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# Inhibition of Peroxynitrite-Mediated Tyrosine Nitration by Catechin Polyphenols

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**Peroxynitrite is a cytotoxic species generated by the reaction between superoxide and nitric oxide. The ability of catechins and their gallate esters to decrease peroxynitrite-induced nitration of tyrosine and to limit surface charge alteration of low density lipoprotein (LDL) was investigated. All compounds tested were found to be potent peroxynitrite scavengers preventing the nitration of tyrosine. The ability of the catechin polyphenols at 10 $\mu$M to minimise tyrosine nitration induced by peroxynitrite (500 $\mu$M) was ECG (38.1 $\pm$ 3.6%) $\approx$ EGCG (32.1 $\pm$ 7.5%) $\approx$ gallic acid (32.1 $\pm$ 1.9%) $>$ catechin (23.9 $\pm$ 5.4%) $\approx$ epicatechin (22.9 $\pm$ 3.3%) $\approx$ EGC (19.9 $\pm$ 2.0%). Trolox (10 $\mu$M) was used as the standard for comparative purposes and was found to be less effective than the polyphenols in inhibiting tyrosine nitration (13.6 $\pm$ 2.9%). The catechin polyphenols were also found to offer protection from peroxynitrite-induced modification of critical amino acids of apolipoprotein B-100 of LDL which contribute towards its surface charge.** 

Diets rich in fruit and vegetables are known to play a role in protection against coronary heart disease and certain types of cancer [1]. This has been attributed to a variety of cardioprotective and anticarcinogenic mechanisms of the individual constituents, including the free radical scavenging properties of the antioxidant nutrients vitamins C and E, and possibly the carotenoids. Recent studies are also beginning to highlight the potential role of the phenolic constituents, the flavonoids, hydroxycinnamates and phenolic acids as contributors to the antioxidant properties. Polyphenols have been reported to function as antioxidants by virtue of their hydrogen-donating properties [2-5] as well as their transition metal-chelating potentials [6]. Flavanols, epicatechin, epigallocatechin and their gallate esters (Fig. 1) have been shown to scavenge both aqueous and lipophilic radicals and to protect low density lipoprotein from oxidation by acting as chain-breaking antioxidants [7-9].

Peroxynitrite is a highly toxic oxidising and nitrating species which can be produced *in vivo* by the interaction of superoxide and nitric oxide. The second order rate constant of the reaction between nitric oxide and superoxide is $6.7 \times 10^9$ $M^{-1}$ $s^{-1}$ [10]. Stimulated macrophages, neutrophils and endothelial cells have been shown to generate peroxynitrite [11-13] and recent data have provided evidence for *in vivo* formation of peroxynitrite in, for example, human atherosclerotic coronary vessels, acute lung injury and chronic inflammation [14-17]. Peroxynitrite at physiological pH (pKa - 6.8) protonates to form peroxynitrous acid which decays rapidly to form a mixture of reactive products; peroxynitrite and products derived from it have been reported to induce lipid peroxidation [18] and modify amino acids of proteins. For example, tyrosine is especially susceptible to peroxynitrite-induced nitration reactions. The ability to inhibit peroxynitrite dependent nitration provides a useful assay to screen various compounds for their ability to scavenge peroxynitrite and the nitrating species derived from it [19]. DNA bases have been reported to undergo peroxynitrite-induced base modification which can lead to strand breakage [20].

Several studies have been devoted to characterising oxidative and non-oxidative changes in apolipoprotein B-100 of LDL, and they indicate the critical involvement of specific amino acids in causing recognition of modified LDL by the macrophage scavenger receptor - modifications to lysine residues and changes in charge have a major influence [21-24]. Recent evidence suggests that peroxynitrite is capable of modifying more than half of the lysine residues on apolipoprotein B-

Abbreviations used: LDL, low density lipoprotein; $ONOO^-$, peroxynitrite anion; ONOOH, peroxynitrous acid; EGC, epigallocatechin; ECG, epicatechin gallate; EGCG, epigallocatechin gallate; REM, relative electrophoretic mobility; CV%, percentage coefficient of variation; M%D, mean percentage difference.

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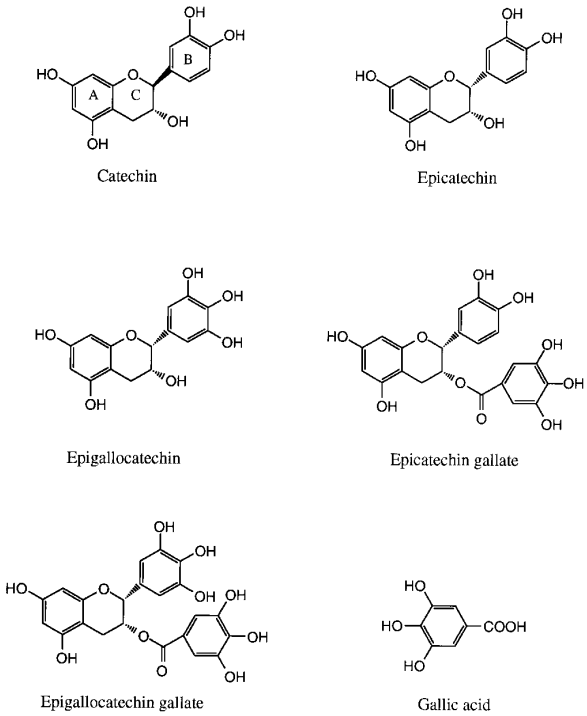


**FIG. 1.** Chemical structures of catechins and catechin-gallate esters.

100 [25] but the end-products have not been identified to date. In addition to interaction with lysine residues, modification of other aromatic and basic amino acids such as tyrosine, tryptophan, phenylalanine, histidine and arginine may also be significant in enhancing the recognition and uptake of modified LDL by macrophages.

Due to the increasing interest in the biological importance of catechin polyphenols (catechin, epicatechin, ECG, EGC and EGCG) their ability to act as peroxynitrite scavengers was investigated in this study. Two assays were used, the decrease in the nitration of tyrosine and ability to limit modification of low density lipoprotein (LDL) induced by peroxynitrite. The established antioxidants trolox and gallic acid were used as reference standards for comparative activities.

## MATERIALS AND METHODS

*Chemicals.* The flavanols were obtained from Unilever Research Laboratories (Colworth, UK). Sodium dihydrogen orthophosphate dihydrate, disodium hydrogen orthophosphate dihydrate, potassium hydroxide, hydrochloric acid were obtained from BDH (Poole, Dorset, UK). Tyrosine, 3-nitrotyrosine, 4-hydroxy-3-nitro benzoic acid, hydrogen peroxide, sodium nitrite were obtained from Sigma Chemical Company (Poole, Dorset, UK). HPLC grade acetonitrile was purchased from Rathburn Chemicals Ltd. (Walkerburn, Scotland). All other reagents used were of analytical grade. All the reagents were prepared using deionised water (Waters Milli-Q system).

*Peroxynitrite synthesis.* Peroxynitrite synthesis was carried-out by modifying the method described by Beckman *et al.* [26]. Acidified hydrogen peroxide (1 M in 0.5 M HCl, 20 ml) and sodium nitrite (200 mM, 20 ml) solutions were drawn into two separate syringes, analogous to a stop flow set up. Simultaneous injection of the contents of both syringes into an ice-cooled beaker containing 1.5 M potassium hydroxide (40 ml) through a 'Y'–shaped junction - leading to rapid mixing to form peroxynitrous acid followed by stabilisation of the resulting peroxynitrite anion. Excess hydrogen peroxide was removed by passing the solution through a manganese dioxide column. The concentration of peroxynitrite was determined by measuring the absorbance at 302 nm ($\epsilon = 1670\ M^{-1}\ cm^{-1}$). The typical yield of freshly prepared peroxynitrite ranged from 45 - 80 mM. Higher concentrations (> 400 mM) of peroxynitrite can be obtained by freeze fractionation. However, in the present study only freshly prepared peroxynitrite solutions were used to minimise nitrite ion contamination.

*Tyrosine nitration assay.* A 50 $\mu$L aliquot of peroxynitrite (500 $\mu$M) was added to a solution containing tyrosine (100 $\mu$M) in the presence of varying concentrations (1 - 100 $\mu$M) of the catechin polyphenols in 0.2 M phosphate buffer, pH 7 giving a final volume of 1 ml. Concentrated buffer solution was utilised to ensure the pH of the samples was not altered by the addition of alkaline peroxynitrite. Appropriate controls, without the antioxidants, were carried out to estimate levels of tyrosine nitration.

The samples were then analysed by HPLC using a Hypercarb column (10 cm × 4.6 mm. i.d.). A Hewlett Packard Model 1090M-II HPLC system with an autoinjector, auto sampler and diode array detector linked to a HP 900-300 data station was used to analyse the samples. The mobile phase (50 mM phosphate buffer, pH 7) and acetonitrile (MeCN) were pumped through the column at a flow rate of 1 ml/ min. The following gradient system was used (min/% MeCN): 0/10, 4/10, 14/60, 15/10, 20/10.

The amount of untreated tyrosine and 3-nitrotyrosine formed were determined from calibration plots constructed using authentic samples. 4-Hydroxy-3-nitrobenzoic acid (100 $\mu$M) was used as an internal standard. Tyrosine was monitored at 275 nm while 3-nitrotyrosine formation and the internal standard were monitored at 430 nm. The peroxynitrite scavenging ability of the flavanols is expressed as the percentage decrease in 3-nitrotyrosine formation compared to control samples. Calibration plots of 3-nitrotyrosine and tyrosine were constructed over the range of 0 - 10 $\mu$M (low calibration) and 0 - 100 $\mu$M (high calibration). Known concentrations of tyrosine and 3-nitrotyrosine solutions were spiked in pH 7 phosphate buffer to which was added 100 $\mu$l of the internal standard (100 $\mu$M). Peak area ratios (PAR) of tyrosine:internal standard ($\lambda$ = 275 nm) and 3-nitrotyrosine:internal standard ($\lambda$ = 430 nm) were plotted against the spiked concentration of both species. Linear behaviour with correlation coefficient values $\geqslant$ 0.995 were obtained. The assay developed for the quantification of both tyrosine and 3-nitrotyrosine were found to be precise, accurate and reproducible over the calibration range of 0 - 100 $\mu$M with CV% and M%D of less than 5%. The minimum quantifiable level for tyrosine and 3-nitrotyrosine was 1 $\mu$M.

*Isolation of LDL.* Blood was collected by venepuncture into vials containing acid-citrate dextrose and 100 $\mu$M EDTA. Plasma was obtained by centrifugation. LDL was isolated from plasma by density gradient ultracentrifugation (d = 1.063 g/ml) at 16 °C on a Beckman L-70 centrifuge using a fixed angle rotor (Ti-70) working at 150,000 *g* according to modified method of Chung *et al.* [27]. After isolation, LDL was filtered through a 0.22 $\mu$m pore size filter (Millipore) followed by dialysis in phosphate buffered saline (10 mM, pH 7.4) containing 10 $\mu$M EDTA at 4 °C. Protein concentration was estimated using a modified Lowry method with bovine serum albumin used as the standard [28]. In all experiments LDL was used at a final concentration of 0.125 mg LDL protein/ml.

*Gel electrophoresis.* Modification of LDL was assessed by measuring the altered surface charge of the apolipoprotein B of the LDL by using agarose-gel electrophoresis. Varying concentrations of catechin polyphenols (10, 50 or 100 $\mu$M) were added to LDL (125 $\mu$g of protein/

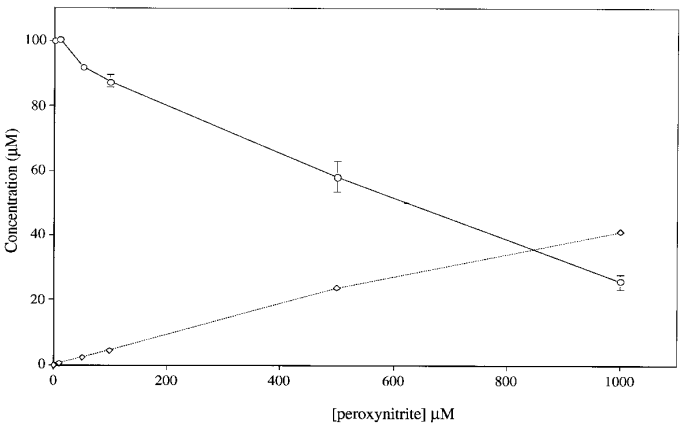


**FIG. 2.** The extent of tyrosine nitration at increasing concentrations of peroxynitrite. Peroxynitrite was mixed with tyrosine (100 $\mu$M) in 0.2 M phosphate buffer, pH 7. Unreacted and nitrated tyrosine were quantified as described in Methods. Nitrotyrosine (◇) and tyrosine (○). Data points represent means ± SD (n = 3).

ml) in pH 7 phosphate buffer, 0.2 M followed by the addition of peroxynitrite (100 $\mu$M). A control sample without the antioxidant was also included for each run. Samples were allowed to react with peroxynitrite for 2 hr prior to analysis. Gel electrophoretic analyses of peroxynitrite treated samples analysed immediately were not significantly different from those incubated for 2 hr, implying that the reaction of LDL with peroxynitrite occurs very rapidly. An aliquot (5 $\mu$L) of the sample was subsequently applied to the gels at 100 V in pH 8.6 barbital buffer (Beckman Paragon Lipo Gel electrophoresis system). The electrophoretic mobility of the LDL samples was measured relative to the mobility of untreated LDL and expressed as the percentage decrease of the relative electrophoretic mobility of peroxynitrite treated LDL in the presence and absence of the catechin polyphenols. Lipoproteins were visualised by staining with Sudan Black B.

*Statistical analysis.* Statistical analysis was determined by Students paired and unpaired *t* test (Statworks); $p \leq 0.05$ was considered to be statistically significant.

## RESULTS

*Tyrosine nitration.* Tyrosine, when exposed to peroxynitrite at pH 7, undergoes nitration to form 3-nitrotyrosine. The identity of 3-nitrotyrosine was confirmed by comparison with an authentic standard. Both the retention time and spectroscopic properties of the peroxynitrite generated product and authentic 3-nitrotyrosine were identical. 3,4-dihydroxyphenylalanine (DOPA), a possible hydroxylation product of the reaction between tyrosine and peroxynitrite, was not detected at any concentration of peroxynitrite. Exposure of tyrosine (100 $\mu$M) to increasing concentrations of peroxynitrite (0-1000 $\mu$M) resulted in an increase in the production of 3-nitrotyrosine and a subsequent decrease in the levels of tyrosine (Fig. 2). The total recovery of tyrosine and 3-nitrotyrosine in each sample, was close to 100% at low concentrations of peroxynitrite (10-50 $\mu$M), but decreased to 80% and 60% at 500 and 1000 $\mu$M of peroxynitrite respectively. No additional chromatographic peaks were detected at these higher concentrations of peroxynitrite.

*Inhibition of tyrosine nitration.* The ability of flavanol antioxidants to decrease peroxynitrite-mediated tyrosine nitration was determined. The catechin polyphenols were co-incubated with tyrosine prior to the addition of 500 $\mu$M peroxynitrite followed by quantification of 3-nitrotyrosine formation. Results (Fig. 3) indicate that all the catechin polyphenols tested were potent scavengers of peroxynitrite due to their ability to prevent the nitration of tyrosine. None of the compounds interfered with the HPLC analysis of tyrosine and 3-nitrotyrosine. All compounds tested had a greater ability to reduce nitration of tyrosine than Tro-

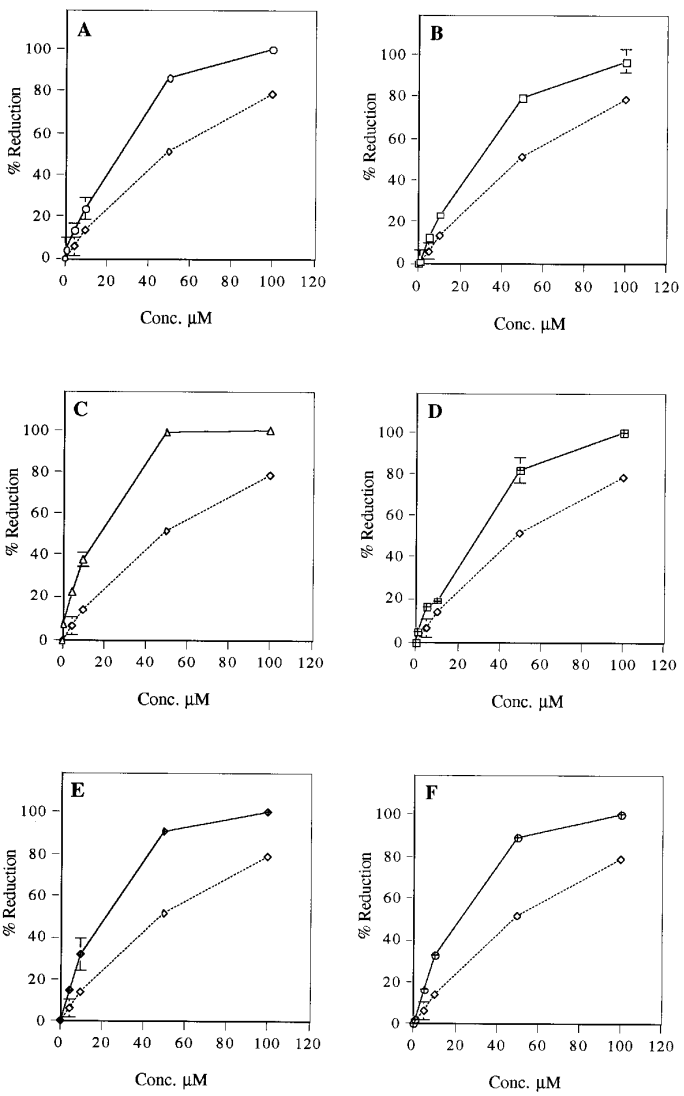


**FIG. 3.** Effect of catechins and catechin-gallate esters on peroxynitrite (500 $\mu$M) mediated tyrosine (100 $\mu$M) nitration. A, catechin; B, epicatechin; C, ECG; D, EGC; E, EGCG; F, gallic acid. Trolox (◇) was used as the standard drug for comparison. Data points represent means ± SD (n = 3).

**TABLE 1**

Effect of Peroxynitrite (100 $\mu$M) on the REM of LDL (0.125 mg of Protein/ml) in the Presence of Catechinpolyphenols (50 $\mu$M)

| | % Reduction in REM |
|---|---|
| Epicatechin | 43 ± 14 |
| ECG | 100 |
| EGC | 32 ± 22 |
| EGCG | 46 ± 12 |
| Gallic acid | 64 ± 1 |

Data are mean ± SD (n = 3).

lox which was used as a standard for comparative purposes. At higher concentrations (50 and 100 $\mu$M) of the polyphenols, reduction of tyrosine nitration was close to 100%. At lower concentrations (10 $\mu$M) the abilities of the catechin polyphenols to minimise tyrosine nitration were: ECG (38.1 ± 3.6%) ≈ EGCG (32.1 ± 7.5%) ≈ gallic acid (32.1 ± 1.9%) > catechin (23.9 ± 5.4%) ≈ epicatechin (22.9 ± 3.3%) ≈ EGC (19.9 ± 2.0%). Inhibition of tyrosine nitration by Trolox at 10 $\mu$M was 13.6 ± 2.9%.

*Inhibition of low density lipoprotein modification.* When human LDL was incubated with peroxynitrite (100 $\mu$M), a small but significant increase in the REM of the LDL was observed (170 ± 10%). The relative effectiveness of the catechin polyphenols in decreasing LDL oxidation is shown in Table 1. Peroxynitrite was added to LDL (final concentration 0.125 mg protein/ml) after pre-treatment with the catechin polyphenolic antioxidants (final concentration 50 $\mu$M). The results (Table 1) show that ECG was able to prevent completely the modification of LDL by peroxynitrite. While gallic acid showed significant activity (64 ± 1%), there was no clear distinction between EGC, EGCG and epicatechin (32 - 46%).

## DISCUSSION

The antioxidant activities of the polyphenols have been discussed in relation to their structural characteristics [2,4,5]. The reduction potentials of the polyphenolic antioxidants, through the extended conjugation and the increasing number of hydroxyl groups define their free radical scavenging activities. In addition, their abilities to chelate transition metal ions can also retard iron- and copper- mediated free radical production [6]. Other studies have shown the sequence of reactivities of the flavanols as H-donating antioxidants against $ABTS^{\Sigma+}$ radicals generated in the aqueous phase as ECG ≈ EGCG > EGC > epicatechin ≈ catechin [7], suggesting that incorporation of the gallate moiety through esterification enhances the antioxidant activity of the catechins, as do increasing number of hydroxyl groups on the phenolic B ring. A similar pattern of reactivity is observed for the $DPPH^{\Sigma}$ radical [8] and the superoxide radical [5]. An enhancing effect of the gallate moiety on the antioxidant activity has also been shown with the theaflavins [30]. The present investigation shows yet another potential effect of the ability of the polyphenols to prevent damage by peroxynitrite at molar concentrations much lower than that of peroxynitrite. This is important because reactive nitrogen species such as peroxynitrite have been implicated in inducing cancer at sites of chronic inflammation and oxidising LDL in human atherosclerotic lesions and hence the putative protective effects of these agents *in vivo* could be due to multiple mechanisms.

Findings from the present investigation indicate that ECG, EGCG and gallic acid are the most effective peroxynitrite scavengers from the catechin polyphenolic series. The antioxidant protection imparted by these compounds is probably mediated by the direct competition with tyrosine for nitration. This is supported by the observation that there is an increase in absorbance at 430 nm when the catechin polyphenols are exposed to peroxynitrite in the absence of tyrosine, indicative of the formation of a nitrophenol (data not shown). For all the catechin polyphenols, the possibility of nitration occurring on the chromanol ring is diminished due to decreased delocalisation resulting from the presence of two hydroxyl groups which are meta to each other. It is therefore likely that the majority of any nitration reactions involving the antioxidant will occur on ring B, predominantly at the 2′- and 5′- positions and to a lesser extent at the 6′- position. Of all the compounds tested, EGC is the least likely to undergo nitration due to the presence of an additional hydroxyl group at the 5′- position. This is reflected by the observation that EGC is the least effective compound in preventing tyrosine nitration. The corresponding gallate esters, in addition to nitration on the chromanol and catechol rings, can also undergo further nitration reactions on the gallic acid moiety and hence enhance their peroxynitrite-scavenging properties. It therefore appears that the greater the number of sites available for nitration the higher the antioxidant activity with respect to peroxynitrite.

The flavonoid antioxidants were also tested for their ability to minimise modification of LDL by peroxynitrite. By and large, findings parallel those obtained in the tyrosine nitration analysis and show that the catechin polyphenols are able to divert the effects of peroxynitrite and minimise the modification of LDL as determined by the decrease in the electrophoretic mobility. Many reports, as well as findings from our laboratory, indicate that in addition to tyrosine nitration, peroxynitrite is capable of modifying a number of other amino acids, including basic amino acids such as lysine, arginine and histidine [31-33]. It is likely that there may be a more complex mechanism of protection by antioxi-

dants is involved in minimising oxidative changes to amino acids within the protein structure of LDL when exposed to peroxynitrite.

## ACKNOWLEDGMENTS

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