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# Antioxidative Activity of Amino Acids on Tissue Oxidative Stress in Human Intestinal Epithelial Cell Model

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The protective effects of amino acids against $H_2O_2$-induced oxidative stress were investigated in an in vitro assay using human intestinal epithelial cells. Caco-2 cells were pretreated with amino acids (1, 2, and 5 mM) for 2 h and then stimulated with 1 mM $H_2O_2$ for 6 h. The secretion of IL-8, a proinflammatory mediator, was determined by ELISA as an indicator of tissue oxidative stress. The inhibition of $H_2O_2$-induced IL-8 secretion from Caco-2 cells was observed by pretreatment with Cys, Val, Ile, Leu, Trp, His, Lys, and Ala. Cys enhanced glutathione (GSH) biosynthesis enzyme activity and increased cellular GSH levels. Branched-chain amino acids such as Val, Ile, and Leu elevated activities of GSH S-transferase (GST) and catalase. Trp, His, and Lys caused increases in GST activity. Ala enhanced GSH reductase activity. These data suggest that specific amino acids exert protective effects against tissue oxidative stress in intestinal epithelial cells based on the structure.

**KEYWORDS: Amino acids; antioxidant enzymes; tissue oxidative stress; glutathione; Caco-2**

## INTRODUCTION

Oxidative stress is a state characterized by an imbalance between generation of reactive oxygen species (ROS) and the cellular antioxidant system. Under physiological conditions, ROS play multiple positive roles in energy production, phagocytosis, cell growth, and intercellular signaling regulation (*1*). Low levels of ROS are effectively scavenged by the cellular antioxidant defense system; however, excessive generation of ROS has a deleterious effect on human health since they can damage cell structure involved in the cellular lipids, membranes, proteins, and DNA and consequently lead to loss of their biological function. Oxidative stress contributes to the pathogenesis of various degenerative diseases, including atherosclerosis, cardiovascular disease, type 2 diabetes, and cancer, as well as aging (*1–4*). The human body is equipped with antioxidative defense systems that protect cellular macromolecules from detrimental effects of exogenous or endogenous ROS. These consist of enzymatic antioxidant defenses (superoxide dismutase, SOD; glutathione peroxidase, GPx; catalase), nonenzymatic antioxidants (glutathione, GSH; thioredoxin), and dietary antioxidants (*5*). A variety of naturally occurring antioxidants has been discovered in various food sources. Polyphenols are known as powerful antioxidants due to its favorable reduction, and several flavonoids such as red wine resveratrol (*6*) and turmeric curcumin (*7*) have been demonstrated to enhance endogenous antioxidants and detoxifying enzymes, leading to increased resistance to oxidative tissue injury.

An amino acid is a basic structural unit of proteins that plays important roles in a variety of biological functions, including protein synthesis maintenance, potentiation of immune function, tissue repair acceleration after trauma, stimulation of insulin, or growth hormone secretion (*8*, *9*). Recent studies have suggested that amino acids have potential as therapeutic agents for the treatment of a number of medical conditions, including liver failure, anorexia, cachexia, chronic inflammatory disease, and muscle wasting of aging (*10–12*). However, there was little information concerning the protective effects of amino acids against tissue oxidative stress. Recently, Son et al. (*13*) have showed that His and Lys inhibit oxidative stress induced IL-8 production in intestinal epithelial cells (Caco-2). Specific amino acids such as His exerted as inactivator of transcription factor, nuclear factor-kappaB (NF-$\kappa$B), which is a regulatory element for IL-8 gene expression. We hypothesized that some amino acids modulate the cellular antioxidative systems and thereby suppress oxidative stress induced inflammatory response. Induction of antioxidants and detoxifying enzymes in the cells appears to be a promising strategy for protecting against oxidative tissue injury.

In the present study, we evaluated the protective effect of amino acids against $H_2O_2$-induced oxidative stress in an in vitro assay using Caco-2 cells (a widely studied human intestinal epithelial cell model). We further measured GSH content and activities of GSH biosynthesis-associated enzyme and antioxidant enzymes to examine the enhancing effects of the cellular antioxidative defense systems. We also assessed whether amino acids have any effect on the modulation of the defense systems under nonstressed condition.

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## MATERIALS AND METHODS

**Materials.** GSH, $H_2O_2$, glutathione reductase (EC 1.6.4.2; GR), pyruvate kinase/lactatedehydrogenase (PK/LDH), and all other chemicals were purchased from Sigma Chemical (St. Louis, MO). Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and molecular biology reagents were purchased from Gibco Invitrogen (Burlington, ON, Canada). The flasks for growing cells were obtained from Corning Costar (Cambridge, MA).

**Cell Culture.** Caco-2 cell lines were purchased from the American type Culture Collection (Rockville, MD). The cells were cultured in DMEM medium with 20% FBS in the presence of penicillin and streptomycin (Gibco Invitrogen), and incubated at 37 °C in 5% $CO_2$. Passages 20–50 were used in all monolayers employed in tissue oxidative-stress experiments. The cells were grown in a flask for 5–8 days after seeding and then transferred (at $2 \times 10^5$ cells/mL) to 24-well culture plates (Corning Costar). The medium was changed twice per week. The cells were cultivated for 5–7 days until confluent monolayers formed.

**Induction of Oxidative Stress.** The tissue oxidative stress was induced in confluent cell cultures by the addition of $H_2O_2$. Caco-2 monolayers were washed twice with Hank's balanced salt solution without calcium and magnesium and placed in 5% serum-containing media. The cells were then incubated for 2 h with various concentrations of amino acids (0–5 mM) and next added 50 mM $H_2O_2$ (final concentration, 1 mM). At least 95% of the cells remained viable for the above treatments, as assessed by trypan-blue exclusion. The culture supernatants were collected to measure IL-8 production and stored at −80 °C. The cells were then washed twice with 100 mM phosphate-buffered saline (PBS), pH 7.2, containing 100 mM NaCl. For measurement of GSH, PBS containing 0.1% Triton X-100 was added into each well to extract intracellular GSH and stored at −80 °C until required. For the enzymes assay, 100 mM potassium phosphate solution (pH 7.5) with 1 mM EDTA was added, sonicated on ice for 30 s with a Branson Sonifier 250 (Danbury, CT), and centrifuged at 10000*g* and 4 °C for 15 min. The supernatant was stored at −80 °C until use. The protein concentration in the cell lysate was determined by the Bio-Rad DC protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin (BSA) as the standard.

**Enzyme-Linked Immunosorbent Assay (ELISA) for IL-8.** The determination of IL-8 in culture supernatants was carried out by sandwich ELISA. Ninety six well ELISA microtiter plates (Corning Costar) were coated with 100 μL/well of mouse antihuman IL-8 antibody (1 μg/mL) (BD Bioscience, San Diego, CA) in 100 mM sodium phosphate buffer (pH 9.0) and incubated at 4 °C overnight. The plate was then washed three times with PBS containing 0.05% Tween-20 (PBST) and blocked with 200 μL/well of 2% BSA in PBS at 37 °C for 1 h. The plate was washed with PBST three times, and the culture supernatant samples (100 μL/well) were added into each well and incubated for 2 h at 37 °C. After the samples were washed four times, 100 μL/well of biotinylated mouse antihuman IL-8 antibody (0.5 μg/mL) (BD Bioscience) in PBS containing 1% BSA was added and incubated for 1 h at 37 °C. The plate was further washed four times with PBST and incubated with avidin-horseradish peroxidase conjugate (100 μL/well, diluted 1:2000 in PBS containing 1% BSA) (BD Bioscience) for 1 h at 37 °C. The plate was washed six times and developed with 100 μL of 3,3′,5,5′-tetramethylbenzidine (TMB) (Sigma) for 15 min at 37 °C. The reaction was terminated with 0.5 N $H_2SO_4$ (50 μL/well), and the absorbance at 450 nm was read by a microplate reader (model 550, Bio-Rad Laboratories).

**Measurement of Intracellular GSH.** Intracellular total GSH was determined according to the method of Allen et al. (*14*) with a modification. The cell lysate was added to a 10% sulfosalicylic acid solution to precipitate proteins and centrifuged at 11000*g* for 10 min at 4 °C. The supernatant (25 μL) was mixed with 125 μL of 100 mM PBS containing 4 mM EDTA, 0.2 mM NADPH, 0.5 mM DTNB, and 100 U/mL GR. The mixture was incubated for 5 min at 25 °C, and the absorbance was measured at 412 nm using a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). The concentration of GSH in the cell lysate was calculated using a standard curve and expressed as moles of GSH per milligram of protein.

**Measurement of γ-Glutamylcysteine Synthetase (γ-GCS) Activity.** γ-GCS activity was assayed by the method described by Seelig and Meister (*15*) by following the oxidation of NADH at 340 nm and 25 °C. Briefly, 0.6 mL of lower case solution, which consists of 0.25 M Tris-HCl (pH 8.2) containing 1.25 mM EDTA, 166 mM KCl, and 0.1 mM NADH, was added with 0.31 mL of 10 mM ATP, 10 mM PEP, 10 mM L-glutamate, PK/LDH, and 5 mM L-α-aminobutyric acid (L-α-AB). The cell lysate (0.09 mL) was added to the solution, and the decrease in absorbance at 340 nm was recorded every 1 min for 5 min using a spectrophotometer. Enzyme-specific activity was defined as mmol $min^{-1}$ NADH oxidized $mg^{-1}$ of protein, which is equal to 1 IU.

**Isolation of RNA and Reverse Transcription.** After the sample was incubated for 2 h with amino acids followed by the addition of 50 mM $H_2O_2$ (final concentration, 1 mM) for 1 h in six-well plates, the total RNA was isolated from the Caco-2 cells using Trizol reagent. The concentration of total RNA in the final eluate was determined by spectrophotometry. cDNA was synthesized using 5 μg of total RNA and a cDNA synthesis kit according to the manufacturer's instructions. The resultant cDNA was stored at −20 °C until required.

**Analysis of γ-GCS-HS mRNA by the Polymerase Chain Reaction (PCR).** To quantitate γ-GCS mRNA expression, the reverse transcriptase PCR assay was used. The γ-GCS holoenzyme exists as a dimer composed of heavy (γ-GCS-HS; 73 kDa) and light (γ-GCS-LS; 28 kDa) subunits, and the heavy subunit possesses all of the catalytic activity (*16*). Oligonucleotide primers were chosen using the published sequence of human γ-GCS-HS (*17*), cDNA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*18*). The sequences of the primers used in the PCR were as follows: γ-GCS-HS (forward primer 5′-GTG GTA CTG CTC ACC AGA GTG ATC CT-3′ and reverse primer 5′-TGA TCC AAG TAA CTC TGG ACA TTC ACA-3, 531 bp); GAPDH (forward primer 5′-CC ACC CAT GGC AAA TTC CAT GGC A-3′ and reverse primer 5′-TC TAG ACG GCA GGT CAG GTC AAC C-3′, 600 bp). The reverse transcribed mRNA mixture (2 μL) was added directly to the PCR mixture, which consisted of buffer (10 mM Tris-HCl, pH 8.4, 50 mM KCl, 50 mM $MgCl_2$), 10 mM dNTPs and 10 mM of each primer, and 5 U of Taq DNA polymerase in a final volume of 50 μL. Thirty five and thirty cycles were repeated for the amplification of γ-GCS-HS and GAPDH, respectively. Conditions for the thermal cycles were as follows: γ-GCS, denaturated at 94 °C for 2 min, annealing at 56 °C for 30 s, primer extension at 72 °C for 2 min, and final extension at 72 °C for 7 min; GAPDH, denaturated at 94 °C for 1 min, annealing at 62 °C for 1 min, primer extension at 72 °C for 27 s and final extension at 72 °C for 7 min. The amplified PCR products (10 μL) were subjected to electrophoresis on 2% agarose gels containing ethidium bromide. DNA band sizes were confirmed using a Gene Ruler 100-bp DNA ladder (Invitrogen Gibco).

**Determination of Glutathione *S*-Transferase (GST) Activity.** GST activity was determined according to the procedure of Habig et al. (*19*), by following the formation of 1-chloro-2,4-dinitrobenzene (CDNB)-GSH conjugate at 340 nm and 25 °C. Briefly, 0.05 mL of cell lysate was mixed with 0.95 mL of 0.1 M potassium phosphate buffer (pH 6.5) containing 2 mM GSH, 1 mM CDNB, and 0.1% Triton X-100. The increase in absorbance was monitored every 1 min for 5 min using a spectrophotometer. Changes in the rate of absorbance were converted into units of GST per milligram of protein using a molar extinction coefficient of 9.6 $mM^{-1}$ $cm^{-1}$. One unit of activity was expressed as the conjugation of 1 nmol $min^{-1}$ CDNB with GSH.

**Determination of GR Activity.** GR activity was determined according to the procedure of Carlberg and Mannervik (*20*), by following the decomposition of NADPH at 340 nm and 25 °C. Briefly, 0.1 mL of cell lysate was mixed with 0.85 mL of 100 mM PBS (pH 7.5) containing 1 mM EDTA and 0.02 mM oxidized GSH (GSSG). A total of 0.05 mL of 0.1 mM NADPH was added to the solution, and the decrease in absorbance was recorded every 1 min for 10 min using a spectrophotometer. Changes in the rate of absorbance were converted into units of GR per milligram of protein using a molar extinction coefficient of 6.22 $mM^{-1}$ $cm^{-1}$. One unit of activity was defined as the oxidization of 1 mmol $min^{-1}$ NADPH.

**Determination of Catalase Activity.** Catalase activity was determined according to the procedure of Johansson and Borg (*21*), by

following the decomposition of $H_2O_2$ at 340 nm and 25 °C. Briefly, 0.02 mL of cell lysate was mixed with 0.1 mL of 100 mM phosphate buffer (pH 7.0) and 0.03 mL of methanol. The reaction was initiated with 0.02 mL of 352.8 mM $H_2O_2$. The mixture was incubated on a shaker for 20 min and terminated by the addition of 0.03 mL of 10 M potassium hydroxide solution. Immediately thereafter the mixture was supplied with 0.03 mL of 34.2 mM Purpald in 0.5 N hydrochloric acid and then incubated on a shaker for 10 min. Potassium periodate was added to the mixture solution, which was further incubated for 5 min. The absorbance of the purple formaldehyde adduct produced was measured at 570 nm. The catalase activity was calculated using a standard curve with formaldehyde and converted to milligrams of protein. One unit of activity was defined as the formation of 1.0 mmol $min^{-1}$ formaldehyde.

**Statistical Analysis.** The statistical significance of the data was determined by Student's *t* test. A *p* value of less than 0.05 was taken as significant.

## RESULTS

**Effects of Amino Acids on IL-8 Secretion in $H_2O_2$-Treated Caco-2 Cells.** IL-8 is a major proinflammatory mediator and plays an important role in mucosal inflammation of the gastrointestinal tract. Caco-2 cells have been reported to secrete IL-8 when stimulated by oxidative stress or proinflammatory cytokine (*22*). Indeed, we observed that $H_2O_2$-treated Caco-2 cells released IL-8 in a time- and concentration-dependent manner (data not shown). To study the effects of amino acids on this secretion, Caco-2 cells were pretreated with different concentrations of amino acids or GSH (1, 2, and 5 mM) for 2 h, and then stimulated with 1 mM $H_2O_2$ for 6 h. GSH, a powerful antioxidant, was used as a positive control. As shown in **Figure 1**, the pretreatment with Cys, Ala, Ile, Leu, Trp, Val, His, and Lys significantly inhibited IL-8 secretion from $H_2O_2$-treated Caco-2, and their inhibitory effects were almost identical to that of GSH. In contrast, the other amino acids exerted no significant inhibitory effect. We thus focused on the above eight amino acids to characterize the protective effects of amino acids against $H_2O_2$-induced oxidative stress.

**Effect of Amino Acids on GSH Levels in $H_2O_2$-Treated Caco-2 Cells.** To clarify whether the cellular antioxidant defense system was modulated by amino acids, we first investigated the effect of amino acids on intracellular GSH levels. GSH is the main endogenous antioxidant in cells, and it plays important roles in antioxidant defense, including radical scavenge, elimination of lipid peroxidation products, preservation of the reduced state of cellular protein thiols, and oxidative damage repair (*23*). It is usually assumed that GSH depletion reflects oxidative damage. As shown in **Figure 2A**, cells treated with $H_2O_2$ significantly decreased GSH contents compared with the control cells. Pretreatment with Cys, Ile, Trp, His, and Ala significantly increased intracellular GSH levels. In particular, we observed that the GSH levels of Cys- and Trp-pretreated cells were clearly higher than that of the control cells. On the other hand, when the cells were treated with amino acids for 2 h without the following addition of $H_2O_2$, a marked increase in GSH levels was observed in the treatment with only Cys (**Figure 2B**). The GSH synthesis is regulated by $\gamma$-GCS activity and a heavy subunit of $\gamma$-GCS ($\gamma$-GCS-HS) is a key domain. We further investigated the levels of cellular $\gamma$-GCS-HS mRNA expression and $\gamma$-GCS activity in cells treated with Cys and Trp. As shown in **Figure 3**, $\gamma$-GCS-HS mRNA expression was elevated by treatment and pretreatment with Cys compared to those of the control and $H_2O_2$-treated cells, respectively; however, there were no statistical significant differences. Trp did not show any increases in the $\gamma$-GCS-HS mRNA expression. As shown in **Figure 4**, $\gamma$-GCS activity was significantly elevated by treatment with Cys regardless of $H_2O_2$ stimulation. Thus, these data suggest that the increase in GSH levels by Cys is mainly due to up-regulation of $\gamma$-GCS activity; however, the increase in the GCS activity would not be due to the transcriptional regulation but could be associated with other potential pathways. The mechanism remains to be proven.

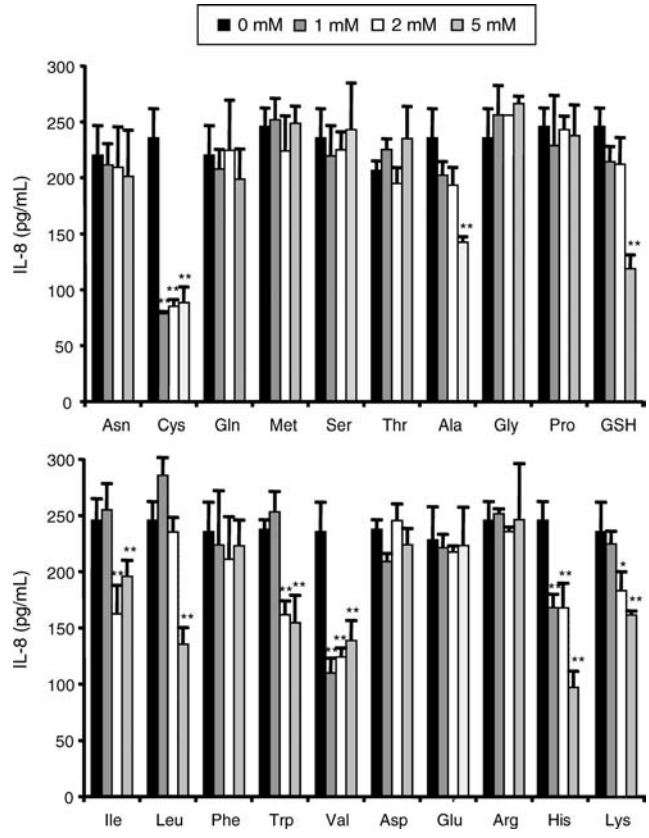


**Figure 1.** Effects of amino acid pretreatment on IL-8 secretion in $H_2O_2$-treated Caco-2 cells. Cells were cultured with 5% FBS-DMEM, treated with various concentrations of amino acids and GSH (0–5 mM) for 2 h at 37 °C, and they were then incubated with $H_2O_2$ (1 mM) for 6 h. $^*P < 0.05$ and $^{**}P < 0.01$, compared with cells treated with $H_2O_2$ alone. Data are presented as mean ± standard deviation of three experiments performed in triplicate.

**Effect of Amino Acids on Antioxidant Enzyme Activity.** We next examined the effect of amino acids on the activities of antioxidant enzymes to determine whether amino acids regulate the enzymatic antioxidant defense systems in cells. Oxidative stress induced by 1 mM $H_2O_2$ for 6 h did not alter GST activity profiles (**Figure 5A**). Pretreatment with 5 mM Val, Ile, Leu, Trp, His, and Lys significantly elevated GST activity in $H_2O_2$-treated cells. On the other hand, $H_2O_2$ stimulation caused decreases in GR and catalase activities compared to that of the control (parts **B** and **C** of Figure 5). The decrease in the GR activity induced by the $H_2O_2$ treatment was suppressed by pretreatment with His and Ala at 5 mM. Additionally, the decrease in the catalase activity induced by the $H_2O_2$ treatment was suppressed by Val, Ile, Leu and Lys.

To investigate the effects of amino acids on the enzymes under nonstressed condition, the activities of GST, GR, and catalase after 2 h of pretreatment with amino acids were measured (**Figure 6**). GST activity was elevated by His and Lys, but the rest of treatment showed no effect under nonstressed condition. The increasing effect of amino acids on GST activity was higher effective under oxidatively stressed conditions compared with nonstressed conditions (**Figures 5A** and **6A**).

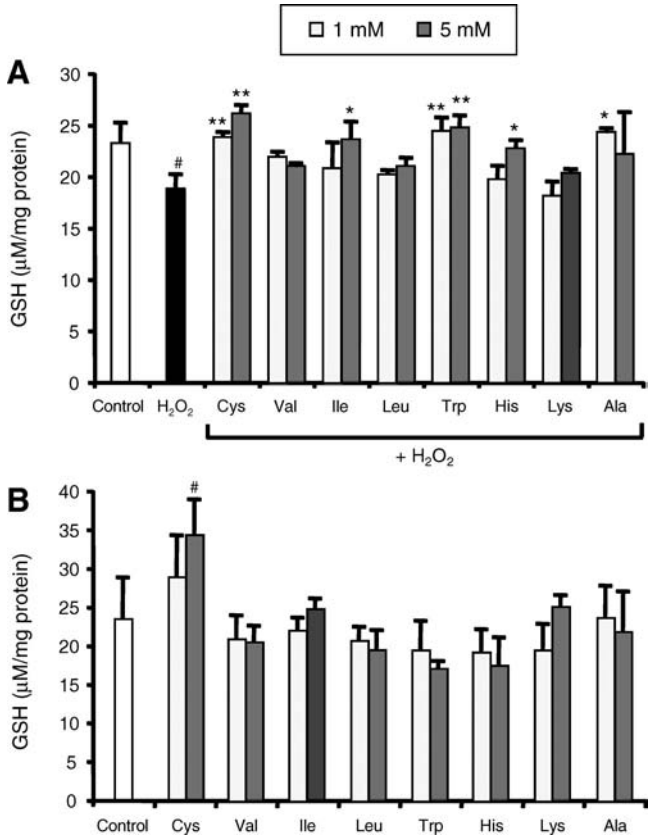


**Figure 2.** Effects of amino acids on GSH content in $H_2O_2$-treated or nontreated Caco-2 cells. (**A**) The cells treated with amino acids (0–5 mM) for 2 h at 37 °C and then incubated with $H_2O_2$ (1 mM) for 6 h. (**B**) The cells treated with amino acids (0–5 mM) for 2 h at 37 °C. Control refers to cell cultures that were not treated with amino acids and not subjected to oxidative stress treatment by $H_2O_2$. $^{\#}P < 0.05$ compared with control cells. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with cells treated with $H_2O_2$ alone. Data are expressed as mean ± standard deviation of three experiments performed in triplicate.

GR activity was enhanced by only Ala treatment (**Figure 6B**), whereas Val, Ile, and Leu significantly increased catalase activity, compared to those of the control (**Figure 6C**). This indicates that several amino acids could elevate antioxidant enzyme activities under physiological conditions.

In addition, we measured the activity after prolonged incubation (additional 3 and 6 h without $H_2O_2$), but any significant alteration in the enzyme activity was not observed (data not shown).

## DISCUSSION

We here demonstrated that eight amino acids (Cys, Ala, Ile, Leu, Trp, Val, Lys, and His) suppressed oxidative stress induced inflammatory response in intestinal epithelial cells. The suppressive effect of His and Lys is in agreement with those recently obtained by Son et al. (*13*) in cultured Caco-2 cells, although their experimental conditions of amino acids were 10 times higher than those of the current study. The antioxidative effects of Ala, Ile, Leu, Trp, and Val are first reported in this study. In the present study, the inhibitory effect by several amino acids might be mainly due to increase of the cellular GSH pool and induction of antioxidant enzyme activities. This study is the first to compare the protective effects of amino acids against oxidative stress in view of enhancement of the cellular antioxidant defense systems, including nonenzymatic antioxidants

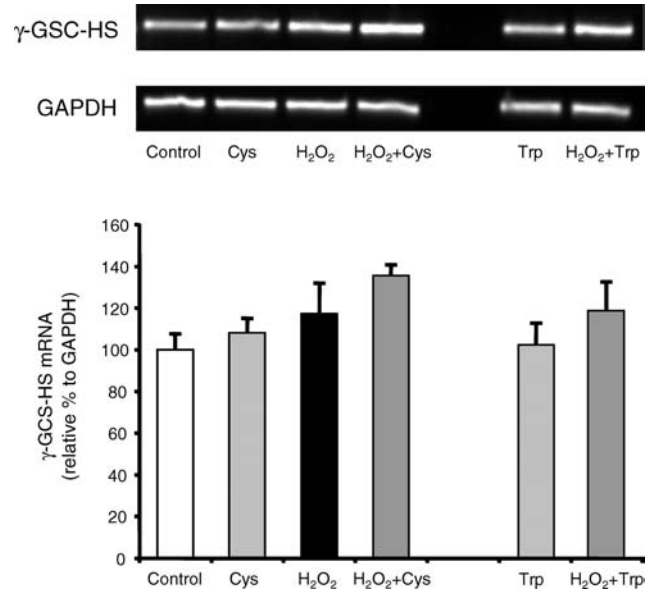


**Figure 3.** Effects of Cys on γ-GCS-HS mRNA expression in $H_2O_2$-treated Caco-2 cells. Total RNA was isolated from control cells and cells treated with Cys and Trp (5 mM) for 2 h or pretreated with Cys and Trp (5 mM) for 2 h and then incubated with $H_2O_2$ (1 mM) for 1 h. The experiments were carried out three times, with similar results being obtained.

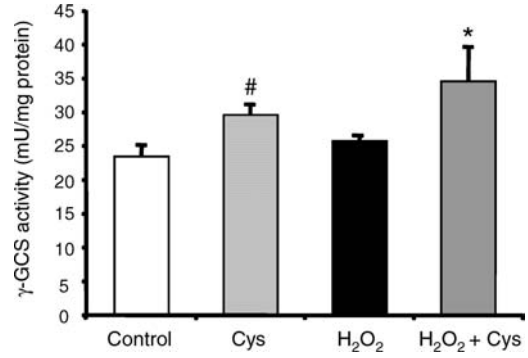


**Figure 4.** Changes in γ-GCS activity in $H_2O_2$-treated Caco-2 cells. The cells were treated with Cys (5 mM) for 2 h or pretreated with Cys (5 mM) and then incubated with 1 mM $H_2O_2$ for 6 h. $^{\#}P < 0.05$ compared with control cells. $^{*}P < 0.05$ compared with cells treated with $H_2O_2$ alone. Data are presented as mean ± standard deviation of two experiments performed in triplicate.

and antioxidant enzymes. From our findings, amino acids having antioxidative stress activities were divided into four groups; sulfhydryl-containing amino acids (Cys), branched-chain amino acids (BCAAs: Val, Ile and Leu), heterocyclic group-containing amino acids (Trp and His), and the others (Lys and Ala) based on the side chain structure (**Table 1**).

Cys is a sulfhydryl group containing amino acid that is an antioxidant precursor of GSH. We here showed that pretreatment of Caco-2 cells with Cys increased de novo GSH synthesis by elevating γ-GCS activity under oxidatively stressed and nonstressed conditions. GSH is synthesized in two steps: Cys and Glu are combined to form γ-Glutamyl-Cys by γ-GCS, then Gly is added to the dipeptide by GSH synthetase (*24*). Cellular GSH is involved in scavenging $H_2O_2$ and lipid hydroperoxides via the GPx- and GST-catalyzed reactions. Thus, this indicates that increased GSH levels by Cys treatment contributes to the protection against $H_2O_2$-induced oxidative stress. Tateishi et al. (*25*) reported that intracellular Cys levels were consistently and

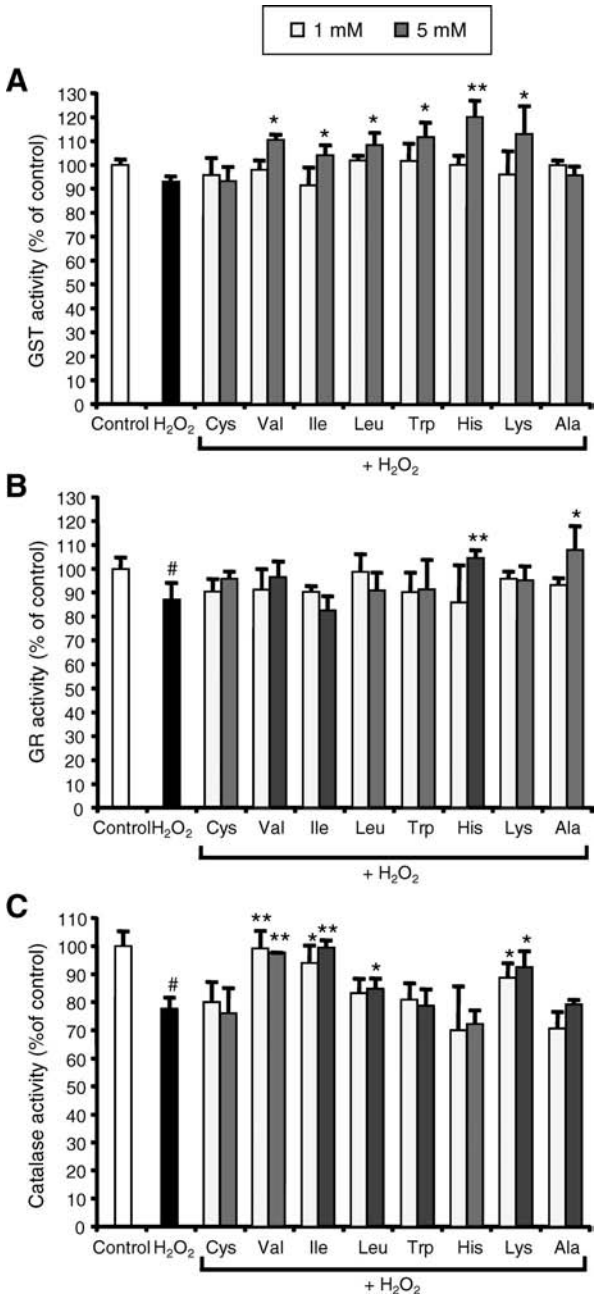


**Figure 5.** Effects of amino acids pretreatment on GST (**A**), GR (**B**), and catalase (**C**) activities in $H_2O_2$-treated Caco-2 cells. The cells were pretreated with amino acids (0–5 mM) for 2 h at 37 °C and then incubated with 1 mM $H_2O_2$ for 6 h. $^{*}P < 0.05$ and $^{**}P < 0.01$ compared with cells treated with $H_2O_2$ alone. $^{\#}P < 0.05$ compared with control cells. Data are expressed as mean ± standard deviation of three experiments performed in triplicate.

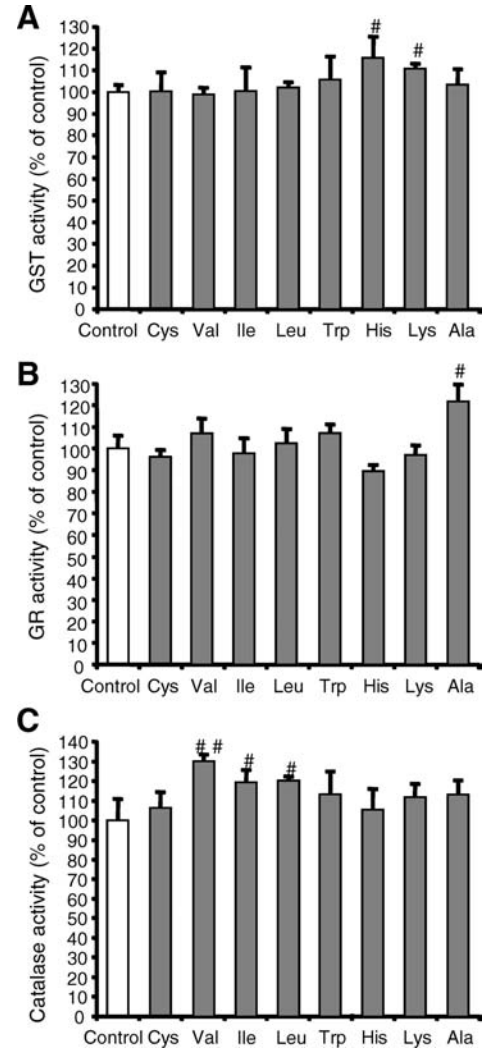


**Figure 6.** Effects of amino acids treatment on GST (**A**), GR (**B**), and catalase (**C**) in Caco-2 cells. The cells were treated with amino acids 5 mM for 2 h. $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ compared with control cells. Data are presented as mean ± standard deviation of three experiments performed in triplicate.

substantially lower than those of Glu and Gly, and they proposed that Cys availability was a general limiting factor for GSH synthesis. In this regard, supplementation with Cys would be a reasonable strategy for inducing de novo GSH synthesis.

BCAAs play an important role in regulating protein metabolism and serving as an energy source (*26*). In the current study, we demonstrated that BCAAs have potential to up-regulate the activities of GST and catalase in $H_2O_2$-treated Caco-2 cells, indicating that BCAAs can act as antioxidant enzyme inducers. In biological systems, catalase is responsible for decomposing $H_2O_2$, while GST is involved in detoxification of lipid hydroperoxides (*27*). Thus, the potent induction of GST and catalase may largely contribute to the protective effects of BCAAs against $H_2O_2$-induced oxidative stress in cells. Transcriptional regulation of antioxidant genes is predominantly mediated by a redox-sensitive transcriptional factor NF-E2 related factor-2 (Nrf2) (*28*). Recent studies have reported that a variety of phytochemicals, including curcumin, resveratrol, and epigallocatechin gallate, are able to activate Nrf2 signaling pathway, thereby up-regulating a variety of cytoprotective genes in various types of cells/tissues (*29*). The mechanism of Nrf2 in intestinal epithelial cells remains unclear; however, it is likely that BCAAs could up-regulate a set of antioxidant enzymes by stimulating the Keap1/Nrf2 signaling pathway. In this study, we did not investigate their effect on the regulation of redox-sensitive transcriptional factors. Thus, the mechanism underlying the induction of cellular antioxidant enzymes needs to be further explored at the transcriptional level.

Trp has an indole ring that is the precursor of niacin and serotonin. It is necessary for optimal growth in infants and for nitrogen equilibrium in human adults. We here showed that pretreatment with Trp enhanced GST activity in the $H_2O_2$-treated cells. Recently, many researchers have studied the cancer chemopreventive properties of natural indoles such as indole-3-carbinol (I3C) and ascorbigen (*30*, *31*), and it has been showed

**Table 1.** Summary of Effects of Amino Acids on GSH Level and Antioxidant Enzyme Activities in Caco-2 Cells[a]

| | GSH level | | γ-GCS activity | | GST activity | | GR activity | | catalase activity | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 2 h[b] | 2 h + 6 h[c] | 2 h[b] | 2 h + 6 h[c] | 2 h[b] | 2 h + 6 h[c] | 2 h[b] | 2 h + 6 h[c] | 2 h[b] | 2 h + 6 h[c] |
| thiol | | | | | | | | | | |
| Cys | ↑ | ↑↑ | ↑ | ↑ | – | – | – | – | – | – |
| BCAAs | | | | | | | | | | |
| Val | – | – | | | – | ↑ | – | – | ↑↑ | ↑↑ |
| Ile | – | ↑ | | | – | ↑ | – | – | ↑ | ↑↑ |
| Leu | – | – | | | – | ↑ | – | – | ↑ | ↑ |
| heterocyclic | | | | | | | | | | |
| Trp | – | ↑↑ | | | – | ↑ | – | – | – | – |
| His | – | ↑ | | | ↑ | ↑↑ | – | ↑↑ | – | – |
| others | | | | | | | | | | |
| Lys | – | – | | | ↑ | ↑ | – | – | – | ↑ |
| Ala | – | ↑ | | | – | – | ↑ | ↑ | – | – |

[a] Summary of results of the present study: –, no significant alteration (*t* test, $P < 0.05$); ↑, significant increase (*t* test, $P < 0.05$); ↑↑, more significant increase (*t* test, $P < 0.01$). [b] After pretreatment with amino acids for 2 h. [c] After pretreatment with amino acids for 2 h and then treatment with $H_2O_2$ for 6 h.

that in rodents, I3C can increase GST, cytochrome P450, and NQO1 activities (*32*). Thus, the amine-heterocyclic group in Trp might be an important factor in an inducer of transcriptionally GST genes. This hypothesis may also help to explain the increasing GST activity observed for His. Treatment with His increased GST activity under nonstressed and stressed conditions; however, Trp showed the effect only under stressed condition. This difference is now being explored at molecular levels including signaling pathway in our laboratory.

Pretreatment with His and Ala elevated GR activity in $H_2O_2$-treated cells. GR plays an important role in GSH homeostasis by recycling the GSH oxidized form (GSSG) into GSH. Thus, induction of GR may contribute to maintain a high intracellular GSH concentration. Indeed, intracellular GSH depletion caused by $H_2O_2$ treatment was recovered by His and Ala (**Figure 2**). In our previous study, oligophosphopeptides derived from hen egg yolk phosvitin up-regulated GR activity in $H_2O_2$-treated Caco-2 cells (*33*). Several amino acids have been reported as antioxidants that delay lipid oxidation in certain food systems (*34*, *35*). Pazos et al. (*36*) have reported that Trp is the amino acid with the highest antiradical activity, followed by His > Gly ∼ Met. They demonstrated that Trp and His showed the higher metal-chelating and radical-scavenging properties, while Gly and Met mainly exhibited chelating properties. In the present study, pretreatment of Met and Gly did not show any inhibitory effect on IL-8 secretion from $H_2O_2$-treated cells, whereas Trp and His significantly suppressed IL-8 production (**Figure 1**). Trp and His might directly scavenge free radicals, possibly causing the decrease in actual concentration of $H_2O_2$, which could lead to the decreased production of IL-8. Moreover, His could also decrease IL-8 production at the transcriptional level in intestinal epithelial cells by abolishing the NF-κB-dependent activation of the IL-8 promoter (*13*). Nevertheless, it is worthy to note that Trp and His can affect the antioxidative defense systems in epithelial cells under oxidative stress.

In conclusion, the present study indicates that several amino acids have the potential to protect against oxidative stress in the intestinal epithelial cells. Coordinate enhancement of cellular antioxidant enzymes by amino acids may represent an effective approach to the protection of oxidative stress-induced gut inflammation. It is thus necessary to confirm those findings by in vivo studies to obtain useful information for eventual therapeutic or dietary intervention.

## ABBREVATIONS USED

BCAAs, branched-chain amino acids; BSA, bovine serum albumin; CDNB, 1-chloro-2,4-dinitrobenzene; DMEM, Dulbecco's modified Eagle's medium; ELISA, enzyme-linked immunosorbent assay; FBS, fetal bovine serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; γ-GCS, γ-glutamylcysteine synthetase; γ-GCS-HS, heavy subunit of γ-GCS; γ-GCS-LS, light subunit of γ-GCS; GR, glutathione reductase; GSH, glutathione; GSSG, oxidized form of GSH; GST, glutathione *S*-transferase; NF-κB, nuclear factor-kappa B; NQO1, quinone oxidoreductase-1; Nrf2, NF-E2 related factor-2; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; ROS, reactive oxygen species; SOD, superoxide dismutase.

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