Procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the consumption of a flavanol-rich cocoa[1–3]

Roberta R Holt, Sheryl A Lazarus, M Cameron Sullards, Qin Yan Zhu, Derek D Schramm, John F Hammerstone, Cesar G Fraga, Harold H Schmitz, and Carl L Keen

ABSTRACT

Background: Epidemiologic studies have linked flavonoid-rich foods with a reduced risk of cardiovascular mortality. Some cocoas are flavonoid-rich and contain the monomeric flavanols (−)-epicatechin and (+)-catechin and oligomeric procyanidins formed from these monomeric units. Both the monomers and the oligomers have shown potential in favorably influencing cardiovascular health in in vitro and preliminary clinical studies. Although previous investigations have shown increasing concentrations of (−)-epicatechin in human plasma after cocoa consumption, no information is available in the published literature regarding the presence of procyanidins in human plasma.

Objective: This study sought to determine whether procyanidins can be detected and quantified in human plasma after acute consumption of a flavanol-rich cocoa.

Design: Peripheral blood was obtained from 5 healthy adult subjects before (baseline, 0 h) and 0.5, 2, and 6 h after consumption of 0.375 g cocoa/kg body wt as a beverage. Plasma samples were analyzed for monomers and procyanidins with the use of reversed-phase HPLC with coulometric electrochemical array detection and liquid chromatography–tandem mass spectrometry.

Results: Procyanidin dimer, (−)-epicatechin, and (+)-catechin were detected in the plasma of human subjects as early as 0.5 h (16 ± 5 nmol/L, 2.61 ± 0.46 μmol/L, and 0.13 ± 0.03 μmol/L, respectively) after acute cocoa consumption and reached maximal concentrations by 2 h (41 ± 4 nmol/L, 5.92 ± 0.60 μmol/L, and 0.16 ± 0.03 μmol/L, respectively).

Conclusion: Dimeric procyanidins can be detected in human plasma as early as 30 min after the consumption of a flavanol-rich food such as cocoa. *Am J Clin Nutr* 2002;76:798–804.

INTRODUCTION

A diet rich in fruit, nuts, and vegetables has been associated with a reduced risk of cardiovascular disease (1, 2). In addition to vitamins C, E, and the carotenoids, fruit, nuts, and vegetables contain a complex array of phenolic compounds that may contribute to increases in the plasma antioxidant capacity (3–5). Although the extent to which an enhanced plasma antioxidant capacity may contribute to improved health is a subject of debate, most investigators view increases in oxidant defense as positive. Flavonoids represent one class of phenolic compounds that has attracted considerable attention. Epidemiologic studies suggest that the consumption of a flavonoid-rich diet reduces an individual's risk of cardiovascular morbidity and mortality (6–8). Cocoa can be a rich source of flavonoids and was historically used as a medicine to combat inflammation, pain, and numerous other ailments (9). Like wine and tea, cocoa flavanols consist of a complex mixture of the monomeric (+)-catechin (catechin) and (−)-epicatechin (epicatechin) and the oligomers of these monomeric base units known as procyanidins (10).

In in vitro systems, extracts of cocoa flavanols and their oligomers have the ability to inhibit LDL oxidation (11), prevent peroxynitrite-dependent oxidation and nitration (12, 13), promote endothelium-dependent relaxation (14), and modulate the production of inflammatory cytokines (15, 16). The in vitro effects observed with the monomers are often significantly different from those observed with the oligomers, suggesting that there may be considerable structure specificity for these compounds. Although the potent in vitro effects of the flavanols and their oligomers have generated considerable interest in these compounds, it has been suggested that their in vivo effects may be minimal because of gastric degradation (17). Thus, it is important to determine whether the oligomers can be absorbed from the diet.

In a recent study (18) we used a sensitive and selective method to measure plasma epicatechin concentrations after acute cocoa consumption. In the current study we modified this method to enable us to simultaneously measure epicatechin, catechin and the procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] in human plasma after the acute consumption of a flavanol-rich cocoa. Furthermore, the chemical identity of these compounds

was assessed by liquid chromatography–tandem mass spectrometry (LC-MS/MS).

SUBJECTS AND METHODS

Subjects and clinical study design

Five nonsmoking adult volunteers (3 men and 2 women aged 23–34 y with an average body weight of 70.5 ± 4.6 kg) with no apparent disease participated in the study. The health status of all participants was evaluated via questionnaire, and written, informed consent was given before participation in the study. The study protocol was conducted, and approved, according to guidelines set by the Human Subjects Review Committee of the University of California, Davis.

Subjects were asked to abstain from alcohol, analgesics, and flavonoid-rich foods (eg, fruit, vegetables, nuts, coffee, tea, juice, and chocolate) on the day before the experiment. Baseline samples were drawn after a 12-h evening fast. The subjects then consumed 0.375 g cocoa (Cocoapro; Mars Inc, Hackettstown, NJ)/kg body wt in 300 mL water. One gram of the cocoa provided 12.2 mg monomers, 9.7 mg dimers, and 28.2 mg procyanidins (trimers through decamers) (3). On average, the subjects ingested a total dose of 26.4 g cocoa, providing 323 mg monomers and 256 mg dimers. In addition to the cocoa beverage, the subjects were allowed to consume white bread.

Blood samples were drawn via venipuncture at baseline (0 h) and 0.5, 2, and 6 h after cocoa consumption. Blood was collected into two 10-mL Vacutainer tubes (Becton Dickinson, Franklin Lakes, NJ) containing sodium heparin as an anticoagulant. Blood was centrifuged at $1500 \times g$ at $4\,°C$ for 10 min, and the plasma was separated and frozen at $-80\,°C$ until analyzed.

Determination of plasma catechin, epicatechin, and procyanidin dimers

Chemicals were purchased from Sigma Chemical Co (St Louis) unless otherwise stated. Plasma samples were extracted as described by Richelle et al (19) and Rein et al (18). The resulting solution was filtered with a 0.22-µm Ultrafree-MC low-binding Durapore centrifugal filter (Millipore, Bedford, MA) and centrifuged ($10\,000 \times g$, 5 min, $4\,°C$); 50 µL of the filtered solution was analyzed for catechin, epicatechin, and procyanidin dimers by reversed-phase HPLC with coulometric multiple-array detection.

Chromatography was carried out by using an HP 1100 HPLC system with Chemstation software, equipped with a quaternary pump, temperature-controlled autosampler, column oven, and diode array detector (Hewlett-Packard, Wilmington, DE) in series with a CoulArray 5600 detector (ESA, Chelmsford, MA). Separation was achieved by using a reversed-phase Alltima C_{18} column (5 µm, 150 mm $\times$ 4.6 mm; Alltech Associates, Deerfield, IL) with a C_{18} 5-µm guard column (Alltech Associates) . The mobile phase was mixed with 2 solvents: solvent A [40% methanol, 60% 100 mmol sodium acetate/L in water (EM Sciences, Darmstadt, Germany), pH 5.0] and solvent B (7% methanol, 93% 100 mmol sodium acetate/L in water, pH 5.0). A gradient elution was used at a flow rate of 1 mL/min with the initial concentration of solvent A set at 20%, which was held until 2 min. This was followed by a linear increase to 40% solvent A by 10 min, immediately followed with another linear increase to 85% solvent A by 13 min. The system was held at 85% of solvent A until 17 min, at which time a linear increase to 100% solvent A was achieved by 20 min. The system

was then linearly decreased to 40% solvent A by 23 min, followed by another linear decrease to 20% solvent A by 25 min.

Coulometric electrochemical array detection was carried out by using the following cell settings: −50, 65, 150, 200, 250, 300, 700, and 800 mV. The resulting chromatographs were analyzed by using CoulArray for WINDOWS software (ESA, Chelmsford, MA). Identification of epicatechin and catechin at 150 mV was based on coelution with authentic standards and quantified by using external standards. Identification of the procyanidin dimer peak at 700 mV was based on coelution with authentic standards and quantified by using external standards extracted from cocoa (Cocoapro) (3, 20).

Confirmation of procyanidin dimers with liquid chromatography–tandem mass spectrometry

Experiments were performed on a Sciex API 3000 triple quadrupole mass spectrometer (Perkin-Elmer, Norwalk, CT) equipped with a turbo ionspray source. All experiments were performed in the negative ion mode. The ionspray needle was held at −4500 V while the inlet voltage (orifice) was kept at −65 V to minimize collisional decomposition of molecular ions before entry into the first quadrupole.

Molecular ions of dimer were identified by simple MS analysis of standard solutions. Product ion spectra of these species were acquired by using Q1 to pass the molecular ion of interest. Nitrogen was used to collisionally activate precursor ion decomposition in the second quadrupole, which was offset from the first quadrupole by 35 eV. Subsequently, formed product ions were then detected by scanning the third quadrupole.

Quantitative MS/MS data for procyanidin dimers was acquired by multiple reaction monitoring experiments in which the first quadrupole was set to pass a specific precursor ion mass-to-charge ratio (m/z) and the third quadrupole was set to pass a structurally distinctive product ion m/z. The dimer transitions were 577.3/407.0, 577.3/289.0, and 577.3/125.0. Each transition was optimized with regard to both ionization and collision energy to minimize collisional decomposition of molecular ions before entry into the first quadrupole and maximize formation of structurally distinctive product ions. The dwell time for multiple reaction monitoring transitions of each individual molecular species was 30 ms when both monomer and dimer were monitored and 60 ms when only the dimer was monitored.

Procyanidin dimer was confirmed in the plasma by using reversed-phase LC-MS/MS. A 100-µL aliquot of the plasma extract in 0.25 mol perchloric acid/L in water was diluted to a final volume of 200 µL with water:methanol (90:10 by vol). A 50-µL volume was injected onto a 150 $\times$ 2.1 mm Discovery C_8 column (Supelco, Bellefonte, PA) at a flow rate of 300 µL/min. Solvent A was water:methanol (90:10 by vol) and solvent B was 100% methanol. The column eluent was directly infused into the ion source of the mass spectrometer, which was operated at $500\,°C$ with a 6 L/min nebulizing nitrogen gas flow. The elution protocol consisted of column preequilibration with 100% solvent A for 3 min followed by sample injection, 7 min at 100% solvent A, a 1-min linear gradient to 100% solvent B, and a 7-min wash with 100% solvent B.

Statistical analyses

Data were analyzed for differences by one-way analysis of variance or Kruskal-Wallis one-way analysis of variance on ranks (SIGMASTAT for WINDOWS, version 2.03; SPSS, Richmond, CA). When appropriate, Tukey's or Dunn's all-pairwise-comparison tests were used to identify differences between baseline and results 0.5, 2, and 6 h after cocoa consumption. Statistical significance was set at $P < 0.05$.

RESULTS

Analytic determination of epicatechin, catechin, and procyanidin dimers

The sensitivity and selectivity of coulometric electrochemical array detection allowed us to measure not only epicatechin but also catechin and the procyanidin dimers B2 and B5. A multiple-array chromatograph for standards of catechin and epicatechin [peaks 1 (12.6 min) and 2 (16.9 min), respectively] is depicted in **Figure 1**A. Two procyanidin dimer peaks were found in the standard that was purified from cocoa; we previously characterized these 2 peaks by nuclear magnetic resonance (21) as dimers B2 and B5 [peak 3 (14.7 min) and peak 4 (20.3 min), respectively; Figure 1B]. Both epicatechin and catechin exhibit peak oxidation responses at 150 mV followed by a second peak oxidation response at 700 mV. The sample preparation and chromatography did not fully resolve desired analytes from other coeluting plasma components. However, the detection system selectively detected catechin and epicatechin at the lower (150 mV) potential. A similar strategy was used with the procyanidin dimers; however, to achieve greater sensitivity, 700 mV was chosen as the analytic potential. By choosing these 2 analytic potentials we were able to measure plasma epicatechin and catechin and to simultaneously detect low concentrations of dimer. A typical chromatograph at 150 and 700 mV for a 2-h plasma sample is depicted in Figure 1C.

The coulometric detection response was linear for on-column standard curves with the use of the purified dimer B2 extract between 290 and 29 ng. When plasma was spiked with purified extracts of dimer, the recovery of B2 dimer was 103%. As previously reported, the recovery for catechin and epicatechin was between 70% and 90% (18). The extraction procedure did not yield additional dimer from plasma spiked with epicatechin and catechin. The relative SDs for unspiked samples of 7 consecutive analyses of dimer B2, catechin, and epicatechin were 12%, 7%, and 4%, respectively. The limits of detection for dimer B2 and dimer B5 on-column were 290 and 723 pg, respectively. These detection limits allow for the measurement of plasma concentrations of dimer B2 and B5 as low as 10 and 25 nmol/L, respectively.

Mass spectral identification of procyanidin dimers in plasma

To enhance the MS signal response, chromatography was not used to resolve procyanidin dimers B2 and B5 in the purified standard. The molecular ions of the procyanidin dimers were identified by simple MS analysis of a standard solution (*m/z*: 577). Because of background chemical noise or other intrinsic interferences that may generate ions having the same molecular ion (*m/z*),
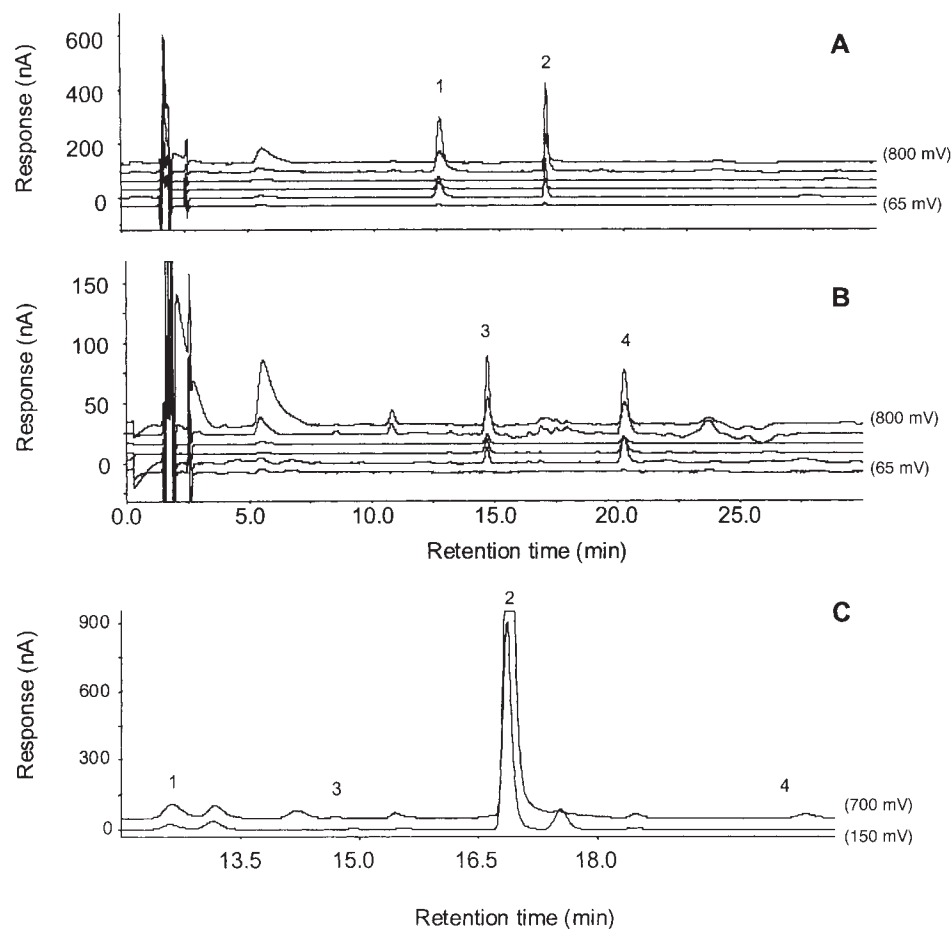

FIGURE 1. Coulometric multiple-array chromatograms. A: 10 nmol (on-column) catechin standard [peak 1, 12.6 min; molecular weight (MW) = 289] and 10 nmol epicatechin standard (peak 2, 16.9 min; MW = 289). B: 12.5 nmol procyanidin dimer B2 standard [epicatechin-(4β-8)-epicatechin; peak 3, 14.7 min; MW = 578] and 12.5 nmol dimer B5 standard [epicatechin-(4β-6)-epicatechin; peak 4, 20.3 min; MW = 578]. C: an extracted plasma sample from one subject 2 h after cocoa consumption. The HPLC conditions are as described in the text.
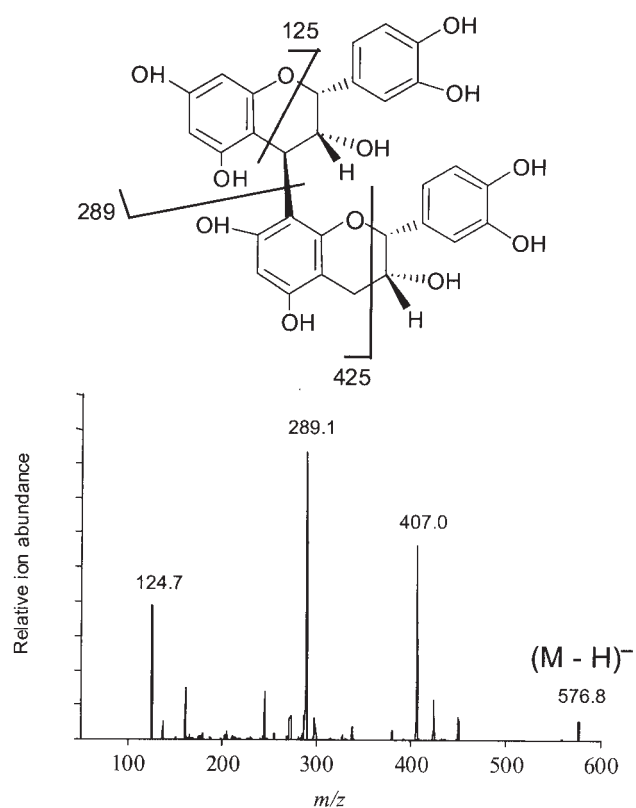
FIGURE 2. The structure of procyanidin dimer B2 [epicatechin-(4β-8)-epicatechin] with major fragmentations and the relative abundance of product ion spectrum determined with tandem mass spectrometry. *m/z*, mass-to-charge ratio; (M-H)⁻, molecular ion in negative mode.

FIGURE 3. Results of liquid chromatography–tandem mass spectrometry of the dimer standard, containing both dimer B2 and B5, spiked into control plasma at 9.86 nmol/L (A) and dimers in an extracted plasma sample (unspiked) from one subject 2 h after consumption of 0.375 g cocoa/kg body wt (B). The data for both panels A and B represent the mass–to–product ion pairs (577.3/407.0, 577.3/289.0, and 577.3/125.0) produced from both dimer B2 and dimer B5. A plasma dimer B2 concentration of 42.5 nmol/L was quantified in the sample represented in panel B with the use of multiple-array coulometric detection.

we used MS/MS to further identify the ion (*m/z*: 577) as dimer. MS/MS is used to fragment the ion of interest. Once fragmented, the MS/MS product ion spectra of the dimer standard showed 3 structurally distinctive ion products of 125, 289, and 407 *m/z* (**Figure 2**). It is important to note that when ionized, the 425-*m/z* fragment represented in Figure 2 will lose a molecule of water, producing the product ion of 407 *m/z* (Figure 2). The major product ions that are produced from the dimer standard indicate that fragmentation occurs at the linkage (*m/z*: 289) of the oligomer as well as opening of the C rings (*m/z*: 125 and 407). The fragmentation pattern of dimer B2 is represented in Figure 2. Although its fragmentation pattern is not shown, the fragmentation dimer of B5 also involves simple opening of the C rings and linkage cleavage, producing product ions similar to dimer B2.

These data were used to determine the transitions used in the multiple reaction monitoring experiments to identify procyanidin dimers in the plasma. Thus, the dimers are detected by pairing a specific precursor ion (*m/z*: 577.3) with a structurally distinct product ion (either *m/z* 407.0, 289.0, or 125.0). The LC-MS/MS results of the purified procyanidin dimer standard are shown in **Figure 3**A, and the procyanidin dimers in the plasma extract of one subject 2 h after consumption of the cocoa beverage are represented in Figure 3B.

Plasma monomer and dimer B2 concentrations

An unknown peak with a retention time of 14.7 min was detected in the plasma extract 0.5, 2, and 6 h after consumption of
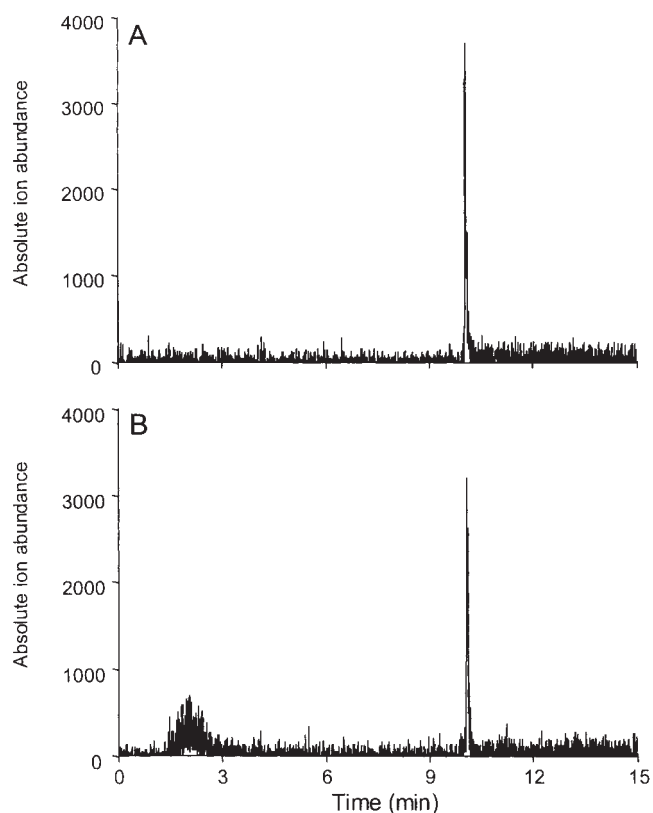
0.375 *g* cocoa/kg body wt. We later confirmed by coelution with the purified dimer standards that this unknown peak in the plasma extract corresponded with the first peak observed in the purified dimer standard (retention time: 14.7 min). As stated above, we characterized the dimer peak at this retention time as dimer B2.

Representative chromatographs at 700 mV from one subject are shown in **Figure 4**. These chromatographs show the presence of the dimer B2 peak in the plasma at 0.5, 2, and 6 h. Dimer B5 (retention time: 20.3 min) was not detected in the plasma by coulometric detection at any time point in any of the subjects. The plasma concentrations of the dimer B2 were significantly ($P < 0.05$) elevated at 0.5, 2, and 6 h relative to baseline (16 ± 5, 41 ± 4, and 15 ± 2 nmol/L, respectively) (**Figure 5**).

Thirty minutes after the subjects consumed 0.375 *g* cocoa/kg body wt, plasma epicatechin and catechin concentrations increased significantly ($P < 0.05$) above baseline (0.08 ± 0.46 compared with 2.61 ± 0.46 μmol/L and 0.00 compared with 0.13 ± 0.03 μmo/L, respectively). Plasma concentrations of epicatechin and catechin continued to increase, with values at 2 h being 5.92 ± 0.60 and 0.16 ± 0.03 μmol/L, respectively. Plasma concentrations of epicatechin and catechin
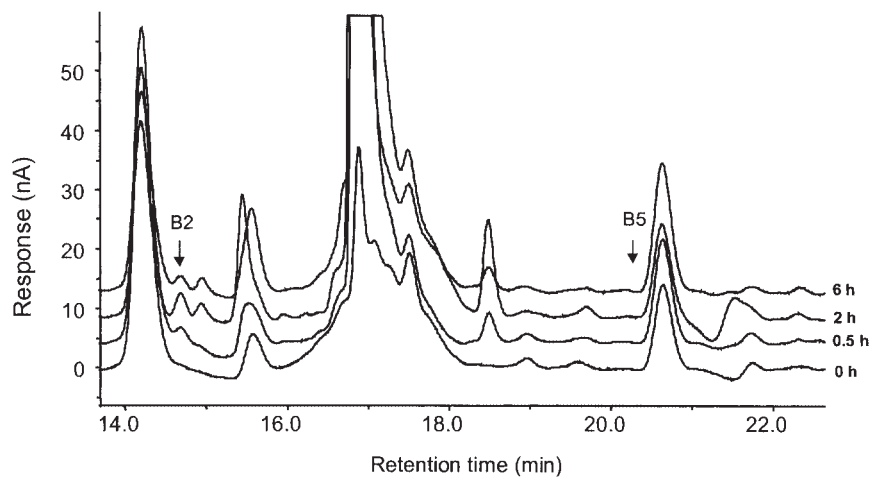
FIGURE 4. The detection of dimer B2 in one subject 0, 0.5, 2, and 6 h after consumption of 0.375 g cocoa/kg body wt. At baseline (0 h), dimer B2 was undetectable, increasing in concentration to 17.5 nmol/L at 0.5 h, to 51.5 nmol/L at 2 h, and to 21.5 nmol/L 6 h after cocoa consumption. The limit of detection for dimer B2 was 10 nmol/L. The treatment of samples and HPLC conditions are as described in the text.

were lower 6 h after cocoa consumption: 1.05 ± 0.01 and 0.02 ± 0.002 µmol/L, respectively (Figure 5).

DISCUSSION

The flavanols and the related oligomers of this basic subunit, known as the procyanidins, can be found in a variety of foods, including apples, wine, tea, peanuts, almonds, and cocoa (3, 10). Although many papers have determined the time course of monomer absorption into plasma (19, 22–26), there is limited information concerning the metabolism of the procyanidins. Some studies have used radiolabeled techniques to indicate that extracts containing procyanidins are bioavailable (27, 28). However, the question remains whether the procyanidins are depolymerized or remain intact before absorption. Deprez et al (29) reported that procyanidins, with an average polymerization of 6, could be degraded after 48 h incubation with human colonic microflora into low-molecular-weight aromatic acids. As for the smaller oligomers, it was reported that radiolabeled monomers, dimers, and trimers can be transported across an in vitro cell layer of Caco-2 cells, whereas the larger oligomers adhere to the cell surface (30). Complementing the above, using procyanidins isolated from cocoa, Spencer et al (31) perfused a 50-µmol/L dimer solution for 90 min through the small intestine of rats. Small amounts (< 1%) of the procyanidin dimers B2 and B5 were shown to pass through the rat enterocytes, in vitro, with 95.8% of the total passing flavanols being unconjugated epicatechin. These data support the concept that the dimers can be absorbed in vivo, albeit to a lesser extent than the monomer subunits of the depolymerized dimers. In the current study, we identified and quantified dimer B2 using coulometric multiple-array detection in the plasma after the subjects consumed a flavanol-rich cocoa beverage. We confirmed the presence of the procyanidin dimers in extracted plasma samples by using LC-MS/MS. The use of LC-MS/MS enabled us to confirm that unpolymerized dimers were present in the plasma samples by locking onto ions that had an m/z of 577 and were associated with structure-dependent product (dimer) ion fragments that are unique to this procyanidin oligomer.

Consistent with the in vitro findings of Spencer et al (31), we detected relatively modest concentrations of dimer B2 in the
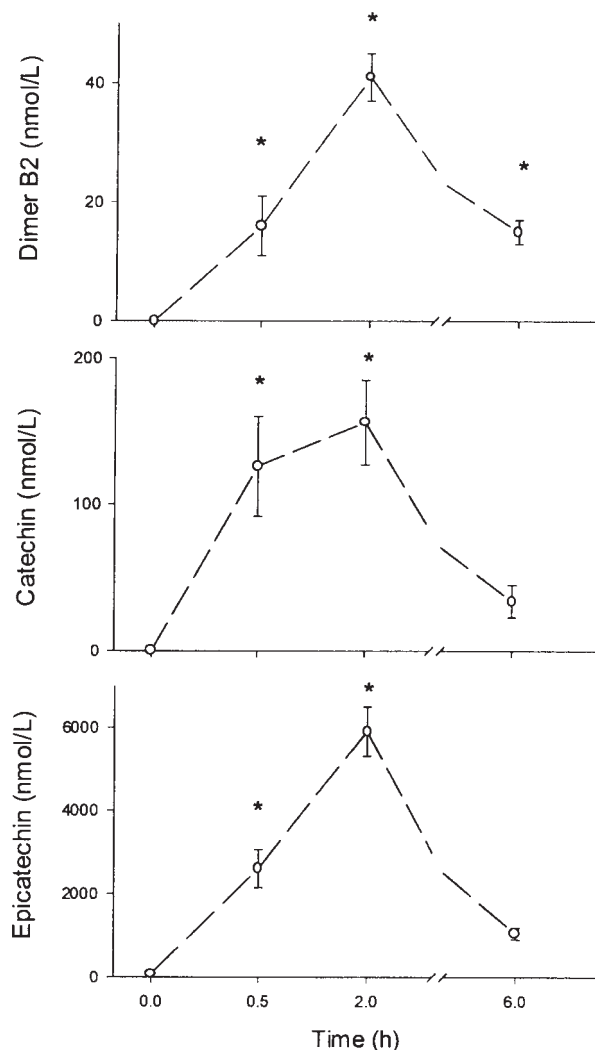

FIGURE 5. Mean ($\pm$ SEM) plasma dimer B2, catechin, and epicatechin concentrations 0, 0.5, 2, and 6 h after consumption of 0.375 g cocoa/kg body wt ($n = 5$). *Significantly different from baseline (0 h), $P < 0.05$.

plasma relative to the monomers. In the current study, a mean plasma dimer B2 concentration of 41 nmol/L was observed 2 h after the consumption of ≈256 mg dimers in the cocoa; the dimer represented < 1% of the circulating nonmethylated flavanols compared with an average plasma epicatechin concentration of 5.92 μmol/L at the same time point. Interestingly, although the measured ratio of epicatechin to catechin was 1:1 in the cocoa preparation, epicatechin was the predominant plasma flavanol, with plasma catechin concentrations being only 3% of the plasma epicatechin concentration. A similar observation was previously made by Rein et al (18). Complementing these findings, epicatechin was reported to be the primary bioavailable form of the procyanidin dimers B2 and B5 in an isolated intestine model (31). The combination of these results suggests that dimers and other procyanidin oligomers may be degraded in the gut into epicatechin monomers that are then adsorbed. On the basis of this concept, only a limited amount of oligomer is absorbed. These observations provide an explanation for the high ratio of epicatechin to catechin observed in the current study. Further research on this issue is warranted. It will be interesting to characterize the effects of catechin-containing dimers such as B1 [epicatechin-(4β-8)-catechin] and B3 [catechin-(4α-8)-catechin], which are predominant in red wine and grape juice, on ratios of plasma epicatechin to catechin. Information on the above will provide insight as to whether the observed high ratio of epicatechin to catechin in the current study is due to the absorption of the monomers after the degradation of the oligomers in the gut or whether the ratio represents a preferential absorption of epicatechin.

With respect to the physiologic relevance of the in vivo plasma concentrations observed in this study, in vitro studies have reported an IC_{50} (concentration that yields 50% inhibition, expressed as μmol monomer) of a cocoa extract of dimer B2 as ≈5 μmol/L for an azo compound–dependent liposome oxidation and 2 μmol/L for an iron- and ascorbate-dependent liposome oxidation (32). A dimer extract increased the lag time of conjugated diene formation by 47% from control and at a concentration of 290 nmol/L (32). These data support the concept that low nanomolar concentrations of dimers may have significant in vivo effects.

We previously reported increases in plasma antioxidant capacity and reductions in lipid oxidation products with concurrent increases of plasma epicatechin concentrations (33). Dose-response increases in plasma epicatechin after the feeding of various amounts of cocoa flavonoids have also been reported by Richelle et al (19). There was considerable disparity between the reported plasma epicatechin concentrations in the current study and in that by Richelle et al. In a recent report by Baba et al (26), the consumption of cocoa flavonoids was associated with a rise in plasma epicatechin concentrations to concentrations similar to those in the current study.

In the current study, we pretreated the plasma samples with a solution of β-glucuronidase (EC 3.2.1.31) and arylsulfatase (EC 3.1.6.1). With this approach we measured nonmethylated (free, glucuronide, sulfide, and glucuronide-sulfide) plasma conjugates of epicatechin, catechin, and dimer B2; the individual conjugates were not analyzed. Consistent with our findings, Baba et al (26) reported nonmethylated plasma epicatechin concentrations of 3.46 μmol/L in healthy subjects 2 h after they consumed a cocoa drink that provided 220 mg epicatechin. Baba et al (26) reported a concentration of 1.46 μmol methylated epicatechin/L in the plasma at the 2-h time point; free epicatechin represented 4.5% of the total of methylated and nonmethylated epicatechin.

Only a limited amount of studies have examined the biological effects of the conjugates. Harada et al (34) reported that glucuronide conjugates of epicatechin and catechin have similar superoxide anion scavenging activities compared with the aglycones; however, reduced activities were measured with the methylglucuronide conjugates of both catechin and epicatechin. Similarly, Da Silva et al (35) reported that rat plasma containing predominantly nonmethylated conjugates was more effective in inhibiting radical-generated hydroperoxides than was plasma containing primarily methylated conjugates. Finally, Manach et al (36) observed that quercetin glucuronides and sulfides increased the inhibition of copper-induced LDL oxidation, however, to a lesser extent than that of aglycone. The abovementioned studies suggest that the nonmethylated conjugates possess, although possibly to a lesser extent, the biological effects that are observed in vitro with free epicatechin, catechin, and dimer.

In conclusion, although several investigators have observed positive vascular effects in healthy adults given flavonoid-rich foods such as whole grape juice (37–39) and cocoa (33, 40–42); the components in these foods that are responsible for these effects have not been definitively identified. The evidence presented here establishes that the procyanidin oligomer dimer B2, as well as the flavanol monomers epicatechin and catechin, can be absorbed into the circulation. Additional work is needed to determine whether even larger procyanidins can be absorbed. Future investigation is also needed to determine whether the reported in vitro effects of these procyanidin oligomers occur in vivo. ✿

REFERENCES

1. Rimm EB, Ascherio A, Giovannucci E, Spiegelman D, Stampfer MJ, Willett WC. Vegetable, fruit, and cereal fiber intake and risk of coronary heart disease among men. JAMA 1996;275:447–51.
2. Krauss RM, Eckel RH, Howard B, et al. AHA dietary guidelines revision 2000: a statement for healthcare professionals from the nutrition committee of the American Heart Association. Circulation 2000;102:2284–99.
3. Adamson GE, Lazarus SA, Mitchell AE, et al. HPLC method for the quantification of procyanidins in cocoa and chocolate samples and correlation to total antioxidant capacity. J Agric Food Chem 1999;47:4184–8.
4. Prior R, Cao G. Analysis of botanicals and dietary supplements for antioxidant capacity: a review. J AOAC Int 2000;83:950–6.
5. Richelle M, Tavazzi I, Offord E. Comparison of the antioxidant activity of commonly consumed polyphenolic beverages (coffee, cocoa, and tea) prepared per cup serving. J Agric Food Chem 2001;49:3438–42.
6. Hertog MG, Feskens EJ, Hollman PC, Katan MB, Kromhout D. Dietary antioxidant flavonoids and risk of coronary heart disease: the Zutphen Elderly Study. Lancet 1993;342:1007–11.
7. Hertog MG, Kromhout D, Aravanis C, et al. Flavonoid intake and long-term risk of coronary heart disease and cancer in the Seven Countries Study. Arch Intern Med 1995;155:381–6.
8. Knekt P, Jarvinene R, Reunanen A, Maatela J. Flavonoid intake and coronary mortality in Finland: a cohort study. BMJ 1996;312:478–81.
9. Dillinger TL, Barriga P, Escarcega S, Jimenez M, Lowe DS, Grivetti LE. Food of the gods: cure for humanity? A cultural history of the medicinal and ritual use of chocolate. J Nutr 2000;130:2057S–72S.
10. Lazarus SA, Adamson GE, Hammerstone JF, Schmitz HH. High-performance liquid chromatography/mass spectrometry analysis of proanthocyanidins in foods and beverages. J Agric Food Chem 1999;47:3693–701.

11. Pearson DA, Schmitz HH, Lazarus SA, Keen CL. Inhibition of in vitro low-density lipoprotein oxidation by oligomeric procyanidins present in chocolate and cocoas. In: Packer L, ed. Methods in enzymology. New York: Academic Press, 2001:350–60.

12. Arteel GE, Schroeder P, Sies H. Reactions of peroxynitrite with cocoa procyanidin oligomers. J Nutr 2000;130:2100S–4S.

13. Arteel GE, Sies H. Protection against peroxynitrite by cocoa polyphenol oligomers. FEBS Lett 1999;462:167–70.

14. Karim M, McCormick K, Kappagoda CT. Effects of cocoa procyanidins on endothelium-dependent relaxation. J Nutr 2000;130:2105S–8S.

15. Mao T, Van De Water J, Keen CL, Schmitz H, Gershwin M. Cocoa procyanidins and human cytokine transcription and secretion. J Nutr 2000;130:2093S–9S.

16. Mao T, Powell J, Van de Water J, Keen CL, Schmitz H, Gershwin M. The influence of cocoa procyanidins on the transcription of interleukin-2 in peripheral blood mononuclear cells. Int J Immunother 1999;15:23–9.

17. Spencer JP, Chaudry F, Pannala AS, Srai SK, Debnam E, Rice-Evans C. Decomposition of cocoa procyanidins in the gastric milieu. Biochem Biophys Res Commun 2000;272:236–41.

18. Rein D, Lotito S, Holt RR, Keen CL, Schmitz HH, Fraga CG. Epicatechin in human plasma: In vivo determination and effect of chocolate consumption on plasma oxidation status. J Nutr 2000;130:2109S–14S.

19. Richelle M, Tavazzi I, Enslen M, Offord E. Plasma kinetics in man of epicatechin from black chocolate. Eur J Clin Nutr 1999;53:22–6.

20. Hammerstone JF, Lazarus SA, Mitchell AE, Rucker R, Schmitz HH. Identification of procyanidins in cocoa (*Theobroma cacao*) and chocolate using high-performance liquid chromatography/mass spectrometry. J Agric Food Chem 1999;47:490–6.

21. Zhu QY, Holt RR, Lazarus SA, et al. Stability of the flavan-3-ols epicatechin and catechin and related dimeric procyanidins derived from cocoa. J Agric Food Chem 2002;50:1700–5.

22. Bell JR, Donovan JL, Wong R, et al. (+)-Catechin in human plasma after ingestion of a single serving of reconstituted red wine. Am J Clin Nutr 2000;71:103–8.

23. Lee M-J, Wang Z-Y, Li H, et al. Analysis of plasma and urinary tea polyphenols in human subjects. Cancer Epidemiol Biomarkers Prev 1995;4:393–9.

24. Piskula MK, Terao J. Accumulation of (−)-epicatechin metabolites in rat plasma after oral administration and distribution of conjugation enzymes in rat tissues. J Nutr 1998;128:1172–8.

25. Chen L, Lee M-J, Yang CS. Absorption, distribution, and elimination of tea polyphenols in rats. Drug Metab Dispos 1997;25:1045–50.

26. Baba S, Osakabe N, Yasuda A, et al. Bioavailability of (−)-epicatechin upon intake of chocolate and cocoa in human volunteers. Free Radic Res 2000;33:635–41.

27. Laparra J, Michaud J, Masquelier J. Etude pharmacocinetique des oligomeres flavanoliques. (Pharmacokinetic study of flavonic oligomers.) Plant Med Phytother 1977;11:133–42 (in French).

28. Jimenez-Ramsey LM, Rogler JC, Housley TL, Butler LG, Elkin RG. Absorption and distribution of ^{14}C-labeled condensed tannins and related sorghum phenolics in chickens. J Agric Food Chem 1994;42:963–7.

29. Deprez S, Brezillon C, Rabot S, et al. Polymeric proanthocyanidins are catabolized by human colonic microflora into low-molecular-weight phenolic acids. J Nutr 2000;130:2733–8.

30. Santos-Buelga C, Scalbert A. Proanthocyanidins and tannin-like compounds—nature, occurrence, dietary intake and effects on nutrition and health. J Sci Food Agric 2000;80:1094–117.

31. Spencer JP, Schroeter H, Shenoy B, Srai SK, Debnam E, Rice-Evans C. Epicatechin is the primary bioavailable form of the procyanidin dimers B2 and B5 after transfer across the small intestine. Biochem Biophys Res Commun 2001;285:588–93.

32. Lotito SB, Actis-Goretta L, Renart L, et al. Influence of oligomer chain length on the antioxidant activity of procyanidins. Biochem Biophys Res Commun 2000;276:945–51.

33. Wang JF, Schramm DD, Holt RR, et al. A dose-response effect from chocolate consumption on plasma epicatechin and oxidative damage. J Nutr 2000;130:2115S–9S.

34. Harada M, Kan Y, Naoki H, et al. Identification of the major antioxidative metabolites in biological fluids of the rat with ingested (+)-catechin and (−)-epicatechin. Biosci Biotechnol Biochem 1999;63:973–7.

35. Da Silva EL, Piskula MK, Terao J. Enhancement of antioxidative ability of rat plasma by oral administration of (−)-epicatechin. Free Radic Biol Med 1998;24:1209–16.

36. Manach C, Morand C, Crespy V, et al. Quercetin is recovered in human plasma as conjugated derivatives which retain antioxidant properties. FEBS Lett 1998;426:331–6.

37. Keevil JG, Osman HE, Reed JD, Folts JD. Grape juice, but not orange juice or grapefruit juice, inhibits human platelet aggregation. J Nutr 2000;130:53–6.

38. Stein JH, Keevil JG, Wiebe DA, Aeschlimann S, Folts JD. Purple grape juice improves endothelial function and reduces the susceptibility of LDL cholesterol to oxidation in patients with coronary artery disease. Circulation 1999;100:1050–5.

39. Freedman JE, Parker CI, Li L, et al. Select flavonoids and whole juice from purple grapes inhibit platelet function and enhance nitric oxide release. Circulation 2001;103:2792–8.

40. Osakabe N, Baba S, Yasuda A, et al. Daily cocoa intake reduces the susceptibility of low-density lipoprotein to oxidation as demonstrated in healthy human volunteers. Free Radic Res 2001;34:93–9.

41. Rein D, Paglieroni, Teresa G, et al. Cocoa inhibits platelet activation and function. Am J Clin Nutr 2000;72:30–5.

42. Schramm DD, Wang JF, Holt RR, et al. Chocolate procyanidins decrease the leukotriene-prostacyclin ratio in humans and human aortic endothelial cells. Am J Clin Nutr 2001;73:36–40.