Biochemical Pharmacology, Vol. 39, No. 11, pp. 1743–1750, 1990.
Printed in Great Britain.

0006-2952/90 $3.00 + 0.00

FLAVONOIDS INHIBIT THE OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEINS BY MACROPHAGES

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(*Received* 21 *November* 1989; *accepted* 15 *January* 1990)

Abstract—Low density lipoproteins (LDL) can be oxidatively modified *in vitro* by macrophages and certain other cell types so that macrophages will take them up much faster. This process may be important in the formation of cholesterol-laden foam cells derived from macrophages in atherosclerotic lesions. In this study, we have shown that certain flavonoids, plant constituents found in the diet, are potent inhibitors of the modification of ^{125}I-labelled LDL by macrophages, with IC_{50} values in the micromolar range (e.g. morin and fisetin 1 μM; quercetin and gossypetin 2 μM). The potencies of individual flavonoids in inhibiting LDL modification did not correlate with their previously determined potencies as inhibitors of 5-lipoxygenase and cyclo-oxygenase. The modification of LDL by macrophages exhibits a lag period of about 4–6 hr before enhanced uptake is detected. During this time, there is a rapid depletion in its content of α-tocopherol (an endogenous antioxidant found in lipoproteins) followed by a large increase in the level of hydroperoxides. The flavonoids conserved the α-tocopherol content of LDL and delayed the onset of detectable lipid peroxidation. Flavonoids also inhibited the cell-free oxidation of LDL mediated by $CuSO_4$. These findings raise the possibility that flavonoids may protect LDL against oxidation in atherosclerotic lesions and may therefore be natural anti-atherosclerotic components of the diet, although this will depend to a large extent on their pharmacokinetics.

The presence of lipid-laden macrophages within the intima of arteries is a characteristic feature of atherosclerotic lesions. In humans, the lipid, mainly cholesteryl esters, is believed to be derived from circulating low density lipoproteins (LDL‖). The uptake of native LDL by macrophages *in vitro* is only slow, however, and does not usually lead to significant lipid accumulation, due to the relatively low number and affinity of LDL receptors on these cells and their down-regulation by accumulating intracellular cholesterol [1]. Macrophages do, however, express abundant receptors that mediate the uptake of various polyanions, including certain modified forms of LDL but not native LDL [2]. These receptors are not down-regulated. Certain cells in culture, such as arterial endothelial cells [3, 4], arterial smooth muscle cells [4] or macrophages [5–8], have been shown to modify LDL to a form recognised and taken up efficiently by these receptors. One of these receptors is known as the acetyl-LDL receptor or scavenger receptor, but others may exist as well [9]. There is increasing evidence that modified LDL is present in atherosclerotic lesions but not in normal arterial walls [10–14].

Cells modify LDL by an oxidative mechanism [15]. The polyunsaturated lipids of LDL are peroxidized, either by free radicals released from the cells or by oxidized lipids transferred to them by the cells. The exact mechanism of the modification is not yet clear but one possibility is that when the lipid hydroperoxides fragment highly reactive aldehydes are released which may bind to the lysyl residues of apolipoprotein B-100, the protein moiety of LDL, thus abolishing their positive charge [16]. These, and possibly other changes to the LDL brought about by oxidation, somehow lead to the recognition of the protein by scavenger receptors on macrophages.

LDL contains a number of endogenous anti-oxidants, including α- and γ-tocopherols, β-carotene, lycopine and retinyl stearate. It is only when these have been largely consumed that peroxidation takes place [17–20]. LDL oxidation can be inhibited by adding lipophilic antioxidants such as α-tocopherol, butylated hydroxytoluene or probucol [5, 7, 15, 18, 19, 21]. Probucol protects against spontaneous atherosclerosis in Watanabe hereditable hyperlipidaemic rabbits [22]. This provides additional evidence that oxidized LDL is involved in the early stages of atherogenesis. Consistent with this is epidemiological evidence that the plasma levels of the natural antioxidants, ascorbic acid and α-tocopherol are higher in European populations in which there is a low incidence of coronary heart disease [23].

We describe here the effects on the oxidative modification of LDL of another type of antioxidant, the

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‖ Abbreviations: DMEM, Dulbecco's modified Eagle's medium; LDL, low density lipoproteins.

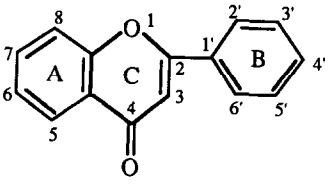

Fig. 1. General structure of 4-oxo-flavonoids. The structure illustrated is flavone, the parent compound of the flavonoids. The flavonoids contain a number of hydroxyl groups at various positions around the rings (see Table 1). The lettering of the rings is shown.

flavonoids. Flavonoids form a large class of naturally occurring 2-phenylbenzo- γ-pyrone (or flavone) derivatives, some of which are glycosylated (Fig. 1). Some 4000 have now been identified from both higher and lower plants [24]. The average daily human intake of these compounds in the U.K. and U.S.A. has been estimated to be 1 g or more [25]. Flavonoids have many properties including scavenging free radicals [26–31] preventing lipid peroxidation [26, 27, 30–32], anti-inflammatory actions [33] and the inhibition of many enzymes, including cyclo-oxygenase and lipoxygenase [34, 35] and phospholipase A_2 [35]. We have now shown that they are potent inhibitors of LDL oxidation.

Some of the results of this study have been published briefly before [7, 36].

MATERIALS AND METHODS

Isolation and radioiodination of LDL. LDL (1.019–1.063 g/mL) was isolated by sequential density ultracentrifugation from normal human blood collected in 3 mM Na_2 EDTA, as described previously [37]. It was labelled with 37 MBq (1 mCi) of $Na^{125}I$ (Code IMS. 30; Amersham International PLC, Aylesbury, U.K.) by the method of McFarlane as modified by Bilheimer *et al.* [38]. It was found that the labelling efficiency was much greater when 2 mg of LDL-protein was iodinated than when 10–20 mg was iodinated. The 2 mg of ^{125}I-labelled LDL was dialysed at 4° over 2 nights against four batches of 5 L of 154 mM NaCl, 16.7 mM NaH_2PO_4, 21.1 mM Na_2HPO_4 and 100 μM Na_2 EDTA (pH value adjusted to 7.4 with NaOH solution). It was then sterilized by membrane filtration (Millex-GV, pore size 220 nm; Millipore, Watford, U.K.), its protein content determined by a modification of the Lowry procedure [39] and its radioactivity measured. It was then diluted with nonlabelled LDL and stored at 4°.

Isolation and culture of macrophages. Resident macrophages were isolated by peritoneal lavage of female Swiss T.O. mice weighing about 20–30 g (from A. Tuck & Son, Battlesbridge, U.K.) using ice-cold Dulbecco's phosphate buffered saline without Ca^{2+} and Mg^{2+} (Gibco Ltd, Uxbridge, U.K.) [40]. The cells were plated in 22.6 mm diameter wells in 12-well cluster plates (Costar; supplied by Northumbria Biologicals Ltd, Cramlington, U.K.) at two million peritoneal cells per well for the macrophages that were to modify the LDL (modifying macrophages) and one million for the macrophages that were to have modified LDL added to them to measure its rate of degradation (recipient macrophages). The culture medium consisted of 9 vol. of Dulbecco's-modified Eagle's medium (DMEM; containing 1 g glucose/L; Catalogue No. 041-1885; Gibco) and 1 vol. of foetal calf serum (not heat-inactivated; Gibco) and contained 50 μg of gentamicin/mL (Catalogue No. 043-5710; Gibco). The cells were incubated at 37° under water-saturated air/CO_2 (19:1). After 2 hr, the macrophages were washed several times with DMEM to remove the contaminating cells. They were then used immediately, if they were to modify the LDL, or after an overnight incubation, if they were to be used to measure the degradation of modified LDL.

LDL modification by macrophages or $CuSO_4$. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated for up to 24 hr with macrophages (macrophage-modified LDL) or cell-free wells (control LDL). The culture medium (0.5 mL/well) consisted of Ham's F-10 medium (Catalogue No. 12-412-54; Flow Laboratories Ltd, Rickmansworth, U.K.) containing gentamicin (50 μg/mL). If a bottle of F-10 that had been stored unopened for some time was used, it was supplemented with 3 μM of freshly dissolved $FeSO_4$ [6]. If a new bottle was used, it was unnecessary to add additional $FeSO_4$ and, in fact, this could cause some degree of modification of LDL in the cell-free wells.

After incubation, the medium was centrifuged (250 *g* for 10 min at 4°) to remove any detached cells. If its α-tocopherol and hydroperoxide contents were to be determined, butylated hydroxytoluene and Na_2 EDTA (final concentrations 20 μM and 2 mM, respectively) were added to a portion of it, to prevent any further oxidation before it could be assayed [7]. The remaining medium was diluted to 10 μg LDL-protein/mL with DMEM containing 10% (v/v) foetal calf serum and 50 μg gentamicin/mL. (In this medium, the further modification of LDL by macrophages should be totally inhibited, as DMEM does not support the modification of LDL and foetal calf serum is a powerful inhibitor of it [7].) It was then incubated with recipient macrophages or cell-free wells to measure its rate of degradation.

^{125}I-Labelled LDL (100 μg protein/mL) was oxidized by copper by incubating it was 24 hr at 37° under aseptic conditions with 100 μM $CuSO_4$ added to Ham's F-10 medium containing 50 μg gentamicin/mL.

Measurement of LDL degradation by macrophages. The rate of uptake of ^{125}I-labelled LDL by macrophages was estimated by measuring the release of its noniodide degradation products into the medium [41]. After 20–24 hr incubation with macrophages or cell-free wells in a total volume of 0.5 mL, the degradation products were determined immediately or after being stored frozen.

A sample (100 μL) of the medium was taken and its total radioactivity was determined (in a total volume of 1 mL, so as to preserve the standard counting geometry), to check that the amounts of ^{125}I-labelled LDL added to each dish were the same. To the remaining medium on ice, was added 100 μL of bovine serum albumin (30 mg/mL; Catalogue No. A7906; Sigma Chemical Co., Poole, U.K.), as a carrier, and 250 μL of ice-cold 3 M trichloroacetic

acid. The tubes were centrifuged at 1500 *g* for 10 min at 4°. $AgNO_3$ (250 μL of 700 mM) was then added to the same tube (to precipitate free $^{125}I^-$) and the samples were again centrifuged at 1500 *g* for 10 min at 4°. A sample (1 mL) of the supernatant was removed and its radioactivity determined. The measured degradation products of the cell-free wells were subtracted from those of the medium from the corresponding macrophages.

The macrophages were washed three times with Dulbecco's phosphate buffered saline containing Ca^{2+} and Mg^{2+} and lysed by incubation for 20 min at room temperature with 600 μL of 200 mM NaOH followed by shaking the wells. The protein content of 500 μL of the lysate was determined [39].

Determination of hydroperoxides and α-tocopherol. The hydroperoxide content of LDL-containing media was measured by an automated triiodide assay [42]. Measurements were made of both the aqueous samples and of heptane extracts [43] of them. The lipid hydroperoxides (as measured in the heptane extracts) usually represented in excess of 95% of the total hydroperoxides. Only data for the lipid hydroperoxides are shown here.

α-Tocopherol was determined in heptane extracts of the media by HPLC [8] using a Merck Lichrocart CN column (250 × 4 mm) and hexane/propan-2-ol (99:1, v/v) as the mobile phase. The fluorescence detector was set to an excitation wavelength of 295 nm and emission of 325 nm.

Flavonoids. All the flavonoids were obtained from Sigma, except for flavone, 3-hydroxyflavone, galangin and fisetin which were from Aldrich Chemical Co. (Gillingham, U.K.). Myricetin was either from Sigma or Aldrich. Gossypetin, hypolaetin and hypolaetin-8-glucoside were kindly provided by Dr M. J. Alcaraz of the University of Valencia, Spain. They were added to the macrophages dissolved in ethanol (whose final concentration was 1% (v/v) or less) or in the case of gossypin or hypolaetin-8-glucoside dissolved as a concentrated solution in 100 mM NaOH.

RESULTS

Effect of flavonoids on LDL modification by macrophages

We and others have previously shown that mouse peritoneal macrophages incubated in the presence of micromolar amounts of iron can oxidatively modify LDL so that it is degraded up to 10–20 times more rapidly by recipient macrophages by means of the acetyl-LDL or other receptors [5–8]. We show here that this modification can be prevented completely by flavonoids. Each of the flavonoids tested inhibited the modification of ^{125}I-labelled LDL in a dose-dependent manner, although their potencies varied considerably. Figure 2 shows the effects of three of these compounds. The IC_{50} values for nine aglycone flavonoids (i.e. flavonoids without a carbohydrate moiety) are shown in Table 1. The Table also shows previously published IC_{50} values for the inhibition of 5-lipoxygenase and cyclo-oxygenase in rat elicited peritoneal leukocytes by these nine compounds [34]. There was no obvious correlation between the potencies of individual flavonoids in inhibiting LDL

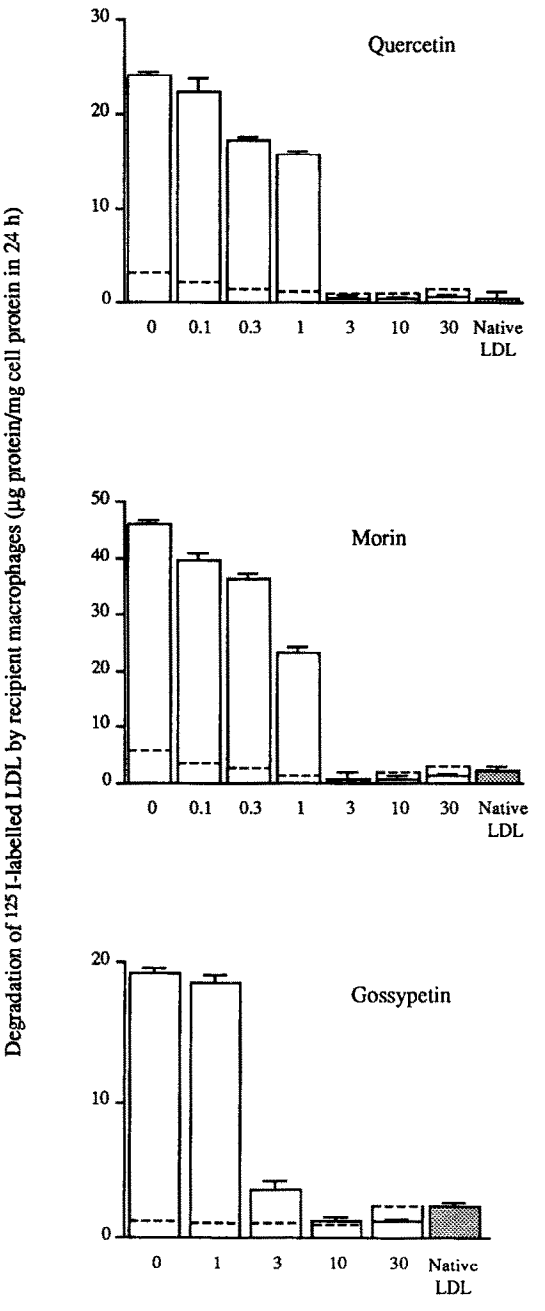

Fig. 2. Inhibition by flavonoids of ^{125}I-labelled LDL modification by macrophages. ^{125}I-Labelled LDL (100 μg protein/mL) was incubated for 24 hr with macrophages (macrophage-modified LDL) or cell-free wells (control LDL) in the presence of various concentrations of flavonoids. It was then diluted to 10 μg LDL-protein/mL with serum-containing culture medium and incubated for 24 hr with recipient macrophages or cell-free wells and its degradation was measured. The degradation of the macrophage-modified LDL is shown by the continuous lines and that of the control LDL by the dashed lines. The degradation of native (i.e. nonincubated) LDL by the macrophages is also shown. The mean of three wells of cells is shown, together with the SE for the degradation of macrophage-modified LDL.

Table 1. Potencies of flavonoids in inhibiting LDL modification by macrophages

Flavonoid	OH group positions	Approx. IC_{50} values (μM)		
		Inhibition of cyclo-oxygenase	Inhibition of 5-lipoxygenase	Inhibition of LDL modification by macrophages
Flavone	None	8	32	100
3-Hydroxyflavone	3	1	16	5
Chrysin	5,7	5	18	20
Galangin	3,5,7	7	20	15
Fisetin	3,7,3′,4′	80	11	1
Morin	3,5,7,2′,4′	180	160	1
Quercetin	3,5,7,3′,4′	16	3.5	2
Hypolaetin	5,7,8,3′,4′	70	4.5	5
Gossypetin	3,5,7,8,3′,4′	Not active	10	2

Macrophages were incubated for 24 hr with ^{125}I-labelled LDL (100 μg protein/mL) in the presence of various concentrations of flavonoids. The LDL was then diluted to 10 μg LDL-protein/mL with serum-containing medium and its degradation by recipient macrophages was determined, as a measure of its extent of modification. The flavonoids had no effect on the uptake of ^{125}I-labelled LDL by the recipient macrophages when added at the appropriate concentrations (i.e. one tenth of that added to the modifying macrophages). The IC_{50} values for the inhibition of cyclo-oxygenase and 5-lipoxygenase in intact rat elicited peritoneal leucocytes are also shown [34].

modification and in inhibiting either of the two enzymes.

Gossypin, a glycosylated derivative of gossypetin on the 8 position, was less potent than gossypetin in inhibiting the modification of LDL by macrophages (10 μM gossypin gave about 30% inhibition and 100 μM gave total inhibition whereas, as shown in Fig. 2, 3 μM gossypetin gave almost total inhibition).

Effects of quercetin on the time course of LDL oxidation by macrophages

We have investigated the time course of the oxidative modification of LDL by macrophages in relation to its increased uptake by recipient macrophages, the generation of hydroperoxides and the depletion of the endogenous antioxidant α-tocopherol within it [8]. These experiments were performed by sampling the medium at various times after adding ^{125}I-labelled LDL to the macrophages. Figure 3 shows one experiment in which the effects of 1 μM quercetin on these parameters were studied.

In the absence of quercetin, there was a lag period of about 6 hr before the LDL became sufficiently modified so that it was taken up and degraded faster by the recipient macrophages (Fig. 3a). After this time, there was a progressive increase in the ability of this LDL to be taken up by the recipient macrophages, implying a progressive increase in its extent of modification. The addition of 1 μM quercetin prolonged the lag period to at least 10 hr (Fig. 3a), although by 24 hr the LDL had been sufficiently modified to be taken up rapidly by the recipient macrophages. The exact time of onset of the modification of the LDL to a form that could be taken up faster by macrophages could not be assessed from this experiment.

In the absence of quercetin, the hydroperoxides in the medium started to increase rapidly after 2 hr incubation with the macrophages and plateaued after about 8 hr, probably due to a balance being reached between their rates of formation and decomposition (Fig. 3b). The onset of peroxidation was delayed to at least 10 hr in the presence of quercetin but reached a comparable level after 24 hr.

There was a rapid depletion of α-tocopherol in the macrophage-modified LDL, such that it was completely consumed by 4 hr (Fig. 3c). In the presence of quercetin, its levels were maintained for at least 10 hr but it was all consumed by 24 hr.

These results can be explained in terms of the α-tocopherol and flavonoids protecting the LDL against peroxidation. Depletion of α-tocopherol by the oxidative stress created by the macrophages in the presence of micromolar concentrations of iron allows the onset of peroxidation to occur. After a further delay, the peroxidation results in an alteration of the apolipoprotein B-100 of the LDL which results in its recognition by the scavenger receptors of macrophages and to its rapid uptake. Quercetin delays the consumption of the α-tocopherol in LDL, thereby delaying the formation of hydroperoxides and the generation of a form of LDL that is taken up rapidly by macrophages.

When ^{125}I-labelled LDL was incubated with Ham's F-10 medium in cell-free wells, the levels of α-tocopherol in the LDL decreased much more slowly than in the presence of cells (Fig. 3f). There was a slow increase in the hydroperoxide levels over this time but even after 24 hr it was much less than in the presence of cells (Fig. 3e) and there was no increase in the degradation of the LDL when it was added to macrophages (Fig. 3d). When 1 μM quercetin was added to the cell-free wells, it delayed for at least 10 hr the slow decrease of α-tocopherol (Fig. 3f) and slowed down the gradual increase in the levels of hydroperoxides (Fig. 3e).

Effects of flavonoids on LDL modification by copper

LDL can be oxidized by $CuSO_4$ in Ham's F-10

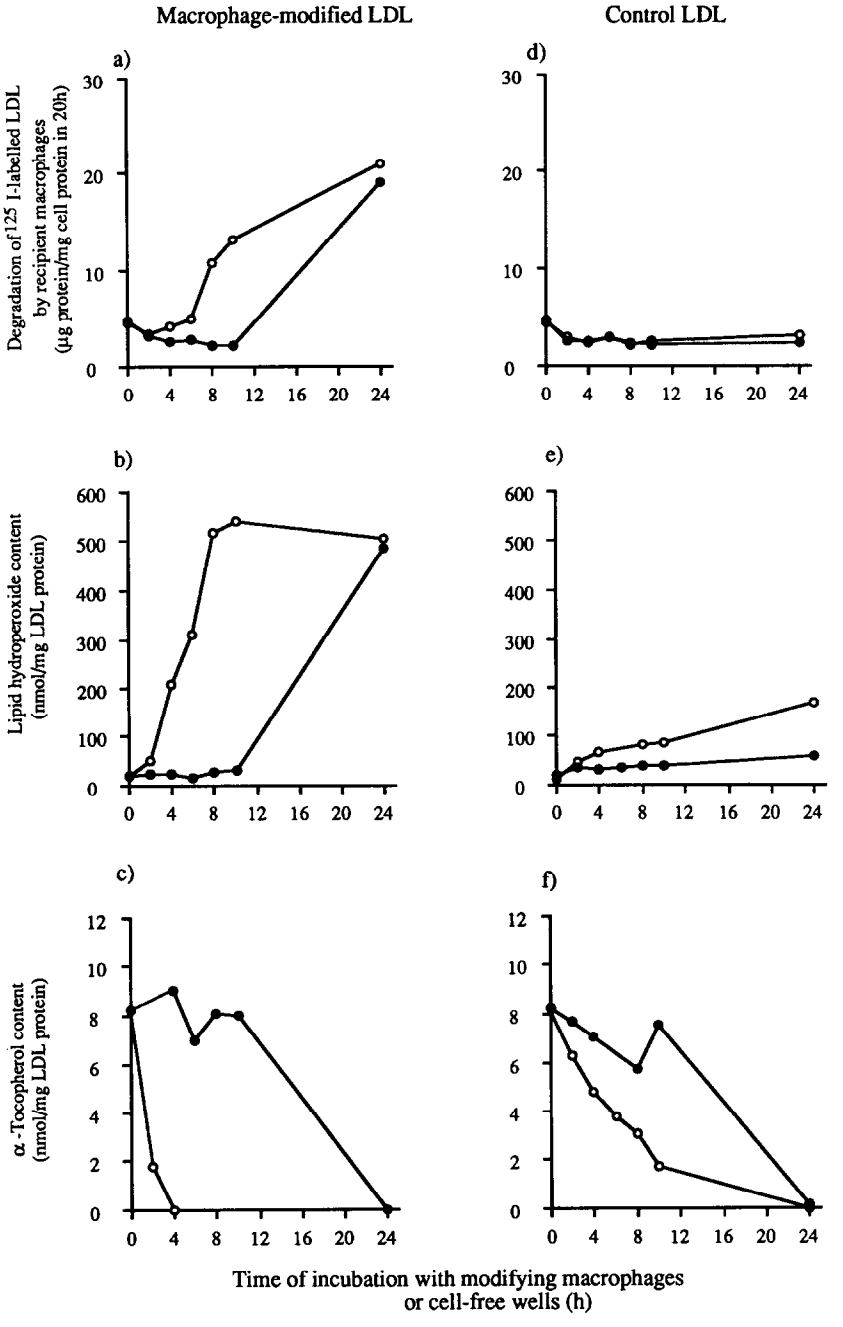

Fig. 3. Inhibition of LDL oxidation by quercetin. Macrophages (a, b and c) or cell-free wells (d, e and f) were incubated with ^{125}I-labelled LDL (100 μg protein/mL) for various times up to 24 hr with (●) or without (○) 1 μM quercetin. The medium was then assayed for hydroperoxides (b and e) and α-tocopherol (c and f) or diluted to 10 μg LDL-protein/mL with serum-containing medium and added to recipient macrophages or cell-free wells. Its degradation at the end of 20 hr was measured (a and d). Each point for the degradation of LDL is the mean of three wells of cells, which did not differ by more than about 5%. Each point for the hydroperoxide and α-tocopherol levels is the mean of duplicate determinations, which did not differ by more than about 5%, from a pooled sample from three wells.

medium in the absence of cells so that it is taken up much faster by macrophages by the acetyl-LDL receptor and possibly other receptors [8, 15, 44]. A range of glycosylated and aglycone flavonoids inhibited LDL oxidation mediated by 100 μM Cu^{2+} (Table 2). Gossypetin was again more potent than its glycosylated derivative gossypin. Flavone (100 μM) had no inhibitory effect against the $CuSO_4$-mediated oxidation.

DISCUSSION

There is increasing evidence that the localized

Table 2. Inhibition by flavonoids of LDL oxidation by Cu^{2+}

Flavonoid	Degradation of ^{125}I-labelled LDL by macrophages (μg protein/mg cell protein in 24 hr)
None	42.6 ± 2.5
Flavone (100 μM)	46.5 ± 0.5
Hypolaetin-8-glucoside* (10 μM)	0.9 ± 0.1
Gossypetin (10 μM)	1.5 ± 0.1
Gossypin (10 μM)	42.5 ± 1.9
Gossypin (100 μM)	1.0 ± 0.1
Myricetin (10 μM)	41.2 ± 1.0
Myricetin (100 μM)	7.3 ± 0.2

^{125}I-Labelled LDL (100 μg protein/mL) was incubated at 37° for 24 hr in Ham's F10 medium containing 100 μM freshly dissolved $CuSO_4$ with or without a flavonoid. It was then diluted to 10 μg LDL-protein/mL with serum-containing medium and incubated for 24 hr with macrophages or cell-free wells. Its degradation by the cells was then measured. The mean ± SE of three wells of cells is shown.

* Hypolaetin-8-glucoside is hydroxylated at the 5,7,3′ and 4′ positions.

oxidation of LDL in the intima of arteries plays an important role in the development of fatty streaks, an early type of atherosclerotic lesion [10–14, 22]. Macrophages, which enter the intima from the blood as monocytes, may well contribute to this oxidation [5–8].

In this study, we have shown that various aglycone flavonoids are effective inhibitors of the modification of LDL by macrophages. The flavonoids appear to act by protecting the LDL against oxidation caused by the macrophages, as they inhibit the generation of hydroperoxides and protect the α-tocopherol, a major lipophilic antioxidant carried in lipoproteins, from being consumed by oxidation in the LDL. Many, but not all, aglycone flavonoids are lipophilic [45, 46] and if they were to partition into lipoproteins this would help to explain why they are so potent in inhibiting LDL oxidation.

The conversion of LDL by macrophages to a form that is taken up more rapidly by macrophages exhibits a lag phase of about 4–6 hr. This is probably due in part to the time required for the α-tocopherol and other antioxidants in LDL to be consumed due to the oxidative stress created by the macrophages. The chain reaction of peroxidation of the polyunsaturated fatty acyl groups in the lipoprotein can then continue unrestricted. The flavonoids protect the α-tocopherol (and possibly other endogenous antioxidants) in LDL from oxidation, maintain their levels for longer and delay the start of lipid peroxidation.

The fact that flavonoids prolong the lag phase before LDL is converted to a form that is taken up rapidly explains the very steep concentration–response relationships that were observed over a narrow range of flavonoid concentrations. Thus, if a certain concentration of a flavonoid were to extend the lag phase just beyond the time of incubation used in an experiment, no increased uptake would be seen at all (i.e. a complete inhibition of LDL modification), whereas a slightly lower concentration would not extend the lag phase beyond the end of the incubation and some increased uptake would be observed. If flavonoids, together with other natural anti-oxidants, were to extend the lag phase for LDL oxidation beyond the residence time of LDL in the arterial wall *in vivo*, then LDL particles would be able to enter the wall and leave it without being oxidized. This would prevent the rapid uptake of oxidized LDL by macrophages and their conversion into cholesterol-laden foam cells.

The mechanisms by which flavonoids inhibit LDL oxidation are uncertain. They may reduce the formation or release of free radicals in the macrophages or they may protect the α-tocopherol in LDL from oxidation by being oxidized by free radicals themselves in preference to it. Flavonoids have been shown to react with superoxide anions [31], hydroxyl radicals [29, 30] and lipid peroxyl radicals [26, 27, 31]. Another possibility is that the flavonoids may have regenerated active α-tocopherol by donating a hydrogen atom to the α-tocopheryl radical, which is formed when it transfers its own hydroxyl hydrogen atom to a lipid peroxyl radical to terminate the chain reaction of lipid peroxidation. Such a mechanism has been demonstrated for the maintenance of α-tocopherol levels by ascorbic acid [47].

Flavonoids are known to bind iron [31] and copper [48] ions and it is possible that they may exert part of their effect by reducing the formation of free radicals mediated by iron ions in the medium of the macrophages and by copper ions in the cell-free system. It is doubtful, however, if this could explain all their effect, as some flavonoids at a concentration of only 10 μM completely inhibited the modification of LDL by 100 μM Cu^{2+}.

Another mechanism by which flavonoids could inhibit LDL oxidation by macrophages would be by inhibiting the generation or release of free radicals from the macrophages. These radicals could be derived from a number of sources, two of which are as byproducts of the activities of 5-lipoxygenase and cyclo-oxygenase. There were no obvious correlations between the known potencies of individual flavonoids in inhibiting these two enzymes and their potencies in inhibiting LDL modification, but more work will be needed to determine the source of the free radicals that oxidize LDL.

We have not tested enough individual flavonoids to make detailed statements about their structure–

activity relationships, but we can say that the substitution of the flavone nucleus with hydroxyl groups is important, as flavone itself was not a good inhibitor of LDL modification whereas the polyhydroxylated aglycone flavonoids were potent inhibitors. Flavone has been shown to be a poor free radical scavenger [29].

Relatively little is known about the absorption, metabolism and excretion of individual flavonoids in man [49] and whether or not they are transported in the bloodstream dissolved in lipoproteins. Some flavonoids appear to be absorbed after oral administration [50] whereas others do not appear to reach the general circulation, at least in an unchanged form, to any measurable extent [51]. It may be interesting to note that the lipophilic drug probucol, an anti-oxidant which inhibits LDL oxidation by cells with a similar potency to some of our flavonoids [52] is carried in the bloodstream in the lipoproteins [52, 53].

In summary, we have shown that flavonoids inhibit the oxidative modification of LDL by macrophages or $CuSO_4$. As flavonoids are consumed in the normal human diet, this raises the possibility that they may protect to a certain extent against atherosclerosis, but this will depend to a large degree on their pharmacokinetics.

Acknowledgements—We are grateful for financial support to the Wellcome Trust, British Heart Foundation and Medical Research Council and to Mrs S. F. Dalton for processing the manuscript. C.V. de W. and S.M.R. were supported by Collaborative Awards in Science and Engineering studentships from the Science and Engineering Research Council, in collaboration with Smith Kline & French Research Ltd.

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