JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Enzymatic *O*-Methylation of Flavanols Changes Lag Time, Propagation Rate, and Total Oxidation during in Vitro Model Triacylglycerol-Rich Lipoprotein Oxidation

JUN YU,[†,§] GABE SMITH,[‡] HEIDRUN B. GROSS,[‡,#] ROBERT J. HANSEN,[‡] JOHN LEVENBERG,[‡] AND ROSEMARY L. WALZEM*[,§,‡]

Departments of Horticultural Sciences and Poultry Science, Texas A&M University, College Station, Texas 77843, and Department of Molecular Biosciences, School of Veterinary Medicine, University of California, Davis, California 95616

3′-*O*-Methyl derivatives of flavan-3-ols, (+)-catechin (C), (−)-epicatechin (EC), and (−)-catechin gallate (CG) were prepared enzymatically. Hexanal (EC and CG family, 5 mmol/L) and conjugated diene (C and EC family, 0.25−10 mmol/L) formation following $CuSO_4$-mediated triacylglycerol-rich lipoprotein oxidation was measured. All EC and CG compounds significantly reduced hexanal formation ($p < 0.02$). *O*-Methylation improved the ability of CG (more polar) while reducing the ability of EC (less polar) to limit hexanal formation. 3′-*O*-methyl EC was 18% ($p < 0.001$) and 4′-*O*-methyl 65% ($p < 0.001$) less able than EC to suppress hexanal formation. At >1 μmol/L all EC and C compounds significantly increased lag time. Parent compounds were more effective (>4-fold increase) than metabolites (1.5-fold increase). Parent compounds did not influence propagation rate (ΔOD/min). At >1 mmol/L *O*-methylated EC and C reduced propagation by 20−40% ($p < 0.01$). Notably, at 0.25 mmol/L *O*-methylated EC and C increased propagation rates 22% ($p < 0.01$) despite prolonging lag time.

KEYWORDS: Catechins; methylated catechins; conjugated dienes; hexanal; VLDL*y*

INTRODUCTION

Dietary flavanols alter metabolism in a number of ways, many of which may improve long-term health and reduce morbidity and mortality from a variety of chronic diseases (*1*). Flavanol-rich foods, such as red wine, tea, cocoa, and apples, contain multiple, chemically distinct compounds that are further modified by xenobiotic enzyme systems in vivo (*2*). Common xenobiotic modifications in animals include methylation, glucuronidation, and sulfation. Whereas methylated derivatives, flavonoid glucuronides, and sulfates are all plant products, methyl or sulfate esters or glucuronides of (+)-catechins (C) and (−)-epicatechins (EC) are not known as plant products (*3*). More recent studies determined that 3″-, 4″-, and 4′,4″-methyl derivatives of (−)-epigallocatechin gallate are minor components of several tea cultivars (*4*).

Flavanols contain a chromanol ring system with the capacity to stabilize unpaired electrons. The redox potential of individual flavonoid molecules depends upon the number and location of hydroxyl groups (*5*, *6*). Within multiphase systems such as emulsions, the polarity of both antioxidant and oxidizing species is important. For example, in model emulsion systems, polarity controls the partitioning of antioxidant within both the bulk phase and the oil-in-water phase (*7*, *8*). Oxidation in multiphase systems occurs readily at the interface of lipid and aqueous phases; thus, the interfacial properties of antioxidants can influence their ability to protect lipids. Lipophilic antioxidants generally protect best against lipophilic initiators, hydrophilic antioxidants protect best against hydrophilic initiators, and additive or synergistic effects occur with combinations of antioxidants of disparate polarities (*6*).

The partition coefficients of flavanol aglycones are positive, although published values vary. For example, the determined P^a for C was reported to be 2.93 (*9*) and calculated as 1.10 (*10*). Similar disparities occur in literature values for P^a of EC with a determined value of 1.45 (*9*) and a calculated value of 1.18 (*11*), respectively. In one study (*11*), the calculated value of $P^a = 1.18$ for EC was estimated to change to −0.67 by positing the addition of a −*O*-β-D-glucuronide moiety in the 5- or 7-position, whereas positing a 3′- or 4′-*O*-methylation increased the calculated P^a by 25% to 1.48. Variation in literature values for flavanol P^a values encourages empirical testing of the effects that structural changes such as *O*-methylation have on defined flavanol actions.

The flavanol antioxidant activity changes with each modification of ring structure and/or removal of a hydroxyl group.

* Author to whom correspondence should be addressed [telephone (979) 845-7537; fax (979) 845-1921; e-mail rwalzem@poultry.tamu.edu].
[†] Department of Horticultural Sciences, Texas A&M University.
[§] Department of Poultry Science, Texas A&M University.
[‡] University of California.
[#] H.B.G. is currently affiliated with the Department of Nutrition, University of California.

10.1021/jf060690b CCC: $33.50
Published on Web 10/06/2006

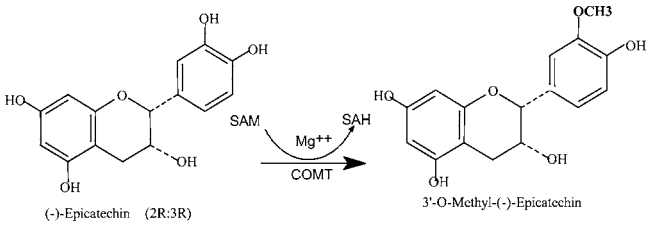

Figure 1. *O*-Methylation of catechol-containing polyphenols. COMT, catechol-*O*-methyl transferase; SAM, *S*-adenosyl methionine; SAH, *S*-adenosyl homocysteine.

Studies with humans show that ingested flavonoids are rapidly converted to glucuronide and sulfate conjugates (*12*). Following the ingestion of a single glass of red wine (~120 mL) containing 35 mg of C within a total phenolic dose of 103 mg, C was rapidly metabolized to a number of products, including its 3′-*O*-methyl derivative, glucuronide or sulfate conjugates, or complex conjugates containing varying combinations of all possible modifications (*2*, *12*). 3′-*O*-Methylcatechin accounted (3′-*O*-MC) for 20−22% of total plasma C 1 h after the consumption of wine and 12−14% 4 h after consumption. *O*-Methylation is a common modification that occurs at the 3′- and, less frequently, the 4′-positions (*13*). Catechol-*O*-methyl transferase (COMT, EC 2.1.1.6), a phase II enzyme, generates methylated derivatives of catechol-containing polyphenols (**Figure 1**), including *O*-methylated EC, C, (−)-catechin gallate **(**CG**)**, (−)-epigallocatechin, and quercetin (*14*). The enzyme COMT is a component of the normal xenobiotic metabolizing systems present in a variety of tissues, particularly in the gut, liver, and kidney (*15*, *16*).

Hydroxyl group loss from flavonoid molecules in vivo becomes particularly significant for biological activities that rely on redox potential because as the number of free hydroxyl groups decreases, so does the free radical sequestering capacity (*5*, *6*). *O*-Methylation of a hydroxyl group reduces the polarity of some flavonoids, which could result in closer interactions with the nonpolar components of individual lipoprotein particles. The present study sought to determine whether and how *O*-methylation affected the ability of flavanols to act as antioxidants in an in vitro assay of $CuSO_4$-catalyzed oxidation of model triacylglycerol-rich lipoproteins (VLDL*y*) (*17*, *18*). It was determined that *O*-methylation differently affected the antioxidant action on the basis of the polarity of parent compound and test concentration, notably, that *O*-methylated C and EC delayed the propagation rate of lipid oxidation at concentrations >1 μmol/L while increasing this same rate when tested at 0.25 μmol/L despite prolonging lag time.

MATERIALS AND METHODS

Chemicals. The stereoisomers, C and EC, which have an identical number of hydroxyl substituents, and CG, which is more polar due to its gallate substituent containing three hydroxyl groups, were tested for antioxidant activity. (+)-Catechin [2-(3,4-dihydroxyphenyl)-3,4-dihydro-2*H*-1-benzopyran], EC ([2*S*,3*S*]-2-[3,4-dihydro-1[2*H*]-benzopyran-3,5,7-triol]), CG ([2*S*,3*R*]-2-[3,4-dihydroxyphenyl]-3,4-dihydro-1[2*H*]-benzopyran-3,5,7-triol 3-[3,4,5-trihydroxybenzoate]), COMT (from porcine liver, 1000 units/mg, EC 2.1.1.0), *S*-adenosyl-L-methionine *p*-toluenesulfonate salt (SAM), and dithiothreitol (DTT) were obtained from Sigma Chemical Co. (St. Louis, MO). $MgCl_2$, ethyl acetate (HPLC grade), and acetyl nitrile (HPLC grade) were obtained from Fisher Scientific Co. (Fair Lawn, NJ). A 10% HCl solution was obtained from Mallinckrodt, Inc. (Paris, KY). Ammonium dihydrogen phosphate and hexanal (Spectra grade) were obtained from Aldrich Chemical Co., Inc. (Milwaukee, WI). Hexanal (Aldrich Chemical Co.) was purified prior to use by passing a sample of about 0.5 mL through a Sep-Pak silica cartridge (Waters Associates, Milford, MA) and sealing it in a sample bottle under nitrogen (*19*).

Table 1. Mobile Phase Composition during Separation of Parent Flavan-3-ol Compounds and Their *O*-Methyl Derivatives

elution time (min)	0–5	5–8	8–20	20–25	25–35	35–45	45–50	50–65
solvent A:B[a] (v/v)	100:0	92.8	86:14	83.5:16.5	78.5:21.5	67.5:32.5	50:50	100:0

[a] Solvent A was 50 mM ammonium dihydrogen phosphate, pH 2.6. Solvent B was 20% solvent A and 80% acetonitrile.

Table 2. Retention Times of Individual Flavan-3-ol Compounds Separated by HPLC

compound	retention time (min)
catechin	16.9
epicatechin	18.8
3′-*O*-methylcatechin	19.9
4′-*O*-methylcatechin	21.6
3′-*O*-methylepicatechin	22.1
4′-*O*-methylepicatechin	23.7

COMT-Catalyzed *O*-Methylations of Flavanols. *O*-Methylated flavanol derivatives were produced enzymatically using a modification of the method described by Zhu et al. (*14*). The reaction mixture consisted of 250 units of COMT, 1.2 μM $MgCl_2$, 2 mM SAM, 1 mM DTT, and 40 mM Tris buffer (pH 7.64) in a final volume of 1.0 mL. The reaction mixture was incubated at 37 °C for 5 min. The methylation reaction was started by the addition of 10 μmol of parent flavanol and was then incubated for 20 h in a light-free environment. The reaction was stopped by adding 15 μL of 10% HCl and cooling rapidly to 0 °C. Assay mixtures were centrifuged (Eppendorf 5415C) at 10000 rpm for 5 min at 4 °C. Blanks were prepared by addition of boiled COMT to reaction mixtures prior to incubation and used to facilitate identification of the retention times of novel peaks, that is, *O*-methylated compounds.

Supernatant fractions were filtered through a 0.45 μm filter prior to application to the HPLC for separation, identification, and collection of reaction products. About 30% of the parent compound was recovered as *O*-methylated product in this system.

Chromatographic Techniques. Parent compounds were separated from their *O*-methyl derivatives using a modification of the method described by Donovan et al. (*18*). Samples were injected in 20 μL aliquots using a Waters Wisp 712 autosampler with cooler. A Hewlett-Packard model 1050 quaternary pump was coupled to an ISCO V4 detector set for 280 nm (Agilent Technologies, Palo Alto, CA). Separation was accomplished on a stainless steel Lichrosphere 100 RP18 column, 250 mm × 4.6 mm, and with a 5 μm particle size (Alltech, Deerfield, IL). The mobile phase was heated to 40 °C and delivered at a rate of 0.5 mL/min and consisted of a multilinear gradient as shown in **Table 1**. The primary solvent (A) was 50 mM ammonium dihydrogen phosphate adjusted to pH 2.6. The secondary solvent (B) consisted of 20% of the primary solvent and 80% acetonitrile.

Purification of Methylated Flavonoids. Replicate assay mixtures were pooled into 6-mL aliquots and twice extracted with 12 mL of ethyl acetate. Extracts were evaporated to dryness under low temperature and vacuum (Buchi Rotary Evaporator, Rinco, Flawil, Switzerland) and redissolved in 0.5 mL of 0.01 N HCl, (pH 2). Fifty-microliter aliquots of the concentrated extract were injected into the HPLC, and peaks of interest were collected manually. Retention times for individual peaks are shown in **Table 2.** For each batch of pooled concentrated extract, absorption spectra (λ = 200−800 nm) were determined for individual peaks using a Beckman DU-50 series spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) to verify flavanol spectral profiles and the absence of contaminants prior to separation and collection of the material in the remaining nine aliquots. Except for EC, a single novel peak was found in the concentrated extract when compared to the elution profile of similarly concentrated extracts of reaction mixtures containing boiled COMT enzyme. Peaks collected

from individual chromatographic separations were pooled. *O*-Methylated EC yielded two novel peaks, which were collected separately for further testing. Within this chromatographic separation system the 4′-*O*-methyl derivative of EC elutes later than the 3′-*O*-methyl derivative (*18*). The late-eluting peak was assumed to be 4′-*O*-MEC. To collect sufficient material for kinetic analyses several HPLC fractionations were conducted for each compound. The eluting fractions containing a single novel peak from separate HPLC fractionations were pooled (~5 mL) and concentrated by extracting twice with 10 mL of ethyl acetate and evaporation to dryness. The residue was redissolved in 0.5 mL of 0.01 N HCl, and aliquots of the isolated compounds were rechromatographed to assess purity. Standard curves were prepared from parent compounds of the *O*-methyl flavanols. Area under the curve (AUC) was used as the response variable following HPLC separation under standard conditions. The AUC were assumed to be similar for equivalent molar amounts of parent compound and *O*-methylated derivative (*20*).

VLDL*y* Isolation. Blood from Hy-Line W-36 egg-laying hens (Hy-Line International, Lakeview, CA) was collected according to an approved protocol from the brachial vein using a 22-G needle and a 3-mL syringe and immediately transferred into 3-mL tubes containing EDTA placed on ice. Plasma was harvested within 2 h of collection following whole blood centrifugation at 1500*g* and 4 °C. Concentrated VLDL*y* was isolated as the $d < 1.02$ g/mL fraction of plasma by sequential density gradient ultracentrifugation exactly as described (*21*). The VLDL*y* particle is a lipoprotein particle with demonstrated utility in mechanistic investigations of biologic oxidation (22). The VLDL*y* lipoprotein is triacylglycerol-rich and of similar size to human remnant lipoproteins. Hens lacking receptors for VLDL*y* develop severe atherosclerosis (23). During the course of these studies VLDL*y* were more readily available and easily prepared than human material. Prior to use, isolated VLDL*y* was dialyzed in the dark for 30 h at 4 °C against three changes of 0.01 M phosphate−0.15 M NaCl (PBS), pH 7.4. The protein content of dialyzed VLDL*y* was determined according to the method described by Markwell et al. (*24*), using bovine serum albumin as a standard.

Hexanal Formation and Measurement. In an initial experiment, VLDL*y* oxidative stability was assessed by measuring hexanal formation resulting from the oxidative decomposition of n-6 fatty acids. Incubations conducted in duplicate contained 0.25-mL aliquots of VLDL*y* containing 1 mg/mL protein and 80 μM $CuSO_4$ within 6-mL headspace bottles held at 37 °C. Six-hour incubations were used as all were within the propagation phase of lipid oxidation and so gave comparable data. Incubations were conducted in the absence (control incubations) and presence of 5 μM flavanols **(**EC and CG**)** and their *O*-methyl derivatives. Incubated sample vials were inserted into the headspace sampler, heated to 40 °C, and pressurized with carrier gas for 30 s prior to injection of a 3 mL aliquot of the gas phase (headspace) by a stationary needle into a Perkin-Elmer Sigma 3B gas chromatograph fitted with an H-6 headspace sampler (Norwalk, CT; instrumentation generously provided by E. N. Frankel). Injected volatiles were separated using a DB-1701 capillary column (30 m × 0.32 mm, 1-μm thickness, J&W Scientific, Folsom, CA) heated isothermally to 80 °C. Injector temperature was held at 180 °C, and the detector temperature was 200 °C (*22*). Hexanal was identified by comparison of retention times with those of authentic reference compounds. Analyses were standardized daily with solutions of 10 μmol/L hexanal.

Conjugated Diene Formation and Measurement. Copper-catalyzed oxidation of VLDL*y* (50 μg of protein/mL in PBS) was monitored with a DU-640 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) with the temperature regulated to maintain 37 °C, by continuous measurement of absorbance at 234 nm following the addition of $CuSO_4$ (40 μM final concentration) as an initiator. Increases in absorbance at 234 nm are due to the formation of conjugated dienes in polyunsaturated fatty acids and provide an index of reversible lipid oxidation (*25*). The amount of time prior to an increase in Abs234 nm, termed lag time (minutes), and the rate of increase in Abs234 nm, termed propagation rate (ΔAbs234nm/min), were recorded (**Figure 2**). The minimum three replicates used for each determination exhibited uniform patterns of oxidation. Total assay volume was 1 mL with compound or sample blank being a 0.1 mL addition to the final assay volume. Flavanol additions were prepared by weighing the appropriate amount of material into a 5 mL volumetric flask for dissolution in 0.5 mL of acetonitrile; following dissolution, the material was brought to a final volume of 5 mL with deionized water. The final assay concentration of acetonitrile was held constant. Blank (control) assays were prepared to contain the same amount of water and acetonitrile as for flavanol (test) assays.

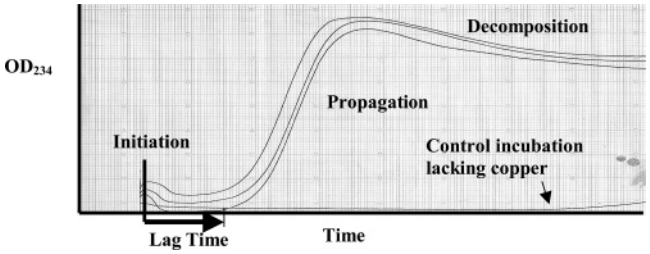

Figure 2. Conjugated diene formation in replicate VLDL*y* samples. The graph is labeled to show stages of oxidation and lack of oxidation in VLDL*y* incubations lacking $CuSO_4$. Initial tracing offsets can be seen at time 0.

Table 3. Effect of Parent Flavan-3-ol *O*-Methylation on Hexanal Formation during Copper-Initiated VLDL*y* Oxidation[a,b]

5 μM	parent	3′-*O*-methyl derivative	4′-*O*-methyl derivative
catechin gallate[c]	5954 ± 362 a	3929 ± 271 a,z	ND
epicatechin[c]	476 ± 70 a	2501 ± 152 a,z	7859 ± 648 a,z

[a] Values are expressed as mean ± SEM, $n = 3$. [b] Hexanal formation in vehicle control incubations lacking flavanols: 11907 ± 809 peak area standardized to a 10 μM hexanal solution. [c] a, significantly different from vehicle control incubation, $p < 0.01$; z, significantly different from parent compound, $p < 0.05$.

Statistical Analysis. Values are presented as means ± SEM of triplicate assays. Differences between means were evaluated by two-tailed *t* test assuming equal variances (Excel, Redmond, WA).

RESULTS

Hexanal Formation. Hexanal production showed two patterns of response to compound methylation. As shown in **Table 3**, addition of 5 μM EC prevented 96% of the hexanal formation observed in control samples incubated without flavanols. For EC, *O*-methylation reduced total antioxidant action as 5 μM 3′-*O*-MEC and 4′-*O*-MEC suppressed only 79 and 34% of control hexanal production, respectively, significantly less ($p < 0.05$) than parent EC. However, all EC compounds caused significant reductions in hexanal formation compared to control. In contrast, CG was able to suppress 50% of the hexanal formed in samples lacking flavanols ($p < 0.05$ compared to control), but *O*-MCG suppressed 67%, significantly more than CG (**Table 3**, $p < 0.05$ compared to CG).

Conjugated Diene Formation. C, EC, 3′-*O*-MC, and 3′-*O*-MEC increased lag time prior to lipid oxidation in VLDL*y*, albeit to differing degrees. As shown in **Figure 3**, 5 μM C increased lag time 6-fold, whereas an equivalent concentration of 3′-*O*-MC increased lag time 3-fold. Increasing 3-*O*-MC concentration to 10 μM did not further increase lag time (**Figure 3**). Both 5 and 10 μM 3′-*O*-MC reduced the propagation rate to ~40% of that observed in the control assays lacking flavanols or those assays containing 5 μM C (**Figure 3**). **Table 4** shows the relative abilities of 1.0 μM C, EC, 3′-*O*-MC, and 3′-*O*-MEC to prolong lag time and delay propagation rate. At this concentration, C and EC increased lag time >4-fold ($p < 0.001$), with the 5-fold increase observed with C being greater than the 4.3-fold increase observed with EC ($p < 0.01$). Both 3′-*O*-MC and 3′-*O*-MEC increased lag time by 1.6-fold (**Table 4**; $p < 0.002$**)**. Although

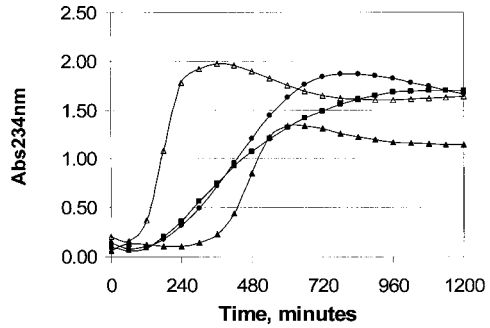

Figure 3. Effect of flavanols and their *O*-methyl metabolites on conjugated diene formation during copper-initiated VLDL*y* oxidation: (△) control without catechin; (▲) 5 μM catechin, C; (■) 5 μM 3′-*O*-methylcatechin, 3′-*O*-MC; (●) 10 μM 3′-*O*-MC.

Table 4. Effect of 1.0 μM Selected Flavan-3-ols and *O*-Methylated Metabolites on Lag Time and Propagation Rate of Conjugated Diene Formation in Copper-Initiated VLDL*y* Oxidation[a]

compound	lag time[b] (min)	propagation rate[b] ($Abs_{234} \times 10^{-3}$/min)
catechin	730 ± 6.6 a	1.5 ± 0.02 a
epicatechin	628 ± 5.8 b	1.5 ± 0.02 a
3′-*O*-methylcatechin	230 ± 3.3 c	1.2 ± 0.02 b
3′-*O*-methylepicatechin	230 ± 2.9 c	0.9 ± 0.02 c
control[c]	146 ± 1.9 d	1.5 ± 0.02 a

[a] Values are expressed as mean ± SEM, $n = 3$. [b] Values within a column not sharing a common letter are significantly different, $p < 0.05$. [c] Vehicle control incubations lack flavanols.

Table 5. Effect of Increasing Concentrations of *O*-Methylated Flavanols on Lag Time (Minutes) Prior to Conjugated Diene Formation during Copper-Initiated VLDL*y* Oxidation[a,b]

	0.25 μM	0.5 μM	1.0 μM	2.0 μM
3′-*O*-methylcatechin[c,d]	173 ± 1.9	180 ± 3.3	183 ± 1.9 a	190 ± 3.3 a
3′-*O*-methylepicatechin[c,d]	175 ± 1.7	180 ± 1.7	180 ± 2.9	192 ± 4.2 a

[a] Values are expressed as mean ± SEM, $n = 3$. [b] Lag time in vehicle control incubations lacking flavanol: 123 ± 3 min. [c] All values are significantly different from control, $p < 0.05$. [d] a, value significantly different from 0.25 μM.

1.0 μmol/L *O*-methylated flavanols suppressed the propagation rate of lipid oxidation ($p < 0.001$), the parent flavanols did not (**Figure 3**; **Table 4**). A dose−response analysis at lower concentrations (**Table 5**) showed that within more physiologically relevant concentrations (0.25−2.0 μM), both 3′-*O*-MC and 3′-*O*-MEC produced modest but significant 1.4−1.55-fold increases in lag time compared to control incubations. The degree of propagation inhibition caused by 5 μM 3′-*O*-MC was similar to that produced with 2 μM 3′-*O*-MC (**Figure 3**; **Table 6**). Interestingly, propagation rate increased by 24% ($p < 0.01$) in the presence of 0.25 μM 3′-*O*-MC and was unaffected by 0.50 μM 3′-*O*-MC (**Table 6**). At 1.0−2.0 mM 3′-O-MC propagation rates were significantly ($p < 0.001$) and progressively reduced to 87 and 53% of control assay values, respectively (**Table 6**). Similar effects were seen with 3′-*O*-MEC, with which 0.25 μM increased the propagation rate by 20% ($p < 0.01$), 0.50 μM had no significant effect (7% decrease), but concentrations of 1.0−2.0 μM 3′-*O*-MEC decreased propagation rates to 73% ($p < 0.001$) and 40% ($p < 0.001$) of control values, respectively. The degree of reduction in propagation rates for 3′-*O*-MEC was significantly less ($p < 0.05$) than that of 3′-*O*-MC at comparable concentrations.

Table 6. Effect of Increasing Concentrations of *O*-Methylated Flavanols on Propagation Rates ($Abs_{234} \times 10^{-3}$/min) of Conjugated Diene Formation in Copper-Initiated VLDL*y* Oxidation[a,b]

	0.25 μM	0.5 μM	1.0 μM	2.0 μM
3′-*O*-methylcatechin[c,d]	1.86 ± 0.05 a	1.53 ± 0.02 b	1.30 ± 0.02 c,x	0.80 ± 0.03 d,x
3′-*O*-methylepicatechin[c,d]	1.80 ± 0.10 a	1.40 ± 0.10 a	1.10 ± 0.04 b,y	0.60 ± 0.02 c,y

[a] Values are expressed as mean ± SEM, $n = 3$. [b] Propagation rate in vehicle control incubations lacking flavanols: 1.50 ± 0.02. [c] a–d, values across rows not sharing a common letter are significantly different, $p < 0.01$. [d] x,y, values within a column not sharing a common letter are significantly different, $p < 0.05$.

Neither C or EC inhibited the propagation rate at any concentration tested.

DISCUSSION

The results of this study indicate that *O*-methylation can impair or improve antioxidant action depending on parent compound structure and placement of the *O*-methylated group and compound concentration. Differences in the ability of parent compounds to modify lipid oxidation following the addition of a nonpolar substituent is also seen in comparisons between retinol and retinyl palmitate (*26*). We selected several flavanols, C, EC, and CG, on the basis of chemical and nutritional properties. Catechin and EC are predominant dietary flavan-3-ols for which substantial bioavailability information exists. Catechin gallate is a significant component of many black teas, but a minor component of green teas (*27*). There is less information on CG absorption and metabolism, although simultaneous estimates of pharmacokinetic parameters of C, EC, and CG from rats fed green tea are available (*28*).

At higher concentrations (5 μmol/L) the net effect of *O*-methylation appeared to reflect the counterbalancing of two effects, namely, reduced hydroxyl number and polarity change. For a compound such as CG, it appears that loss of one of its eight hydroxyl groups from participation in electron stabilization was less important than the reduction in polarity that likely improved target−antioxidant interactions in this in vitro assay system. However, *O*-methylation of even one of the five hydroxyl groups found on EC reduced its ability to inhibit oxidative decomposition as measured by hexanal formation in vitro. Moreover, the effect of *O*-methylation appears to be position dependent as 4′-*O*-MEC was much less able to prevent hexanal formation than 3′-*O*-MEC. As was observed with conjugation reactions such as glucuronidation (*29*), conversion of a hydroxyl to an *O*-methyl group reduces the ability of C and EC to prevent initiation of lipid oxidation in vitro as indicated by lag time prior to detectable conjugated diene formation. However, unlike glucuronidation, *O*-methylation changed flavanol chemical antioxidant functionality such that propagation rate was increased at 0.25 μmol/L and reduced at concentrations ≥1.0 μmol/L, despite nearly equivalent increases in lag time at all concentrations tested. This observation is novel and suggests that 0.25 μmol/L represents a concentration at which oxidative stability is enhanced but allows accumulation of radicals that absorb at wavelengths other than 234 nm (*30*) and subsequently promotes a higher propagation rate for lipid oxidation. Other possibilities exist and may be particularly important. In the more complex biological matrices, such as those of intact animals and humans, other interactions and outcomes may exist (*31*). Assessing the biological relevance of these various possibilities requires experiments designed to specifically address these questions.

There are significant implications for these observations, despite the seeming inevitability of propagation and decomposition phases after free radical initiation in vitro. Whereas extrapolation of in vitro results directly to in vivo situations rarely succeeds, reduced propagation rates may become biologically significant. Oxidant-associated chronic diseases such as atherosclerosis arise from in vivo oxidative stress that is not constant in its intensity (*32*) and varies in source (*11*). These oxidative processes occur within the context of active repair processes (*33*). Reduced propagation of lipid oxidation in lipoproteins such as VLDL*y* or LDL could allow sufficient time for repair mechanisms to operate and limit the damage caused by oxidative insult (*34*, *35*). Improvement in the antioxidant activity of CG following *O*-methylation supports the hypothesis that a closer association between lipoprotein particles and *O*-methylated metabolites of polar parent flavonoids can enhance oxidative protection despite loss of a free hydroxyl moiety. Flavonoids and phenolic compounds including C, CG, EC, and quercetin as shown in the present study and by others (*2*, *15*, *16*, *29*) are metabolized by COMT to produce *O*-methylated derivatives. *O*-Methylated quercetin derivatives retain some bioactivity in cell-based assays (*29*). This is notable as the biological actions of flavonoids are achieved by a variety of mechanisms in addition to the direct chemical antioxidant actions assessed in the present study (*1*, *36*).

Dietary intake and compound pharmacokinetics are other important considerations when in vivo events are hypothesized from in vitro observations. It is known that C, in the amounts in a typical glass of wine (~35 mg), is rapidly converted to a number of metabolites, including methylated derivatives. Nonconjugated 3′-*O*-MC (i.e., lacking glucuronide or sulfate moieties) constituted 7% of plasma total *O*-MC derivatives 1 h after the conumption of wine and 5% 3−4 h after (*12*). However, total amounts of nonconjugated C and 3′-*O*-MC never amounted to more than 2% of plasma total C derivatives. Total *O*-methylated derivatives of C accounted for about 20% of plasma total C in that study (*12*). Moreover, in that study, the concentration of 3′-O-MC remained in the nanomolar range. Manach et al. (*17*) surveyed the available literature and found values for C plasma concentrations of 0.14−0.49 μmol/L in humans given 0.36 mg of pure catechin/kg of body weight. Reported concentrations in excess of 1.0 μmol/L (2−7.8 μmol/L) were achieved with relatively large flavanol doses (>500 mg). Plasma EC concentrations reportedly exceed 1.0 μmol/L more often (*17*), with published concentrations reaching 5.9 μmol/L. Individual metabolite forms were not reported in this review, and it is noteworthy that the majority of *O*-methyl derivatives are also conjugated to either glucuronide or sulfate moieties (*3*) in vivo. Thus, a first step in moving the present observations to the in vivo setting would be to assess the chemical antioxidant actions of glucuronide and sulfate conjugates of *O*-methyl derivatives.

Species differences occur in flavonoid metabolism, and care must be taken in cross-species extrapolations. Rats have been widely used to evaluate flavonoid absorption (*10*, *37*), and these efforts have greatly improved our general understanding of flavonoid absorption. However, rat metabolites have a different chemical structure than those of humans (*38*), and unlike humans, rats do not have a gall bladder and the liver is not a site of flavonoid accumulation (*10*). Comparisons between rats and pigs, the latter have gall bladders, showed that pig liver accumulated more of the flavonol quercetin than did rats, although *O*-methylated flavonoids were widely distributed in rat and pig tissues in a dietary-concentration-dependent fashion (*39*). Reduced risk for atherosclerosis is most commonly associated with higher flavanol intakes, but rats are a relatively poor model for this disease. Hamsters are a better model for this disease process (*40*) due to similarities between human and hamster lipoprotein metabolism and dietary responses. Data on flavonoid bioavailability in hamsters is limited (*41*) but exhibited similarities to humans'. More information on flavonoid absorption and metabolism in this animal model would be very helpful in understanding the relationship between dietary flavanols and atherosclerosis.

The results of the present study and growing information on the bioavailability, metabolism, and pharmacokinetics of individual metabolites of absorbed flavonoids underscore the need to expand the assessment of biological activities to include the testing of physiological concentrations of flavonoid metabolites in addition to parent compounds. In this regard it is exciting to speculate that the increase in lipoprotein lipid propagation at more physiological metabolite concentrations despite a prolongation of lag time represents a sensitive model system to test additional interacting factors that may tip the balance between protection and oxidative stress. Such studies could improve our understanding of the chemical processes that contribute to or limit lipoprotein oxidative stability.

ABBREVIATIONS USED

C, catechin; CG, catechin gallate; COMT, catechol-*O*-methyl transferase; EC, epicatechin; 3′-*O*-MC, 3′-*O*-methylcatechin; 3′-*O*-MCG, 3′-*O*-methylcatechin gallate; 3′-*O*-MEC, 3′-*O*-methylepicatechin; 4′-*O*-MEC, 4′-*O*-methylepicatechin; VLDL*y*, yolk targeted very low-density lipoprotein.

LITERATURE CITED

(1) German, J. B.; Walzem, R. L. The health benefits of wine. *Annu. Rev. Nutr.* **2000**, *20*, 561−593.

(2) Bell, J. R.; Donovan, J. L.; Wong, R.; Waterhouse, A. L.; German, J. B.; Walzem, R. L.; Kasim-Karakas, S. E. (+)-Catechin in human plasma after ingestion of a single serving of reconstituted red wine. *Am. J. Clin. Nutr.* **2000**, *71* (1), 103−108.

(3) Williamson, G.; Barron, D.; Shimoi, K.; Terao, J. In vitro biological properties of flavonoid conjugates found in vivo. *Free Radical Res.* **2005**, *39* (5), 457−469.

(4) Chiu, F. L.; Lin, J. K. HPLC analysis of naturally occurring methylated catechins, 3″- and 4″-methyl-epigallocatechin gallate, in various fresh tea leaves and commercial teas and their potent inhibitory effects on inducible nitric oxide synthase in macrophages. *J. Agric. Food Chem.* **2005**, *53*, 7035−7042.

(5) Cao, G.; Sofic, E.; Prior, R. L. Antioxidant and prooxidant behavior of flavonoids: structure−activity relationships. *Free Radical Biol. Med.* **1997**, *22* (5), 749−760.

(6) Frankel, E. N. *Lipid Oxidation*; The Oily Press: Dundee, U.K., 1998.

(7) Schwarz, K.; Frankel, E. N.; German, J. B. Partition behaviour of antioxidative phenolic compounds in heterophasic systems. *Fett. Lipid* **1996**, *98*, 115−121.

(8) Frankel, E. N. Interfacial lipid oxidation and antioxidation. *J. Oleo Sci.* **2001**, *50*, 387−391.

(9) Schroeder, P.; Klotz, L. O.; Sies, H. Amphiphilic properties of (−)-epicatechin and their significance for protection of cells against peroxynitrite. *Biochem. Biophys. Res. Commun.* **2003**, *307* (1), 69−73.

(10) Crespy, V.; Morand, C.; Besson, C.; Cotelle, N.; Vezin, H.; Demigne, C.; Remesy, C. The splanchnic metabolism of flavonoids highly differed according to the nature of the compound. *Am. J. Physiol. Gastrointest. Liver Physiol.* **2003**, *284* (6), G980−G988.

(11) Yeum, K. J.; Aldini, G.; Chung, H. Y.; Krinsky, N. I.; Russell, R. M. The activities of antioxidant nutrients in human plasma depend on the localization of attacking radical species. *J. Nutr.* **2003**, *133* (8), 2688−2691.

(12) Donovan, J. L.; Bell, J. R.; Kasim-Karakas, S.; German, J. B.; Walzem, R. L.; Hansen, R. J.; Waterhouse, A. L. Catechin is present as metabolites in human plasma after consumption of red wine. *J. Nutr.* **1999**, *129* (9), 1662−1658.

(13) Donovan, J. L.; Kasim-Karakas, S.; German, J. B.; Waterhouse, A. L. Urinary excretion of catechin metabolites by human subjects after red wine consumption. *Br. J. Nutr.* **2002**, *87* (1), 31−37.

(14) Zhu, B. T.; Patel, U. K.; Cai, M. X.; Lee, A. J.; Conney, A. H. Rapid conversion of tea catechins to monomethylated products by rat liver cytosolic catechol-*O*-methyltransferase. *Xenobiotica* **2001**, *31* (12), 879−890.

(15) Scalbert, A.; Williamson, G. Dietary intake and bioavailability of polyphenols. *J. Nutr.* **2000**, *130* (8S Suppl.), 2073S−2085S.

(16) Manach, C.; Texier, O.; Morand, C.; Crespy, V.; Regerat, F.; Demigne, C.; Remesy, C. Comparison of the bioavailability of quercetin and catechin in rats. *Free Radical Biol. Med.* **1999**, *27* (11−12), 1259−1266.

(17) Manach, C.; Williamson, G.; Morand, C.; Scalbert, A.; Remesy, C. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **2005**, *81* (1 Suppl.), 230S−242S.

(18) Donovan, J. L.; Luthria, D. L.; Stremple, P.; Waterhouse, A. L. Analysis of (+)-catechin, (−)-epicatechin and their 3′- and 4′-O-methylated analogs. A comparison of sensitive methods. *J. Chromatogr. B: Biomed. Sci. Appl.* **1999**, *726* (1−2), 277−283.

(19) Frankel, E. N.; Hu, M. L.; Tappel, A. L. Rapid headspace gas chromatography of hexanal as a measure of lipid peroxidation in biological samples. *Lipids* **1989**, *24* (11), 976−981.

(20) Studer, A.; Stupperich, E.; Vuilleumier, S.; Leisinger, T. Chloromethane: tetrahydrofolate methyl transfer by two proteins from *Methylobacterium chloromethanicum* strain CM4. *Eur. J. Biochem.* **2001**, *268* (10), 2931−2938.

(21) Walzem, R. L.; Davis, P. A.; Hansen, R. J. Overfeeding increases very low density lipoprotein diameter and causes the appearance of a unique lipoprotein particle in association with failed yolk deposition. *J. Lipid Res.* **1994**, *35* (8), 1354−1366.

(22) Walzem, R. L.; Watkins, S.; Frankel, E. N.; Hansen, R. J.; German, J. B. Older plasma lipoproteins are more susceptible to oxidation: a linking mechanism for the lipid and oxidation theories of atherosclerotic cardiovascular disease. *Proc. Natl. Acad. Sci. U.S.A.* **1995**, *92* (16), 7460−7464.

(23) Ho, K. J.; Lawrence, W. D.; Lewis, L. A.; Liu, L. B.; Taylor, C. B. Hereditary hyperlipidemia in nonlaying chickens. *Arch. Pathol.* **1974**, *98* (3), 161−172.

(24) Markwell, M. A.; Haas, S. M.; Bieber, L. L.; Tolbert, N. E. A modification of the Lowry procedure to simplify protein determination in membrane and lipoprotein samples. *Anal. Biochem.* **1978**, *87* (1), 206−210.

(25) Esterbauer, H.; Striegl, G.; Puhl, H.; Rotheneder, M. Continuous monitoring of in vitro oxidation of human low density lipoprotein. *Free Radical Res. Commun.* **1989**, *6* (1), 67−75.

(26) Livrea, M. A.; Tesoriere, L.; Bongiorno, A.; Pintaudi, A. M.; Ciaccio, M.; Riccio, A. Contribution of vitamin A to the oxidation resistance of human low density lipoproteins. *Free Radical Biol. Med.* **1995**, *18* (3), 401−409.

(27) Henning, S. M.; Fajardo-Lira, C.; Lee, H. W.; Youssefian, A. A.; Go, V. L.; Heber, D. Catechin content of 18 teas and a green tea extract supplement correlates with the antioxidant capacity. *Nutr. Cancer* **2003**, *45* (2), 226−235.

(28) Chu, K. O.; Wang, C. C.; Chu, C. Y.; Rogers, M. S.; Choy, K. W.; Pang, C. P. Determination of catechins and catechin gallates in tissues by liquid chromatography with coulometric array detection and selective solid phase extraction. *J. Chromatogr. B: Anal. Technol. Biomed. Life Sci.* **2004**, *810* (2), 187−195.

(29) Spencer, J. P.; Kuhnle, G. G.; Williams, R. J.; Rice-Evans, C. Intracellular metabolism and bioactivity of quercetin and its in vivo metabolites. *Biochem. J.* **2003**, *372* (Part 1), 173−181.

(30) Filipe, P.; Morliere, P.; Patterson, L. K.; Hug, G. L.; Maziere, J. C.; Maziere, C.; Freitas, J. P.; Fernandes, A.; Santus, R. Mechanisms of flavonoid repair reactions with amino acid radicals in models of biological systems: a pulse radiolysis study in micelles and human serum albumin. *Biochim. Biophys. Acta* **2002**, *1572* (1), 150−162.

(31) Filipe, P.; Haigle, J.; Silva, J. N.; Freitas, J.; Fernandes, A.; Maziere, J. C.; Maziere, C.; Santus, R.; Morliere, P. Anti- and pro-oxidant effects of quercetin in copper-induced low density lipoprotein oxidation. Quercetin as an effective antioxidant against pro-oxidant effects of urate. *Eur. J. Biochem.* **2004**, *271* (10), 1991−1999.

(32) Sies, H.; Stahl, W.; Sevanian, A. Nutritional, dietary and postprandial oxidative stress. *J. Nutr.* **2005**, *135* (5), 969−972.

(33) Martinet, W.; Knaapen, M. W.; De Meyer, G. R.; Herman, A. G.; Kockx, M. M. Oxidative DNA damage and repair in experimental atherosclerosis are reversed by dietary lipid lowering. *Circ. Res.* **2001**, *88* (7), 733−739.

(34) Mackness, M. I.; Durrington, P. N.; Mackness, B. How high-density lipoprotein protects against the effects of lipid peroxidation. *Curr. Opin. Lipidol.* **2000**, *11* (4), 383−388.

(35) Fuhrman, B.; Aviram, M. Preservation of paraoxonase activity by wine flavonoids: possible role in protection of LDL from lipid peroxidation. *Ann. N.Y. Acad. Sci.* **2002,** *957*, 321−324.

(36) Shay, N. F.; Banz, W. J. Regulation of gene transcription by botanicals: novel regulatory mechanisms. *Annu. Rev. Nutr.* **2005**, *25*, 297−315.

(37) Donovan, J. L.; Crespy, V.; Manach, C.; Morand, C.; Besson, C.; Scalbert, A.; Remesy, C. Catechin is metabolized by both the small intestine and liver of rats. *J. Nutr.* **2001**, *131* (6), 1753−1757.

(38) Natsume, M.; Osakabe, N.; Oyama, M.; Sasaki, M.; Baba, S.; Nakamura, Y.; Osawa, T.; Terao, J. Structures of (−)-epicatechin glucuronide identified from plasma and urine after oral ingestion of (−)-epicatechin: differences between human and rat. *Free Radical Biol. Med.* **2003**, *34* (7), 840−849.

(39) de Boer, V. C.; Dihal, A. A.; van der Woude, H.; Arts, I. C.; Wolffram, S.; Alink, G. M.; Rietjens, I. M.; Keijer, J.; Hollman, P. C. Tissue distribution of quercetin in rats and pigs. *J. Nutr.* **2005**, *135* (7), 1718−1725.

(40) El-Swefy, S.; Schaefer, E. J.; Seman, L. J.; van Dongen, D.; Sevanian, A.; Smith, D. E.; Ordovas, J. M.; El-Sweidy, M.; Meydani, M. The effect of vitamin E, probucol, and lovastatin on oxidative status and aortic fatty lesions in hyperlipidemic-diabetic hamsters. *Atherosclerosis* **2000**, *149* (2), 277−286.

(41) Chen, C. Y.; Milbury, P. E.; Lapsley, K.; Blumberg, J. B. Flavonoids from almond skins are bioavailable and act synergistically with vitamins C and E to enhance hamster and human LDL resistance to oxidation. *J. Nutr.* **2005**, *135* (6), 1366−1373.

Received for review March 10, 2006. Revised manuscript received August 30, 2006. Accepted September 3, 2006. We gratefully acknowledge the support of an unrestricted gift from the E&J Gallo Co. Support was also provided by the Texas Agricultural Experiment Station, Project 8738 (R.L.W.).

JF060690B