Reactions of Peroxynitrite with Cocoa Procyanidin Oligomers1,2

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ABSTRACT Peroxynitrite is a mediator molecule in inflammation, and its biological properties are being studied extensively. Flavonoids, which are natural plant constituents, protect against peroxynitrite and thereby could play an anti-inflammatory role. Procyanidin oligomers of different sizes (monomer through nonamer), isolated from the seeds of Theobroma cacao, were recently examined for their ability to protect against peroxynitrite-dependent oxidation of dihydrorhodamine 123 and nitration of tyrosine and were found to be effective in attenuating these reactions. The tetramer was particularly efficient at protecting against oxidation and nitration reactions. Epicatechin oligomers found in cocoa powder and chocolate may be a potent dietary source for defense against peroxynitrite. J. Nutr. 130: 2100S—2104S, 2000.

KEY WORDS: • flavonoids • inflammation • procyanidins • cocoa • peroxynitrite • antioxidants

Oxidative stress is an imbalance between the steady-state fluxes of pro-oxidants and antioxidants in the biological system, with the balance being shifted in favor of the pro-oxidants, so that oxidative damage may be inflicted (Sies 2000). One form of oxidative stress is that associated with enhanced production of reactive nitrogen species, nitric oxide and peroxynitrite. This condition, which is also called nitrosative stress, may occur in states of inflammation, because inflammatory cells produce enhanced amounts of nitric oxide and of superoxide, both of which react rapidly to form peroxynitrite. While this is used by the organism to attack invading microorganisms, there may be an overproduction of these oxidants, which could be hazardous to surrounding healthy tissue. Indeed, as a potent oxidizing and nitrating species, peroxynitrite leads to tissue damage in a number of pathological conditions in humans and experimental animals (Beckman 1996, Beckman et al. 1990). Therefore, there is a need for defense against peroxynitrite. The physiological and pharmacological strategies for protection against peroxynitrite are organized into three categories: prevention, interception and repair (Arteel et al. 1999, Sies 1993). Flavonoids occur in different classes, including procyanidins, as natural products in plants, and these polyphenols are ingested with the diet (Haslam 1998, Lazarus et al. 1999). Flavonoids are general free radical scavengers (Bors and Michel 1999, Kondo et al. 1999). Possible health benefits of polyphenols include the suppression of inflammatory cytokine production (Mao et al. 1999, Sanbongi et al. 1997), protection against cardiovascular disease (Kondo et al. 1996, Waterhouse et al. 1996) and anticarcinogenic effects (Stoner and Mukhtar 1995, Yang et al. 1998).

Flavonoids react with nitric oxide (van Acker et al. 1995) and superoxide (Girard et al. 1995, Robak and Gryglewski 1988) and protect against peroxynitrite oxidation and nitration reactions (Haenen et al. 1997, Pannala et al. 1997). Recent work with the procyanidin (−)-epicatechin and the respective procyanidin oligomers, ranging up to the nonamer isolated from Theobroma cacao, showed that these compounds effectively prevent oxidation and nitration reactions of peroxynitrite (Arteel and Sies 1999), with the tetrameric compound being of particular interest. It is likely that these compounds react with nitrating and oxidizing intermediate species formed during peroxynitrite decay and not with peroxynitrite proper, similar to simple phenolic compounds (Ramezanian et al. 1996). Here, we present some characteristics of the reaction of peroxynitrite with epicatechin and the tetrameric compound.

MATERIALS AND METHODS

Reagents. The tetrameric procyanidin oligomer was isolated and purified from CocoaPro cocoa from the seeds of T. cacao (Adamson et al. 1999, Hammerstone et al. 1999) and was kindly supplied by Mars (Hackettstown, NJ). Stock solutions (10 mg/ml) of the tested procyanidin preparations were made in methanol but were readily soluble in aqueous solutions used in the studies. Diethyletheramine pentaacetic acid (DTPA),4 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 5,5'-dithiobis-2-nitrobenzoic acid (DHR-123) and cyanidin preparations were made in methanol but were readily soluble in aqueous solutions used in the studies. Diethyletheramine pentaacetic acid (DTPA), 5,5-dithiobis-2-nitrobenzoic acid (DTNB), 5,5'-dithiobis-2-nitrobenzoic acid (DHR-123) and cyanidin preparations were made in methanol but were readily soluble in aqueous solutions used in the studies. Diethyletheramine pentaacetic acid

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To determine the effect of peroxynitrite on glutathione, peroxynitrite (500 \mu mol/L) was added via bolus addition under constant vortexing to 100 \mu mol/L tyrosine in 0.1 mol/L phosphate buffer (pH 7.3) containing 0.1 mmol/L DTPA. Under these conditions, the effects of the tested compounds (0–20 \mu mol/L) were determined and quantified with HPLC (see later).

**HPLC analysis.** To quantify hydroxylated benzoic acid formation by peroxynitrite, samples (50 \mu L) were injected onto a C18 reversed phase column (150 \times 4.6 mm; Merck, Darmstadt, Germany) with a Waters (Milford, MA) 720 WISP autosampler. Separation was performed with 50 mmol/L ammonium acetate (pH 7.0)/methanol (68:32) at a flow rate of 1.0 ml/min. The fluorescent product (2-hydroxybenzoic acid) formation was monitored with a Merck-Hitachi F-1000 fluorescence detector (\lambda_e = 330 nm, \lambda_m = 410 nm) equipped with a D-2500 Chromato-Integrator. Calibration curves with 2-hydroxybenzoic acid were used to determine concentrations. Under these conditions, the effects of epicatechin or of the tetrmeric oligomer (50 \mu mol/L) were determined relative to the effect of peroxynitrite alone (“No addition”).

To determine the effect of peroxynitrite on free tyrosine, samples (50 \mu L; containing 100 \mu mol/L 3-hydroxy-4-nitrobenzoic acid as an internal standard) were injected onto a C18 reversed phase column (150 \times 4.6 mm; Merck) with a Waters 720 WISP autosampler. Separation was performed with a 50 mmol/L potassium phosphate buffer (pH 7.0)/acetonitrile step gradient on a Merck-Hitachi L-655A 12 HPLC unit coupled with a Merck-Hitachi L-5000 controller unit at a flow rate of 1.0 ml/min. The initial ratio of buffer to acetonitrile was 95:5, follow by a stepwise decrease to 50:50 at 5 min; after 13 min, the ratio was returned to 95:5 and was maintained for an additional 13 min. Such a step gradient was necessary to achieve separation of the compounds of interest and then to elute the fla-

### Table 1

<table>
<thead>
<tr>
<th>Compound</th>
<th>IC50 (nmol/L)</th>
<th>DHR-123 oxidation</th>
<th>Tyrosine nitration</th>
</tr>
</thead>
<tbody>
<tr>
<td>Epigallocatechin</td>
<td>410</td>
<td>0.9</td>
<td>4.3</td>
</tr>
<tr>
<td>Gallate</td>
<td>50</td>
<td>1.11</td>
<td>4.3</td>
</tr>
<tr>
<td>Epicatechin</td>
<td>290</td>
<td>12.0</td>
<td>ND</td>
</tr>
<tr>
<td>Tetrimer</td>
<td>1154</td>
<td>4.3</td>
<td>ND</td>
</tr>
<tr>
<td>Ebselen</td>
<td>274</td>
<td>160</td>
<td>ND</td>
</tr>
</tbody>
</table>

The oxidation of dihydrorhodamine 123 (DHR-123) and nitration of tyrosine by peroxynitrite was determined as described in the text. See Figure 2. ND, not determined. Data from Arteel and Sies (1999).
vonoids. The formation of 3-nitrotyrosine was monitored with a Merck-Hitachi L-4200 UV/Vis detector equipped with a D-2500 Chromato-Integrator at 430 nm, whereas the disappearance of tyrosine was monitored at 280 nm. Calibration curves of the ratio of peak area of 3-nitrotyrosine standard to internal standard were used to determine concentrations (Fig. 1).

RESULTS AND DISCUSSION

Protection by procyanidin oligomers against the oxidation of dihydrorhodamine during bolus addition of peroxynitrite. Table 1 summarizes the protection of the tested compounds against oxidation of free tyrosine by peroxynitrite (see Figs. 2, 3). When peroxynitrite (500 μmol/L) was added to tyrosine-containing buffer, a peak that absorbs at 430 nm appeared (Fig. 2B) with a concomitant decrease in tyrosine (Fig. 3B); this effect was blunted with low micromolar concentrations of epicatechin or epigallocatechin gallate (Table 1), leading to protection of tyrosine (Fig. 3C). Similar to protection against DHR-123 oxidation, the tetrameric compound was more effective on a molar basis (Table 1, Fig. 3D). It is of interest that the peak corresponding to procyanidin parent compound (e.g., Fig. 3E) disappeared on the addition of peroxynitrite, leading to the formation of new product peaks (Fig. 3F). These data suggest that the procyanidins are modified during reaction with peroxynitrite, leading to new products that appear to be more water soluble.

It is suggested that DHR-123 oxidation is a second-order reaction (Kooy et al. 1994); therefore, the results of the DHR-123 oxidation assay could suggest a competition-type reaction. However, simpler phenolic compounds tend to react via a first-order pathway (Ramezanian et al. 1996), dependent only on peroxynitrite concentrations. To further determine protection by procyanidin oligomers against nitration of tyrosine by peroxynitrite. Table 1 summarizes the protection of the tested compounds against nitration of free tyrosine by peroxynitrite (see Figs. 2, 3). When peroxynitrite (500 μmol/L) was added to tyrosine-containing buffer, a peak that absorbs at 430 nm appeared (Fig. 2B) with a concomitant decrease in tyrosine (Fig. 3B); this effect was blunted with low micromolar concentrations of epicatechin or epigallocatechin gallate (Table 1), leading to protection of tyrosine (Fig. 3C). Similar to protection against DHR-123 oxidation, the tetrameric compound was more effective on a molar basis (Table 1, Fig. 3D). It is of interest that the peak corresponding to procyanidin parent compound (e.g., Fig. 3E) disappeared on the addition of peroxynitrite, leading to the formation of new product peaks (Fig. 3F). These data suggest that the procyanidins are modified during reaction with peroxynitrite, leading to new products that appear to be more water soluble.

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the mechanism of protection of the tested compounds against peroxynitrite, we investigated whether epicatechin can protect against the oxidation of GSH, another second-order reaction (Radi et al. 1991). Peroxynitrite (10 μmol/L), added via bolus addition to GSH (10 μmol/L), caused a 40% oxidation of this thiol compound, as assayed by the DTNB-reactive content (Table 2). Molar ratios of epicatechin as high as 20-fold had no protective effect on GSH oxidation. Indeed, lower concentrations tended to increase GSH oxidation (Table 2). The lack of competitive protection against GSH oxidation by peroxynitrite with procyanidin oligomers suggest that these compounds do not react directly with peroxynitrite, similar to simpler phenolics (Ramezanian et al. 1996). Conversely, epicatechin and the tetrameric compound were both effective at protecting against hydroxylolation of benzoic acid by peroxynitrite (Table 3), also a first-order reaction (Ramezanian et al. 1996), with the tetrameric compound again more effective than epicatechin (Table 3). Furthermore, although epicatechin did not enhance the decay rate of peroxynitrite, an increase in absorbance at 430 nm was observed that occurred with a time frame longer than peroxynitrite decay (data not shown).

**Implications.** The role of dietary polyphenols in health and disease has received recent attention (Rice-Evans and Packer 1998, Ursini et al. 1999). These compounds have been shown to inhibit nitration (Pannala et al. 1997) and oxidation reactions (Haenen et al. 1997), as well as DNA damage and strand breakage (Fiala et al. 1996, Ohshima et al. 1998), caused by peroxynitrite. Previous studies have shown that polyphenolic compounds may be both oxidized and nitrated by the addition of peroxynitrite (Kerry and Rice-Evans 1998, Pannala et al. 1998), similar to simpler phenolics (Ramezanian et al. 1996). It has been previously suggested that polyphenolic compounds scavenge peroxynitrite (Haenen et al. 1997), suggesting a second-order type of reaction, dependent on concentration of both procyanidin and peroxynitrite. However, the results of this current study, coupled with previous work (Arteel and Sies 1999), suggest that procyanidins do not directly react with peroxynitrite but most likely with reactive oxidizing/nitrating intermediates. Currently, it is not known whether the product or products formed from this reaction can be recycled in vivo (i.e., to maintain a catalytic cycle of defense) or whether this reaction is a one-time event. However, because the daily dietary intake of procyanidins is quite high, it may be unnecessary for these compounds to be recycled. Furthermore, destruction/modification of procyanidins during the reaction with peroxynitrite may protect more important sites from damage (e.g., tyrosine residues on proteins). Although epicatechin from chocolate was found to reach a concentration of 0.7 μmol/L in plasma after the intake of 80 g of black chocolate (Richelle et al. 1999), it is not yet known how well cocoa procyanidin oligomers are absorbed into the bloodstream. Recent work (Spencer et al. 1999) using the isolated rat intestine showed that for certain flavonoids, glucuronidation and possibly other metabolism may occur at the level of the intestinal mucosa. Future research will involve further investigation of some of these issues.

**TABLE 2**

**Oxidation of GSH by peroxynitrite showing the effect of epicatechin**

<table>
<thead>
<tr>
<th>Addition</th>
<th>GSH</th>
<th>% of control</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10.5 ± 0.7</td>
<td>100 ± 6</td>
</tr>
<tr>
<td>Peroxynitrite, 10 μmol/L</td>
<td>6.4 ± 0.1</td>
<td>61 ± 1</td>
</tr>
<tr>
<td>+Epicatechin, 10 μmol/L</td>
<td>5.2 ± 0.2</td>
<td>49 ± 2</td>
</tr>
<tr>
<td>20 μmol/L</td>
<td>5.1 ± 0.2</td>
<td>48 ± 2</td>
</tr>
<tr>
<td>100 μmol/L</td>
<td>5.6 ± 0.6</td>
<td>53 ± 6</td>
</tr>
<tr>
<td>200 μmol/L</td>
<td>6.1 ± 0.3</td>
<td>58 ± 3</td>
</tr>
</tbody>
</table>

Peroxynitrite (10 μmol/L) was added to 10 μmol/L glutathione (GSH) and different concentrations of epicatechin in 0.1 mol/L phosphate buffer, 0.1 mmol/L diethylethriammonium pentaacetic acid, pH 7.4, under intense stirring at room temperature. GSH was determined colorimetrically with 5,5′-dithiobis-2-nitrobenzoic acid as described in the text. The concentration of GSH in the presence of epicatechin (200 μmol/L) alone was 9.4 ± 0.2 μmol/L. Results are means ± SD (n = 3).

**TABLE 3**

**Protection against peroxynitrite-induced hydroxylation of benzoate by epicatechin and epicatechin tetramer**

<table>
<thead>
<tr>
<th>Addition</th>
<th>2-Hydroxy-benzoic acid formation</th>
<th>μmol/L</th>
<th>% yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Peroxynitrite, 500 μmol/L</td>
<td>1.60 ± 0.10</td>
<td>0.32</td>
<td></td>
</tr>
<tr>
<td>+Epicatechin, 50 μmol/L</td>
<td>0.51 ± 0.04</td>
<td>0.10</td>
<td></td>
</tr>
<tr>
<td>+Tetramer, 50 μmol/L</td>
<td>0.16 ± 0.01</td>
<td>0.03</td>
<td></td>
</tr>
</tbody>
</table>

Peroxynitrite (500 μmol/L) was added to 1 mmol/L benzoic acid and 50 μmol/L epicatechin or tetramer in 0.1 mol/L phosphate buffer, 0.1 mmol/L diethyletherammonium pentaacetic acid, pH 7.3, under intense stirring at room temperature. 2-Hydroxy-benzoic acid formation was determined with HPLC separation and fluorescence detection as described in the text. Results are means ± SD (n = 3).

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**LITERATURE CITED**


