ABSTRACT Evidence from epidemiological studies suggests that a diet high in plant foods and rich in polyphenols is inversely associated with a risk for cardiovascular and other chronic diseases. Chocolate, like red wine and green tea, is a polyphenol-rich food, primarily containing procyanidin polyphenols. These polyphenols are hypothesized to provide cardioprotective effects due to their ability to scavenge free radicals and inhibit lipid oxidation. Herein, we demonstrate that 2 h after the ingestion of a procyanidin-rich chocolate containing 5.3 mg total procyanidin/g, of which 1.3 mg/g was (−)-epicatechin (epicatechin), plasma levels of epicatechin increased 133 ± 27, 258 ± 29 and 355 ± 49 nmol/L in individuals who consumed 27, 53 and 80 g of chocolate, respectively. That the rise in plasma epicatechin levels was functionally significant is suggested by observations of trends for dose-response increases in the plasma antioxidant capacity and decreases in plasma lipid oxidation products. The above data support the theories that in healthy adults, 1) a positive relationship exists between procyanidin consumption and plasma procyanidin concentration and 2) the rise in plasma epicatechin contributes to the ability of plasma to scavenge free radicals and to inhibit lipid peroxidation. J. Nutr. 130: 2115S—2119S, 2000.
levels increased from baseline values of 22 ± 4 nmol/L to values of 257 ± 66 nmol/L after 2 h. Importantly, this increase in epicatechin was associated with an increase in the antioxidant capacity of the plasma and a decrease in plasma 2-thiobarbituric acid reactive substances (TBARS) (Rein et al. 2000a). In the current work, we extend these observations by examining dose-response changes in plasma epicatechin, plasma antioxidant capacity, plasma TBARS and 8-isoprostane after the acute consumption of different amounts of a procyanidin-rich chocolate.

METHODS

Subjects. Twenty volunteers (14 women and 6 men, age range 20–56 years, weight 67 ± 2.7 kg, body mass index 23.8 ± 0.79 kg/m²) composed the subject pool from which subjects were randomly drawn to participate in each of four chocolate treatment groups. Ten to 12 subjects were studied on four separate occasions, separated by 1-week intervals. Subjects were non-smokers and were free from any apparent diseases. Volunteers signed informed consent before beginning the study, and all procedures were conducted in accordance with the guidelines of the Human Subjects Review Committee of the University of California, Davis.

Chemicals. Chemicals were purchased from Sigma Chemical Co. (St. Louis, MO) unless otherwise stated.

Study design. Participants were asked to refrain from taking vitamin supplements, nonsteroidal anti-inflammatory medications and caffeine-containing polyphenolic foods for 24 h and to fast overnight for 12 h before each feeding trial. Blood was collected at baseline (0 h) and 2 and 6 h after the first blood draw. Subjects were instructed to consume the test foods (chocolate and bread, or bread only) within 15 min of the first blood draw. All subjects were asked to consume an additional 95 g of bread during the next 2 h.

Test foods provided 0, 27, 53 or 80 g of procyanidin-rich chocolate in the form of M&M’s Semi-Sweet Chocolate Minis Baking Bits made with Cacoapo cacao (Mars Incorporated, Hacketstown, NJ) and included a serving of 47 g of bread. The 27-g chocolate portion was in the form of candy-coated M&M’s baking bits, which provided 0.732 MJ, 0.021 g caffeine and 0.18 g theobromine. Each 27-g chocolate portion contained 46 mg epicatechin and a total of 186 mg carbohydrates, 25 g carbohydrate and 4.5 g protein in a 47-g serving.

Collection of plasma samples. Eighteen milliliters of venous blood was drawn into Vacutainer tubes containing EDTA or sodium heparin (Becton Dickinson, Franklin Lakes, NJ). Plasma was separated by low-speed centrifugation (1500 × g at 4°C for 10 min) and stored at −80°C until analysis. For lipid oxidation determinations, aliquots of plasma samples were added with 4% w/v butylated hydroxytoluene/ethanol (for a final ratio of 4:1) before freezing.

Assessment of epicatechin in plasma. Plasma concentration of epicatechin was determined as described by Richelle et al. (1999), with some modifications. In brief, 200 μl of heparinized plasma was vortexed with a solution of 0.2 g/ml ascorbic acid, 1 mg/ml EDTA and 20 μmol β-gluconidase suspension (~200 U of glucuronidase activity and 80 U of sulfatase activity). After a 45-min incubation at 37°C, 0.5 ml of acetonitrile was added, and the samples were vortexed and centrifuged at 10,000 × g for 5 min. The supernatant fraction was combined with 50 mg of alumina and 1 ml of 50 mmol/L Tris-HCl (pH 7.6), vortexed and centrifuged. After discarding the supernatant, the alumina was washed with 1 ml of 50 mmol/L Tris-HCl (pH 7.6), followed by a final wash with 1 ml of methanol, and centrifuged as indicated. After discarding the methanol, residual methanol was evaporated under nitrogen, and epicatechin was extracted from the alumina with 250 μl of 0.25 mol/L perchloric acid. The sample was centrifuged (10,000 × g, 1 min, 4°C), and the supernatant fraction was filtered through a 0.2-μm polyethersulfone membrane (Nalgene).

Samples (50 μl) were chromatographed using a Hewlett Packard (Wilmington, DE) 1100 series system equipped with an ESA (Chelmsford, MA) Coulchem II coulometric detector with a 5011 analytical cell set as follows: guard cell + 550 mV, conditioning cell +100 mV and analytical cell +400 mV. Chromatography was carried out using an Altima C-18 column (5 μm, 150 × 4.6 mm; Deerfield, IL) with a 0.5-μm Frit precolumn filter (Upchurch, WA). The mobile phase was composed of two solvent solutions: solvent A, 40% methanol/60% 50 mmol/L sodium acetate, pH 5.8; and solvent B, 7% methanol/93% 100 mmol/L sodium acetate, pH 5.2.

Epicatechin separation was achieved with a flow rate of 1 ml/min and a gradient elution. For gradient elution, the composition of the mobile phase was first set at 80% B, which was linearly decreased to 60% B by 1 min. This was followed by another linear decrease to 20% B by 3.5 min. The mobile phase composition was maintained at 20% B until 20 min, when B was linearly increased to 80% by 30 min.

Assessment of plasma TBARS. Plasma TBARS determination was conducted as previously described (Oreiza et al. 1997), with modifications for using a plate reader. Briefly, 100 μl of plasma, 200 μl of 3% sodium dodecyl sulfate, 800 μl of 0.1 mol/L HCl, 100 μl of 10% (wt/v) phosphotungstic acid and 400 μl of 0.7% (wt/v) 2-thiobarbituric acid were combined and placed at 95°C for 30 min. Samples were cooled on ice and mixed with 1 ml of 1-butanol. After centrifugation (1800 × g, 10 min, 4°C), a 200-μl aliquot of the butanol phase was separated and analyzed spectrophotometrically (excitation 515 nm and emission 555 nm) using a plate reader attachment (Perkin–Elmer Cetus, Norwalk, CT). TBARS are expressed as malondialdehyde equivalents.

Assessment of plasma antioxidant capacity. Plasma samples (5–10 μl) were assayed for their ability to inhibit the chemiluminescence produced by a mixture of 3 ml of 5.4 mg/ml 2,2′-azobis(2-aminopropane) in 0.1 mol/L phosphate-buffered saline, pH 7.4 (GIBCO BRL, Life Technologies, Grand Island, NY) and 10 μl of 10 mg/ml luminol. The chemiluminescence was measured in a liquid scintillation counter (Wallac 1410; Wallac Oy, Turku, Finland). The plasma antioxidant capacity value was calculated as the lag time before an increase in the chemiluminescence was observed (Lissi et al. 1995). This lag time is proportional to the cumulative amount of antioxidants present in the samples. A reference lag time was obtained by using a known amount of 6-hydroxy-2,5,7,8-tetramethoxychroman-2-carboxylic acid (Trolox; Aldrich Chemical Co, Milwauk ee, WI) with a 0.5-μm Frit precolumn filter (Upchurch, WA). TBARS were determined by using a known amount of 6-hydroxy-2,5,7,8-tetramethoxychroman-2-carboxylic acid (Trolox; Aldrich Chemical Co, Milwauk ee, WI).

Assessment of 8-isoprostane. 8-Isoprostane (8-epi-prostaglandin F₂α) was determined in plasma using 25 μl of plasma added to 40 μl of the supplied enzyme immunoassay buffer from the 8-Isoprostane Enzyme Immunoassay Kit (Cayman Chemical, Ann Arbor, MI). Plates were read using a Shimadzu (Columbia, MD) spectrophotometer at 450 nm.

Statistical analysis. Results in the text and tables are expressed as means ± SEM. Changes between the baseline (0 h) and the 2- and 6-h time points among treatment groups were examined using repeated-measures ANOVA, with control for multiple measurements on the same subjects. Where appropriate, multiple comparisons were made using Tukey-Kramer corrections.

Statistical significance was assessed at the 5% level. All statistical analyses were performed using SAS for Windows Release 7 (SAS Institute, Cary, NC).

RESULTS

Before the consumption of the test foods, 82% of the subjects had baseline values of plasma epicatechin that were nondetectable (<1 nmol/L). The subjects with detectable levels of epicatechin in plasma showed values ranging from 5.5 to 28.3 nmol/L. After the consumption of the test foods, plasma epicatechin levels were higher than baseline values for the three test groups that consumed the procyanidin-rich chocolate. At the 2-h point, the plasma epicatechin concentrations averaged 133, 258 and 355 nmol/L for the subjects who consumed 27, 53 and 80 g of the procyanidin-rich chocolate, respectively (Table 1). There were no significant changes in plasma epicatechin levels in the subjects who consumed bread only. In the procyanidin-rich chocolate groups, the average decrease in epicatechin concentration between the 2- and 6-h point was...
81, 77 and 74% for the 27-, 53- and 80-g groups, respectively. It can be noted that the 6-h epicatechin concentrations in the 53- and 80-g procyanidin-rich chocolate groups were still markedly higher than the baseline concentrations (P < 0.001), and they were higher than plasma epicatechin concentrations in the subjects who consumed bread only (P < 0.0001).

With respect to plasma antioxidant capacity, there was a decrease in the time of inhibition produced by plasma in the subjects who consumed the non–chocolate-containing food from 489 ± 88 s (0 h) to 401 ± 70 s (2 h) and 350 ± 69 s (6 h). These decreases were ameliorated in the subjects who consumed procyanidin-rich chocolate. Although there were no trends toward increased plasma antioxidant capacity 2 h after the consumption of chocolate, 6 h after the consumption of chocolate, there was a trend for an increase in total antioxidant capacity with an increase in the amount of procyanidin-rich chocolate ingested (Fig. 1). Subjects who consumed 27 g had an increase in total antioxidant capacity of 78 s at 2 h and a decrease of 38 s at 6 h compared with baseline values. Subjects who consumed 53 g had a decrease of 57 s at 2 h and an increase of 138 s at 6 h compared with baseline values. Finally, subjects who consumed 80 g of chocolate had increases of 36 and 253 s at 2 and 6 h compared with baseline values, respectively.

With respect to lipid oxidation, there was an increase in plasma TBARS that was dependent on the time after which subjects consumed bread only (0.63 ± 0.12, 0.77 ± 0.11 and 0.83 ± 0.11 μmol/L at the 0-, 2- and 6-h time points, respectively). There was a trend for the increase in plasma TBARS to be reversed, in a dose-dependent manner, in the subjects who consumed procyanidin-rich chocolate (Fig. 2). A second marker of lipid oxidation, plasma 8-isoprostane, was also measured in the subjects. Plasma 8-isoprostane values for the subjects who consumed the different amounts of the procyanidin-rich chocolate were not statistically different at the 2- and 6-h time points (Table 2).

### DISCUSSION

In this study, we confirm previous observations that in healthy adult human subjects, epicatechin is rapidly absorbed after the consumption of procyanidin-rich chocolate (Rein et al. 1999 and 2000). Importantly, the data support the theory that in healthy adults, a linear relationship exists between consumption of procyanidin-rich chocolate and plasma procyanidin concentration. In addition, we have extended this observation by showing dose-dependent increases in plasma epicatechin that are associated with increases in plasma antioxidant capacity and reductions in plasma lipid oxidation.

The highest plasma epicatechin concentration observed in

### TABLE 1

<table>
<thead>
<tr>
<th>Procyanidin-rich chocolate food consumed, g</th>
<th>0</th>
<th>27</th>
<th>53</th>
<th>80</th>
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<td></td>
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</tr>
<tr>
<td>0</td>
<td>1 ± 1 (9) 🅃️</td>
<td>2 ± 2 (13) 🅃️</td>
<td>4 ± 2 (13) 🅃️</td>
<td>4 ± 3 (10) 🅃️</td>
</tr>
<tr>
<td>2</td>
<td>19 ± 1 (9) 🅃️</td>
<td>133 ± 27 (13) 🅃️</td>
<td>258 ± 29 (13) 🅃️</td>
<td>355 ± 49 (13) 🅃️</td>
</tr>
<tr>
<td>6</td>
<td>1 ± 1 (9) 🅃️</td>
<td>26 ± 8 (13) 🅃️</td>
<td>66 ± 8 (13) 🅃️</td>
<td>103 ± 16 (10) 🅃️</td>
</tr>
<tr>
<td>Area under the curve, μmol · h · L⁻¹</td>
<td>0 (9)</td>
<td>0.5 (13)</td>
<td>1.0 (13)</td>
<td>1.5 (10)</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. The area under the curve for epicatechin versus time was calculated from the means of the different time points. Values in parentheses indicate number of subjects. Means within a column not sharing a common superscript number are significantly different at P < 0.05.

#### FIGURE 1
Antioxidant capacity of plasma at baseline and 2 and 6 h after the consumption of procyanidin-rich chocolate. Values are expressed as changes in lag time(s) before chemiluminescence is observed. A weak trend (P = 0.5302) was observed for increasing antioxidant capacity with increasing amounts of procyanidin-rich chocolate consumed. Variability is represented as SEM. Please refer to Table 1 for the number of subjects in each group.

#### FIGURE 2
Plasma TBARS at baseline and 2 and 6 h after the consumption of procyanidin-rich chocolate. Values are expressed as malondialdehyde equivalents (μmol/L). A weak trend (P = 0.3451) was observed for decreasing plasma TBARS with increasing amounts of procyanidin-rich chocolate consumed. Variability is represented as SEM. Please refer to Table 1 for the number of subjects in each group.
across treatments or time. catechin absorption of 330 mL of chocolate. These percentages can be compared with a plasma epicatechin consumed in 80, 53 and 27 g of procyanidin-rich chocolate (et al. 1998). In the current study, we can estimate the amount the blood catechin levels for black tea with milk (van het Hof et al. 1998a). This difference was 104 nmol/L. The estimated area under the curve of epicatechin concentration versus time is 263 nmol/L, whereas in the study of Rein et al. (2000a), this difference was 104 nmol/L. The estimated area under the curve of epicatechin concentration versus time is similar to that reported by Richelle et al. (1999), although it is higher than the average value of 285 nmol/L achieved in a previous study by our group (Rein et al. 2000a). In contrast to the present study, in the study by Rein et al. (2000a), subjects did not consume any additional food (bread) until 4 h after the chocolate test meal. Therefore, we suggest that the difference in results between our two studies is due to subtle changes in the experimental methods that were used. This can be shown by examining the change in plasma epicatechin concentration between the 2- and 6-h time points after chocolate consumption. In the present study, this difference was 263 nmol/L, whereas in the study of Rein et al. (2000a), this difference was 104 nmol/L. The estimated area under the curve of epicatechin concentration versus time is similar to the two studies (1480 versus 1540 nmol · h⁻¹ · L⁻¹), suggesting that although maximum absorption time was delayed with chocolate consumption alone, the total epicatechin absorbed after the consumption of 80 g of procyanidin-rich chocolate was unchanged between the two studies.

The influence of the bread on the bioavailability of epicatechin from chocolate must be determined in future studies. When green tea and black tea were consumed with semi-skimmed milk, the addition of milk did not significantly affect the blood catechin levels for black tea with milk (van het Hof et al. 1998). In the current study, we can estimate the amount of epicatechin present in the plasma, extrapolated to a 6-h postconsumption time, to 1.6, 1.5 and 1.4% of the total epicatechin consumed in 80, 53 and 27 g of procyanidin-rich chocolate. These percentages can be compared with a plasma catechin absorption of 330 μmol · h⁻¹ · L⁻¹ and a recovery of 0.24% after subjects consumed 126 μmol of (+)-catechin in reconstituted red wine (Bell et al. 2000). Collectively, the data from the above studies suggest that catechins from chocolate are more bioavailable than catechins from wine.

Similar to plasma epicatechin kinetics, there were also differences in chocolate-induced changes in plasma antioxidant capacity between our previous work (Rein et al. 2000a) and the current study. In the former study, plasma antioxidant capacity peaked at 2 h after chocolate consumption, whereas in the present study, plasma antioxidant capacity showed a further increase at the 6-h time point from that of the 2-h time point. This response is consistent with the different pattern of epicatechin absorption that was observed in the two studies.

It has been suggested that the measurement of plasma concentrations of F₂-isoprostanes can provide an additional biomarker for oxidative stress in vivo (Pratico, 1999, Roberts and Morrow, 2000). In addition, because of their function as renal and pulmonary vasoconstrictors, the assessment of F₂-isoprostanes may provide insight into the effects of specific food products on vascular health (Morrow and Roberts 1997, Morrow et al. 1999, Practico, 1999). In the current study, we could not document a dose-related change between chocolate consumption and plasma F₂-isoprostane concentrations.

Given the results of the current study, as well as the findings of others, we suggest that the daily consumption of procyanidin-rich foods, such as chocolate, can result in improvements in the antioxidant potential of plasma. A potential mechanism for such improvement would be a sparing capacity that involved other antioxidant molecules such as ascorbate, α-tocopherols and carotenoids (Lotito and Fraga 1998 and 1999 and 2000, Salah et al. 1995).

The physiological relevance of such an increase in plasma epicatechin after the consumption of procyanidin-rich chocolate can be shown by the results presented in this study and in Rein et al. (2000a) on modifying select oxidative stress variables. In addition, it can be speculated that changes in oxidative stress may contribute to the suppressive effects of procyanidins on platelet activation (Rein et al. 2000b). In summary, the results obtained to date demonstrate an in vivo physiological effect of chocolate consumption on several biological variables associated with vascular pathologies, including cardiovascular disease.

ACKNOWLEDGMENT
We would like to thank Janet Peerson for her assistance in the statistical analysis of these data.

LITERATURE CITED


<table>
<thead>
<tr>
<th>Procyanidin-rich chocolate food consumed, g</th>
<th>8-Isoprostane μmol/L</th>
<th>0</th>
<th>27</th>
<th>53</th>
<th>80</th>
</tr>
</thead>
<tbody>
<tr>
<td>Time, h</td>
<td></td>
<td>48 ± 5 (7) 39 ± 8 (8) 52 ± 7 (10) 50 ± 7 (7)</td>
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<td>0</td>
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<td>54 ± 7 (7) 42 ± 5 (8) 49 ± 4 (10) 42 ± 9 (7)</td>
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<td></td>
</tr>
</tbody>
</table>

Values are expressed as means ± SEM. Values in parentheses indicate number of subjects. There were no significant differences across treatments or time.


