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THE INHIBITION OF THE OXIDATION OF LOW DENSITY LIPOPROTEIN BY (+)-CATECHIN, A NATURALLY OCCURRING FLAVONOID

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Abstract—(+)-Catechin inhibited the copper-catalysed oxidation of human low density lipoprotein (LDL) in a dose-dependent manner with complete inhibition at 20 μg/mL. The flavonoid at a concentration of 50 μg/mL also inhibited oxidation of LDL induced by the mouse transformed macrophage J774, human monocyte-derived macrophages and vascular endothelial cells isolated from human umbilical cords. LDL modified by copper-catalysed or cell-induced oxidation was endocytosed and degraded by human macrophages at a much greater rate than native LDL. LDL reisolated from copper or cell incubations in the presence of (+)-catechin was endocytosed and degraded at rates similar to native LDL. (+)-Catechin appeared to inhibit the uptake and degradation by macrophages of cell-modified LDL. The actions of (+)-catechin on cell-induced oxidation of LDL are consistent with the ability of flavonoids of similar structure to inhibit lipoxygenases and with a role for lipoxygenases in cell-induced modification of LDL *in vivo*.

There is much epidemiological evidence to demonstrate a positive correlation between raised plasma low density lipoprotein (LDL§) cholesterol concentrations and accelerated atherogenesis. In the early atheromatous lesions, the fatty streak, cholesterol ester accumulates in macrophages to form foam cells immediately beneath the vascular endothelium. The origin of the cholesterol which accumulates in the macrophage is probably LDL but paradoxically, macrophages have few LDL receptors and these are down-regulated when the LDL concentration rises [1]. Furthermore it has not been possible to convert monocyte-derived macrophages to foam cells *in vitro* even in the presence of high concentrations of LDL cholesterol [1]. Goldstein *et al.* [2] were the first to describe a chemically modified (acetylated) LDL which was recognized by a specific receptor quite distinct from the LDL receptor in the macrophage and which was endocytosed at a rate many times that of LDL. These authors proposed that modifications to LDL *in vivo* might produce a species which is recognized by non-regulated scavenger receptors and rapidly accumulated leading to foam cell formation. Scavenger receptors that recognize chemically modified LDL have recently been isolated [3, 4]. While acetyl LDL is unlikely to be formed *in vivo* there is growing evidence to suggest that oxidized LDL might be the modified LDL species responsible for cholesterol loading of macrophages (reviewed in Ref. 5) and for promoting atherogenesis. Thus, oxidized LDL is (i) chemotactic for macrophages promoting their residence in the intima [6], (ii) cytotoxic to the endothelium [7], (iii) chemo-attractant for monocytes [6] and (iv) rapidly accumulated by resident macrophages [8] and aortic endothelium *in vivo* [9].

The argument for an *in vivo* role for oxLDL is further strengthened by the immunocytochemical detection of oxLDL in atherosclerotic lesions of white rabbits, the isolation of a species of LDL from human and rabbit lesions which are immunologically similar to oxLDL and the detection of autoantibodies to charge modified LDL in human and rabbit serum [10, 11].

Although the mechanism of LDL oxidation *in vivo* has still to be elucidated, observations *in vitro* suggest the involvement of cellular lipoxygenases. Thus, 15-lipoxygenase activity is increased in atherosclerotic lesions of rabbit aorta compared with control tissue [12, 13]. Sparrow *et al.* [14] showed that endothelial cell-mediated oxidation of LDL could be mimicked by incubation of LDL with purified soybean lipoxygenase and phospholipase A_2 and inhibited by lipoxygenase inhibitors [15]. Recently Yla-Herttuala *et al.* [16] demonstrated the co-localization of 15-lipoxygenase mRNA and oxLDL in macrophage-rich areas of atherosclerotic lesion of WHHL rabbits and suggest "that therapy to block macrophage lipoxygenase activity might decrease the rate of development of atherosclerotic lesions". The finding that probucol enhances regression of atherosclerotic plaques in animal models [11, 17, 18] appears to be related to its

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§ Abbreviations: LDL, low density lipoproteins; oxLDL, oxidized LDL; α-MEM, α-modified minimal essential medium; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulphonic acid; TCA, trichloroacetic acid; TBARS, thiobarbituric acid reactive substances.

antioxidant properties and physiological concentrations of both vitamin E and ascorbate inhibit copper-induced oxidation of LDL [19].

Flavonoids are components of a wide variety of edible plants, fruits, vegetables and grains and are an integral part of the human diet. They are enriched in citrus fruits and beverages such as tea and coffee, and wine and beer contain appreciable amounts [20]. Thus, a typical western daily diet may contain 1 g of various flavonoids [21]. This group of compounds appears to have effects on a number of enzyme systems [21] and of particular relevance to the present study is their action as scavengers of free radical oxygen. Certain flavonoids inhibit the 5-lipoxygenase of rat peritoneal macrophages [22] and human neutrophils [23] and both the 12-lipoxygenase of bovine platelets [22] and the 15-lipoxygenase of soybean [24, 25]. (+)-Catechin is a naturally occurring flavonoid with the composition (+)-3′,4′,5,7-tetrahydroxyflavan-3-ol and has been available commercially as (+)-cyanidanol-3 as a pharmaceutical preparation. It is also sold extensively as a health food preparation in continental Europe. (+)-Catechin acts as a free radical scavenger and antioxidant and prevents lipid peroxidation both *in vitro* and *in vivo* [26, 27]. The present study reports on the action of (+)-catechin on oxidative modification of human LDL and the subsequent uptake and degradation of the modified LDL by human monocyte-derived macrophages.

MATERIALS AND METHODS

Iodide-free ^{125}I was purchased from Amersham International (Little Chalfont, U.K.). α-MEM was purchased from Flow Laboratories (Irvine, U.K.) and RPMI 1640 medium from Imperial Laboratories (Salisbury, U.K.). All other chemicals were purchased from the Sigma Chemical Co. (Poole, U.K.). (+)-Catechin was supplied as pyknogenol, a commercial preparation extracted from *Pina maritima* and was a gift from Horphag Research Ltd.

Isolation of human LDL

Human LDL was isolated from pooled serum from healthy volunteers using sequential flotation in a preparative ultracentrifuge [28]. EDTA (10% w/v) was added in a ratio 1:100 to blood to prevent clotting.

Iodination of LDL

A sample of freshly prepared LDL was radiolabelled with ^{125}I as described by Salter *et al.* [29].

Cell culture

Transformed mouse macrophage J774. J774 cells were maintained in α-MEM supplemented with 10% (v/v) foetal calf serum, $NaHCO_3$ (2 g/L) and 4 mM Hepes pH 8.1. A series of antibiotics was included in rotation in the medium. The cells were cultured routinely in large dishes (90 mm diameter) in 10 mL of medium and plated out into smaller dishes (60 mm diameter) containing 2 mL of medium for experimentation. Cultures were maintained in a humidified incubator at 37° and the medium was changed every 48 hr.

Human vascular endothelial cells. These cells were isolated from veins of human umbilical cords by collagenase digestion by the method of Jaffe *et al.* [30] as modified by Van Hinsberg *et al.* [31]. Cultures were maintained for up to seven serial passages.

Human monocyte derived macrophages. Human monocytes were prepared [19] from 40 mL of heparinized blood. The cells were cultured in 24-well plates (Falcon 3047, Becton Dickinson) and incubated with 1 mL per well of RPMI 1640 medium containing 2 mM glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin and 20% autologous serum. The medium was replaced every second day. Monocyte-derived macrophages were usually used 8 days after the preparation of monocytes.

Oxidation of LDL

Copper-mediated oxidation. LDL (333 μg/mL) or ^{125}I-LDL (286 μg/mL) was incubated in phosphate buffered saline containing 5 μM $CuSO_4$, in the absence or presence of varying concentrations of (+)-catechin for up to 24 hr at 37°.

Cell-mediated oxidation. LDL (200 μg/mL) or ^{125}I-LDL (11.8 μg/mL) was incubated in the absence or presence of (+)-catechin for 24 hr at 37° with either J774 cells, human vascular endothelial cells or human monocyte derived macrophages. After this incubation the medium was analysed for the extent of LDL oxidation as described below. In experiments using ^{125}I-LDL the LDL was freed of low molecular weight iodinated products, by passing the media through a 5 mL column of Sephadex G-25 which had been equilibrated with serum-free medium, and was then sterilized by filtration.

Measurement of the extent of oxidation of LDL

TBARS were determined fluorimetrically [32] using an excitation wavelength of 515 nm, an emission wavelength of 550 nm and 1,1,3,3-tetraethoxy propane as standard.

Electrophoresis of lipoproteins

Electrophoresis of oxidized and native LDL was carried out using the Corning agarose system (Corning Medical and Scientific Instruments, Halstead, U.K.).

Degradation of oxLDL by macrophages

The ^{125}I-LDL reisolated by Sephadex G-25 chromatography after incubation with the various cells described above was added to fresh macrophages in 1 mL medium and incubated for 6 hr at 37°. The uptake and degradation of ^{125}I-LDL during this 6 hr incubation was determined by analysing the medium for TCA-soluble ^{125}I-radioactivity and [^{125}I]iodotyrosine as described by Salter *et al.* [29].

Determination of cellular protein

Cell protein was measured by the method of Lowry *et al.* [33] using bovine serum albumin as standard. Intact cell monolayers were solubilized in 0.1 M NaOH (1 mL) for 1 hr at room temperature prior to assay.

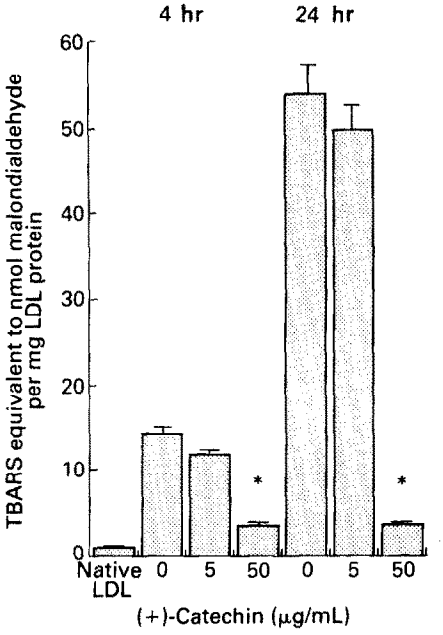

Fig. 1. The effect of (+)-catechin on the copper-catalysed oxidation of LDL. LDL (333 μg/mL) was incubated for 4 or 24 hr at 37° in phosphate buffered saline containing 5 μM $CuSO_4$ in the presence or absence of increasing concentrations of (+)-catechin. At the end of the incubation period TBARS were determined fluorimetrically by the method of Dousset *et al.* [32]. Results are expressed as mean ± SD of triplicate analyses. The significance of difference between (+)-catechin-treated and control values was calculated by an unpaired *t*-test, *P < 0.001.

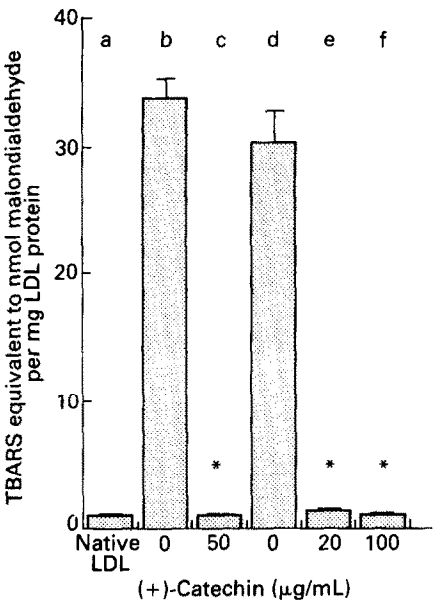

Fig. 2. The effect of (+)-catechin on cell-induced oxidation of LDL. LDL (200 μg/mL) was incubated with human monocyte-derived macrophages (b, c) or J774 cells (d–f) for 24 hr at 37° in the presence or absence of (+)-catechin. The medium was then removed and assayed for TBARS by the method of Dousset *et al.* [32]. Results are expressed as means ± SD of triplicate analyses. The significance of the difference between (+)-catechin-treated and control values was calculated by an unpaired *t*-test, *P < 0.001.

RESULTS AND DISCUSSION

The copper-catalysed oxidation of LDL, as measured by production of TBARS, was inhibited on addition of (+)-catechin to the incubation medium. At a concentration of 50 μg/mL (+)-catechin the oxidation of LDL was approximately 20% of that observed in the absence of (+)-catechin (Fig. 1). The electrophoretic mobility of the oxidized LDL relative to native LDL was 3.6 whereas that of LDL incubated in the presence of (+)-catechin was 1.0 indicating that charge modification had not occurred in this case. Jialal *et al.* [19] showed that oxidation of LDL in the presence of copper was maximal between 2 and 3 hr of incubation but in the present study oxidation after 24 hr incubation was almost 4-fold greater than at 4 hr. This may reflect the level of endogenous antioxidants present in the LDL preparation which may vary with individual donors. For instance, any dietary antioxidant such as vitamin E is likely to partition into the LDL fraction and protect against oxidation and also LDL from donors who smoke is much more susceptible to oxidation than that from non-smokers [34]. Thus, oxidation of LDL lipid will not occur until the endogenous antioxidant is exhausted. In this respect it is interesting to note that in our experiments oxidation in terms of nmol malondialdehyde produced per mg LDL was maximal at about 200 μg LDL/mL of incubation and decreased with increasing LDL concentration. At a concentration of 1.3 mg/mL oxidation was virtually zero (results not shown). This suggests that LDL should be stored as concentrated rather than dilute solutions to minimize oxidation during storage.

Henricksen *et al.* [35] were the first to demonstrate that LDL could undergo endothelial cell-induced oxidation. Many other cell types have since been shown to oxidize LDL *in vitro* (e.g. arterial smooth muscle cells [36]; mouse peritoneal macrophages [37, 38]; human monocyte-derived macrophages [39]). The oxidation of LDL by human monocyte-derived macrophages and also by the transformed macrophage J774 was completely inhibited by the presence of (+)-catechin (50 and 20 μg/mL, respectively) in the culture medium (Fig. 2). Although the mechanism of oxidation is not known, studies by Sparrow *et al.* [14] suggest an involvement of cellular lipoxygenases and the inhibition of cell-induced oxidation by (+)-catechin is consistent with its inhibition of lipoxygenase [40].

Macrophages endocytose and degrade oxidatively modified LDL via scavenger receptors at a much greater rate than native LDL [2, 35] and this property was used to assess the protection afforded LDL by coincubation with (+)-catechin during the oxidation period. LDL which had been subjected to copper-induced oxidation in the absence or presence of (+)-catechin was reisolated by gel filtration and incubation with fresh macrophages. Uptake and

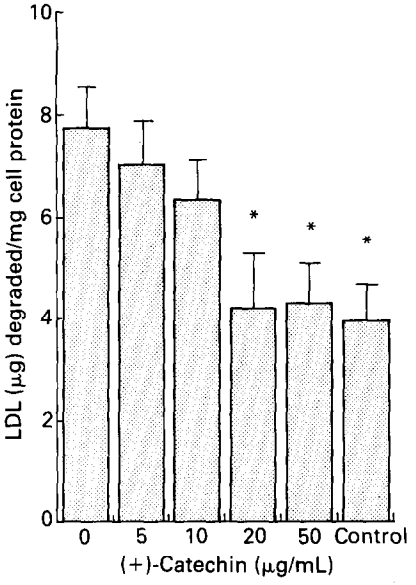

Fig. 3. The uptake and degradation by human macrophages of LDL modified in the presence of increasing concentrations of (+)-catechin. ^{125}I-LDL (286 μg/mL) was incubated in the presence of various concentrations of (+)-catechin with phosphate buffered saline containing 5 μM $CuSO_4$ for 24 hr at 37°. The LDL was reisolated by chromatography on Sephadex-G25 and aliquots (11.4 μg) were incubated with human monocyte-derived macrophages in 1 mL medium for 6 hr at 37°. Uptake and degradation of the modified LDL was measured as [^{125}I]iodotyrosine in the medium as described in Materials and Methods. Results are expressed as means ± SD of triplicate samples. The significance of difference between (+)-catechin-treated or non-oxidized native LDL and LDL modified in the absence of (+)-catechin was calculated using an unpaired *t*-test, *P < 0.01.

Table 1. Uptake and degradation by human monocyte-derived macrophages of LDL oxidized in the presence and absence of catechin

Oxidizing system	(+)-Catechin (μg/mL) 0	50
Macrophages	0.648 ± 0.138	0.0428 ± 0.0212*
Endothelial cells	0.0243 ± 0.007	0.00435 ± 0.00236*
$CuSO_4$	7.72 ± 0.80	4.26 ± 0.81*

Values are μg LDL degraded per mg cell protein.

LDL was preincubated, in the absence or presence of (+)-catechin (50 μg/mL), with macrophages, endothelial cells or phosphate buffered saline containing 5 μM $CuSO_4$, for 24 hr at 37°. The LDL was reisolated by chromatography on Sephadex-G25 and reincubated with fresh macrophages. Uptake and degradation of LDL was determined as the release of ^{125}I-tyrosine into the medium during the incubation period as described in Materials and Methods.

In the experiment shown for preincubation with $CuSO_4$, the degradation of LDL which had not been preincubated was 3.94 ± 0.69 μg/mL cell protein. In the reincubation with fresh macrophages, the amount of LDL which had been preincubated with macrophages or endothelial cells was less than 15% of the amount which had been preincubated with $CuSO_4$.

Results are expressed as means ± SD of triplicate samples of a typical experiment. The significance of difference between (+)-catechin-treated and control values was calculated by an unpaired *t*-test and is indicated by *P < 0.01. Similar results were obtained in two further experiments for preincubation with macrophages and one further experiment for preincubation with endothelial cells or with $CuSO_4$.

subsequent degradation of the lipoprotein was measured as the release of iodide-free TCA soluble radioactivity into the medium during incubation of the radiolabelled lipoprotein with the macrophages. The presence of increasing concentrations of (+)-catechin in the oxidation medium caused a decreased uptake of LDL via the scavenger receptor (Fig. 3). When (+)-catechin was present at 20 μg/mL the rate of uptake and degradation of the reisolated LDL was similar to the native non-oxidized control LDL. Similar effects were seen with LDL isolated after incubation with human vascular endothelial cells and monocyte-derived macrophages (Table 1). The extent of oxidation induced by endothelial cells was much lower than with either copper or macrophages in agreement with observations by Parthasarathy *et al.* [37] but even so in the present study, inclusion of (+)-catechin in the medium gave rise to an LDL species which was only poorly metabolized by macrophages. In all cell-induced oxidation experiments cells were cultured to near confluence in medium containing foetal calf serum (J774 or endothelial cells) or human serum (macrophages) before changing to serum-free medium for the oxidation. Little oxidation was found when serum was present even at low concentrations during the incubation with LDL. Leake and Rankin [38] reported similar effects of serum on the oxidation of LDL by mouse peritoneal macrophages and suggested that some non-dialysable factor such as transferrin, extracellular superoxide dismutase, high density lipoprotein or vitamin E or C might inhibit the oxidation.

In the degradation experiments (+)-catechin was included only in the oxidation medium and not in the subsequent incubation of the reisolated lipoprotein with macrophages to try to separate any effects of the flavonoid on the degradation process from effects on oxidation. As shown in Table 2 catechin also inhibits the uptake of oxidatively modified LDL. In this experiment ^{125}I-LDL was allowed to undergo macrophage-induced oxidation in the absence of (+)-catechin and reisolated prior to incubation with human monocyte-derived macrophages in the presence or absence of the flavonoid. Inclusion of (+)-catechin (50 μg/mL) in the medium decreased degradation of the modified LDL to 27% of that in the absence of (+)-catechin. This was an unexpected and so far unexplained observation and implies that the flavonoid has some effect on endocytosis of oxLDL or its subsequent degradation. It will be of interest to see if endocytosis of LDL is affected in the same way.

Thus, (+)-catechin has marked inhibitory effects on both LDL oxidation and the subsequent degradation of the oxLDL by human monocyte-derived macrophages. Such actions are consistent

Table 2. The effect of (+)-catechin on the uptake and degradation by human monocyte-derived macrophages of LDL oxidized by human macrophages

	ng LDL protein degraded/min/cell protein
Control	648 ± 138
(+)-Catechin	179 ± 57

^{125}I-LDL (11.8 μg/mL) was incubated with human macrophages for 24 hr at 37° and reisolated by chromatography on Sephadex-G25. The cell-modified LDL was then added to monocyte-derived macrophages and incubated for 6 hr at 37° in the presence or absence of (+)-catechin (50 μg/mL). Uptake and degradation of the modified LDL was determined as the non-iodide, TCA-soluble radioactivity as described in Materials and Methods.

Results are expressed as means ± SD of triplicate analyses. The significance of difference between (+)-catechin-treated and control was calculated by Student's *t*-test, $P < 0.01$.

with its free radical scavenging properties. As stated earlier, the cellular 15-lipoxygenase has been implicated in the *in vivo* oxidation of LDL [16] and flavonoids have been shown to be potent inhibitors of cellular lipoxygenases. Thus, they inhibit the 5-lipoxygenase in rat RBL-1 cells [22, 41, 42], rat neutrophils [43], human neutrophils [23] and rat peritoneal macrophages [22]. Interestingly, Welton *et al.* [22] observed that flavonoids with hydroxyl groups at carbons 4′, 3 and 7 were potent inhibitors of the 5-lipoxygenase with IC_{50} values between 10^{-7} and 10^{-6} M. While not included in that study [22], (+)-catechin has hydroxyl groups on each of these carbons. Similar inhibitions have been demonstrated for the soybean 15-lipoxygenase [25] and while there are no reports as yet of actions on the cellular 15-lipoxygenase, it is likely that this enzyme too will be inhibited in a similar manner. The present results suggest a potentially useful antioxidant role for flavonoids and (+)-catechin in particular, providing the molecule can be delivered to the subendothelium where damage occurs. Probucol may achieve its antioxidant, antiatherogenic effect by partitioning itself into the LDL particle itself thereby being carried directly to sites of lipid deposition. The amphipathic nature of (+)-catechin suggests that it too might partition into lipoproteins and the availability of a radiolabelled molecule should allow plasma concentrations and distribution to be measured.

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