JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Article

pubs.acs.org/JAFC

Differential Effects of Grape (*Vitis vinifera*) Skin Polyphenolics on Human Platelet Aggregation and Low-Density Lipoprotein Oxidation

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ABSTRACT: Antioxidant and antiplatelet properties of grape products are thought to be responsible for observed antiatherosclerotic effects. Diverse classes of phenolics are derived from the seed and skin (GSK) of grapes. The relative contributions of the classes of phenolics to observed properties of grape products are unknown. In this paper, GSK fractions were used to examine effects on platelet aggregation, low-density lipoprotein (LDL) oxidation in vitro, and relative binding of phenolics to LDL. GSK was separated into six fractions (fractions 1−6), and primary phenolics were characterized using high-performance liquid chromatography and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Fractions 4, 5, and 6, enriched in polygalloyl polyflavan-3-ols (PGPFs) with 3−6, 4−8, and 6−15 degrees of polymerization, respectively, inhibited platelet aggregation. Fractions 1−3, containing various amounts of oligosaccharides, hydroxycinnamic acids, anthocyanins, flavanols, and low molecular weight PGPFs, significantly increased platelet aggregation. Fractions 4−6 were most effective in binding LDL and inhibiting LDL oxidation. Fractions 5 and 6 exhibited the greatest inhibition of platelet aggregation and LDL oxidation, suggesting that polymeric PGPFs are responsible for the beneficial effects of grape products. Conversely, phenolics in fractions 1−3 may reduce the net biological potency of the grape products and have undesirable effects on cardiovascular disease risk factors.

KEYWORDS: *antioxidant, antiplatelet, cardiovascular, grape, LDL, oxidation, platelets, polygalloyl polyflavan-3-ols, polyphenolics, Vitis vinifera*

INTRODUCTION

Atherosclerosis begins with the accumulation and retention of low-density lipoprotein (LDL) within the arterial wall.[1,2] The subsequent oxidation of LDL triggers a series of atherogenic mechanisms that result in the formation of the atherosclerotic plaque.[3−5] The oxidation of LDL also stimulates platelet hyperactivity,[6] increasing the involvement of platelets in the disease process.[7] The eventual rupture of the plaque causes further activation and aggregation of the hyperactive platelets. Platelet-mediated thrombi are often responsible for the clinical complications of cardiovascular disease (CVD).[7] Thus, the use of antioxidants[5] and antiplatelet[7] compounds is a common therapeutic approach for reducing the development of CVD and protecting against thrombosis.

The antioxidant and antiplatelet properties of dietary polyphenolic compounds are, in part, responsible for the observed inverse association between dietary polyphenolic intake and the incidence of CVD.[8,9] Polyphenolics, especially those found in grape products such as red wine and grape juice, inhibit platelet aggregation[10−13] and LDL oxidation[14−20] and thus attenuate the development of atherosclerosis.[21−24] The majority of the phenolics in grape products are derived from the seed[25] and skin[26] of grapes. We have previously illustrated that extracts of grape seed and grape skin (GSK) are effective inhibitors of platelet aggregation.[13] The characterization of these extracts revealed that GSK contains a greater diversity of phenolic compounds, including those found in grape seed; grape seed is primarily composed of polygalloyl polyflavan-3-ols (PGPFs).[13]

In this study, we generated phenolic fractions of various compositions from GSK. The effects of the fractions on LDL oxidation and platelet aggregation in vitro were then compared. The ability of the phenolics to bind LDL was also examined. Although water-soluble antioxidants such as ascorbate inhibit LDL oxidation,[27] lipid-soluble antioxidants such as α-tocopherol and probucol that strongly associate with LDL are believed to offer greater protection against LDL oxidation in vivo within the arterial wall, away from water-soluble antioxidants, where LDL is predominantly oxidized.[28] As previous studies examining the effects of red wine on LDL oxidation suggest that some, but not all, grape phenolics bind LDL,[29,30] we attempted to identify the class(es) of grape phenolics that bind LDL and protect it against oxidation.

MATERIALS AND METHODS

GSK was provided by Melaleuca Inc., Idaho Falls, ID. The obtaining of blood from healthy human subjects for use in platelet aggregation and

Special Issue: 2011 Berry Health Benefits Symposium

Received: August 30, 2011
Revised: December 16, 2011
Accepted: December 26, 2011
Published: December 26, 2011

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LDL oxidation studies in vitro was approved by and in compliance with the requirements of the Institutional Review Board at the University of Wisconsin—Madison.

Fractionation of Grape Skin Phenolics. Sephadex LH-20 (Thermo Fisher Scientific Inc., Waltham, MA) was equilibrated in water for 2 h. A Kontes glass preparative column (2.5 cm i.d. × 10 cm long) was filled with LH-20 slurry to a height of 10 cm. GSK (0.5 g in 0.01 L of H_2O) was applied to the column and eluted sequentially with water (0.1 L; fraction 1), 50% water/ethanol (v/v; 0.1 L; fraction 2), ethanol (0.1 L; fraction 3), 50% ethanol/methanol (v/v; 0.1 L; fraction 4), methanol (0.1 L; fraction 5), and 80% aqueous acetone (v/v; 0.3 L; fraction 6). Each fraction was dried by vacuum evaporation at 30 °C. Subsamples of the fractions were solubilized in water (0.5 mL) prior to HPLC and matrix-assisted laser desorption/ionization time-of-flight MS (MALDI-TOF MS). The total phenolic contents of the unfractionated GSK and the six fractions were determined according to the modified Folin–Ciocalteu method[31] (reported as gallic acid equivalents (GAE)); the total phenolic concentrations of GSK and the fractions were then adjusted to 11.8 mmol GAE/L, and the solutions were stored in aliquots at −70 °C until use.

Analysis of Phenolic Fractions by HPLC. Each of the fractions (50 μL) was injected onto a C-18 column (60 A, 8 μm, 25 cm × 0.45 cm; Ranin Dynamax, Columbus, OH). The solvents for elution were 0.1% trifluoroacetic acid in water (solvent A) and methanol (solvent B). A linear gradient starting with 100% solvent A and finishing with 100% solvent B was run over 40 min. The HPLC system consisted of a Waters automated gradient controller, two Waters 501 HPLC pumps, and a Rheodyne 7125 manual injector. The flow rate was maintained at 2 mL/min, and the elution was monitored by a Waters 996 diode array detector using Waters Millennium software for collecting and analyzing three-dimensional chromatograms.

Analysis of Phenolic Fractions by MALDI-TOF MS. Mass spectra were collected on a Bruker Reflex II- MALDI-TOF mass spectrometer (Billerica, MA) equipped with delayed extraction and a N_2 laser (337 nm). In the positive mode, an accelerating voltage of 25.0 kV and a reflectron voltage of 26.5 kV were used. Spectra were the sum of 300 shots. Spectra were calibrated with bradykinin (1060.6 MW) and glucagon (3483.8 MW) as external standards.

In accordance with previously published results,[32] *trans*-3-indoleacrylic acid (*t*-IAA; 50 g/L 80% aqueous acetone) was used as a matrix. Analytes were mixed with the matrix solution (1:2) and applied directly (0.2 μL) to a stainless steel target and dried at room temperature. Bradykinin, glucagon (Sigma Chemical Co., St. Louis, MO), and *t*-IAA (Aldrich Chemical Co., Milwaukee, WI) were used as received.

Platelet Aggregation Studies. Blood samples from eight healthy human subjects (20 mL each) were drawn into syringes containing 3.8% sodium citrate (1 sodium citrate:9 blood) using 19G butterfly needles. The blood samples were then diluted with equal volumes of preservative-free saline. Collagen (2 mg/L; Chrono-log Corp., Havertown, PA)-induced platelet aggregation was determined in 1 mL aliquots of blood samples incubated (for 5 min) with preservative-free saline (solvent for gallic acid, GSK or the fractions), gallic acid, GSK, or each of the fractions. Equal amounts of total phenolics of gallic acid, GSK, and the fractions were used in each experiment such that the final cuvette concentrations were 0.341 mmol GAE/L. This concentration, selected from series of preliminary experiments, ensured the comparison of the relative effect of the fractions on platelet aggregation. The whole blood platelet aggregometry was performed using a Chrono-log 4-Channel Whole Blood Impedance Aggregometer (model 590). A detailed methodology of our platelet aggregation studies has been previously published.[33]

Isolation and Purification of LDL from Plasma. Blood samples from the eight healthy human subjects were drawn into vacutainer tubes containing 15% (w/v) K_3EDTA (BD, Franklin Lake, NJ). The tubes were centrifuged at 2000*g* for 20 min at 16 °C to obtain plasma. Sucrose was added to the plasma (0.06% w/w) to stabilize the lipoproteins during plasma storage;[34] aliquots of plasma were stored at −70 °C until use. LDL was isolated from the stored plasma from each human subject as needed using a modification of the density gradient centrifugation method previously described by Graham et al.[35] Briefly, an Optiprep density gradient medium (Sigma Chemical Co.) was used to generate the required density gradients. Six milliliters of a 9% (w/v) Optiprep solution was layered under 1.5 mL of HEPES–saline buffer (10 mmol/L HEPES–NaOH, 0.85% (w/v) NaCl, pH 7.4) in a 13.5 mL ultracentrifugation tube (Sorvall Polyallomer Ultracrimp tubes, Sorvall, Newtown, CT). Then, 6 mL of a 12% (w/v) solution containing Optiprep and the human plasma was layered under the 9% (w/v) Optiprep solution. The tube was then centrifuged (Sorvall Discovery 90SE, Thermo Electron Corp., Asheville, NC) in a vertical rotor (Sorvall StepSaver 65 V13, Thermo Electron Corp.) at 362000*g* for 2 h at 16 °C (the slowest acceleration and deceleration settings were used during centrifugation). The LDL was carefully extracted using a needle pushed through the side of the centrifugation tube directly below the LDL band. The isolated LDL was washed repeatedly (three to four times over an hour) with PBS (0.22 mmol/L KH_2PO_4, 0.76 mmol/L Na_2HPO_4, 37.5 μmol/L NaCl, pH 7.4) using a 100 000 MW cutoff centrifugation filter (Amicon Ultra-4, Millipore, Billerica, MA). The filters retain LDL particles while allowing smaller impurities and Optiprep to pass through. The protein concentration of the washed LDL was adjusted to 0.5 g/L with PBS. The purity of the washed isolated LDL was confirmed using Invitrogen NuPAGE nondenaturing 3–8% Tris–acetate gels and Western blotting using goat anti-apo B-48/100 as the primary antibody (Biodesign International, Saco, ME) in an Invitrogen WesternBreeze Chromogenic blotting system (Invitrogen, Carlsbad, CA).

LDL Oxidation Studies. In these studies, we refer to "pre-incubated LDL" and "co-incubated LDL". "Pre-incubated LDL" is LDL that is incubated with the test substance or phenolic fraction and then repeatedly washed with PBS to remove unbound test substance or phenolic fraction before initiation of the oxidation of the LDL, whereas "co-incubated LDL" is LDL incubated with the test substance or phenolic fraction in the buffer media in which oxidation is initiated.

Aliquots of the isolated LDL were pre-incubated with PBS (control), gallic acid, or fractions 1–6 (99.9 μmol GAE/g LDL or 5 μM GAE) for 15 min. The pre-incubated LDL samples were then washed repeatedly with PBS using a 100 000 MW cutoff centrifugation filter (Amicon Ultra-4, Millipore). The filter, which retains LDL, allows unbound/unassociated phenolics to pass through. We confirmed that even large polymeric grape polyphenolics found in grape seeds were able to pass through the filter; there was <1% loss of polyphenolics to the filter membrane. The protein concentrations of the washed LDL samples were again adjusted to 0.5 g/L with PBS.

The in vitro susceptibility of pre-incubated LDL to oxidation was then compared to that of LDL co-incubated with PBS (control), gallic acid, or fractions 1–6 (99.9 μmol GAE/g LDL or 5 μM GAE in the oxidation media). This concentration, selected from series of preliminary experiments, ensured the comparison of the relative effect of the fractions on LDL oxidation. The oxidation of LDL (50 mg/L) was continuously measured by monitoring conjugated diene formation (absorption at 234 nm) induced by the addition of Cu^{2+} (5 μmol/L) using a modified microplate version of a previously described method.[36,37] The length of the lag phase (lagtime) before the oxidation of LDL was taken to inversely reflect the susceptibility of the LDL to oxidation; lagtime measurements were made using a Microsoft Excel-(Microsoft Corp., Redmond, WA)-based macro software developed by our laboratory. The retention (%) of the antioxidant effect after washing of the LDL was calculated as (% change in lagtime (pre-incubation) × 100)/(% change in lagtime (co-incubation)).

Statistical Analysis. The data are reported as the mean ± SEM. A statistical software package, SPSS (version 11; SPSS Inc., Chicago, IL), was used to perform statistical analysis. The data were tested for normality and homogeneity of variance. A paired or unpaired Student's *t* test was used accordingly to assess statistical significance. Statistical significance was accepted at $p < 0.05$.

RESULTS

Phenolic Composition of Grape Skin Fractions. The LH-20 water fraction (fraction 1) contained oligosaccharides

Table 1. Phenolic Composition of Fractions (1−6) Obtained by Sequential Elution of Grape Skin Extract (GSK) from Sephadex LH-20[a]

fraction	elution solvent	relative abundance of phenolics in fraction (%)	class of phenolic compound	UV absorbance max (nm)
1	H_2O	2.0	7 hydroxycinnamic acids	between 280 and 320
			oligosaccharides	
2	ethanol/H_2O (1:1)	35.1	6 hydroxycinnamic acids	between 280 and 330
			12 anthocyanins	between 530 and 550
			5 flavonols	between 350 and 372
			monomeric flavan-3-ols	280
			PGPFs (2−3 DP)	280
3	ethanol	17.6	3 hydroxycinnamic acids	between 280 and 330
			3 flavonols	between 350 and 372
			monomeric flavan-3-ols	280
			PGPFs (2−4 DP)	280
4	ethanol/methanol (1:1)	20.1	PGPFs (3−6 DP)	280
5	methanol	20.1	PGPFs (4−8 DP)	280
6	acetone/H_2O (4:1)	5.2	PGPFs (6−15 DP)	280

[a]The classes of phenolic compounds were determined by HPLC, whereas the characterization (degrees of polymerization, DP) of the polygalloyl polyflavan-3-ol (PGPFs) was performed using matrix-assisted laser desorption/ionization time-of-flight mass spectrometry. Relative abundances of phenolics in the fractions were determined by Folin−Ciocalteu method.

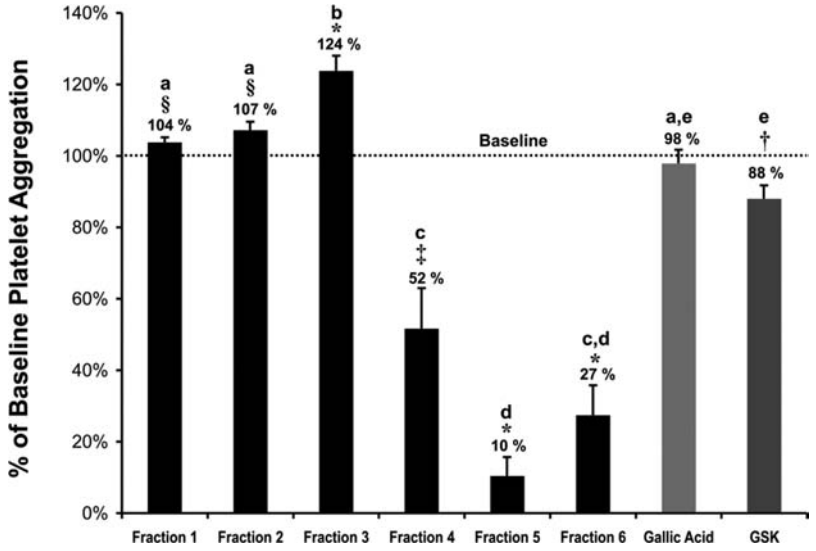

Figure 1. Effect of phenolic fractions (fractions 1−6), gallic acid, and unfractionated grape skin (GSK) co-incubated with whole blood (n = 8) on collagen-induced platelet aggregation. Platelet aggregation after co-incubation with grape skin fractions (fractions 1−6), gallic acid, or GSK is expressed as a percentage (mean ± SEM) of baseline platelet aggregation (co-incubation with preservative-free saline). An equal amount of total phenolics of each preparation was used (0.341 mmol GAE/L). Symbols §, †, ‡, and ∗ indicate significant ($p \leq 0.01$, $p \leq 0.05$, $p \leq 0.005$, and $p \leq 0.001$, respectively) change in platelet aggregation compared to baseline platelet aggregation. Means with different lower case letters (a−e) are significantly different from each other. Fractions 1−3 significantly increased platelet aggregation, whereas fractions 4−6 significantly inhibited platelet aggregation. Whereas GSK produced a slight, but significant, inhibition of platelet aggregation, gallic acid had no effect.

and 7 peaks that were characteristic of hydroxycinnamic acids (hydroxycinnamic acids and their esters to tartaric and quinic acid have characteristic maxima between 280 and 330 nm). The LH-20 water/ethanol fraction (fraction 2) contained 6 peaks characteristic of hydroxycinnamic acids, 12 peaks characteristic of anthocyanins (absorbance maxima between 530 and 550 nm), 5 peaks characteristic of flavonols (absorbance maxima between 350 and 372 nm), monomeric flavan-3-ols (catechin or epicatechin units, esterified to gallic acid having a UV maxima at 280 nm), and a series of low molecular weight (2−3 degrees of polymerization, DP) PGPFs (oligomers of repeating catechin or epicatechin units, esterified to gallic acid and having a UV maxima at 280 nm). The LH-20 ethanol fraction (fraction 3) contained 2 hydroxycinnamic acids, 2 flavanols, monomeric flavan-3-ols, and a series of PGPFs (2−4 DP). The LH-20 ethanol/methanol (fraction 4), methanol (fraction 5), and 80% aqueous acetone (fraction 6) fractions contained a series of PGPFs with 3−6, 4−8, and 6−15 DP, respectively. Data are summarized in Table 1. The detailed characterization of PGPFs found in GSK has been previously published.[13,26]

Effect of Phenolic Fractions on Platelet Aggregation in Vitro. Most unexpectedly, fractions 1, 2, and 3 significantly increased collagen-induced platelet aggregation by 3.8 ± 1.4% ($p < 0.05$), 7.2 ± 2.4% ($p < 0.05$), and 23.8 ± 4.2% ($p < 0.001$),

dx.doi.org/10.1021/jf203487g | J. Agric.Food Chem. 2012, 60, 5787−5794

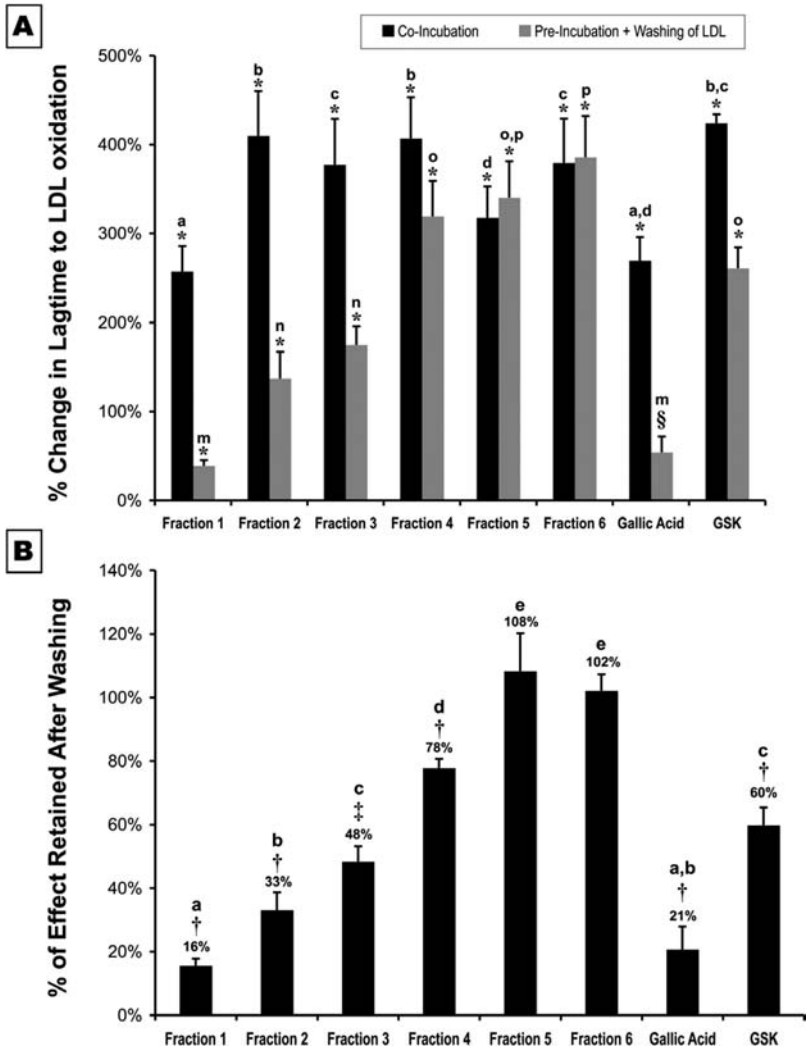

Figure 2. Effect of phenolic fractions (fractions 1−6), gallic acid, and unfractionated grape skin (GSK) on Cu^{2+}-induced LDL oxidation (n = 8). (A) Effect of co-incubation and pre-incubation (followed by washing of the LDL to remove unbound polyphenolics) of LDL with grape skin fractions (fractions 1−6), gallic acid, or GSK on the susceptibility of LDL to Cu^{2+}-induced oxidation. All incubations were conducted such that a total phenolic concentration of 99.9 μmol GAE/g LDL was achieved. The lagtime to LDL oxidation was taken to inversely represent the susceptibility of LDL to oxidation. Thus, the extent to which the substances tested increased lagtime reflected their ability to protect LDL against oxidation. The data are presented as percentage change in lagtime (mean ± SEM). Symbols * and § indicate significant ($p \leq 0.001$ and $p \leq 0.05$, respectively) change in lagtime compared to lagtime to oxidation of control LDL (LDL co-incubated or pre-incubated with PBS). Means from the co-incubation or pre-incubation studies with different lower case letters (a−d) or (m−o), respectively, are significantly different from each other. (B) Percentage (mean ± SEM) of antioxidant effect of grape skin fractions (fractions 1−6), gallic acid, or GSK retained after the LDL was washed to remove unbound polyphenolics. Symbols † and ‡ indicate significant ($p \leq 0.001$ and $p \leq 0.05$, respectively) loss of antioxidant effect after the LDL was washed. Means with different lower case letters (a−e) are significantly different from each other.

respectively (Figure 1). In contrast, fractions 4, 5, and 6 significantly inhibited platelet aggregation by 48.4 ± 11.4% (p < 0.005), 89.6 ± 5.3% (p < 0.001), and 72.5 ± 8.3% (p < 0.001), respectively. The unfractionated GSK produced only a slight inhibition of platelet aggregation (12.0 ± 3.8% (p < 0.01)), whereas gallic acid had no significant effect (Figure 1).

Effect of Phenolic Fractions on LDL Oxidation in Vitro. When co-incubated with LDL, fractions 1−6 significantly increased the lagtime to LDL oxidation by 257.4 ± 28.5% (p < 0.001), 410.0 ± 50.3% (p < 0.001), 377.3 ± 51.7% (p < 0.001), 406.9 ± 46.1% (p < 0.001), 318.0 ± 35.2% (p < 0.001), and 379.6 ± 49.6% (p < 0.001), respectively (Figure 2A). However, the greatest inhibition of LDL oxidation (423.9 ± 10.1% (p < 0.001) was observed with the co-incubation of the unfractionated GSK with LDL (Figure 2A). In contrast, gallic acid produced only a 269.3 ± 26.6% (p < 0.001) increase in lagtime (Figure 2A).

When pre-incubated with LDL, fractions 1−6 also significantly increased the lagtime to LDL oxidation by 38.9 ± 6.2% (p < 0.001), 136.9 ± 30.1% (p < 0.001), 174.6 ± 21.4% (p < 0.001), 319.6 ± 39.3% (p < 0.001), 340.4 ± 40.9% (p < 0.001), and 385.9 ± 46.4% (p < 0.001), respectively (Figure 2A). GSK and gallic acid produced 261.0 ± 23.4% (p < 0.001) and 54.0 ± 17.9% (p < 0.05) increases in lagtimes, respectively (Figure 2A).

The comparison of the co-incubation and pre-incubation data revealed that washing of LDL to remove unbound phenolics after pre-incubation with fractions 5 and 6 did not produce any significant loss in the antioxidant property of these two fractions (i.e., they retained approximately 100% of their

activity). This suggests that the phenolics in these two fractions were completely bound to the LDL (Figure 2B). In contrast, fractions 1−4 showed significant loss of activity ($p < 0.001$ for fractions 1, 2, and 4; $p < 0.005$ for fraction 3) after the washing of LDL and retained only 15.6 ± 2.2, 33.1 ± 5.6, 48.3 ± 4.8, and 77.8 ± 2.9%, respectively (Figure 2B). GSK and gallic acid also showed significant losses of activity ($p < 0.001$) after washing of LDL and retained 59.8 ± 5.6 and 20.7 ± 7.1%, respectively (Figure 2B).

DISCUSSION

The major finding of this study is that polyphenolic fractions of GSK enriched in PGPFs with >4 DP (fractions 5 and 6) are most effective as inhibitors of platelet aggregation and LDL oxidation in vitro. An increasing association of the polyphenolics with LDL was observed with fractions enriched in PGPFs with increasing degrees of polymerization. Although fractions 1−3 were moderate inhibitors of LDL oxidation, they significantly increased platelet aggregation; the greatest increase was observed with fraction 3 containing hydroxycinnamic acids, flavonols, monomeric flavan-3-ols, and low molecular weight PGPFs.

Effect of Phenolic Fractions on Platelet Aggregation. We have previously illustrated the platelet inhibitory properties of grape products in vitro,[12,13,38] in vivo,[11,39] and ex vivo.[10,12,24,39] We have also demonstrated that grape seed extract, composed of only PGPFs, is a more potent inhibitor of platelet aggregation than GSK in vitro.[13] In the current study, the observation that GSK phenolic fractions composed of only PGPFs (fractions 4−6) were effective at inhibiting platelet aggregation suggests that PGPFs may be primarily responsible for the antiplatelet effects of grape products. Although fractions 4−6 (only PGPFs) contribute nearly 50% of the polyphenolics in GSK,[13] the unfractionated GSK inhibited platelet aggregation by only 12% (Figure 1). We believe that the presence of pro-aggregatory phenolics in fractions 1−3 (Figure 1) significantly lowers the net antiplatelet property of the unfractionated GSK.

A recent study reported that a phenolic fraction from Concord grape juice containing anthocyanins, cinnamic acids, and monomeric flavan-3-ols increased the aggregation of washed resuspended platelets and decreased the production of nitric oxide by the platelets.[12] Interestingly, all three of our fractions that increased platelet aggregation also contained hydroxycinnamic acids. Whereas anthocyanins were detected only in fraction 2, fractions 2 and 3 contained monomeric flavan-3-ols. Although we did not detect monomeric flavan-3-ols in fraction 1, trace amounts of these may have been present. Monomeric flavan-3-ols with a galloyl group in the 3′-position, catechin gallate, epicatechin gallate, and epigallocatechin gallate (EGCG), were observed by Lill et al. to induce platelet aggregation via a tyrosine-phosphorylation-dependent pathway without the need for a platelet agonist.[40] However, it should be pointed out that several other studies[41−43] with EGCG seem to contradict these findings. As we did not identify the specific monomeric flavan-3-ols present, we cannot confirm whether the fractions contained the above-mentioned catechin forms. Furthermore, our analysis of the phenolics in the fractions was limited to the classes of phenolics that were primarily observed. Thus, we did not detect all of the classes of grape phenolics that are usually present.[25,26,44] Future studies with subfractionation and detailed characterization of fractions 1−3 are required to identify the pro-aggregatory grape phenolics.

Effect of Phenolic Fractions on the Susceptibility of LDL to Oxidation. Polyphenolics reduce the formation of free radicals by affecting multiple pathways.[45−51] Whereas some polyphenolics act as scavengers of superoxide anions and hydroxyl radicals,[46,52] inhibiting the initiation of free radical-mediated chain reactions, others donate hydrogen atoms to peroxyl radicals, forming polyphenolic radicals,[46,52] which in turn react with other free radicals to terminate chain reactions.[49,52] In addition, certain polyphenolics chelate transition metal ions, such as iron and copper, preventing free radical generation by Fenton-type reactions.[53] Thus, polyphenolics have shown great potential for inhibiting free radical-mediated oxidation of LDL.

When equal amounts of total phenolics were co-incubated with the LDL, all six fractions significantly protected LDL against Cu^{2+}-induced oxidation (Figure 2A). As the amounts of total phenolics were determined by the Folin−Ciocalteu method,[31] a method that inherently measures reduction capacity, it could be said that the concentrations of the fractions were adjusted to have equal reduction capacities. Despite this, there were statistically significant differences between the effects of the fractions, suggesting that variations in the structures of the phenolics in the fractions influence the effects on LDL oxidation independently of the reduction capacities.

Generally, in vitro studies examining the effect of polyphenolics on LDL oxidation do so by co-incubating polyphenolics with LDL during the oxidation reactions. However, polyphenolic compounds or sources that are effective in inhibiting oxidation under such experimental conditions may not necessarily be effective in reducing LDL oxidation in vivo and attenuating atherosclerosis. LDL oxidation in vivo predominantly occurs within the vessel wall in regions of increased oxidative stress, away from water-soluble antioxidants in circulation. Therefore, antioxidants that bind LDL are believed to offer better protection against oxidation in vivo than those that do not, as they can enter the vessel wall with the LDL.[28]

When the fractions were pre-incubated with LDL and then washed to remove unassociated polyphenolics, the degrees to which they inhibited LDL oxidation varied dramatically (Figure 2A). The fractions that were enriched in PGPFs with greater degrees of polymerization were observed to protect the LDL to a greater extent. When fractions 5 and 6, enriched in PGPFs with 4−15 DP, were pre-incubated with LDL, they completely retained their antioxidant protection. This suggests that PGPFs with >4 DP strongly bind LDL.

The ability of polyphenolics to bind proteins, lipids, and lipoproteins has been previously illustrated in vitro and in vivo.[54−61] The consumption of red wine polyphenolics results in an increase in total polyphenolics associated with LDL.[62,63] However, Ivanov et al. observed that only a portion of the phenolics in wine bind LDL.[12] In a study in which LDL was pre-incubated with red wine, the authors observed that certain polyphenolics (quercetin and catechin) formed ether (glycosidic) bonds with the LDL particle, and acid hydrolysis of the LDL was required to release the polyphenolics from the lipoprotein.[64] Porter et al., examining the effect of cranberry polyphenolics on LDL oxidation, observed, as we did, that fractions containing polyflavan-3-ols strongly associated with LDL;[65] fractions containing polyflavan-3-ols with greater degrees of polymerization had a greater affinity for LDL. This supports the observation that sources primarily composed of

PGPFs attenuated atherosclerosis.[21,66] In fact, the effect of PGPFs on atheroma development in one of the studies was similar to that of probucol, a lipid-soluble antioxidant that strongly associates with LDL.[21] In contrast, fraction 1, primarily composed of hydroxycinnamic acids, showed the least affinity for LDL; its affinity was similar to that of gallic acid. The low affinity of hydroxycinnamic acids for LDL may explain why the consumption of hydroxycinnamic acids has failed to attenuate atherosclerosis, despite increasing plasma antioxidant activity.[67]

Limitations of the Study: Bioavailability and Detection of PGPFs. The concentrations of GSK fractions used to produce significant effects on platelet aggregation in our studies were above published physiological concentrations of phenolics.[68] Although inhibition of LDL oxidation was achieved at lower concentrations, when particularly considering fractions primarily consisting of PGPFs, the concentrations utilized were well above those reported as physiological.[68] Whereas we observed fractions primarily composed of PGPFs to be most effective as inhibitors of platelet aggregation and LDL oxidation in vitro in an experimental design that allows for direct interaction of these compounds with platelets and LDL, respectively, it raises questions as to whether indirect mechanisms are involved when these compounds are orally consumed.

Although PGPFs and their dietary sources show a range of biological effects in human interventional studies[68] and in animal models,[21,69−75] their bioavailability is a highly controversial topic.[68,76,77] Several studies suggest that polymerization greatly reduces intestinal absorption of PGPFs.[78−80] Whereas studies utilizing Caco-2 cells have observed the transport of PGPFs with 2−3 DP across the cell monolayer, PGPFs of greater degrees of polymerization adhered to the epithelial surface, reducing their transport across the monolayer.[78] Thus far, PGPFs up to only 2 DP have been detected in human plasma.[81,82] Furthermore, the kinetics of the metabolism of PGPFs that are absorbed are also not known. In our binding studies, we incubated phenolic fractions with LDL for 15 min, which may not be sufficient time for the compounds to reach equilibrium for binding. With the lack of knowledge of the kinetics of PGPF metabolism in circulation, it is challenging to discern whether equilibrium of binding is reached in vivo.

Studies to examine bioavailability of PGPFs have been hindered by the lack of methods for the detection and accurate quantification of PGPFs in biological tissue and fluids. Furthermore, the highly interactive nature of PGPFs presents challenges to detection methods. For example, Hayek et al. were unable to detect red wine polyphenolics bound to LDL until the lipoprotein was treated with acid to hydrolytically dissociate the polyphenolics.[64]

Fractionation of GSK revealed distinct variations in the effects of grape phenolics on platelet aggregation and LDL oxidation. Certain fractions, which account for about 50% of the total phenolics in GSK, were disturbingly found to increase platelet aggregation and perhaps reduce the net platelet inhibitory effect of the unfractionated GSK. In contrast, fractions composed of PGPFs, especially those with >3 DP, were very effective inhibitors of platelet aggregation and LDL oxidation. Furthermore, the polyphenolics in these fractions strongly bound LDL and thus may be able to better protect LDL against oxidation in vivo and attenuate the development of atherosclerosis. Future studies in animals examining the effects of oral consumption of the GSK fractions on platelet aggregation and LDL oxidation are needed to confirm whether the in vitro effects can be observed in vivo.

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Funding

The studies were supported by the Coronary Thrombosis Research and Prevention Laboratory Multidonor Fund at the University of Wisconsin—Madison and funding from Melaleuca Inc.

ACKNOWLEDGMENTS

Technical assistance was provided by Areyl D. Goff, Derrick L. Woodward, Bryan J. Henslin, David J. Mun, and Erich L. Brauer.

ABBREVIATIONS USED

CVD, cardiovascular disease; DP, degree of polymerization; EGCG, epigallocatechin gallate; GAE, gallic acid equivalents; GSK, grape skin/grape skin extract; LDL, low-density lipoprotein; MALDI-TOF MS, matrix-assisted laser desorption/ionization time-of-flight mass spectrometry; PGPF, polygalloyl polyflavan-3-ol.

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