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THE MODIFICATION OF LOW DENSITY LIPOPROTEIN BY THE FLAVONOIDS MYRICETIN AND GOSSYPETIN

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Abstract—Myricetin and gossypetin, two hexahydroxylated flavonoids, are capable of modifying low density lipoprotein (LDL) to increase greatly its uptake by macrophages. When human ^{125}I-labelled LDL was incubated with 100–1000 μM myricetin or gossypetin, it was subsequently endocytosed much faster by mouse peritoneal macrophages. This modification did not occur at a concentration of 10 μM. Nine other flavonoids containing up to five hydroxyl substituents did not modify LDL to any great extent at 100 μM. The modification of LDL by 100 μM myricetin was time-dependent and complete by 6 hr. Flavonoids can sometimes act as pro-oxidants but myricetin did not act by oxidizing the LDL, as the LDL lipid hydroperoxide content was not increased by myricetin, nor did it promote the depletion of the endogenous antioxidant α-tocopherol in the LDL. High concentrations of myricetin caused the aggregation of LDL particles, as judged by light microscopy, agarose gel electrophoresis, retention by a membrane filter and sedimentability by centrifugation. SDS–PAGE indicated that the apolipoprotein B-100 molecules of LDL particles were covalently crosslinked. The uptake and degradation by macrophages of myricetin-modified ^{125}I-labelled LDL reached saturation at about 10 μg protein/mL, suggesting the existence of a high affinity uptake process for the modified LDL. The uptake of myricetin-modified ^{125}I-labelled LDL was not competed for by a large excess of non-labelled native LDL or acetylated LDL. We conclude that myricetin and gossypetin at high concentrations are capable of modifying LDL by a novel non-oxidative mechanism to a form taken up by macrophages by a high affinity process.

There is increasing evidence that oxidized low density lipoprotein (LDL**) may be involved in the pathogenesis of atherosclerosis [1]. Oxidized LDL is internalized rapidly by macrophages by means of their scavenger receptors [2] and may contribute to their conversion into cholesterol-laden foam cells in atherosclerotic lesions. Arterial endothelial cells [3], arterial smooth muscle cells [4], macrophages [5–7] and lymphocytes [8], as well as redox-active transition metal ions [7,9], are capable of oxidizing LDL to forms taken up more avidly by macrophages.

Flavonoids are 2-phenylbenzo-γ-pyrone derivatives which are ubiquitous in plants and therefore present in the normal human diet [10]. In a previous study, we have shown that flavonoids inhibit the oxidation of LDL by macrophages or copper ions with IC_{50} values of 1–20 μM [11]. During the course of that study, we were surprised to observe that two hexahydroxylated flavonoids, myricetin and gossypetin (Fig. 1), appeared to be able to modify LDL themselves at a concentration of 100 μM so that it was taken up much faster by macrophages. Lower concentrations of myricetin or gossypetin did not modify LDL in this way. We have investigated the modification of LDL by these flavonoids in more detail. Some of these results have already been published in abstract form [12].

MATERIALS AND METHODS

Isolation and radioiodination of LDL. LDL (1.019–1.063 g/mL) was isolated from normal human blood by sequential density ultracentrifugation, as described previously [13]. EDTA was present throughout to inhibit the oxidation of LDL. LDL was radioiodinated by the iodine monochloride method as described before [11]. The LDL was stored at 4° in the presence of 100 μM EDTA under aseptic conditions. LDL was no longer used about 1 month after isolation.

LDL modification by myricetin or gossypetin. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for up to 24 hr with myricetin or gossypetin in (1) Ham's F-10 medium (Catalogue No. 12-412-54; Flow Laboratories, High Wycombe, U.K.); (2) Dulbecco's modified Eagle's medium (DMEM; containing 1 g glucose/L; Catalogue No. D41-1885; Gibco., Uxbridge, U.K.) or (3) Dulbecco's phosphate-buffered saline without Ca^{2+} or Mg^{2+} (Gibco). Gentamicin (50 μg/mL) was added to all

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** Abbreviations: DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoprotein; apo B-100, apolipoprotein B-100.

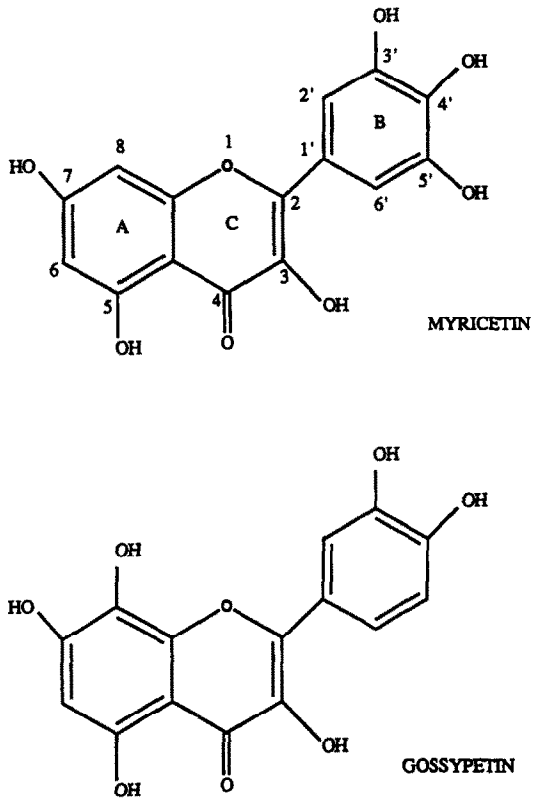

Fig. 1. Structure of myricetin and gossypetin. The 4-oxo-flavonoids are based on the parent molecule flavone, which can be hydroxylated or glycosylated at various positions. The lettering of the rings is shown.

these solutions. Myricetin was obtained from either the Sigma Chemical Co. (Poole, U.K.) or the Aldrich Chemical Co. (Gillingham, U.K.). Gossypetin was kindly provided by Dr M.J. Alcaraz of the University of Valencia, Spain. Flavonoids were dissolved in ethanol or 70% (v/v) ethanol before being added to the LDL. The final concentration of ethanol was 1% (v/v) or less.

Isolation and culture of macrophages. Resident macrophages were isolated from mice by peritoneal lavage and cultured as described previously [11]. One million peritoneal cells were added to each 22.6 mm well and cultured in DMEM supplemented with foetal calf serum (10% of final volume) and gentamicin (50 μg/mL).

Measurement of LDL degradation and cell association in macrophages. After incubation with or without flavonoids, the ^{125}I-labelled LDL was diluted to 10 μg of LDL-protein/mL with DMEM containing 10% (v/v) foetal calf serum and gentamicin (50 μg/mL) and incubated with macrophages or in cell-free wells. LDL degradation by the cells was determined by measuring the release of its non-iodide trichloroacetic acid-soluble degradation products into the medium [14]. The medium was made up to 1 mL with water and the degradation products determined as described previously [11]. The small amount of non-iodide acid-soluble radioactivity found in the medium of the cell-free wells was subtracted from that of the medium from the macrophages. The cells were washed, lysed in 0.2 M NaOH and their protein content measured [11]. The degradation was expressed per milligram of cell protein.

To determine the amount of ^{125}I-labelled LDL remaining associated with the cells, the radioactivity of a portion of the NaOH lysates was measured (after being made up to a total volume of 1 mL to preserve the normal counting geometry and efficiency). The small amount of radioactivity present in the NaOH "lysate" of the cell-free dishes was subtracted from that found associated with the cells.

Determination of lipid hydroperoxides and α-tocopherol. The lipid hydroperoxides were deter-

Table 1. Modification of LDL by myricetin and gossypetin

Flavonoid	Concentration (μM)	Medium	Degradation of ^{125}I-labelled LDL by macrophages (μg protein/mg cell protein in 24 hr)
Myricetin	0*	Ham's F-10	0.9 ± 0.5
	0		0.5 ± 0.1
	10		0.6 ± 0.2
	100		6.7 ± 1.5
	1000		27.8 ± 7.5
Gossypetin	0	Ham's F-10	1.2 ± 0.2
	1		1.0 ± 0.3
	3		1.0 ± 0.3
	10		1.1 ± 0.3
	30		2.1 ± 0.3
	100		10.6 ± 0.4

^{125}I-Labelled LDL (100 μg protein/mL) was incubated for 24 hr at 37° with Ham's F-10 medium containing increasing concentrations of myricetin or gossypetin. The flavonoids were dissolved in ethanol and the final ethanol concentration was 1% (v/v). A control incubation containing 1% v/v ethanol was included. The LDL was then diluted to 10 μg protein/mL and its rate of degradation by macrophages measured.

The mean ± SEM for triplicate determinations is shown.

* No solvent added.

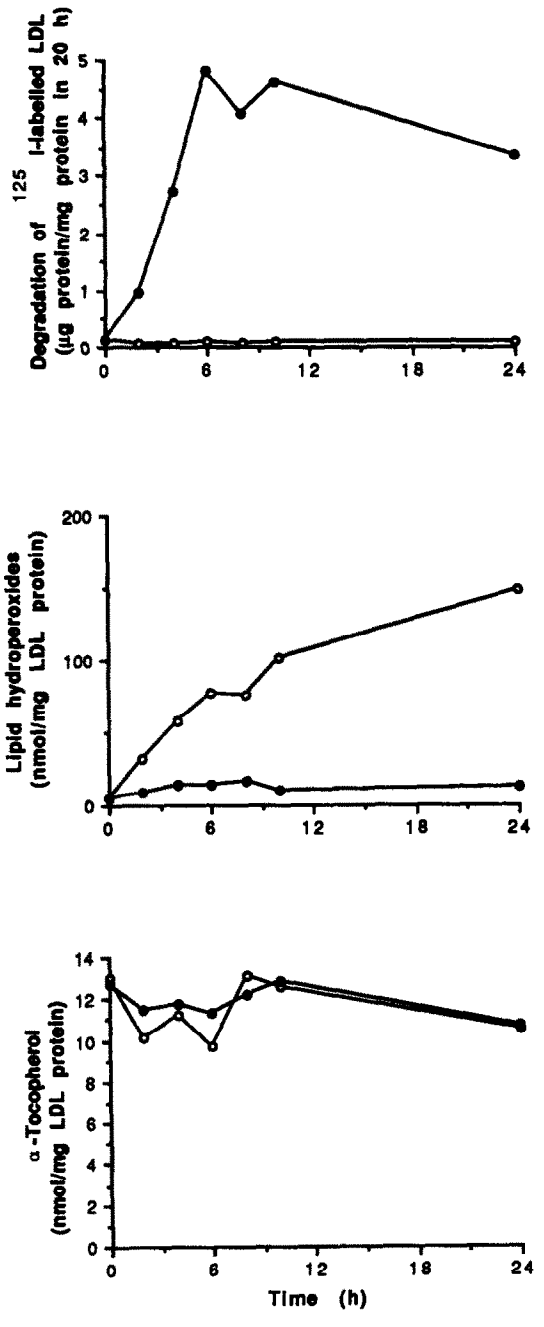

Fig. 2. Time course of LDL modification by 100 μM myricetin. ^{125}I-Labelled LDL (100μg protein/mL) was incubated at 37° for various times up to 24 hr in Dulbecco's phosphate-buffered saline (without Ca^{2+} or Mg^{2+}) with (●) or without (○) 100 μM myricetin. The LDL was then assayed for lipid hydroperoxides or α-tocopherol or was diluted to 10 μg LDL-protein/mL with serum-containing medium and incubated for 20 hr with macrophages and its degradation measured (the final concentration of myricetin in the macrophage cultures would thus have been 10 μM). Each point for the hydroperoxide or α-tocopherol content is the mean of duplicate determinations and each point for the degradation of LDL is the mean of three wells of cells. Generally, the variation between the replicates was in the range of 5–10% of the mean.

mined in the LDL-containing media (after addition of 20 μM butylated hydroxytoluene and 2 mM Na_2 EDTA to prevent any further LDL oxidation) by extraction into heptane [15] and an automated triiodide assay [16]. α-Tocopherol was also determined in the heptane extracts by HPLC on a Merck Lichrocart CN column (250 mm × 4 mm) with hexane/propan-2-ol (99:1, v/v) as the mobile phase, as described by Jessup *et al.* [17].

Polyacrylamide gel electrophoresis. SDS–PAGE of LDL samples was performed using precast 4–15% gradient gels (Bio-Rad Mini Protean II ready gels: Catalogue No. 161-0902). Twenty microlitre LDL samples (100 μg protein/mL) were mixed with 5 μL sample buffer which contained 0.125 M Tris–HCl (pH 6.8), 2% (w/v) SDS, 10% (v/v) glycerol, 0.1% (w/v) bromophenol blue and 2% (v/v) 2-mercaptoethanol and heated at 100° for 2 min. Twenty microlitre samples were loaded and the gels run for 50 min at 150 V and 100 mA. Gels were fixed in ethanol (38%, v/v) acetic acid (10%, v/v) and stained with silver according to Henkeshoven and Dernick [18]. M_r markers were phosphorylase B (94,000), bovine serum albumin (67,000), ovalbumin (43,000), carbonic anhydrase (30,000), soybean trypsin inhibitor (20,000) and alpha-lactalbumin (14,000).

Agarose gel electrophoresis. ^{125}I-Labelled LDL (100 μg protein/mL) was loaded directly on to 1% (w/v) Universal agarose gels (Ciba Corning, Palo Alto, CA, U.S.A.) and electrophoresed in barbitone buffer (pH 8.6) for 45 min at 90 V and 50 mA. The gel was then fixed in methanol, stained with Oil Red 0 and destained in 70% (v/v) methanol. The gel was dried and exposed to X-ray film for 24 hr, which was then developed.

RESULTS

Increased uptake of myricetin- or gossypetin-modified LDL by macrophages

Myricetin at 100–1000 μM modified ^{125}I-labelled LDL so that it was endocytosed very much faster by mouse peritoneal macrophages than control LDL (Table 1). In general, an increase in the rate of degradation of ^{125}I-labelled LDL paralleled an increased rate of uptake. There was no increase in the rate of degradation of LDL that had been treated with 10 μM myricetin. The myricetin was dissolved in ethanol, whose final concentration in the medium was 1% (v/v). This concentration of ethanol did not significantly affect the LDL in terms of its degradation by macrophages (Table 1).

Figure 2 shows the time course of LDL modification by 100 μM myricetin in Dulbecco's phosphate-buffered saline. The modification, as regards increased degradation by macrophages, appeared to be complete by 6 hr (Fig. 2). Lipid hydroperoxide levels increased gradually in the absence of myricetin (Fig. 2) but were much lower than those observed during the oxidation of LDL over the same time period by macrophages (typically about 600 nmol/mg LDL protein at 6 hr; [17]). No detectable peroxidation of LDL lipids occurred in the presence of myricetin, consistent with its antioxidant properties. Moreover, in the buffer that was used in

Table 2. Effect of LDL concentration and of serum on LDL modification by myricetin

Concn of myricetin (μM)	Conditions during the incubation with myricetin: Presence of foetal calf serum (10%, v/v)	Conditions during the incubation with myricetin: ^{125}I-Labelled LDL concn (μg protein/mL)	Degradation of ^{125}I-labelled LDL by macrophages (μg protein/mg cell protein in 24 hr)
0	−	50	0.65 ± 0.02
100	−	50	2.01 ± 0.03
0	−	100	0.66 ± 0.03
100	−	100	8.69 ± 0.33
0	+	100	0.32 ± 0.02
100	+	100	0.31 ± 0.02

^{125}I-Labelled LDL was incubated for 24 hr with DMEM in the presence or absence of myricetin or foetal calf serum (not heat-inactivated; 10% v/v). The LDL was then diluted to 10 μg protein/mL with DMEM containing 10% (v/v) foetal calf serum and added to macrophages. Its degradation by the cells was measured after 24 hr.

The mean ± SEM for three wells of cells is shown.

this experiment, there was no detectable depletion over the 24 hr incubation period of the endogenous antioxidant α-tocopherol in the LDL, either in the presence or absence of myricetin (Fig. 2).

^{125}I-Labelled LDL was modified by 100 μM myricetin much more extensively at 100 μg LDL-protein/mL than at 50 μg protein/mL (Table 2). The modification of LDL was completely inhibited by 10% (v/v) foetal calf serum (Table 2).

Gossypetin also modified LDL to increase its degradation by macrophages at 100 μM but not at 10 μM (Table 1). The following flavonoids did not modify LDL to any great extent at 100 μM: flavone (0), 3-hydroxyflavone (1), chrysin (2), galangin (3), fisetin (4), morin (5), quercetin (5) and hypolaetin (5). The numbers in parentheses indicate the number of hydroxyl substituents on the flavone nucleus. Furthermore, gossypin (5), the C7-glycosylated derivative of gossypetin, did not modify LDL at a concentration of 100 μM.

The effects of myricetin and gossypetin in increasing LDL degradation by macrophages cannot be explained by any actions they may have had on the macrophages because the degradation of native ^{125}I-labelled LDL by macrophages was not increased when 10 μM myricetin or gossypetin was added to the cells (results not shown).

Aggregation of myricetin-modified LDL

When LDL was incubated with 100 μM or 1 mM myricetin, aggregated material was observed, by the naked eye, to develop gradually. When the myricetin-modified LDL was examined by bright field light microscopy (× 200), aggregates were seen when concentrations of myricetin of 10 μM or above were used, the size of the aggregates increasing as the concentration of myricetin increased. This aggregation of LDL by myricetin was confirmed in a number of ways. Control LDL and LDL that had been incubated with 1 μM myricetin migrated as a single band when subjected to agarose gel electrophoresis (Fig. 3). Very little LDL that had been incubated with 10 μM myricetin migrated out of the sample well and no LDL that had been incubated with 100 μM myricetin was observed to have migrated out.

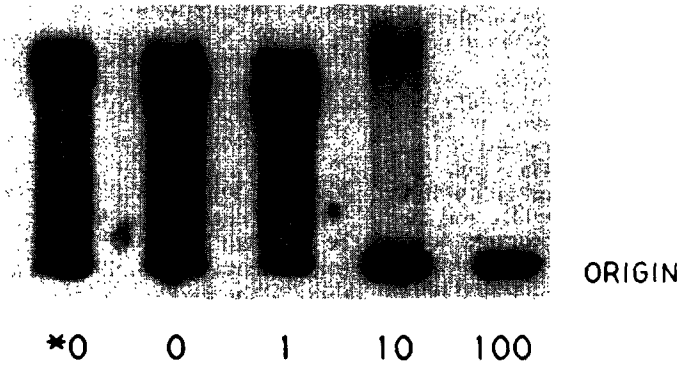

Fig. 3. Agarose gel electrophoresis of myricetin-modified LDL. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for 24 hr in Ham's F-10 medium alone (*0) or containing 1% (v/v) ethanol (0), as a solvent control, or 1, 10 or 100 μM myricetin, as indicated. The LDLs were then subjected to agarose gel electrophoresis at pH 8.6 and an autoradiogram of the gel was taken.

Table 3. Retention of myricetin-modified LDL by filters

Treatment	Retention of ^{125}I-labelled LDL by 0.45 μm filter (%)
None	4.6
Ethanol (1% v/v)	3.5
Myricetin (1 μM)	5.3
Myricetin (10 μM)	39.6
Myricetin (100 μM)	77.8

^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for 24 hr with various concentrations of myricetin in Ham's F-10 medium. The myricetin was dissolved in ethanol and so a control containing ethanol (final concentration 1% v/v) was also included. Separate samples were taken and their radioactivity measured before and after passage through a membrane filter with a nominal pore size of 0.45 μm (Millipore). Retention of ^{125}I-labelled material is expressed as a percentage of the total radioactivity in each sample.

The results are the means of two separate experiments.

A large proportion of ^{125}I-labelled LDL treated with 10 or 100 μM myricetin was retained by a filter with a nominal pore size of 0.45 μm (Table 3).

When ^{125}I-labelled LDL was incubated with 1 mM myricetin and centrifuged at 11,600 *g* for 10 min, 85% of it was sedimented, whereas only 2% or less was sedimented at lower concentrations of myricetin (Table 4). The sedimentability correlated with the degradation of the LDL by macrophages.

Myricetin-modified LDL was subjected to SDS–PAGE to investigate if there was any intermolecular cross-linking of its apolipoprotein B-100 (apo B-100) molecules (the protein component of LDL), as has been described for LDL modified by 4-hydroxynonenal [19]. ^{125}I-Labelled LDL was modified with 0, 1, 10, 100 or 1000 μM myricetin for 24 hr, then applied to a 4–15% polyacrylamide gel. By silver staining, the only major band detectable in control samples was apo B-100 (Fig. 4). In LDL modified by 10, 100 or 1000 μM myricetin, however, increasing amounts of apo B-100 failed to enter the gels and this was accompanied by a progressive decrease in the intensity of staining of the apo B-100 band.

Characteristics of myricetin-modified LDL uptake by macrophages

The degradation and cell association of ^{125}I-labelled LDL modified by various concentrations of myricetin were compared (Table 4). With 10 or 100 μM myricetin, over 80% of the total ^{125}I-labelled LDL that had become associated with the cells over 24 hr was degraded by the cells, whereas with 1 mM myricetin only 37% of the ^{125}I-labelled LDL was degraded with large amounts of radioactive material accumulating in the cells.

When increasing concentrations of LDL modified by 1 mM myricetin were added to macrophages, its degradation plateaued at about 10 μg protein/mL, whereas the degradation of control LDL continued to increase up to at least 100 μg protein/mL, the

Table 4. Sedimentability of myricetin-modified LDL

Treatment	Sedimentability of ^{125}I-labelled LDL by centrifugation (%)	Degradation of ^{125}I-labelled LDL by macrophages (μg protein/mg) cell protein in 24 hr)	Cell-associated ^{125}I-labelled LDL (μg protein/mg) cell protein after 24 hr)	Total ^{125}I-labelled LDL (μg protein/mg cell protein in 24 hr)	Degraded ^{125}I-labelled LDL as % of total
Ethanol (1% v/v)	0.65	0.78	0.15	0.93	84.0
Myricetin (10 μM)	1.21	0.90	0.20	1.10	82.2
Myricetin (100 μM)	1.95	4.30	0.72	5.01	85.7
Myricetin (1 mM)	84.8	43.6	74.1	118	37.0

^{125}I-Labelled LDL (100 μg protein/mL) was incubated for 17 hr at 37° with various concentrations of myricetin or with 1% (v/v) ethanol as a control in Ham's F-10 medium. One portion of the incubated LDL was then centrifuged at 11,600 *g*/13,000 rev./min for 10 min at room temperature in a MSE Microcentaur centrifuge and the supernatants from near the top of the tubes sampled for the measurement of their radioactivities. The radioactivity that was sedimented was calculated by subtracting the radioactivity in the supernatant from the radioactivity present before centrifugation. Another portion of the myricetin-incubated LDL was diluted to 10 μg protein/mL, incubated with macrophages in triplicate or in cell-free dishes and its rate of degradation and cell association determined. The total ^{125}I-labelled LDL shown in the table is the sum of the degraded and cell-associated radioactivities.

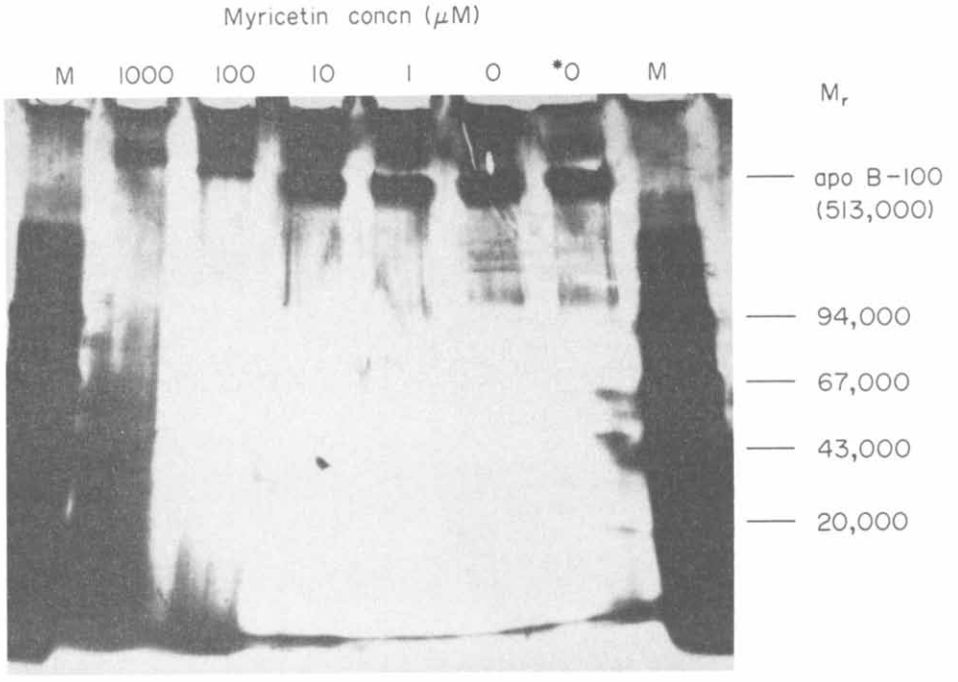

Fig. 4 SDS–PAGE of myricetin-modified LDL. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for 24 hr in Ham's F-10 medium alone (*0) or containing 1% (v/v) ethanol (0), or 1, 10, 100 or 1000 μM myricetin, as indicated. The LDLs were subjected to SDS–PAGE using a 4–15% gradient gel. The gels were then stained with silver. The lanes with the relative molecular mass standards are denoted by M. Similar results were obtained when LDL was incubated with myricetin in phosphate-buffered saline rather than Ham's F-10 and in another experiment in Ham's F-10 with a different batch of LDL.

highest concentration used (Fig. 5). At concentrations below 100 μg protein/mL, the degradation of myricetin-modified ^{125}I-labelled LDL was much greater than that of the control ^{125}I-labelled LDL. Large amounts of radiolabelled material remained associated with the cells when they were incubated with myricetin-modified LDL and again this plateaued at about 10 μg protein/mL (Fig. 5). As mentioned above, more radioactivity from myricetin-modified ^{125}I-labelled LDL was associated with the cells than was degraded by them. The amounts of radioactivity associated with the macrophages when they were incubated with control ^{125}I-labelled LDL were very much less than when they were incubated with myricetin-modified ^{125}I-labelled LDL.

Competition experiments were performed to investigate the nature of the binding site for myricetin-modified ^{125}I-labelled LDL by using non-labelled native LDL or acetyl-LDL to compete for the native LDL and acetyl-LDL receptors, respectively. When macrophages were incubated for 6 hr with myricetin-modified ^{125}I-labelled LDL in the presence of up to a 20-fold excess of non-labelled native LDL, there was no decrease in the degradation or cell association of the labelled myricetin-modified LDL (Fig. 6). The degradation of myricetin-modified ^{125}I-labelled LDL was reduced by about 25% by 10 μg of non-labelled acetyl-LDL/mL but this modest inhibition did not increase with increasing acetyl-LDL concentrations. Moreover, the cell-associated radioactivity was not decreased by non-labelled acetyl-LDL and the total radioactivity (i.e. degraded plus cell-associated radioactivity) was not decreased by it in a consistent manner (Fig. 6).

DISCUSSION

We have found that two hexahydroxylated flavonoids, myricetin and gossypetin, at a concentration of 100 μM or above modify LDL so that macrophages take it up much faster. Lower concentrations of myricetin or gossypetin did not modify LDL to nearly the same extent. None of the other nine flavonoids we have investigated modified LDL appreciably at 100 μM, including gossypin, a glycosylated derivative of gossypetin. These results suggest that the number of hydroxyl substituents may be important for this effect on LDL and it would therefore be relevant to examine other phenolic polyols.

The modification of LDL by 100 μM myricetin appeared to be complete by 6 hr. Flavonoids are well known to have antioxidant properties but it has been shown more recently that some of them, including myricetin and gossypetin, can also have pro-oxidant effects under certain conditions [20, 21]. This raised the possibility that the myricetin and gossypetin at 100 μM or above may have been acting by oxidizing the LDL. However, several lines of evidence argue against this explanation. Firstly,

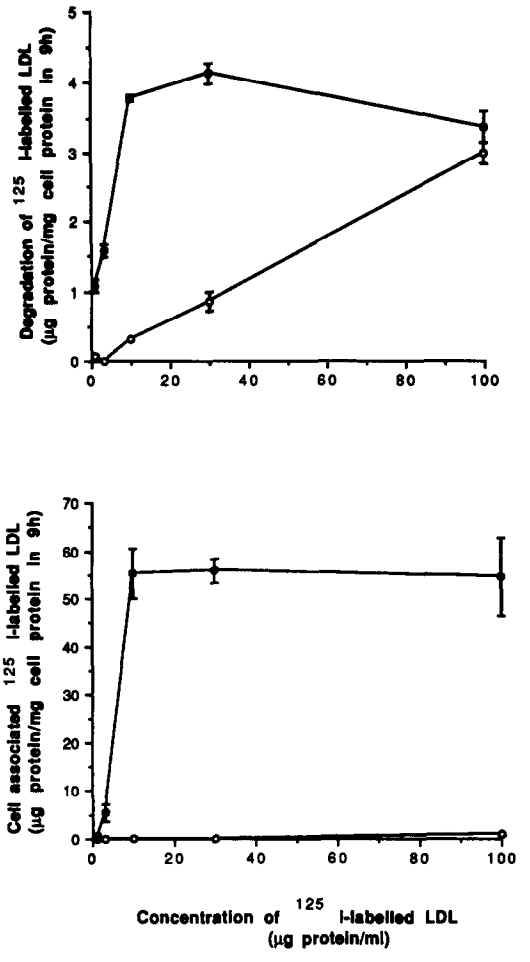

Fig. 5. Effect of myricetin-modified LDL concentration on its degradation and cell association in macrophages. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated for 16 hr with (●) or without (○) 1 mM myricetin in DMEM. It was diluted to various concentrations with serum-containing DMEM and incubated for 9 hr in triplicate with macrophages or in cell-free wells. Radioactive degradation products in the medium and the amounts of radioactivity remaining associated with the cells were then measured. The SEM is shown by the error bars, except where it is smaller than the symbol.

100 μM myricetin did not increase the levels of lipid hydroperoxides in LDL; in fact, it prevented the slow increase in lipid hydroperoxides that occurred in its absence. This is consistent with the inhibition by myricetin of microsomal lipid peroxidation induced by Fe^{3+}-ascorbate (50% inhibition at about 1.5 μM myricetin [22]) and with the inhibition by myricetin of LDL oxidation by macrophages [23] or copper ions [11]. Secondly, when LDL is oxidized by copper ions or macrophages, there is a rapid decrease in its α-tocopherol content [17], but in the experiments described here the α-tocopherol content of the LDL remained constant both in the presence and absence of 100 μM myricetin. This is consistent with the lack of any pro-oxidant effect of myricetin in this system. Finally, the LDL could be modified extensively in DMEM, in addition to Dulbecco's phosphate-buffered saline and Ham's F-10 medium. We have shown previously that LDL in DMEM is highly resistant to oxidation by macrophages or copper ions [7]. Taken together, this evidence suggests that LDL modification by myricetin is not due to an oxidative process as observed when LDL is modified by cells or copper ions, a process which involves the loss of α-tocopherol and the generation of lipid hydroperoxides.

The development of a form of LDL that was taken up faster by macrophages was correlated with the formation of aggregated LDL. This was shown by several techniques, including light microscopy, agarose gel electrophoresis, membrane filtration, sedimentation by centrifugation and SDS–PAGE.

Proteins of higher M_r than apo B-100 were seen in myricetin-treated LDL in the polyacrylamide gels. Apo B-100 was the only major protein present in the control incubations and the formation of higher M_r species accompanied the loss of protein of the M_r of native apo B-100. As each LDL particle contains only one apo B-100 molecule, we conclude that myricetin-modified LDL includes LDL particles whose apo B-100 molecules are covalently cross-linked by myricetin. Similar results have been shown for 4-hydroxynonenal-modified LDL [19]. The capacity of polyphenolic compounds such as flavonoids to form covalent complexes with proteins is already known [24] and is believed to involve the reaction of quinones with nucleophilic groups, such as sulphydryl and amino groups on proteins. It may be of interest that in the experiments in which we extracted myricetin-modified LDL with heptane to measure lipid hydroperoxides and α-tocopherol, we noticed a purple-coloured layer rather than the usual white layer at the interface between the heptane and aqueous phases. This would be consistent with the postulated binding of myricetin to apo B-100.

The correlation between the appearance of aggregates of LDL, the formation of higher M_r forms of apo B-100 and the accelerated uptake of LDL suggests that cross-linking of LDL particles is an important factor in the formation by myricetin of a form of LDL that is taken up faster by macrophages. It would also be consistent with the LDL being modified by myricetin to a much greater extent at a concentration of 100 μg LDL-protein/mL than at 50 μg/mL. It is possible, however, that other physical effects of flavonoids on the LDL particles may also be involved [24].

It has been shown that aggregated LDL produced by vortexing [25], by phospholipase C treatment [26] or by incubation with 4-hydroxynonenal [19] is rapidly taken up by macrophages by phagocytosis and this may possibly have been the case here.

The myricetin-modified LDL was taken up by macrophages by a high affinity binding site of some kind, as its degradation and association with the cells plateaued at about 10 μg LDL-protein/mL. Competition experiments between myricetin-modified ^{125}I-labelled LDL and non-labelled acetyl-LDL suggested that the uptake of myricetin-modified LDL was largely independent of the scavenger receptor on macrophages, which mediates the uptake of acetyl-LDL and, at least in part, oxidized LDL.

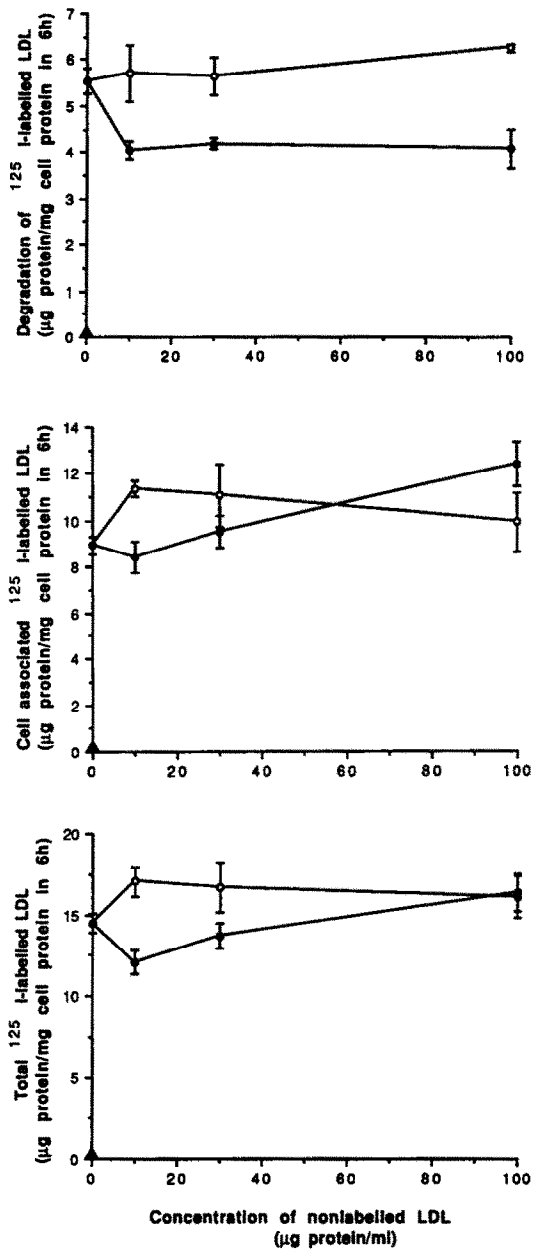

Fig. 6. Competition studies between myricetin-modified ^{125}I-labelled LDL and non-labelled native or acetylated LDL for degradation by macrophages. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for 18 hr with 1 mM myricetin in phosphate-buffered saline without Ca^{2+} or Mg^{2+}. It was then diluted in DMEM containing 10% (v/v) lipoprotein-deficient foetal calf serum and gentamicin (50 μg/mL) and added to macrophages or cell-free dishes at a concentration of 5 μg protein/mL, in the presence of various concentrations of non-labelled native (○) or acetylated (●) LDL. The degradation and cell association of the ^{125}I-labelled LDL was measured after 6 hr incubation. The low rate of degradation and cell association of control ^{125}I-labelled LDL is shown on the ordinate (▲). In the same experiment, the rate of degradation (followed by cell association in parentheses) of acetylated ^{125}I-labelled LDL (5 μg protein/mL) alone was 20.6 ± 1.6 μg protein/mg cell protein in 6 hr (3.6 ± 0.1); for acetylated ^{125}I-labelled LDL (5 μg protein/mL) plus non-labelled native LDL (100 μg protein/mL) it was 19.7 ± 1.5 (3.5 ± 0.05) and for acetylated ^{125}I-labelled LDL (5 μg protein/mL) plus non-labelled acetylated LDL (100 μg protein/mL) it was 2.14 ± 0.26 (0.47 ± 0.02). The mean ± SEM for three wells of cells is shown (except when the SEM is smaller than the symbol). The myricetin-modified ^{125}I-labelled LDL bound to the cell-free wells more than did the control or acetylated ^{125}I-labelled LDL, but its binding was only about 5% of the radioactivity associated with the cells.

A 20-fold excess of non-labelled native LDL did not compete for the uptake of myricetin-modified radioactive LDL. Macrophages possess a receptor for native LDL, but it has a low affinity for native LDL compared to that of the classical human fibroblast LDL receptor and it should therefore be borne in mind that it may be difficult to compete effectively using native LDL for the binding of a high affinity ligand to this receptor [27]. Hoff *et al.* [19] found that the degradation of labelled 4-hydroxynonenal-modified LDL by macrophages was not decreased by excess non-labelled native or acetyl-LDL and also suggested that the binding of a multivalent ligand may be of such a high affinity that univalent native LDL or acetyl-LDL are unable to compete for it.

The ratio of degraded LDL to that associated with the cells was much less for LDL that had been modified by 1 mM myricetin than for unmodified LDL or LDL incubated with lower concentrations of myricetin. The cell-associated myricetin-modified LDL may either have been bound to the surface of the cells or it may have been internalized by them. If it had been internalized, its reduced degradation may have been due to either a decrease in its delivery to the lysosomes or to a decrease in its rate of degradation following its delivery to the lysosomes. A decreased susceptibility to lysosomal proteases may possibly be explained by the cross-linking of the apo B-100 molecules in myricetin-modified LDL.

The modification of LDL by 100 μM myricetin was entirely inhibited by 10% (v/v) foetal calf serum. The reason for this is unclear, although one possibility is that the serum proteins may have competed with the LDL apo B-100 for binding to the myricetin.

Little is known of the pharmacokinetics of flavonoids in humans, but it would seem unlikely that normal levels of dietary myricetin would reach concentrations of the order of 100 μM in the plasma. Even if this were to happen, however, it is unlikely that the myricetin would modify the LDL in the general circulation because the plasma would be expected to inhibit the modification totally. Thus, if flavonoids are present in the circulation and interstitial fluid, their inhibitory effects on LDL oxidation [11] would be expected to predominate over any possible effects of myricetin and gossypetin in modifying LDL themselves. Some antioxidants that inhibit LDL oxidation *in vitro*, for instance probucol [28] and butylated hydroxytoluene [29], suppress experimental atherosclerosis in animals when administered in the diet. Whether or not dietary flavonoids have any effect on LDL oxidation *in vivo*, however, and therefore influence the atherosclerotic process, remains to be established.

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REFERENCES

1. Steinberg D, Parthasarathy S, Carew TE, Khoo JC and Witztum JL, Beyond cholesterol: modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* **320**: 915–924, 1989.
2. Sparrow CP, Parthasarathy S and Steinberg D, A macrophage receptor that recognizes oxidised low density lipoprotein but not acetylated low density lipoprotein. *J Biol Chem* **264**: 2599–2604, 1989.
3. Henriksen T, Mahoney EM and Steinberg D, Enhanced macrophage degradation of low density lipoprotein previously incubated with cultured endothelial cells—recognition by receptors for acetylated low density lipoproteins. *Proc Natl Acad Sci USA* **78**: 6499–6503, 1981.
4. Henriksen T, Mahoney EM and Steinberg D, Enhanced macrophage degradation of biologically modified low density lipoprotein. *Arteriosclerosis:* **3**: 149–159, 1983.
5. Parthasarathy S, Printz DJ, Boyd D, Joy L and Steinberg D, Macrophage oxidation of low density lipoprotein generates a modified form recognized by the scavenger receptor. *Arteriosclerosis* **6**: 505–510, 1986.
6. Rankin SM and Leake DS, The modification of low-density lipoproteins by macrophages in relation to atherosclerosis. *Biochem Soc Trans* **15**: 485–486, 1987.
7. Leake DS and Rankin SM, The oxidative modification of low density lipoproteins by macrophages. *Biochem J* **270**: 741–748, 1990.
8. Lamb DJ, Wilkins GM and Leake DS, The oxidative modification of low density lipoprotein by human lymphocytes. *Atherosclerosis* **92**: 187–192, 1992.
9. Steinbrecher UP, Parthasarathy S, Leake DS, Witztum JL and Steinberg D, Modification of low density lipoprotein by endothelial cells involves lipid peroxidation and degradation of low density lipoprotein phospholipids. *Proc Natl Acad Sci USA* **81**: 3883–3887, 1984.
10. Harborne JB (Ed.), *The Flavonoids: Advances in Research Since 1980*. Chapman and Hall, London, 1988.
11. de Whalley CV, Rankin SM, Hoult JRS, Jessup W and Leake DS, Flavonoids inhibit the oxidative modification of low density lipoproteins by macrophages. *Biochem Pharmacol* **39**: 1743–1750, 1990.
12. de Whalley, CV, Rankin SM, Hoult JRS, Jessup W, Wilkins GM, Collard J and Leake DS, Modification of low density lipoproteins by flavonoids. *Biochem Soc Trans* **18**: 1182–1183, 1990.
13. Rankin SM, Knowles ME and Leake DS, Macrophages possess both neutral and acidic protease activities toward low density lipoproteins. *Atherosclerosis* **79**: 71–78, 1989.
14. Drevon CA, Attie AD, Pangburn SH and Steinberg D, Metabolism of homologous and heterologous lipoproteins by cultured rat and human skin fibroblasts. *J Lipid Res* **22**: 37–46, 1981.
15. Burton GW, Webb A and Ingold KU, A mild, rapid, and efficient method of lipid extraction for use in determining vitamin E/lipid ratios. *Lipids* **20**: 29–39, 1985.
16. Thomas SM, Jessup W, Gebicki JM and Dean RT, A continuous-flow automated assay for iodometric estimation of hydroperoxides. *Anal Biochem* **176**: 353–359, 1989.
17. Jessup W, Rankin SM, de Whalley CV, Hoult JRS, Scott J and Leake DS, α-Tocopherol consumption during low-density-lipoprotein oxidation. *Biochem J* **265**: 399–405, 1990.
18. Henkeshoven J and Dernick R, Improved silver staining procedure for fast staining in Phast System Development Unit 1. Staining of SDS gels. *Electrophoresis* **9**: 28–33, 1988.
19. Hoff HF, O'Neil J, Chisolm GM III, Cole TB, Quehenberger O, Esterbauer H and Jürgens G, Modification of low density lipoprotein with 4-hydroxynonenal induces uptake by macrophages. *Arteriosclerosis* **9**: 538–549, 1989.
20. Hodnick WF, Kung FS, Roettger WJ, Bohmont CW and Pardini RS, Inhibition of mitochondrial respiration and production of toxic oxygen radicals by flavonoids: A structure–activity study. *Biochem Pharmacol* **35**: 2345–2357, 1986.
21. Laughton MJ, Halliwell B, Evans PJ and Hoult JRS, Antioxidant and pro-oxidant actions of the plant phenolics quercetin, gossypol and myricetin. Effects on lipoperoxidation, hydroxyl radical generation and bleomycin-dependent damage to DNA. *Biochem Pharmacol* **38**: 2859–2865, 1989.
22. Laughton MJ, Evans PJ, Moroney MA, Hoult JRS and Halliwell B, Inhibition of mammalian 5-lipoxygenase and cyclo-oxygenase by flavonoids and phenolic dietary additives. Relationship to antioxidant activity and to iron ion-reducing ability. *Biochem Pharmacol* **42**: 1673–1681, 1991.
23. Rankin SM, Hoult JRS and Leake DS, Effects of flavonoids on the oxidative modification of low density lipoproteins by macrophages. *Br J Pharmacol* **95**: 727P, 1988.
24. Haslam E and Lilley TH, Interactions of natural phenols with macromolecules. In: *Plant Flavonoids in Biology and Medicine: Biochemical, Pharmacological and Structure–Activity Relationships* (Eds. Cody V, Middleton E Jr and Harborne JB), pp. 53–65. Alan R. Liss, New York, 1986.
25. Khoo JC, Miller E, McLoughlin P and Steinberg D, Enhanced macrophage uptake of low density lipoprotein after self-aggregation. *Arteriosclerosis* **8**: 348–358, 1988.
26. Suits AG, Chait A, Aviram M and Heinecke JW, Phagocytosis of aggregated lipoprotein by macrophages: Low density lipoprotein receptor-dependent foam-cell formation. *Proc Natl Acad Sci USA* **86**: 2713–2717, 1989.
27. Koo C, Wernette-Hammond ME and Innerarity TL, Uptake of canine β- very low density lipoproteins by mouse peritoneal macrophages is mediated by a low density lipoprotein receptor. *J Biol Chem* **261**: 11194–11201, 1986.
28. Carew TE, Schwenke DC and Steinberg D, Anti-atherogenic effect of probucol unrelated to its hypocholesterolemic effect: evidence that antioxidants *in vivo* can selectively inhibit low density lipoprotein degradation in macrophage-rich fatty streaks and slow the progression of atherosclerosis in the Watanabe heritable hyperlipidemic rabbit. *Proc Natl Acad Sci USA* **84**: 7725–7729, 1987.
29. Björkhem I, Henriksson-Freyschuss A, Breuer O, Diczfalusy U, Berglund L and Henriksson P, The antioxidant butylated hydroxytoluene protects against atherosclerosis. *Arteriosclerosis* **11**: 15–22, 1991.