Inhibition of LDL Oxidation and Oxidized LDL-induced Cytotoxicity by Dihydropyridine Calcium Antagonists

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Purpose. The antioxidant activity of dihydropyridine calcium channel antagonists was evaluated based on LDL oxidation kinetics, oxidative cell injury associated with reactive species generation, and increases in free intracellular calcium (Ca^{2+}) levels. Interactions with ascorbic acid were studied under conditions representative of LDL oxidation in plasma and tissue.

Methods. Analysis of antioxidant activity utilized measurements of one-electron oxidation potentials and scavenging of peroxy radical-mediated oxidation. LDL antioxidant potency was determined spectrophotometrically using copper-mediated oxidation kinetics in the absence and presence of 100 μM ascorbic acid. Prevention of oxidant-induced endothelial cell injury was determined from the formation of reactive oxygen species generation and increases in intracellular free calcium concentrations following addition of oxidized LDL or linoleic acid hydroperoxide.

Results. Felodipine and amlodipine effectively inhibit peroxyl radical-mediated oxidation in lipoproteins and cells that is markedly enhanced in the presence of ascorbic acid. In the presence of ascorbic acid, inhibition of LDL oxidation is over four times greater than in LDL treated without antioxidants, and oxidized LDL and linoleic acid hydroperoxide-induced reactive oxygen species formation is effectively suppressed in cells. Inhibition of intracellular calcium increases was achieved using nM concentrations of felodipine or amlodipine.

Conclusions. The additive effect for ascorbic acid and the calcium channel antagonist is postulated to involve a combination of peroxide-degrading and peroxyl radical scavenging reactions, demonstrating the importance of lipid peroxides during LDL oxidation and oxidized LDL-induced cytotoxicity. Cytoprotection is associated with inhibition of oxidant-induced increases in intracellular free calcium. Both the cytoprotective and LDL antioxidant activity for these compounds is manifested at concentrations approaching the therapeutic levels found in plasma.

KEY WORDS: lipid peroxides; ascorbic acid; vitamin E; felodipine; amlodipine; atherosclerosis.

INTRODUCTION

Calcium channel antagonists have been shown to reduce the progression of coronary atherosclerosis in humans. The inhibition of lipoprotein oxidation is considered as an important aspect of their mechanism of action and whereby the formation of potentially atherogenic modified lipoproteins is prevented. The antioxidant potencies of calcium channel blockers of the T and L-type have been previously studied, particularly the ability to inhibit LDL oxidation in various ways (1–4). Important criteria for antioxidant activity include the 2-substitution of the phenyl ring, an essential role for the dihydropyridine ring (5) and lipophilicity (6). The relative potency of these compounds was previously investigated based on substitutions on the phenyl- as well as the dihydropyridine- ring having important effects on antioxidant activity (7).

The antioxidant potency of the dihydropyridine calcium antagonists (DHP) is generally weak compared to other biological antioxidants, such as the radical trapping activity of vitamin E. Based on inhibitory effects on membrane lipid peroxidation or by analysis of competitive kinetics for reactions with reactive oxygen species, the DHP are approximately two to three orders of magnitude less reactive than vitamin E and are effective antioxidants only at micromolar concentrations (2,7,8). These concentrations are far above the therapeutic levels and suggest that antioxidant activity is unlikely when employed in the nanomolar concentration range. However, a consideration of the antioxidant mechanism of action for the DHP should take into account their ability to participate in redox reactions that are initiated by peroxyl radicals (7). Electrochemical studies show that upon formation of dihydropyridine radicals, these species behave thermodynamically only as reductants to form a pyridinium ion (9). Consequently, the potential to induce radical mediated oxidation reactions, such as for vitamin E radical (10), are unlikely. Rather, once oxidation of a dihydropyridine takes place, the strong 1-electron reducing potential of the product intermediate should facilitate reduction of other oxidants—thus behaving as an antioxidant. Based on these properties, it is conceivable that the DHP can interact with other redox active compounds thereby potentiating the antioxidant activity of the biological matrix in which they exist.

In this report we describe the antioxidant activity of specific DHP in terms of their ability to inhibit LDL oxidation. In the presence of ascorbic acid the efficacy of the DHP is markedly enhanced with inhibition of LDL oxidation evident at sub-micromolar concentrations. The ability of these agents to protect endothelial cells from oxidized LDL and lipid peroxide-induced increases in intracellular free Ca^{2+} concentrations, reactive oxygen species formation (ROS) and cytotoxicity is also demonstrated.

METHODS

Chemicals and Reagents

Felodipine was provided by Astra/Merck (Wayne, PA), Amlodipine from Pfizer (New York, NY), and Nisoldipine from Bayer (West Haven, CT). Verapamil was purchased from Sigma Chemical Company (St. Louis, MO) as were L-ascorbic acid (Vitamin C), 2,4,6-tripyridyl-s-triazine, ferric chloride, sodium acetate, NaBr, TRIS buffer, trichloroacetic acid, metaphosphoric acid, triethanolamine, 2-vinylpyridine, N, N-dimethyl formamide, $MnCl_2$, $CaCl_2$, EDTA, EGTA, and t-butyl hydroperoxide. Linoleic hydroperoxide (LOOH) was prepared from linoleic acid (Nu-Chek Prep, Elysian, MN) as described previously (11), then dissolved in absolute ethanol (USI Chemicals, Co., Tuscola, IL) and kept at −20°C under argon. LOOH purity was

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checked by HPLC. Calcium Green and 2,7-dichlorohydrofluorescin diacetate were obtained from Molecular Probes, Inc. (Eugene, OR). Vitamin E (*dl*-α-tocopherol) was a gift from Henkel Corp. (Elysian, MN). Trolox was purchased from Calbiochem Corp. (La Jolla, CA). All organic solvents and copper sulfate were HPLC grade and purchased from J.T. Baker Chemical Co. (Phillipsburg, NJ). Triton X-100 was purchased from BioRad (Hercules, CA). Benzoyl leucomethylene blue was from TCI (Japan).

Lipoprotein Separation and Analysis

Citrated venous blood was obtained from fasting adult human volunteers and plasma was immediately separated by centrifugation at 1500 g for 10 minutes at 4°C. LDL (δ = 1.019–1.063 g/mL) was isolated from plasma by preparative ultracentrifugation using a Beckman L8-55 ultracentrifuge and a SW-41 rotor. The technique used for separating LDL is previously described (12). The isolated LDL was dialyzed against argon-sparged 0.01 M Tris buffer, pH 7.2, containing 10 μM EDTA, sterilized by filtration (0.2 μm Millipore membrane) and stored at 4°C under nitrogen.

In Vitro Oxidation of LDL

LDL oxidation kinetics were analyzed after adding 10 μM $CuSO_4$ to 200 μg LDL protein/mL in PBS. Oxidized LDL (oxLDL) was prepared by incubating freshly isolated LDL with 10 μM $CuSO_4$ for 24 hr followed by ultrafiltration and resuspension in PBS. This oxLDL was used for cell culture experiments described below. Formation of conjugated dienes was monitored continuously at 234 nm for up to eight hours using a Beckman DU-650 spectrophotometer. Oxidation kinetics were analyzed on the basis of the oxidation lag phase and the rate of oxidation during the lag phase and propagation phase (13). Figure 1, lower insert, shows a typical kinetic profile and the method for measuring the various phases of LDL oxidation. The rate of oxidation during the lag phase and its duration represent the initial and antioxidant protected phase of oxidation before the onset of the rapid propagation phase (or *log rate*). Emphasis was placed in this study on the initial kinetics of oxidation, involving the rate and length of the lag phase. The kinetics of LDL oxidation were analyzed in the presence or absence of calcium channel antagonists (added in either buffer or ethanol), and/or ascorbic acid. The amount of ethanol vehicle was the same for all experiments (0.2% v/v).

Lipid Peroxide Measurements

Lipid peroxide levels were measured by a modified method of Auerbach (14). Briefly, samples were added to a solution containing leucomethylene blue in the presence of hemoglobin. The reaction between hemoglobin and peroxides converts leucomethylene blue to methylene blue, generating a color that is read at 650 nm using a 96 well plate reader. The amounts of peroxides were determined by standard calibration using t-butyl hydroperoxide.

Analysis of Antioxidant Activity by Competition Kinetics

The method of Tubaro *et al.* (15) was used to compare the antioxidant activity of the study compounds. Carbon-centered radicals generated by the thermal decomposition of 2,2′-azobis (2-amidinopropane) dihydrochloride (ABAP) were utilized to measure the rate of crocin bleaching (monitored at 443 nm) in the absence and presence of the study compounds which served as competitive scavengers. The crocin bleaching rate was measured in the presence of different concentrations of the test compounds and plotted to fit the competition kinetics equation:

$$Vb/Va = 1 + Ka/Kc \times [A]/[C]$$

Where Vb is the bleaching rate of crocin in the absence of antioxidant, Va is the bleaching rate in the presence of antioxidant, Ka is the rate constant for interaction of the antioxidant with free radicals, Kc is the rate constant for the reaction between crocin and free radicals, A is the concentration of antioxidant and C is the crocin concentration.

Vitamin E Measurements

LDL fractions were collected during the kinetic assay and extracted for vitamin E (α-tocopherol) analysis. Cultures from 35 mm dishes were harvested by trypsinization and the cell suspension extracted according to a modified method of Bui (17). Cell suspensions or LDL samples (500 μL) were mixed with 50 μL of internal standard (α-tocopherol acetate) and extracted twice with 500 μL of hexane containing BHT. The pooled phases were evaporated under nitrogen, the residue dissolved in 200 μL of ethanol, and injected into a Perkin Elmer Series 4 HPLC equipped with a BioRad reverse-phase column Bio-Sil ODS-5S, 250 × 4 mm (BioRad Instruments, CA). Samples were eluted with acetronitrile-tetrahydrofuran-H_2O at 0.9 mL/min, the eluent monitored using a UV/Vis monitor model 1706 (BioRad instruments, CA), and the peak areas integrated with Axxi-chrom 747 analytical chromatography software.

Cell Culture

Rabbit aortic endothelial cells (REC) were obtained from New Zealand albino rabbits and used between passages 9 and 13. For fluorescence analysis of intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$), cells were grown to confluence on polymethacrylate fluorometer cuvettes (Sigma, St Louis, MO) and maintained in 80/20 DMEM/M199 with 15% fetal bovine serum (Gibco, NY), endothelial cell growth factor (5 μg/mL) and gentamicin (50 μg/mL)—this is otherwise referred to as complete medium. Cells were grown in 35 mm multiwell culture dishes that were routinely used to assess plating efficiency, cell growth and survival after treatment with various agents. The cells were characterized as endothelial on the basis of morphology and by measurement of angiotensin converting enzyme activity using commercially available radioassay kits. Stock cells were passaged using a one to three split ratio with mechanical disruption of the monolayer and cultures maintained with weekly media changes.

In some experiments cells were pretreated with 10 μM vitamin E for 24 hr as described previously (18). Vitamin E supplemented REC were then used for measurements of $[Ca^{2+}]_i$ and formation of reactive oxygen species as described below, and for determination of vitamin E content as described above.

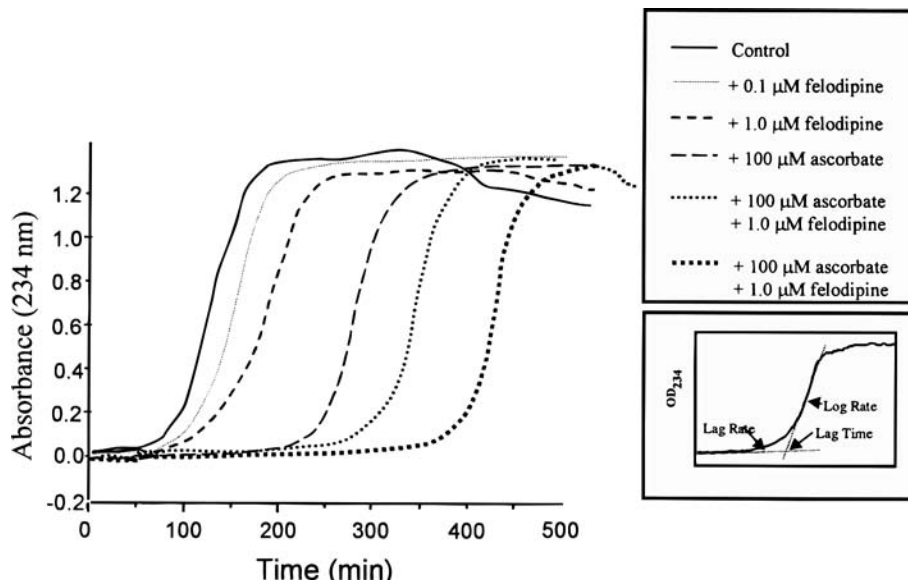

Fig. 1. Representative oxidation kinetic profiles are shown for LDL. LDL oxidation was initiated by adding 10 μM $CuSO_4$ to 200 μg LDL protein/mL in PBS and monitored over an 8 hr interval at 234 nm. The upper figure legend insert indicates the treatment condition for each kinetic profile shown. The lower figure insert shows the three parameters used to assess the oxidative susceptibility of LDL. The components measured are the lag time (or lag phase), the lag rate, and the log rate (or rate of the propagation phase of oxidation). The intercept for the slopes of the lag and log rates is used to estimate the lag time.

Measurement of Intracellular Free Calcium Concentrations

For determinations of $[Ca^{2+}]_i$ cell suspensions were centrifuged and resuspended in 10 mL fresh complete medium ($\sim 3 \times 10^6$ cells/10 mL). Suspensions were distributed as 1.0 mL aliquots into sterilized polymethacrylate fluorescence cuvettes which were placed into 100 mm Petri dishes (at a 15 to 30 degree inclination) and maintained until confluent. Confluent monolayers were obtained by five days on the inner surface of the cuvette. REC were incubated with 5 μM Calcium Green (added in DMSO) for 60 min at 21°C. After washing with fresh media, cells were treated with the indicated concentrations of LOOH or LDL that were suspended in ethanol or PBS, respectively, and added to cells maintained in HEPES assay buffer (10 mM) containing 0.9% NaCl and 1.0 mM Ca^{2+}. The cuvettes were kept in the dark during the treatment periods to prevent fluorescence artifacts. The fluorescence signal was monitored at set intervals using a Hitachi F-2000 fluorometer at 480 nm excitation and an emission signal at 500 nm. Determination of maximum fluorescence was accomplished by saturating the dye with $MnCl_2$ (2 mM) after addition of the ionophore, ionomycin. Further details on measurements of $[Ca^{2+}]_i$ are provided elsewhere (19,20) and in the figure legends. All agents were dissolved in either ethanol, DMSO or PBS, and added to the culture/treatment medium in 5–10 μl aliquots 2 hr prior to LOOH or LDL.

Analysis of Cytotoxicity

Cells were seeded into 24 well dishes one day prior to measurements of cytotoxicity as described previously (21). Cytotoxicity produced by DHP, LDL or LOOH was determined using nearly confluent cultures grown in the presence of 2% serum. Cell numbers were measured after replating and 24 hr incubation using a Coulter counter (Model ZB). Treatments typically used 2×10^4 cells per well. The parameters used to determine cytotoxicity included: plating efficiency (PE) and growth curves based on the surviving fraction (SF).

Determination of 2,7-Dichlorohydrofluorescin Oxidation in Cells

The extent of ROS, representing induction of peroxidation after addition of LOOH, was determined from the oxidation of 2,7-dichlorohydrofluorescein to the fluorescent product 2,7-dichlorofluorescein (22). 2,7-dichlorohydrofluorescin diacetate (DCF-DA, 2 μM in ethanol vehicle) was added to 5×10^5 cells grown on sterile plastic fluorescence cuvettes for 1 hr. During this time the probe becomes hydrolyzed and entrapped in cells. The cells were washed and treated with LOOH (30 min) and fluorescence of the product, 2′-7′-dichlorofluorescein (DCF), monitored at 506 ex/526 em in a thermostatically controlled fluorometer (Hitachi F200IC) at 37 °C. The extent of oxidation was measured in absolute fluorescence units (AFU), normalized to cell number and compared to the total oxidizable DCF (AFU_{max}) by harvesting cells, lysing with detergent and measuring total fluorescence after incubating lysate with 20 mM H_2O_2 plus 250 ng/mL horseradish peroxidase for 15 min. Each treatment condition was then expressed as percent maximal fluorescence at specific intervals.

Statistics

All results are expressed as mean and standard errors determined from at least 3 independent experiments with all

measurements performed in duplicate unless otherwise stated. Determinations of statistical significance between various treatment groups were made using the paired two-tailed student t-test or by analysis of variance (ANOVA).

RESULTS AND DISCUSSION

The antioxidant activity of some typical DHP was evaluated on the basis of reaction rates with peroxyl ($ROO^{\cdot}$) radicals as described previously (15). As determined from the ratios between the rate constants for reaction between $ROO^{\cdot}$ and the oxidizable target chromophore crocin (Vb/Va), reaction rates for felodipine, amlodipine and lacidipine with $ROO^{\cdot}$ were in the range of 3×10^{-3} to 4×10^{-4} (being approximately 500-fold lower than the reaction with trolox $-$Vb/Va = 1.8). However, reaction rates for DHP varied widely, where verapamil and nisoldipine were essentially inactive (reaction rates being $<2 \times 10^{-4}$). Although the relative order of reactivity and potency differs somewhat from previously reported findings (2,8), a moderate to weak antioxidant activity was confirmed, as compared to vitamin E (represented by trolox).

Figure 1 shows typical oxidation kinetic profiles for LDL after addition of 10 μM $CuSO_4$ and compares the rates of conjugated diene formation in the presence of different felodipine concentrations, and in the absence and presence of ascorbic acid. Similar determinations were made for LDL oxidation in the presence of amlodipine and verapamil, representing calcium channel antagonists with similar or no antioxidant activities.

Table 1 presents the effects of the calcium channel antagonists on copper-mediated LDL oxidation with or without 100 μM ascorbic acid. Felodipine alone effectively inhibited LDL oxidation at concentrations greater than 10 μM. A 30% reduction in LDL oxidation rates and doubling of the lag phase was achieved at concentrations between 10 and 25 μM. However, when combined with 100 μM ascorbic acid, felodipine was a much more effective antioxidant. Using 100 nM felodipine plus 100 μM ascorbic acid, the LDL oxidation lag time was more than four times longer than that measured for LDL alone. Although 100 μM ascorbic acid alone increased the oxidation lag phase by approximately three-fold, the combination of ascorbic acid and felodipine produced an additive antioxidant effect. The rate of peroxide accumulation was markedly reduced in the presence of the DHP (indicated by the lag rates), was inversely related to the concentration of the added DHP, and this inhibition was most augmented in the presence of ascorbic acid. None of the compounds tested affected the propagation rate for LDL oxidation regardless of the methods of oxidation employed. These findings show that the ability of DHP to react with and inhibit LDL oxidation is manifested primarily during the slow or antioxidant protected phase for lipid peroxidation wherein initiation of chain reactions and accumulation of peroxides takes place. Under these conditions, ascorbic acid dramatically suppresses the accumulation of peroxides and prolongs the lag phase via a metal-dependent degradation of peroxides (23).

Table I. Effect of Calcium Channel Antagonists on LDL Susceptibility to Copper-Mediated Oxidation

	Lag time (min)		Lag rate (OD × 10^{-3}/min)	
Addition	− Ascorbate	+ Ascorbate	− Ascorbate	+ Ascorbate
None	58 ± 16	190 ± 26	1.88 ± 0.19	1.20 ± 0.05
Fel. (0.1)*	76 ± 22	265 ± 32	1.61 ± 0.20	1.22 ± 0.09
Fel. (1.0)*	85 ± 20	342 ± 41	1.50 ± 0.11	1.04 ± 0.08
Fel. (10)*	100 ± 22	ND#	1.41 ± 0.15	ND
Fel. (25)*	134 ± 30	ND#	1.29 ± 0.09	ND
Aml. (0.1)*	66 ± 17	280 ± 44	1.80 ± 0.24	1.33 ± 0.06
Aml. (1.0)*	97 ± 32	315 ± 35	1.46± 0.07	1.39 ± 0.11
Verap. (10)*	71 ± 18	280 ± 28	1.81 ± 0.16	1.17 ± 0.05

Note: LDL oxidative susceptibility was determined by kinetic analysis of the progression of conjugated diene formation, reflecting the peroxidation of LDL lipids as described under Methods. The lag time preceding the propagation phase for lipid peroxidation is shown in minutes and is assumed to represent the resistance of LDL to oxidation.
* The concentration of the calcium channel blocker is shown in μM. Fel. Felodipine Aml. Amlodipine Verap. Verapamil.
# Not determined.

LDL vitamin E content (α-tocopherol) after addition of $CuSO_4$ was completely depleted by the end of the lag phase. Copper-mediated LDL oxidation in the presence of ascorbic acid (100 μM) produced only a 20–30% loss of vitamin E, and only when vitamin E was fully depleted did the lag phase end and propagation of lipid peroxidation begin. By contrast, none of the DHP prevented the loss of vitamin E which was consumed at a rate proportional to the duration of the lag phase. Apparently, both ascorbic acid and felodipine or amlodipine inhibit LDL oxidation, at least in part, by reacting with peroxyl radicals. Whereas the DHP react slowly with peroxyl radicals and can inhibit the accumulation of lipid hydroperoxides during the slow (lag) phase of peroxidation that eventually depletes LDL antioxidants, ascorbic acid reacts with and degrades pre-existing peroxides to non-radical products (23) thereby sparing LDL antioxidants as well as the DHP. The combined effects of ascorbic acid and DHP are, therefore, manifested through reductive degradation of LDL peroxides to non-radical products by ascorbic acid, and the quenching of nascent peroxyl radical reactions by the DHP. The latter continues as long as the rate of peroxide formation remains low.

The effect of LOOH on $[Ca^{2+}]_i$ is shown in Fig. 2. LOOH produced a rapid "spike" in the levels of $[Ca^{2+}]_i$ that returned to baseline within 30 sec as described previously (20). The primary rise in $[Ca^{2+}]_i$ is reported to be due largely to the influx of extracellular Ca^{2+} while the secondary increase, or tailing, is attributed to release of Ca^{2+} from intracellular stores (20). A second addition of 10 μM LOOH also produced a similar spike, however, the $[Ca^{2+}]_i$ did not return to baseline but instead continued to rise after the spike. At a cumulative concentration of 20 μM, LOOH produces significant cytotoxicity (20,21). Although both nLDL and oxLDL increased $[Ca^{2+}]_i$, the latter produced larger increases when added to REC at 10 μg/mL, while at 100 μg/mL both LDL preparations were equally effective (Table 2). Table 2 also shows that pretreatment of oxLDL with the selenoperoxidase mimetic, ebselen, reduced the ability to induce increases in $[Ca^{2+}]_i$ such that 10 μg/mL of ebselen-treated oxLDL and nLDL were equally effective. Measurement of peroxide content in the LDL preparations indicated that nLDL contained ~ 30 nmol/mg LDL protein, oxLDL contained 1.0–1.5 μmol/mg LDL protein, and peroxides were undetectable in ebselen-treated oxLDL. This indicates that stimulation of Ca^{2+} influx, and/or release of intracellular Ca^{2+} into the cytoplasm, may be attributed to the lipid peroxides associated with LDL. Based on the levels of peroxides measured in oxLDL,

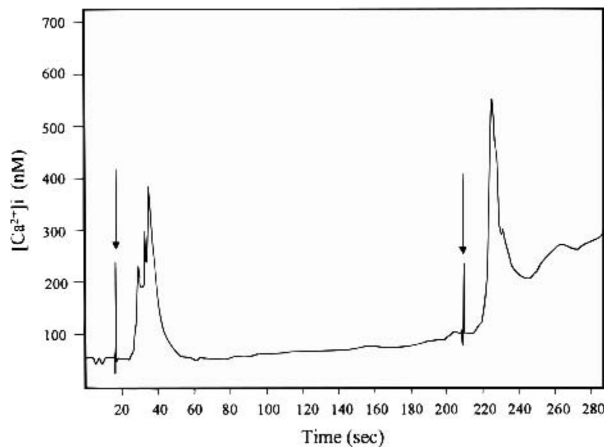

Fig. 2. Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) as monitored by Calcium Green fluorescence following LOOH treatment of REC in 10 mM HEPES/0.9% NaCl containing 1 mM Ca^{2+}. Confluent monolayers comprised of 1×10^5 cells were loaded with Calcium Green by pretreating cultures in 1 mL complete media with the fluorescent dye (5 μM) for 30 min, followed by rinsing twice with buffer. The basal fluorescence signal was monitored prior to addition of LOOH (indicated by arrows) at treatment concentrations of 10–20 μM/10^5 cells. The times of LOOH addition are indicated by arrows.

the LOOH equivalent concentration resulting from addition of 10 μg/mL LDL protein is estimated to be ~12 μM. Addition of 10 μM LOOH produced a large increase in $[Ca^{2+}]_i$ that peaked within 20 sec, reaching levels of over 300% above baseline. By contrast, oxLDL was less effective despite the comparable levels of LDL-associated peroxides administered, however, peak levels of $[Ca^{2+}]_i$ were achieved between 90–150 sec.

As shown in Fig. 3, the DHP were able to inhibit LOOH- and oxLDL-induced increases in $[Ca^{2+}]_i$. Furthermore, the effective concentrations were similar to those described above for LDL (ie. maximum inhibition produced with 100 nM DHP). However, at 1 μM there was no further inhibition of LOOH-induced increases in $[Ca^{2+}]_i$, while at 10 μM both felodipine and amlodipine were toxic to REC. Neither vitamin E nor verapamil at 10 μM inhibited LOOH-induced increases in $[Ca^{2+}]_i$, indeed, effective inhibition was only achieved in both cases at μM concentrations. At 100 nM, both felodipine and amlodipine inhibited oxLDL-induced increases in $[Ca^{2+}]_i$, however, further studies with higher concentrations of DHP were not done since at concentrations greater than 100 nM these compounds had relatively little effect on LOOH induced increases in $[Ca^{2+}]_i$. Based on the effective range for inhibiting LOOH-induced increases in $[Ca^{2+}]_i$, an ID_{50} concentration was estimated for a number of DHP and compared to trolox. The results are presented in Table 3. Lacidipine, felodipine and amlodipine were the most potent inhibitors, followed by other weaker DHP antioxidants (eg. nisoldipine and verapamil), however, trolox (representing the vitamin E chromanol) was ~1000-fold less potent despite being a better antioxidant than the DHP.

Table 4 compares the effects of vitamin E, felodipine and amlodipine in terms of the inhibition of DCF fluorescence after addition of 20 μM LOOH or 100 μg/ml oxLDL. Measurements of DCF fluorescence were made in control cultures and compared to cells pretreated with 10 μM vitamin E for 24 hr, or with 100 nM or 1.0 μM felodipine or amlodipine for 4 hr. The maximal DCF fluorescence was 18.6% after treating control cells with LOOH for 30 min at 37°C. In vitamin E pretreated cells, the level of DCF oxidation was reduced to 9% of maximum, respresenting a significant inhibition. Although the level of vitamin E in the treated cells was not determined, previous studies have shown that pretreatment with 10 μM vitamin E increased the cell content by approximately 20-fold (18,21). Felodipine or amlodipine at 100 nM produced non-significant effects on DCF fluorescence while treatments with 1.0 μM felodipine or amlodipine resulted in significant reductions in the maximal fluorescence following LOOH treatment. Thus, lower levels of DHP produced only marginal inhibitory effects on ROS production, but at higher concentrations the extent of inhibition approximated that afforded by vitamin E. Significant inhibition of DCF fluorescence by DHP was also found after treatments with 100 μg/ml oxLDL, a dose of oxLDL that induced marked increases in $[Ca^{2+}]_i$ and cytotoxicity. As seen with LOOH, the DHP significantly inhibited ROS production only at 1.0 μM.

Janero and Burghardt (24) previously reported an "antiperoxidant" effect of felodipine and suggested that 2,3 dichloro-substitution of the phenyl ring enhanced antioxidant activity. In addition, the extent to which the DHP associate with the lipid components of LDL or membrane bilayers appears to favor antioxidant activity. Thus, these compounds exhibited increasing activity relative to vitamin E when analyzed using membrane systems rather than homogeneous solutions to measure antioxidant potency (15). Indeed, lacidipine was only half

Table II. Intracellular Free Calcium Concentrations Following Additions of Normal or Oxidized LDL to Endothelial Cells

Treatment	Peak# $[Ca^{2+}]_i$ (nM)	% Change*
None	85 ± 7	100
nLDL (10 μg/mL)	97 ± 12	114[c]
nLDL (100 μg/mL)	202 ± 20	238[a]
oxLDL (10 μg/mL)	153 ± 18	180[b,c]
oxLDL (100 μg/mL)	213 ± 27	251[a]
nLDL (10 μg/mL) + ebselen	90 ± 8	106
oxLDL (10 μg/mL) + ebselen	108 ± 12	128
LOOH (20 μM)	328 ± 64	385

Note: Rabbit aortic endothelial cells were treated with the indicated concentrations of LDL that was suspended in PBS and added to cells maintained in HEPES assay buffer (10 mM) containing 1.0 mM Ca^{2+}. Prior to treatment, 5 μl of 1 mM Calcium Green in DMSO was added directly to cuvettes containing cells grown on coverslips in 1 mL of complete media (buffered with 10 mM HEPES) resulting in a final dye concentration of 5 μM. After 60 min at 21°C, unincorporated probe was removed by washings using the assay buffer and then treated with the LDL preparations in assay buffer.

\# Peak concentrations represent the maximum levels in $[Ca^{2+}]_i$ achieved after addition of the LDL preparation. Values shown are in nM and represent the mean and standard error calculated from 3 independent measurements. The time course for the Ca^{2+} increase is shown in Figure 2.

* The change in $[Ca^{2+}]_i$ are indicated as a percent of the levels found in control cells to which only buffer was added—indicated as "None" as set at 100%.

[a] Significantly different from controls (None) − $p < 0.001$.

[b] Significantly different from controls (None) − $p < 0.05$.

[c] oxLDL significantly different from nLDL − $p < 0.05$.

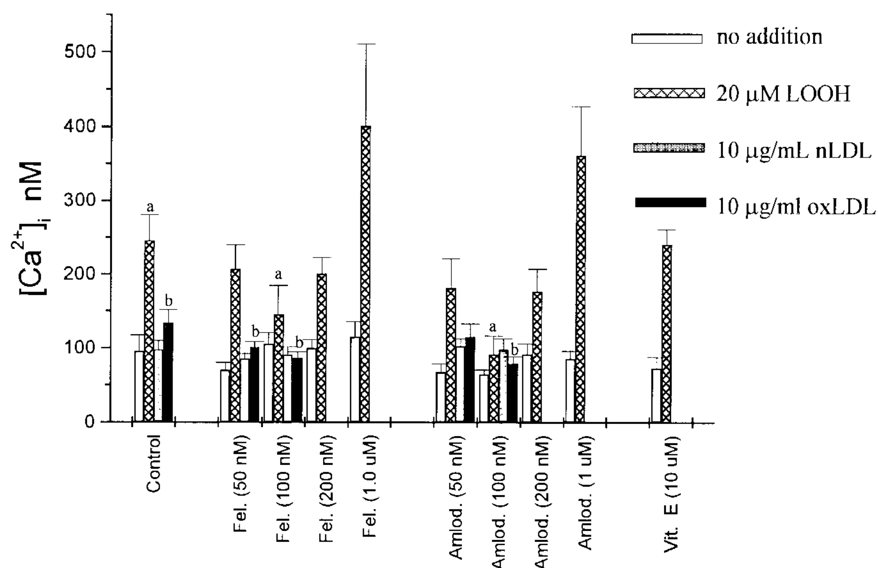

Fig. 3. Intracellular free Ca^{2+} concentrations ($[Ca^{2+}]_i$) as monitored by Calcium Green fluorescence following treatments with LOOH, normal LDL (nLDL), and oxLDL. Values shown are the mean and standard error from three independent experiments with each analysis performed in duplicate. The type of agent added is indicated in the legend insert. Comparisons of the effects of felodipine (Fel), amlodipine (Amlod), and vitamin E (Vit. E) are made according to concentrations added to cultures. The DHP were dissolved in PBS and added to the culture/treatment medium in 5–10 μl aliquots 2 hr prior to treatments with LOOH or LDL. Vitamin E was added to the cells 24 hr prior to treatments with LOOH or LDL. The $[Ca^{2+}]_i$ represent the maximal levels achieved after addition of LOOH or LDL. For LOOH, this was typically within 20 sec while for LDL maximal $[Ca^{2+}]_i$ were achieved within 2 min. a) Significantly different from control cells for LOOH treatment, $p < 0.05$. b) Significantly different from control cells for oxLDL treatment, $p < 0.05$.

as potent as vitamin E in a model system utilizing rat cortical membranes subjected to autoxidation whereas it was 2–3 orders of magnitude less potent in the DPPH test (15). These findings indicate that lipohilicity and interaction with peroxyl radicals in organized lipids such as membranes favors the antioxidant behavior of DHP. In addition, partitioning of DHP into lipid domains could increase local concentrations and far exceed the levels indicated (or assumed) in homogeneous solutions.

The ability of the DHP to prevent LOOH- or oxidized LDL-induced toxicity was examined under similar conditions to those described for the measurement of $[Ca^{2+}]_i$. Figure 4 compares the cytotoxic effects of LOOH and oxLDL. This was measured as the surviving fraction of cells after 24 hr treatment with 10 μg/mL or 100 μg/ml oxLDL, or 30 min treatment in PBS with 20 μM LOOH followed by washing with complete medium and incubation for 24 hr. Treatment with LOOH produced marked toxicity with a mean SF being 0.66, as compared to untreated cells for which the SF was arbitrarily set at 100%. Neither felodipine nor amlodipine afforded significant protection under these conditions. Treatment with 10 μg/ml oxLDL produced only marginal toxicity with the mean SF being 86%. Under these conditions both felodipine and amlodipine were protective. Treatments with 100 μg/ml oxLDL reduced the SF to 0.61 but felodipine and amlodipine both afforded significant protection. The reason for the apparent proliferative effect of the DHP in the presence of the low dose of oxLDL is unclear but may be due to the mitogenic action reported for oxLDL (25, 26) that possibly involves low-level ROS generation following uptake (26), and its modulation in the presence of DHP. Also, it is reasonable to expect the cells to grow better upon addition of LDL, provided that the levels of LDL are not excessive and if pro-oxidant effects are minimized.

Table III. Inhibition of LOOH-induced Increases in Intracellular Free Calcium Levels by DHP

Compound	ID_{50}*
Felodipine	90 nM
Lacidipine	45 nM
Amlodipine	70 nM
Verapamil	400 nM
Nisoldipine	125 nM
Trolox	90 μM

* Concentration of compound at which the rise in $[Ca^{2+}]_i$ was inhibited 50%. The results represent the average of three independent measurements. See Methods text and Figure 3 for details.

The findings above are consistent with the effects of LOOH and oxLDL on $[Ca^{2+}]_i$ as shown in Fig. 3. Although both felodipine and amlodipine inhibited LOOH-induced increases in $[Ca^{2+}]_i$, the effects were not dose dependent and the maximum levels of intracellular Ca^{2+} were considerably higher than those attained after addition of oxLDL. This is in partial agreement with previous studies using smooth muscle cells exposed to H_2O_2 (27). H_2O_2 also induced a rapid increase in $[Ca^{2+}]_i$ followed by a decrease to an elevated basal level as seen with repeated LOOH treatment (Fig. 2), however, unlike the effects reported here, DHP did not inhibit the primary increase (or

Table IV. Inhibition of LOOH-induced and oxLDL-induced Oxidation of DCF by DHP and Vitamin E

Treatment	+ 10 μM LOOH (AFU)/(AFU_{max})	+ 100 μg/ml oxLDL (AFU)/(AFU_{max})	% LOOH max	% oxLDL max
None	0.066 ± 0.009/0.355	0.053 ± 0.010/0.460	18.6 ± 4.7	11.5 ± 3.6
Vitamin E (10 μM)	0.026 ± 0.004/0.290	0.020 ± 0.005/0.419	9.0 ± 2.1†	4.8 ± 3.3†
Felodipine (0.1 μM)	0.052 ± 0.006/0.397	0.036 ± 0.005/0.440	13.0 ± 2.2	8.2 ± 2.1
Amlodipine (0.1 μM)	0.062 ± 0.018/0.408	0.027 ± 0.003/0.320	15.1 ± 5.7	8.4 ± 1.6
Felodipine (1.0 μM)	0.075 ± 0.017/0.696	0.016 ± 0.004/0.355	10.8 ± 1.9†	4.5 ± 2.0†
Amlodipine (1.0 μM)	0.077 ± 0.013/0.737	0.016 ± 0.006/0.383	10.4 ± 1.5†	4.2 ± 1.3†

Note: Two independent measurements for each treatment condition are shown where the fluorescence intensity was monitored at 506 ex/526 em at 37 °C. The extent of 2′-7′-dichlorofluorescin oxidation was measured in absolute fluorescence units, normalized to cell number (~ 5×10^5 cells). The average of the two measurements is expressed as the percent of total oxidizable DCF fluorescence (AFU_{max}) as described under Methods. Values for AFU_{max} varied less than 10% for each set of determinations.
† $P < 0.05$, ANOVA with post hoc pairwise comparisons to control (none).

spike) in $[Ca^{2+}]_i$. The secondary increase and new level of $[Ca^{2+}]_i$ was also reported to be due to release of intracellular stores. Thus, thiol reducing agents, DHP and some antioxidants effectively prevent the secondary increase in $[Ca^{2+}]_i$. Disparate effects of DHP on the primary rise in $[Ca^{2+}]_i$ may be due to the use of different cell types and H_2O_2, rather than lipid peroxides, as oxidants. Taken together, these results suggest that DHP and certain antioxidants may prevent the increased influx of Ca^{2+} on the basis of membrane thiol oxidation.

It also appears that part of the protection afforded by the DHP is based on inhibition of ROS production (Table 4). Thus, vitamin E was the most potent antioxidant but had relatively little effect on LOOH-induced increases in $[Ca^{2+}]_i$. Since the effectiveness of these compounds at limiting LOOH induced increases in $[Ca^{2+}]_i$ was similar at 1.0 μM and 100 nM, it is surmised that maximal effects on calcium flux reflect the threshold at which production of ROS and toxicity are manifested. Considering the results presented in Table 3, these findings show that oxidant-induced effects on Ca^{2+} influx or release of intracellular Ca^{2+} stores involve specific sites or oxidant sensitive proteins that are not directly affected by antioxidants that merely inhibit lipid peroxidation (e.g., vitamin E). Rather, a

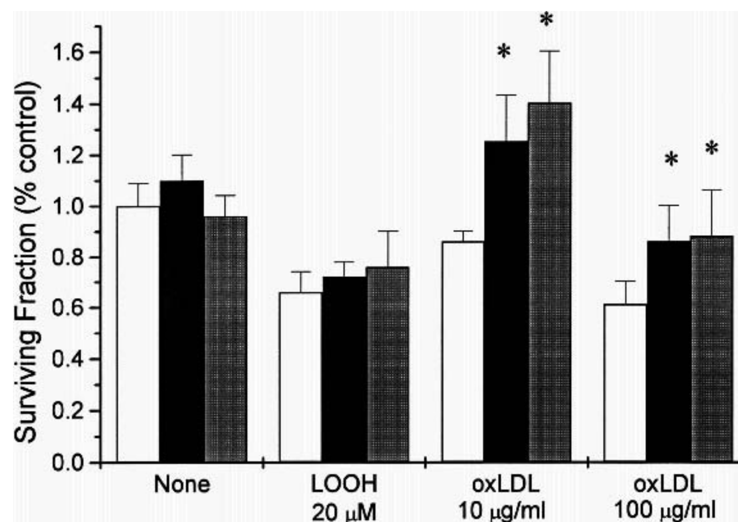

Fig. 4. Determination of LOOH and oxLDL cytotoxicity in the absence and presence of DHP. Cytotoxicity was estimated by means of the surviving fraction (SF) of cells after a 30 min treatment with 20 μM LOOH (in PBS) or 24 hr treatment with 10 μg/ml or 100 μg/ml oxLDL. Cells were pretreated with 100 nM felodipine (black bars) or amlodipine (gray bars) for 4 hr prior to addition of LOOH or oxLDL. Control cultures that were treated with buffer instead of the DHP are shown as white bars. Values shown are the mean and standard error from two independent experiments with each analysis performed in duplicate. Measurement of the SF was performed by replating cells after LOOH treatment followed by culture in complete medium for 24 hr. SF measurment for oxLDL treated cells involved measuring the number of surviving cells at the end of the 24 hr treatment period. Plating efficiencies for control cultures were >95% and the SF obtained 24 hr after plating was arbitrarily set at 100%. All other treatment conditions are expressed as a percent of the control SF. * Significantly different from control (oxLDL-treated) − $p < 0.05$.

specific interaction of the DHP with membrane proteins or Ca^{2+} channels appears to afford protection against oxidant-induced disruption of membrane Ca^{2+} permeability or flux. Increased $[Ca^{2+}]_i$ may trigger a series of signaling events that are associated with Ca^{2+}-mediated cell death (28). The relatively lower stimulation of $[Ca^{2+}]_i$ following treatments with oxLDL corresponds to the lower acute cytotoxicity compared to LOOH, and with the ability of the DHP to provide a margin of protection that is at least in part related to preventing increases in $[Ca^{2+}]_i$ beyond a cytotoxic threshold.

Potential anti-atherosclerotic effects of the DHP studied may be manifested through inhibition of oxidized LDL formation in the vasculature, thereby reducing the oxidative burden to vascular cells. It was also demonstrated for the first time that the antioxidant effects for the DHP are enhanced in the presence of ascorbic acid suggesting that prevention of lipoprotein oxidation may be facilitated by interactions with other native antioxidants which contribute to an antioxidant network (29,30). Under these conditions, antioxidant activity for these compounds is manifested at concentrations approaching the therapeutic levels found in plasma.

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