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(−)-Epigallocatechin gallate, a polyphenolic tea antioxidant, inhibits peroxynitrite-mediated formation of 8-oxodeoxyguanosine and 3-nitrotyrosine

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Abstract. Reaction with peroxynitrite at pH 7.4 and 37 °C was found to increase the 8-oxodeoxyguanosine levels in calf thymus DNA 35–38-fold. This oxidation of deoxyguanosine, as well as the peroxynitrite-mediated nitration of tyrosine to 3-nitrotyrosine, was significantly inhibited by ascorbic acid, glutathione and (−)-epigallocatechin gallate, a polyphenolic antioxidant present in tea. For 50% inhibition of the oxidation of deoxyguanosine to 8-oxodeoxyguanosine, 1.1, 7.6 or 0.25 mM ascorbate, glutathione or (−)-epigallocatechin gallate, respectively, was required. For 50% inhibition of tyrosine nitration, the respective concentrations were 1.4, 4.6 or 0.11 mM. Thus, (−)-epigallocatechin gallate is a significantly better inhibitor of both reactions than either ascorbate or glutathione. Reaction of (−)-epigallocatechin gallate with peroxynitrite alone resulted in the formation of a number of products. Ultraviolet spectra of two of these suggest that the tea polyphenol and/or its oxidation products are nitrated by peroxynitrite.
Key words. Antioxidant; epigallocatechin gallate; tea polyphenols; 3-nitrotyrosine; 8-oxodeoxyguanosine.

Nitric oxide and superoxide, produced by a variety of cells including activated neutrophils and macrophages, react to form peroxynitrite (ONOO-) [1, 2], a bactericidal agent [3], at a near-diffusion-limited rate [4, 5]. The protonated form of peroxynitrite, peroxynitrous acid ($pK_a = 6.8$, $t_{1/2} \sim 1$ s) [6, 7], decomposes rapidly to form powerful oxidizing and nitrating species. These are known to react with lipids, proteins and nucleic acids with reactivities similar to those of the hydroxyl radical and nitrogen dioxide [8–11]. While the production of peroxynitrite by leukocytes contributes to the destruction of foreign microorganisms, its excess or uncontrolled production may be harmful. For instance, the nitration of tyrosine to 3-nitrotyrosine and oxidation of low-density lipoprotein by peroxynitrite have been suggested as initiating steps in atherosclerosis [12, 13]. Protection against tissue damage by peroxynitrite and reactive oxygen species is afforded by various cellular antioxidants, including glutathione, α-tocopherol and ascorbic acid [14]. However, endogenous antioxidants may become depleted to levels insufficient for complete cellular protection. If this occurs, additional antioxidants from dietary sources could become important in limiting damage [15].

Due to the wide variety of beneficial biological effects ascribed to them, the powerful naturally occurring polyphenol antioxidants (catechins) present in tea (*Camellia sinensis*) are attracting increasing attention [16]. These antioxidants have been reported to inhibit hypercholesterolemia, hypertension, mutagenesis and tumorigenesis in several experimental models [17, 18]. Of the tea catechins examined, (−)-epigallocatechin gallate (EGCg; fig. 1) is the most effective, and also has the most powerful antioxidant properties. In some respects, EGCg is an even better antioxidant than α-tocopherol, butylated hydroxyanisole or butylated hydroxytoluene [18]. The exceptional antioxidant characteristics of EGCg and its extensive human consumption in the form of tea prompted us to examine its effects on two of the reactions by which peroxynitrite could produce cell injury: oxidation of guanine residues in DNA and the nitration of tyrosine. We find that both reactions are inhibited by the antioxidants ascorbic acid and glutathione as well as by the tea polyphenol EGCg. Of the three antioxidants, however, EGCg is by far the most effective.

Materials and methods

Chemicals. Sodium azide was purchased from Aldrich Chemical Co. (Milwaukee, WI). L-Tyrosine, 3-nitro-L-

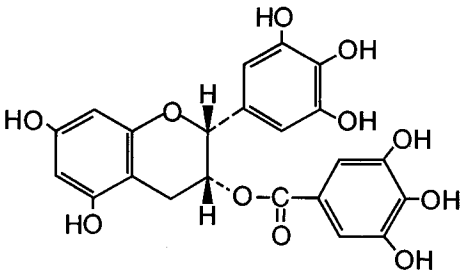

Figure 1. Structure of (−)-epigallocatechin gallate (EGCg).

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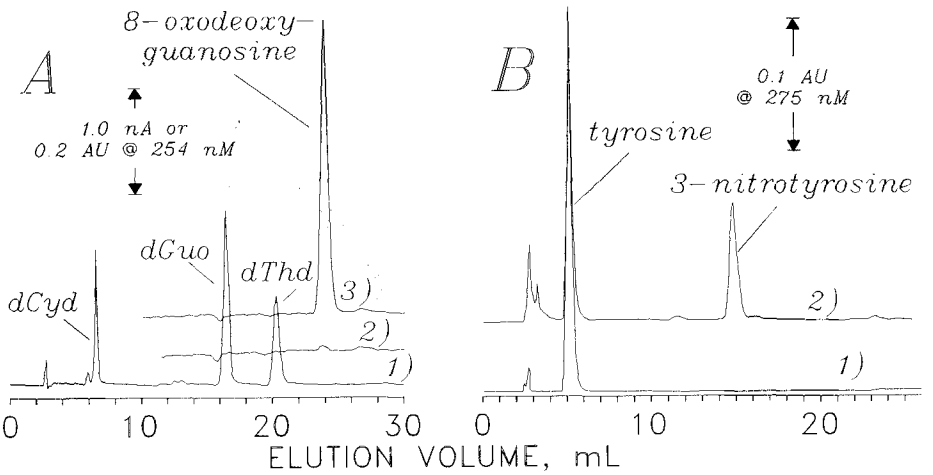

Figure 2. (*A*) Determination, by HPLC-EC, of 8-oxodeoxyguanosine formed in calf thymus DNA by reaction with peroxynitrite at pH 7.4. Solvent system 2 was used. Trace 1, UV absorption at 254 nm; traces 2 and 3, electrochemical detector response. Trace 2, control; DNA was incubated with decomposed peroxynitrite. Trace 3, DNA was incubated with peroxynitrite. (*B*) Determination, by HPLC with UV detection, of peroxynitrite-mediated formation of 3-nitrotyrosine from tyrosine. Solvent system 1 was used. Trace 1, control; tyrosine was incubated with decomposed peroxynitrite; trace 2, tyrosine was incubated with peroxynitrite.

tyrosine standard, calf thymus DNA, diethylenetriaminepentaacetic acid (DTPA), ascorbic acid, glutathione and all of the enzymes used in this work were purchased from Sigma Chemical Co. (St Louis, MO). Beckman Ultrasphere ODS columns were obtained from Alltech (Avondale, PA). EGCg $\geq$ 97% pure by high-performance liquid chromatograph (HPLC) analysis, was the kind gift of Dr Y. Hara through Dr John H. Weisburger.

Preparation of peroxynitrite. *Caution: Ozone is extremely toxic by inhalation.* The ozone generator, Fischer Model 500, was purchased from Fischer Associates (Deerfield, IL). Peroxynitrite, 0.06–0.08 M, was prepared by the method of Pryor et al. [19], by passing ozone through a solution of sodium azide in 0.01 N NaOH in a fume hood. Excess ozone was destroyed by reaction with potassium iodide solution in traps that were connected in series with the effluent end of the reaction flask. Stock solutions of 60–80 mM peroxynitrite showed no significant loss when stored at −80 °C over a period of 4 months. The stock preparations were thawed just before use and were diluted to the required concentrations with 0.01 N NaOH. The concentration of peroxynitrite was determined by ultraviolet (UV) absorbance at 301 nm using the extinction coefficient 1670 M^{-1} cm^{-1} [19].

HPLC systems. Either Shimadzu Model LC-600 pumps with the Shimadzu Model SCL-6B solvent system controller and the Waters Model 996 photodiode array detector, or the Waters HPLC Model 510 pumps with the Waters automated gradient controller and the Model 990 photodiode array detector, were used. An Ultrasphere ODS column (4.5 × 250 mm) connected in series with an Ultrasphere ODS guard column (4.5–50 mm) was eluted at 1 ml/min with one of the following solvent systems: solvent system 1, isocratic elution with 10% methanol in water containing 0.1% acetic acid; solvent system 2, isocratic elution with 12.5 mM sodium citrate, 25 mM sodium acetate, pH 5.1, 5% in methanol; solvent system 3, 12.5 mM sodium citrate, 25 mM sodium acetate, pH 5.2, 4% in methanol for 5 min, then linear gradient with same buffer to 24% methanol in 30 min, followed by a 15-min linear gradient to 60% methanol (same buffer system).

Reaction of peroxynitrite with calf thymus DNA. While the mixture was vortexed gently, peroxynitrite, 12–15 μmol, was added to ~3 mg of calf thymus DNA that had been dissolved in 1.0 ml of 0.5 M potassium phosphate buffer, pH 7.4, 0.5 mM in DTPA, pre-equilibrated at 37 °C. The reaction mixture was incubated at 37 °C for 5 min more and then was dialyzed against several large volumes of distilled water at 4 °C for 24–48 h. The desalted DNA was hydrolyzed using nuclease P1 at pH 5.2 and then alkaline phosphatase at pH 7.4. For the quantitation of 8-oxodeoxyguanosine, a modification of the method described by Floyd and co-workers [20] was used. Analyses were performed using solvent system 2, with a Princeton Applied Research Model 400 electrochemical detector (ECD) connected downstream of the photodiode array detector. The potential at the working electrode of the ECD was set to +600 mV vs the Ag/AgCl reference electrode. To provide controls, DNA was incubated in exactly the same way with peroxynitrite that had been allowed to decompose at pH 7.4. The DNA was then processed as above.

Reaction of peroxynitrite with L-tyrosine. Peroxynitrite, 6–8 μmol, was added to a vigorously stirred solution consisting of 0.9 ml of 2.8 mM L-tyrosine in 0.5 M potassium phosphate buffer, pH 7.4, 0.5 mM in DTPA, at 37 °C. For the quantitation of 3-nitrotyrosine, HPLC

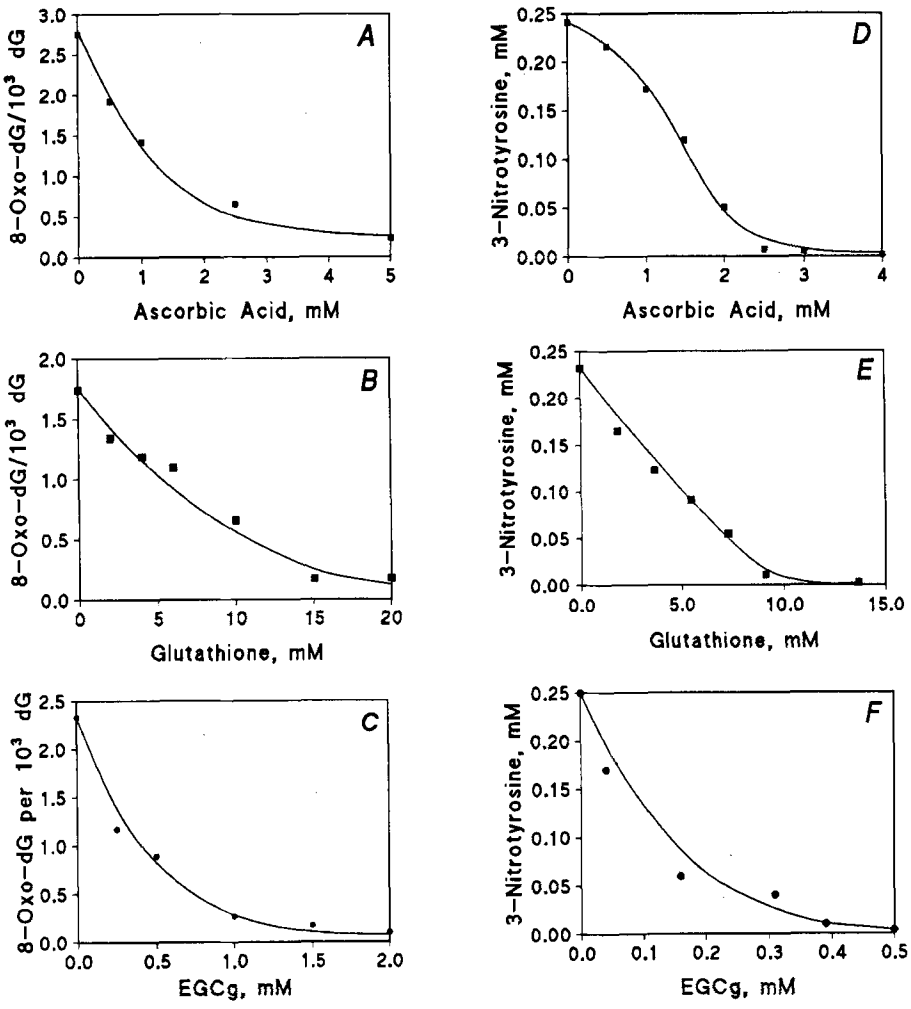

Figure 3. Concentration-dependent inhibition by ascorbic acid, glutathione or EGCg of peroxynitrite-mediated oxidation of calf thymus DNA deoxyguanosine to 8-oxodeoxyguanosine (panels *A*, *B* and *C*) and of peroxynitrite-mediated nitration of tyrosine to 3-nitrotyrosine (panels *D*, *E* and *F*).

and solvent system 1 were used, with detection by UV light absorption at 275 nm. Standard curves were constructed using commercial 3-nitrotyrosine. Controls involved exactly the same operations, except that decomposed peroxynitrite was used.

Effects of antioxidants. To study the effect of antioxidants on the peroxynitrite-mediated formation of 3-nitrotyrosine or 8-oxodeoxyguanosine, appropriate aliquots of the freshly prepared concentrated solutions (10–50 mM) of ascorbic acid, glutathione or EGCg were added to the solutions of calf thymus DNA or L-tyrosine just prior to the addition of peroxynitrite. The reaction mixtures were incubated and analyzed for 8-oxodeoxyguanosine or 3-nitrotyrosine as above. Data shown are representative of two to four separate experiments.

Results and discussion

8-Oxodeoxyguanosine, a weakly mutagenic DNA modification [21], is normally present in DNA from various sources at low levels [22]. As shown in fig. 2A, at pH 7.4 and 37 °C, peroxynitrite was found to increase 8-oxodeoxyguanosine in calf thymus DNA 35–38 times the basal level (70 μmol/mol guanine). Essentially no increase in 8-oxodeoxyguanosine was observed in control experiments in which either decomposed peroxynitrite, or sodium nitrite plus sodium nitrate, were incubated with DNA at pH 7.4. Addition of the antioxidants ascorbic acid or glutathione, or the tea polyphenol EGCg, to DNA solutions prior to the addition of peroxynitrite produced a concentration-dependent inhibition of 8-oxodeoxyguanosine formation (fig. 3A–C).

While thiols such as cysteine and glutathione have been reported to react with peroxynitrite at rates higher than ascorbic acid [23, 24], under our conditions glutathione (fig. 3B), was less effective than ascorbic acid (fig. 3A) in inhibiting the oxidation of deoxyguanosine to 8-oxodeoxyguanosine. For a 50% inhibition of the reaction, the concentration of glutathione required was ~7.6 mM (fig. 3B), compared to ~1.1 mM required for

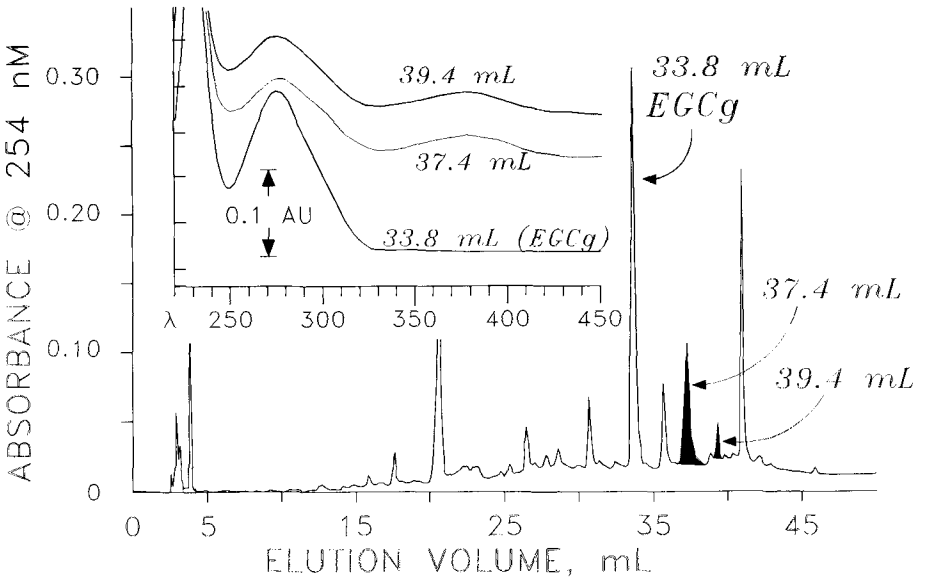

Figure 4. Typical HPLC profile of products formed during the incubation of peroxynitrite (3.9 mM) with EGCg (2 mM) at 37 °C for 5 min. HPLC solvent system 3 was used. The UV spectra of peaks (shaded) eluting at 37.4 and 39.4 mL are compared in insert with the spectrum of EGCg eluting at 33.8 ml. The presence of a maximum at 380–390 nm suggests that these species contain a nitro group.

ascorbic acid (fig. 3A). EGCg, which decreased the oxidation of deoxyguanosine by 50% at a concentration of ~0.25 mM under identical conditions, proved to the most effective inhibitor (fig. 3C).

Peroxynitrite can nitrate tyrosine to 3-nitrotyrosine [13, 25, 26]. In vitro studies with the gas phase of cigarette smoke, which presumably gives rise to peroxynitrite, have suggested that the nitration of tyrosine can be inhibited by glutathione, ascorbic acid and uric acid [27]. In chronic diseases such as human acute lung injury [28] and atherosclerosis [13], the detection of 3-nitrotyrosine in proteins at the sites of the lesions has been considered evidence for the involvement of peroxynitrite. Figure 2B illustrates the extensive nitration of tyrosine (2.8 mM) which occurs during incubation with 7 mM peroxynitrite at pH 7.4 and 37 °C; the yield under these conditions is 7.6–9.4% of the tyrosine present.

Both ascorbic acid and glutathione inhibited the formation of 3-nitrotyrosine in a concentration-dependent manner, but ascorbic acid was more effective than was glutathione (fig. 3D and 3E). The polyphenolic tea antioxidant EGCg again was more effective than either of the two endogenous antioxidants, producing a 50% inhibition of tyrosine nitration (fig. 3F) at a concentration of 0.11 mM. The same degree of inhibition was achieved by ascorbic acid at ~1.4 mM, and by glutathione at ~4.6 mM. In this case, EGCg appears to function as a true antioxidant as defined by Halliwell [22], since we find that it was effective at concentrations much lower (~10 times) than the substrates tyrosine and the deoxyguanosine moiety in calf thymus DNA.

When EGCg (2 mM) alone was incubated with peroxynitrite (3.9 mM) in phosphate buffer, pH 7.4, at 37 °C for 5 min, we noted the formation of a variety of reaction products detectable by HPLC with UV detection (fig. 4). These have not yet been identified. However, it is interesting that at least two of the products showed UV light absorption maxima at 275 and 380–390 nm (fig. 4, insert), characteristic of nitroaromatic compounds. This suggests that EGCg and/or its oxidation products are nitrated by peroxynitrous acid. Thus, although the detailed mechanism whereby EGCg inhibits peroxynitrous acid-mediated reactions such as the oxidation of guanine to 8-oxoguanine and the nitration of tyrosine requires further study, competition with other reactants for the oxidizing and nitrating species derived from peroxynitrous acid may be at least partially involved. The kinetic parameters of the inhibition are currently being studied.

Our observation that the tea polyphenolic antioxidant EGCg strongly inhibits peroxynitrite-mediated oxidation and nitration in model systems in vitro raises the possibility that EGCg could play a similar role in vivo. EGCg may be well qualified for that role. The antioxidant is soluble in both water and organic solvents and might be able to inhibit these reactions in hydrophilic as well as hydrophobic cell compartments. Whether this indeed occurs, and the mechanisms responsible for the protective effects of EGCg on hypertension, hypercholesterolemia and tumorigenesis in animal models previously reported by others [14, 18], are subjects deserving further investigation.

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