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Effects of Wheat Antioxidants on Oxygen Diffusion−Concentration Products in Liposomes and mRNA Levels of HMG-CoA Reductase and Cholesterol 7α-Hydroxylase in Primary Rat Hepatocytes

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Three wheat antioxidant fractions were investigated for their potential effects on oxygen diffusion−concentration products in liposomes prepared with egg yolk phosphatidycholine (yolk PC) and rat liver PC (liver PC), using the electron spin resonance (ESR) oximetry method with 2,2′-azobis(2-aminopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) as radical generators. Both water-soluble wheat antioxidant (WWA) and the second lipophilic antioxidant (LWA2) fractions were able to inhibit oxygen diffusion−concentration product induced by either AAPH or AMVN. The first lipophilic wheat antioxidant (LWA1) fraction only showed antioxidant activity in yolk PC liposomes with AAPH as the radical initiator but had pro-oxidant activity under other testing conditions. Both liposome composition and radical initiator altered the antioxidative properties of WWA, LWA1, and LWA2. WWA also showed the strongest DPPH$^{\bullet}$ scavenging capacity on a per grain weight basis. HPLC analysis showed that WWA had a much higher level of total phenolic acids, which may partially explain their antioxidant properties. In addition, wheat antioxidants significantly down-regulated the mRNA of HMG-CoA reductase, the key enzyme for cholesterol biosynthesis, and up-regulated the mRNA of cholesterol 7α-hydroxylase (CYP7A1), the key enzyme for cholesterol metabolism, in primary rat hepatocytes. These data indicated the potential of wheat antioxidants in reducing the risk of atherosclerosis through multimechanisms.

KEYWORDS: Wheat antioxidants; ESR; liposomes; cholesterol; lipid peroxidation

INTRODUCTION

Atherosclerosis is a leading cause of coronary heart disease (CHD), which probably resulted in 42% death of CHD in the United States (*1*). Prevention appears very important for CHD and atherosclerosis, since they have no medicine to affect a cure once they are contracted. It is widely accepted that oxidative stress and hypercholesterolemia play causal roles in the pathogenesis of atherosclerosis and CHD, although some other mechanisms, such as diabetes, aging, hypertension and lifestyle, may also be involved (*2, 3*). Oxidative stress, such as overproduction of free radicals, is associated with endothelial dysfunction, the earliest signal of the atherosclerotic process (*4*). Free radical-mediated peroxidation of low density lipoprotein (LDL), in both lipid and protein fractions, is also a key factor in the early development of atherosclerosis (*5*). In addition, lipid peroxidation of injured human arterial wall was revealed as a significant consequence of atherosclerosis development (*6*). Epidemiological studies suggested that dietary antioxidants might scavenge the free radicals in the biological systems and reduce the risk of atherosclerosis and CHD (*7*). Development of functional foods rich in natural antioxidants is a practical approach to reduce the oxidative stress and consequently the risk of chronic human diseases including atherosclerosis and CHD. The key for such functional foods is to develop food ingredients rich in antioxidants and other health beneficial components, as well as to understand the mechanisms involved in their biological actions.

As an important food ingredient across the world, wheat grain is gradually being revealed to be rich in health beneficial components such as antioxidants and dietary fibers (*5, 8–11*).

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Previous studies showed that wheat antioxidants may directly react with and quench DPPH$^{\bullet}$, ABTS$^{+\bullet}$, peroxyl (ROO$^{\bullet}$), and hydroxyl (HO$^{\bullet}$) radicals and chelate transition metal ions such as Fe^{2+} and Cu^{2+}. In 2005, a study also showed that wheat antioxidants may suppress Cu^{2+}-induced lipid peroxidation in human LDL (*5*). In addition, it is widely recognized that testing systems may alter the antioxidant activity estimation (*9, 11, 12*). Many efforts have been taken to develop antioxidant activity assays that may be performed under physiological conditions for better predicting the possible actions of these antioxidants in biological systems (*13*). Liposomes are considered artificial membranes and are commonly employed to evaluate the potential inhibitory activity of antioxidants on lipid peroxidation in membranes (*14*). Liposomes prepared from different phospholipids may serve as excellent model systems for investigating wheat antioxidants for their potential inhibition of lipid oxidation in membranes such as injured human arterial membrane. To our knowledge, the inhibitory effect of wheat antioxidants on lipid oxidation in membranes has not been evaluated.

In addition to their antioxidant activities, it is possible that wheat antioxidants may reduce the risk of atherosclerosis and CHD through other mechanisms. Whole wheat flours were shown to reduce the plasma and liver cholesterol and triglyceride levels in male Wistar rats (*15, 16*). It was also reported that different grain fractions may differ in their capacities to modulate plasma and liver lipid profiles (*15*). The whole wheat flour significantly reduced both plasma cholesterol and triglyceride concentrations, while bran and white flour could not as compared to the control group (*15*), suggesting that the insoluble fiber may not be the primary contributor for the observed hypolipidemic activity and wheat grain may contain other cholesterol-lowering components. It is well accepted that dietary components capable of inhibiting cholesterol de novo biosynthesis, stimulating conversion of cholesterol to bile acids, and/or up-regulating the liver LDL receptor may reduce plasma and LDL cholesterol, and they appear to be of primary importance for CHD prevention. To date, little is known whether wheat antioxidants may directly alter cholesterol and fatty acid biosynthesis and metabolism at the molecular level.

As part of our continuous efforts to promote the valued-added production and utilization of wheat for health promotion and disease prevention, the objective of the present study was to investigate (1) the potential effect of wheat antioxidants on lipid peroxidation in liposomes, prepared from egg yolk and rat liver phosphatidycholine (PC), and (2) their possible effect on expression of key genes involved in cholesterol biosynthesis and metabolism. Choptank wheat was selected in this study because of its excellent antioxidant activity according to a previous comparative study on the antioxidant components and properties of Maryland-grown soft wheat (*9*). Choptank wheat is a soft red winter variety (*Triticum aestivum* L.) registered in 2004 (*17*).

MATERIALS AND METHODS

Materials. Egg yolk PC (yolk PC) and rat liver PC (liver PC) were obtained from Avanti Polar Lipids Inc. (Alabaster, AL). The fatty acid compositions of these natural PCs are shown in **Table 1**. 2,2′-Azobis-(2-aminopropane) dihydrochloride (AAPH) and 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) were purchased from Polysciences (Warrington, PA). 4-Oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl (^{15}N-PDT), and 2,2-diphenyl-1-picrylhydrazyl radical (DPPH$^{\bullet}$) were acquired from Sigma-Aldrich (St. Louis, MO). Ribonuclease protection assay (RPA II) kits were from Ambion (Austin, TX). Gene expression kits for rat LDL-R, CYP7A1, and HMG-CoA-R were from Applied Biosystems (Foster City, CA). All other chemicals and solvents were of the highest commercial grade and used without further purification.

Table 1. Fatty Acid Compositions of the Natural PCs Used in This Study[a]

fatty acid	egg PC	liver PC
16:0	34.0	11.5
16:1	1.7	ND
18:0	11.0	29.3
18:1	32.0	13.4
18:2	18.0	12.1
20:3	ND	8.5
20:4	3.3	9.3
22:6	ND	1.4
other	ND	14.5

[a] The natural PCs were purchased from Avanti Polar Lipids Inc. Egg PC and Liver PC stand for egg yolk PC and rat liver PC, respectively. Fatty acid compositions in this table were expressed as g/100 g of total fatty acids by FAME-GC/FID and were those provided by the supplier.

Extraction and Testing Sample Preparation. *Preparation of Antioxidant Samples for ESR Oxygen Diffusion−Concentration Product Study.* Choptank wheat grain samples from Dr. Jose Costa in the Department of Natural Resource Sciences and Landscape Architecture at the Univesity of Maryland (College Park, MD) were ground to a fine powder using a micromill manufactured by Bel Art Products (Pequannock, NJ). Wheat antioxidants were extracted and fractionated according to their water solubility and polarity as shown in **Figure 1**. Briefly, the wheat grain powders (14 g) were extracted with 140 mL of 50% aqueous acetone at ambient temperature for 12 h under nitrogen as described previously (*9*). The supernatant was separated from the solid residue by centrifugation. Both supernatant and the solid residue were collected for further fractionation or extraction, respectively. Acetone in the supernatant was removed by evaporation at 50 °C under reduced pressure until the total volume reduced to approximately 70 mL. The resulting suspension was partitioned three times in a separatory funnel with an equal volume of chloroform each time. The chloroform layers were combined and evaporated to dryness at 50 °C under reduced pressure. Then the dry residue was redissolved in 5 mL of chloroform to form a liposoluble wheat antioxidant stock solution. This antioxidant fraction was named as LWA1. The aqueous layer was lyophilized to dryness. The dry powder was dissolved in 25 mL of distilled−deionized water to form a water-soluble stock solution of wheat antioxidants. This antioxidant fraction was named as WWA.

The solid residue from 50% acetone extraction was re-extracted three times using 30 mL of chloroform with sonication at ambient temperature. The combined chloroform extraction was also evaporated to dryness at 50 °C under reduced pressure. Five milliliter of chloroform was used to redissolve the residue to obtain the second lipophilic antioxidant stock solution, labeled as LWA2. The concentrations of WWA, LWA1, and LWA2 stock solutions were all adjusted to 560 mg of dry wheat grain equivalents/mL. All the three solutions were stored in a refrigerator until further analysis.

Preparation of Antioxidant Sample for Cholesterol-Lowering Study. Briefly, 20 g of ground Choptank wheat grain was extracted by 20 mL of 50% aqueous acetone at ambient temperature for 12 h under nitrogen as described previously (*9*). The acetone was removed under reduced pressure at 50 °C. The resulting aqueous suspension was freeze-dried. The freeze-dried antioxidants were kept in light-protected vials at 4 °C and redissolved in DMSO for the cholesterol-lowering capacity study.

ESR Measurement of Oxygen Diffusion−Concentration Products. Lipid peroxidation is induced by either hydrophilic AAPH or lipophilic AMVN. For the lipophilic antioxidants using AMVN as a lipophilic radical initiator, the chloroform solutions of individual PCs, lipophilic antioxidants, and AMAN were mixed before liposome preparation. The mixture contained 375 μL of 10 mg/mL rat liver PC or 187.5 μL of 20 mg/mL egg yolk PC, 12.5 μL lipophilic antioxidant testing solution, and 10 or 15 μL of 100 mM AMVN for liver or yolk PC, respectively. After being vortexed for 1 min, the mixture was

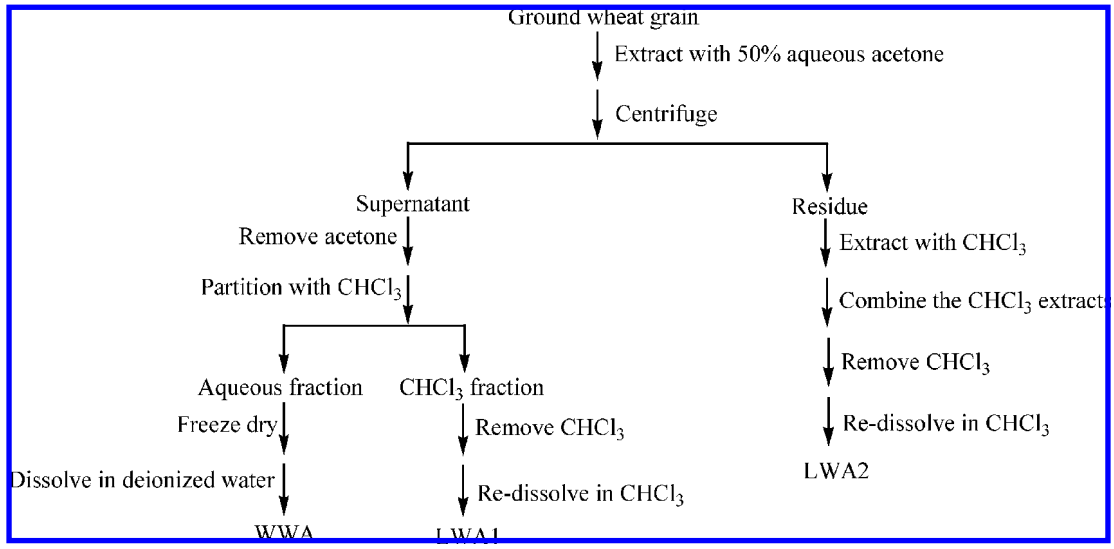

Figure 1. Preparation of wheat antioxidant fractions for ESR study.

coevaporated under a steady stream of argon for 30 min and then in vacuo for 12 h to remove the trace solvent. The resulted thin film was suspended in 112.5 μL of 50 mM HEPES buffer (pH 7.4) containing 12.5 μL of 0.6 mM ^{15}N-PDT. The suspension was vortexed for 1 min and then incubated in a light-protected water bath for 40 min at 37 °C with continuous shaking at 50 oscillations/min. After incubation, the suspension was transferred to two 50 μL capillary tubes and subjected to ESR examination. The final concentrations in the liposome suspensions were as follows: 30 mg/mL of PC, 12 mM (for yolk PC) or 8 mM (for liver PC) of AMVN, 0.06 mM of ^{15}N-PDT, and 22.4−179.2 mg of dry wheat grain equivalents/mL for lipophilic wheat antioxidants LWA1 and LWA2.

In the case of lipophilic antioxidants using AAPH as the water-soluble radical initiator, the liposomes were prepared following the same protocol described above without adding AMVN. AAPH and ^{15}N-PDT were added to the liposome suspension.

For the hydrophilic antioxidants using AAPH as the initiator, 150 μL of 25 mg/mL liver PC or 375 μL of 10 mg/mL yolk PC liposomes were prepared by evaporating chloroform from their solutions for 30 min using argon, followed by drying overnight under reduced pressure to form thin films. The thin film was resuspended in a mixture of 90 μL of 50 mM HEPES buffer (pH 7.4), 12.5 μL of 0.4 mM ^{15}N-PDT, and 10 μL of water soluble wheat antioxidants (WWA) and vortexed for 1 min to obtain a liposome suspension. The liposome suspension was coincubated in a light-protected water bath for 40 min at 37 °C with continuous shaking at 50 oscillations/min. After incubation for 45 min, a 12.5 μL aqueous solution of 400 mM (for liver PC) or 200 mM (for yolk PC) AAPH was added and vortexed instantaneously to initiate lipid peroxidation. The solution was then transferred to two 50 μL glass capillary tubes for ESR determinations. The final concentrations in the mixture were as follows: 30 mg/mL of PCs, 20 and 40 mM of AAPH for yolk or liver PC liposomes, respectively; 0.04 mM of ^{15}N-PDT; and 22.4−179.2 mg of dry wheat grain equivalents/mL for WWA.

Also, the WWA were tested in the liposomes made from both PCs with AMVN as the radical initiator. The liposome system was prepared following the same protocol described above except that AMVN was mixed in PC solutions. Solutions of AAPH and AMVN were freshly prepared before each experiment. Controls were prepared in the same way, but without the addition of any antioxidant sample.

All the ESR measurements were carried out at 37 °C using a Varian E-109 X-band ESR spectrometer (Varian, Inc., Palo Alto, CA), equipped with a variable gas flow temperature controller. Typical spectroscopic parameters were microwave power (1 mW), modulation amplitude (0.05 G), and scan range (1 G). Spectra were recorded, stored, and manipulated using a special computer program (VIKING obtained from the Nation Biomedical ESR Center, MCW, Milwaukee, WI). The 50 μL glass capillary tube containing liposome suspension was sealed at both ends with Citroseal sealant and placed inside the ESR Dewar cavity, and a flow of nitrogen was employed to control cavity temperature at 37 °C. All experiments were performed in duplicate.

ESR Determination of DPPH$^\bullet$ Scavenging Capacity of the Three Wheat Antioxidant Fractions. An aliquot of the antioxidant solution (LWA1, LWA2, or WWA, 100 μL) at a concentration of 280 mg of wheat grain equivalents/mL was added to 100 μL of 0.5 mM DPPH$^\bullet$ in ethanol. The mixture was then vortexed for 10 s and transferred to a 50 μL glass capillary tube for ESR determination. Equal volumes of chloroform or deionized water were used for controls of LWA1 and LWA2, and WWA, respectively. ESR signals were recorded at 5 min of the reaction, with 15 mW incident microwave power and 100 kHz field modulation of 1.6 G.

Rat Primary Hepatocyte Isolation and Cell Culture. Hepatocytes were isolated from adult male rats (250−300 g of body weight) according to the method described previously (*18*). Viability of the cell (>90%) was determined by trypan blue exclusion. The isolated hepatocytes were cultured in a serum-free Williams medium E containing dexamethasone (0.1 μM), insulin (100 nM), penicillin (100 units/mL), and thyroxine (1 μM). They were resuspended into collagen-coated plate (60 mm), and incubated for 12−24 h at 37 °C under an atmosphere of 95% air and 5% CO_2 before treatments of wheat antioxidants at a final concentration of 0.12 mg/mL, equivalent to 2 mg of wheat grain/mL of medium, for 24 h.

RNA Isolation and Ribonuclease Protection Assay (RPA). Total RNA was isolated from rat primary hepatocytes using the guanidine thiocyanate cesium chloride centrifugation method. All ribonuclease protection assay (RPA) probes for rat CYP7A1, HMG-CoA-R, LDL-R, and cyclophilin were synthesized using a MAXIscript T7 kit from Ambion with a probe-specific DNA fragment which was cloned into a pSP72 vector. The RPA probes were labeled with [α-^{32}P]UTP and isolated using Qiaquick columns. Overnight hybridization was carried out with 8×10^4 counts per minute (cpm) for CYP7A1, HMG-CoA-R, LDL-R, and 4×10^4 cpm for cyclophilin, which was used as an internal control. Twenty microgram of total RNA was used in all RPA assays. Following RNase digestion, samples were fractionated on 5% acrylamide/8 M urea gels and bands visualized by autoradiography using Kodak Biomax MS film. The density of bands was analyzed using Image J computer software (NIH) and normalized to rat cyclophilin.

Phenolic Acid Composition Analyzed by HPLC. The three wheat antioxidant samples were analyzed for their free and conjugated phenolic acid compositions using a previously reported procedure (*9*). Briefly, WWA was separated into the free and the conjugated phenolic acids, while LWA1 and LWA2 were directly subjected to HPLC analysis for free phenolic acids without any further purification. The free and conjugated phenolic acids in WWA were separated based on their water solubility under acidic conditions (pH 2). The free phenolic acids were extracted into ethyl acetate and ethyl ether (1/1, v/v) under

acidic condition, while the conjugated phenolic acids in the aqueous phase were hydrolyzed with 2 M NaOH prior to being re-extracted with ethyl acetate and ethyl ether (1/1, v/v) under acidic condition. After evaporation of ethyl acetate and ethyl ether by a nitrogen evaporator, each phenolic acid extract as well as LWA1 and LWA2 was quantitatively redissolved in MeOH and analyzed by reverse phase HPLC using a Phenomenex C_{18} column (250 mm × 4.6 mm) according to an established laboratory procedure (*9*). The phenolic acids were detected at 280 nm and separated using a linear gradient elution program with a mobile phase consisting of solvent A (acetic acid/H_2O, 2:98, v/v) and solvent B (acetic acid/acetonitrile/H_2O, 2:30:68, v/v/v). The solvent gradient was programmed from 10 to 100% B in 42 min with a flow rate of 1.0 mL/min. Identification of individual phenolic acids was accomplished by comparing the retention time and UV spectra of peaks in the samples to those of the standards under the same HPLC conditions. The concentration of each phenolic acid was determined using external standards and total area under each peak.

RESULTS AND DISCUSSION

Lipid peroxidation is believed to contribute to the development of several aging-associated human chronic diseases, such as CHD. Generally, lipid peroxidation is estimated by measuring the disappearance of reactants, such as unsaturated fatty acids, and the formation of primary or secondary lipid peroxidation products (*6*). In contrast to these commonly used assays, the ESR spin-label oximetry method measures the molecular oxygen concentration in the biological microenvironments such as liposome suspensions and is well accepted as a diagnostic indicator of lipid peroxidation because a slower local oxygen consumption is associated with a lower level of oxygen diffusion−concentration products, which reflects a lower degree of lipid peroxidation (*19, 20*). A stronger antioxidant activity results in a slower reduction of molecular oxygen concentration in the testing system under the experimental conditions. In the present study, this method was employed to investigate the effects of water-soluble and lipophilic wheat antioxidants on oxygen diffusion−concentration product in liposomes prepared using both liver PC and yolk PC by measuring the reduction of molecular oxygen concentration in the liposome systems sealed in a capillary.

Effect of Water Soluble Wheat Antioxidants (WWA) on Oxygen Diffusion−Concentration Products in Liposomes. It is well-known that AAPH is a water-soluble radical generator and produces peroxyl radical in the aqueous phase of the liposome suspension, whereas the fat-soluble AMVN generates peroxyl radicals within the lipophilic lipid bilayers of the liposome. Antioxidants with different polarity are distributed differently in the lipid and water phase of the liposome suspension and contribute differently to the overall protection of phospholipid peroxidation. **Figure 2** reports the effects of WWA on oxygen diffusion−concentration products in liver PC and yolk PC liposome suspensions when peroxyl radicals are generated in the aqueous phase using the water-soluble radical initiator AAPH, whereas **Figure 3** represents the effect of WWA on oxygen diffusion−concentration products in liver PC and yolk PC liposome suspensions when peroxyl radicals are generated in lipid phase using the fat-soluble radical initiator AMVN (**Figure 3**). As shown in **Figure 2A**, WWA at all tested concentrations significantly slowed down the rate of molecular oxygen consumption in the sealed capillary containing the liver PC liposomes at all testing time points, indicating that WWA was able to reduce the level of oxygen diffusion−concentration products in the liver PC liposomes. Furthermore, WWA was able to inhibit oxygen diffusion−concentration product in liver PC liposomes in a dose- and time-dependent matter. A similar inhibitory effect was also observed in the yolk PC liposome

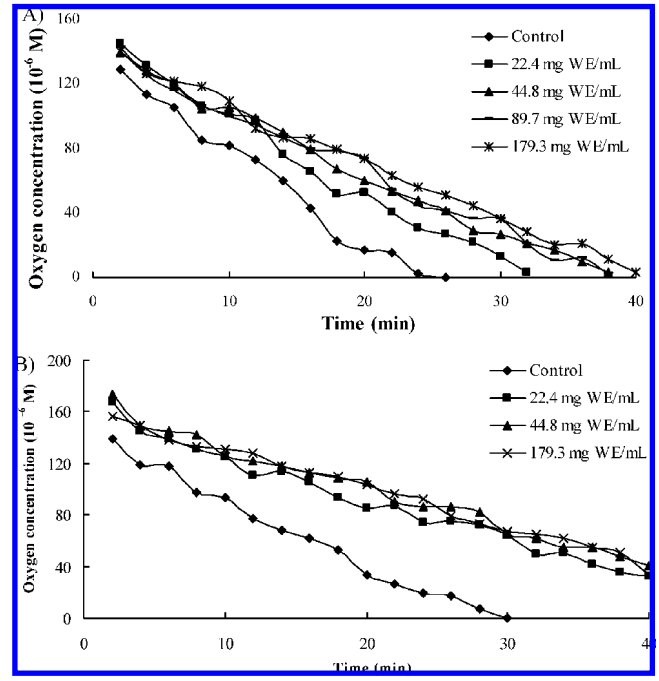

Figure 2. Effect of water soluble wheat antioxidants (WWA) on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC liposomes initiated by AAPH. All the measurements were performed at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL PCs, 20 mM (for egg yolk PC) or 40 mM (for rat liver PC) AAPH, 22.4−179.3 mg of dry wheat grain equivalents/mL wheat antioxidants, and 0.04 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration for sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

system (**Figure 2B**). In contrast to that observed in the liver PC liposomes, three concentrations of WWA, including 22.4, 44.8, and 179.3 mg of wheat grain equivalents per mL, had no difference in their capacity in suppressing oxygen diffusion−concentration products in yolk PC liposomes. These results suggest that water-soluble or hydrophilic wheat antioxidants in aqueous phase may inhibit lipid peroxidation in liposome induced by AAPH-derived peroxyl radicals in the water phase. These data also indicate that liposome composition may influence the effectiveness of WWA in suppressing lipid peroxidation under the experimental conditions.

It is interesting whether WWA may suppress lipid peroxidation in liposomes induced by AWVN, a fat-soluble radical initiator. As shown in **Figure 3**, WWA in the aqueous phase might be able to inhibit the oxygen diffusion−concentration product in both liver PC and yolk PC liposome suspensions induced by a lipophilic radical generator AWVN, which was present in the nonpolar lipid bilayer. These results are the first demonstration that water-soluble wheat antioxidants may prevent lipid peroxidation in liposomes induced by lipophilic radical generator. In the liver PC liposome suspension, WWA at a concentration ranging from 44.8 to 179.3 mg of wheat grain equivalents/mL of suspension solution (mg WE/mL) inhibited lipid peroxidation in the testing system but had no apparent inhibitory activity at the final concentration of 22.4 mg WE/mL (**Figure 3A**). In the yolk PC liposome suspension, WWA exhibited clear dose-dependent inhibition of lipid peroxidation at concentrations of 89.7 and 179.3 mg WE/mL (**Figure 3B**). Interestingly, WWA at 22.4 and 44.8 mg WE/mL concentrations enhanced oxygen consumption in the sealed testing systems (**Figure 3B**), indicating that WWA components may have pro-oxidant activity at lower concentrations under the experimental

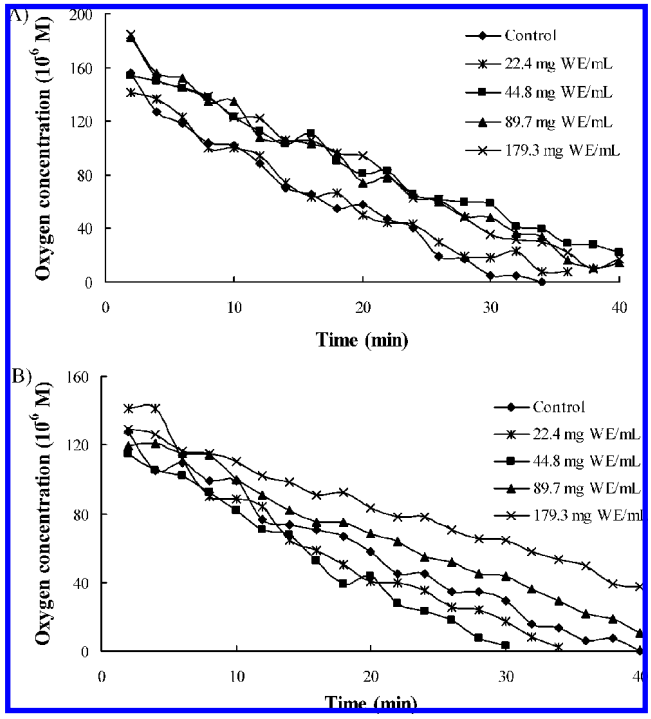

Figure 3. Effect of water-soluble WWA on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC initiated by AMVN. All the measurements were carried out at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL PCs, 12 mM (for egg yolk PC) or 40 mM (for rat liver PC) AMVN, 22.4−179.3 mg of dry wheat grain equivalents/mL antioxidants, and 0.04 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration of sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

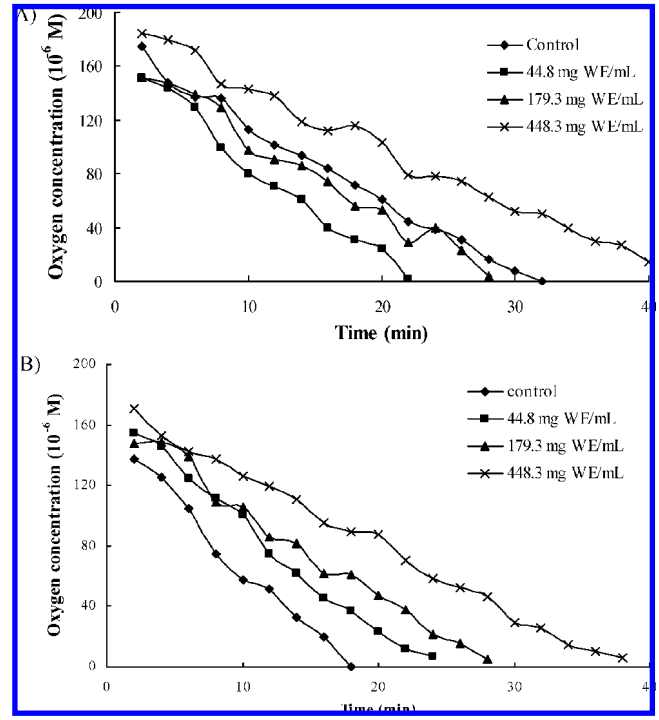

Figure 4. Effect of lipophilic wheat antioxidants (LWA1) on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC initiated by AAPH. All the measurements were carried out at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL PCs, 20 mM (for egg yolk PC) or 40 mM (for rat liver PC) AAPH, 44.8−448.3 mg WE/mL antioxidants, and 0.04 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration of sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

conditions. These data suggested that different liposome systems, such as rat liver PC and egg yolk PC liposomes, may alter the effectiveness of water-soluble wheat antioxidants in reducing the oxygen diffusion−concentration product. This may be explained by the fact that liposomes prepared with different PCs may have different fatty acid compositions. Different fatty acid compositions in the lipid bilayers may result in different membrane fluid properties, which may lead to the different oxygen diffusion rate and different local molecular oxygen concentration (*20*). This may alter oxygen diffusion−concentration product or lipid peroxidation in the testing systems. Two liposome systems with different fatty acid compositions are required to better investigate the effect of wheat antioxidants in suppressing oxygen diffusion−concentration product or lipid peroxidation.

Effect of the First Lipid−Soluble Wheat Antioxidant Preparation (LWA1) on Oxygen Diffusion−Concentration Products in Liposomes. The first lipid-soluble wheat antioxidant fraction (LWA1) was 50% acetone extractable but less polar than those in WWA. LWA1 was incorporated in both rat liver and egg yolk PC liposomes to evaluate its potential inhibitory activity against AAPH-induced lipid peroxidation in liposome suspensions. Under this testing condition, antioxidants were incorporated in the liposome particles, and the free radicals were produced in the aqueous phase. As shown in **Figure 4A**, LWA1 exhibited a pro-oxidant activity at a lower concentration of 44.8 mg WE/mL, had no effect at a medium concentration of 179.3 mg WE/mL, and showed antioxidant activity at a higher concentration of 448.3 mg WE/mL in liver PC liposome systems. Furthermore, LWA1 was able to reduce the oxygen diffusion−concentration product in yolk PC liposomes in a dose- and time-dependent matter under the same experimental conditions (**Figure 4B**).

LWA1 was also evaluated for its effect on oxygen diffusion−concentration product in liposomes induced by lipid-soluble radical generator AMVN. Under this testing condition, antioxidants were incorporated in liposome particles and free radicals were initiated in the lipid phase. LWA1 at concentrations up to 448 mg WE/mL exhibited no effect on oxygen consumption rate or oxygen diffusion−concentration products in liver PC liposomes (**Figure 5A**). Moreover, LWA1 significantly accelerated oxygen consumption rate in yolk PC liposomes under the same experimental conditions (**Figure 5B**), indicating that LWA1 increased the oxygen diffusion−concentration products induced by AMVN under the same experimental condition. It could be concluded from these data that LWA1 may not exhibit antioxidant activity but may act as pro-oxidant in liposomes when lipid-soluble radial generator is present.

Effect of the Second Lipid-Soluble Wheat Antioxidant Preparation (LWA2) on Oxygen Diffusion−Concentration Products in Liposomes. The second lipid-soluble wheat antioxidant fraction (LWA2), which was not extractable with 50% acetone, was also investigated for its possible effect on oxygen diffusion−concentration product in liposomes induced by AAPH. In liver PC liposome system, LWA2 exhibited dose-dependent inhibition of oxygen diffusion−concentration product at all tested time points (**Figure 6A**). LWA2 showed its strongest antioxidant activity at the concentration of 179.3 mg WE/mL and had similar antioxidant activity at concentrations of 44.8 and 448.3 mg WE/mL in yolk PC liposomes (**Figure 6B**). Taking into account the data in **Figure 6A**, and **6B**, LWA2 was able to suppress lipid peroxidation in liposomes induced

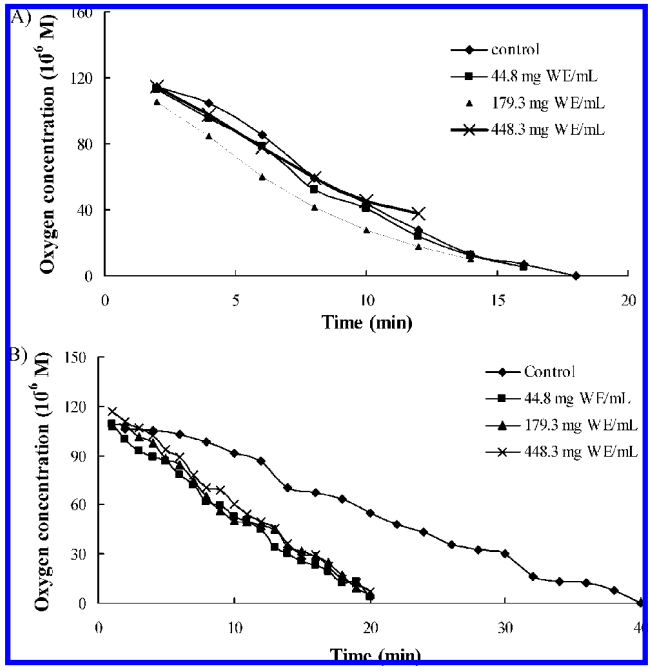

Figure 5. Effect of lipophilic wheat antioxidants (LWA1) on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC initiated by AMVN. All the measurements were carried out at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL PCs, 12 mM (for egg yolk PC) or 8 mM (for rat liver PC) AMVN, 44.8−448.3 mg WE/mL antioxidants, and 0.06 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration of sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

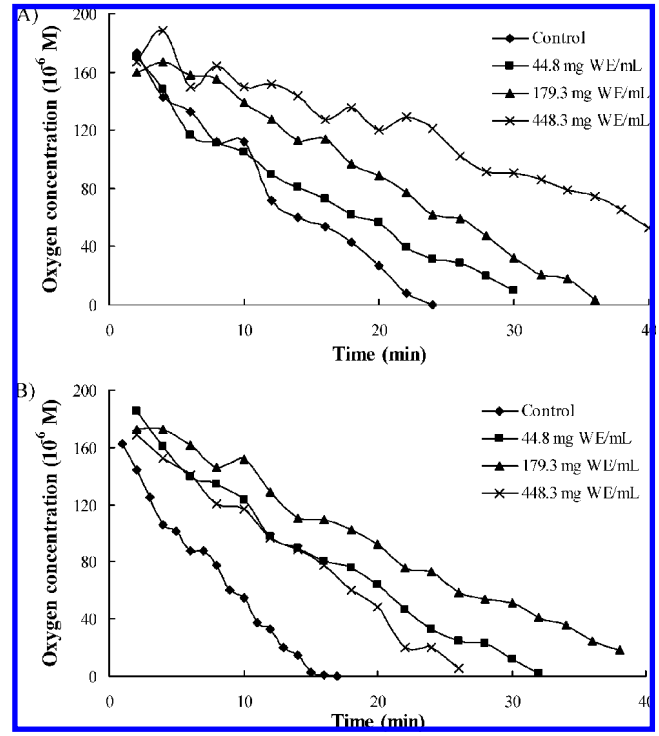

Figure 6. Effect of lipophilic wheat section (LWA2) on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC initiated by AAPH. All the measurements were carried out at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL PCs, 20 mM (for egg yolk PC) or 8 mM (for rat liver PC) AAPH, 44.8−448.3 mg WE/mL antioxidants, and 0.06 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration of sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

by water-soluble radical initiator AAPH, and the liposome systems used in the testing altered the effectiveness of the antioxidants.

In contrast to that observed for LWA1, LWA2 at the concentrations of 179.3 and 448.3 mg WE/mL reduced oxygen consumption in liver PC liposomes when lipophilic radical initiator AMVN was used to induce the oxidative reactions but had no influence on oxygen consumption in the liver PC liposomes at a lower concentration of 44.8 mg WE/mL under the same experimental conditions (**Figure 7A**). The higher LWA2 concentration was associated with a lower oxygen diffusion−concentration product in liver PC liposomes, suggesting the possible dose-dependent manner of its antioxidant activity. Furthermore, LWA2 had no influence on oxygen diffusion−concentration products in yolk PC liposomes at a concentration range of 44.8−448.3 mg WE/mL (**Figure 7B**).

The oxygen concentrations in each sealed capillary containing individual liposome suspensions with different wheat antioxidants and different free radical initiators after 10 min of reaction were compared using normalized arbitrary ESR signal values in **Figure 8**. The comparison was also on a same per grain weight equivalent basis. A greater positive value in **Figure 8** is associated with a stronger antioxidant activity, and a larger negative value represents a stronger pro-oxidant activity. Data in **Figure 8** clearly indicated that WWA, LWA1, and LWA2 differed in their actions in the same liposome oxidation reaction, regardless of PC source and free radical initiator. These data also demonstrated that the liposome system altered the antioxidant and pro-oxidant properties of wheat antioxidants. For instance, LWA1 exhibited antioxidant activity in egg yolk PC liposome with AAPH as the radical generator, whereas it acted as a pro-oxidant in rat liver PC liposomes under the same experimental conditions (**Figure 8**). Also noted was that LWA2 was much more effective than WWA in suppressing lipid peroxidation in egg yolk PC liposomes with AAPH as the radical initiator, but WWA was slightly more effective than LWA2 in rat liver PC liposomes under the same testing conditions. This observation was supported by the observation in an earlier study that the natural PCs including those prepared from rat liver and egg yolk PCs markedly altered lipid peroxidation or local oxidative status in the liposomes prepared from these PCs (*20*). The different fatty acid composition of rat liver PC and egg yolk PC (**Table 1**) might affect the liposome structure and might result in different oxygen diffusion status in the liposomes, which might partially explain the observation (*21*). This speculation was supported by the observation that the antioxidative activity of WWA was less influenced by the liposome systems than that of LWA1 and LWA2, regardless of the radical generator used in the study (**Figure 8**), because LWA 2 and LWA1 are incorporated in the lipid bilayers of liposome particles and their location or distribution may be highly dependent on their polarity and structure, as well as the liposome structure and fatty acid composition. In addition, data in **Figure 8** suggested the possible influence of radical initiators on antioxidant activity estimation using liposomes. It is highly recommended that both lipophilic and hydrophilic radical generators are included in the future studies of lipid oxidation in liposomes. It is also important for these studies to prepare liposomes using phospholipid from at least two sources, such as egg yolk and rat liver.

It is well-accepted that antioxidants at higher level may act as pro-oxidants. In the present study, both liposome composition and the type of radical initiator used to induce the oxidative stress altered the pro-oxidant properties of LWA1 (**Figure 8**).

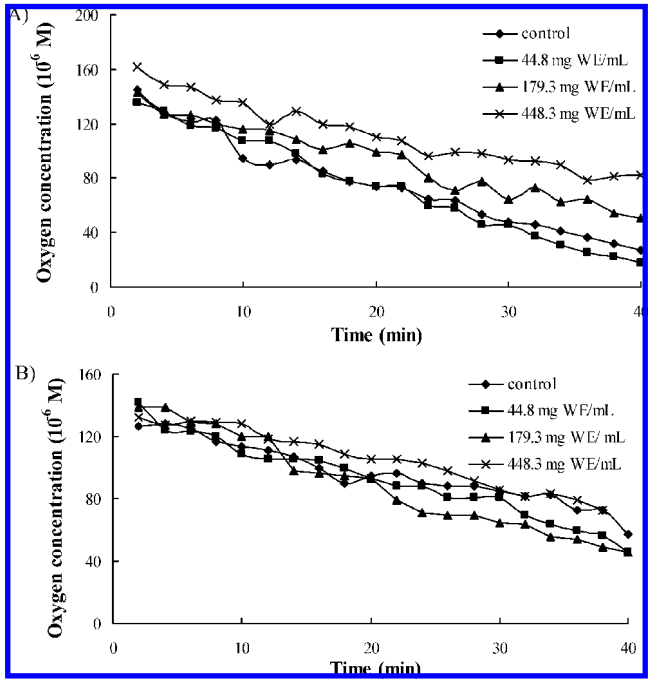

Figure 7. Effect of lipophilic wheat section (LWA2) on oxygen consumption in (**A**) rat liver PC and (**B**) egg yolk PC initiated by AMVN. All the measurements were carried out at 37 °C in HEPES buffer (pH 7.4) containing 30 mg/mL liposomes, 12 mM (for egg yolk PC) or 8 mM (for rat liver PC) AMVN, 44.8−448.3 mg WE/mL antioxidants, and 0.06 mM ^{15}N-PDT. The ESR spectra were recorded 2 min after the temperature equilibration of sealed samples in the cavity. Spectrometer settings: microwave power, 1 mW; modulation amplitude, 0.05 G.

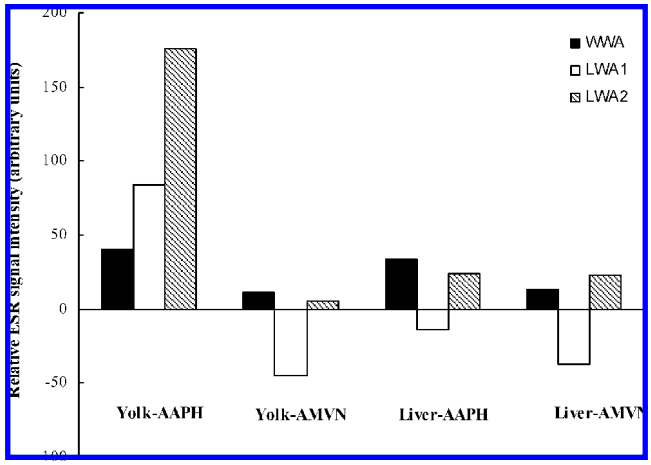

Figure 8. Effect of hydrophilic and lipophilic wheat antioxidants on lipid peroxidation of two natural PCs (egg yolk and rat liver PC). All the PCs were treated with 179.3 mg WE/mL antioxidants. Yolk PC-AAPH and liver PC-AAPH represent the lipid peroxidation reactions in yolk PC and in rat liver PC liposomes with AAPH as the radical initiator, respectively. Yolk PC-AMVN and liver PC-AMVN represent the lipid peroxidation reactions in yolk PC and liver PC liposomes initiated by AMVN. ESR spectra were recorded after 10 min of reaction.

Furthermore, LWA1 had a stronger pro-oxidant activity when the lipid-soluble AMVN was used to generate free radicals and introduce the oxidative stress in liposomes (**Figure 8**). In contrast, no apparent pro-oxidant activity was determined in liposomes containing LWA2. These observations may be explained by the fact that AMVN concentration might be higher at the location where LWA1 was primarily distributed to in the liposomes. In other words, LWA1 concentration at the location of AMVN was relative higher. This might result in a relatively high local LWA1 concentration and lead to higher concentration of secondary free radicals formed during the interaction of AMVN−LWA1 components and accelerated the lipid peroxidation in the liposome systems. Additional study using a group of spin labels which may be inserted at different depth of liposome particles is required to advance our understanding of antioxidant behavior in cellular membranes and their antioxidant benefits.

Scavenging Capacities of the Wheat Antioxidant Preparations against DPPH$^\bullet$ in Solution. In order to further confirm the relative ability of WWA, LWA1, and LWA2 in their reactions with free radicals, these three antioxidant preparations were tested for their capacities to directly react with and quench DPPH$^\bullet$ in solution. In agreement with the observation in liposome systems, WWA scavenged almost all DPPH$^\bullet$ in the solution at an initial concentration of 280 mg WE/mL (**Figure 9A**), whereas LWA1 and LWA2 quenched about 1.5 and 12.7% DPPH$^\bullet$ in the solution under the experimental conditions, on a per wheat grain weight basis. The results of liposome oxidation prevention and DPPH$^\bullet$ scavenging capacity assays suggested that wheat grain contains more water-soluble natural antioxidants on a per grain weight basis. These antioxidant components with different polarity may act differently in individual microenvironments or nanoenvironments such as in liposomes prepared from rat liver and egg yolk phospholipids and in solution. Combining findings from both the present research and our previous study that wheat bran extract rich in antioxidants can suppress human LDL oxidation, wheat antioxidants might delay the progress of atherosclerosis and reduce the risk of CHD (*5*) by inhibiting the lipid peroxidation in cellular membrane and in human LDL. Additional research is required to further investigate the possible synergic effects among different groups of wheat antioxidants with different polarity in their interactions with free radicals in the biological systems.

Phenolic Acid Composition of WWA, LWA1, and LWA2. A reverse-phase HPLC analysis of phenolic acid composition was performed to better understand the phenolic acid composition in WWA, LWA1, and LWA2. As shown in **Table 2**, five phenolic acids, including ferulic, *p*-coumaric, syringic, 4-hydroxybenzoic, and vanillic acids in both free and conjugated forms, were detected in the WWA. The LWA1 had only free ferulic and *p*-coumaric acids, and the LWA2 contained free ferulic, *p*-coumaric, and vanillic acids. No conjugated phenolic acid was presented in LWA1 or LWA2, since they are highly water-soluble and could not be extracted by chloroform.

Data in **Table 2** showed that WWA had much higher level of total phenolic acids (123.5 nmol/g) and total conjugated phenolic acids than LWA1 and LWA2. Also note that the level of free phenolic acids in WWA is comparable to that in LWA2 and was much higher than that in LWA1. The conjugated phenolic acids may orientate at the lipid−water interface of the liposomes, while the free phenolic acids may be inserted in the lipid bilayers of liposomes. This may partially explain the observation that WWA was able to suppress lipid peroxidation in both yolk and liver PC liposomes induced by either water-soluble or lipophilic free radical initiators, as well as that the inhibitory effectiveness of WWA was not altered by either PCs or radical inducers as much as that of LWA1 or LWA2 (**Figure 8**). In addition, the difference in total phenolic acid contents provides an excellent explanation of the DPPH radical scavenging capacities of WWA, LWA1, and LWA2 (**Figure 9**).

LWA2 contained about 3 times more ferulic acid and total phenolic acids than LWA1 (**Table 2**). This concentration difference may explain why LWA2 had much higher antioxidant

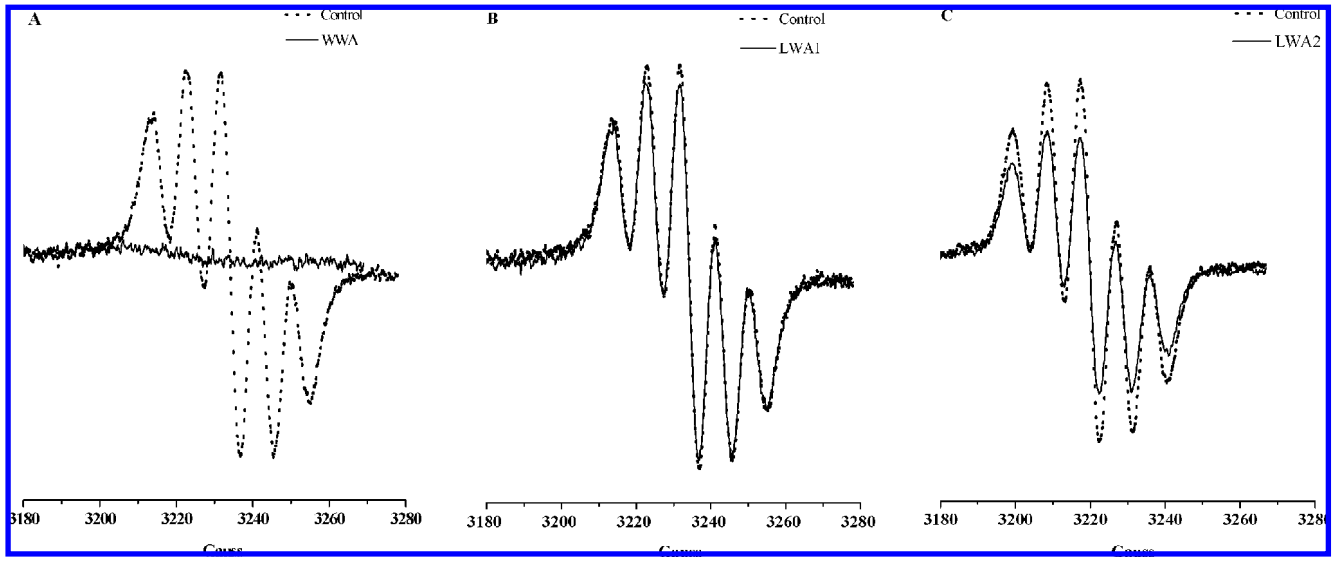

Figure 9. Effects of wheat antioxidants on DPPH• scavenging capacities assayed by ESR: (**A**) hydrophilic wheat antioxidants (WWA), (**B**) first lipophilic wheat antioxidants (LWA1), and (**C**) second lipophilic wheat antioxidants (LWA2). The reactions were carried out at ambient temperature for 5 min before ESR determination. The finial concentration was 0.25 mM DPPH• and 280 mg WE/mL antioxidants in all reaction mixtures. ESR signals were recorded with 15 mW incident microwave and 100 kHz field modulation of 1.6 G at ambient temperature.

Table 2. Phenolic Acid Compositions of Choptank Wheat Grain[a]

	phenolic acid	free (μg/g)	conjugated (μg/g)	total (μg/g)	total (nmol/g)
WWA	ferulic acid	0.73 ± 0.00	12.64 ± 0.01	13.37 ± 0.01	123.54 ± 0.27
	p-coumaric acid	0.10 ± 0.00	0.53 ± 0.00	0.64 ± 0.00	
	syringic acid	nd	2.82 ± 0.02	2.82 ± 0.02	
	4-OH benzoic acid	nd	2.66 ± 0.01	2.66 ± 0.01	
	vanillic acid	0.63 ± 0.00	2.29 ± 0.00	2.92 ± 0.01	
LWA1	ferulic acid	0.32 ± 0.01	nd	0.32 ± 0.01	1.96 ± 0.06
	p-coumaric acid	0.05 ± 0.00	nd	0.05 ± 0.00	
LWA2	ferulic acid	1.16 ± 0.01	nd	1.16 ± 0.01	7.24 ± 0.12
	p-coumaric acid	0.10 ± 0.00	nd	0.10 ± 0.00	
	vanillic acid	0.10 ± 0.02	nd	0.10 ± 0.02	

[a] Data expressed as mean ± standard deviation (SD) ($n = 2$). nd, not detected.

activity in yolk PC liposomes with AAPH as the radical inducer and LWA2 acted as antioxidant but LWA1 acted as pro-oxidants in the other testing systems (**Figure 8**). In summary, the phenolic acid profiles of WWA, LWA1, and LWA2 support the observations of their inhibitory effects on lipid peroxidation in liposomes and their DPPH• scavenging capacities.

Cholesterol-Lowering Effect of Wheat Antioxidants. Growing evidence indicates that whole grain intake is inversely associated with the total and LDL cholesterol levels and the risk of CHD (*22*). Wheat antioxidants and the insoluble fiber are considered as the possible primary contributors for this beneficial effect (*22, 23*). In 2005, wheat antioxidants were shown to suppress lipid peroxidation in human LDL (*5*), which may contribute to the overall cholesterol-lowering property of wheat grain, because oxidized LDL cannot be taken up again by liver LDL receptor and lead to accelerated plasma and LDL cholesterol concentrations. To date, little is known about whether wheat antioxidants may reduce plasma total and LDL cholesterol levels by altering cholesterol biosynthesis and metabolism other than simply acting as antioxidative agents.

In the present study, wheat antioxidants were examined for their possible effects on expression of the key genes involved in cholesterol biosynthesis and metabolism. Primary rat hepatocytes were treated with wheat antioxidants at a final concentration of 0.12 mg/mL, equivalent to 2 mg of wheat grain/mL of medium, for 24 h, and the mRNA levels of HMG-CoA reductase (HMG-CoA-R), cholesterol 7α-hydroxylase (CYP7A1), and LDL receptor (LDLR) were examined using a ribonuclease protection assay (RPA). HMG-CoA-R and CYP7A1 are the rate-limiting enzymes for cholesterol biosynthesis and converting cholesterol to bile acids, respectively (*24*). As shown in **Figure 10**, wheat antioxidants significantly reduced HMG-CoA-R mRNA but increased CYP7A1 mRNA expression, while showing no effect on LDLR mRNA. Further investigation found that wheat antioxidants significantly increased the degradation of HMG-CoA-R mRNA but increased the CYP7A1 mRNA stability in a time-dependent matter (**Figure 11**). Taken together, these data suggest that regulation of the key genes involved in cholesterol biosynthesis and metabolism may be a possible cellular/molecular mechanism by which wheat antioxidants reduce plasma cholesterol and warrants further in vivo investigations. This effect agreed with a previous study that wheat bran supplements can decrease the total cholesterol and LDL cholesterol in human subjects (*25*).

In summary, the present study demonstrates that wheat antioxidants may reduce oxygen diffusion−concentration products and lipid peroxidation in cellular membranes, indicating their potential in prevention of aging-associated chronic diseases such as CHD, which involves ROS or oxidative stress in their pathologic development. This study also showed for the first time that wheat antioxidants may down-regulate HMG-CoA gene expression and up-regulate CYP7A1 expression, as well as their mRNA stability, which may reduce the liver and plasma cholesterol levels. Wheat antioxidants might reduce the risk of

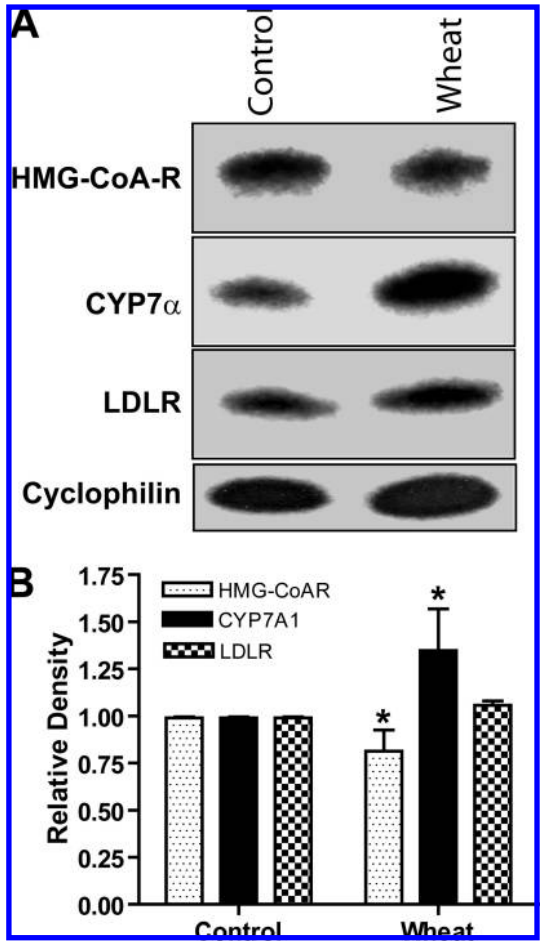

Figure 10. Effects of wheat antioxidants on mRNA levels of genes involved in cholesterol biosynthesis and metabolism in rat primary hepatocytes. (**A**) RPA and (**B**) relative mRNA levels of the genes. Rat primary hepatocytes were treated with wheat antioxidants for 24 h. Total RNA was isolated. The mRNA levels of cholesterol 7α-hydroxylase (CYP7A1), 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMG-CoA-R), and low-density lipoprotein receptor (LDL-R), were determined by RPA analysis, and relative amounts of mRNA were determined by quantifying the densities of the radioactive bands using Image J software and normalized to rat cyclophilin mRNA as loading control.

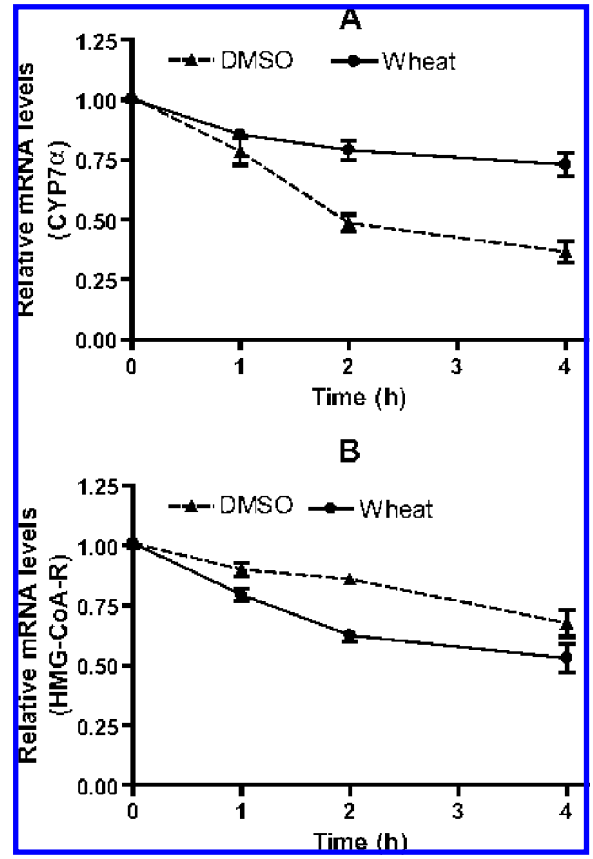

Figure 11. Kinetics of (**A**) CYP7α and (**B**) HMG-CoA reductase (HMG-CoA-R) mRNA expression in the rat primary hepatocytes after wheat antioxidant treatments. The mRNA was determined by RPA analysis. Values were expressed as mRNA levels relative to the levels in sham operation. For detailed preparation, see **Figure 10**.

atherosclerosis through multiple mechanisms, including their antioxidant activities and their capacity to directly alter the expression of key genes involved in cholesterol biosynthesis and metabolism. This study demonstrates the potential of a common food staple in disease prevention and the possible reward of nutraceutical and functional food research and development for human health and life quality.

ABBREVIATIONS USED

^{15}N-PDT, 4-oxo-2,2,6,6-tetramethylpiperidine-d_{16}-1-oxyl; AAPH, 2,2′-azobis(2-aminopropane) dihydrochloride; AMVN, 2,2′-azobis(2,4-dimethylvaleronitrile); CHD, coronary heart disease; CYP7A1, cholesterol 7α-hydroxylase; DPPH$^{\bullet}$, 2,2-diphenyl-1-picrylhydrazyl radical; ESR, electron spin resonance; HMG-CoA-R, HMG-CoA reductase; LDL, low-density lipoprotein; LDLR, LDL receptor; liver PC, rat liver PC; LWA1, the first lipophilic wheat antioxidant; LWA2, the second lipophilic wheat antioxidant; PC, phosphatidycholine; RPA, ribonuclease protection assay; WWA, water-soluble wheat antioxidant; yolk, egg yolk PC.

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