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Calcium antagonists as inhibitors of *in vitro* low density lipoprotein oxidation and glycation

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Abstract

The peroxidation step in lipid transformation is considered to be essential in the pathogenesis of atherosclerosis. Calcium antagonists (CA) appear to have antioxidant effects in addition to their potent vasorelaxant properties. In the present study, we compared the antioxidative efficacy of CA (amlodipine, lacidipine, nifedipine, isradipine, diltiazem, and semotiadil) in the copper-catalysed oxidation of low-density lipoprotein (LDL) with that of glycated(g)/glycoxidated(go) LDL. This issue is of great importance when considering the potential therapeutic use of antioxidant drugs in diabetes-associated vasculopathy. Oxidation of native LDL was inhibited most efficiently (>90%) by lacidipine and semotiadil in the concentration range 10^{-4}–10^{-3} M. We found, however, a dramatic decrease in antioxidant activity towards g/goLDL as compared to native LDL in all the CA tested. Only lacidipine significantly inhibited copper-mediated oxidation of g/goLDL in the whole concentration range tested (10^{-5} M–10^{-3} M). This probably resulted from the increased auto-oxidative potential introduced by early and advanced glycation end products (AGE) into the g/goLDL. We noted that coincubation of LDL with 10^{-3} M CA and 0.5 M glucose under oxidative/non-oxidative conditions partially or fully restored the antioxidant capacity of the different CA to inhibit the subsequent copper-catalysed oxidation of the modified LDL. This is a clear indication that CA inhibit glycative or glycoxidative LDL changes during the preceding long-term glycation period. The notion that both oxidative changes and long-term glycation effects were reduced by CA was corroborated by fluorescence analysis, AGE–ELISA, quantitation of lipid peroxidation, and thiobarbituric acid reactive substance (TBARS) measurement of long-term g/goLDL. The strongest antioxidative effects during long-term glycation of LDL were seen with isradipine, lacidipine, nifedipine, and semotiadil. Diltiazem was the only CA that could not prevent TBARS formation in LDL during the long-term glycation period. In contrast, Amadori product formation, as measured by the generation of fructosamines, was not significantly reduced by any CA tested. Thus CA, like other antioxidants, significantly retard AGE formation, while initial glycation reactions, such as Amadori product formation, are only weakly inhibited.

Keywords: Low-density lipoprotein; Calcium antagonists; Glycation; Glycoxidation; Diabetes mellitus; Atherosclerosis

1. Introduction

Oxidation of LDL has been suggested to play a central role in atherogenesis, as evidenced by the presence of oxidised LDL in atherosclerotic lesions and by the demonstration of autoantibodies against this antigen, e.g. in patients with carotid atherosclerosis or diabetes mellitus [1–5]. In part, these autoantibodies circulate as immune complexes [6]. In addition to its involvement in foam cell generation, oxidised LDL is considered responsible for triggering several processes underlying the formation and progression of atheromatous lesions [7–10].

An accelerated rate of LDL oxidation as a consequence of long-term hyperglycemia, which causes the formation of protein modifications termed AGE, may be a unique risk factor for accelerated atherosclerosis in diabetic subjects, as manifested by an increased risk of coronary heart disease [11–13]. Thus, *in vitro*, glucose, and/or glycated proteins enhance the rate of lipid peroxidation [14–17], and glycated LDL itself is more easily oxidised than native LDL [18,19]. In line with these findings, diabetic LDL has been shown to be more glycated and more susceptible to oxidation than

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Abbreviations: Apo B, apolipoprotein B; AGE, advanced glycation end products; CA, calcium antagonists; FA, fructosamine; gLDL, glycated low-density lipoprotein; goLDL, glycoxidated low-density lipoprotein; LDL, low-density lipoprotein; LPO, lipid peroxides; and TBARS, thiobarbituric acid reactive substances.

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non-diabetic LDL [20–22]. Lipoproteins from diabetic rats are more extensively oxidised *in vivo* and are more toxic than LDL from normal rats, abnormal properties that could be reduced by correction of hyperglycemia [23].

The peroxidation step in lipid transformation is considered to be essential for the pathogenesis of atherosclerosis. Although data concerning the mechanisms by which lipid peroxidation occurs *in vivo* are scarce, several lines of evidence suggest that some endogenous and exogenous compounds with antioxidant activity could have beneficial effects in the prevention of atherosclerosis. Ascorbic acid and α-tocopherol act as the most important hydrophilic and lipophilic antioxidants, respectively, *in vivo*. Several studies show that antioxidants such as probucol and butylated hydroxytoluene can inhibit the development of atherosclerotic lesions in Watanabe and cholesterol-fed rabbits.

Potentially useful antiatherosclerotic drugs would be those that not only have a direct effect on the arterial wall, but also demonstrate antioxidant and free radical scavenging properties. CA appear to have at least *in vitro* antioxidant effects in addition to their potent vasorelaxant properties. Thus, CA inhibit LDL oxidation by oxygen radicals [24] and preserve Apo B-100 integrity against oxygen radical attack. Copper-induced lipid hydroperoxidation of LDL was also blocked by CA [25]. Using a radical-generating system, the CA nifedipine was shown to exert free radical trapping activity [26]. CA of different chemical structure have a concentration-dependent effect as antioxidants against LDL oxidation [24,27,28]. It is generally accepted that particularly CA of the dihydropyridine type can prevent LDL oxidation [27]. 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 [28]. In addition to the structure of surface apolipoproteins, the factors determining the interaction of CA with plasma lipoproteins and their antioxidative effects include the lipophilicity of the drug [29,30]. CA may potentially exert antiatherosclerotic properties via reduction of the oxidative modification of LDL, thereby reducing foam cell formation and the pathophysiological cellular activities of oxidised lipids [26]. However, interventions aiming to decrease LDL oxidative susceptibility have not been shown to attenuate atherogenesis when cholesterol levels remain markedly elevated [31].

In the present paper, we investigated the role of CA as antioxidants in the *in vitro* oxidation of LDL. The potential inhibition of long-term glycation of LDL by these drugs was also studied.

2. Materials and methods

2.1. Preparation and characterisation of LDL and its modifications

Human LDL (d 1.019–1.063 g/mL) was isolated from EDTA-treated plasma by density gradient centrifugation. The supernatant was collected and dialysed extensively at 4° against PBS containing 1 mM EDTA. The isolated LDL was kept at 4° for no more than 16 hr before all further experiments or analysis. For oxidation, EDTA-stabilised LDL samples were dialysed overnight against PBS at 4°, diluted to 0.25 mg/mL of LDL protein, and then incubated under air with 5.0 μM $CuSO_4$ for 4 hr at 37°. After this time interval, maximal LDL oxidation was obtained. CA were dissolved in DMSO to obtain a stock solution of 10^{-2} M and further diluted to the required concentrations. DMSO at the end concentration does not influence the oxidation process. Oxidation was stopped by addition of 20 μL of 0.1 M EDTA per mL LDL solution, followed by dialysis against PBS containing 1 mM EDTA at 4° for 24 hr. For glycation/glycoxidation, LDL was incubated under air with 0.5 M glucose in phosphate-buffered saline (pH 7.4) for four weeks in the presence or absence of antioxidants at 37° in the dark. As control, native LDL was incubated without glucose in the presence or absence of antioxidants. LDL glycation/glycoxidation was monitored by measuring the fluorescence at 370 nm/440 nm, by AGE–ELISA [32], and by FA assay. In some biphasic experiments, CA effects were studied after long-term glycation/glycoxidation in the presence of 10^{-3} M CA for four weeks. Then, after dialysis, new CA was added and copper-mediated oxidation was performed for 4 hr.

2.2. Lipid peroxides

LPO were quantitated by a commercial test (LPO-CC, Kamiya Biomedical Co.). In the presence of haemoglobin, lipid hydroperoxides are reduced to hydroxyl derivatives (lipid alcohols), and a chromogen is oxidatively cleaved to form methylene blue in an equimolar reaction. Methylene blue is measured at 675 nm.

2.3. AGE–ELISA

AGE–RNase was prepared by incubating 25 mg/mL of bovine RNase (Sigma-Aldrich) in 0.2 M phosphate buffer, pH 7.4, containing 0.02% sodium azide and 0.5 M glucose 6-phosphate for six weeks at 37°. The solution was filtered through a 0.2-μm filter at the start of the incubation period. At the end of the incubation, the unbound carbohydrate was removed by extensive dialysis against PBS. White female New Zealand rabbits were immunised with AGE–RNase (10 or 25 mg/mL) which was emulsified with an equal volume of complete Freund's adjuvant (Sigma-Aldrich). About 250 mL of immunogen was injected subcutaneously every two weeks at two different sites on the back of the animals. A total of six injections were given, all booster injections being performed with the immunogen in incomplete adjuvans. High titers of antibodies developed after ten weeks. Nunc Maxisorp plates were coated with 50 mg/mL of AGE–Apo-B in 0.2 M bicarbonate buffer, pH 9.0, for 3 days at 4°. This was obtained by incubating Apo-B (Sigma)

with 0.5 M glucose for six weeks at 37°. AGE–LDL samples were preincubated at dilutions ranging from 1:5 to 1:100 with anti-AGE–RNase antibody (1:1000 in PBS containing 1.0% BSA) for 1 hr at 37° and 16 hr at 4°. The antibody/antigen mixtures were then incubated with the solid-phase AGE–Apo B for 1 hr at 37° and 16 hr at 4°. Binding of free anti-AGE antibody was quantitated with peroxidase labelled goat anti-rabbit immunoglobulin G (IgG) antibody (Amersham) and 2,2′-azino-di-[3-ethyl-benzthiazoline sulfonate(6)] (ABTS) (Boehringer Mannheim) as colouring substrate. As standard, goLDL was used. One unit of AGE activity was defined as the amount of antibody-reactive material equivalent to 1 μg of AGE–LDL.

2.4. Fructosamine assay

Early glycation products were determined with the Roche fructosamine test (Hoffmann-LaRoche). Unimate 5 FRUC is an *in vitro* diagnostic reagent based on the ability of ketoamines to reduce nitroblue tetrazolium in alkaline medium. The rate of formazan formation is directly related to the FA concentration and is measured photometrically at 550 nm. We obtained an intra-assay coefficient of variation (CV) of 6.7% and an inter-assay CV of 9.8% for this assay.

2.5. TBARS assay

Lipid peroxidation was determined by quantitation of TBARS with a microtiter plate modification of the method devised by Buege and Aust [33]. To 120 μL oxidised LDL were added 70 μL PBS and 50 μL trichloroacetic acid (50%), followed by 75 μL thiobarbituric acid (1.3%). The reaction mixture was incubated for 40 min at 60°. The tubes were placed on ice for 5 min and the samples then centrifuged at 2000 *g* for 10 min. The supernatants were transferred to microtiter plates and the absorbance (540 nm) was measured in an Anthos microplate reader. TBARS concentration was calculated using a malondialdehyde (MDA) standard curve and expressed as nM MDA-equivalent per mg LDL protein.

2.6. Statistical analysis

Statistical differences were tested using Student's *t*-test. A *P* value of 0.05 or less was considered statistically significant. Data are presented as means ± SD.

3. Results

CA in the concentration range 10^{-5} to 10^{-3} M dose-dependently inhibited the oxidation of native LDL (Fig. 1). In the concentration range 10^{-4} M to 10^{-3} M, the degree of oxidation inhibition by lacidipine and by semotiadil, the most powerful antioxidants in the group of CA tested by us, was above 90%. Glycoxidation or glycation of LDL entailed an increased *in vitro* copper-mediated oxidation, as measured by the TBARS assay (Fig. 1). This generally increased oxidation of glucose-modified LDL (g/goLDL) as compared to native or control LDL incubated for 4 weeks without glucose ± antioxidants was efficiently inhibited only by the CA lacidipine over the *whole* concentration range tested by us. Semotiadil and diltiazem showed a significant antioxidative activity towards modified LDL only at 10^{-3}–10^{-4} M, while the other three CA investigated (amlodipine, isradipine, and nifedipine) were ineffective as antioxidants in the copper-mediated oxidation of g/goLDL (Fig. 1).

Long-term coincubation of LDL with glucose and CA (10^{-3} M) in the absence of other antioxidants resulted in the partial inhibition of LDL glycoxidation: thus, generation of AGE, as measured by ELISA, of lipid peroxides and of TBARS was drastically reduced by CA, while non-oxidative changes such as early glycation product formation as measured via the FA test were only weakly inhibited (Table 1). AGE formation as determined by fluorescence was also strongly impaired by CA (data not shown). AGE-specific fluorescence data are, however, hampered by non-specific fluorescence generated, for example, by semotiadil and lacidipine, as well as by the fact that oxidation of LDL also results in fluorescence, peaking very near to the emission peak of AGE-specific fluorescence (430 nm vs. 440 nm). The most powerful antioxidants were nifedipine and lacidipine, which dramatically inhibited TBARS formation and the generation of AGE and hydroperoxides (Table 1). Control incubation of LDL without glucose resulted in minimal oxidation as measured by TBARS and LPO (2 nM/mg of LDL protein and 8 nM LPO/mg of LDL).

Long-term glycation in the presence of EDTA, i.e. in an anaerobic milieu, showed less significant CA-dependent effects (Table 2), as oxidative changes are only marginally involved in this glycation mode. Of all the CA tested, only diltiazem did not completely abrogate TBARS formation during long-term glycation/glycoxidation of native LDL (Tables 1 and 2). Control incubation of LDL without glucose in the presence of antioxidants virtually inhibited LDL oxidation (less than 1 nM/mg of LDL protein and 4 nM LPO/mg of LDL), nearly corresponding to the original values of native LDL used as starting material).

Glycoxidation of LDL in the presence of CA partially restored their antioxidative potential in the subsequent copper-catalysed LDL oxidation step (Table 3), evidently through a CA-dependent inhibition of LDL *glycoxidation* in the previous glycation period of four weeks (biphasic experiments). The regeneration of the antioxidative potential of CA was most significant for semotiadil, nifedipine, and diltiazem. The effect was, however, much less evident after long-term *glycation* of LDL in the presence of CA and EDTA (Table 4).

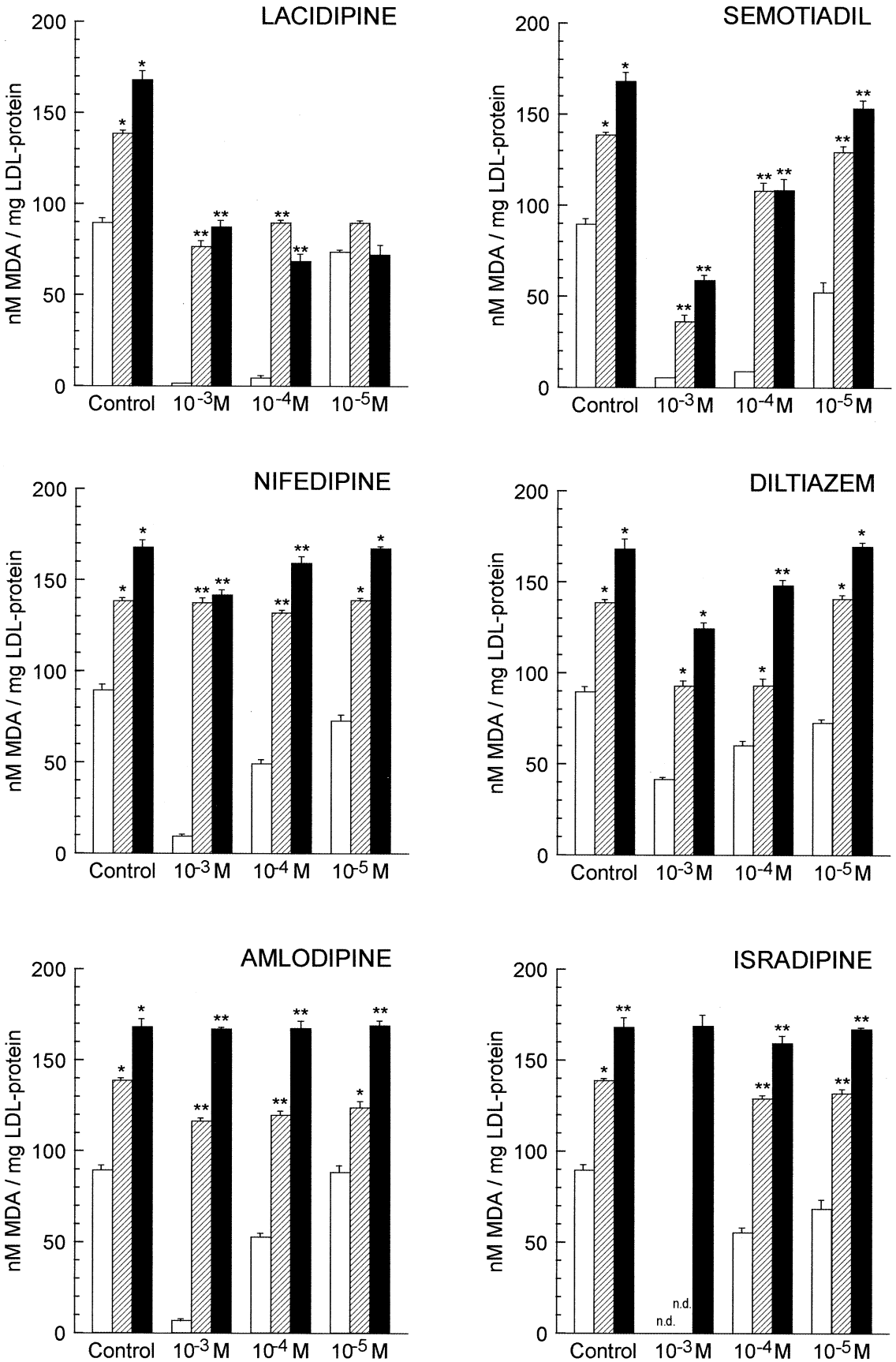

Fig. 1. Influence of calcium antagonists on copper-mediated oxidation of native, glycated, and glycoxidated low-density lipoprotein. Open bars: native LDL; hatched bars: glycated LDL; solid bars: glycoxidated LDL. Data represent the mean values ± SEM of four parallel oxidation experiments. *, larger with $P < 0.01$ than the respective oxidation of native LDL; **, with $P < 0.001$.

Table 1
Effects of CA on long-term glycoxidation of LDL

CA	TBARS nM/mg LDL prot	LPO nM/mg LDL	AGE (u/mg prot)	FA (M/M)
Amlodipine	—	16.0 ± 1.6*	633 ± 13*	95 ± 2
Diltiazem	6.2 ± 0.3	47.6 ± 5.1	857 ± 96	92 ± 5
Isradipine	—	6.2 ± 0.3*	212 ± 32*	84 ± 7
Lacidipine	—	5.7 ± 1.2*	103 ± 14*	87 ± 3
Nifedipine	—	7.3 ± 2.4*	107 ± 17*	98 ± 9
Semotiadil	—	6.2 ± 1.0*	180 ± 4*	91 ± 2
none	7.5 ± 0.9	47.3 ± 4.7	1000	98 ± 13

LDL was glycated for four weeks in the absence of antioxidants and in the presence or absence of 1.0 mM CA. Data represent the mean values of four determinations ± SD.

* $P < 0.01$, as compared to glycoxidation without CA.

4. Discussion

Our results clearly confirm the dose-dependent antioxidative activity of CA. An antioxidative effect was found at a CA concentration of 1.0 mM in the following order: lacidipine > semotiadil > amlodipine > nifedipine > diltiazem. At 10^{-4} M, lacidipine and semotiadil showed far stronger inhibitory effects than the other CA tested. These CA concentrations are higher than therapeutic plasma concentrations of CA attained *in vivo*, which are in the range 10^{-7}–10^{-5} M [34,35]. Following local accumulation, however, the most lipid-soluble CA, such as lacidipine, might even reach 10^{-3} M *in vivo* in the microenvironment [27]. As most other authors studying the *in vitro* antioxidant activity of CA against LDL oxidation [24,26–28,36–38] found inhibitory effects in the range 10^{-5}–10^{-3} M, we also used a concentration range of 10^{-5} M to 10^{-3} M for our *in vitro* studies. Our findings are consistent with the results of other groups [27,28], both in the order of antioxidant capacity and in that the benzothiazepine derivative diltiazem showed the lowest antioxidative effect. No phenylalkylamine CA (e.g. verapamil) was investigated in the present study.

Table 2
Effects of CA on long-term glycation of LDL

CA	TBARS nM/mg LDL prot	LPO nM/mg LDL	AGE (u/mg prot)	FA (M/M)
Amlodipine	—	18.0 ± 0.9*	413 ± 17	105 ± 3
Diltiazem	4.5 ± 0.6	18.3 ± 1.7*	505 ± 42	110 ± 6
Isradipine	—	8.1 ± 0.3*	120 ± 14*	98 ± 9
Lacidipine	—	3.1 ± 0.6*	233 ± 21*	89 ± 13
Nifedipine	—	3.4 ± 0.2*	367 ± 45**	100 ± 9
Semotiadil	—	7.1 ± 0.5*	351 ± 27**	92 ± 12
none	5.0 ± 0.5	35.1 ± 3.9	539 ± 67	118 ± 16

LDL was glycated for four weeks in the presence of antioxidants and different CA at a concentration of 1.0 mM. Data represent the mean values ± SD of four determinations.

* $P < 0.01$.

** $P < 0.05$ as compared to control without CA.

Table 3
Inhibition of LDL glycoxidation by calcium antagonists partially restores their antioxidative potential in copper-catalysed LDL oxidation

CA (10^{-3} M)	TBARS (nM/mg LDL protein)		
	before	after glycoxidation with CA	without CA
Amlodipine	6.8 ± 1.2	81.7 ± 3.1*	167.0 ± 1.1*
Diltiazem	41.5 ± 0.6	68.0 ± 2.5*	124.4 ± 3.0*
Isradipine	ND	118.4 ± 5.3	168.1 ± 4.2
Lacidipine	1.4 ± 0.3	19.8 ± 2.6*	21.6 ± 2.8*
Nifedipine	9.4 ± 0.8	11.5 ± 1.2	141.9 ± 2.4*
Semotiadil	5.4 ± 0.1	3.1 ± 0.7	58.8 ± 2.9

LDL was oxidised with copper ions for 4 hr in the presence of CA before or after glycoxidation. Glycoxidation itself was performed in the presence or absence of the same CA as were used in the subsequent oxidation. Data represent the mean values of four determinations ± SD. ND, not determined.

* Significantly higher than the respective oxidation values of native LDL; $P < 0.01$.

We furthermore compared the antioxidative efficacy of CA in the copper-catalysed oxidation of native LDL with that of g/goLDL. To our knowledge, this is the first report describing the modification of the antioxidative potential of CA by the *in vitro* long-term glycation/glycoxidation of the substrate LDL. This issue is of great importance when considering the potential therapeutic use of antioxidant drugs in diabetes-associated atherosclerosis. We generally found a dramatic decrease in the antioxidant activity of all the CA tested towards g/goLDL as compared to native LDL. This reduced efficacy in inhibiting g/goLDL oxidation has also been noted using other therapeutic agents such as taprostene [39]. This probably results from the increased auto-oxidative potential introduced by early and advanced glycation end products into the g/goLDL [17]. In the presence of transition metal catalysts, early glycation products would be expected to amplify their increased rate of oxygen radical production even further via Fenton reaction chemistry. In addition, metal-catalysed decomposition of lipid hydroperoxides and amino acid hydroperoxides, formed

Table 4
Ineffectual inhibition of LDL glycation by CA does not restore their antioxidative potential in copper-catalysed LDL oxidation

CA (10^{-3} M)	TBARS (nM/mg LDL protein)		
	before	after glycation with CA	without CA
Amlodipine	6.8 ± 1.2	109.4 ± 3.3	116.5 ± 1.9
Diltiazem	41.5 ± 0.6	88.1 ± 3.5	92.9 ± 2.5
Lacidipine	1.4 ± 0.3	69.3 ± 1.9	76.5 ± 1.8
Nifedipine	9.4 ± 0.8	121.7 ± 2.8	137.6 ± 2.7
Semotiadil	5.4 ± 0.1	34.4 ± 1.2	36.1 ± 3.1

LDL was oxidised with copper ions for 4 hr before or after a glycation period of four weeks in the presence or absence of 1.0 mM CA. Data represent the mean values of four determinations ± SD. Oxidation of gLDL in all experiments significantly higher than LDL values; $P < 0.01$.

during glycation under aerobic conditions in Apo B [40], might contribute to the induction of oxidative stress. The function of these hydroperoxides is to reoxidise Cu^{+} under formation of alkoxyl or "primordial" radicals [41] such as tocopheroxyl and lipid peroxyl radicals. Their formation rates govern the total rate of initiation, which is essential for the whole oxidation process [41].

Although the antioxidative mechanisms of CA have not been elucidated yet, it is evident that at least the dihydropyridine CA belong to the chain-breaking group of antioxidants [36], not to the group of "preventive" or chelating antioxidants, e.g. EDTA. The dihydropyridine ring can donate electrons to the propagating radicals to reduce them to a non-reactive form. Due to the increased generation of reactive oxygen species by glucose-modified LDL, however, the chain-breaking capacity of CA may be overridden. In addition, the antioxidant action of CA depends on their lipophilicity and their ability to become efficiently incorporated into the LDL particle, i.e. to reach the site of peroxidation [27,30]. Such an interaction could be hampered by the reduced lipophilic character of the LDL core as seen in long-term g/goLDL, which shows a predominant AGE formation in the lipid part of LDL [18] and further loses lipophilicity by lipid peroxidation and malondialdehyde (MDA) formation, especially in the course of glycoxidation.

We noted that coincubation of LDL with 10^{-3} M CA and 0.5 M glucose under oxidative/non-oxidative conditions partially or fully conserved the antioxidant capacity of the diverse CA to inhibit the subsequent copper-catalysed oxidation of the modified LDLs (biphasic experiments). This is a clear indication that CA inhibit glycative or glycoxidative LDL changes during the preceding long-term glycation period. The notion that both oxidative changes and at least long-term glycation effects were indeed drastically reduced by CA is corroborated by fluorescence analysis, AGE–ELISA, quantitation of lipid peroxidation, and TBARS measurement of long-term g/goLDL. Thus, all CA tested with the exception of diltiazem completely abrogated TBARS formation during long-term glycation/glycoxidation of LDL. In contrast, early glycation product formation, as measured by the generation of FA, was not significantly reduced by any CA tested. It is a general finding that antioxidants significantly retard AGE formation, while glycation itself is only weakly inhibited. Fu *et al*. term this phenomenon "uncoupling" of glycation from oxidative AGE formation [42].

In the presence of glucose or (early) glycosylated Apo B, the lipoperoxidation/peroxidative cascade is complicated by chain reactions [17]. Lipid hydroperoxides normally accumulating at a slow rate under not strictly anaerobic conditions are able to react with the enol form of glucose or the eneaminol form of glycosylated Apo B to form alkoxy and $LO^{\cdot}$ radicals as well as glucose/glycosylated peptide radicals. Additionally, superoxide anions and hydroxyl radicals are formed, especially in the presence of trace amounts of transition metals [15]. These reactive species can initiate further peroxidative chain reactions, resulting in a rapid increase in peroxidation rates: goLDL is formed. CA can deactivate these reactive intermediates and may therefore inhibit glycoxidation of LDL. In contrast to verapamil, which shows a relative preference for $LO^{\cdot}$ radicals, lacidipine is equally effective against $LOO^{\cdot}$ and $LO^{\cdot}$ [36], explaining its high efficiency in blocking both copper-catalysed LDL oxidation and glycoxidation of LDL. The chain-breaking quality of CA is manifested in their overall antioxidative effects, as evidenced, for example, by reduced peroxide formation and the complete blocking of TBARS generation. Especially effective in this respect in our hands were isradipine, lacidipine, and nifedipine.

As shown in experimental models of atherosclerosis, formation of circulating TBARS/hydroperoxides can be suppressed by CA, e.g. lacidipine [39]. This CA reduced atherosclerosis in hypercholesterolemic rabbits, hamsters, and hypertensive rats [39]. Whether CA can beneficially affect atherosclerosis in *humans* is doubtful, however, as clinical trials such as MIDAS have evidenced [43].

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