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CALCIUM ANTAGONISTS PREVENT MONOCYTE AND ENDOTHELIAL CELL-INDUCED MODIFICATION OF LOW DENSITY LIPOPROTEINS

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Low density lipoprotein (LDL) incubated in the presence of the calcium antagonists verapamil, nifedipine and flunarizine were more resistant than control LDL to human monocyte- or endothelial cell-induced modification, as assessed by electrophoretic mobility in agarose gel, thiobarbituric acid reactive substance content, and degradation by J774 macrophages. The efficiency of the drugs was: flunarizine > nifedipine > verapamil. Moreover, a 24 h preculture with calcium antagonists significantly impaired the ability of cells to modify LDL in the absence of the drugs. All the studied drugs also inhibited copper-induced autooxidation of LDL. None of the studied calcium antagonists, at concentrations up to 10^{-4} M, significantly reacted with free radicals as assessed by the 1,1-diphenyl-2-picrylhydrazyl test. It is suggested that such a protective effect of calcium antagonists against LDL peroxidation could play a role in the previously reported antiatherogenic effect of these drugs.

KEY WORDS: Calcium antagonists, LDL peroxidation.

ABBREVIATIONS: **DMPO**: 5,5′-dimethyl-1-pyrroline-*N*-oxide; **DPPH**, 1,1-diphenyl-2-picrylhydrazyl; **LDL**, low density lipoprotein; **TBARS**: thiobarbituric acid reactive substances.

INTRODUCTION

Cholesterol delivery to cells is achieved in most tissues via the low density lipoprotein (LDL) receptor-mediated pathway.[1] In the seventies, the protective role of the LDL pathway against atherosclerosis has been largely recognized.[2] More recently, it has been shown that LDL can be modified by incubation with cultured endothelial cells,[3,4] smooth muscle cells,[5] monocytes[6] or macrophages.[7] These modified LDL are no longer recognized by their specific apo B/E receptor but are avidly taken up by the scavenger receptor of macrophages, leading to cholesteryl ester accumulation. Such a mechanism has been admitted to be probably a key factor in the progress of

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atherogenesis.[8,9] The recent demonstration of the existence *in vivo* of oxidatively modified LDL supports this hypothesis.[10]

In the last years, works from the group of Steinberg demonstrated that probucol, an hypolipidemic agent, can prevent LDL peroxidation induced by incubation with either endothelial cells or copper ions.[11] Such a protective effect of probucol against LDL oxidative modification has been implicated in the antiatherogenic effect of this drug.[12,13] In a previous report, we showed that other drugs, which are not free radical scavengers such as phenothiazines, are almost as efficient as probucol in protecting LDL from copper or endothelial cell-induced oxidative modification.[14] We suggested that these properties could be related to modification of the LDL physicochemical characteristics by the drug, which results in a decreased sensitivity of the particle to the oxidative attack.[14] In the present work, we show that calcium antagonists, other amphipathic compounds used in the treatment of cardiovascular diseases,[15] are also able to protect LDL against human monocyte- and endothelial cell-induced modification.

MATERIALS AND METHODS

Chemicals

Verapamil, nifedipine, flunarizine, vitamin E, $CuSO_4$ and 1,1-diphenyl-2-phenyl-hydrazyl (DPPH) were from Sigma Chemical Co. (St Louis, MO, U.S.A.). Ham F10, RPMI 1640 and Dulbecco's MEM media were from Gibco (Grand Island, NY, U.S.A.). The U937 human monocyte-like cell line [16] and J774 murine monocyte-macrophage cell line [17] were from the American Type Culture Collection (Rockville, Maryland, U.S.A.). The serum substitute Ultroser G was from Industries Biologiques Françaises (Villeneuve la Garenne, France). Flasks and Petri dishes were from Nunc. Na-^{125}I 13–17 Ci/mg was purchased from Amersham (Buckinghamshire, U.K.).

Cell culture

The U937 cell line was maintained in suspension in RPMI medium supplemented with 10% heat-inactivated fetal calf serum. Monolayers of this cell line was obtained when cells were shifted to Ham F10 medium containing 10^{-7} 12-O-tetradecanoyl phorbol 13-acetate. The human endothelial cells were obtained from umbilical vein by collagenase digestion, as previously described,[18] and maintained in Ham F10 medium supplemented with 10% fetal calf serum. The J774 cell line was maintained in suspension culture with RPMI 1640 medium supplemented with 10% heat inactivated fetal calf serum. For studies on LDL degradation, J774 cells were seeded in 3.5 cm Petri dishes. All experiments were performed on subconfluent cultures.

LDL preparation and labeling

The LDL (d = 1.024–1.050) was prepared from human normal serum by sequential ultracentrifugation according to Havel *et al.*,[19] and dialyzed against 0.005 M Tris, 0.05 M NaCl, 0.02% EDTA pH 7.4 for conservation. Prior to oxidation by cells, EDTA was removed by dialysis. ^{125}I-labeling of LDL was performed as described by Bilheimer *et al.*[20] The specific activity was about 250 CPM/ng LDL protein.

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LDL oxidation

The cellular modification of LDL was achieved by incubating 100 μg/ml LDL protein during 24 h with U937 monocyte-like cells or endothelial cells in 3.5 cm Petri dishes, in Ham F10 medium supplemented with the serum substitute Ultroser G, in the presence or absence of the drugs (10^{-5} to 10^{-4} M). Verapamil was introduced in aqueous solution, nifedipine and flunarizine in ethanolic solution (final ethanol concentration 0.5%; controls with 0.5% ethanol alone were done for experiments concerning nifedipine or flunarizine). The drugs were introduced at the beginning of the incubation of cells with LDL. In some experiments ("pretreatment" experiments), cells were first cultured for 24 h in the presence of the drugs, then washed, and their ability to subsequently modify LDL was tested in the absence of drugs. Copper-induced auto-oxidation of the LDL was achieved by incubation of 50 μg LDL protein in 500 μl Ham F10 medium in the presence of 5×10^{-6} M $CuSO_4$ during 24 h.

The presence of lipid peroxidation products (TBARS) was checked by the fluorometric assay of Yagi.[21] Results are expressed in nmol equivalent malondialdehyde/mg LDL protein, using tetraethoxypropane as standard, and calculated as % of control. The modification of the negative net charge of LDL was assessed by agarose gel electrophoresis at pH 8.6 in a Ciba Corning system.

^{125}I-LDL degradation by J774 macrophages

Oxidation of ^{125}I-LDL by U937 cells was first carried out with 20 μg/ml LDL protein in Ham F10 medium supplemented with 2% Ultroser G during 24 h. The medium was then transferred to J774 cells in 6 cm Petri dishes. The LDL degradation by J774 cells was determined as the trichloroacetic acid-soluble non iodide radioactivity,[22] and expressed in ng LDL protein degraded/mg cellular protein. Blank values from parallel incubations without U937 cells were also determined.

Evaluation of the free radical scavenging properties of the drugs by the DPPH test

DPPH is a stable free radical which is currently used for determination of the antioxidant properties of drugs.[23] It exhibits a strong absorption at 516 nm which is reduced when it reacts with a free radical scavenger. A 10^{-4} M DPPH solution was prepared in ethanol just before experiments. 3 ml of the DPPH solution were mixed with 0.03 ml of a 10^{-2} M solution of the studied drug or vitamin E for comparison, and the time-course of the decrease in O.D. at 516 nm was followed up to 20 min. using a Hewlett-Packard HP 8452 spectrophotometer.

Each experiment was performed at least twice in duplicate.

RESULTS

Figure 1 shows the effect of verapamil, nifedipine and flunarizine 10^{-5} to 10^{-4} M on the TBARS content (Figure 1a) and on the relative electrophoretic mobility (Figure 1b) of LDL submitted to oxidative modification by the human monocytic cell line U937. It clearly appears that all the studied drugs reduced in a dose-dependent manner both the TBARS level and the relative electrophoretic mobility of the particle, flunarizine being the most efficient, inducing about 60 and 95% reduction of the TBARS

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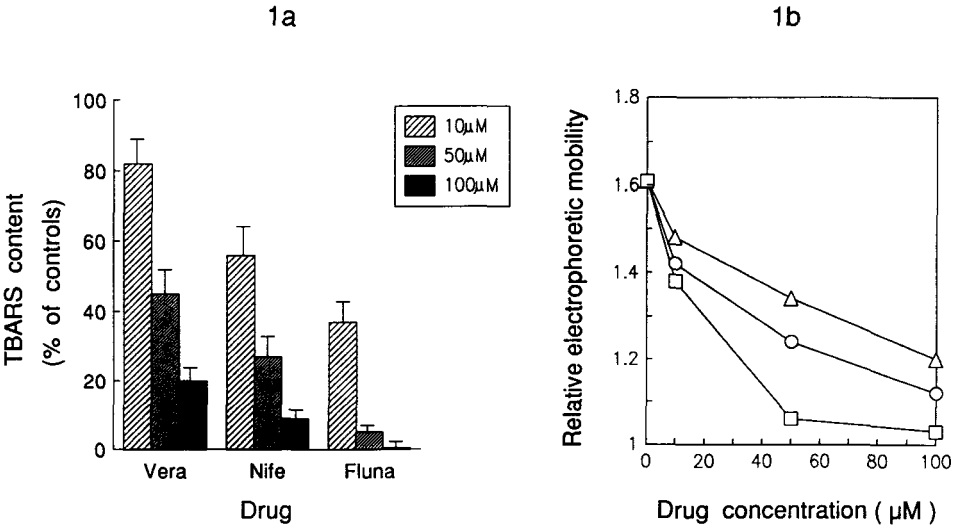

FIGURE 1 Effect of verapamil, nifedipine and flunarizine on U937 cell-induced modification of human LDL. 100 μg/ml LDL protein were incubated during 24 h with U937 cells in Ham 10 medium supplemented with the serum substitute Ultroser G, in the presence or absence of the drugs (10^{-5} to 10^{-4} M). The drugs were introduced at the beginning of the incubation of cells with LDL. (a) TBARS content of LDL assessed by the fluorometric method of Yagi (see Ref. 21). 100%: 73 ± 14 nmol equivalent MDA/mg LDL protein. (b) relative electrophoretic mobility of the particle measured by agarose gel electrophoresis (see experimental section). △: verapamil; ○: nifedipine; □: flunarizine. Means of 6 experimental values.

content of the LDL at the concentration of 10^{-5} and 10^{-4} M, respectively (Figure 1a). The relative electrophoretic mobility of the particle was similarly decreased (Figure 1b).

Table I shows that verapamil, nifedipine and flunarizine also protected LDL against human umbilical endothelial cell-induced modification, albeit the drugs were less effective than in experiments conducted with U937 monocyte-like cells (see Figure 1).

The effect of a 24 h pretreatment of U937 cells by the drugs on their ability to oxidize LDL was also investigated (subsequent incubation of LDL with cells was performed in the absence of the drugs). It can be observed in Table II that

TABLE I

Effect of verapamil, nifedipine and flunarizine on human endothelial cell-induced modification of LDL

Addition	TBARS formation	Relative electrophoretic mobility
None	47 ± 6	1.33 ± 0.01
Verapamil 5×10^{-5} M	31 ± 5	1.21 ± 0.01
Ethanol 0.5%	49 ± 7	1.33 ± 0.02
Nifedipine 5×10^{-5} M	17 ± 2	1.14 ± 0.01
Flunarizine 5×10^{-5} M	7 ± 1	1.05 ± 0.01

100 μg/ml LDL protein were incubated during 24 h with endothelial cells in Ham F10 medium supplemented with 2% Ultroser G, in the presence or absence of the drugs. The drugs were introduced at the beginning of the incubation of cells with LDL. Results for TBARS production are expressed in nmol equivalent MDA/mg LDL protein (means of 4 experimental values ± s.d.).

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TABLE II
Effect of pretreatment of U937 human monocytes with calcium antagonists on subsequent LDL-modification

Addition	TBARS formation	Relative electrophoretic mobility
None	69 ± 12	1.61 ± 0.02
Verapamil 5×10^{-5} M	52 ± 7	1.50 ± 0.01
Ethanol 0.5%	71 ± 10	1.61 ± 0.03
Nifedipine 5×10^{-5} M	36 ± 4	1.37 ± 0.01
Flunarizine 5×10^{-5} M	13 ± 2	1.16 ± 0.01

Cells were pretreated for 24 h with the indicated drugs before subsequent LDL modification, which was performed during the following 24 h in the absence of drugs. Results for TBARS production are given in nmol equivalent MDA/mg LDL protein. Means of 4 experimental values ± s.d.

pretreatment with nifedipine and especially with flunarizine significantly reduced the ability of U937 cells to oxidize LDL (about 50 and 80% decrease in TBARS formation for 5×10^{-5} M nifedipine and flunarizine, respectively). In contrast, verapamil was less effective, resulting in only 20–25% reduction of the TBARS of the LDL.

As it has been demonstrated that copper- or cell-modified LDL are actively catabolized by macrophages, we tested the effect of calcium antagonists on the degradation of U937-modified LDL by the murine monocyte-macrophage cell line J774. For this purpose, ^{125}I-labeled LDL was first incubated for 24 h with U937 cells in the presence or absence of the drugs, then transferred to cultured macrophages. Table III shows that all the studied calcium antagonists significantly reduced the degradation of ^{125}I-labeled LDL previously modified by the U937 monocyte-like cells. This effect was well correlated with the preventive action of the drugs against U937 cell-induced modification, flunarizine and nifedipine being more efficient than verapamil.

In order to investigate the mechanism(s) of the preventive action of calcium antagonists against cell-induced modification of LDL, we also studied the effect of verapamil, nifedipine and flunarizine on copper-induced LDL autooxidation.

TABLE III
Effect of calcium antagonists on the degradation by J774 macrophages of U937 cell-modified ^{125}I-LDL

Conditions	^{125}I-LDL degradation (ng/mg cell protein)
without U937 cells	232 ± 35
with U937 cells:	1162 ± 172
+ verapamil 5×10^{-5} M	645 ± 75
+ ethanol 0.5%	1093 ± 190
+ nifedipine 5×10^{-5} M in ethanol	440 ± 55
+ flunarizine 5×10^{-5} M in ethanol	270 ± 24

Oxidation of ^{125}I-LDL by U937 cells was carried out during 24 h with 20 μg/ml LDL protein in Ham F10 medium supplemented with the serum substitute Ultroser G in the presence or absence of the studied drugs. The medium was then transferred to J774 macrophages for determination of ^{125}I-LDL degradation during 6 h. Results are means of 4 experimental values ± s.d.

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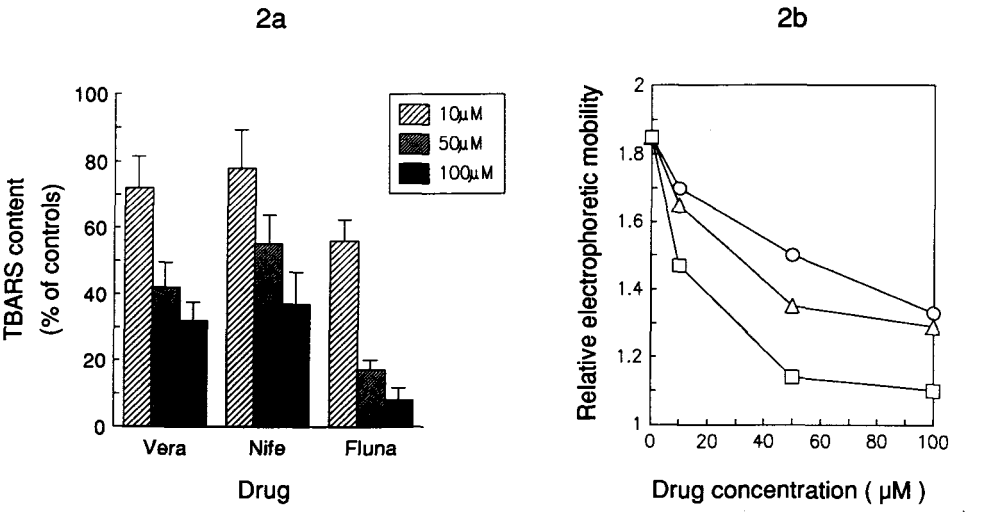

FIGURE 2 Effect of verapamil, nifedipine and flunarizine on copper-induced auto-oxidation of human LDL. 100 μg/ml LDL protein were incubated for 24 h in Ham F10 medium with 5×10^{-6} M $CuSO_4$ in the absence or in the presence of the drugs (10^{-5} to 10^{-4} M). The drugs were introduced at the beginning of the incubation time. (a) TBARS content of LDL assessed by the fluorometric method of Yagi (see Ref. 21). 100%: 89 ± 11 nmol equivalent MDA/mg LDL protein. (b) relative electrophoretic mobility of the particle measured by agarose gel electrophoresis (see experimental section). △: verapamil; ○: nifedipine; □: flunarizine. Means of 6 experimental values.

Figure 2 shows that all the studied drugs were also able to inhibit LDL oxidation induced by a 24 h incubation in the presence of 5×10^{-6} M $CuSO_4$, albeit this protective effect was less marked than that observed in experiments conducted with U937 cells. Table IV shows that the three drugs also reduced the degradation of ^{125}I-labeled LDL previously modified by copper ions.

TABLE IV
Effect of calcium antagonists on the degradation by J774 macrophages of copper-modified ^{125}I-LDL

Conditions	^{125}I-LDL degradation (ng/mg cell protein)
without Cu^{2+}	282 ± 38
with Cu^{2+}, alone:	885 ± 125
+ verapamil 5×10^{-5} M	450 ± 82
+ ethanol 0.5%	860 ± 140
+ nifedipine 5×10^{-5} M in ethanol	392 ± 77
+ flunarizine 5×10^{-5} M in ethanol	318 ± 45

Oxidation of ^{125}I-LDL by 5×10^{-6} M $CuSO_4$ was carried out during 24 h with 20 μg/ml LDL protein in Ham F10 medium supplemented with the serum substitute Ultroser G in the presence or absence of the studied drugs. The medium was then transferred to J774 macrophages for determination of ^{125}I-LDL degradation during 6 h. Results are means of 4 experimental values ± s.d.

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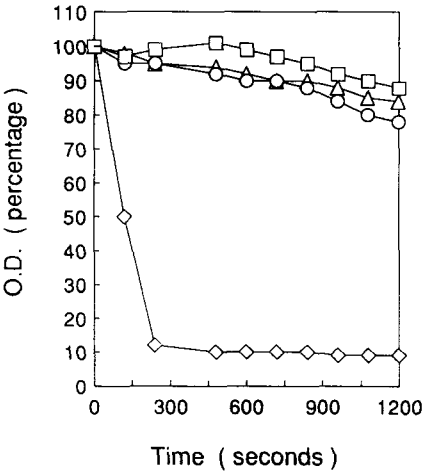

FIGURE 3 Evaluation of the free radical scavenging properties of verapamil, nifedipine, flunarizine and vitamin E using the DPPH test. A 10^{-4} M DPPH solution was prepared in ethanol just before experiments. 3 ml of the DPPH solution were mixed with 0.03 ml of a 10^{-2} M solution of the studied drug or vitamin E, and the time-course of the decrease in O.D. at 516 nm was followed up to 20 min using a Hewlett-Packard HP8452 spectrophotometer. △: verapamil 10^{-4} M; ○: nifedipine 10^{-4} M; □: flunarizine 10^{-4} M; ◇: vitamin E 10^{-4} M (means of 4 experimental values).

Finally, the efficiency of verapamil, nifedipine and flunarizine as free radical scavengers was assessed by the DPPH test, and compared to that of the well known antioxidant vitamin E. Figure 3 shows that at the maximal concentration used (10^{-4} M) all the studied calcium antagonists did not exhibit any significant free radical scavenging properties under our experimental conditions, whereas, as expected, vitamin E was very efficient.

DISCUSSION

The results of the present study clearly demonstrate that calcium antagonists from the phenylalkylamine series (verapamil), dihydropyridine series (nifedipine) and diphenylpiperazine series (flunarizine) are able to prevent monocyte and endothelial cell-induced modification of LDL. Although calcium antagonists have been previously reported to inhibit lipid peroxidation in liposomes,[24] the present report is to our knowledge the first demonstration of a protective effect of these drugs against LDL peroxidation. It must be noted that in another experimental model, Janero *et al.* described a protective effect of verapamil, flunarizine, felodipine and nisoldipine against iron-promoted peroxidation of cardiac membrane lipids, but did not observe any significant action of nifedipine.[25,26]

The mechanisms by which calcium antagonists can protect LDL against cell-induced modification are probably complex and multifocal. The fact that the studied drugs are also able to reduce copper-induced LDL autooxidation (Fig. 2) suggests that a direct protection of the particle is probably partly involved in the observed phenomenon. But it must also be stressed that pretreatment with nifedipine or flunarizine notably impaired the ability of cells to subsequently modify LDL (Table II), which suggests that, besides their direct protective effect, probably related

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to the insertion of the drugs into the LDL particle, these compounds can also modulate the mechanisms by which cells are capable of altering LDL. It can be supposed, for example, that nifedipine or flunarizine could decrease the production and/or the secretion of active oxygen species by the cells. Experiments are now in progress in order to explore this hypothesis. Moreover, it is of note that in pretreatment experiments, verapamil was only poorly effective in preventing subsequent modification of the LDL (Table II), whereas it exhibited a protective effect when the drug and the particle were simultaneously introduced in the culture medium. In contrast, nifedipine and flunarizine prevented LDL modification in both conditions, however with decreased efficiency in pretreatment experiments. These observations therefore suggest that significant differences could exist between the mechanisms of action of drugs from the three studied series on cell-induced modification of LDL.

Results in Figure 3 show that at concentrations up to 10^{-4} M, neither verapamil nor nifedipine or flunarizine appeared to be efficient free radical scavengers using the DPPH test. In a previous report on the inhibition of phosphatidylcholine liposome peroxidation by verapamil and nifedipine, Ondrias *et al.* found that verapamil competes with DMPO in trapping $OH^{\cdot}$ radicals, although this effect was observed for very high (above millimolar) concentrations of the drug.[24] In another experimental system (measurement of the $O_2^{\cdot -}$ scavenging ability of the drugs by the decrease in the rate of superoxide dismutase-inhibitable ferricytochrome c reduction), Janero *et al.* did not find any oxy-radical scavenging properties for felodipine, nicardipine and nisoldipine at 5×10^{-4} M, whereas these compounds inhibited cardiac membrane lipid peroxidation by about 50% at 10^{-4} M.[26] The data about the "free radical scavenging properties" of calcium antagonists thus appear to be very dependent upon the experimental models used, and to our opinion there is no convincing evidence which allows to conclude that, in the range of concentrations for which they inhibit lipid peroxidation, these compounds are effectively able to trap active oxygen species.

Thus, it is conceivable that in our experimental model, the studied calcium antagonists prevent LDL modification by more complex mechanisms than a simple free radical scavenging action. In a previous study, we reported that phenothiazines, other amphiphathic compounds, were very efficient in inhibiting cell and copper-induced LDL peroxidation.[14] With the DPPH test (data not shown) or using peroxy radicals generated by gamma radiolysis of aerated ethanol,[14] we failed to demonstrate any free radical scavenging properties for phenothiazines. We thus suggested that LDL oxidative modification can be prevented by other mechanisms than an actual "antioxidant" action, and we proposed that structural modification of the LDL particle induced by the insertion of some hydrophobic drugs (such as phenothiazines, or even probucol) could decrease its sensitivity to the oxidative process.[14] It is of note that the role of the physical state of the lipid phase in the susceptibility of lipids to peroxidation has been already pointed out in liposome models.[27] Moreover, in a recent report on the antioxidant activity of probucol against phosphatidylcholine liposome peroxidation, Mc Lean *et al.* also suggested that the "antioxidant" effect of this drug could be at least partially related to structural modification of the lipid phase.[28] Thus, it can be conceived that, although not being effective free radical scavengers as compared to vitamin E, calcium antagonists and other drugs such as phenothiazines can inhibit LDL oxidative modification.

Regardless of the mechanisms which remains to be specified, the inhibition by calcium antagonists of LDL oxidative modification appears to be of great interest as

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these drugs, which are currently used in the therapy of angina pectoris,[15] a coronary heart disease closely linked to perturbations of cholesterol metabolism, have been reported to exhibit a protective effect against experimental atherosclerosis in animal models.[29,30]. In fact, calcium antagonists appear to act at various levels of cholesterol and lipoprotein metabolism. They have been shown to increase LDL[31-33] and HDL[34] catabolism, and to enhance cholesterol efflux from macrophages.[35] Combined with a possible protective effect against LDL peroxidation, which remains to be demonstrated *in vivo*, all these properties suggest that calcium antagonists could effectively have a preventive action against the progression of atherosclerotic lesions.

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