Biochemical Pharmacology, Vol. 51, pp. 141–150, 1996.

ISSN 0006-2952/96/$15.00 + 0.00
SSDI 0006-2952(95)02162-0

Structure-Function Relationships of Calcium Antagonists

EFFECT ON OXIDATIVE MODIFICATION OF LOW DENSITY LIPOPROTEIN

*Nurit Rojstaczer and David J. Triggle**

SCHOOL OF PHARMACY, STATE UNIVERSITY OF NEW YORK AT BUFFALO, BUFFALO, NY 14260, U.S.A.

ABSTRACT. Human low density lipoprotein (LDL) incubated with active Ca^{2+} antagonists from three different chemical groups, 1,4-dihydropyridines that are of reduced activity as Ca^{2+} antagonists, vitamin E, and probucol, was more resistant than control to copper- or human monocyte-induced oxidation, as assessed by thiobarbituric acid reactive substance (TBARS) content, degradation by J774 macrophages, and relative electrophoretic mobility on agarose gel. In the copper-induced oxidation system, the drugs tested reduced the TBARS levels of LDL in a concentration-dependent manner. The order of potency was vitamin E > felodipine > 2-chloro analog of nifedipine > nifedipine > amlodipine, nitrendipine, verapamil > diltiazem. In agreement with the results of the TBARS assay, felodipine (25 μM) was also the most effective calcium antagonist in the degradation assay, inducing a significant ($P < 0.05$) 97 ± 2% reduction in the amount of oxidized [^{125}I]LDL degraded by J774 macrophages compared with nifedipine and its 4-nitro analog, amlodipine, and verapamil. The relative mobility of oxidized LDL on agarose gel was reduced significantly ($P < 0.05$) by felodipine (50 μM) and amlodipine (25 and 50 μM) when compared with control, and was similar to that of native LDL, suggesting an effect of these drugs on the net negative charge of oxidized LDL. In the cell-induced oxidation system, both nifedipine and felodipine (25 μM) induced significant ($P < 0.05$) reductions in the TBARS content of LDL (96 ± 2 and 65 ± 9%, respectively) compared with amlodipine, verapamil and the 4-nitro analog of nifedipine. However, in this oxidation system nifedipine was a more effective antioxidant than felodipine. Analysis of the structure–function relationships for the effect of 1,4-dihydropyridines on the oxidative modification of LDL suggests an important role for the 2-substitution of the phenyl ring, and an essential role for the dihydropyridine ring. This study clearly shows that Ca^{2+} antagonists from different chemical groups have a concentration-dependent effect as antioxidants against LDL oxidation. However, the order of potency of the drugs depends on the oxidation system and the assay used to measure the antioxidant effect. Our data suggest that such a protective effect of Ca^{2+} antagonists against LDL oxidation could play a role in the antiatherosclerotic effect of these drugs. BIOCHEM PHARMACOL 51;2:141–150, 1996.

KEY WORDS. Ca^{2+} antagonists; atherosclerosis; LDL; oxidized-LDL; LDL oxidation; 1,4-dihydropyridines

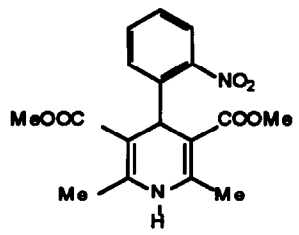
The clinically available Ca^{2+} antagonists, including nifedipine, verapamil, and diltiazem, are currently used to treat cardiovascular disorders, including hypertension and angina pectoris. The primary mode of action of these agents is at voltage-gated L-type Ca^{2+} channels where they inhibit calcium influx into arterial smooth muscle cells to decrease vascular contractility and arterial tone [1, 2]. Additional pharmacological activities have also been proposed [2]. Thus, both experimental and clinical studies indicate that the Ca^{2+} antagonists are, as a group, antiatherogenic [3, 4]. However, the mechanism(s) of this action has not been defined adequately, and both Ca^{2+} channel-dependent and -independent processes have been proposed [5, 6]. Recent studies indicate that the Ca^{2+} channel antagonists possess lipid antioxidant properties, a property that may contribute to their role in atherosclerosis [4] and other diseases, including inflammatory vascular diseases and central nervous system trauma and stroke [7–11].

The earliest recognizable gross lesion in the atherosclerotic process is the "fatty streak," characterized by an accumulation of monocyte-derived macrophages loaded with cholesteryl esters ("foam cells") in the subendothelial space [reviewed in Refs. 12–14]. The cholesterol that accumulates in the fatty streak originates primarily from elevated levels of plasma LDL† [13]. Several theories have been proposed to explain the initiating factors involved in the genesis of the fatty streak lesion. The lipid-infiltration hypothesis, proposed by Steinberg *et al.* [14], suggests that the initiating event in the development of the fatty streak is an oxidative modification of LDL that markedly increases its uptake into the arterial intima. There is strong evidence that oxidative modification of LDL is critically important and possibly a prerequisite for uptake and accumulation of cholesteryl ester by macrophages in the arterial wall [15]. When LDL first enters the subendothelial space and

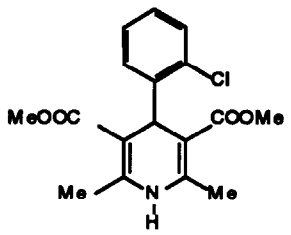
* Corresponding author. Tel. (716) 645-2823; FAX (716) 645-3688.
Received 28 March 1995; accepted 9 August 1995.

† *Abbreviations:* LDL, low density lipoprotein; DMEM, Dulbecco's modified Eagle's medium; TBARS, thiobarbituric acid reactive substances; and MDA, malondialdehyde.

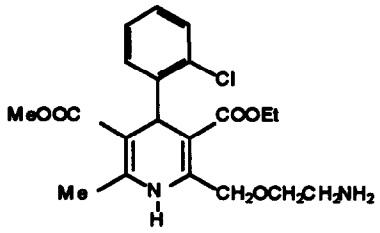
a.



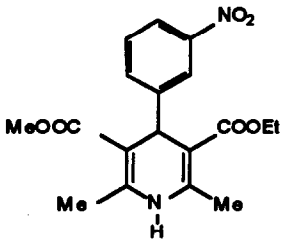
Nifedipine



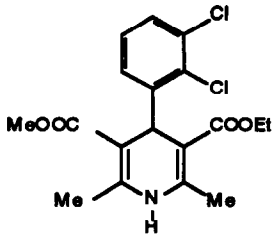
2-chloro analog of nifedipine



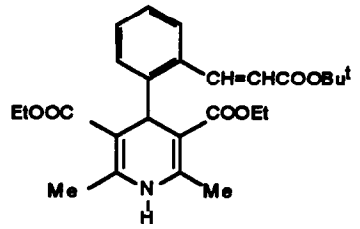
Amlodipine



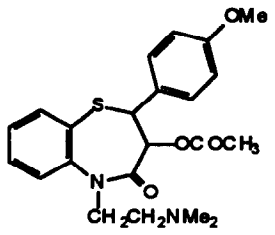
Nitrendipine



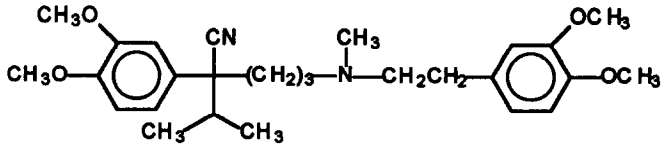
Felodipine



Lacidipine

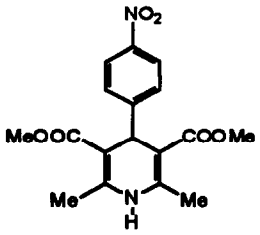


Diltiazem

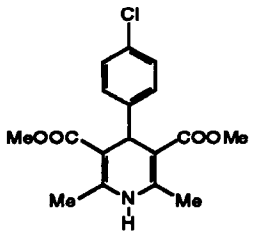


Verapamil

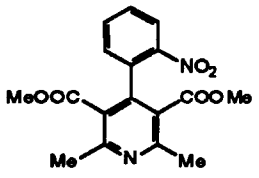
b.



4-nitro analog of nifedipine



4-chloro analog of nifedipine



Oxidized metabolite of nifedipine

FIG. 1. Chemical structures of Ca^{2+} antagonists. (a) Chemical structures of active Ca^{2+} antagonists. (b) Chemical structures of compounds that are of reduced activity as Ca^{2+} antagonists.

is exposed to pro-oxidant conditions, it undergoes the earliest steps of oxidation ("minimally oxidized") [15]. The minimally oxidized LDL is capable of releasing products that affect gene expression in arterial cells, leading to a mechanism for monocyte chemoattraction [16]. Oxidized LDL facilitates the activation-differentiation of monocytes resulting in their conversion to macrophages [17].

We have compared the effects of several calcium antagonists from different chemical groups on the oxidation of human LDL. We have included three compounds, the 4-nitro- and the 4-chloro-analogs, and the oxidized metabolite of nifedipine, which are of reduced activity as calcium antagonists, to determine whether the same structure–function relationships apply to calcium channel antagonism and antioxidant activity. We have also included two known antioxidants, vitamin E and probucol, for comparison.

MATERIALS AND METHODS

Chemicals

Ham F10, RPMI 1640 and DMEM were purchased from Gibco (Grand Island, NY). The U937 human monocyte-like cell line and J774A.1 murine monocyte-macrophage cell line were purchased from the American Type Culture Collection (Rockville, MD). Flasks and petri dishes were purchased from Fisher Scientific, (Pittsburgh, PA). Na-^{125}I (carrier free) was purchased from Amersham (Arlington Heights, IL). All reagents and chemicals were from commercial sources at the highest available grade. Calcium antagonists were available in our laboratory or were gifts from Pfizer Inc. (Sandwich, U.K.) (amlodipine) and Astra-Merck (Rochester, NY) (felodipine). The oxidized metabolite of nifedipine was purchased from Research Biochemicals International (Natick, MA).

Cell Culture

The U937 human monocyte-like cell line was grown and maintained in monolayers in RPMI 1640 medium supplemented with 10% fetal bovine serum. The J774A.1 cells were grown and maintained in monolayers in DMEM supplemented with 10% fetal bovine serum. For each experiment the cells were plated in 35 mm dishes and incubated at 37° in an atmosphere containing 5% CO_2/95% air. All experiments were performed on subconfluent cultures.

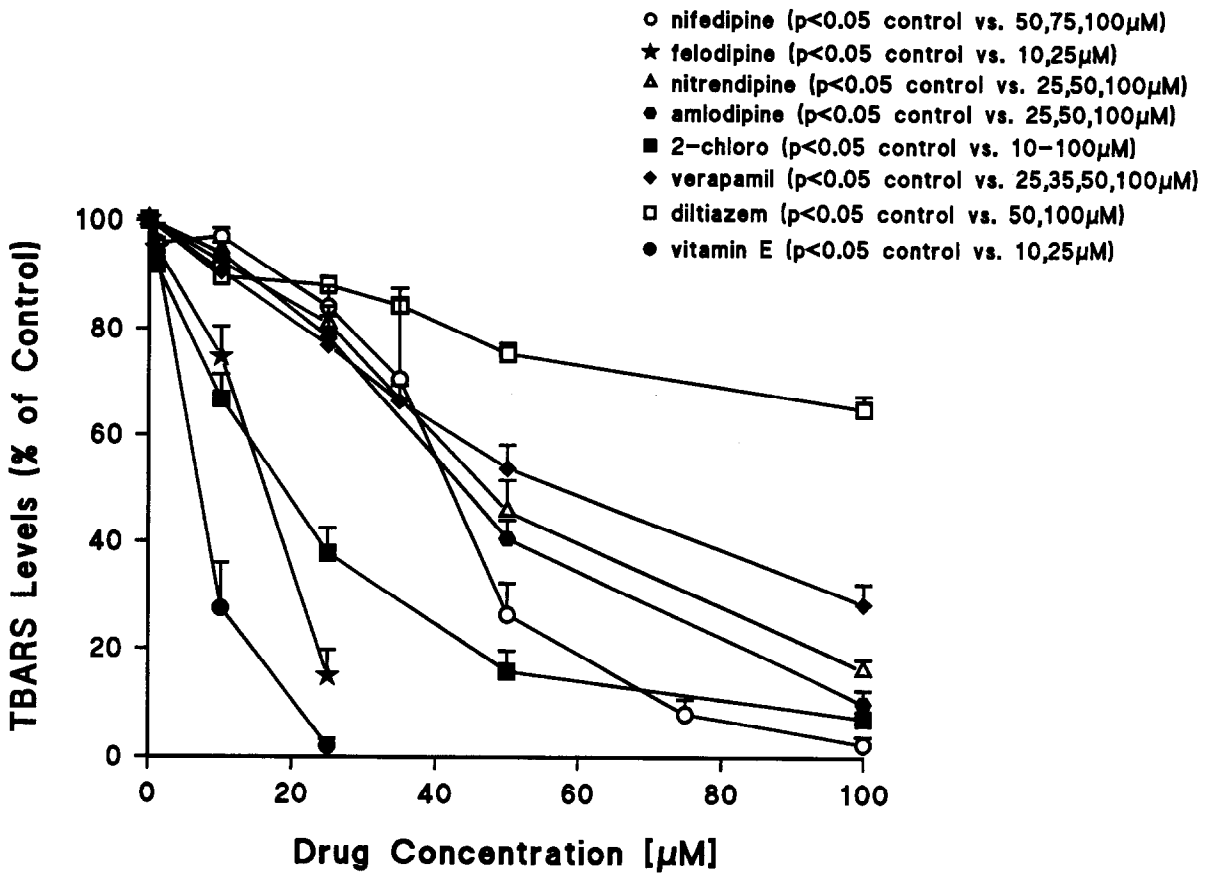


FIG. 2. Concentration-effect relationships for active Ca^{2+} antagonists on the TBARS levels of human LDL submitted to copper-induced oxidation. LDL protein (50–100 μg/mL) was preincubated with or without the drugs (1–100 μM), followed by its oxidation as described under Materials and Methods. The data shown are means ± SEM of 4–8 experiments, each performed in triplicate. Control levels: 76 ± 2 nmol of MDA equivalents produced/mg LDL protein.

LDL Preparation and Labeling

Human LDL (d = 1.006 g/mL) was isolated by discontinuous NaCl/KBr density gradient ultracentrifugation in a vertical rotor (VTi 50, Beckman) as described by Chung *et al.* [18], and dialyzed against 150 mM NaCl, 5 mM HEPES, 10 μM EDTA, pH 8.25. The purified sample was filtered through a 0.22 μm filter paper and stored in the dark under nitrogen at 4°, and used within 2 weeks. The protein content was determined by the BCA protein assay (Pierce, Rockford, IL). LDL was radioiodinated using the iodine monochloride method as described by Goldstein *et al.* [19]. In brief, Na-^{125}I was added to 0.3 to 0.5 M glycine buffer (pH 10.0). The ^{125}I/glycine mixture was added to the LDL sample, and then diluted iodine monochloride (0.7 μL of 33 mM stock/mg LDL protein) was injected quickly and vigorously into the LDL/^{125}I/glycine solution. The bound ^{125}I was separated from the free ^{125}I by a Sephadex G-25 column (PD-10; Pharmacia, Piscataway, NJ). The specific activity of [^{125}I]LDL was 200–600 cpm/ng LDL protein, and >90% of the radioactivity was precipitated by 16% trichloroacetic acid. The iodinated LDL was found to be similar to native LDL in its electrophoretic mobility. [^{125}I]ox-LDL was prepared by iodination performed before the modification.

LDL Oxidation

The cellular modification of LDL was achieved by preincubation of 100 μg/mL LDL protein with or without the drugs (1–50 μM) in HAM F10 medium without serum, at room temperature for 30 min, in the dark. Oxidation was carried out by applying the medium to U937 human monocyte-like cell monolayers in 35 mm petri dishes and incubating at 37° for 24 hr. Copper-induced auto-oxidation of LDL was achieved by preincubation of LDL (50–100 μg LDL protein) with or without the drugs for 30 min in the dark in EDTA-free NaCl buffer, pH 8.25. Oxidation was carried out by adding 5 μM $CuSO_4$ to the LDL sample and incubating it at 37° for 2 hr. The oxidation was terminated by the addition of EDTA (2 mM final concentration). Verapamil and diltiazem were introduced in aqueous solution. 1,4-Dihydropyridines, vitamin E, and probucol were introduced in ethanolic solution, and control experiments with ethanol for each concentration of drug were done. The concentrations of ethanol ranged to 5% at the highest concentration of antagonist employed. These concentrations caused from 2 to 10% inhibition of LDL oxidation.

Measurement of Lipid Oxidation

Lipid oxidation was measured by using the TBARS assay as described by Kleinveld *et al.* [20] with modification. Briefly, 0.5 mL of 100 g/L trichloroacetic acid and 1.0 mL of 6.7 g/L thiobarbituric acid were added to 0.5 mL of LDL-containing samples. The samples were heated in boiling water for 20 min, cooled on ice, and then centrifuged at 3000 g for 10 min. The absorbance of the supernatant was measured at 530 nm, and the amount of MDA equivalents formed was calculated using a molar extinction coefficient of 1.56×10^5 M^{-1} cm^{-1} [21]. The results are expressed as nanomoles of MDA equivalents produced per milligram of LDL protein and presented as percent of control of TBARS levels.

LDL Degradation by J774A.1 Macrophages

Prior to its oxidation, [^{125}I]LDL (10 μg/mL [^{125}I]LDL protein) was incubated with or without the drugs for 30 min in the dark in PBS. Oxidation was carried out by the addition of 10 μM $CuSO_4$ and incubation at 37° for 24 hr, and terminated by refrigeration and addition of 0.1 mM EDTA. Cellular degradation of the [^{125}I]-ox-LDL was determined after 5 hr of cell incubation at 37° with the labeled lipoproteins, with or without Ca^{2+} antagonists, by measuring the trichloroacetic acid-

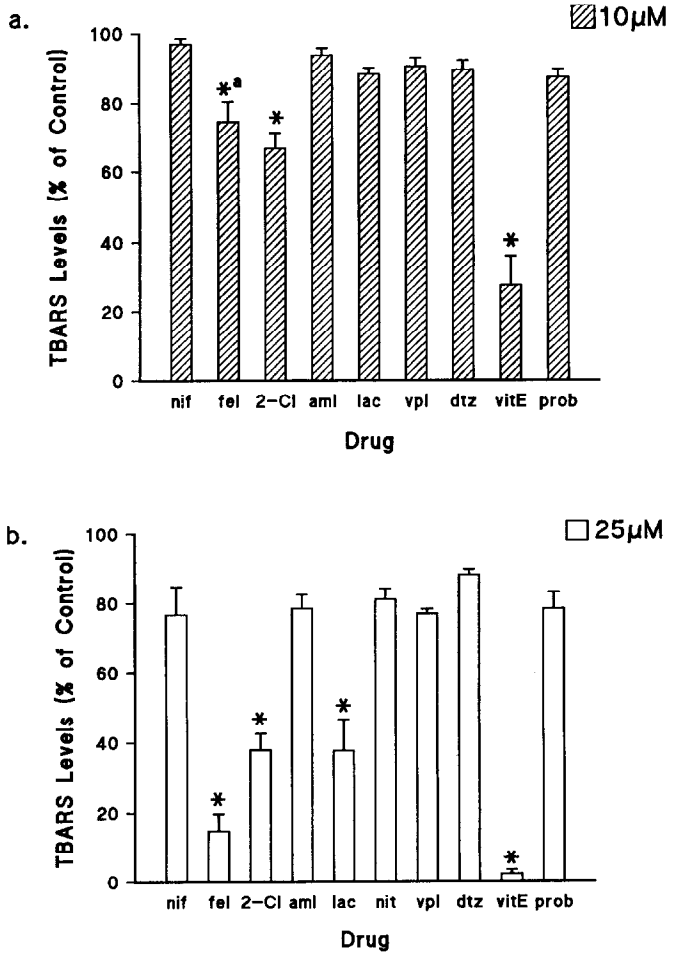


FIG. 3. Comparison at 10 and 25 μM concentrations of the effects of active Ca^{2+} antagonists on the TBARS levels of LDL submitted to copper-induced oxidation. (a) (*) $P < 0.05$ when compared with all other drugs; and (*[a]) $P < 0.05$ when compared with nif, aml, vpl, dtz, and vitE. (b) (*): $P < 0.05$ when compared with all the drugs, except when 2-Cl is compared to lac and fel to vitE. Abbreviations: nif, nifedipine; fel, felodipine; 2-Cl, the 2-chloro analog of nifedipine; aml, amlodipine; lac, lacidipine; nit, nitrendipine; vpl, verapamil; dtz, diltiazem; vitE, vitamin E; and prob, probucol. The data shown are means ± SEM of 4–6 experiments. Control levels: 76 ± 2 nmol of MDA equivalents produced/mg LDL protein.

soluble noniodide radioactivity in the medium [19]. LDL degradation was expressed as nanograms of LDL protein degraded per milligram of cellular protein. Blank values from parallel incubations without J774A.1 cells were also determined.

Agarose Gel Electrophoresis

The modification of the net negative charge of oxidized LDL was assessed by agarose gel electrophoresis as described by Reid and Mitchinson [22]. Briefly, samples of LDL were oxidized in the presence or absence of calcium antagonists as described under "*LDL oxidation.*" Samples of ox-LDL and native LDL were run on agarose gels for 3.5 hr in barbitone buffer (0.05 M sodium barbitone and 1 mM EDTA), pH 8.6, at a constant 90 V at room temperature. The gels were stained with amido black 10B and destained with 5% acetic acid.

Statistical Analysis

Results are expressed as the means ± SEM. The data were analyzed by Student's *t*-test and one-way ANOVA, using SigmaStat Statistical software (Jandel Scientific). Following ANOVA, the statistically significant differences between means were calculated by either the Student-Newman–Keuls test or Dunnett's test. Significance was accepted at $P < 0.05$.

RESULTS

TBARS formation during copper-induced oxidation of LDL was assayed in the presence of eight active Ca^{2+} antagonists from three different chemical groups, three compounds of reduced activity as Ca^{2+} antagonists (Fig. 1), and two known antioxidants, vitamin E and probucol (1–100 μM). The active Ca^{2+} antagonists tested reduced the TBARS level of LDL in a concentration-dependent manner (Fig. 2). Among the active drugs, the order of potency, ranked by apparent IC_{50} values, was vitamin E > felodipine > 2-chloro analog of nifedipine > nifedipine > amlodipine, nitrendipine, verapamil > diltiazem (Fig. 2).

Figure 3 shows a comparison of the effects of vitamin E, probucol, five 1,4-dihydropyridines, a phenylalkylamine (verapamil), and a benzothiazepine (diltiazem), on the TBARS content of LDL submitted to oxidation by 5 μM $CuSO_4$. At 10 and 25 μM, vitamin E, a known antioxidant, was significantly (Dunnett's test; $P < 0.05$) more effective than each drug

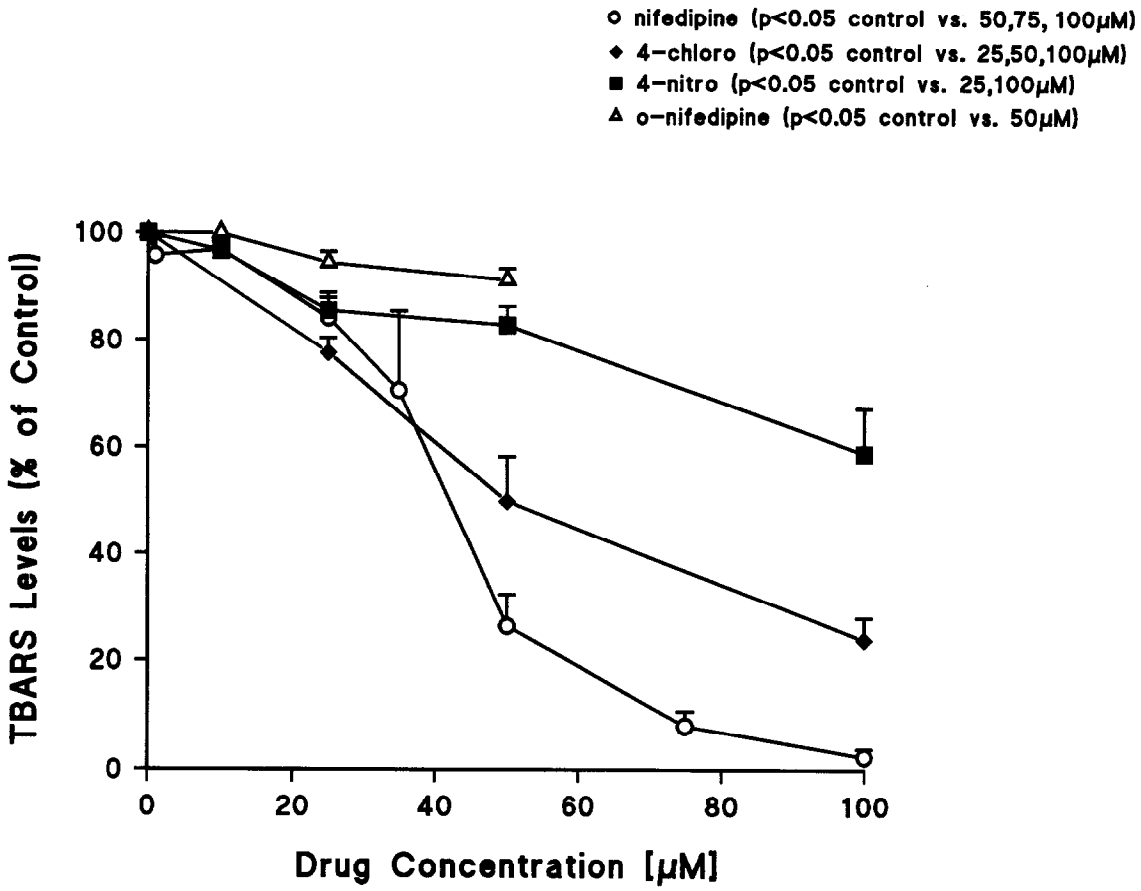


FIG. 4. Effects of compounds with reduced activity as Ca^{2+} antagonists on the TBARS levels of LDL submitted to copper-induced oxidation. LDL protein (50–100 μg/mL) was preincubated with or without the drugs (1–100 μM), followed by its oxidation as described under Materials and Methods. The data shown are mean ± SEM of 4–8 experiments, each performed in triplicate. Control levels: 85 ± 2 nmol of MDA equivalents produced/mg LDL protein.

tested, except for felodipine (25 μM). At 25 μM, the effects of felodipine and vitamin E were not significantly (Dunnett's test; $P < 0.05$) different, suggesting a similar activity of the two drugs at this concentration. Comparison of the antioxidant activity of the Ca^{2+} antagonists (Student–Newman–Keuls test) showed that at 10 μM, the antioxidant activities of the 2-chloro analog of nifedipine and vitamin E were significantly ($P < 0.05$) higher than those of the other tested drugs (the 2-chloro analog was not different from felodipine). However, felodipine induced a significant ($P < 0.05$) reduction in TBARS levels only when compared with nifedipine, amlodipine, verapamil, diltiazem and vitamin E (Fig. 3a). At 25 μM, felodipine, lacidipine, the 2-chloro analog of nifedipine, and vitamin E induced significant ($P < 0.05$) reductions in the TBARS levels of LDL compared with the other tested drugs (lacidipine was not different from the 2-chloro analog, and felodipine was not different from vitamin E) (Fig. 3b). The effect of probucol (10–25 μM) was similar to those of nifedipine, amlodipine, nitrendipine, verapamil and diltiazem, and significantly ($P < 0.05$) lower than that of vitamin E and the 2-chloro analog (10 and 25 μM), felodipine, and lacidipine (25 μM) (Fig. 3).

1,4-Dihydropyridines that are of reduced activity as Ca^{2+} antagonists also reduced the TBARS level of LDL submitted to copper-induced oxidation in a concentration-dependent manner. The order of potency was nifedipine > the 4-chloro analog of nifedipine > the 4–nitro analog of nifedipine > the oxidized metabolite of nifedipine (Fig. 4). However, all of these compounds showed weak antioxidant activity, being less effective than nifedipine. At 25 μM, the 4–chloro analog induced a significant ($P < 0.05$) reduction in the TBARS content of LDL only when compared with the oxidized metabolite of nifedipine (22 ± 2 vs 5 ± 2% reduction), but not when compared with nifedipine or its 4-nitro analog (16 ± 4 and 14 ± 3%, respectively) (Fig. 5). At 50 μM, both nifedipine and its 4-chloro analog induced significant ($P < 0.05$) 73 ± 6 and 50 + 8% reductions in the TBARS content of oxidized LDL, respectively, when compared with the 4-nitro analog or the oxidized metabolite of nifedipine (17 ± 3 and 9 ± 2% reduction, respectively) (Fig. 5). At 100 μM, both nifedipine and its 4-chloro analog induced significant ($P < 0.05$) 97 ± 1 and 76 ± 4% reductions in the TBARS levels of oxidized LDL, respectively, compared with the 4-nitro analog of nifedipine (41 ± 8% reduction). However, nifedipine at both 50 and 100 μM, was a significantly ($P < 0.05$) more effective antioxidant than its 4-chloro analog (Figs. 4 and 5).

TBARS formation during U937 human monocyte cell-induced oxidation of LDL was assayed in the presence of different concentrations of four active Ca^{2+} antagonists and one compound of reduced activity as Ca^{2+} antagonist from two different chemical groups. It appears that nifedipine and felodipine reduced the TBARS level of LDL in a concentration-dependent manner, nifedipine being more effective than felodipine (Fig. 6). However, the 4-nitro analog was not an effective inhibitor.

Figure 7 shows a comparison between the antioxidant effects of four selected 1,4-dihydropyridines and verapamil, on the TBARS level of LDL submitted to oxidative modification by U937 human monocyte cells. At 10 μM, the effect of amlodipine was significantly ($P < 0.05$) lower than that of the other drugs. At 25 μM, both nifedipine and felodipine induced significant ($P < 0.05$) reductions in the TBARS content of LDL (96 ± 2 and 65 ± 9%, respectively) compared with verapamil, amlodipine and the 4-nitro analog of nifedipine (29 ± 5, 7 ± 5, and 24 ± 5%, respectively). In this set of experiments, the most effective drug was nifedipine, followed by felodipine, while verapamil, amlodipine and the 4-nitro analog of nifedipine were not effective antioxidants.

Since the TBARS assay is not specific, we performed two additional assays to investigate the antioxidant activity of Ca^{2+} antagonists. We followed the same experimental design as in the TBARS assays: preincubation of LDL in the presence or absence of the drugs for 30 min in the dark at room temperature, followed by its oxidation (addition of $CuSO_4$), and incubation at 37° for 2 hr. Following the oxidative modification of LDL, we tested the effects of Ca^{2+} antagonists on the degradation of [^{125}I]LDL by the J774A.1 murine macrophage cell line, and their effects on the relative mobility of oxidized LDL in agarose gel.

Figure 8 shows a comparison of the effects of five selected drugs on the degradation of copper-modified [^{125}I]LDL by J774A.1 macrophage cell monolayers. At 25 μM, both felodipine and amlodipine induced significant ($P < 0.05$) reductions in the amount of oxidized [^{125}I]LDL degraded by J774A.1 macrophages (97 ± 2 and 63 ± 7% reduction, respectively), compared with nifedipine, verapamil and the 4-nitro analog of nifedipine. However, there was no significant difference be-

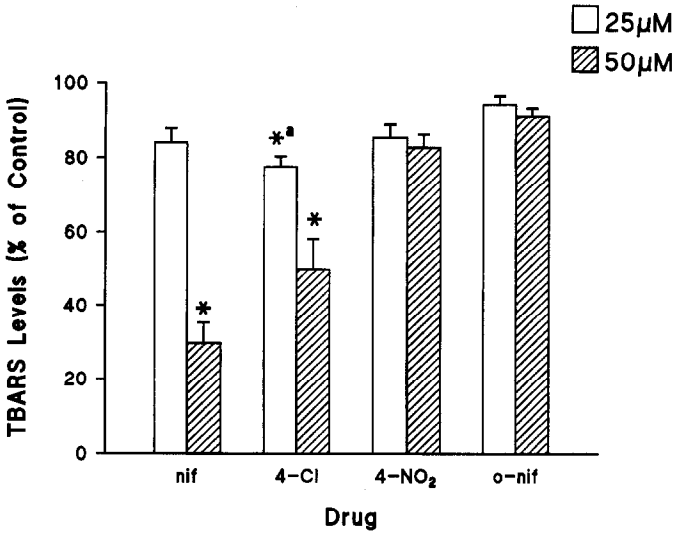


FIG. 5. Comparison of the effects of nifedipine and its analogs on the TBARS levels of LDL submitted to copper-induced oxidation. Key (*a) $P < 0.05$ when the 4-chloro analog (4-Cl) is compared with the oxidized metabolite of nifedipine (o-nif); and (*) $P < 0.05$ when nifedipine (nif) is compared with its three analogs, and when the 4-chloro analog is compared with the oxidized metabolite and the 4-nitro analog (4-NO_2) of nifedipine. The data shown are means ± SEM at 4–6 experiments. Control levels: 85 ± 2 nmol of MDA equivalents produced/mg LDL protein.

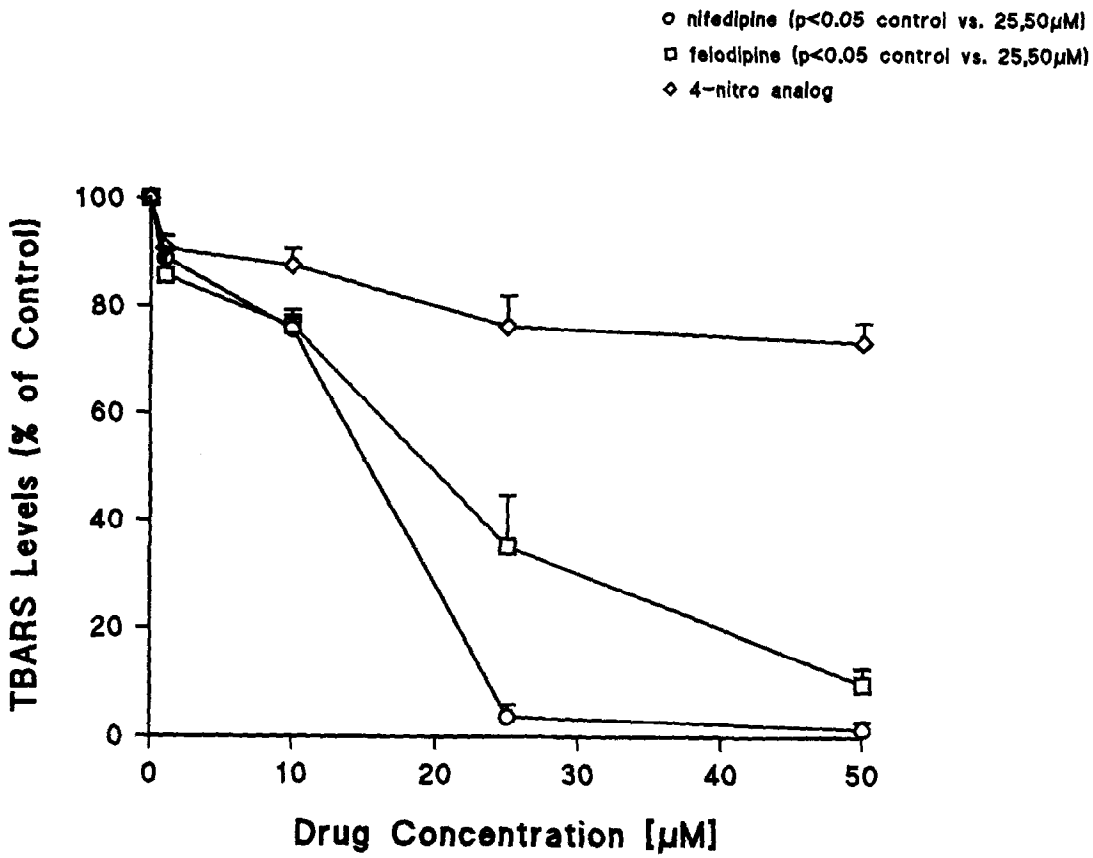


FIG. 6. Concentration–effect relationships for Ca^{2+} antagonists (active and of reduced activity) on the TBARS levels of human LDL submitted to U937 monocyte cell-induced oxidation. LDL protein (100 μg/mL) was preincubated with or without the drugs (1–50 μM) in Ham F10 medium without serum, for 30 min at room temperature in the dark. Oxidation was carried out by applying the LDL-containing medium to U937 human monocyte-like cell monolayers in 35 mm petri dishes and incubating at 37° for 24 hr. The data shown are means ± SEM of 4–8 experiments, each performed in triplicate. Control levels: 28 ± 0.9 nmol of MDA equivalents produced/mg LDL protein.

tween the effects of verapamil, nifedipine, and its 4-nitro analog on the degradation of oxidized [^{125}I]LDL by J774A.1 macrophages. In this set of experiments, felodipine was the most effective drug, followed by amlodipine. At 10 μM, all the tested drugs induced similar antioxidant effects.

The modification of the net negative charge of oxidized LDL, treated with Ca^{2+} antagonists, was assessed by agarose gel electrophoresis. Among the 1,4-dihydropyridines tested, amlodipine (25 and 50 μM) and felodipine (50 μM) significantly reduced ($P < 0.05$) the relative mobility of oxidized LDL compared with control (assay conditions but without Ca^{2+} antagonists), but not compared with native LDL (Fig. 9), suggesting an effect of these drugs on the net negative charge of LDL. The effects of nifedipine and its 4-nitro analog (25 and 50 μM), and felodipine (25 μM) on the relative mobility of oxidized LDL were similar to that of control (Fig. 9), and also significantly ($P < 0.05$) different from native LDL, suggesting no effect of these drugs on the net negative charge of LDL submitted to copper-induced oxidation.

DISCUSSION

The results of this study clearly demonstrate that calcium antagonists from three different chemical groups, including the 1,4-dihydropyridines, phenylalkylamines, and benzothiazepines, are able to prevent copper-induced and human monocyte-induced oxidation of LDL in a concentration-dependent manner. The order of potency of the drugs as antioxidants depends on the oxidation system and the assay used. In the copper-induced oxidation system, felodipine was the most effective antioxidant and in the monocyte-induced oxidation system nifedipine was more effective than felodipine. However, in both systems the 1,4-dihydropyridines were more effective antioxidants than verapamil or diltiazem. The mechanisms by which Ca^{2+} antagonists can protect LDL against copper- or cell-induced modification are yet to be established. The fact that the studied drugs are also able to reduce copper-induced LDL auto-oxidation suggests that a direct protection of the LDL particle itself is probably involved.

Since the mechanism of cell-induced oxidation of LDL is not known, one can only speculate why the order of potency of Ca^{2+} antagonists varies in the different oxidation systems. It was suggested by Breugnot *et al.* [23] that structural modification of the LDL particle could occur by the insertion of a hydrophobic drug, causing a change in the physical state of the lipid phase, leading to a decrease in the susceptibility of the LDL lipids to oxidation. Although the antioxidant effect of

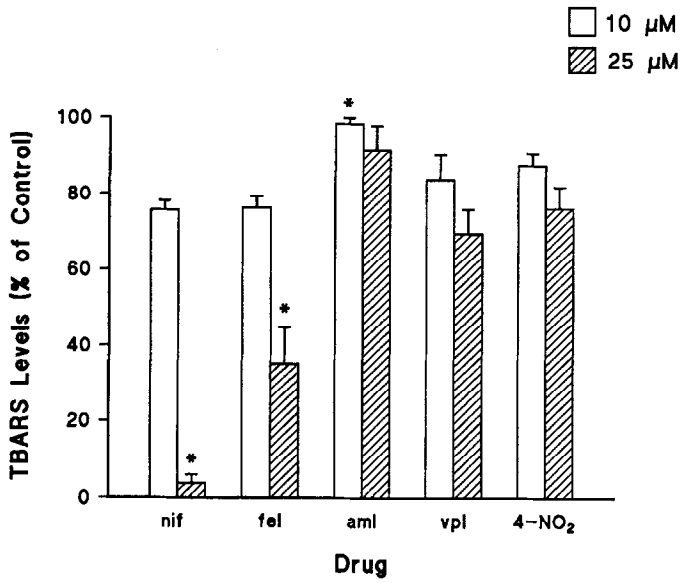


FIG. 7. Comparison at 10 and 25 μM concentrations of the effects of Ca^{2+} antagonists (active and of reduced activity) on the TBARS levels of LDL submitted to U937 monocyte-cell-induced oxidation. Key: (*) $P < 0.05$ when amlodipine (aml) is compared with the other drugs at 10 μM, and when nifedipine (nif) and felodipine (fel) are compared with the other drugs and with each other at 25 μM. Abbreviations: nif, nifedipine; fel, felodipine; aml, amlodipine; vpl, verapamil; and 4-NO_2, the 4-nitro analog of nifedipine. The data shown are means ± SEM of 4–6 experiments. Control levels: 28 ± 0.9 nmol of MDA equivalents produced/mg LDL protein.

certain Ca^{2+} antagonists can be enhanced by their capacity to concentrate in the lipid layer (felodipine being more lipophilic than nifedipine), another mechanism(s) must be involved in the protective effect of these drugs against the oxidation of LDL, thereby rendering nifedipine a more effective antioxidant overall. For example, if LDL-cell contact is essential for LDL oxidation, perhaps nifedipine, being more hydrophilic, somehow physically interferes with and disrupts this contact in a more efficient way than felodipine.

The concentration-dependency of the antioxidant activity of Ca^{2+} antagonists and vitamin E against copper-induced oxidation of LDL agrees with the results of Lupo *et al.* [24], who have shown that at 10 and 50 μM, the order of potency is vitamin E > lacidipine > nifedipine > verapamil, amlodipine, while diltiazem (1–50 μM) has no antioxidant effect.

The results with nifedipine are consistent with those of Negre-Salvayre and Salvayre [25] who compared the abilities of nifedipine, verapamil, and diltiazem (0–100 μM) to protect LDL against UV-promoted oxidation and copper-induced auto-oxidation, and showed that nifedipine has an inhibitory effect (EC_{50} values of 10 and 4 μM, respectively), while diltiazem and verapamil are poorly active or completely ineffective (estimated EC_{50} values of 200 and ≥400 μM, respectively). Breugnot *et al.* [23] demonstrated that nifedipine was more potent than verapamil (10–100 μM) as an inhibitor of human monocyte- and endothelial cell-induced modification of LDL. In agreement with our results and the data of Breugnot *et al.* [23] and Negre-Salvayre and Salvayre [25], Li *et al.* [26] have shown that nifedipine is more potent than verapamil (1–100 μM) in inhibiting LDL lipid oxidation by the activated U937 human monocyte cell line. In another experimental model, an ascorbic acid-induced brain membrane and liposome lipid oxidation system, the results of Goncalves *et al.* [27] with the antioxidant effect of nifedipine were also consistent with our data. However, in yet another experimental model, Janero *et al.* [28] and Janero and Burghardt [10] did not observe any protective action of nifedipine (≤500 μM) against oxidative injury of cardiac membrane phospholipids. These data agree with the literature, where comparison of the antioxidant activity of a drug in different oxidation systems yields a very wide range of effective concentrations (low micromolar through millimolar concentrations), depending on the oxidation system, the lipid preparation, lipid oxidation measurement, and drug concentration tested [reviewed in Ref. 29]. Since nifedipine is unstable under daylight conditions, this may have an influence on its antioxidant activity [30].

Our results with probucol are not consistent with those of Parthasarathy *et al.* [31], who have shown that probucol reduces LDL oxidation and macrophage uptake, and slows down the progression of atheroma in the Watanabe heritable hyperlipidemic rabbit [32]. These discrepancies could be due to

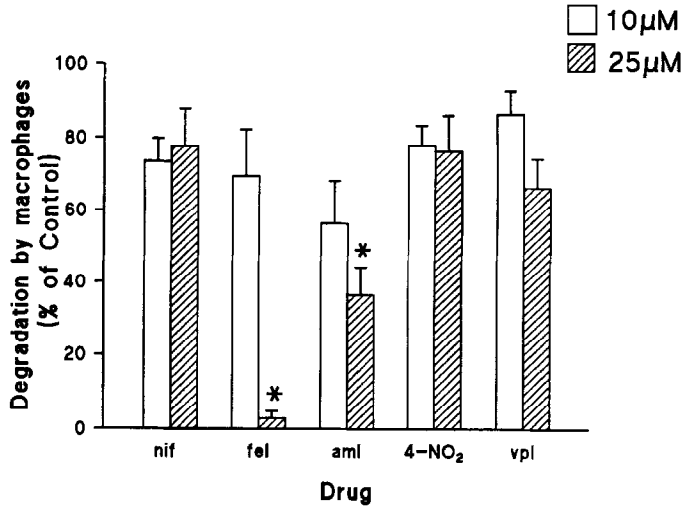


FIG. 8. Comparison of the effects of Ca^{2+} antagonists (10 and 25 μM) on the degradation of [^{125}I]LDL by J774 macrophages. [^{125}I]LDL (10 μg/mL) was preincubated with or without the drugs for 30 min in the dark in PBS. Oxidation was carried out by the addition of 10 μM $CuSO_4$ and incubation at 37° for 24 hr, and terminated by refrigeration and addition of 0.1 mM EDTA. Cellular degradation of the [^{125}I]LDL was determined after 5 hr of macrophage cell incubation at 37° with the drug-treated, oxidized [^{125}I]LDL, by measuring the trichloroacetic acid-soluble noniodide radioactivity in the medium [19]. The data shown are means ± SEM of 6 experiments, each performed in triplicate. Key: (*) $P < 0.05$ when felodipine (fel) and amlodipine (aml) are compared with the other drugs at 25 μM. Abbreviations: nif, nifedipine; fel, felodipine; aml, amlodipine; 4-NO_2, the 4-nitro analog of nifedipine; and vpl, verapamil. Control levels: 822 ± 146 ng LDL degraded/mg cellular protein.

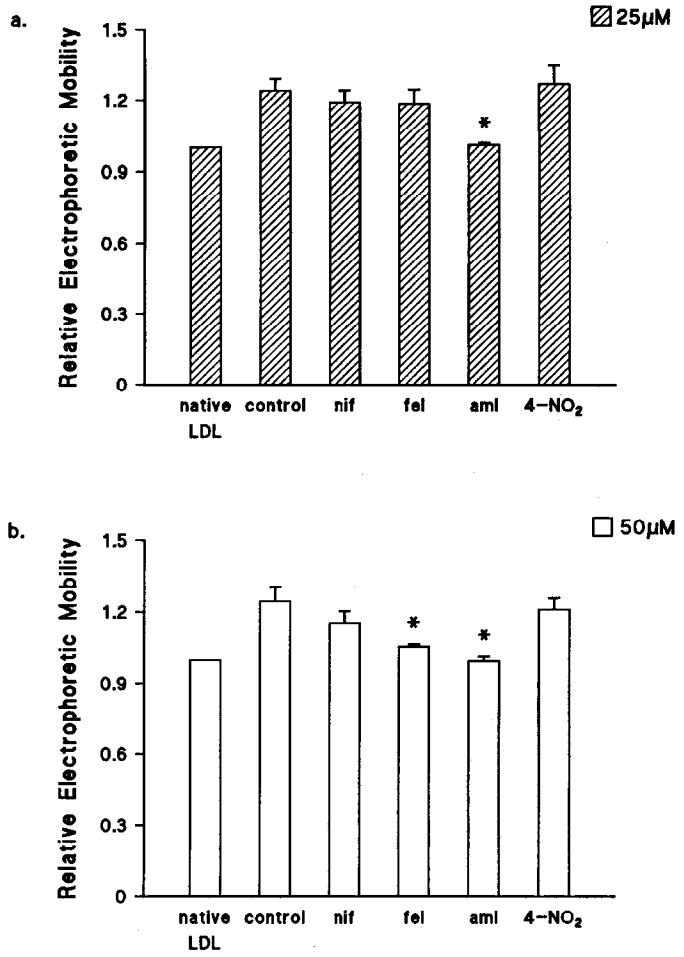


FIG. 9. Effects of 1,4-dihydropyridines on the relative electrophoretic mobility of oxidized LDL on agarose gel. LDL was oxidized in the absence or presence of four selected 1,4-dihydropyridines as described under Materials and Methods, and then was run on agarose gel (1% gel) for 3.5 hr in barbitone buffer, pH 8.6, at a constant 90 V at room temperature. Electrophoretic mobility of native LDL was 42.8 ± 2.17 mm (defined as 1.0). Key: (*) $P < 0.05$ vs control (assay conditions but without Ca^{2+} antagonists). The data shown are means ± SEM of 4–5 experiments. Abbreviations: nif, nifedipine; fel, felodipine; aml, amlodipine; and 4-NO_2, the 4-nitro analog of nifedipine.

some methodological differences in the oxidation conditions and the assays used to measure oxidation.

A comparison between the structural requirements for Ca^{2+} channel antagonism and antioxidant activity of the 1,4-dihydropyridines demonstrates some similarities. The requirement for 2-substitution (or *o*-substitution) on the phenyl ring is similar to that of Ca^{2+} channel antagonism, since substituents on the phenyl ring enhance both activities in the same sequence $o > m \gg p$ [33]. In addition, the requirement for the 1,4-dihydropyridine ring [33] is the same for both antioxidant activity and Ca^{2+} channel antagonism, as seen by our results with the oxidized metabolite of nifedipine. The latter had a very weak antioxidant activity against the oxidation of LDL as tested by the TBARS assay and is inactive on L-type Ca^{2+} channels at 10^{-4} M in the 1,4-dihydropyridine binding assay.

Structural comparison between amlodipine and felodipine and the 2-chloro analog of nifedipine suggests that a charged substituent at the 2-position of the 1,4-dihydropyridine ring may reduce the antioxidant activity. Amlodipine has a positively charged amine at that position, which might render it less lipophilic. Reduced lipophilicity could contribute to its decreased antioxidant activity. However, the presence of the positively charged amino group at the 2-position of the 1,4-dihydropyridine ring is not critical to Ca^{2+} channel antagonism activity. Replacement of a methyl by an ethyl group on the ester groups at C-3 and C-5 positions on the 1,4-dihydropyridine ring does not seem to increase the antioxidant activity of the tested drugs in our model system.

However, despite the relative differences in Ca^{2+} channel and antioxidant activities of the 1,4-dihydropyridines, it is important to note that this would not lend to selective antioxidant activity of the compounds listed. At the concentrations necessary to demonstrate antioxidant activity, all of the 1,4-dihydropyridines would have shown significant Ca^{2+} channel antagonism.

In summary, Ca^{2+} antagonists from three different chemical groups have been shown to increase LDL resistance to copper- and monocyte-induced oxidation. However, the 1,4-dihydropyridines that are active as Ca^{2+} channel antagonists were shown to be more effective than verapamil (a phenylalkylamine) or diltiazem (a benzothiazepine). Among the 1,4-dihydropyridines, felodipine was found to be the most effective antioxidant in the copper-induced oxidation system, while nifedipine seemed to be the most effective in the cell-induced oxidation system, followed by felodipine. However, in the latter system, felodipine still showed a significant effect when compared to with amlodipine, verapamil, and the 4-nitro analog of nifedipine. These results are in agreement with the order of potency of the agents tested as Ca^{2+} channel antagonists. Analysis of the structure–function relationships for the 1,4-dihydropyridine effect on the oxidative modification of LDL suggests an important role for the 2-substitution of the phenyl ring, and an essential role for the dihydropyridine ring. These structural requirements are also critical for Ca^{2+} channel antagonism. All these properties suggest that LDL oxidation is one potential target at which Ca^{2+} antagonists may regulate the development of atherosclerosis.

This work was supported by grants from the National Institute of Health (HL 16003) and Miles Inc. (West Haven, CT). Nurit Rojstaczer held a predoctoral fellowship from NIH (GM 07145).

References

1. Kloner RA and Przyklenk K, Progress in cardioprotection: The role of calcium antagonists. *Am J Cardiol* **66:** 2H–9H, 1990.
2. Triggle DJ, Calcium antagonists. In: *Cardiovascular Pharmacology* (Ed. Antonaccio M), pp. 107–160. Raven Press, New York, 1990.
3. Triggle DJ, Calcium antagonists and atherosclerosis. *Drugs Today* **27:** 147–155, 1991.
4. Henry PD, Antiperoxidative actions of calcium antagonists and atherogenesis. *J Cardiovasc Pharmacol* **18** (Suppl 1): S6–S10, 1991.
5. Nomomoto A, Mutoh S, Hagihara H and Yamaguchi I, Smooth muscle cell migration induced by inflammatory cell products and

its inhibition by a potent calcium antagonist, nivaldipine. *Atherosclerosis* **72:** 213–219, 1988.
6. Daugherty A, Rateri DL, Schonfeld G and Sobel BE, Inhibition of cholesteryl ester deposition in macrophages by calcium entry blockers: An effect dissociable from calcium entry blockade. *Br J Pharmacol* **91:** 113–118, 1987.
7. Belch JJF, Chopra M, Hutchinson S, Lorimer R, Sturrock RD, Forbes CD and Smith WE, Free radical pathology in chronic arterial disease. *Free Radic Biol Med* **6:** 375–378, 1989.
8. Braughler JM and Hall ED, Central nervous system trauma and stroke I. Biochemical consideration for oxygen radical formation and lipid peroxidation. *Free Radic Biol Med* **6:** 289–301, 1989.
9. Halliwell B, Oxidants and human disease: Some new concepts. *FASEB J* **1:** 358–364, 1987.
10. Janero DR and Burghardt B, Antiperoxidant effects of dihydropyridine calcium antagonists. *Biochem Pharmacol* **38:** 4344–4348, 1989.
11. Rubanyi GM, Vascular effects of oxygen derived free radicals. *Free Radic Biol Med* **4:** 107–120, 1988.
12. Ross R, The pathogenesis of atherosclerosis: A perspective for the 1990s. *Nature* **362:** 801–809, 1993.
13. Sowers JR, Zemel PC, Zemel MB, Kasim SE and Khoury S, Hypertension and atherosclerosis: Calcium antagonists as antiatherogenic agents. *J Vasc Med Biol* **2:** 1–6, 1990.
14. 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.
15. Witztum JL, Role of oxidised low density lipoprotein in atherogenesis. *Br Heart J* **69** (Suppl): S12–S18, 1993.
16. Schwartz CJ, Valente AJ and Sprague EA, A modern view of atherogenesis. *Am J Cardiol* **71:** 9B–14B, 1993.
17. Steinberg D, Antioxidant and atherosclerosis. *Circulation* **84:** 1420–1425, 1991.
18. Chung BH, Wilkinson T, Geer JC and Segrest' JP, Preparative and quantitative isolation of plasma lipoproteins: Rapid, single discontinuous density gradient ultracentrifugation in a vertical rotor. *J Lipid Res* **21:** 284–291, 1980.
19. Goldstein JL, Basu SK and Brown MS, Receptor-mediated endocytosis of low-density lipoprotein in cultured cells. *Methods Enzymol* **98:** 241–260, 1983.
20. Kleinveld HA, Hak-Lemmers HLM, Stalenhoef AFH and Demacker PNM, Improved measurement of low-density lipoprotein susceptibility to copper-induced oxidation: Application of a short procedure for isolating low-density lipoprotein. *Clin Chem* **38:** 2066–2072, 1992.
21. Wills ED, Lipid peroxide formation in microsomes. General considerations. *Biochem J* **113:** 315–319, 1969.
22. Reid VC and Mitchinson MJ, Toxicity of oxidized low density lipoprotein towards mouse peritoneal macrophages *in vitro*. *Atherosclerosis* **98:** 17–24, 1993.
23. Breugnot C, Maziere C, Auclair M, Mora L, Ronveaux MF, Salmon S, Santus R, Morliere P, Lenaers A and Maziere JC, Calcium antagonists prevent monocyte and endothelial cell-induced modification of low density lipoproteins. *Free Radic Res Commun* **15:** 91–100, 1991.
24. Lupo E, Locher R, Weisser B and Vetter W, *In vitro* antioxidant activity of calcium antagonists against LDL oxidation compared with α-tocopherol. *Biochem Biophys Res Commun* **203:** 1803–1808, 1994.
25. Negre-Salvayre A and Salvayre R, UV-treated lipoproteins as a model system for the study of the biological effects of lipid peroxides on cell culture. 4. Calcium is involved in the cytotoxicity of UV-treated LDL on lymphoid cell lines. *Biochim Biophys Acta* **1003:** 238–245, 1992.
26. Li Q, Tallant A and Cathcart MK, Dual calcium requirement for optimal lipid peroxidation of low density lipoprotein by activated human monocytes. *J Clin Invest* **91:** 1499–1506, 1993.
27. Goncalves T, Carvalho AP and Oliveira CR, Antioxidant effect of calcium antagonists on microsomal membranes isolated from different brain areas. *Eur J Pharmacol* **204:** 315–322, 1991.
28. Janero DR, Burghardt B and Lopez R, Protection of cardiac membrane phospholipid against oxidative injury by calcium antagonists. *Biochem Pharmacol* **37:** 4197–4203, 1988.
29. Rojstaczer N and Triggle JT, Ca^{2+} antagonists as antioxidants. *Cardiovasc Drug Reviews* **12:** 70–84, 1994.
30. Misik V, Stasko A, Gergel D and Ondrias K, Spin-trapping and antioxidant properties of illuminated and nonilluminated nifedipine and nimodipine in heart homogenate and model system. *Mol Pharmacol* **40:** 435–439, 1991.
31. Parthasarathy S, Young SG, Witztum JL, Pittman RC and Steinberg D, Probucol inhibits oxidative modification of low density lipoprotein. *J Clin Invest* **77:** 641–644, 1986.
32. Kita T, Nagano Y, Yokode M, Ishii K, Kume N, Ooshima A, Yoshida H and Kawai C, Probucol prevents the progression of atherosclerosis in Watanabe heritable hyperlipidemic rabbit, an animal model for familiar hypercholesterolemia. *Proc Natl Acad Sci USA* **84:** 5928–5931, 1987.
33. Triggle DJ, Langs DA and Janis RA, Ca^{2+} channel ligands: Structure–function relationships of the 1,4-dihydropyridines. *Med Res Rev* **9:** 123–180, 1989.