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Red Blood Cells and Lipoproteins: Important Reservoirs and Transporters of Polyphenols and Their Metabolites

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ABSTRACT: Dietary polyphenols are protective for chronic diseases. Their blood transport has not been well investigated. This work examines multiple classes of polyphenols and their interactions with albumin, lipoproteins, and red blood cell (RBC) compartments using four models and determines the % polyphenol in each compartment studied. The RBC alone model showed a dose−response polyphenol association with RBCs. A blood model with flavanones determined the % polyphenol that was inside RBCs and bound to the surface using a new albumin washing procedure. It was shown that RBCs can methylate flavanones. The whole blood model separated the polyphenol into four compartments with the aid of affinity chromatography. More polyphenols were found with albumin and lipoproteins (high-density lipoproteins and low-density lipoproteins) than with RBCs. In the plasma model, the polyphenols associated almost equally between lipoproteins and albumin. RBCs and lipoproteins are shown to be important reservoirs and transporters of polyphenols in blood.

KEYWORDS: *polyphenols, metabolites, lipoproteins, albumin, red blood cells, plasma*

INTRODUCTION

Dietary polyphenols have been well documented in epidemiology studies since 1993[1] to the present day[2] to reduce the risk of chronic diseases such as cardiovascular disease. Polyphenols are extensively metabolized in both the GI tract (microbial catabolites) and the systemic circulation so that recently the emphasis has been on the investigation of these metabolites. GI metabolites are primarily phenolic acids and these systemic metabolites are methylated, sulfated, and glucuronidated polyphenols following phase II conjugation primarily in the liver. The concentration of the polyphenol aglyones and their metabolites ranges from nM to 10 μM in human plasma. The hypothesis that will be investigated in this article is that blood polyphenols are transported in both lipoproteins and RBCs.

One of the big questions in polyphenol research is how they and their metabolites are transported to cells and tissues from the systemic circulation. Over 40 polyphenols of multiple classes have been shown in this Journal to be bound by in vitro experiments to plasma proteins including albumin and lipoproteins.[3] The binding constants were calculated, and it was found that albumin bound significantly more strongly than LDL. Recently, there has been evidence for the presence of polyphenols and their metabolites in platelets after coffee consumption and their concentration correlated with ex vivo inhibition of platelet aggregation.[4] Consumption of quercetin-3-glucoside (found in onions) was also shown to significantly inhibit platelet aggregation, which is indirect evidence for occurrence in platelets.[5] There are multiple pieces of evidence of polyphenol interaction with red blood cells (RBCs).[6−8] Fiorani et al. investigated a single polyphenol and found that quercetin could quickly enter human RBCs using a nonspecific and nonseparation UV quantitation[9] but had no data on what the intracellular RBC polyphenol concentration was in blood and whether any polyphenols are bound to the surface membrane.

This work focuses on polyphenol and RBCs as well as polyphenols/metabolites present in plasma. The polyphenols investigated in this article are shown in Figure 1. Traditionally, plasma proteins have been viewed as the carriers of drugs and endogenous compounds, in equilibrium with plasma water, while the RBCs have been regarded as a transport system for blood gases, which bind to hemoglobin within the RBC. Early studies with drugs indicate that RBCs are a transport system with a high capacity and a low affinity compared to plasma proteins.[10] Four models have been studied in this article in chronological order. We will examine three models with RBCs and/or blood with increasing number of compartments analyzed. This will determine polyphenols residing in various compartments in the blood, and we will use a new albumin washing procedure to distinguish surface binding and internalization of polyphenols. The fourth model will involve compartments in plasma. Multiple classes of polyphenols will be examined including anthocyanins, flavanols, flavonols, flavones, flavanones, phenolic acids, and glucuronide metabolites.

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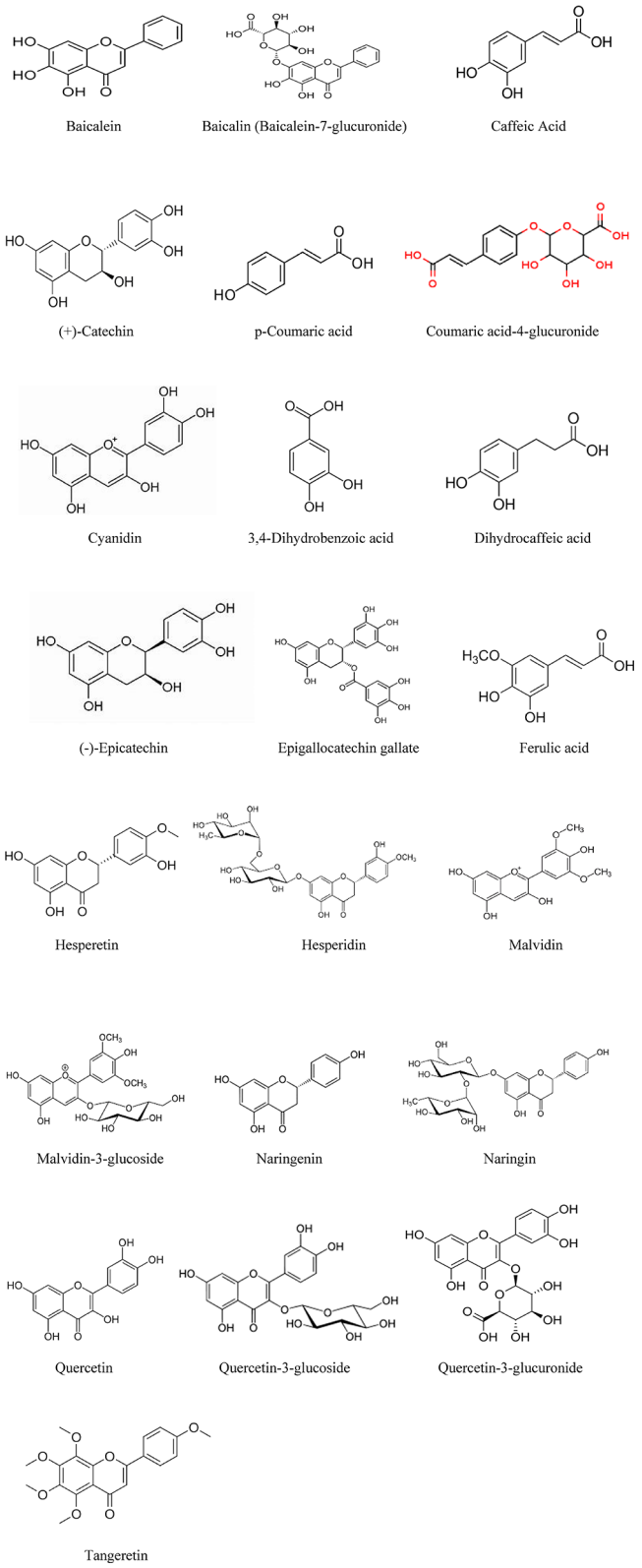

Figure 1. Polyphenol structures.

MATERIALS AND METHODS

Materials. All chemicals (>95% purity), resins and phosphate-buffered saline (PBS) were obtained from Sigma Chemical Company. PBS and affinity column elution solutions were run through a column of Chelex-100 resin (sodium form, 50−100 mesh) to remove ppm amount of heavy metal ions such as cupric and ferric ions which are pro-oxidants. Heparin-agarose type I and Cibacron Blue 3GA agarose were from Sigma/Aldrich. Bio Gel P-6 desalting gel was from Bio-Rad. Coumaric acid-4-glucuronide was a gift from Olivier Dangles of the University of Avignon. Quercetin-3-glucuronide was a generous gift from Paul Needs of the Institute of Food Research (IFR) in Norwich, U.K. as a solid sodium salt. 3′-Methylcatechin was synthesized by a published method and purified by preparative HPLC with the technical assistance of Patrick Donnelly. Fresh pig blood stabilized with EDTA and shipped at ice temperatures and stored at 4 °C was supplied by Lampire Biological Products (Pipersville, PA) and Pel-Freez (Rogers, AR) and used within 7 days of receipt. Pig plasma obtained from Innovative Research (Novi, MI) was shipped in dry ice and kept at −80 °C until use.

HPLC (Shimadzu LC-20 AD) was done with photodiode detection (Shimadzu SPD-M20A) using either a gradient of 0.4% phosphoric acid water in 100% water to 0.4% phosphoric acid in 100% methanol or 0.1% formic acid in 100% water to 0.1% formic acid in 100% methanol for 15 min with a 10 cm C18 column (10 μm) and a flow rate of 1 mL/min. Injection volumes ranged from 1 to 100 μL, and isolated fractions were directly injected. Standard curves were made using polyphenols dissolved in methanol. Reproducibility of injection was less than 10%. The limit of quantitation was 20 nM for catechin and 5 nM for detection. All samples were done in duplicate. Polyphenols were selected to represent a wide variety of classes.

LC-electrospray ionization/mass spectrometric analysis (LC-ESI/MS) was carried out using a Shimadzu 2010 LC/MS system with a Shimadzu autosampler, Shimadzu binary LC pumps, and a Shimadzu PDA detector. The same HPLC column was used for the LC/MS analysis as well as for the HPLC method. The MS analysis was done in negative ion mode, with nebulizing gas (nitrogen) flow at 1.5 L/min, a collision energy of 1.4 kV, mass range of 150−600 amu, heat block temperature of 200 °C, and curved desolvation line heater temperature of 200 °C. Methylated metabolites were characterized by molecular weight (but not the position of the substitution) using a Shimadzu LC−MS (model 2010) courtesy of Professor Deanne Garver at our sister institution, Marywood University.

Model I: RBC/PBS (One RBC Compartment Analyzed: Intracellular + Surface Membrane-Bound). Ten milliliters of pig blood/EDTA from Pel-Freez (Rogers, AR) was centrifuged at 2000*g* for 10 min at 4 °C. After removal of plasma, buffy coat, and ∼15% of the RBCs, the remaining portion of RBCs was immediately used for the incubation studies. RBCs were immediately washed twice with Chelex-treated PBS (150 mM NaCl, 5 mM Na_2HPO_4 in nano-pure water adjusted to pH 7.4). Stock solutions of polyphenols were prepared in DMSO and diluted to increasing micromolar concentrations with PBS. Five milliliters of RBCs was incubated in a plastic tube with 5 mL of polyphenol in PBS (50% hematocrit with maximum concentration of 0.5% DMSO) for 30 min at 25 °C. RBCs were washed twice with 5 mL of fresh PBS. A measured volume of packed RBCs after centrifugation was resuspended in 15 mL of PBS and lysed by alternate freezing and thawing cycles. Polyphenols in both PBS and RBC were extracted three times with 5 mL of ethyl acetate, which was pooled and evaporated under nitrogen and reconstituted with methanol (1 mL). Methanol extracts were analyzed for polyphenols by HPLC, and the results were calculated for moles of RBCs on a volume basis.

Model II: Whole Blood (Two RBC Compartments Analyzed; Intracellular and Surface Membrane-Bound)). Fresh blood was centrifuged at 2000*g* at 25 °C for 10 min. The plasma fraction was observed for signs of hemolysis. Nine milliliters of whole blood was aliquoted into multiple 10 mL plastic centrifuge tubes, which were kept in an ice bath. Various amounts of stock solutions of polyphenols in DMSO and diluted with PBS were added to give the desired micromolar concentrations (1−20 μM). Tubes were gently mixed for 10 s and then placed in a water bath at 25 °C for 30 min. Pelleted cells were washed three times with 2 mL of PBS. Membrane-bound polyphenols were extracted by washing cells three times with 1 mL of 0.5% bovine serum albumin in PBS. This fraction and the lysed cells were separately extracted three times with 1 mL of ethyl acetate, and the ethyl acetate extracts were pooled and then evaporated under

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nitrogen at 50 °C in a water bath. The solid residue was reconstituted with methanol (250 to 1000 μL) and stored in an amber vial at 4 °C until analysis with HPLC.

Model III: Blood and Plasma Compartments (Four Compartments Analyzed; Intracellular, Surface Membrane-Bound, LDL, and HDL + Albumin). This is the same procedure as model II in which whole blood is equilibrated with 1−20 μM polyphenol for 30 min at 25 °C (optimized polyphenol recovery temperature and equilibration time with 20 μM quercetin), and both the RBCs and plasma were isolated. The RBCs were treated the same way as in model II. Then, 200 μL of the plasma was also subjected to separation by our published heparin-agarose affinity column chromatography method[12] with all samples and eluent solutions kept on ice to provide an α fraction consisting of polyphenols bound to albumin and HDL and a β fraction of polyphenols bound to LDL. The fractions were bubbled with nitrogen and then stored at 4 °C until they were analyzed by HPLC. If needed, the fractions were analyzed for proteins using Coomassie Blue as the reagent and bovine serum albumin as the standard.

Model IV: Plasma Compartments Including HDL (Three Compartments Analyzed; HDL, LDL, and Albumin). This is the same procedure as model III except that pig plasma was used instead of whole blood for the equilibration with polyphenols. The plasma was then subjected to heparin-agarose gel separation. The LDL fraction was bubbled with nitrogen and stored at 4 °C until the assay by HPLC. The α fraction (500 μL aliquot) was immediately desalted with 4 g of pre-prepared Bio-Gel-6-P placed in a disposable column, and the column was centrifuged at 1000*g* at 4 °C for 4 min. The desalted α fraction was then added to 3 mL of pre-prepared Cibacron Blue gel, and 5 mL of 20 M PBS was added to elute HDL, which was kept cold. Albumin was eluted from the gel with 5.0 mL of 1.5 M ammonium thiocyanate and kept on ice. The fractions were bubbled with nitrogen and then stored at 4 °C until they were analyzed for polyphenols by HPLC. The Cibacron Blue column was regenerated with ammonium thiocyanate solution as per instructions from the manufacturer.

RESULTS AND DISCUSSION

In the human body, 84% of the cells are RBC and almost 50% of the volume of blood (hematocrit) consists of RBCs.[13] We used pig blood as our surrogate human blood due to safety concerns. Pigs are anatomically similar to humans although their blood lipids are lower than in humans. The hematocrit of an adult pig is 40%, which is similar to that of humans.[14] RBCs have an overall negative charge.[15] A total of 21 polyphenols of multiple classes were examined (see Figure 1) but not in every model due to time and resource constraints. Comparisons for the same polyphenol in different models were not possible due to different sources and different lots of pig blood that were used.

Model I RBC/PBS. We determined the kinetics of quercetin at 10 μM in RBC/PBS (physiological conditions) from 30 min to 2 h at 25 °C and found that maximal association occurred at 30 min. The association was stable for 2 h before the concentration in RBCs started to slowly decline (results not shown). We decided to conduct 30 min incubations to minimize hemolysis of RBCs and possible polyphenol decomposition. There was some hemolysis during incubation as shown by the slight pink color of the PBS wash. Table 1 shows the results of differing concentrations of polyphenols in the media and equilibrium polyphenol concentration associated with RBCs after lysing (membrane surface-bound plus intracellular). The results for the 12 compounds (flavanols, flavonols, flavanones, phenolic acids, and glucuronides, the latter of which are in vivo metabolites) indicate an overall linear increase in RBC association as the polyphenol concentration in the RBC/PBS media increases. The data shows that at 20 or 50 μM PP, the RBCs are not saturated in this model and thus there is a passive diffusion mechanism of transport. The more lipophilic methyl polyphenols associate more with RBCs than the parent aglycones at the four concentrations ($p < 0.05$), probably indicative of greater transport. The more hydrophilic and ionic flavanone glucuronide baicalin, which has a negative charge at pH 7.4,

Table 1. Model I: Determination of Polyphenols Associated with RBCs (Intracellular + Surface-Bound) after Equilibrating in RBCs in Isotonic Buffer at Physiological pH

polyphenol	RBC incubation polyphenol concentration (μM)	RBC-associated polyphenol concentration (μM)
quercetin	1	0.19 ± 0.08
	10	4.7 ± 1.6
	20	8.0 ± 4.2
	50	26.4 ± 0.02
3′-methylquercetin	1	0.7 ± 0.4
	5	3.2 ± 0.01
	10	5.9 ± 1.9
	20	2.3 ± 1.6
catechin	1	0.3 ± 0.2
	5	1.8 ± 1.3
	10	5.0 ± 1.1
	20	9.4 ± 0.9
3′-methylcatechin	1	0.3 ± 0.5
	5	2.5 ± 0.1
	10	4.9 ± 0.1
	20	11.3 ± 0.1
baicalein	1	0.5 ± 0.3
	5	2.9 ± 0.9
	10	5.8 ± 1.3
	20	11.9 ± 1.2
baicalin (baicalein-7-glucuronide)	1	0.4 ± 0.3
	5	2.0 ± 1.0
	10	4.7 ± 2.2
	20	11.5 ± 1.8
coumaric acid	5	1.9 ± 0.2
	10	3.7 ± 0.5
	20	8.9 ± 0.3
	50	16.4 ± 0.3
coumaric acid-4-glucuronide	5	3.0 ± 0.2
	10	5.9 ± 0.5
	20	11.6 ± 0.6
	50	24.5 ± 0.02
caffeic acid	1	0.06 ± 0.05
	5	0.5 ± 0.0
	10	5.9 ± 0.1
	20	11.6 ± 0.1
ferulic acid	5	2.4 ± 0.1
	10	4.9 ± 0.2
	20	9.7 ± 0.4
	50	18.0 ± 1.7
hesperidin	1	0.4 ± 0.4
	5	0.5 ± 0.0
	10	4.9 ± 0.1
	20	10.7 ± 0.1
cyanidin	1	0.4 ± 0.1
	5	0.5 ± 0.0
	10	4.9 ± 1.3
	20	10.5 ± 1.3

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associates similarly with RBCs to the parent undissociated aglycone baicalein. However, coumaric acid glucuronide (with two negative charges) associates more than does coumaric acid. The polyphenols and their metabolites (glucuronides) associated with RBCs from 30 to 70% of the added amount put into the RBC media. Glucuronides and phenolic acids at pH 7.4 are charged and are still able to associate with the RBC in this model. Cyanidin, a positively charged polyphenol at physiological pH, also associates with RBCs. These results suggest that the magnitude and sign of the charge of the polyphenol apparently have little effect on the binding in this isolated RBC model. The average recovery of polyphenols by analyzing PBS + intracellular polyphenol was 61% using our multiple extraction procedure. The loss is due to hemolysis producing oxidants and degradation under basic conditions (physiological) as shown by Xiao and Högger for a wide variety of polyphenols.[16]

Using the same RBC model, Fiorani et al.[6] found that quercetin is taken up by passive diffusion (85%) at 50 μM and by very fast kinetics as we have found. This earlier study showed that 90% of the RBC cystolic quercetin fraction was bound to hemoglobin. Model I demonstrates the ability of RBCs to capture polyphenols in the absence of competing proteins from plasma, and thus these in vitro results indicate a higher and perhaps maximal association compared to the in vivo situation. The calculated quercetin concentration in a single pig RBC with a volume of 61 fL[17] after 20 μM quercetin incubation is 345 μM. It is evident that the RBC concentrates polyphenols compared to blood levels. The major intracellular antioxidant glutathione in the average human RBC has a concentration of 1400 μM.[18] In this model, the concentration of RBC-associated polyphenols is high enough to function as important antioxidants for the RBC.

Model II Whole Blood RBC Compartments. Flavanone polyphenols in whole blood (*ex vivo*) were equilibrated at different concentrations, and the surface membrane-bound and intracellular polyphenols were separated using the albumin wash and cell lysis. Albumin was utilized to quantitatively remove and isolate the surface-bound polyphenols as it binds more strongly to polyphenols than the polyphenols bind to the surface membrane of the RBC. PBS, the usual cell washing solution used prior to lysing, only removes adventitious polyphenols. We used the published albumin procedure described for endothelial cells with quercetin and quercetin glucuronide, where three washings with albumin quantitatively removed the surface-bound compounds without lysing the cells.[11] Table 2 shows the values normalized to 100% of the recovered amounts for ease of interpretation. We found for the first time that methylated metabolites can be formed in blood under these ex vivo conditions for hesperetin and baicalein. Hesperidin and naringin did not undergo methylation. These two polar and large-molecular-mass polyphenol glycosides were only found on the RBC surface and were not found in the intracellular space. These two had a molecular mass of over 600 and were therefore too large to enter the cell, which supports the classic drug permeability rules. There are no literature references indicating that these glycosidic compounds can undergo metabolic methylation either in vitro or in vivo. Tangeretin does not methylate as all the −OH groups are already methylated. Another study used a different lot of pig blood and equilibrated quercetin and quercetin-3-glucoside with RBCs. The identity in the compartments was demonstrated by LC−MS, but there was no determination of the concentration of methylquercetin-3-glucoside and methylquercetin-3-glucuronide, which are both in the intracellular space and also bound to RBCs (see Figure 1 for structures). A dose−response curve for RBC compartmentalization of the polyphenol baicalein is shown in Figure 2. There is a linear increase in the amount bound to the surface membrane and also in the intracellular space with no indication of saturation at higher concentrations. This is also the case for the other polyphenols examined in this model. Ginsburg and co-workers were the first to show by direct observation with confocal laser microscopy that four autofluorescing polyphenols (morin,

Table 2. Model II: Determination of Polyphenols (Flavanones) and Their Methyl Metabolites (Me) in RBC Compartments after Equilibrating in Whole Blood

polyphenol	blood concentration	% bound to RBC	% intracellular RBC
hesperetin (H)	1	MeH 60.1	MeH 39.9
	5	MeH 38.8	MeH 61.2
	10	MeH 32.0	MeH 69.0
	20	MeH 32.1	MeH 67.9
hesperidin (hesperitin-7-rutinoside)	1	[a]	[a]
	5	100	[a]
	10	100	[a]
	20	100	[a]
tangeretin	1	[a]	[a]
	5	[a]	1.91
	10	[a]	1.20
	20	38.8	61.2
baicalein (B)	1	[a]	MeB 100
	5	B 10.1, MeB 4.6	B 17.7, MeB 67.6
	10	B 12.4, MeB 2.9	B 24.6, MeB 60.1
	20	B 15.8, MeB 10.8	B 26.1, MeB 47.3
naringenin	1	32.9	67.1
	5	29.7	70.3
	10	26.4	73.6
	20	39.1	60.9
naringin (naringenin-7-*O*-neohesperidoside)	1	[a]	[a]
	5	100	[a]
	10	100	[a]
	20	100	[a]

[a]Not detectable.

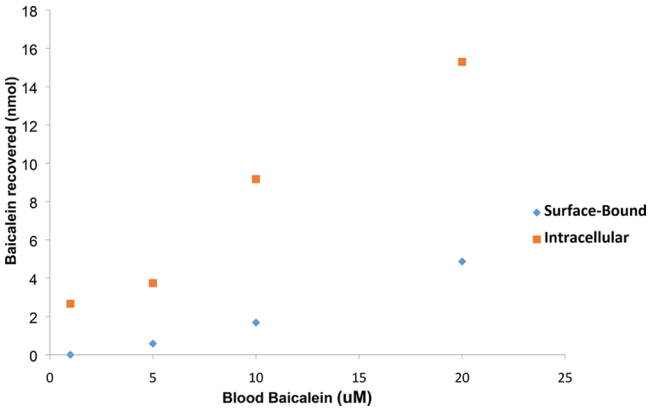

Figure 2. Dose−response of baicalein for RBC surface membrane and intracellular compartmentalization.

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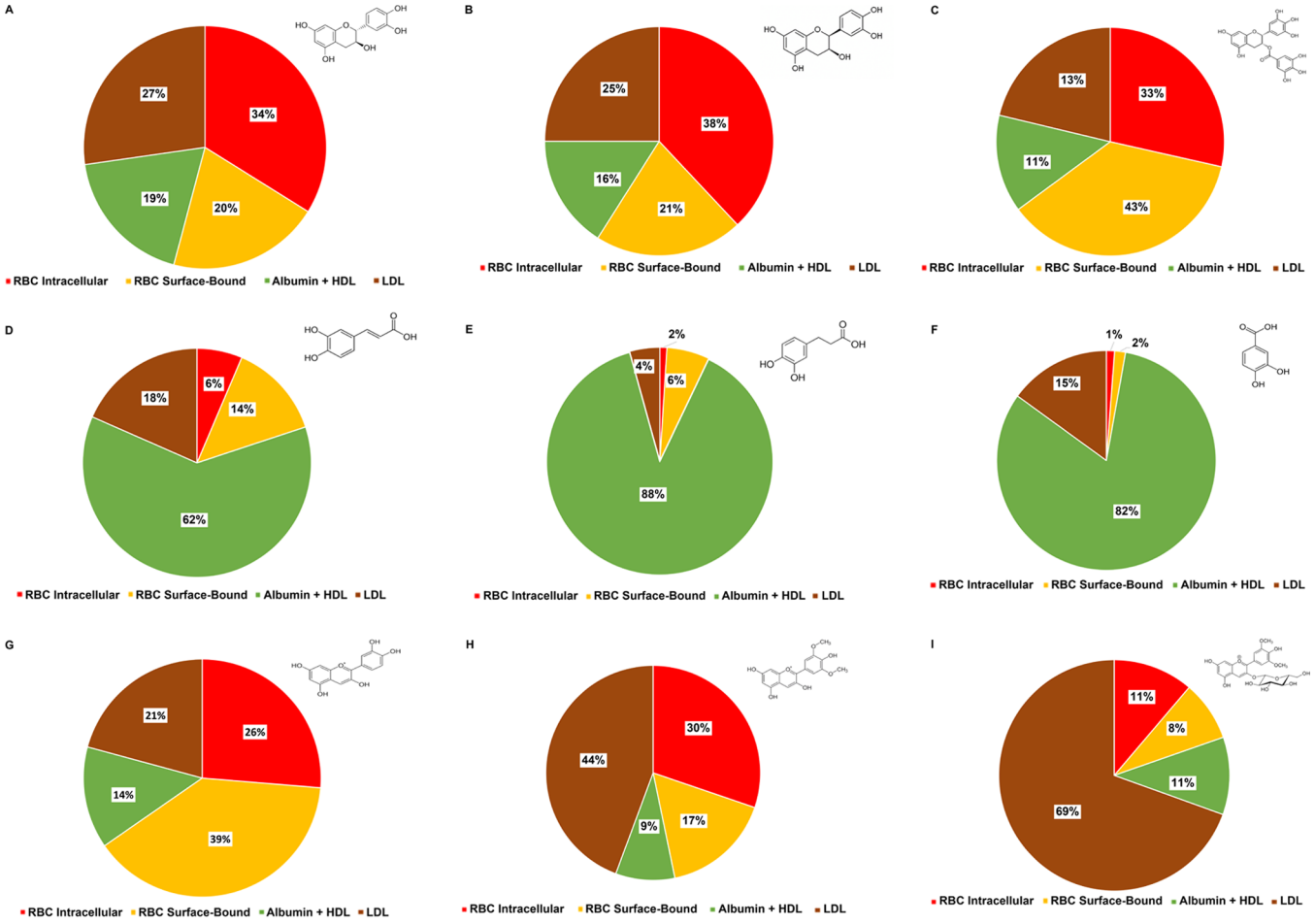

Figure 3. Polyphenol (20 μM) compartmentalization in whole blood. (A) Catechin, (B) epicatechin, (C) epigallocatechin gallate, (D) caffeic acid, (E) dihydrocaffeic acid, (F) 3,4-dihydroxybenzoic acid, (G) cyanidin, (H) malvidin, and (I) malvidin-3-glucoside.

resveratrol, curcumin, and tannic acid) were bound to the surface of RBCs.[9] Our model allowed the quantitation of this phenomenon.

RBCs are known to have catechol-*O*-methyltransferase (COMT) present within the cell, which catalyzes methylation, but RBCs also have membrane-bound COMT.[19] Methyl quercetin and three other methylated polyphenols were found to be in vitro polyphenol metabolites in human hepatocytes.[20] Platelets also produce methyl metabolites of polyphenols.[21] Interestingly, we find that all of the polyphenols and methylated metabolites bind to the surface of RBCs. This indicates that membrane methylation can occur in this model and it also occurs in vivo. Tangeretin, which has all the phenolic groups methylated, did not undergo metabolism. Surprisingly, no hesperetin, only its methyl metabolite, was found inside or bound to the surface of the cell. This indicates complete monomethylation metabolism for this polyphenol. Naringenin, which has no 4′-OH group, was not methylated, which is consistent with its chemical structure. In addition to RBCs in this model, quercetin mixed with isolated platelets was found to undergo methylation. Confocal microscopy demonstrated that methylated metabolites were localized in the cystolic compartment of the cell.[21] However, this microscopic technique did not allow the determination of surface-bound polyphenol but rather the sum of surface-bound and intracellular polyphenol.

It is our hypothesis that methylation did not occur in method I due to the longer travel time (1200 miles vs 80 miles) and storage time for the blood, which deactivated COMT, co-factors, or its precursors prior to use of the blood in the model. This hypothesis is borne out by the very poor absolute recovery (less than 30%) in method I, which produced pro-oxidative products of hemolysis in the blood. Experiments with control pig blood showed that there were no polyphenols detectable. This was the case in spite of the fact that pig food contains grains with phenolic acids present. We hypothesize that hemolysis of RBCs occurred during transport (2−3 days in ice packs and including vibration) and storage, which oxidized the phenolic acids. Precision of duplicates was <10%.

Model III Blood Compartments. In this whole blood model, the amount of polyphenol in two plasma compartments was determined: (1) albumin + high density lipoprotein (HDL) and (2) LDL and two compartments for RBCs (surface membrane-bound and intracellular). Three classes of polyphenols by three different investigators were examined in duplicate at 1−20 μM; catechins, anthocyanins and phenolic acids for a total of nine polyphenols (Figure 3). Multiple lots of blood were used from the same manufacturer and no methyl derivatives were detected. There was an increase in the amount of the polyphenol in the compartments as the spiked concentration increased. The results for 20 μM are shown in the form of pie charts for the relative % recovered polyphenol

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for comparison purposes. The average precision for this model is 5.7%.

Figure 3a–c shows the catechins (catechin, epicatechin, and epigallocatechin gallate), which are all un-ionized at pH 7.4. Catechin and epicatechin, being diastereomers, gave almost identical results as expected. The largest molecule, epigallocatechin gallate (EGCG), is the major polyphenol in green tea. It binds more on the surface of the RBC than the other two catechins and more in fact than any of the nine polyphenols studied, yet EGCG is able to enter the RBC similarly to the other catechins. Epicatechin and catechin bind much more to LDL and albumin + HDL than does ECGC, perhaps due to their smaller size and greater polarity.

Figure 3d–f illustrates the results for the phenolic acids (caffeic acid, dihydrocaffeic acid, and 3,4-dihydroxybenzoic acid). These compounds are completely ionized at pH 7.4. This group of phenolic acids binds much more strongly to albumin + HDL than the other classes of compounds tested in this model. Only caffeic acid and the benzoic acid bind appreciably to LDL. There are very little of these compounds bound to the surface or inside the RBC. This can be attributed to the fact that the surface of RBC has a negative charge[15] (sialic acids), which repels the negative charges of these phenolic carboxylates.

Figure 3g–i shows the compartmentalization of three anthocyanins: cyanidin, malvidin, and malvidin-3-glucoside. Cyanidin is associated more with RBCs than the other two compounds, which much prefer the plasma (LDL and albumin + HDL). These anthocyanins on the average bind much more to LDL than any of the other compounds studied in this model due to their positive charge and the fact that LDL has a net negative charge.[22]

Among the three classes of polyphenols in this model, the catechins associate more with RBC and bind and enter the RBC more than any other class. They are unionized and the other classes are ionized. The phenolic acids bind more to the albumin + HDL, and the anthocyanins bind more to LDL than any other class.

This blood model best represents the in vivo condition of the four models as it contains all the cells and the normal physiological levels of all of the proteins. A typical HDL cholesterol level for the normal pig in our study is 44 mg/dL (32 μM of lipoprotein), LDL is 56 mg/dL (0.6 μM lipoprotein), and albumin is 465 μM. These values fit the normal range for pigs.[23] Normal human levels are 600 μM albumin, 33 μM HDL, and 1.5 μM for LDL.[24] The β fraction of pig plasma, which contains both LDL and VLDL, is 99 mol % LDL, so it is referred to as LDL.[25] Human plasma contains 95 mol % LDL.[24] The nine polyphenols examined in this model (see Figure 3 caption) associate with LDL on an average of 26% and a range from 13 to 69%. The model also contains RBCs, which as can be seen from the results compete with the albumin and lipoproteins for binding of polyphenols. Association with RBCs accounts for 39% of the polyphenols, and the 61% associate with the three proteins we examined in plasma. Thus, the previous practice of discarding RBCs in polyphenol in vivo research has led to a loss of information of a significant fraction of polyphenols and their metabolites. In a previous investigation with whole blood, 50% of the spiked resveratrol and 76% of the quercetin were lost when analyzing only the plasma.[26]

Model IV Plasma Compartments. Plasma polyphenol compartmentalization was investigated in this model after incubation at a single polyphenol concentration in plasma (20 μM) using two affinity columns and a desalting column separation. The plasma compartments' isolation scheme is shown in Figure 4. Of all the models, this gave the best total

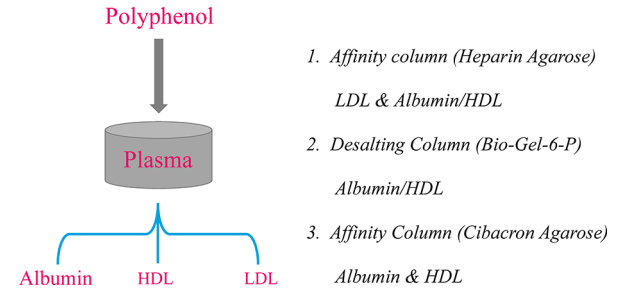

Figure 4. Scheme for isolating polyphenols in plasma compartments in model IV.

recovery of the three polyphenols, ranging from 94 to 100%. This was due to the absence of oxidation of the polyphenol in this plasma milieu as compared with the other models in which blood and/or RBCs were present.

The results are shown in Figure 5a–d for four polyphenols at 20 μM in plasma. The percent of recovered polyphenol in each compartment is shown in the pie charts. The average precision for this model is 1.8%. Baicalein had the highest percent in both albumin and LDL. Baicalin, the much more hydrophilic glucuronide metabolite of baicalein, had almost the same percent in LDL as baicalein but much greater percent in HDL. HDL has more protein and less lipids than LDL, rendering it more hydrophilic. Catechin had the highest amount in HDL of 49% of the four polyphenols followed by quercetin, baicalin, and lastly baicalein. The average of the four polyphenols was 54% in albumin and 46% in the lipoproteins LDL and HDL. For LDL, the order of binding was baicalein = baicalin > quercetin > catechin. Interestingly, the average for LDL binding was 24%, which is the same (26%) as the LDL average for the nine blood polyphenols (albeit different compounds). This model does not include association of polyphenols with RBCs due to their absence in the plasma model. As expected for the polyphenols, catechin, which binds appreciably to HDL and albumin in the plasma model, associates less with these proteins with RBCs present in the blood model. In spite of the much higher concentration of albumin (600 μM), in plasma, almost half of the polyphenols/metabolites bind to lipoproteins.

When proteins are also analyzed with polyphenols in the protein fractions, the plasma recovery is over 90% and the mole percent of polyphenols in the proteins can be calculated. This was done for the plasma model, and results are as follows: (1) quercetin, 1.7 mol % in albumin, 8.3 mol % in LDL, and 25 mol % in HDL; (2) catechin, 0.5 mol % in albumin, 6.5 mol % in LDL, and 29 mol % in HDL; (3) baicalein, 0.6 mol % in albumin, 2.7 mol % in LDL, and 3.3 mol % in HDL; (4) baicalin, 1.2 mol % in albumin, 2.2 mol % in LDL, and 3.3 mol % in HDL. The mol % numbers divided by 100 also represent the molecules of polyphenols associated with one particle, i.e. one molecule of LDL. After these direct measurements, the conclusion is that there are no free polyphenols in the plasma as found by Walle et al.[27] using radioactive quercetin in human plasma. Thus, it is hypothesized that there are no free polyphenols or metabolites in blood where RBCs are also present.

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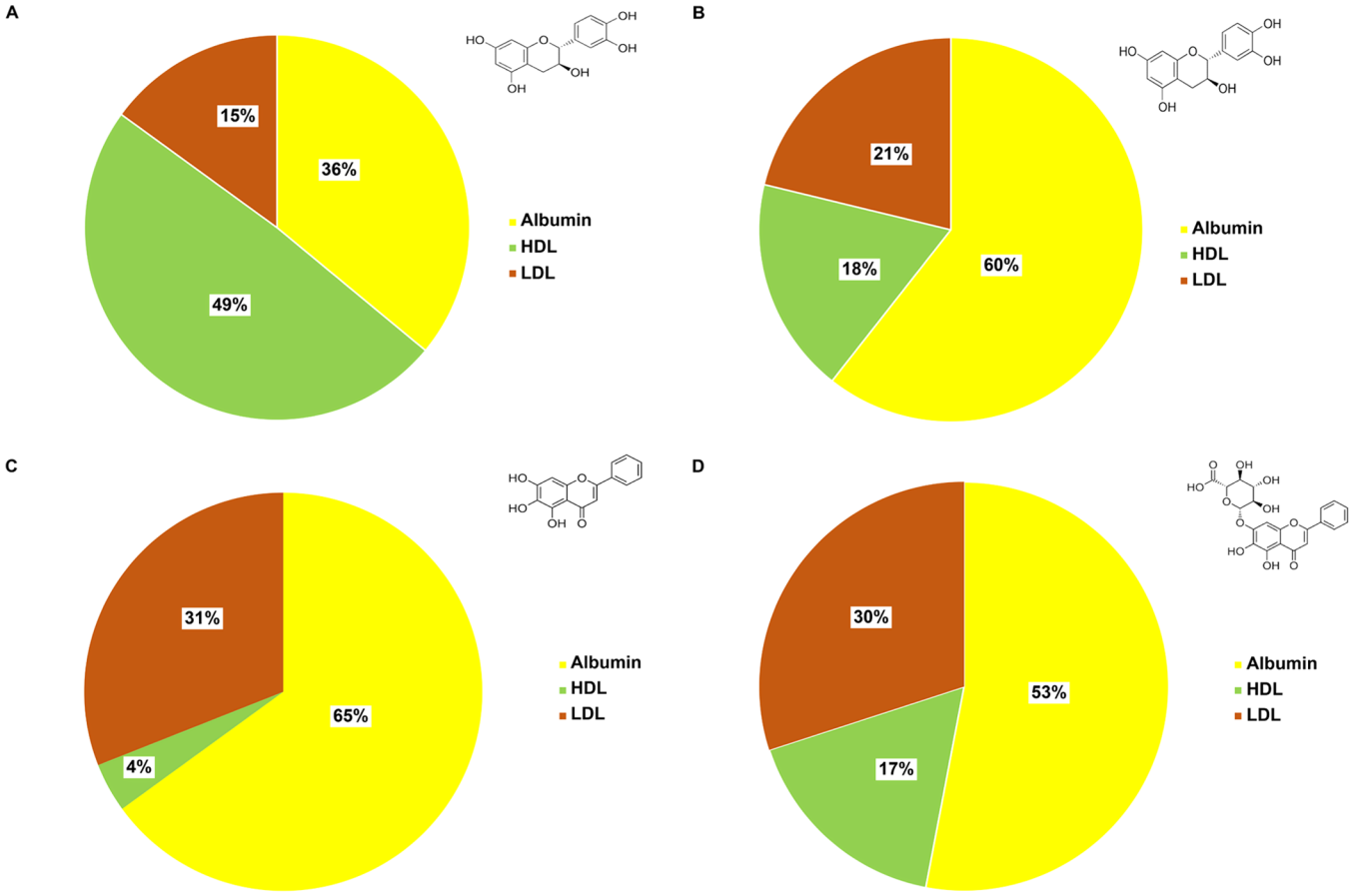

Figure 5. Polyphenol (20 μM) compartmentalization in plasma compartments. (A) Catechin, (B) quercetin, (C) baicalein, and (D) baicalin.

Significance of Polyphenols Binding to Proteins and Cells. Polyphenols bind to cells and proteins in blood including RBCs, albumin, and lipoproteins described in this work. This potentially benefits both proteins and RBCs as polyphenols bind to amino acids as well as lipids in lipoproteins. They can also (we hypothesize) bind to and enter other cells in the bloodstream. These areas are only beginning to be explored. Binding to RBC surface proteins, albumin, LDL, and HDL can occur specifically on the proteins in a 1:1 molecule fashion as determined by indirect methods such as fluorescence quenching[3] and also non-specifically with both proteins and lipids, which has not been well investigated. With the albumin wash described in this work, researchers can determine accurately how much of a polyphenol (or a drug) can bind to the cell and how much can enter the cell. Since polyphenols are antioxidants, they can protect cells and proteins in the blood from oxidation by their association with the particles. This has been well demonstrated for RBCs[9] and for many other cell types. Published examples include the review of polyphenols and mitochondria oxidant protection in neurons[28] and a current review of resveratrol and endothelial cells involved in vascular function.[29] However, the effect of cell surface-bound versus intracellular polyphenols on cell oxidation has not been studied due to the fact that traditional isolation methods do not separate polyphenols in the two compartments but rather lump them together incorrectly as "intracellular". Binding of polyphenol to human serum albumin stabilizes the polyphenol at the physiological pH[30] and provides protection to the transported polyunsaturated fatty acids.[31]

As mentioned in the Introduction, epidemiology studies with polyphenols have provided evidence that their consumption reduces the risk of heart disease. It is hypothesized that one of the mechanisms of polyphenols for benefitting heart disease is their antioxidant effect when bound to lipoproteins. Our group showed that polyphenols added in vitro to LDL[32] or ex vivo to plasma[33] were bound to LDL (after affinity column isolation) and protected the lipids in the particle from oxidation. This result follows the oxidative theory of atherosclerosis of Steinberg,[34] which states that LDL oxidation in the subendothelial space is the initiating event in atherosclerosis. Polyphenols bound to the LDL are transported with the LDL and thus are antioxidants for inhibiting LDL oxidation in the blood and in the endothelium. As evidence, the oxidized LDL in plasma is inversely correlated with subclinical carotid atherosclerosis in middle-aged men.[35] As further proof, we conducted an animal atherosclerosis study with citrus extract polyphenols and found that LDL oxidation and oxidative susceptibility were both significantly and indirectly correlated with the extent of atherosclerosis.[36]

There is growing evidence in vivo that polyphenols and their metabolites are found in both LDL and HDL from plasma collected after human consumption of foods and beverages high in polyphenols. One of the roles of HDL is the protection of LDL from oxidation and thus antioxidants bound to HDL could protect this particle as well as LDL from being oxidized. This hypothesis has been corroborated by LDL isolated from plasma 12 h after human olive oil consumption, which produced olive oil polyphenol metabolites bound to HDLDL. A less atherogenic LDL and HDL profile occurred

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after 3 weeks of olive oil consumption.[37,38] The researchers found ~1 mol % polyphenol metabolites in the LDL and HDL by direct LC/MS analysis. Our ex vivo plasma spike experiment with 20 μM polyphenols led to bound polyphenol levels of 2.7 to 8.3 mol % in LDL, probably an upper limit due to the superphysiologcial concentration of polyphenol spiked in the plasma. Recently, we have shown that saturation of LDL with polyphenols can result in up to 13 molecules of polyphenols bound per molecule of lipoprotein.[3] Even very low levels of polyphenols in LDL and HDL are protective for heart disease risk in vivo.

Polyphenols are amphiphilic and thus can bind to both proteins and lipids in particles such as lipoproteins or RBCs. From the results of this work, the binding of polyphenols and their metabolites to lipoproteins and the significant physiological reservoir RBCs need to be further studied ex vivo and in vivo using the RBC washing and affinity column isolation methodology described herein. The most complete study would involve human blood and combining model III and model IV to obtain data on polyphenols/metabolites in all five compartments: intracellular RBC, membrane-bound RBC, HDL, LDL, and albumin.

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Notes

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REFERENCES

(1) Hertog, M. G. L.; Feskens, E. J. M.; Kromhout, D.; Hollman, P. C. H.; Katan, M. B. Dietary antioxidant flavonoids and risk of coronary heart disease: The Zutphen Elderly Study. *Lancet* **1993**, *342*, 1007−1011.

(2) Cassidy, A.; Mukamal, K. J.; Liu, L.; Franz, M.; Eliassen, A. H.; Rimm, E. B. High Anthocyanin intake is associated with a reduced risk of myocardial infarction in young and middle-aged women. *Circulation* **2013**, *127*, 188−196.

(3) Poloni, D. M.; Dangles, O.; Vinson, J. A. Binding of Plant Polyphenols to Serum Albumin and LDL: Healthy Implications for Heart Disease. *J. Agric. Food Chem.* **2019**, *67*, 9139−9147.

(4) Natella, F.; Nardini, M.; Belelli, F.; Pignatelli, P.; Di Santo, S.; Ghiselli, A.; Violi, F.; Scaccini, C. Effect of coffee drinking on platelets: inhibition of aggregation and phenols incorporation. *J. Geophys. Res. Oceans* **2008**, *100*, 1276−1282.

(5) Hubbard, G. P.; Wolffram, S.; Lovegrove, J. A.; Gibbins, J. M. Ingestion of quercetin inhibits platelet aggregation and essential components of the collagen-stimulated platelet activation pathway in humans. *J. Thromb. Haemost.* **2004**, *2*, 2138.

(6) Fiorani, M.; Accorsi, A.; Cantoni, O. Human red blood cells as a natural flavonoid reservoir. *Free Radical Res.* **2003**, *37*, 1331−1338.

(7) Fiorani, M.; De Sanctis, R.; De Bellis, R.; Dachà, M. Intracellular flavonoids as electron donors for extracellular ferricyanide reduction in human erythrocytes. *Free Radical Biol. Med.* **2002**, *32*, 64−72.

(8) Fiorani, M.; Accorsi, A. Dietary flavonoids as intracellular substrates for an erythrocyte trans-plasma membrane oxidoreductase activity. *J. Geophys. Res. Oceans* **2005**, *94*, 338−345.

(9) Koren, E.; Kohen, R.; Ginsburg, I. Polyphenols enhance total oxidant-scavenging capacities of human blood by binding to red blood cells. *Exp. Biol. Med. (Maywood)* **2010**, *235*, 689−699.

(10) Highley, M. S.; De Bruijn, E. A. Erythrocytes and the transport of drugs and endogenous compounds. *Pharm. Res.* **1996**, *13*, 186−195.

(11) Wittig, J. *Bioverfügbarkeit und metabolismus von flavonoiden-analytische und biochemische untersuchungen*. 2002. Julius-Maximillans-Universität Würzburg, PhD Dissertation.

(12) Vinson, J. A.; Proch, J.; Bose, P. Determination of quantity and quality of polyphenol antioxidants in foods and beverages. *Methods Enzymol* **2001**, *335*, 103−114.

(13) Sender, R.; Fuchs, S.; Milo, R. Revised Estimates for the Number of Human and Bacteria Cells in the Body. *PLoS Biol.* **2016**, *14*, No. e1002533.

(14) Miller, E. R.; ULlrey, D. E.; Ackermann, I.; Schmidt, D. A.; Luecke, R. W.; Hoefer, J. A. Swine hematology from birth to maturity. II. Erythrocyte population, size and hemoglobin concentration. *J. Anim. Sci.* **1961**, *20*, 890−897.

(15) Van Damme, M. P. I.; Tiglias, J.; Nemat, N.; Preston, B. N. Determination of the charge content at the surface of cells using a colloid titration technique. *Anal. Biochem.* **1994**, *223*, 62−70.
(16) Xiao, J.; Högger, P. Stability of dietary polyphenols under the cell culture conditions: avoiding erroneous conclusions. *J. Agric. Food Chem.* **2015**, *63*, 1547−1557.
(17) Namdee, K.; Carrasco-Teja, M.; Fish, M. B.; Charoenphol, P.; Eniola-Adefeso, O. Effect of variation in hemorheology between human and animal blood on the binding efficacy of vascular-targeted carriers. *Sci. Rep.* **2015**, *5*, 11631.
(18) Van't Erve, T. J.; Wagner, B. A.; Ryckman, K. K.; Raife, T. J.; Buettner, G. R. The concentration of glutathione in human erythrocytes is a heritable trait. *Free Radical Biol. Med.* **2013**, *65*, 742−749.
(19) Schultz, E.; Nissinen, E.; Kaakkola, S. Determination of catechol-O-methyltransferase activity in erythrocytes by high performance liquid chromatography with electrochemical detection. *Biomed. Chromatogr.* **1989**, *3*, 64−67.
(20) Vacek, J.; Papoušková, B.; Kosina, P.; Vrba, J.; Křen, V.; Ulrichová, J. Biotransformation of flavonols and taxifolin in hepatocyte in vitro systems as determined by liquid chromatography with various stationary phases and electrospray ionization-quadrupole time-of-flight mass spectrometry. *J. Chromatogr., B* **2012**, *899*, 109−115.
(21) Wright, B.; Gibson, T.; Spencer, J.; Lovegrove, J. A.; Gibbins, J. M. Platelet-mediated metabolism of the common dietary flavonoid, quercetin. *PLoS One* **2010**, *5*, e9673.
(22) Rodríguez, M.; Guardiola, M.; Oliva, I.; Carles Vallvé, J.; Ferré, R.; Masana, L.; Parra, S.; Ribalta, J.; Castro, A. Low-density lipoprotein net charge is a risk factor for atherosclerosis in lupus patients independent of lipid concentrations. *Int. J. Rheum. Dis.* **2018**, *22*, 480−487.
(23) Hellwing, A.; Tauson, A.-H.; Skrede, A. Blood parameters in growing pigs fed increasing levels of bacterial protein meal. *Acta Vet. Scand.* **2007**, *49*, 33.
(24) Colhoun, H. M.; Otvos, J. D.; Rubens, M. B.; Taskinen, M. R.; Underwood, S. R.; Fuller, J. H. Lipoprotein subclasses and particle sizes and their relationship with coronary artery calcification in men and women with and without type 1 diabetes. *Diabetes* **2002**, *51*, 1949−1956.
(25) Janado, M.; Martin, W. G.; Cook, W. H. Separation and properties of pig-serum lipoproteins. *Can. J. Biochem.* **1966**, *44*, 1201−1209.
(26) Biasutto, L.; Marotta, E.; Garbisa, S.; Zoratti, M.; Paradisi, C. Determination of quercetin and resveratrol in whole blood–implications for bioavailability studies. *Molecules* **2010**, *15*, 6570−6579.
(27) Boulton, D. W.; Walle, U. K.; Walle, T. Extensive binding of the bioflavonoid quercetin to human plasma proteins. *Pharm. Pharmacol.* **1998**, *50*, 243−249.
(28) Naoi, M.; Wu, Y.; Shamoto-Nagai, M.; Maruyama, W. Mitochondria in neuroprotection by phytochemicals: bioactive polyphenols modulate mitochondrial apoptosis system, function and structure. *Int. J. Mol. Sci.* **2019**, *20*, 2451.
(29) Li, H.; Xia, N.; Hasselwander, S.; Daiber, A. Resveratrol and vascular function. *Int. J. Mol. Sci.* **2019**, *20*, 2155.
(30) Xiao, J.; Chen, T.; Cao, H.; Chen, L.; Yang, F. Molecular property-affinity relationship of flavanoids and flavonoids for HSA in vitro. *Mol. Nutr. Food Res.* **2011**, *55*, 310−317.
(31) Dufour, C.; Loonis, M. Flavonoids and their oxidation products protect efficiently albumin-bound linoleic acid in a model of plasma oxidation. *Biochim. Biophys. Acta, Gen. Subj.* **2007**, *1770*, 958−965.
(32) Vinson, J. A.; Dabbagh, Y. A.; Serry, M. M.; Jang, J. Plant flavonoids, especially tea flavonols, are powerful antioxidants using an in vitro oxidation model for heart disease. *J. Agric. Food Chem.* **1995**, *43*, 2800−2802.
(33) Vinson, J. A.; Jang, J.; Yang, J.; Dabbagh, Y.; Liang, X.; Serry, M.; Proch, J.; Cai, S. Vitamins and especially flavonoids in common beverages are powerful in vitro antioxidants which enrich lower density lipoproteins and increase their oxidative resistance after ex vivo spiking in human plasma. *J. Agric. Food Chem.* **1999**, *47*, 2502−2504.
(34) Steinberg, D. The LDL modification hypothesis of atherogenesis: an update. *J. Lipid Res.*, **2009**, Suppl:S376−381. *J Lipid Res.*, **2009**, Suppl:S376.
(35) Metso, S.; Loimaala, A.; Mercuri, M. F.; Nenonen, A.; Vuori, I.; Oja, P.; Bond, M. G.; Laine, S.; Rontu, R.; Lehtimäki, T. Circulating oxidized low-density lipoprotein and common carotid artery intima-media thickness in a random sample of middle-aged men. *J Biomed Sci.* **2004**, *11*, 356−361.
(36) Vinson, J. A.; Hu, S.-J.; Jung, S.; Stanski, A. M. A citrus extract plus ascorbic acid decreases lipids, lipid peroxides, lipoprotein oxidative susceptibility, and atherosclerosis in hypercholesterolemic hamsters. *J. Agric. Food Chem.* **1998**, *46*, 1453−1459.
(37) Fernández-Castillejo, S.; Valls, R. M.; Castañer, O.; Rubió, L.; Catalán, Ú.; Pedret, A.; Macià, A.; Sampson, M. L.; Covas, M. I.; Fitó, M.; Motilva, M. J.; Remaley, A. T.; Solà, R. Polyphenol rich olive oils improve lipoprotein particle atherogenic ratios and subclasses profile: A randomized, crossover, controlled trial. *Mol. Nutr. Food Res.* **2016**, *60*, 1544−1554.
(38) Hernáez, Á.; Fernández-Castillejo, S.; Farràs, M.; Catalán, Ú.; Subirana, I.; Montes, R.; Solà, R.; Muñoz-Aguayo, D.; Gelabert-Gorgues, A.; Díaz-Gil, Ó.; Nyyssönen, K.; Zunft, H. J. F.; de la Torre, R.; Martín-Peláez, S.; Pedret, A.; Remaley, A. T.; Covas, M.-I.; Fitó, M. Olive oil polyphenols enhance high-density lipoprotein function in humans. *Arterioscler., Thromb., Vasc. Biol.* **2014**, *34*, 2115−2119.

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J. Agric. Food Chem. XXXX, XXX, XXX−XXX