values were expressed as mean \pm SEM and were statistically analyzed by 1- or 2-factor ANOVA, using SAS (SAS Institute Inc.). Differences between means were determined by using the Student-Newman-Keuls multiple-comparison test. For 2-factor ANOVA, the PROC general linear model was used, with L-glutamine concentration and 4-HNE as the main factors. A *P* value <0.05 was considered significant.

Results

Effects of L-glutamine and/or 4-HNE on cell viability. 4-HNE treatment led to a 21% reduction in cell viability ($P < 0.05$), compared with the control (basal medium containing 0.05 mmol L-glutamine/L), which was attenuated ($P < 0.05$) by 0.25 mmol L-glutamine/L (Figure 1). Consistently, we observed an increase in the number of floating cells, the appearance of cell shrinkage, and boundary contraction in 4-HNE-treated cells, which were abolished by 0.25 mmol L-glutamine/L (Supplemental Figure 1). Cells incubated with 0.25 mmol L-glutamine/L had 20% greater viability than the controls.

L-Glutamine attenuated 4-HNE-induced apoptosis in IPEC-1 cells. The addition of 4-HNE to culture medium resulted in greater apoptosis than with the control (19.6% compared with 6.6%) (Figure 2A and B). Interestingly, the 4-HNE-induced apoptosis was abrogated ($P < 0.05$) by 0.25 mmol L-glutamine/L (19.6% compared with

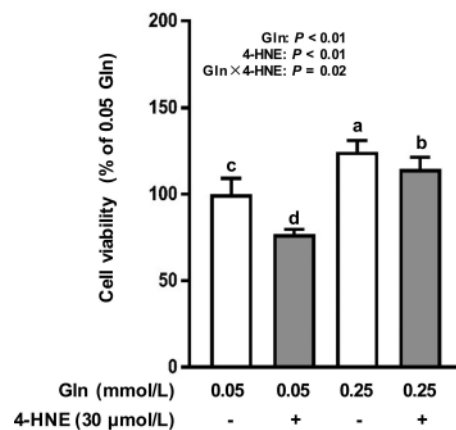


FIGURE 1 Effects of L-glutamine and/or 4-HNE on the viability of IPEC-1 cells. IPEC-1 cells were incubated with 4-HNE (30 $\mu\text{mol/L}$) for 8 h in the presence of 0.05 (the control) or 0.25 mmol L-glutamine/L. Data, expressed as the percentage of control cells, are means \pm SEMs, $n = 6$ independent experiments. Means without a common letter differ, $P < 0.05$. Gln, L-glutamine; IPEC-1, intestinal porcine epithelial cell line 1; 4-HNE, 4-hydroxy-2-nonenal.

9.8%), indicating a protective effect of glutamine on cell survival. In agreement with the phenotype observed, 4-HNE promoted the accumulation of the cleaved-caspase-3 and the cleaved PARP, 2 characteristics of apoptosis, but this effect of 4-HNE was reversed by 0.25 mmol L-glutamine/L (Figure 2C and D). In the absence of 4-HNE, L-glutamine supplementation did not affect cell death in our IPEC-1 model when compared with the control group.

The protective effect of L-glutamine on 4-HNE-induced apoptosis was associated with mitochondrial depolarization. Cells treated with 4-HNE had enhanced ($P < 0.05$) mitochondrial depolarization (Figure 3A and B). This effect of 4-HNE was abolished ($P < 0.05$) by 0.25 mmol L-glutamine/L. Western blot analysis demonstrated that 4-HNE reduced ($P < 0.05$) the abundance of the BCL2 protein (Figure 3C), and enhanced ($P < 0.05$) the abundance of the BAX protein (Figure 3D), as compared with the control cells. These effects of 4-HNE were partially reversed ($P < 0.05$) by L-glutamine supplementation.

L-Glutamine blocked 4-HNE-induced ROS generation in IPEC-1 cells. Cells treated with 4-HNE had higher ($P < 0.05$) levels of ROS, as evidenced by enhanced fluorescence intensity, compared with the controls (Figure 4A). This effect of 4-HNE was confirmed by flow cytometry analysis, with a peak shift observed at 2 h that was maintained until 8 h posttreatment (Figure 4B). The 4-HNE-induced ROS generation was abolished by L-glutamine supplementation (Figure 4A and B). Consistently, 4-HNE treatment led to a decrease in intracellular GSH concentration, the main redox molecule against oxidative stress, and this effect of 4-HNE was reversed ($P < 0.05$) by 0.25 mmol L-glutamine/L (Figure 5A). Furthermore, we determined mRNA levels for the following: 1) the catalytic subunit of glutamate-cysteine ligase (*GCLC*), glutamate-cysteine ligase modifier subunit (*GCLM*), and glutathione synthetase (*GSS*), 3 genes involved in GSH biosynthesis; and 2) glutathione reductase (*GSR*), a gene for the maintenance of GSH in the reduced form. The mRNA levels for

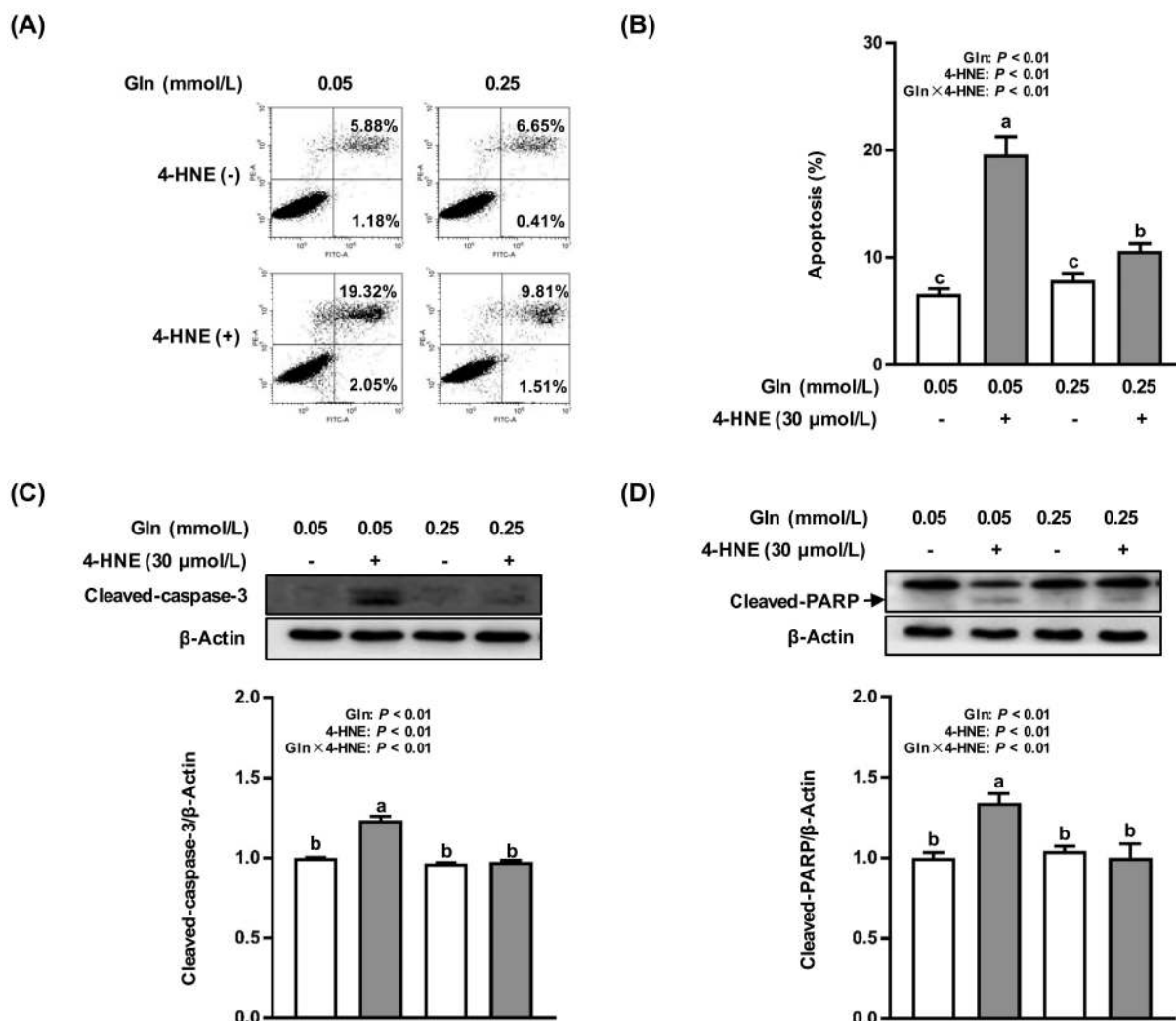


FIGURE 2 L-Glutamine attenuated 4-HNE-induced apoptosis in IPEC-1 cells. IPEC-1 cells were treated as in Figure 1. (A) Apoptosis was determined and (B) analyzed as described. Protein abundances for (C) the cleaved caspase 3 and (D) the cleaved PARP were determined by Western blot. β -Actin was used as the loading control. Values are expressed as means \pm SEMs, $n = 3$ independent experiments. Means without a common letter differ, $P < 0.05$. Gln, L-glutamine; IPEC-1, intestinal porcine epithelial cell line 1; PARP, poly ADP-ribose polymerase; 4-HNE, 4-hydroxy-2-nonenal.

GCLC, *GCLM*, *GSR*, and *GSS* were enhanced by 4-HNE, as compared with the control (Figure 5B and C). The effects of 4-HNE on *GCLC*, *GCLM*, and *GSR*, but not *GSS*, were further enhanced by 0.25 mmol L-glutamine/L (Figure 5B and C). Considering a critical role of cysteine in the biosynthesis of GSH under oxidative conditions, the mRNA levels for cystathionine β -synthase (*CBS*) and cystathionine γ -lyase (*CTH*), 2 important providers of intracellular cysteine for the generation of GSH, were analyzed (36). Of note, the 4-HNE-induced upregulation of these 2 genes was further enhanced by L-glutamine supplementation (Figure 5D). We also determined mRNA levels for thioredoxin reductase (*TXNRD1*), *TXNRD2*, thioredoxin (*TXN1*), and *TXN2*, which encode key enzymes in the thioredoxin antioxidative system (37). Cells treated with 4-HNE had an increased ($P < 0.05$) mRNA level of *TXNRD1* (Supplemental Figure 2A) and decreased ($P < 0.05$) mRNA level of *TXNRD2* (Supplemental Figure 2C), and these effects of 4-HNE were not affected by 0.25 mmol L-glutamine/L. The mRNA levels of *TXN1* and *TXN2* were not influenced by either 4-HNE or 4-HNE plus L-glutamine (Supplemental Figure 2B and 2D).

L-Glutamine enhanced expression of genes related to 4-HNE metabolism. To explore the effect of L-glutamine on the metabolism of 4-HNE, quantitative real-time PCR was performed to determine mRNA levels for glutathione *S*-transferase A (*GSTA1*), *GSTA4*, alcohol dehydrogenase 5 (*ADH5*), aldehyde dehydrogenase 2 (*ALDH2*), aldo-keto reductase family 1 member C1 (*AKR1C1*), and aldo-keto reductase family 1 member A1 (*AKR1A1*) (38), key genes involved in 4-HNE metabolism. As illustrated, the addition of 4-HNE to culture medium enhanced ($P < 0.05$) the mRNA levels for *GSTA1* and *GSTA4* (Figure 6A and B) compared with the control. This effect of 4-HNE was further augmented ($P < 0.05$) by 0.25 mmol L-glutamine/L. In the absence of 4-HNE, glutamine treatment did not affect the expression of *GSTA1* and *GSTA4*. However, 4-HNE resulted in upregulated ($P < 0.05$) mRNA levels for *AKR1C1* and *AKR1A1* (Supplemental Figure 3A, 3C, and 3D) compared with the controls, and this effect of 4-HNE was not affected by 0.25 mmol L-glutamine/L. The mRNA levels for *ALDH2* and *ADH5* were not altered by either 4-HNE or 4-HNE plus L-glutamine supplementation (Supplemental Figure 3B).

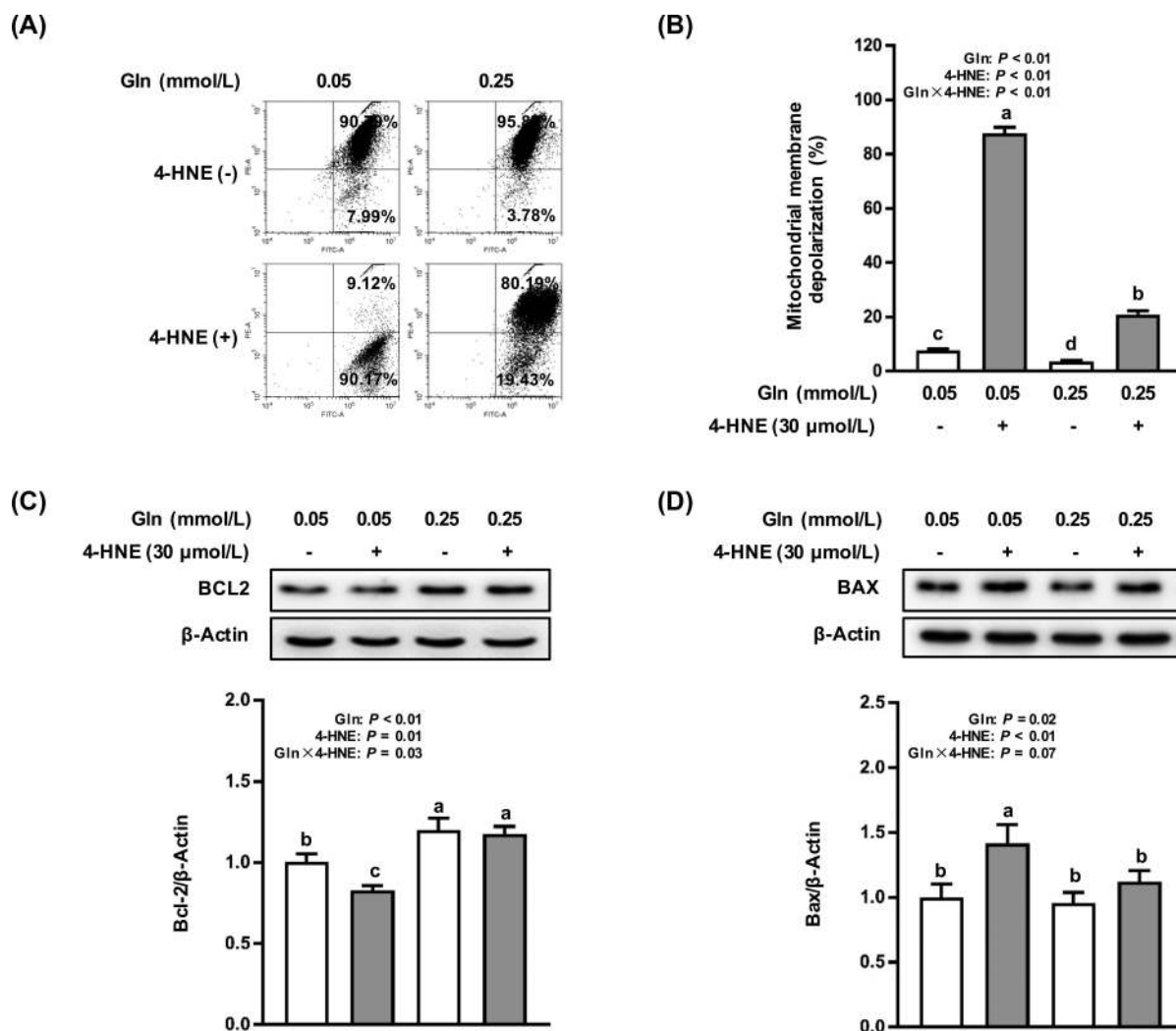


FIGURE 3 The protective effect of L-glutamine on 4-HNE-induced apoptosis and mitochondrial depolarization in IPEC-1 cells. Cells were treated as in Figure 1. Mitochondrial depolarization was determined by JC-1 staining (A) and was analyzed (B). Protein abundances for (C) BCL2 and (D) BAX were determined by Western blot. β -Actin was used as the loading control. Values are expressed as means \pm SEMs, $n = 3$ independent experiments. Means without a common letter differ, $P < 0.05$. BAX, B cell lymphoma 2-associated X protein; BCL2, B cell lymphoma 2 protein; Gln, L-glutamine; IPEC-1, intestinal porcine epithelial cell line 1; JC-1, 1,1',3,3'-tetraethylbenzamidazolcarbocyanin iodide; 4-HNE, 4-hydroxy-2-nonenal.

Discussion

In the present study, we found that 4-HNE, an end product of lipid peroxidation (18), promoted caspase-3-dependent apoptosis in IPEC-1 cells. This effect of 4-HNE was greatly attenuated by 0.25 mmol L-glutamine/L. Further studies showed that the protective effect of L-glutamine was primarily mediated through enhancing the production of GSH and the transcriptional upregulation of *GSTA1* and *GSTA4*, critical genes involved in 4-HNE metabolism. To the best of our knowledge, this is the first study to report an important role for L-glutamine in regulating GSH-related redox homeostasis and GSTA-mediated metabolism in animal cells. The findings highlight a critical role for enteral L-glutamine intake in neonatal intestinal health.

L-Glutamine is known to inhibit apoptosis in intestinal cells (31, 32, 34). However, how L-glutamine exerts this protective role remains largely unknown. In our previous study, we found that 4-HNE concentration in the jejunal tissue of weaned piglets was higher than that in suckling piglets (data not shown), which is accompanied by an increased intestinal permeability and

decreased mucosal barrier function (23), indicating a deleterious effect of 4-HNE on the intestinal barrier of piglets. Furthermore, we demonstrated that the 4-HNE-induced apoptosis was mediated mainly through reducing intracellular GSH biosynthesis in enterocytes (21). Considering a high catabolism rate of L-glutamine by the intestinal epithelium and its role in the maintenance of gut function, IPEC-1 cells were treated with 4-HNE in the presence or absence of physiologic concentrations of L-glutamine (0.25 mmol/L) found in the lumen of the piglet small intestine. In agreement with oxidative damage, as described in previous studies (18, 21), 4-HNE caused mitochondrial depolarization and release of the BCL2 family of proteins, primary characteristics of mitochondrial apoptosis. These biochemical alterations were translated into apoptosis, as shown by decreased cell viability, the cleavage of caspase-3, and the release of PARP, which are well-known markers of apoptosis. Importantly, 4-HNE-induced cell death, mitochondrial depolarization, and ROS accumulation were substantially attenuated by L-glutamine supplementation, indicating a protective effect of this nutrient against oxidative injury.

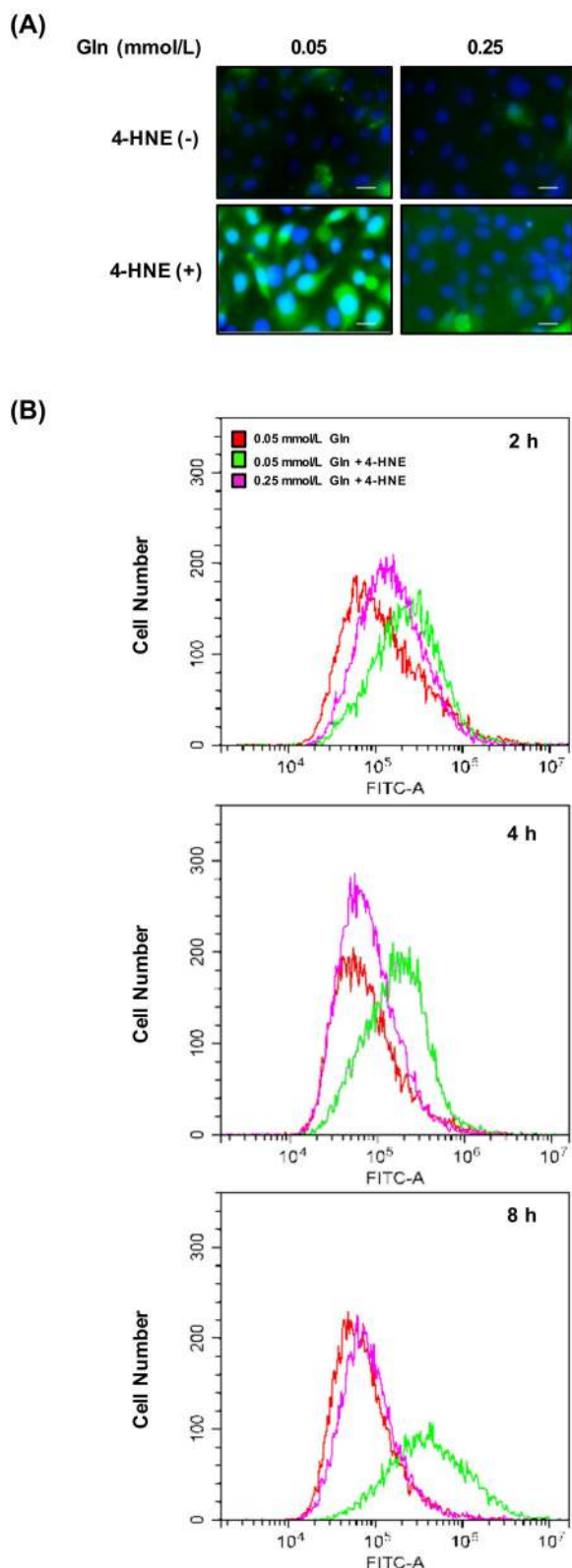


FIGURE 4 L-Glutamine blocked 4-HNE-induced ROS generation in IPEC-1 cells. IPEC-1 cells were treated as in Figure 1. (A) ROS content was determined by the DCFH-DA assay, and images were taken under a fluorescence microscope (magnification $\times 400$). (B) DCFH-DA-positive populations were determined by flow cytometry analysis in a time course. DCFH-DA, 2',7'-dichlorofluorescein diacetate; Gln, L-glutamine; IPEC-1, intestinal porcine epithelial cell line 1; ROS, reactive oxygen species; 4-HNE, 4-hydroxy-2-nonenal.

GSH and TXN are major antioxidant systems, responsible for oxidant detoxification (39–41). In agreement with a previous study (21), IPEC-1 cells treated with 4-HNE exhibited a decrease in GSH concentrations, which, in turn, led to the impairment of antioxidative defense, the accumulation of intracellular ROS and, ultimately, mitochondrial apoptosis. Following 4-HNE exposure, the mRNA levels for *GCLC*, *GCLM*, and *GSR*, genes involved in GSH de novo synthesis and reduction of oxidative glutathione to GSH, were upregulated, indicating the activation of the adaptive response for survival. Considering that L-glutamine is a major fuel for cellular functions, including antioxidative defense, cell proliferation, and ATP-consuming biochemical reactions (24), the insufficiency of L-glutamine (0.05 mmol/L) in the basal medium may limit the adaptive response and contribute to cell death when cells are exposed to 4-HNE. In contrast, the addition of a physiologic concentration of L-glutamine (0.25 mmol/L) rescued oxidative stress-induced cellular damage, by further enhancing the mRNA levels of genes involved in GSH biosynthesis (Figure 5). It has been reported that cysteine is a limiting amino acid for GSH synthesis in response to oxidative damage (36). In the present study, we also observed upregulation of *CBS* and *CTH*, 2 genes encoding key enzymes that catalyze the provision of intracellular cysteine, in 4-HNE-treated IPEC-1 cells. Of particular interest, L-glutamine supplementation enhanced the expression of *CBS* and *CTH* in the cells, indicating a role of the transsulfuration pathway in cell survival by generating cysteine, a substrate for GSH synthesis.

In addition to reacting with GSH, 4-HNE can be conjugated with GSH to form less reactive metabolites, which can be readily excreted (15). This conjugation process is mainly catalyzed by *GSTA1* and *GSTA4* (39, 42). Exposure of IPEC-1 cells to 4-HNE resulted in GSH depletion (Figure 5A), which limited the generation of GST-conjugated 4-HNE and contributed to detrimental effects on the cells. In contrast, L-glutamine supplementation reversed the 4-HNE-induced GSH depletion, which was accompanied by increased mRNA levels of *GSTA1* and *GSTA4*, thereby reducing the cytotoxic effect of 4-HNE and rescuing 4-HNE-induced cell death. These results indicated that a GSH-related redox homeostatic regulation of the cell fate decision plays a critical role in protecting enterocytes from 4-HNE challenge.

The TXN system is another key antioxidant system that modulates redox signaling and related apoptosis under various conditions (39, 40, 43). In mammalian cells, 3 isoforms of TXN have been identified: TXN1, a cytoplasmic protein; TXN2, a mitochondrial protein; and spermatid-specific thioredoxin, which is expressed mainly in spermatozoa (44). Correspondingly, there are 3 thioredoxin reductase isoforms (TXNRDs): the cytosolic protein TXNRD1, the mitochondrial protein TXNRD2, and the testis-specific TXNRD3 (also known as thioredoxin glutathione reductase), which act to maintain thioredoxins in their reduced forms (39, 40, 43). It has been reported that lower levels of oxidative stimuli, including UV irradiation, inflammatory cytokines, and hydrogen peroxide, induce the expression of TXN (45), whereas higher concentrations of 4-HNE inhibit TXN/TXNRD activities and contribute to the cytotoxic effect of 4-HNE and oxidative stress (42). In our study, both the cytosolic (TXN1) and mitochondrial isoforms (TXN2) were not affected by either 4-HNE or 4-HNE plus glutamine supplementation. In contrast, the mRNA level for *TXNRD1* was augmented, but that for *TXNRD2* was suppressed, by 4-HNE, which was not consistent with results of a previous study involving HeLa cells (42). The reason for this discrepancy is unknown. Considering that

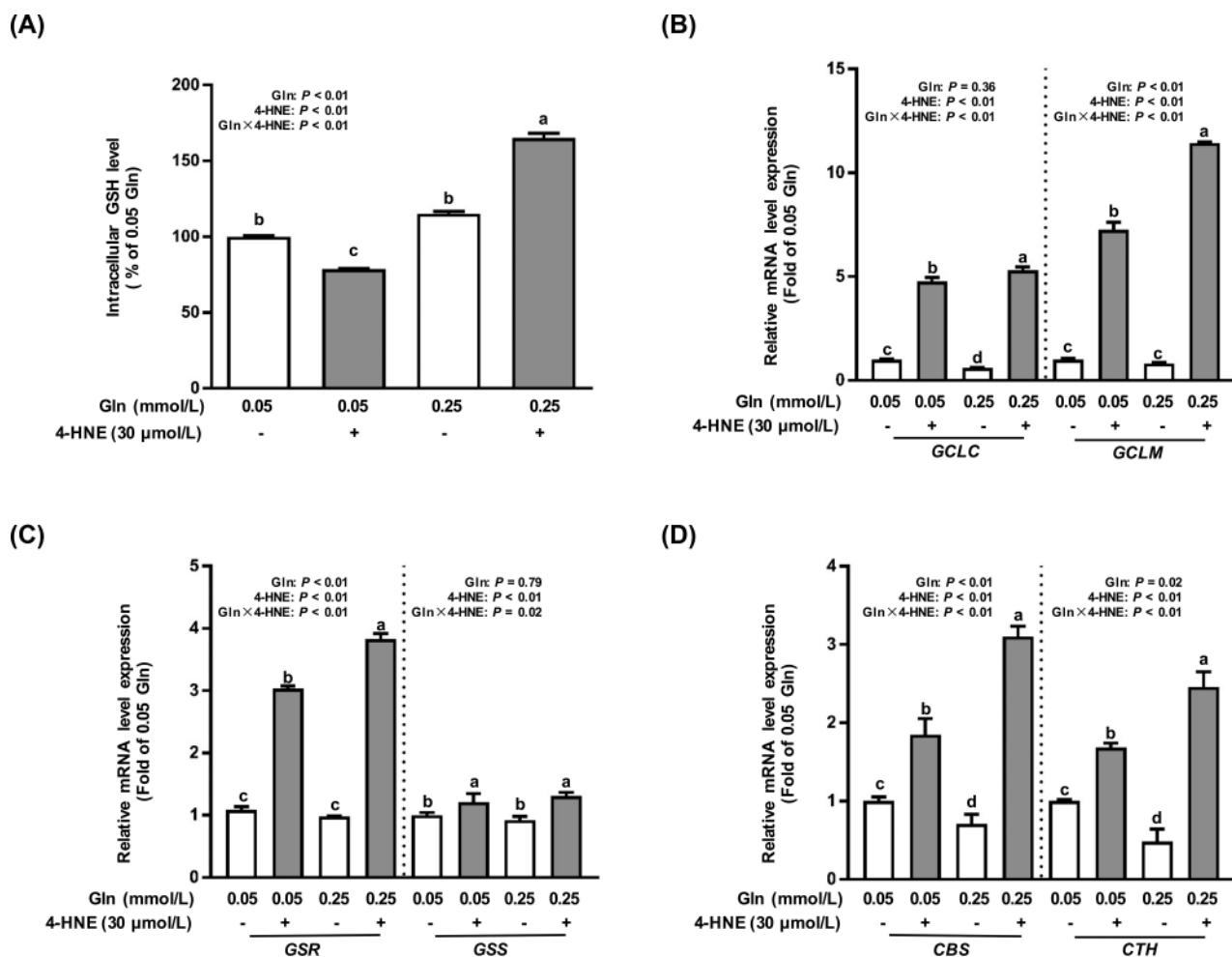


FIGURE 5 Effects of L-glutamine on GSH production in IPEC-1 cells. IPEC-1 cells were treated with 4-HNE (30 μmol/L) for 8 h in the presence of 0.05 (the control) or 0.25 mmol L-glutamine/L. Intracellular GSH (A) and mRNA levels for *GCLC* and *GCLM* (B), *GSR* and *GSS* (C) and *CBS* and *CTH* (D) were determined by the quantitative real-time PCR. Values are means ± SEMs, $n = 6$ independent experiments. Means without a common letter differ, $P < 0.05$. CBS, cystathionine β-synthase; CTH, cystathionine γ-lyase; *GCLC*, catalytic subunit of glutamate-cysteine ligase; *GCLM*, glutamate-cysteine ligase modifier subunit; Gln, L-glutamine; GSH, glutathione; *GSR*, glutathione reductase; *GSS*, glutathione synthetase; IPEC-1, intestinal porcine epithelial cell line 1; 4-HNE, 4-hydroxy-2-nonenal.

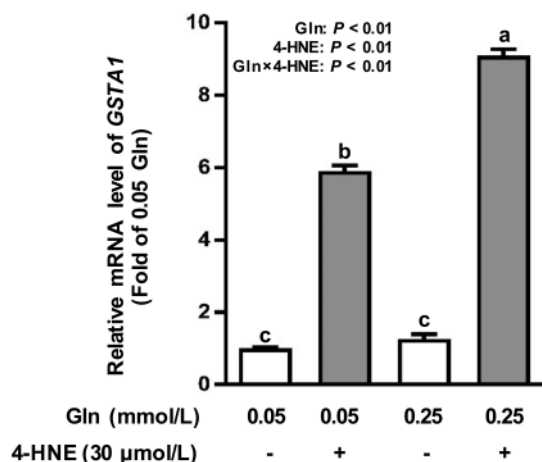
the inhibitory effect of 4-HNE on the activity of TXR was observed at 3.8 μmol/L in HeLa cells, which is much lower than the concentration of 4-HNE used in our study, a difference in the sensitivity of various cell lines to 4-HNE might be a plausible explanation for this inconsistency (16, 20).

In addition to the antioxidant systems, 4-HNE can also be metabolized and detoxified by ALDH (46) and AKR1A1 and AKR1C1 (37, 47). ALDH2 is a mitochondrial enzyme that metabolizes toxic aldehydes (e.g., 4-HNE and acetaldehyde) in a variety of tissues, including the gastrointestinal tract, liver, kidney, and heart (48, 49). Overexpression of the *ALDH2* gene has been reported to protect gastric mucosa cells against oxidative stress-induced apoptosis (50). In our study, the mRNA levels for *ALDH2* were not affected by either 4-HNE or 4-HNE plus L-glutamine, excluding the involvement of *ALDH2* in the detrimental effect of 4-HNE in intestinal epithelial cells. In contrast, the mRNA levels for *AKR1A1* and *AKR1C1*, which encode critical ALDHs to degrade 4-HNE in the liver and other tissues (47), were upregulated upon exposure to 4-HNE, indicating a metabolic response in intestinal epithelial cells. Of interest, the 4-HNE-induced upregulation of *AKR1C1*, instead

of *AKR1A1*, was upregulated by L-glutamine supplementation, indicating a potential role of *AKR1C1* in the protective effect of L-glutamine.

In conclusion, the results from the present study demonstrated that 4-HNE, a lipid peroxidation endproduct, induced cell injury and activation of mitochondrial apoptosis signaling. This effect of 4-HNE was accompanied by a decrease in GSH synthesis and transactivation of genes involved in 4-HNE catabolism, including *GSTAs* and *AKR*. L-Glutamine supplementation reversed 4-HNE-induced GSH depletion and enhanced the mRNA levels for *GSTA1* and *GSTA4*, and therefore conferred a protective effect. These findings uncover a novel functional role of L-glutamine on oxidative stress-induced cellular injury and apoptosis by regulating GSH-mediated detoxification. Considering the involvement of 4-HNE in various intestinal disorders in humans and animals, provision of L-glutamine might be a nutritional strategy to prevent intestinal injury induced by oxidative stress. At present, it is unknown how L-glutamine regulates the expression of genes involved in intestinal GSH synthesis and further studies are warranted to answer this question.

(A)



(B)

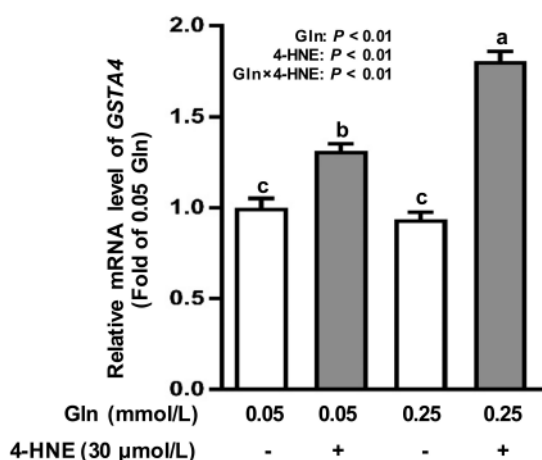


FIGURE 6 Effects of L-glutamine on the mRNA levels of GST in IPEC-1 cells. mRNA levels for *GSTA1* (A) and *GSTA4* (B) were analyzed by real-time PCR. Values are means \pm SEMs, $n = 6$ independent experiments. Means without a common letter differ, $P < 0.05$. Gln, L-glutamine; *GSTA1*, glutathione *S*-transferase A1; *GSTA4*, glutathione *S*-transferase A4; IPEC-1, intestinal porcine epithelial cell line 1; 4-HNE, 4-hydroxy-2-nonenal.

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