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Egg Yolk Peptides Up-regulate Glutathione Synthesis and Antioxidant Enzyme Activities in a Porcine Model of Intestinal Oxidative Stress

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Long-term oxidative stress in the gastrointestinal tract can lead to the development of chronic intestinal disorders. Many food-derived antioxidants are effective in vitro, but the variable reports of in vivo efficacy and the pro-oxidant nature of some antioxidants necessitate alternative strategies for the reduction of in vivo oxidative stress. Compounds that up-regulate the production of endogenous antioxidants such as glutathione (GSH) and antioxidant enzymes provide novel approaches for the restoration of redox homeostatis. Egg yolk peptides (EYP) prepared from Alcalase and protease N digestion of delipidated egg yolk proteins were found to exhibit antioxidative stress properties. The effect of EYP supplementation was examined in a hydrogen peroxide-induced human colon cell line and in an animal model of intestinal oxidative stress. EYP significantly reduced the pro-inflammatory cytokine, IL-8, in Caco-2 cells. In piglets given intraperitoneal infusions of hydrogen peroxide, EYP treatment increased GSH and γ-glutamylcysteine synthetase mRNA expression and activity, significantly increased antioxidant enzyme activities, in particular catalase and glutathione *S*-transferase activities, and reduced protein and lipid oxidation in the duodenum, jejunum, ileum, and colon. Furthermore, EYP boosted the systemic antioxidant status in blood by increasing the GSH concentration in red blood cells. These results suggest that EYP supplementation is a novel strategy for the reduction of intestinal oxidative stress.

KEYWORDS: Egg yolk peptides; hydrogen peroxide; oxidative stress; antioxidant enzymes; glutathione; γ-glutamylcysteine synthetase

INTRODUCTION

Intestinal oxidative stress has been associated with the initiation and propagation of chronic intestinal pathologies such as inflammatory bowel diseases, ischemic-reperfusion disorders, and intestinal cancers (*1*−*3*). Oxidative stress is a consequence of an excess of reactive oxygen species (ROS), which participates in cellular damage by reacting with lipid membranes, proteins, and DNA. The ingestion of dietary pro-oxidants including lipid hydroperoxides, heme, and transition metal ions can lead to deleterious effects within the gastrointestinal tract (*4*). In response to these threats, mucosal cells utilize a variety of enzymatic and nonenzymatic mechanisms that tightly control unwanted ROS accumulation. Nonenzymatic antioxidants such as glutathione (GSH), ascorbate, and α-tocopherol, as well as antioxidative enzymes such as superoxide dismutase (SOD), catalase (CAT), glutathione peroxidase (GPx), glutathione reductase (GR), and glutathione *S*-transferase (GST), act to coordinately detoxify ROS (*5*). During oxidative stress, GSH concentration rapidly decreases while oxidized GSH (GSSG) increases, shifting the thiol redox status of the cell and activating oxidant response transcriptional elements, which lead to the up-regulation of antioxidant enzymes and γ-glutamylcysteine synthetase (γ-GCS), the rate-limiting enzyme in GSH synthesis (*6*).

Antioxidants such as polyphenols, ascorbate, and α-tocopherol physically detoxify ROS; however, in certain conditions, they promote oxidative reactions, and this likely contributes to their limited therapeutic efficacy (*7*, *8*). A novel approach to reducing oxidative stress involves influencing the cellular production of glutathione and endogenous antioxidative enzymes. Food-derived compounds such as curcumin and flavonoids and olive oil biophenols have been shown to up-regulate intracellular GSH synthesis (*9*−*11*) and increase antioxidant enzyme activities (*12*, *13*). Antioxidative stress proteins and peptides have been identified from plants, milk, and eggs (*14*−*16*). Recently, a phosvitin oligophosphopeptide fraction, PPP3, prepared from hen egg yolk phosvitin, inhibited interleukin-8 secretion, suppressed lipid peroxidation, increased GSH levels and synthesis, and elevated antioxidant enzyme activities in hydrogen peroxide-stimulated intestinal cell culture (*16*, *17*).

Delipidated egg yolk protein is a byproduct of lecithin extraction from egg yolk in the egg-processing industry. After the sequential ethanol and hexane removal of phospholipids, proteins are left behind in a denatured state with limited aqueous solubility and functionality (*18*). In vivo studies have demonstrated that this protein is of superior quality with higher protein efficiency ratio,

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biological value, and net protein utilization as compared with milk casein (*19*). The enzymatic hydrolysis of fat-free yolk proteins has been reported to increase protein solubility and improve foaming and emulsification properties (*20*). Egg yolk protein hydrolysates have also been found to have antioxidant activity in a linoleic acid oxidation system (*21*, *22*). Although the prevention of lipid peroxidation is beneficial to the protection of cellular membranes, these peptides may not necessarily display similar activities in vivo.

Cell culture and animal studies are often used for assessing the bioactivity of food-derived peptides. Human intestinal Caco-2 cells stimulated with hydrogen peroxide result in an abundant production of the pro-inflammatory cytokine interleukin-8 (IL-8) (*23*). IL-8 is a useful biomarker of oxidative stress and inflammation, and IL-8 reduction in epithelial cells is often associated with antioxidative protection (*16*, *17*). Although hydrogen peroxide is commonly used to induce oxidative stress in cell culture, it is seldom used in animal models of intestinal oxidative stress. Its high cellular diffusibility and participation in the Fenton reaction to form the extremely damaging hydroxyl radicals (*24*) suggest that enteral administration is likely to cause cellular damage to all tissue contact surfaces. In fact, hydrogen peroxide was once used in the treatment of fecal impaction in humans; however, this practice was terminated due to the development of colitis (*25*). Pigs are increasingly used as models of chronic gastrointestinal disease because their intestinal tracts are morphologically and physiologically similar to that of humans (*26*). The delivery of hydrogen peroxide into the pigs' peritoneal cavity is expected to limit oxidative damage to the small and large intestines and provide an intestinal oxidative stress model in which to evaluate the efficacy of ingested bioactive compounds in the gastrointestinal tract.

The primary objective of this study was to produce antioxidative stress peptides from lipid-free egg yolk protein using a combination of bacterial proteases. The in vitro bioactivity and cytotoxicity of these peptides were evaluated in Caco-2 cells, whereas the in vivo antioxidative stress properties were assessed in tissues harvested and blood drawn from piglets subjected to intestinal oxidative stress. Gastrointestinal tissues including the duodenum, jejunum, ileum, and colon were isolated and individually analyzed for glutathione, antioxidative enzyme activities, and protein and lipid oxidation. Furthermore, the systemic effect of the peptides was determined by the glutathione concentrations in red blood cells.

MATERIALS AND METHODS

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

Preparation of Egg Yolk Peptides. A variety of egg yolk peptides were produced for the eventual selection of an Alcalase- and protease-digested egg yolk protein hydrolysate that demonstrated antioxidative stress properties and which could be used in in vivo studies. Egg yolk peptides were prepared from the digestion of ethanol-delipidated egg yolk protein using a combination of Alcalase (from *Bacillus subtilis*, EC 3.4.21.62; HBI Enzymes Inc., Osaka, Japan) and a variety of proteases including protease S (from *Bacillus sterothermophilus*, 10 ATEE U/mg), protease N (from *B. subtilis*, 150 ATEE U/mg, EC 3.4.24.28), protease A (from *Aspergillus oryzae*, 20 ATEE U/mg, EC 3.4.24.39), protease P (from *Aspergillus melleus*, 60 ATEE U/mg), and trypsin (from porcine pancreas, 1420 BAEE U/mg, EC 3.4.21.4). All proteases were purchased from Amano Enzymes USA (Elgin, IL) with the exception of trypsin, which was obtained from Sigma-Aldrich. Defatted egg yolk protein (50 g; Taiyo Kagaku Ltd., Yokkaichi, Japan), composed of 86% protein (w/w), 0.5% lipids, 4.5% water, and 10% others (carbohydrate and ash), was dissolved in 1 L of 0.1 N NaOH and incubated at 37 °C for 3 h with constant shaking. The mixture was adjusted to pH 10 with 1 N HCl, and 0.5% Alcalase (w/w) was added to the solution and incubated for 3 h at 45 °C. After Alcalase digestion, the pH of the solution decreased, but was adjusted to 8.0 using 2 M NaOH. Protease (0.5%, w/w) was incorporated, and the mixture was held at 45 °C for 15−17 h with constant shaking. The enzymatic reaction was stopped by heating at 85 °C for 20 min, and the digest was centrifuged at 6082*g* for 40 min at room temperature. After centrifugation, the supernatant was separated, lyophilized, and stored in sealed bags at −20 °C until use.

Physicochemical Characterization of Egg Yolk Peptides. *Nitrogen Analysis*. The percent nitrogen in a sample was determined by the rapid combustion of a sample into gases according to the Dumas method (*27*). The LECO FP-528 (Leco, St. Joseph, MI) was first calibrated with blanks and ethylenediamine tetraacetate (EDTA) standards. Approximately 0.2 g of the egg yolk hydrolysate was weighed into foil cups, sealed, and analyzed.

Phosphate Analysis. Egg yolk peptides (approximately 5 mg/mL in Milli-Q water) were dialyzed for two consecutive days with daily changes of water using a 500 Da cutoff membrane (Spectrum Laboratories Inc., Rancho Dominguez, CA). Phosphate content was determined according to the method of Bartlett (*28*). Briefly, dialyzed samples (50 and 100 μL each) and potassium hydrogen phosphate (KH_2PO_4) standards (0−15.3 mg/mL) were dispensed into glass tubes (Corning Costar, Cambridge, MA) and were evaporated completely in a block heater. A 70% perchloric acid solution (0.4 mL) was added, and samples were heated for 60 min at 130−140 °C. A mixture of water, 5% (w/v) ammonium molybdate, and diaminophenol dyhydrochloride (21:1:1, v/v/v; 4.6 mL) was incorporated into each tube, and samples were heated at 100 °C for 10 min. Once the samples were cooled to room temperature, their absorbances were measured at 830 nm in a spectrophotometer (UV-1201, Shimadzu, Kyoto, Japan). A standard curve of absorbance versus micrograms of phosphate was prepared, and the phosphate content in each sample was extrapolated accordingly. The protein concentration of each sample was determined by the microplate Bio-Rad detergent compatible (DC) protein assay (Bio-Rad Laboratories, Hercules, CA) using bovine serum albumin as a protein standard. Phosphate content was expressed as micrograms of phosphate per milligram of protein.

Degree of Hydrolysis. The percent degree of hydrolysis (%DH) was determined by the 2,4,6-trinitrobenzenesulfonic acid (TNBS) method in which the reaction of TNBS with primary amino groups forms a chromophore with a maximum absorbance at 340 nm (*29*). Egg yolk hydrolysates and delipidated egg yolk protein (10 μL each) of approximately 1 mg/mL in 1% sodium dodecyl sulfate (SDS, w/v) were pipetted into a 96-well plate. Sodium phosphate buffer (40 μL, 0.2125 M, pH 8.2) and 40 μL of 0.1% TNBS (w/v) were added to each well and mixed. The sample plate was covered with aluminum foil and incubated at 50 °C for 60 min. The reaction mixture was cooled at room temperature for 10 min in the dark, after which the absorbance was read at 405 nm. A standard curve of 0−15 mM glycine was used to extrapolate the amino nitrogen present in the samples. Because the percent protein in the egg yolk protein was previously analyzed by the manufacturer (86%), the nitrogen content of the protein was estimated by dividing by 6.38. The %DH was determined by subtracting the amino nitrogen content of the delipidated egg yolk protein from that of the egg yolk hydrolysate, dividing this value by the nitrogen content of the peptide bonds in the yolk protein, and then multiplying by 100.

Induction of in Vitro Antioxidative Stress. *Caco-2 Cell Culture*. The Caco-2 human intestinal cell line (American Type Culture Collection, Rockville, MD) was used in all in vitro oxidative stress studies. Cells were grown in Dulbecco's modified Eagle's medium/F12 (DMEM/F12; Gibco, Burlington, ON, Canada) supplemented with 20% fetal bovine serum (FBS; Hyclone, UT) and 50 units/mL of penicillin−streptomycin (Gibco) and were incubated at 37 °C in a 5% CO_2 chamber. Cell passages 15−45 were used to form the confluent monolayers required for the oxidative stress experiments. Cell confluency was based on microscopic examination with the same monolayer cell concentration existing in all wells. These monolayers were formed from 1×10^5 cells (0.5 mL) cultivated in a 48-well culture plate (Corning Costar) and were grown for 5−7 days with fresh medium replacements every 2−3 days.

Induction of Oxidative Stress. Confluent cells were incubated in a 5% FBS−DMEM/F12 medium with 0.1 and 1 mg/mL of protease-derived egg yolk peptides for 2 h. One millimolar H_2O_2 was added to the cells to induce

oxidative stress for 6 h. Previous tryptan-blue exclusion studies confirmed at least 95% of the cells remained viable during oxidant treatment. At the end of 8 h, the cell supernatants were pipetted into Eppendorf tubes and were frozen at −80 °C for future interleukin-8 assays. Cell viability was determined on the aspirated cell monolayer.

Interleukin-8 Assay. The interleukin-8 concentration in the cell supernatants was determined using an IL-8 enzyme-linked immunosorbent assay (ELISA). Mouse anti-human IL-8 antibodies (100 μL; BD Biosciences, San Diego, CA) were coated at a 1:1000 dilution in 100 mM sodium phosphate buffer (pH 9.0) in a 96-well microtiter plate (Corning Costar) held overnight at 4 °C. All incubations from this point forward proceeded at 37 °C. The microtiter wells were washed three times with phosphate-buffered saline (PBS) containing 0.05% Tween-20 (PBST) and then blocked with 200 μL of 1% (w/v) bovine serum albumin (BSA) in PBS for 1 h. After blocking, the plate was washed, and 100 μL of supernatant samples or IL-8 standards (BD Biosciences) was dispensed into the wells and incubated for 2 h. The plate was again washed, and 100 μL of biotinylated mouse anti-human IL-8 antibodies (BD Biosciences) was added to each well and incubated for 1 h. After the plate was washed, 100 μL of avidin−horseradish peroxidase conjugate (BD Biosciences) was placed into each well and incubated for 0.5 h. The plate was washed and color was developed using 50 μL of 3,3′,5,5′-tetramethylbenzidine. The reaction was terminated using 25 μL/well of 0.5 N H_2SO_4, and the absorbance was read at 450 nm. IL-8 concentrations of samples were extrapolated from the IL-8 standard calibration curve and are expressed as picograms of IL-8 per milliliter of sample.

Cell Viability. The water-soluble tetrazolium-1 (WST-1) cell viability assay is based on the cleavage of tetrazolium salts by mitochondrial succinate reductase (NADPH dependent) to a corresponding formazan dye, which can be photometrically detected (*30*). Because reductases are present in living cells, this assay provides a nonspecific indicator of metabolic activity and viability. After the removal of supernatant for the IL-8 assays, 290 μL of 5% FBS−DMEM/F12 mediaum and 10 μL of WST-1 solution were added to each well and mixed. The plate was incubated for 5−10 min at 37 °C until color development. Supernatants (200 μL/well) were pipetted into a 96-well microtiter plate for measurement at 450 nm in a plate reader (Bio-Rad model 550, Bio-Rad Laboratories).

Induction of in Vivo Oxidative Stress. A pilot study was first conducted to determine the optimal conditions for the induction of intestinal oxidative stress in piglets. The trial duration and concentration of hydrogen peroxide used in the actual animal study were based upon the pilot study's biomarker results.

Yorkshire male and female piglets (5−7 days old) were obtained from the Arkell Swine Research Station (University of Guelph, Guelph, ON, Canada) on the first day of the trial (day 1). The piglets were individually housed in an animal facility maintained at 26 °C with a 12 h light/dark cycle. Piglets were fed three times a day with a commercial milk replacement formula (Soweena Litter Life; Merrick's Inc., Middleton, WI) at ad libitum intake volumes. All animal trials were approved by the University of Guelph's Animal Care Committee and were conducted in accordance with the Canadian Council of Animal Care Guide for the Care and Use of Experimental Animals.

After a 2-day acclimatization period, piglets underwent surgery for the placement of an intraperitoneal catheter (Micro-Renathane; Braintree Scientific Inc., Braintree, MA). The catheter was anchored to a trimmed inert silicone patch (about 8 × 12 mm; Access Technologies, Skokie, IL), which was further sutured onto the muscular right side of the inner abdominal cavity with the catether end positioned toward the intestines. A custom-made vest was fitted dorsally on each animal for temporary storage of the exterior segment of the catheter. Following a 3-day recovery period, animals were randomly assigned into one of three groups [negative control (NEG), positive control (POS), and egg yolk peptide (EYP); $n = 5$ per group] and infused with H_2O_2 or saline while receiving daily doses of EYP or L-alanine. During the 10 days of oxidative stress induction, POS and EYP groups were infused with 60 mL of 0.2 mM H_2O_2/kg of body weight (BW)/day, whereas the NEG group was given saline infusions. Hydrogen peroxide infusions (10 mL) were administered six times during the course of the day. After each infusion, the catheter was flushed with 2 mL of saline to ensure delivery of the full 10 mL of hydrogen peroxide. EYP piglets were also fed 255 mg of egg yolk peptides/kg of BW/day, whereas NEG and POS animals were given 254 mg of L-alanine/kg of BW/day for isonitrogen balance (40 mg of nitrogen/kg of BW/day) (*31*) during the oxidative stress period.

Whole blood from the periorbital sinus was collected on days 2, 8, 11, 14, and 17 in EDTA tubes and centrifuged at 1000*g* for 10 min at 4 °C. The red blood cells (RBC) were separated from the plasma and buffy coat and were analyzed for glutathione concentrations.

On day 19, all piglets were sedated through an inhaled anesthetic, isoflurane (Aerrane, Anaquest, WI), and sacrificed via an intracardiac injection of Euthanol (pentobarbital) at 0.3 mL/kg of BW. Intestinal tissues from the duodenum, jejunum, ileum, and colon were separated on the basis of morphological features by an animal care technician and were rinsed with 0.1 mM phenylmethanesulfonyl fluoride in physiological saline and flash frozen in liquid nitrogen for further biomarker analyses. Prior to use, tissues were finely ground in liquid nitrogen using a mortar and pestle. All protein concentrations were determined by the microplate Bio-Rad DC protein assay (Bio-Rad Laboratories) using BSA as a protein standard.

Determination of Glutathione Concentration and γ-Glutamylcysteine Synthetase Gene Expression and Activity. Red blood cells were lysed in a 4× volume of ice-cold HPLC-grade water and centrifuged (10000*g*, 15 min, 4 °C). The supernatant was collected and stored on ice. To avoid assay interferences from particulates and protein thiols, the sample was deproteinated using an equal volume of 10% (w/v) metaphosphoric acid (MPA). The solution was incubated at room temperature for 5 min followed by centrifugation at 3000*g* for 10 min at 4 °C. The supernatant was collected and stored at −80 °C until analysis.

Frozen ground tissues (approximately 1 g) were homogenized in 5 mL of cold phosphate buffer with 1 mM EDTA (pH 6.7) and centrifuged (10000*g*, 15 min, 4 °C). The supernatant was removed, and an equal volume of 10% MPA was added. For both RBC and tissues, immediately prior to analysis, the deproteinated samples were neutralized using 50 μL of 4 M triethanolamine/mL of supernatant and vortexed thoroughly.

Total Glutathione Assay. Total GSH was determined according to a modified version of Allen et al.'s (*32*) method. Supernatants (25 μL) were mixed with 125 μL of 100 mM PBS containing 4 mM EDTA, 0.2 mM NADPH, 0.5 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), and 100 units/mL of glutathione reductase. The mixture was incubated for 5 min at room temperature with shaking, and the absorbance was measured at 405 nm. The concentration of GSH in the samples was calculated using a glutathione standard curve and was expressed as nanomoles of GSH per milligram of protein.

γ-Glutamylcysteine Synthetase Activity. The γ-GCS activity was assayed according to the method of Seelig and Meister (*33*) by following the oxidation of NADH at 340 nm and 25 °C. A premixture solution (0.6 mL) consisting of 0.25 M Tris-HCl (pH 8.2) containing 1.25 mM EDTA, 166 mM KCl, and 0.1 mM NADH was mixed with 0.31 mL of 10 mM ATP, 10 mM phosphoenolpyruvate (PEP), 10 mM L-glutamate, pyruvate kinase/lactate dehydrogenase (PK/LDH; 4U/5U per mL), and 5 mM L-α-aminobutyric acid. Tissue supernatant (0.09 mL) was added to this solution, and the decrease in absorbance at 340 nm was recorded at 2 and 5 min. γ-GCS activity was defined as micromoles of NADH oxidized per minute per milligram of protein, which is equivalent to 1 U, and activity was expressed as units per milligram of protein.

γ-Glutamylcysteine Synthetase mRNA Expression. For determining γ-GCS mRNA expression, total RNA was first extracted from frozen intestinal tissues using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. The quantity and quality of the RNA were verified by measuring the $A_{260}A_{280}$ ratio (NanoDrop ND-1000; Thermo Scientific, Wilmington, DE) and by gel electrophoresis. First-strand cDNA was synthesized using the iScript cDNA Synthesis Kit (Bio-Rad Laboratories) according to the manufacturer's instructions. γ-GCS mRNA expression was quantified using real-time Polymerase Chain Reaction (RT-PCR) with porcine γ-GCS and the β-actin housekeeping gene. The primer sequences were as follows: γ-GCS (forward primer 5′-GAG AAA ATC CAC CTG GAC GA-3′ and reverse primer 5′-ATG GGC CGG AAT TCT ACT CT-3′, 134 bp) and β-actin (forward primer 5′-GGA TGC AGA AGG AGA TCA CG-3′ and reverse primer 5′-ATC TGC TGG AAG GTG GAC AG-3′, 130 bp). RT-PCR was carried out using iQ SYBR Green Supermix (Bio-Rad Laboratories) on a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad

Laboratories) using the following conditions: 50 cycles of denaturation at 95 °C for 15 s, annealing at 56 °C for 15 s, and extension at 72 °C for 30 s. Porcine primers were designed using Primer3 v.0.4.0 (*34*) and synthesized by the University of Guelph Laboratory Services Molecular Biology Section (Guelph, ON, Canada). Results were expressed as fold changes relative to the negative control (untreated) animals.

Determination of Antioxidant Enzyme Activity. Intestinal tissues for the SOD assay were homogenized in sucrose buffer (0.25 M sucrose, 10 mM Tris, 1 mM EDTA) and were centrifuged at 10000*g* for 15 min at 4 °C. For the CAT, GR, and GST enzyme activity assays, frozen ground tissues were homogenized in 100 mM cold potassium phosphate buffer (pH 7.5 with 1 mM EDTA) and centrifuged (10000*g*, 15 min, 4 °C). For the GPx assay, tissues were homogenized in a similar potassium phosphate buffer with 1 mM DTT, and tissue solutions were centrifuged accordingly. After all centrifugations, the supernatants were removed, kept on ice, and immediately used for the enzyme assays.

Superoxide Dismutase Activity. The SOD assay is based on the reaction of WST-1 with superoxide anion, which forms a colorimetric formazan dye that can be monitored at an absorbance of 450 nm. Because xanthine oxidase reduces O_2 to $O_2^{\bullet -}$ but is converted to H_2O_2 by SOD, the inhibition activity of SOD can be determined by following the decrease in color development. SOD enzyme activity of samples was quantified using Fluka's SOD determination kit (Sigma-Aldrich) in accordance with the manufacturer's instructions. SOD standards were used to correlate SOD concentration (U/mL) with percent inhibition. SOD activity in tissue samples was expressed as units per milligram of protein.

Catalase Activity. Catalase activity was determined according to the procedure of Johansson and Borg (*35*) by following the decomposition of H_2O_2 at 25 °C. Tissue homogenates (0.02 mL) were mixed with 0.1 mL of 100 mM phosphate buffer (pH 7.0) and 0.03 mL of methanol in a microtiter plate. The reaction was initiated with 0.02 mL of 352.8 mM H_2O_2. The mixture was incubated for 20 min with shaking, and the reaction was terminated by the addition of 0.03 mL of 10 M KOH. Purpald (0.03 mL; 34.2 mM in 0.5 N HCl) was added, and the mixture was incubated for 10 min on a shaker. Potassium periodate (10 μL) was then added and incubated for a further 5 min. The absorbance of the purple formaldehyde adduct was then measured at 570 nm. Catalase activity was calculated using a standard curve with formaldehyde and was expressed per milligram of protein. One unit of activity was expressed as the formation of 1 μmol of formaldehyde/min.

Glutathione Peroxidase Activity. GPx activity was determined according to the procedure of Wendel (*36*) by following the oxidation of NADPH to $NADP^+$ at 340 nm and 25 °C. Tissue homogenates (0.017 mL) were mixed with 1 mL of a reaction cocktail consisting of 48 mM sodium phosphate, 0.38 mM EDTA, 0.12 mM β-NADPH, 0.95 mM NaN_3, 3.2 units of GR, 1 mM GSH, 0.02 mM DTT, and 0.0007% (w/w) H_2O_2. After mixing in a cuvette by inversion, the absorbance at 340 nm was recorded at 2 and 5 min. The changes in absorbance rate were converted into units of GPx per milligram of protein using a molar extinction coefficient of 6.22 mM^{-1} cm^{-1} for β-NADPH. One unit of activity was defined as the oxidation by H_2O_2 of 1 μmol of GSH to GSSG/min.

Glutathione Reductase Activity. GR activity was determined according to the procedure of Carlberg and Mannervik (*37*) by following the decomposition of β-NADPH at 340 nm and 25 °C. Tissue homogenates (0.075 mL) were mixed with 0.875 mL of 1.18 mM oxidized GSH (dissolved in 100 mM PBS, pH 7.5, containing 1 mM EDTA). β-NADPH (0.05 mL; 2 mM) was added ,and the absorbance was recorded at 2 and 7 min. The changes in absorbance rate 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 oxidation of 1 μmol of β-NADPH/min.

Glutathione S-Transferase Activity. GST activity was determined according to the procedure of Habig et al. (*38*) by following the formation of the 1-chloro-2,4-dinitrobenzine (CDNB)−GSH conjugate at 340 nm and 25 °C. Tissue homogenates (0.05 mL) were 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 at 2 and 5 min. The changes in the absorbance rates 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/min.

Determination of Tissue Oxidation. Tissue oxidation was assessed by measuring the malondialdehyde (MDA) and protein carbonyl (PC) content in intestinal samples. For the MDA assay, tissues were homogenized in a 100 mM cold potassium phosphate buffer (pH 7.5 with 1 mM EDTA) with 5 mM butylated hydroxytoluene (BHT), and tissue solutions were centrifuged at 10000*g* for 15 min at 4 °C. For the PC assay, frozen intestinal tissues were homogenized in a 100 mM potassium phosphate buffer and centrifuged accordingly. After all centrifugations, supernatants were removed, kept on ice, and immediately used for the lipid and protein oxidation assays.

Malondialdehyde Assay. MDA was determined according to the method of Janero (*39*) with a slight modification. Tissue homogenates (0.4 mL) were mixed with a reaction solution (1.3 mL) consisting of 10 mM of *N*-methyl-2-phenylindole in acetonitrile and 0.2 mM $FeCl_2$ in methanol. Hydrochloric acid (0.3 mL; 12 N) was added to the mixture, vortexed, and incubated at 45 °C for 1 h. After cooling on ice, the mixture was centrifuged (10000*g*, 10 min, 4 °C), and the supernatant absorbance was measured at 586 nm. The MDA concentration was extrapolated from a standard curve with methanesulfonic acid and converted to nanomoles of MDA per milligram of protein.

Protein Carbonyl Assay. The PC assay is based on the method of Levine et al. (*40*) in which the protein carbonyl content can be quantified via the reaction of carbonyls with 2,4-dinitrophenylhydrazine (DNPH) to form a Schiff base and a corresponding hydrozone, which can be analyzed spectrophotometrically between 360 and 385 nm. Tissue homogenates (0.54 mL) were incubated with streptomycin sulfate (0.06 mL of 10% streptomycin sulfate stock dissolved in 50 mM potassium phosphate, pH 7.2) to remove nucleic acids. The mixture was centrifuged (6082*g*, 10 min, 4 °C), and 0.2 mL of supernatant was transferred into each of two tubes. One tube was designated a sample and the other a control. DNPH (0.8 mL; 10 mM dissolved in 2.5 N HCl) was added to the sample tube and 0.8 mL of 2.5 N HCl to the control tube. The tubes were incubated in the dark at room temperature for 1 h with occasional vortexing every 15 min. One milliliter of 20% trichloroacetic acid (TCA) was added to each tube, vortexed, and then placed on ice for 5 min. The mixture was centrifuged (10477*g*, 10 min, 4 °C), the supernatant was discarded, and the pellet was resuspended in 10% TCA. The tubes were again placed on ice for 5 min and centrifuged again. The supernatant was discarded, and the pellet was resuspended in 1 mL of ethyl acetate/ethanol solution (1:1 ethyl acetate/absolute ethanol), vortexed, and centrifuged. This last step was repeated two more times with the last centrifugation increasing to 13684*g* for 10 min at 4 °C. After the final wash, the pellet was resuspended in 0.5 mL of guanidine−HCl solution (6 M guanidine−HCl with 20 mM potassium phosphate, pH 2.3), vortexed, and centrifuged at 16060*g* for 10 min at 4 °C. The supernatant (0.11 mL) from sample and control tubes was transferred into two wells of a half-area UV microtiter plate (Corning Costar), and the absorbance was measured at 360 nm. The corrected absorbance was determined by subtracting the average control absorbance from the average sample absorbance. The carbonyl concentration was then calculated using a molar extinction coefficient of 0.022 μM^{-1} cm^{-1} and was expressed as nanomoles per gram of tissue.

Statistical Analysis. All analyses were performed in triplicates unless specified otherwise. Statistical analyses were carried out using GraphPad software (San Diego, CA). The statistical significance of the in vitro data was determined by Student's *t* test with a $P \leq 0.05$ taken as significant. For the in vivo data, comparisons between groups were conducted with a one-way analysis of variance (ANOVA) followed by the Tukey−Kramer multiple-comparisons test. Homogeneity of variances was confirmed using Bartlett's test. Groups were considered to be statistically significant when $P \leq 0.05$. Results were reported as mean ± SEM.

RESULTS AND DISCUSSION

Characterization and in Vitro Evaluation of Antioxidative Stress Egg Yolk Protein Digests. To characterize the egg yolk protein hydrolysates from Alcalase and protease S (EYP-S), protease N (EYP-N), protease A (EYP-A), protease P (EYP-P), and trypsin (EYP-T) digestion, the percent nitrogen, phosphate content, and percent degree of hydrolysis were determined. There was no significant difference in the percent nitrogen among all digests

Table 1. Evaluation of Egg Yolk Protein Digests Derived from Protease S (EYP-S), Protease N (EYP-N), Protease A (EYP-A), Protease P (EYP-P), and Trypsin (EYP-T) Hydrolysis[a]

egg yolk protein digest	nitrogen (%)	phosphate (μg of PO_4/mg of protein)	degree of hydrolysis (%)
EYP-S	11.94 ± 0.01	36.54 ± 1.68	22.30 ± 1.29
EYP-N	11.87 ± 0.09	36.40 ± 1.50	18.91 ± 0.55
EYP-A	12.04 ± 0.03	34.38 ± 1.65	41.22 ± 1.61
EYP-P	11.88 ± 0.01	43.95 ± 2.29	38.64 ± 2.48
EYP-T	11.96 ± 0.03	32.25 ± 0.42	15.26 ± 0.28

[a] Digests were analyzed for percent nitrogen, phosphate content, and percent degree of hydrolysis. Data are presented as mean ± SEM; $n = 3$.

(**Table 1**); however, EYP-P had the highest phosphate content (43.95 μg of PO_4/mg of protein) followed by EYP-S, EYP-N, EYP-A, and EYP-T (32.25 μg of PO_4/mg of protein). From previous work (*17*), peptides with a higher percent phosphorus exhibited higher antioxidative stress bioactivity; therefore, this was a consideration in selecting an effective digest. Phosphorus was expressed as phosphate because the residual phosphorus in delipidated egg yolk protein is likely to be found as phosphate moieties monoesterified to serine as in the case of phosvitin. Using a variety of bacterial proteases and trypsin, the highest %DH was found for EYP-A (41.22%) followed by EYP-P (38.64%), EYP-S (22.30%), EYP-N (18.91%), and EYP-T (15.26%). Because trypsin generally cleaves at the carboxyl terminal of lysine and arginine (*41*), its substrate specificity defines the limited number of peptides that can be produced, which is reflected in the lowest % DH. In comparison, the bacterial proteases used in this study have variable substrate specificities resulting in different and higher degrees of hydrolysis.

The antioxidative stress effect of the digests was established after a 2 h peptide pretreatment followed by a 6 h hydrogen peroxide stimulation in Caco-2 cells. In agreement with previous studies, Caco-2 cells exposed to H_2O_2-induced oxidative stress produced an abundance of IL-8 protein (*23*) (**Figure 1A**). Pretreatment of the cells with individual egg yolk digests at either 0.1 or 1.0 mg/mL concentrations significantly reduced ($P < 0.001$) IL-8 secretion without affecting cell viability (**Figure 1B**). The viability of both EYP-treated and POS cells, however, was lower than that of NEG baseline cells. The maintenance of cell viability in the peptide-treated cells confirms the decrease in IL-8 is not a result of apoptosis or necrosis. Furthermore, reduced IL-8 secretion was observed even after removal of the digests followed by the addition of hydrogen peroxide (data not shown), suggesting this effect was due to modulation of IL-8 cell signaling pathways. There was no significant difference in bioactivity among the digests, but a palatable digest was required for the in vivo pig studies because pigs have been shown to detect and avoid taste compounds that humans perceive as bitter-tasting (*42*). From a practical standpoint, nonbitter peptides do not require encapsulation and could potentially be incorporated into an existing product to "add value". Informal taste tests of 5% (w/v) hydrolysate solutions indicated that EYP-P, EYP-S, and EYP-A were bitter but EYP-N and EYP-T were acceptable. Because EYP-N had higher phosphate content, this hydrolysate was selected for use in the oxidative stress piglet trials. Any reference to EYP-N from this point forward will be simply referred to as EYP to denote egg yolk peptides.

Egg Yolk Pepetide Supplementation Up-regulates in Vivo Glutathione Synthesis. Intestinal oxidative stress was induced in piglets for 10 days via intraperitoneal infusions of hydrogen peroxide. The effect of EYP supplementation during concurrent H_2O_2 intraperitoneal infusions was assessed by the total GSH

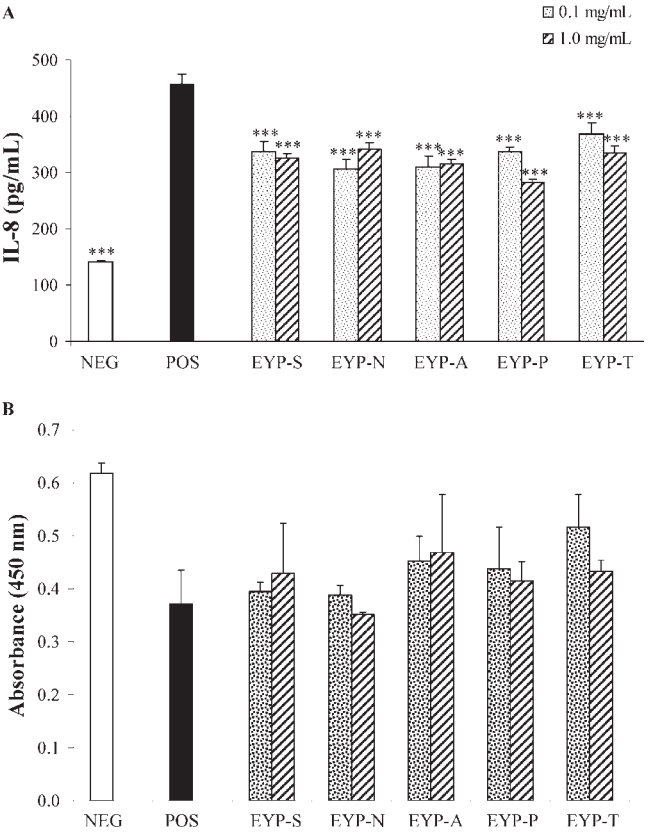

Figure 1. Effect of protease S (EYP-S), protease N (EYP-N), protease A (EYP-A), protease P (EYP-P), and trypsin (EYP-T) digests on (**A**) IL-8 secretion and (**B**) cell viability in H_2O_2-treated Caco-2 cells. Cells were treated with 0.1 or 1.0 mg/mL of protein digests for 2 h at 37 °C and then incubated with 1 mM H_2O_2 for 6 h. Data are presented as mean ± SEM, $n = 3$; ***, $P < 0.001$ compared to H_2O_2-treated cells.

levels, γ-GCS activity, and mRNA expression; antioxidative enzyme activities; and malondialdehyde and protein carbonyl concentrations in the duodenum, jejunum, ileum, and colon tissues.

Glutathione is a naturally occurring cellular reductant and antioxidant (*43*, *44*) that detoxifies reactive oxygen metabolites of endogenous or exogenous origins. The amount of de novo GSH synthesis can be estimated from the intracellular GSH concentration and from the expression level and activity of the key synthesis enzyme, γ-GCS (*45*, *46*). There was no significance in glutathione concentrations among the three treatment groups in the ileum and colon (**Figure 2A**). In the duodenum and jejunum of POS and EYP groups, although total GSH was not significantly different, EYP-fed piglets exhibited higher ($P < 0.05$) glutathione concentrations than NEG pigs. γ-GCS activity in the colon was enhanced ($P < 0.05$ and $P < 0.001$, respectively) in EYP versus POS and NEG animals (**Figure 2B**). Although γ-GCS activity was significantly higher ($P < 0.05$) in the jejunum of positive control animals compared to both NEG and EYP pigs, γ-GCS gene expression was elevated in the peptide-supplemented animals versus both sets of controls (**Figure 2C**).

In humans, the intestinal distribution of GSH and antioxidant enzymes is generally higher in the proximal intestine starting from the duodenum compared to the distal end at the colon (*47*). Levels of these endogenous compounds, however, can be disrupted in the presence of oxidative stress (*48*) as observed in the varied responses among the duodenum, jejunum, ileum, and colon and among the NEG, POS, and EYP groups. In response to mild oxidative stress, tissues often respond by producing more

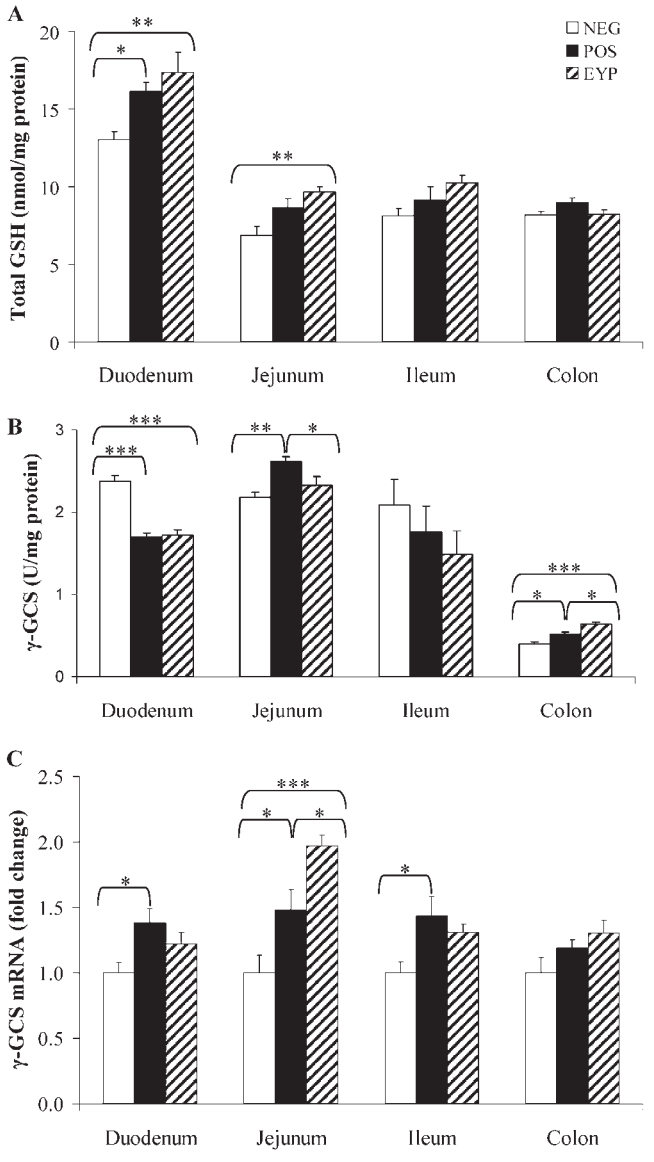

Figure 2. Effect of egg yolk peptides (EYP) on (**A**) total GSH, (**B**) γ-GCS activity, and (**C**) relative mRNA expression of harvested duodenum, jejunum, ileum, and colon tissues. The concentration of GSH is expressed as nanomoles of GSH per milligram of protein, and γ-GCS activity is reported as units per milligram of protein. mRNA was extracted from intestinal tissues, and relative gene expression was measured by real-time RT-PCR. Fold changes were calculated relative to the negative control. Values represent mean ± SEM with $n = 5$ per group; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

antioxidants; however, severe enduring oxidative stress depletes the body's antioxidant resources and overtakes the ability to produce more antioxidants, leading to lower antioxidant levels (*2*). It is plausible, therefore, that tissues may be at different stages of coping with oxidative stress. Duodenum γ-GCS activity may be compromised in POS pigs; however, associated mRNA indicates an effort to re-establish GSH levels by up-regulating γ-GCS gene expression. On the contrary, jejunum γ-GCS activity is higher in positive pigs compared to both NEG and EYP, and this corresponds to the increased γ-GCS mRNA expression, confirming this gene was already induced as an adaptive response to oxidative stress defense and detoxification. The jejunum γ-GCS mRNA expression of EYP-treated pigs is also elevated and is in fact significantly higher than that of the H_2O_2-stressed pigs, suggesting a role in the enhancement of intracellular GSH synthesis. Colon γ-GCS activity is higher in EYP compared to both POS and NEG controls; however, there is no difference in mRNA expression or total GSH among the three groups, which may indicate the beginning of antioxidant defense exhaustion in this tissue. Although the change in antioxidant levels is conflicting among the intestinal segments, the imperative point is the presence of an imbalance in antioxidant concentrations to which EYP supplementation aids in the promotion of antioxidative stress mechanisms to re-establish a stable redox homeostatis at least in the proximal intestine.

Egg Yolk Peptide Supplementation Increases in Vivo Antioxidant Enzyme Activities. During oxidative stress conditions genes encoding antioxidant enzymes such as SOD, CAT, GPx, GST, and GR are preferentially up-regulated in an effort to restore cellular redox homeostatis. Because some dietary components have been found to positively influence these transcription and signaling pathways, the role of egg yolk peptides in affecting antioxidant enzyme activities was investigated. Enzyme activities were measured instead of protein levels or gene expression because this better reflects the state of the enzymes during oxidative stress. The concentration of enzymes can be misleading because some enzymes could be inactivated by oxidants, whereas increased mRNA expression does not always equate to more enzymes (*49*).

The ingestion of EYP during the onset of oxidative stress resulted in significantly increased SOD activity in the ileum ($P < 0.05$) and colon ($P < 0.01$) compared to the POS and NEG groups (**Figure 3A**). SOD activity was significantly higher ($P < 0.01$) in the duodenum of NEG piglets versus both POS and EYP groups; however, there was no significant difference in activity among all groups in the jejunum. Catalase activity was significantly increased in the duodenum ($P < 0.05$), jejunum ($P < 0.001$), and colon ($P < 0.05$) tissues of EYP-supplemented piglets compared to POS and even NEG controls (**Figure 3B**). In the ileum, CAT activity was elevated in both POS and EYP groups versus the NEG control. EYP treatment did not appear to influence GPx activity above endogenously produced levels, because there was no significant difference in GPx activity between EYP and POS piglets, but the activities in both groups were significantly higher than that of the negative controls in all intestinal tissues (**Figure 3C**). EYP supplementation resulted in higher GR activity in the ileum (**Figure 3D**) and elevated GST activities in all intestinal tissues in contrast with the POS group (**Figure 3E**). These elevated GST activities matched or exceeded levels compared to that of the NEG control tissues.

Similar to GSH and GSH-synthesis enzymes, there were intertissue variations and differences among treatment groups for the antioxidant enzymes. EYP supplementation elevated GST and CAT activities in the majority of tissues while exhibiting limited effect on SOD and GR activities, and did not influence GPx activities compared to levels observed in the positive control pigs. The increase in CAT and GST activities is interesting because it appears the cell's priority is to detoxify the exogenously supplied H_2O_2 and protect against lipid peroxidation.

An increase in antioxidant enzymes and their corresponding activities is crucial to the detoxification of ROS and the restoration of tissue health. Under conditions of oxidative or electrophilic stress, the genes encoding γ-GCS and the antioxidative enzymes are controlled by the nuclear factor-E2-related factor 2-antioxidant response element (Nrf2-ARE) signaling pathway (*6*). A low GSH/GSSG ratio triggers the transcription of AREs, which are localized in the promoter region of these genes (*50*). Recent data demonstrate the effect of polyphenols on increasing GSH and γ-GCS, GR, GPx, and GST activities and

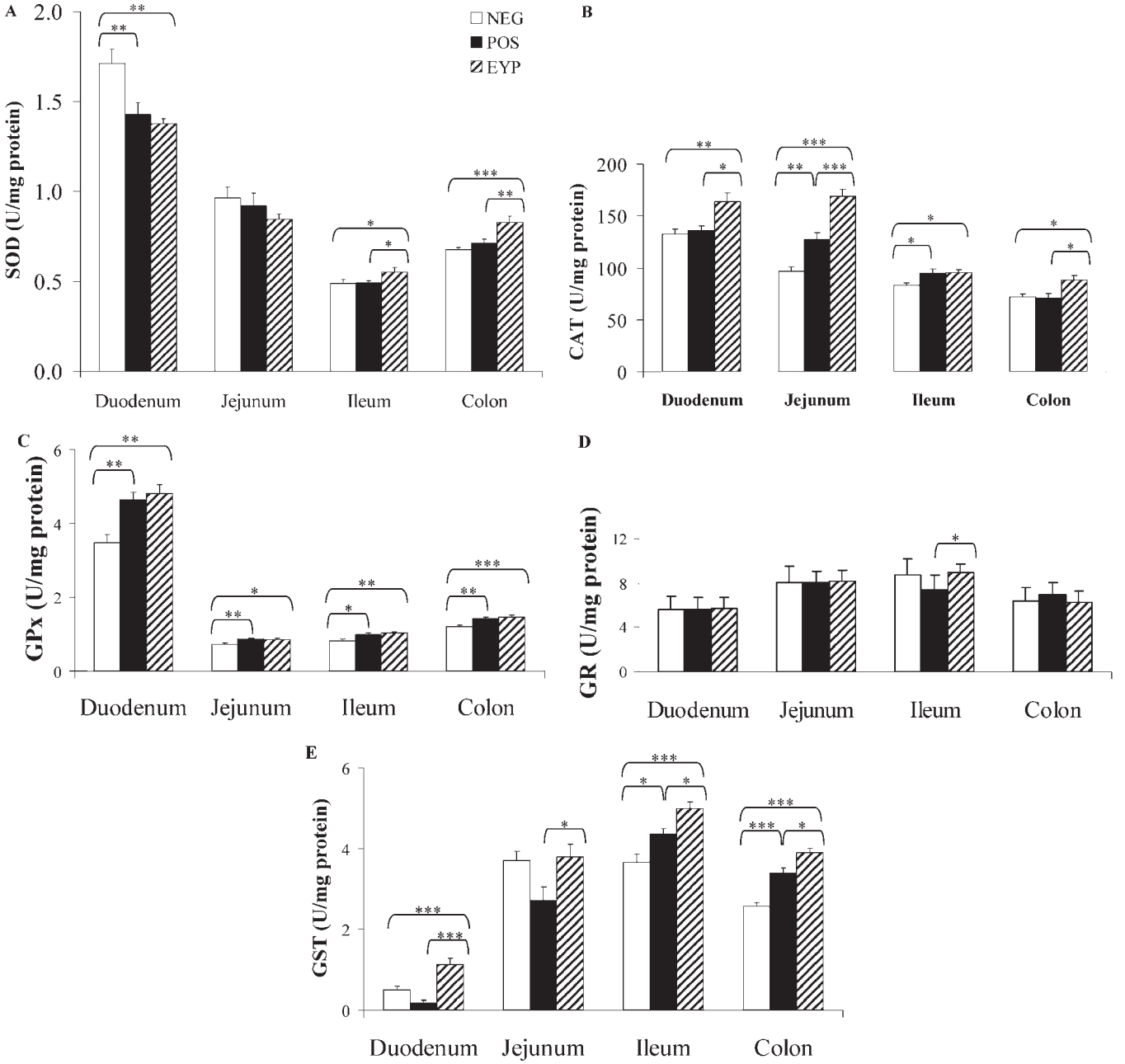

Figure 3. Effect of egg yolk peptides (EYP) on (**A**) superoxide dismutase (SOD), (**B**) catalase (CAT), (**C**) glutathione peroxidase (GPx), (**D**) glutathione reductase (GR), and (**E**) glutathione *S*-transferase (GST) enzyme activities in harvested duodenum, jejunum, ileum, and colon tissues. Enzyme activities are reported as units per milligram of protein. Values represent mean ± SEM with $n = 5$ per group; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

mRNA (*9*−*11*), and it is thought that dietary polyphenols stimulate the transcription of these defense systems through ARE elements (*51*). Researchers (*52*) suggest that polyphenols influence the pathways that regulate ARE activation by modifying the capability of Kelch-like ECH-associated protein1 (Keap1) in sequestering Nrf2 and/or activate the mitogen-activated protein kinase (MAPK) proteins (ERK, JNK, and p38) involved in Nrf2 stabilization, thereby permitting Nrf2 to translocate to the nucleus, where it would transactivate the ARE-containing promoter of antioxidant genes. Egg yolk digests may also be able to enhance Keap1/Nrf2 dissociation during oxidative stress and promote the up-regulation of antioxidative genes.

Egg Yolk Peptide Supplementation Reduces in Vivo Lipid and Protein Oxidation. The extent of oxidative damage to membrane lipids and tissue proteins in the intestinal tissues was evaluated by assaying malondialdehyde and protein carbonyl concentrations, respectively. Malondialdehyde, resulting from the degradation of polyunsaturated lipids by reactive oxygen species, and protein carbonyls, formed from the oxidation of protein side chains, have been shown to increase in parallel during oxidative stress conditions (*53*). EYP supplementation resulted in a significant decrease in MDA ($P < 0.01$) and PC ($P < 0.05$) to levels similar to NEG controls in both jejunum and ileum tissues compared to H_2O_2-stressed animals (panels **A** and **B**, respectively, of **Figure 4**). EYP had no effect on MDA concentrations in the duodenum and colon because there was no significant difference in MDA among all three animal groups. Similarly, EYP did not appear to influence PC levels in the colon, although in the duodenum POS animals exhibited significantly higher ($P < 0.05$) PC versus NEG controls.

The increased CAT and GST detoxification activities supports the lowered malondialdehyde levels seen in the jejunum and ileum of EYP treated animals. The coordinated action of all

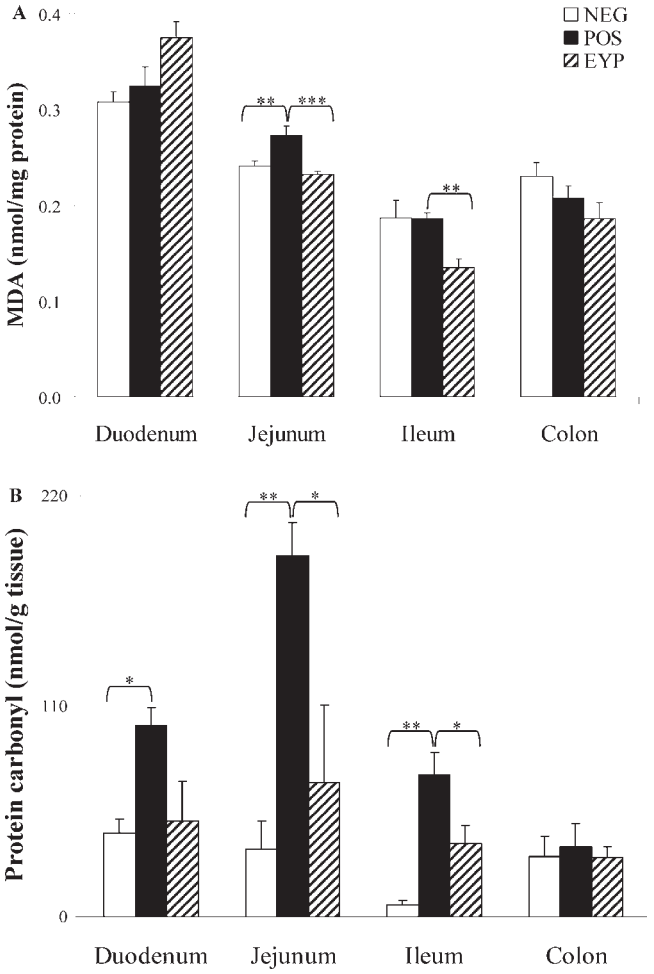

Figure 4. Effect of EYP on (**A**) malondialdehyde (MDA) and (**B**) protein carbonyl (PC) concentrations in the duodenum, jejunum, ileum, and colon. MDA is reported as nanomoles per milligram of protein, whereas PC is reported as nanomoles per gram of tissue. Values represent mean ± SEM with $n = 5$ per group; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

up-regulated antioxidant enzymes also defends against protein damage as reflected by the decreased protein carbonyl concentration of pigs supplemented with egg yolk digests compared to H_2O_2-stressed animals. Interestingly, the majority of effects exhibited by EYP in intestinal tissues are identical to that observed for egg yolk phosvitin phosphopeptides in Caco-2 cell culture (*16*, *17*), which questions the possibility of similarities in peptide composition and structure. Investigations are in progress to determine the amino acid sequence of these two peptide preparations.

Egg Yolk Peptide Supplementation Increases Glutathione Concentrations in Red Blood Cells. In human studies, the efficacy of ingested antioxidants is approximated by measuring the antioxidant status of the blood. Because it is difficult to attain tissue biopsies unless the patient is at a high risk of developing intestinal disease, blood samples are analyzed even though these antioxidants were intended to target the gastrointestinal tract. Glutathione concentrations are typically low in plasma but are quite high in RBC. Analysis of the total glutathione concentration in RBC provides a holistic overview of the body's antioxidant status. At the beginning of the trial, the GSH concentration was not significantly different among all animals (**Figure 5**). The large variation in pig GSH levels on day 2 is likely due to the stress of acclimatizing to a new environment and adapting from sow's milk to a milk replacement formula. On the first

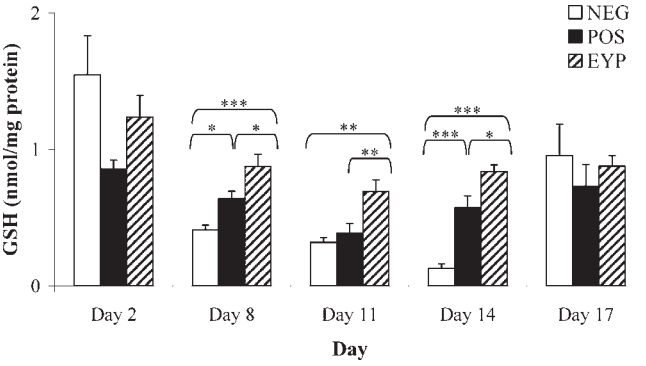

Figure 5. Effect of EYP on total glutathione (GSH) in red blood cells drawn on days 2, 8, 11, 14, and 17 of the animal trial. Glutathione concentration is expressed as nanomoles of GSH per milligram of protein. Values represent mean ± SEM with $n = 5$ per group; ∗, $P < 0.05$; ∗∗, $P < 0.01$; ∗∗∗, $P < 0.001$.

day of EYP supplementation (day 8), GSH levels were significantly elevated ($P < 0.05$) in EYP compared to POS and NEG control piglets. This increase in GSH was also observed on days 11 and 14. On the last day of supplementation (day 17), there was no significant difference in GSH among all three groups of animals.

EYP supplementation maintained high glutathione levels throughout the course of hydrogen peroxide infusions. In comparison, POS animals appear to increase endogenous GSH to cope with the stress and are only able to re-establish levels similar to those of baseline pigs at the end of the study. The dip in GSH levels in NEG pigs is likely due to the oxidative stress incurred after surgery (*54*); however, GSH levels increased by day 17, although not to presurgery levels. Given a longer period of time, NEG pigs may be able to reach presurgery GSH baseline concentrations. Oxidative stress research indicates that during acute oxidative stress, when the increase in ROS is relatively small, the antioxidative response may be sufficient to compensate for the increase in ROS and reset the oxidant/antioxidant balance. However, if ROS production is higher and persists for a longer period of time, as in the case of chronic oxidative stress, the antioxidative response may not be able to rebalance the redox system to a low ROS baseline level (*48*). The intraperitoneal surgery, although a traumatic event, occurred only once, and with the high turnover of cells in the young piglets, it is expected that the tissue antioxidant status will return to presurgery levels. These results suggest that EYP supplementation, even though intended for intestinal oxidative stress, is able to maintain high systemic levels of GSH. Intact peptides can be transported via the PepT1 H^+-coupled transport protein for di- and tripeptides and via the passive transport route through tight junction pores for oligopeptides (*55*, *56*). Past studies have shown that enteral administration of bioactive polypeptides of various amino acid chain lengths can be absorbed intact into the blood without loss of bioactivity (*57*). It is therefore possible for EYP to exert biological activity not only in the gut but on a systemic level as well.

In conclusion, we were able to successfully produce antioxidative stress peptides from egg yolk protein using Alcalase and protease N hydrolysis. Supplementation with these crude egg yolk peptides improved the antioxidant status of intestinal cells and tissues exposed to oxidative stress. Glutathione synthesis and antioxidative stress enzyme systems were induced, thereby preventing oxidative damage to lipids and proteins. The in vivo efficacy of these peptides suggests potential applicability in human trials for the prevention of oxidative stress and subsequent deterrence of gastrointestinal pathologies.

ABBREVIATIONS USED

ARE, antioxidant response element; CAT, catalase; EYP, egg yolk peptides; EYP-A, egg yolk peptides (protease A digest); EYP-N, egg yolk peptides (protease N digest); EYP-P, egg yolk peptides (protease P digest); EYP-S, egg yolk peptides (protease S digest); EYP-T, egg yolk peptides (trypsin digest); γ-GCS, γ-glutamylcysteine synthetase; GPx, glutathione peroxidase; GR, glutathione reductase; GSH, glutathione; GSSG, glutathione disulfide; GST, glutathione *S*-transferase; IL-8, interleukin-8; MDA, malondialdehyde; Nrf2, nuclear factor-E2-related factor 2; PC, protein carbonyl; PPP3, phosvitin oligophosphopeptide fraction 3; RBC, red blood cells; ROS, reactive oxygen species; SOD, superoxide dismutase.

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