Oligophosphopeptides Derived from Egg Yolk Phosvitin Up-regulate γ-Glutamylcysteine Synthetase and Antioxidant Enzymes against Oxidative Stress in Caco-2 Cells

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Previously, we have found phosphopeptides (PPPs) from hen egg yolk phosvitin possess a potent antioxidative activity against oxidative stress in human intestinal epithelial cells, Caco-2. However, their biological activity at the cellular level has not yet fully understood. The objective of this study is to evaluate the regulation of glutathione (GSH) biosynthesis-associated and antioxidant enzymes against oxidative stress in Caco-2 cells using an in vitro model. Treatment of 1 mM H_2O_2-induced Caco-2 cells with PPPs increased cellular GSH levels, concomitant with a significant increase in γ-glutamylcysteine synthetase (γ-GCS) activity and the expression of γ-GCS heavy subunit mRNA. Furthermore, intracellular glutathione reductase, glutathione S-transferase, and catalase activities were elevated by PPPs. In addition, PPPs with high content of phosphorus showed higher induction of these enzyme activities than PPPs without phosphorus. These data indicate that oligophosphopeptides from hen egg yolk phosvitin can up-regulate cellular GSH biosynthesis-associated enzymes activity and antioxidative activities, which play key roles against tissue oxidative stress in the human intestinal epithelial cells.

KEYWORDS: Phosphopeptides; phosvitin; oxidative stress; glutathione; γ-glutamylcysteine synthetase; antioxidant enzymes; Caco-2

INTRODUCTION

Oxidative stress is a state characterized by an excess of reactive oxygen species (ROS) in the body. When the level of ROS exceeds the antioxidant capacity of the cell, the intracellular redox homeostasis is altered and oxidative stress ensues. ROS cause membrane damage, enzyme inactivation, and DNA damage by oxidative modification of lipids, proteins, and DNA. Oxidative stress plays an important role in the pathogenesis of aging and several degenerative diseases, such as cardiovascular, inflammatory diseases, diabetes, and cancer (*1, 2*). Free radicals and lipid hydroperoxides such as ROS may either pre-exist in the diet or arise from polyunsaturated fatty acids (*3−5*). The gastrointestinal mucosa is constantly exposed to ROS, and uncontrolled increase of ROS causes inflammatory or ischemic disorders of the digestive tract (*6, 7*). In order to cope with an excess of ROS upon oxidative stress, humans possess biological defense systems, which are composed of nonenzymatic antioxidants such as vitamin E, vitamin C, glutathione (GSH), and ubiquinols as well as antioxidant enzymes such as superoxide dismutase (SOD), catalase, glutathione reductase (GR), glu-

tathione peroxidase (GPx), and glutathione S-transferase (GST) (*8−10*). These materials function individually and cooperatively as a firm antioxidant network.

GSH (γ-glutamylcysteinylglycine) is the most abundant intracellular antioxidant that plays an essential role in the detoxification of oxidants (*11*). GSH is synthesized intracellularly in two steps: first cysteine is linked with glutamate in an ATP-dependent reaction catalyzed by γ-glutamylcysteine synthetase (γ-GCS), and this intermediate, γ-glutamylcysteine (γ-GC), is further linked with glycine in another ATP-dependent reaction catalyzed by glutathione synthetase (*12*). The level of GSH decreases temporarily under oxidative conditions, but it should be rapidly restored by increased synthesis system. Any continuously decreased state of GSH pool causes serious impairments of the defense system against toxicants and oxidants. Interestingly, recent studies have demonstrated that food-derived components such as curcumin and flavonoids can up-regulate intracellular GSH synthesis (*13, 14*). Moreover, biophenols derived from extra virgin olive oil induced a significant increase in several antioxidant enzyme activities such as GPx and GR (*15*). These suggest that food-derived components can protect our body from oxidative stress by enhancing the intracellular antioxidant defense systems.

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Hen egg yolk phosvitin is a highly phosphorylated protein with a molecular weight (MW) of 35 kDa that comprises 10% phosphorus and 6.5% carbohydrates (*16*). Previously, we have developed phosphopeptides (PPPs) with MWs of 1−3 kDa from hen egg yolk phosvitin by partial alkaline dephosphorylation, tryptic hydrolysis, and anion exchange chromatography. We further demonstrated that PPPs with 35% phosphate retention showed novel protective effects against tissue oxidative stress in intestinal epithelial cells, Caco-2 (*17*). These findings suggest that PPPs represent a new nutraceutical as a powerful antioxidant against oxidative stress on intestinal epithelial. However, their cellular and molecular mechanisms have not yet been fully understood. The objective of this study was to evaluate the regulation of GSH biosynthesis-associated enzymes (γ-GCS) and antioxidatvie enzyme activities (catalase, GR, and GST) against oxidative stress in Caco-2 cells. We also assessed whether PPPs show the protective effects under normal conditions without oxidative stress.

MATERIALS AND METHODS

Materials. Trypsin (EC 3.4.21.4; 11000 units/mg), H_2O_2, GR (EC 1.6.4.2), 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).

Preparation of Phosvitin and Its Derivatives. Phosvitin was prepared from hen egg yolk according to the method of Losso and Nakai (*18*). Partially dephosphorylated phosvitin and its oligophosphopeptides were prepared according to the method of Jiang and Mine (*19*) with the following modifications. Phosvitin (1 g) was dissolved in 40 mL of 0.1 N NaOH and incubated at 37 °C for 3 h to carry out the alkaline treatment. The suspensions were adjusted to pH of 7.0. After overnight dialysis against Milli-Q water, the partially dephosphorylated phosvitin samples were lyophilized. PPPs were prepared by tryptic digestion. Partially dephosphorylated phosvitin (1 g) was suspended in 30 mL of Milli-Q water, and the pH of the solution was adjusted to 8.0 with 0.1 N NaOH. Trypsin was added to sample solutions at an enzyme-to-substrate ratio of 1:50 (w/w) and then incubated overnight at 37 °C. The enzymatic reaction was stopped by heating at 90 °C for 10 min. The tryptic digests were centrifuged at 5900*g* for 15 min at 10 °C, and the supernatant was microfiltrated through a 0.45*μ*m membrane and then ultrafiltrated on a 1 kDa cutoff Diaflo membrane (Amicon, Beverly, MA) with Milli-Q water. The resulting retentate was lyophilized.

PPPs with different phosphorus content were prepared using anion-exchange high-performance liquid chromatography (HPLC) according to the procedure of Katayama et al. (*17*). Briefly, PPPs were injected into a Mono Q HR 5/5 anion exchange column (Pharmacia Biotech, Uppsala, Sweden) and eluted with 20 mM ammonium bicarbonate with a linear NaCl gradient from 0 to 1.0 M. Three fractions were collected, and named PPP-1, PPP-2, and PPP-3. Their phosphorus contents were determined according to the method of Bartlett (*20*), with untreated phosvitin used as a control. In this study, we used PPP-1 and PPP-3 as PPPs without phosphorus (0%) and PPPs with high content of phosphorus (18.9%), respectively (*17*).

Cell Culture. Caco-2 cell lines were used as a model of the human intestinal epithelium, and the cell line was 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 medium was changed twice a week. The cells were grown in a flask for 5−8 days after seeding, and then transferred (at 2×10^5 cells/mL) to 24-well culture plates (Corning Costar). The cells were cultivated for 5−7 days until confluent monolayers formed.
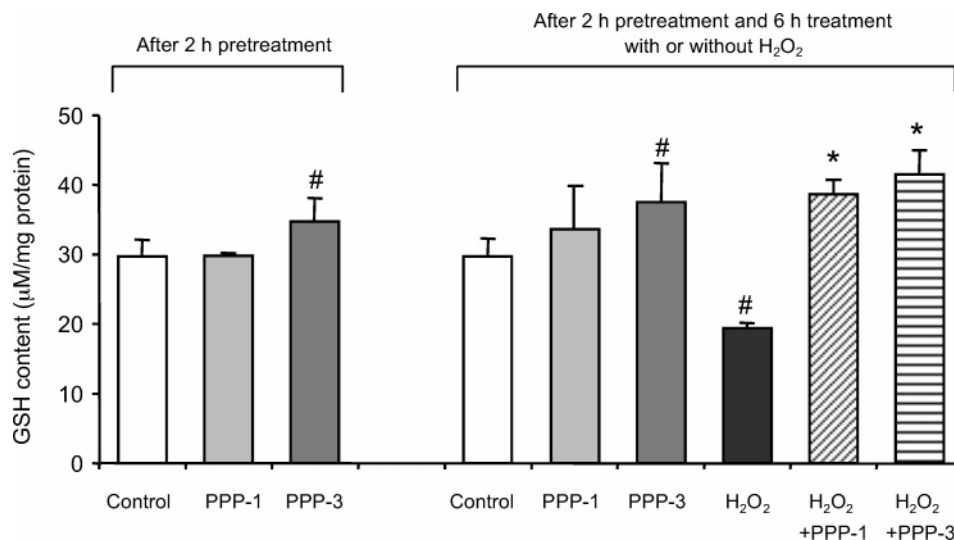
Induction of Oxidative Stress. The tissue oxidative stress was induced in confluent cell cultures by the addition of H_2O_2. Caco-2 monolayers were washed twice with Hank's balanced salt solution without calcium and magnesium and placed in 5% serum-containing media. The cells were then incubated for 2 h with various concentrations of PPP derivates (0−0.5 mg/mL) followed by the addition of 1 mM H_2O_2 for 6 h. At least 95% of the cells remained viable for the above treatments, as assessed by trypan-blue exclusion. The cells were then washed twice with 100 mM phosphate buffered saline (PBS), pH 7.2, containing 100 mM NaCl and suspended in PBS with 0.1% Triton X-100 (for GSH measurement) and 100 mM potassium phosphate solution (pH 7.5) with 1 mM EDTA (for GST, GR, catalase, and γ-GCS assays). Suspended cells were lysed on ice by sonication (Sonifier 250, Branson, 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.

Measurement of Intracellular GSH. Intracellular total GSH was determined according to the method of Allen et al. (*21*) 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 *μ*L) was mixed with 125 *μ*L of 100 mM PBS containing 4 mM EDTA, 0.2 mM NADPH, and 0.5 mM DTNB, and 100 units/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-GCS Activity. γ-GCS activity was assayed by the method of Seelig and Meister (*25*) by following the oxidation of NADH at 340 nm and 25 °C. Briefly, 0.6 mL of Pre-mixture 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 at every 1 min for 5 min using a spectrophotometer. Enzyme specific activity was defined as *μ*mol of NADH oxidized/min/mg protein, which is equal to 1 IU.

Isolation of RNA and Reverse Transcription. Total RNA was isolated from Caco-2 cells using the Trizol reagent after incubation for 1 h with 1 mM H_2O_2 followed by the addition of PPPs. The concentration of total RNA in the final eluate was determined by spectrophotometry. cDNA was synthesized using 5 *μ*g of total RNA and a cDNA synthesis kit (Gibco Invitrogen) 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 (*26*). Oligonucleotide primers were chosen using the published sequence of human γ-GCS-HS (*27*), cDNA, and human glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (*28*). 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 *μ*L) 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, 10 *μ*M of each primer, and 5 U of Taq DNA polymerase in a final volume of 50 *μ*L. 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: denatured 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: denatured at 94 °C for 1 min, annealing at 62 °C for 1 min, primer extension at

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Figure 1. Effects of oligophosphopeptides fractions (PPPs) on GSH content in H_2O_2-treated Caco-2 cells. Cells were replaced to 5% FBS-DMEM media and treated with PPP-1 and PPP-3 (0.5 mg/mL) for 2 h at 37 °C, and then they were incubated with or without H_2O_2 (1 mM) for 6 h. #$P < 0.05$, compared with cells treated with control cells. *$P < 0.05$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in three wells from three separate experiments. Note: data (an increased GSH content by PPP-1 and PPP-3 in the presence of H_2O_2) were adopted from ref *17* for comparison with the current study.

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 GR Activity. GR activity was determined according to the procedure of Carlberg and Mannervik (*23*), 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 μmol of NADPH per minute.

Determination of GST Activity. GST activity was determined according to the procedure of Habig et al. (*22*), by following the formation of 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 of CDNB with GSH per minute.

Determination of Catalase Activity. Catalase activity was determined according to the procedure of Johansson and Borg (*24*), 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 per milligram of protein. One unit of activity was defined as the formation of 1.0 μmol formaldehyde per minute.

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 Phosvitin and Its Derivatives on GSH Content in H_2O_2-Treated Caco-2 Cells. We have previously shown in an in vitro assay using human intestinal epithelial cells that treatment of PPPs suppresses H_2O_2-induced inflammatory response and intracellular lipid peroxidation (*17*). To investigate the possible mechanism by which PPPs could exert the protective effects, we first evaluated the effects of PPPs on the GSH level in H_2O_2-treated or nontreated Caco-2 cells. The cells were pretreated with PPP-1 and PPP-3 (0.5 mg/mL) for 2 h and then stimulated with or without 1 mM H_2O_2 for 6 h. As shown in **Figure 1**, treatment of H_2O_2 decreased intracellular GSH content compared with the control cells; however, pre-treatments of PPP-1 and PPP-3 for 2 h restored GSH levels in H_2O_2-treated Caco2-cells, and in particular, PPP-3 significantly increased the GSH level compared with control cells. The increase in GSH level was also observed when the cells were pretreated with PPP-3 for 2 h without H_2O_2 treatment. Thus, this indicates that PPPs can maintain a high GSH level regardless of oxidative stress. It seems likely that the biosynthesis system of GSH in the cells is activated by PPPs. The levels of GSH are regulated by γ-GCS-HS mRNA expression and γ-GCS activity. We further examined the levels of γ-GCS-HS mRNA expression and γ-GCS activity in H_2O_2-treated Caco-2 cells in presence of PPPs. As shown in **Figure 2**, PPP-3 significantly increased γ-GCS-HS mRNA expression in H_2O_2-treated cells, compared to GAPDH gene expression. The level of γ-GCS-HS mRNA expression was increased 55% by PPP-3 treatment at 2 h. There were no significant different levels of γ-GCS-HS mRNA expression between the control and PPPs without H_2O_2 treatment. As shown in **Figure 3**, γ-GCS activity was significantly elevated by PPP-3 treatment in Caco-2 cells, compared to the values in cells treated with H_2O_2 alone. Thus, our present study suggests that the increase in GSH levels is mainly due to transcriptional up-regulation of γ-GCS-HS mRNA by PPP-3

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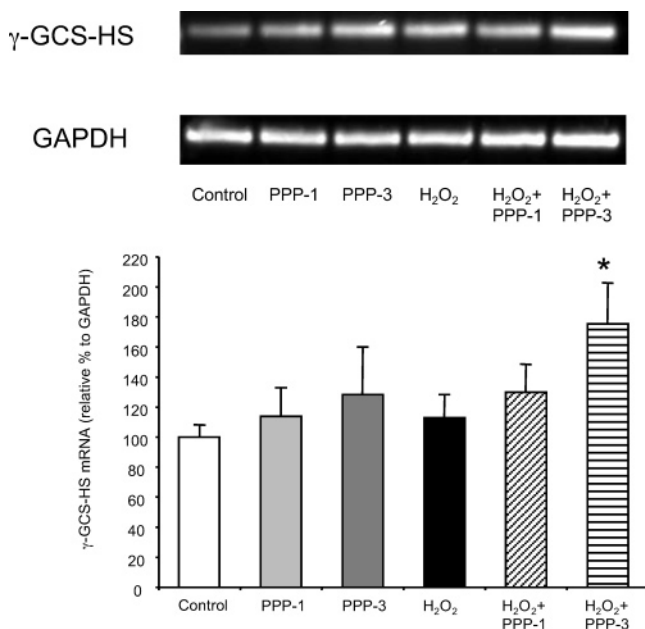
Figure 2. Effect of PPP-1 and PPP-3 on γ-GCS-HS mRNA expression in H_2O_2-treated Caco-2 cells. Total RNA was isolated from control cells and cells exposed with H_2O_2 (1 mM) for 1 h followed by pretreatment with PPP-1 and PPP-3 (0.5 mg/mL) for 2 h. *$P < 0.05$, compared with cells treated with H_2O_2 alone. The experiment was carried out three times, with similar results being obtained on both occasions.

under oxidative stress. These results support the idea that PPP-3 can protect intestinal epithelial cells from H_2O_2-mediated oxidative stress by activation of GSH biosynthesis.

Effects of Oligophosphopeptides on Antioxidant Enzyme Activities in H_2O_2-Treated Caco-2 Cells. We next investigated whether the activities of antioxidant enzymes such as GR, GST, and catalase could be elevated by treatment of PPPs or not. As shown in **Figure 4**, treatment of Caco-2 cells with 1 mM H_2O_2 for 6 h in absence of PPPs decreased the activities of GR and catalase. Pretreatment with PPP-3 for 2 h prior to the induction of oxidative stress enhanced the catalase activity, whereas PPP-3 significantly increased the activities of GR, GST, and catalase in H_2O_2-treated Caco-2 cells. In contrast, PPP-1 showed no significant increase. These results suggest that PPP-3 can exert the protective effect against H_2O_2-induced oxidative stress by enhancing the activities of antioxidant enzymes.

DISCUSSION

Mammalian cells have evolved a variety of antioxidants and antioxidative enzymes to protect against oxidative and electrophilic cell damage (*9, 10*). For example, SOD catalyzes the dismutation of superoxide anion to H_2O_2. The harmful H_2O_2 can further be decomposed by catalase or GPx. In the reaction catalyzed by GPx, the enzyme cofactor GSH is oxidized to the oxidized form of GSH (GSSG), which can be subsequently reduced back to GSH via the action of GR. On the other hand, GST is primarily involved in detoxification of lipid hydroperoxides produced during ROS attack of cellular lipid molecules. Therefore, the coordinate actions of various cellular antioxidants and antioxidative enzymes ensure the effective detoxification of ROS. In this aspect, enhancement of cellular antioxidants and antioxidant enzymes would be an effective method of protecting against oxidative stress-induced tissue injury. Our previous studies have demonstrated that PPP-3, oligophosphopeptides derived from hen egg yolk phosvitin, can exhibit antioxidative effects on stress by suppressing the inflammatory
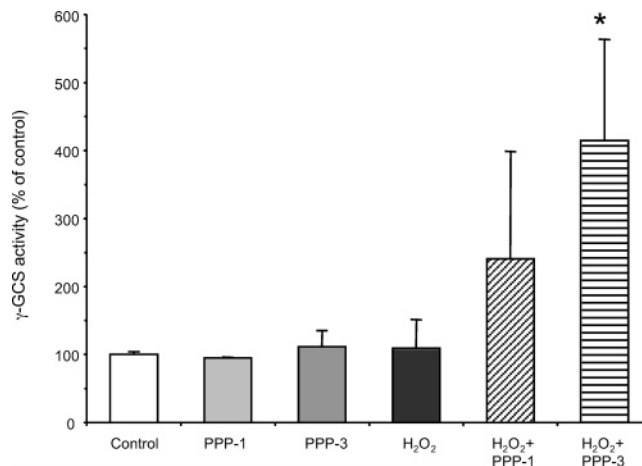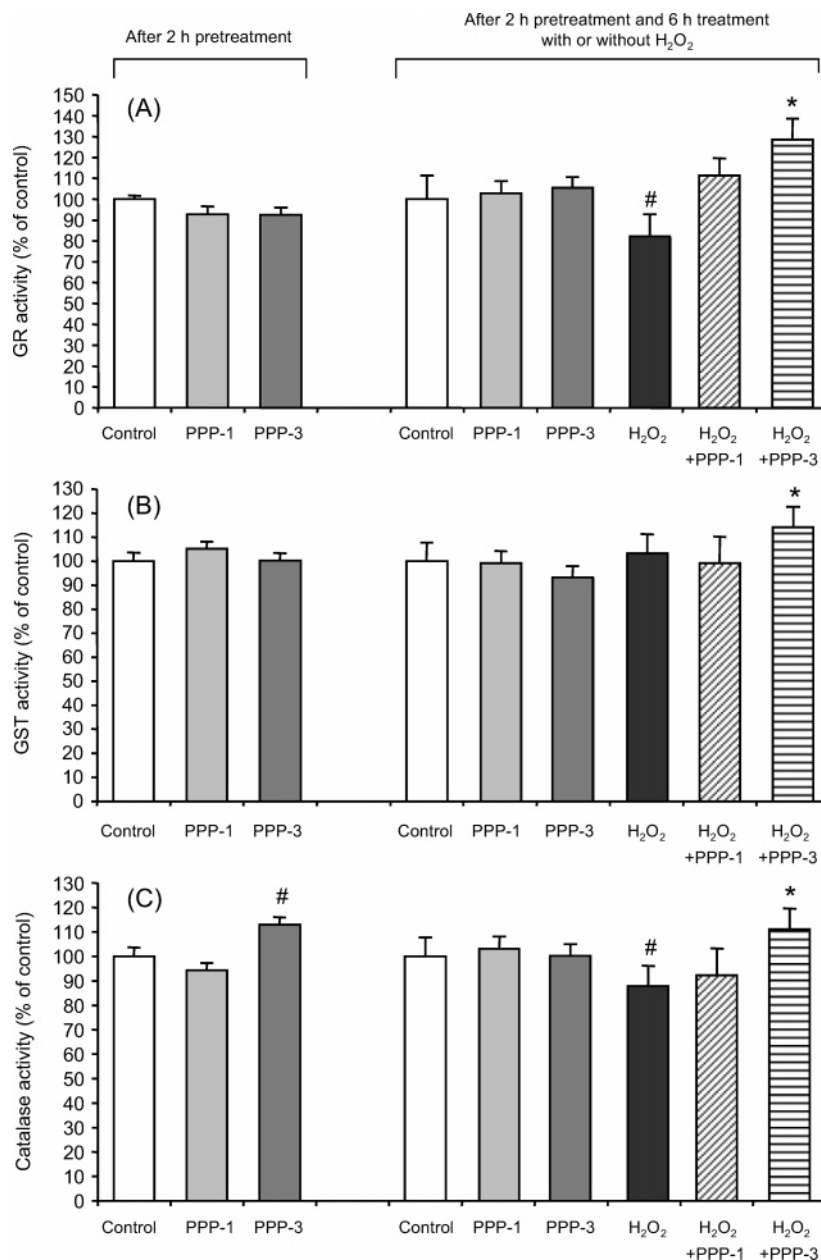

Figure 3. Changes in γ-GCS activity in H_2O_2-treated Caco-2 cells. The cells were treated with PPP-1 and PPP-3 (0.5 mg/mL) for 2 h at 37 °C prior to incubation with 1 mM H_2O_2 for 6 h. *$P < 0.05$, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in triplicates from two separate experiments.

response and acting as a lipid peroxidation inhibitor (*17*). In the present study, we found that PPP-3 increased the intracellular GSH contents via the activation of GSH-biosynthesis system. We also demonstrated that PPP-3 could elevate the enzyme activities of GR, GST, and catalase. These findings imply that the antioxidative effects of PPP-3 result from the elevation of intracellular antioxidants and the enhancement of antioxidative enzymes (see **Figure 5**).

Recent papers have demonstrated the effects of different classes of polyphenols on γ-GCS activity and antioxidant enzyme activities in cell culture systems. Scharf et al. reported that plant-derived phenolic compounds enhanced γ-GCS activity and the consequent concentration of GSH in human hepatoma cells (*29*). Masella et al. also have demonstrated that phenolic compounds contained in extra virgin olive oil remarkably increase GR and GPx activities in murine macrophage (*30*). Van Zanden et al. have further reported that wine and black tea polyphenols can modulate gene expression of GST in colon tumor cells or in breast cancer cell (*31*). These suggest that dietary polyphenols can act as chemoprotective agents against oxidative stress by enhancement of intracellular antioxidative systems. In this regard, it is noteworthy that protein-derived oligopeptides such as PPP-3 as well as polyphenols induced dramatic activation in γ-GCS and antioxidative enzymes in the cells.

Elevation of intracellular GSH levels was induced by upregulation of GCS mRNA expression; however, it is not clear how GR, GST, and catalase activities were activated by PPP-3. Recently, some studies have demonstrated that NF-E2 related factor 2 (Nrf2) is a critical transcription factor for antioxidant responses and Nrf2 controls the expression of antioxidant enzymes (*32*). Under basal conditions, the cytosolic regulatory protein Keap1 (Kelch-like ECH-associated protein1) binds tightly to Nrf2, retaining it in the cytoplasm. Alternation of the Nrf2-Keap1 interaction enables Nrf2 to translocate into the nucleus, bind to the antioxidant response elements (ARE), and initiate the transcription of genes coding for antioxidant enzymes and cytoprotective proteins. This response is triggered by a class of electrophilic compounds, including polyphenols and plant-derived constitutions (*33*). Thus, we assume that PPP-3 might also stimulate the Keap1/Nrf2 signaling pathway thereby upregulating a set of antioxidant enzymes. The mechanism

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**Figure 4.** Effect of PPP-1 and PPP-3 on antioxidative enzyme activities in H_2O_2-treated Caco-2 cells. The cells were treated with PPP-1 and PPP-3 (0.5 mg/ mL) for 2 h at 37 °C, and then exposed with or without 1 mM H_2O_2 for 6 h. #P < 0.05, compared with cells treated with control cells. *P < 0.05, compared with cells treated with H_2O_2 alone. Data are presented as means ± SD in five wells from two separate experiments. Note: data (increased GR activity by PPP-3 in H_2O_2-treated cells—right bar) in part **A** was adopted from ref *17* for the comparison with current study.

underlying the enhancement of antioxidative enzymes by PPP-3 is now being explored at molecular levels in our laboratory.

We found two mechanisms underlying antioxidative stress activity of PPP-3; however, we also assume another mechanism by which the activation of nuclear transcription factors might be suppressed. Persistently elevated ROS activate nuclear-kappaB (NF-κB) and activator protein-1 (AP-1), which are involved in the transcription of proinflammatory mediators such as IL-8 (*34*). Therefore, inactivation of NF-κB or AP-1 by PPP-3 might contribute to the augmentation of cellular antioxidative systems. Son et al. has demonstrated that His inhibits oxidative stress-induced intestinal inflammatory events via the inactivation of NF-κB, and Lys, Pro, Glu, and Ala did not show the significant effect (*35*). Interestingly, according to the amino acid analysis, PPP-3 again contained half as much His as PPP-1, whereas PPP-3 was not rich in Lys, Pro, Glu, and Ala compared with PPP-1 (*17*). At present, however, it is not clear whether

PPPs could interact with the modulation of NF-κB or AP-1, and further studies would be necessary to investigate the protective effect of PPPs at the transcriptional level.

In view of the molecular structure, PPP-3 has a large moiety of phosphorus in contrast with PPP-1 without phosphorus. Our present data clearly demonstrated that PPP-3 exhibited a more significant increase in activities of GSH-synthesis enzyme and antioxidative enzymes than PPP-1. Therefore, it is suggested that up-regulating effects of PPP-3 on intracellular enzyme activities are mainly involved in phosphorus moieties in oligophosphopeptides; however, phosphoserine itself did not markedly induce the antioxidative response (*17*). Peptides' molecular size and amino acid composition as well as phosphorus might play important roles as an enhancer of intracellular antioxidant defense systems. More studies are being conducted in our laboratory to characterize the amino acid sequences and phosphorus content of active components in PPP-3.
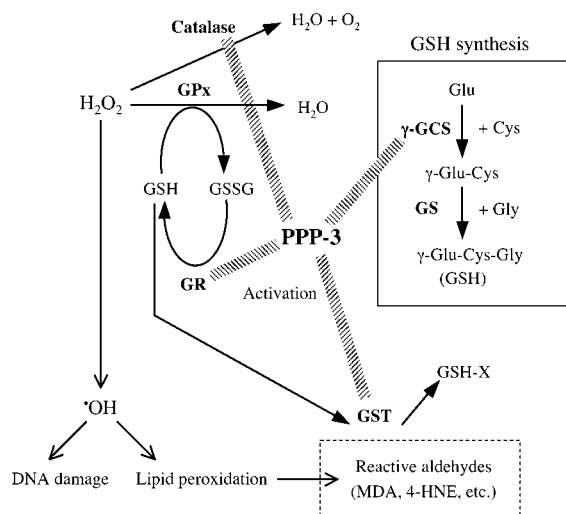
Figure 5. Hypothetical mechanism of protective effect of PPP-3 against H_2O_2-induced oxidative stress. PPP-3 enhances the activation of GR and γ-GCS, key enzymes in GSH biosynthesis, and thereby maintains at the high level of GSH in the cells. PPP-3 also increases catalase and GST activities, so that H_2O_2 and reactive aldehydes derived from lipid peroxidation are detoxified.

In conclusion, this study demonstrated that oligophosphopeptides derived from hen egg yolk phosvitin can up-regulate γ-GCS activity and several antioxidant activities in oxidative stress-induced intestinal epithelial cells. Further investigations would be needed to determine the identification of active components and the activation of which cell pathway in epithelial cells is involved these activities.

ABBREVIATIONS USED

PPPs, phosphopeptides; GSH, glutathione; γ-GCS, γ-glutamyl-cysteine synthetase; ROS, reactive oxygen species; SOD, superoxide dismutase; GR, glutathione reductase; GPx, glutathione peroxidase; GST, glutathione S-transferase; γ-GC, γ-glutamylcysteine; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; HPLC, high-performance liquid chromatography; PBS, phosphate-buffered saline; PCR, Polymerase Chain Reaction; γ-GCS-HS, heavy subunit of γ-GCS; γ-GCS-LS, light subunit of γ-GCS; GSSG, oxidized form of GSH; Nrf2, NF-E2 related factor 2; Keap1, Kelch-like ECH-associated protein1; ARE, antioxidant response elements; NF-κB, nuclear-kappaB; AP-1, activator protein-1.

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