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THE INFLUENCE OF MAGNESIUM-PYRIDOXAL-5′-PHOSPHATE-GLUTAMATE IN COMPARISON WITH PROBUCOL, α-TOCOPHEROL AND TROLOX ON COPPER-INDUCED OXIDATION OF HUMAN LOW DENSITY LIPOPROTEIN *IN VITRO**

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Abstract—Low density lipoprotein (LDL) in the presence of magnesium-pyridoxal-5′-phosphate-glutamate (MPPG), pyridoxal-5′-phosphate (PP), α-tocopherol, probucol or trolox is more resistant against copper-induced oxidation as control-LDL *in vitro*. The efficiency of the drugs is: probucol > MPPG > trolox > α-tocopherol > PP. LDL oxidation is determined by its increasing negative surface charge, fragmentation of apolipoprotein B-100 and changes of the fatty acid content of LDL. The protection of the drugs depends on their concentration and incubation time. Different experiments point to the fact that copper-induced oxidation of LDL *in vitro* starts with the binding of copper at the apolipoprotein B-100, resulting in an increasing negative surface charge and fragmentation of the apolipoprotein B-100. Afterwards a decrease of LDL-bound linoleic acid (18:2) is measurable.

Key words: LDL; magnesium-pyridoxal-5′-phosphate-glutamate; α-tocopherol; probucol; trolox; pyridoxal-5′-phosphate

Human LDL‡ is the main carrier for cholesterol in the blood stream. It is well established that cholesterol deposits in the arteries stem primarily from LDL and that increased levels of plasma LDL and low levels of plasma HDL correlate with an increased risk of arteriosclerosis. The oxidative modification of LDL is suggested to play an important role in the pathogenesis of arteriosclerosis, resulting in a rapid uptake of LDL by macrophages via the scavenger receptor. Native LDL (unmodified) is not recognized by the scavenger receptor, but is taken up via the down-regulated LDL-receptor [1, 2]. Through the uncontrolled uptake of oxidized LDL, macrophages become lipid-laden cells, called foam cells, and may represent the first step in the pathogenesis of arteriosclerosis. LDL can be modified through free radicals, acetylation or incubation by copper ions [3, 4]. Oxidized LDL shows an increased negative surface charge, a decrease of cholesterylester and a fragmentation of apolipoprotein B-100. Furthermore, formation of lipid peroxides which decompose to reactive aldehydes has been shown [5, 6]. Oxidized LDL also functions as a chemoattractant for monocytes and smooth muscle cells, and is cytotoxic for endothelial cells [7].

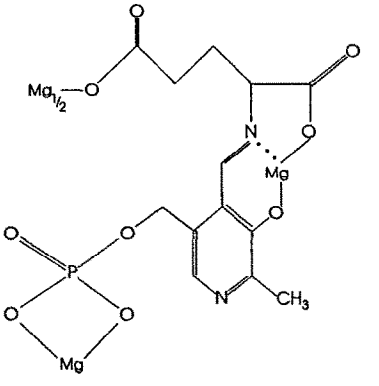

Fig. 1. The chemical structure of MPPG.

Native human LDL has a molecular weight of 2.5 million Da and consists of 20–24% phospholipids, 9–10% free cholesterol, 40–44% cholesterolester, 3–5% triglycerides and 21–26% apolipoprotein B-100 [8]. It also contains different antioxidants, the main component is α-tocopherol, which protects LDL from oxidative damage. *In vitro* α-tocopherol is able to prolong the lag-phase during initiation of LDL oxidation [9]. In a similar manner probucol, a sulphur containing biphenol, prolongs the lag-phase of copper-induced LDL oxidation *in vitro* [10].

MPPG (Fig. 1), ameliorates atherosclerotic symptoms and lowers the blood cholesterol concentration as shown for hypercholesterolemic rats and rabbits [11]. MPPG also prevents degenerative changes and calcification in endothelial and smooth

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§ Abbreviations: LDL, low-density-lipoprotein; MPPG, magnesium-pyridoxal-5′-phosphate-glutamate; PP, pyridoxal -5′-phosphate.

muscle cells of the rabbit aorta [12]. The mechanism of the cholesterol lowering effect of MPPG is still unknown. Furthermore, MPPG acts as a radical scavenger and the reaction of MPPG can be differentiated from the activities of the single components of MPPG [13].

In this study we explored the influence of MPPG and its components on the copper-induced oxidation of LDL *in vitro* compared with α-tocopherol, trolox and probucol. In addition we investigated the mechanism of copper-induced oxidation of LDL.

MATERIALS AND METHODS

Chemicals. PP was obtained from Boehringer Mannheim GmbH (Mannheim, F.R.G.), and trolox from Aldrich-Chemie (Steinheim, F.R.G.). MPPG was from Steigerwald Arzneimittel GmbH (Darmstadt, F.R.G.). All other substances were purchased from Sigma-Chemicals (Deisenhofen, F.R.G.).

LDL preparation. LDL ($d = 1.019$–1.063 g/mL) was isolated from human plasma of healthy donors by sequential ultracentrifugation [14]. To maintain the integrity the isolated LDL was dialysed under argon-atmosphere against 150 mM NaCl, 10 mM Tris–HCl, 0.3 mM EDTA, pH = 7.5 at 4°. The protein content of LDL was determined by the method of Lowry *et al.* [15] with bovine serum albumin as standard.

Cu^{2+}-oxidation of LDL. Prior to oxidation by $CuSO_4$, EDTA was removed by dialysis against 10 mM phosphate, 0.15 M NaCl, pH = 7.4. The modification of LDL was induced by incubation with 10 μM $CuSO_4$ at 37° for 3, 6 and 24 hr. α-Tocopherol and probucol were dissolved in pure ethanol, the final concentration was 5% (v/v). At this concentration ethanol showed no effect on the copper-induced LDL oxidation *in vitro*. The final LDL protein concentration was 0.2 mg/mL.

Electrophoretic mobility was determined by 0.8% agarose-gel-electrophoresis in 50 mM barbital buffer, pH = 8.6. The apolipoprotein B-100 was stained with Sudan Black B [16].

LDL fatty acids were identified and quantified as methylesters by GLC. After stopping oxidation with EDTA the liquid was dried overnight. The following day the lyophiluate was incubated with 300 μL borontrifluoride/methanol (14%) for 30 min at 80°. After cooling down 1 mL aqua dest. was added to destroy the borontrifluoride–methanol complex. The fatty acid methylesters were extracted with diethylether (three times with 1 mL each) and the diethylether was washed twice with 1 mL aqua dest. each. Afterwards, the diethylether was evaporated with nitrogen. For analysis the fatty acid methylesters were dissolved in methanol and detected with a Durabond DB-225 (30 m, 0.25 μm) capilar column from J&W [16].

Statistics. The data are means of at least four experiments with two parallels each showing standard deviations (σ_{n-1}).

RESULTS

The apolipoprotein B-100 has a molecular mass of 500,000 Da and was analysed after removing EDTA by SDS–electrophoresis (separation according to the molecular mass) and by agarose-electrophoresis (separation according to negative surface charge). The fatty acids were analysed by GLC. The composition of the main fatty acids are shown in Table 1. The concentrations of the other fatty acids were below the detection limits of the system.

Table 1. Main fatty acids of native human LDL after removing EDTA

Fatty acid	mol/mol LDL
Palmitic acid	686.6 ± 89.9
Stearic acid	270.9 ± 46.2
Oleic acid	431.2 ± 121.2
Linoleic acid	896.3 ± 103.6

LDL was isolated by density gradient ultracentrifugation and the LDL-bound fatty acids were detected by GLC as described in Materials and Methods.

The data are expressed as means ± SE for N = 8.

LDL is a very unstable molecule which denatures both by freezing and heating and in the presence of oxygen. Therefore, the autoxidation of the LDL-bound fatty acids after removing EDTA have been examined over a 24 hr period at 37°. As shown in Table 2 no autoxidation of the fatty acids was detectable during this time.

In vitro experiments have shown that oxidized LDL is taken up uncontrolled via the scavenger receptor and the incubation with copper ions is widely used to modify native LDL [17].

Through the incubation of LDL with 10 μM $CuSO_4$ *in vitro* increased the negative surface charge of apolipoprotein B-100 visible after 3 hr accompanied by a simultaneous fragmentation of the protein (data not shown). Over a 24 hr period copper induced only a decrease of the LDL-bound linoleic acid. After 3 hr the linoleic acid decreased by 48 ± 11% and after 6 hr the whole linoleic acid was oxidized (see Table 2). Therefore, in the following experiments only the linoleic acid was quantified.

Incubation of free linoleic acid at a concentration of 50 μg/mL with 10 μM Cu^{2+} did not result in a decrease of the linoleic acid over a period of 6 hr at 37° (data not shown).

Influence of MPPG and PP on the copper-induced oxidation of LDL in vitro

Neither MPPG nor PP influenced the electrophoretic mobility of apolipoprotein B-100. During LDL oxidation with 10 μM Cu^{2+}, MPPG (500 μM) inhibited the increase of the negative surface charge over a period of 24 hr. At a concentration of 50 μM MPPG protected for 3 hr. However PP only inhibited copper-induced LDL oxidation by about 6 (500 μM PP) or 3 (50 μM PP) hr (data not shown).

Figures 2 and 3 show the protection by MPPG and PP of the copper-induced oxidation of LDL-bound linoleic acid. MPPG (500 μM) inhibited the oxidation during the whole incubation time. In this concentration PP inhibited for only 6 hr. At a

Table 2. Autoxidation (incubation without copper) and oxidation of the LDL-bound fatty acids by 10 μM $CuSO_4$

Fatty acid [mol/mol LDL]	Cu^{2+} [10 μM]	Incubation time (hr) 3	6	24
Palmitic acid	−	686.6 ± 89.9	710.2 ± 43.9	687.9 ± 52.4
	+	680.4 ± 71.0	723.8 ± 58.3	680.7 ± 99.1
Stearic acid	−	270.9 ± 46.2	311.3 ± 96.7	267.2 ± 74.7
	+	291.7 ± 47.6	289.7 ± 103.5	302.0 ± 78.4
Oleic acid	−	431.2 ± 121.2	451.7 ± 17.4	438.4 ± 97.7
	+	435.8 ± 92.9	465.7 ± 92.0	306.0 ± 150.8
Linoleic acid	−	896.3 ± 103.6	925.0 ± 54.8	900.2 ± 122.7
	+	464.5 ± 230.9	0.0 ± 0.0	0.0 ± 0.0

The reaction was conducted at 37° for 3, 6 and 24 hr and was examined after removing EDTA by dialysis against 10 mM phosphate, 0.15 M NaCl, pH = 7.4. LDL was isolated by density gradient ultracentrifugation and the LDL-bound fatty acids were detected by GLC as described in Materials and Methods.

The data are expressed as means ± SE for four separate experiments with N = 2 each.

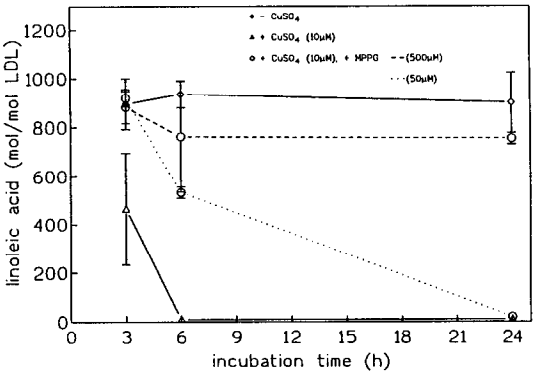

Fig. 2. The influence of MPPG (500 and 50 μM) on the oxidation of human LDL *in vitro*. After removing EDTA by dialysis against 10 mM phosphate, 0.15 M NaCl, pH = 7.4, modification of LDL was induced by incubation with 10 μM $CuSO_4$ at 37° for 3, 6 and 24 hr. LDL was isolated by density gradient ultracentrifugation and the LDL-bound fatty acids were detected by GLC as described in Materials and Methods. The data are expressed as means ± SE for four separate experiments with N = 2 each.

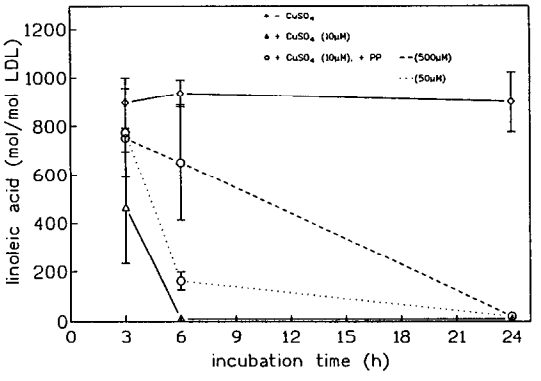

Fig. 3. The influence of PP (500 and 50 μM) on the copper-induced oxidation of human LDL *in vitro*. Conditions are the same as in Fig. 2.

concentration of 50 μM and an incubation time of 6 hr MPPG inhibited oxidation approximately by 58 ± 4% of the control values in the absence of copper and PP by 18 ± 4% of the control values. After 24 hours both compounds showed no influence on the LDL-bound linoleic acid oxidation.

Comparison of MPPG, probucol, α-tocopherol and trolox

Probucol and α-tocopherol were dissolved in 100% ethanol, the final ethanol concentration was for all test substances 5% (v/v). Controls showed that in this concentration ethanol did not influence copper-induced oxidation of LDL *in vitro*. The changes in the electrophoretic mobility of the apolipoprotein B-100 in the presence of the test substances (500 and 50 μM) are shown for an incubation time of 24 hr in Fig. 4. The oxidation of the LDL-bound linoleic acid is documented in Figs 5 and 6.

At a concentration of 500 μM all test substances inhibited the copper-induced oxidation of LDL-bound linoleic acid over a period of 24 hr. In contrast the electrophoretic mobility of apolipoprotein B-100 increased by 50% after 24 hr. Only in the presence of probucol no change of the negative surface charge was observed.

At a concentration of 50 μM only probucol inhibited LDL oxidation by copper over a 24 hr period. MPPG, trolox and α-tocopherol protected for only 3 hr. After 6 hr MPPG protected the LDL-bound linoleic acid from oxidation by about 100%, trolox about 57% and α-tocopherol about 20% of the control values in absence of copper (see Fig. 6). The electrophoretic mobility was increased more than 50% (data not shown). After a period of 24 hr MPPG, trolox and α-tocopherol did not protect LDL from copper-induced oxidation.

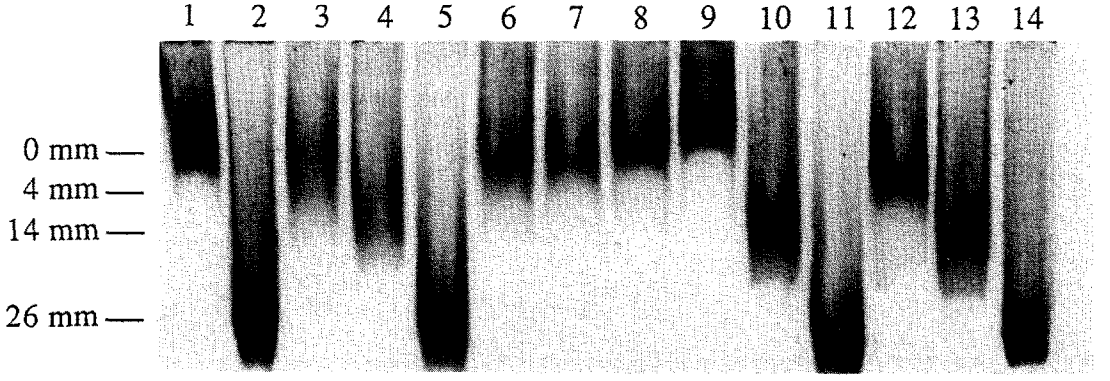

Fig. 4. The influence of the test substances (500 and 50 μM) on the increasing negative surface charge of apolipoprotein B-100 after a 24 hr period. Prior to oxidation, EDTA was removed by dialysis against 10 mM phosphate, 0.15 M NaCl, pH = 7.4. The modification of LDL was induced by incubation with 10 μM $CuSO_4$ at 37°. α-Tocopherol and probucol were dissolved in pure ethanol, the final ethanol concentration for all substances was 5% (v/v). LDL was isolated by density gradient ultracentrifugation and the apolipoprotein was stained with Sudan Black B as described in Materials and Methods. Lane 1: LDL; 37° changing [mm] 0 mm. Lane 2: LDL; 10 μM CuSO4; 37° 26 mm. Lane 3: LDL; 500 μM α-tocopherol; 37° 4 mm. Lane 4: LDL; 10 CuSO4; 500 μM α-tocopherol; 37° 10 mm. Lane 5: LDL; 10 CuSO4; 50 μM α-tocopherol; 37° 26 mm. Lane 6: LDL; 500 μM probucol; 37° 3 mm. Lane 7: LDL; 10 CuSO4; 500 μM probucol; 37° 2 mm. Lane 8: LDL; 10 CuSO4; 50 μM probucol; 37° 0 mm. Lane 9: LDL; 500 μM trolox; 37° 0 mm. Lane 10: LDL; 10 CuSO4; 500 μM trolox; 37° 14 mm. Lane 11: LDL; 10 CuSO4; 50 μM trolox; 37° 26 mm. Lane 12: LDL; 500 μM MPPG; 37° 4 mm. Lane 13: LDL; 10 CuSO4; 500 μM MPPG; 37° 14 mm. Lane 14: LDL; 10 CuSO4; 50 μM MPPG; 37° 26 mm.

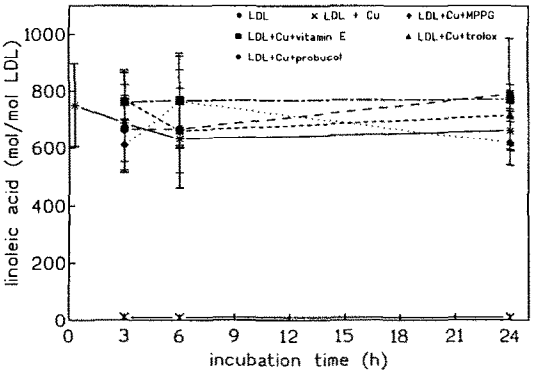

Fig. 5. Comparison of the test substances at a final concentration of 500 μM on the oxidation of LDL. After removing EDTA, the modification of LDL was induced by incubation with 10 μM $CuSO_4$ at 37° for 3, 6 and 24 hr. α-Tocopherol and probucol were dissolved in pure ethanol, the final ethanol concentration for all substances was 5% (v/v). The final LDL protein concentration was 0.2 mg/mL. LDL was isolated by density gradient ultracentrifugation and the LDL-bound fatty acids were detected by GLC as described in Materials and Methods. The data are expressed as means ± SE for four separate experiments with N = 2 each.

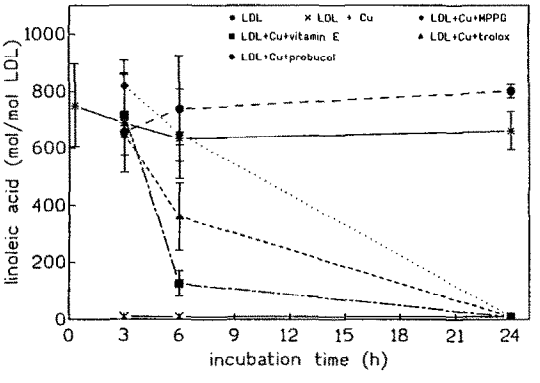

Fig. 6. Comparison of the test substances at a final concentration of 50 μM on the oxidation of LDL. Conditions are the same as in Fig. 5.

DISCUSSION

Different studies show that copper ions modify LDL, forming dienes and thiobarbituric acid-reactive (TBAR) material, accompanied by an increased negative surface charge of the apolipoprotein B-100 [9]. The increasing negative surface charge depends on modification of lysin groups and results in masking LDL for the LDL receptor. The LDL oxidation *in vitro* with Cu^{2+} is a suitable biochemical model, since the content of plasma copper seems to correlate with the risk of arteriosclerosis [18].

In different studies it has been shown that both probucol [10] and α-tocopherol [9] are able to prolong the lag phase during the copper-induced LDL oxidation, but there exists no study comparing these two substances. MPPG, a complex composed of glutamate, pyridoxal-5'-phosphate and magnesium, inhibits the copper-induced LDL oxidation more than its "parent" compound, pyridoxal-5'-phosphate.

Summarizing the efficiency of the drugs the following series in decreasing order is obtained: probucol > MPPG > trolox > α-tocopherol > PP.

The influence of the test substances depends both on concentration and incubation time. It seems that the inhibition of LDL oxidation correlates with a consumption of the test substances. Therefore, both the application dose and the incubation time play important roles. On the other hand, probucol and α-tocopherol are lipophilic substances in contrast to MPPG, PP and trolox. Therefore, α-tocopherol must be compared with trolox because both have the same reactive group, but trolox is missing the lipophilic chain. Over a period of 24 hr trolox is able to inhibit LDL oxidation more effectively than α-tocopherol. The reason could be their different solubility. Probucol and α-tocopherol are comparable, because both are lipophilic and dissolved in the same solvent. MPPG, trolox and PP are comparable, as well. The mechanism by which MPPG inhibits the oxidation is not yet known. A possible mechanism has been described by Meyer *et al.* [13]: MPPG reacts as a radical scavenger or through the binding of reactive aldehydes as Schiff's base. The possibility that MPPG binds copper can not be excluded, but no direct results point to this fact.

The copper-induced oxidation of LDL *in vitro* seems to start at the apolipoprotein B-100. The following results support this assumption.

(a) In different systems apolipoprotein B-100 obtained an increasing negative surface charge although the fatty acids remain intact. Incubation of LDL with 10 μM copper in the presence of 500 μM α-tocopherol, 500 μM trolox or 500 μM MPPG resulted in an increased electrophoretic mobility by about 50% after 24 hr (see Fig. 4); at the same time no decrease of linoleic acid was detectable (see Fig. 5). The substance itself did not increase the electrophoretic mobility (see Fig. 4).

(b) Copper ions (10 μM) did not oxidize free linoleic acid over a period of 6 hr. However LDL-bound linoleic acid decreased in the presence of copper ions by approximately 50% after 3 hr and 100% after 6 hr.

These results point to the possibility that copper reacts with apolipoprotein B-100, generating reactive species, which finally oxidize LDL-bound linoleic acid.

Through the reaction of amino acids with copper ions α-ketoacids, aldehydes and hydroperoxides are produced. All these substances are thiobarbituric acid-reactive (TBAR) [19]. Most of the working groups only take the TBAR-system as an indicator for lipid peroxidation in LDL. But in this system both the peroxidation products of the fatty acids and of the amino acids are reactive. Furthermore free or complexed iron- or copper-ions do not oxidize free fatty acids [9].

Binding of copper ions to proteins or amino acids as described by Kuzuya *et al.* and references therein [17] results in no change of the redox potential of copper. ESR experiments show the binding of copper ions to the apolipoprotein B-100 [20]. Furthermore, the apolipoprotein B-100 has ligands for copper [17] and after 60 min incubation with copper, 28 of 37 tryptophan side-chains are oxidized [21]. Through the reaction of free radicals with proteins, protein hydroperoxides may arise [22], which eventually reduce copper. This seems necessary for the oxidation of fatty acids.

EDTA or other chelaters inhibit the oxidation of amino acids [19] and LDL oxidation by copper. Therefore, the copper-binding to the apolipoprotein B-100 seems to be essential [9]. Furthermore, histidin and BSA, which bind copper, prevent LDL oxidation [23].

Hydroxylradicals and hydroperoxyradicals also lead to an extensive oxidation of polyunsatured fatty acids in LDL, but not to a change of the uptake of this LDL by macrophages [24, 25]. The uncontrolled uptake of LDL through the scavenger receptor requires oxidation of the apolipoprotein B-100 and not necessarily the oxidation of the fatty acids [26]. For a disturbed interaction between LDL and the LDL-receptor the exchange of one amino acid, arginine instead of glutamate on position 3500 in apolipoprotein B-100, seems to suffice [27]. In conclusion apolipoproteins of healthy people and arteriosclerosis patients seems to be different [28], so the apolipoprotein level is also an indicator for the risk of arteriosclerosis in addition to cholesterol, HDL and LDL.

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