

Binding and Uptake of 125 Iodine-Labelled, Oxidized Low Density Lipoprotein by Macrophages: Comparison of the Effects of α -Tocopherol, Probucol, Pyridoxal-5'-phosphate and Magnesium-pyridoxal-5'-phosphate-glutamate

Daniela Selmer^a, Reingard Senekowitsch-Schmidtke^b, W. Schneider^c and E. F. Elstner^a

^a Technische Universität München, Lehrstuhl für Phytopathologie, 85350 Freising-Weißenstephan

^b Nuklearmedizinische Klinik der Technischen Universität München and

^c Steigerwald Arzneimittel, Darmstadt

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Macrophage, Low Density Lipoprotein

Specific and unspecific binding and uptake (internalization) by macrophages of 125 Iodine-labelled, copper-oxidized human low density lipoprotein is differently influenced by the antioxidants α -tocopherol (α -Toc), probucol (Prob), pyridoxal-5'-phosphate (PP) and the magnesium-pyridoxal-5'-phosphate glutamate complex (MPPG). Binding as well as internalization, mediated by the so-called "scavenger receptor" is lower in the presence of MPPG whereas both specific binding and internalization are enhanced. The comparison of the effects *in vitro* allows a rating of the potentially anti-atherogenic and thus protective effects of the tested substances as follows: MPPG > PP > α -Toc > Prob.

Introduction

Oxidation of low density lipoprotein (LDL) and uptake of oxidized LDL by the so-called scavenger receptor of macrophages (Brown and Goldstein, 1979, 1985) is thought to represent one initial step in atherogenesis (Steinbrecher *et al.*, 1989; Esterbauer *et al.*, 1988, 1990, 1991a). Oxidation of LDL may occur enzymatically by lipoxygenase, by reactive oxygen species produced by activated neutrophils, by stimulated endothelial cells or by transition metal ions such as chelated iron or copper (Jürgens *et al.*, 1987; Esterbauer *et al.*, 1988, 1992a; Kuzuya *et al.*, 1991, 1992). Oxidation of LDL has been shown to be inhibited or delayed by antioxidants such as α -tocopherol (Jessup *et al.*, 1990; Esterbauer *et al.*, 1989, 1991b, 1992b, 1993c) or probu-

col (Parthasarathy *et al.*, 1986; Nagano *et al.*, 1991; Kuzuya and Kuzuya, 1993; Kuzuya *et al.*, 1993; Faulkner *et al.*, 1993). Kögl *et al.*, (1994) reported recently that the magnesium-pyridoxal-5'-phosphate-glutamate complex (MPPG) similar to α -tocopherol and probucol inhibited LDL oxidation. In this work electrophoretic mobility was taken as a measure for LDL oxidation and its inhibition. In this communication we used 125 I-labelled LDL in order to directly follow specific and "unspecific" LDL-binding as well as internalization by the adherent murine-leukemic macrophage cell-line RAW 264,7 of LDL_{ox}. Again, the antioxidative activities of α -tocopherol (Esterbauer *et al.*, 1991b), probucol (Kuzuya and Kuzuya, 1993), magnesium-pyridoxal-5'-phosphate-glutamate (Meyer *et al.*, 1992) and pyridoxal-5'-phosphate were compared as to their effects to modify specific and "unspecific" binding by the scavenger receptor and finally internalization after specific binding as compared to total uptake by the used cell-line.

Materials and Methods

Macrophage cell cultures

RAW 264, 7, an adherent monocytic murine leukemic cell-line stems from the ascites of a virus-

Abbreviations: MPPG, magnesium-pyridoxal-5'-phosphate-glutamate; PP, pyridoxal-5'-phosphate; α -Toc, α -tocopherol; Prob, probucol; LDL, low density lipoprotein, LDL_{ox}, Cu-oxidized LDL; LPDS, lipoprotein-free serum; NBS, N-bromosuccinimide; ELAM, endothelial leukocyte adhesion molecule; EDRF, endothelium-derived relaxation factor = NO; MCP-1, monocyte chemoattractant protein; MCSF, monocyte colony stimulating factor.

Reprint requests to Prof. Elstner.
Fax: 08161/71 45 38.

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infected tumor (Abelson-Leucemia-Virus; Raschke 1978). It grows as a monolayer and contains LDL receptors. The cells were stimulated with lipoprotein-free serum, LPDS, leading to an up-regulation of LDL-receptors. More details are described by Selmer (1994).

Isolation and ^{125}I -labelling of LDL

LDL was isolated from the blood serum of six human volunteers by ultracentrifugation essentially as described by Havel *et al.* (1953) as modified by Kögl *et al.* (1994).

Radioactive labelling of LDL was performed according to Sinn *et al.* (1988). Briefly, ^{125}I -labelling by electrophilic substitution using N-bromosuccinimid (NBS) as oxidant at pH 7.5 was done under the following conditions where the reaction mixture contained:

1 μl LDL (1 $\mu\text{g}/\mu\text{l}$)
15 μl Na^{125}I (1.5 mCi) in 1 N NaOH
4.5 μl NBS (1 mg/ml).

The reaction was performed at room temperature and NBS was added in two steps after 7 min. Radioactive labelling efficiency and the specific activity of the ^{125}I -LDL was determined after chromatographic separation of a portion (1 μl) of the reaction product on silica gel (running distance; 10 cm; running time: 15–20 min). The solvent used was: 65% acetone; 20% *n*-butanol; 10% ammonia and 5% water. The iodine-labelled LDL remains at the starting point and the radioactivity at the solvent front represents unreacted, free ^{125}I iodine.

From the signals of the radiation detector the yields of incorporation of ^{125}I into LDL can be calculated. Following this procedure, the protein content of the labelled LDL was determined according to Bradford (1976).

Preparation of lipoprotein-free serum (LPDS)

Uptake of LDL by macrophages is strongly increased after their treatment with LPDS 48 h before LDL exposure due to the upregulation of their LDL receptor density. There seems to be no influence on the scavenger receptor after this upregulation (Esterbauer *et al.*, 1992a).

Human plasma was centrifuged for 36 h at 4 °C in KBr solution of a density of 1.215 g/ml. Lipo-

proteins accumulate in the upper half of the centrifuge tube and were discarded. The lipoprotein-free fraction was dialyzed for 72 h at 4 °C against isotonic NaCl-solution and per ml of plasma solution 10 U of thrombin (Sigma, 82039 Deisenhofen; U = NIH-units as defined in the 1996 Sigma-catalogue p. 1038) were added in order to precipitate the LDL content. After centrifugation (22000 \times g; 2 h; 4 °C) the serum underwent sterile filtration and after protein determination (see above) the protein content was adjusted to 50 mg/ml and stored at -20 °C. "Quality-control" according to lipoprotein deficiency was done electrophoretically.

LDL oxidation

After its isolation from human serum LDL is stored in 150 mM trishydroxymethyl-aminomethane (Tris)-0.3 mM EDTA-buffer, pH 7.5. Concentration of diluted LDL is achieved by Centricon-100-filters (AMICON, 58453 Witten) with subsequent sterile-filtration (Millipore, porewidth 0.45 μm). For copper-catalyzed oxidation the copper chelator, EDTA, has to be removed quantitatively by buffer change via exhaustive dialysis against phosphate buffer pH 7.5 with Visking MG Cutoff 3000 dialysis tubes (SERVA). Stimulation by LPDS occurred 48 h before the oxidation.

Experimental conditions during the oxidation:

500 μg of unlabelled LDL were incubated under the following conditions:

Incubation time: 3 h.

Temperature: 37 °C or 4 °C.

Additions in 2 ml: ^{125}I -LDL (10 $\mu\text{g}/\text{ml}$);

100 μg CuSO_4 .

Effectors to be tested (all 100 μM): MPPG, PP, α -Toc, Prob.

The experiments were performed in titerplates with 3 ml holes containing the 5d cultured adherent RAW 264,7 macrophage cells. Each experiment was done with 6 replicates. The numbers presented under results are means of these six parallel experiments where the bars represent standard deviation (SD), which was between 5 and 10% but always under 10%.

LDL-binding and internalization by macrophages

Differentiation of specific and unspecific binding of ^{125}I -labelled LDL have been studied as outlined by Repke and Liebmann (1987).

Generally it can be assumed that biological membranes only contain a very limited number of specific receptors. Binding of special radioligand molecules thus follows asymptotic saturation kinetics whereas unspecific binding or attachment to cell surfaces is supposed to occur linearly with concentration, since the number of unspecific binding sites by far outnumbers the concentration of specific sites. The affinity towards a specific ligand, however, is much higher for the specific receptors as compared to the unspecific binding sites. Therefore, specifically bound ^{125}I -labelled LDL is less subject to dislocation by high concentrations (10-1000 fold excess) of unlabelled LDL as compared to unspecifically bound, labelled LDL.

Receptor binding studies were done at two temperatures, namely at 4 °C (reduced metabolic activity but with increased LDL receptors) and at 37 °C (high physiological metabolic activity) assuming that LDL-binding to the cell surface is relatively independent of metabolic energy and thus temperature independent as compared to energy-dependent ("active transport") internalization.

The following incubation media have been used: Medium I (4 °C): Dulbeccos modified eagle medium (DMEM) in 20 mM (N-(2-hydroxyethyl) piperazine-Ny2-ethanesulfonic acid)) (Hepes) pH 7.4 buffer

Medium II (37 °C): DMEM in 20 mM hydrogencarbonate buffer.

Both medium I and II contained:

1% non-essential amino acids

1% sodium pyruvate

1% penicillin/streptomycin or gentamycin

5% LPDS.

10 µg/ml ^{125}I -LDL and the corresponding amount of unlabelled LDL in a total volume of 3 ml.

5 d before starting the experiment the RAW 264.7 cells are implanted into 6 different holes (ca. $4-5 \times 10^4$ cells/hole) of the culture plates. At the day of the experiment the cells are in the exponential growth phase. 48 h before start of the experiment the cells were stimulated with LPDS.

The experiment is started by addition of oxidized, labelled ^{125}I -LDL (10 µg/ml) either in the presence or absence of different concentrations of the antioxidants under investigation.

For the binding studies the following, precooled (4 °C) buffers are necessary:

Buffer A: 150 mM NaCl; 50 mM Tris-HCl pH 7.4;

Buffer B: 150 mM NaCl; 50 mM Tris-HCl pH 7.4; 1 mg/ml BSA (freshly prepared)

Buffer C: 50 mM NaCl; 10 mM Hepes pH 7.4; 10 mg/ml heparin

For the 4 °C experiment, the titerplates are precooled 30 min before the experiment; the 37 °C cell cultures are kept at room temperature. At the start of the experiment the culture media are gently removed and exchanged by the experimental media (for 37 °C or 4 °C experiments) followed by a 3 h incubation at 37 °C or at 4 °C. Then the experimental media are removed and the cells are washed 3 times with buffer B containing BSA; then the layers are washed with buffer A followed by an incubation period of 1 h in buffer C.

Buffer C contains heparin which inhibits competitively the binding of LDL to the LDL receptor by binding itself to apolipoprotein B-100. After the incubation 500 µl buffer C is taken from each well and measured for 1 min in the gammacounter in order to estimate the portion of the heparin-soluble membrane-bound LDL.

The monolayers are then washed again with (1 ml per well) buffer A followed by the desintegration procedure of the cells by addition of 1 ml 0.1 N NaOH (30 min, 25 °C) yielding a homogeneous solution. Internalized LDL is determined by measuring 500 µl/well of the NaOH-solution in the gammacounter. Another 500 µl are used for protein determination (Lowry *et al.*, 1951). The data obtained by the gammacounter measurements are converted into ng LDL bound or internalized as follows:

$E = B/T \times \text{ng LDL/mg protein, where}$

$E =$ calculated amount of internalized or bound LDL (ng)/mg cell protein

$B =$ detected amount of internalized or bound LDL

$T =$ detected amount of overall activity in 10 µg LDL/ml added corresponding to total amount of added protein.

Unspecifically bound ^{125}I -LDL is determined by addition of increasing amounts (in µg: 0, 20, 25, 50, 100, 150, 300, 600) of unlabelled LDL in the presence or absence of increasing amounts of CuSO_4 (in µM: 0, 10, 50, 100, 500).

Results

Binding and internalization at 37 °C and 4 °C

The time course of binding and internalization of ^{125}I -labelled LDL was followed after 48 h stimulation with LPDS for 24 h. After 0.5, 1, 2, 3, 4, 5, 6, 8, and 24 h after start of the experiment both specific and unspecific binding as well as internalization at 4 °C and at 37 °C was determined after adding a 50-fold excess of unlabelled LDL. Up to 3 h the cells linearly bind and take up LDL at 37 °C. After this time binding and uptake are decreased (Selmer, 1994). After 3 h at 37 °C or at 4 °C the following values (ng ^{125}I -LDL/mg protein) have been determined (Table I).

Table I. Binding and internalization of LDL (ng/mg cell protein) at 37 °C and 4 °C.

Temperature	37 °C	4 °C
Total binding:	70.99+/- 7.23	70.99+/- 7.12
Specific binding:	65.43+/- 5.9	65.91+/- 6.44
Total internalization:	250.42+/-23.68	115.01+/-11.24
Specific internalization:	223.40+/-20.38	85.37+/- 7.34

Binding at 37 °C or 4 °C in this experiment was almost identical whereas internalization is less than half at low temperature. The difference between higher total and lower specific internalization is clearly expressed at 4 °C where binding is unchanged as compared to 37 °C.

Displacement of ^{125}I -LDL by unlabelled LDL

In order to obtain reliable dislocation of bound untreated and copper-oxidized LDL from unspecific binding sites an excess of unlabelled LDL was applied. For this purpose 10 μg ^{125}I -LDL administered was displaced by a 50-fold excess i.e. 500 μg /ml cold LDL from LPDS-stimulated cells kept at 37 °C or 4 °C (Table II), in order to differentiate between unspecific and specific binding and internalization of LDL and LDL_{ox} .

From Table II one can deduce that copper-oxidation of LDL strongly decreases both specific binding as well as specific internalization. From the data for total binding and internalization increased values of unspecific (scavenger receptor-mediated) binding and internalization can be calculated.

Effects of different antioxidants on LDL binding and internalization by the adherent macrophage cell line RAW 264,7

In the following the effects of different antioxidants on LDL-binding and internalization after copper treatment of LDL was investigated.

Effects of MPPG

The experiment was conducted as follows: part of the administered LDL was preincubated 3 h before starting the cell binding studies

- (A) without additions,
- (A+B) with 100 μM CuSO_4
- (A+C) with 100 μM MPPG and
- (A+B+C) with 100 μM CuSO_4 and 100 μM MPPG.

After this incubation, the additions were removed by buffer change (see Materials and Methods, 4.). Unspecific binding and internalization of ^{125}I -LDL was determined after 3 h incubation with RAW 264,7 cells by the addition of 50-fold excess of cold LDL. The results of this binding experiment is shown in Fig. 1.

As shown in Fig. 1 both total binding and total internalization of LDL is stimulated whereas after Cu-oxidation (100 μM CuSO_4 , LDL_{ox}) specific binding or internalization is reduced (A+B) indicating an unspecific binding and uptake by the scavenger receptor. MPPG (100 μM) in the absence of CuSO_4 has no effect on internalization but reduces both total and specific binding indicat-

Copper-conc. [μM]	Temperature [°C]	Internalized (μg /mg protein +/-10% SD)		Membrane-bound (SD)	
		Total	Specific	Total	Specific
0	37	61.14	35.04	8.58	2.36
500	37	48.26	5.66	8.09	1.03
0	4	30.07	16.21	16.38	5.54
500	4	24.76	4.08	17.01	2.06

Table II. Displacement of untreated and oxidized LDL.

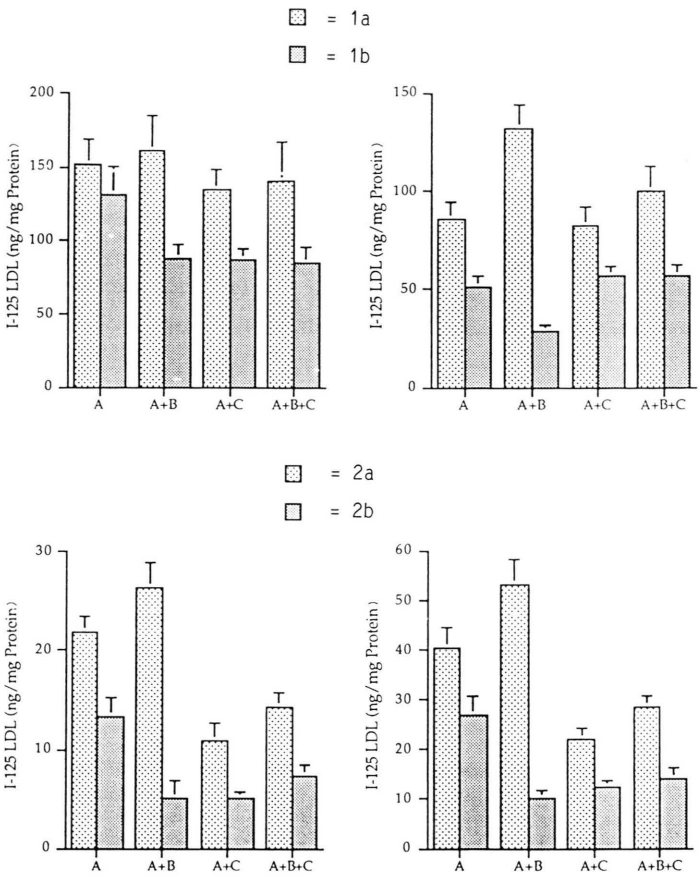


Fig. 1. Influence of MPPG on binding and internalization of Cu-oxidized ¹²⁵I-LDL by macrophages at 37 °C (left side) or 4 °C (right side).
1a = total internalized;
1b = specifically internalized;
2a = total bound;
2b = specifically bound.
A, A+B, A+C, A+B+C = LDL, LDL_{ox}, LDL+MPPG, LDL_{ox}+MPPG, respectively (LDL_{ox}= LDL+100 μM CuSO₄).

ing that MPPG does not influence autoxidation of LDL during incubation (A+C). MPPG clearly reduces both unspecific binding and internalization of Cu-oxidized LDL (visible as reduction of total binding and internalization) where the effects are clearer at 4 °C than at to 37 °C. At 4 °C total and specific internalization decreased to 29 ng. MPPG decreased the copper effect by reducing total internalization down to 100 ng and increasing specific internalization up to 56 ng, i.e. slightly above control-(51 ng)levels. Similar results are obtained for binding.

The effects of MPPG are concentration-dependent. Since LDL binding to the cells is favoured at low temperature (4 °C) and internalization at higher temperature (37 °C) the protective properties (A+B+C in Fig. 1) are compared for MPPG on a per cent basis with LDL+Cu (A+B).

From Table III we learn that increasing concentrations of MPPG stimulate increasingly both specific

binding as well as internalization while total (and thus unspecific) binding and internalization are reduced.

Effects of PP

Similar to MPPG, PP also influences total binding and internalization of Cu-oxidized LDL both at 37 °C and 4 °C; the effects are less pronounced,

Table III. Comparison of the effects of different concentrations of MPPG on LDL-binding at 4 °C and LDL internalization at 37 °C (per cent of control: LDL + CuSO₄).

MPPG-concentration [μM]					
10	100	500	10	100	500
Binding at 4 °C (5)			Internalization at 37 °C (%)		
(specific)			(specific)		
120	141	194	85	96	112
(total)			(total)		
61	53	50	107	87	86

however (data not shown; see however summarizing Table IV with respective percentage data).

Effects of α -Tocopherol

The presence of (100 μM) α -tocopherol during copper-catalyzed LDL oxidation only increases specific internalization both at 37 °C (from 23ng to 49ng) and 4 °C (from 13ng to 30ng) but neither total binding nor internalization were reduced (data not shown; see however Table IV).

Effects of Probucol

As shown in Fig. 2 addition of Prob to the Cu-LDL oxidation experiment clearly reduces both total binding and total internalization whereas effects on the "specific" properties of Cu-oxidized LDL are scarcely pronounced. An increase by Prob of oxidation-resistance in the absence of copper, however, may be especially expressed as

increased specific binding of LDL at both temperatures namely, A=2.5ng as compared to A+F=4.1ng at 37 °C and A=5.2ng as compared to A+F=7.3ng at 4 °C (standard deviation always between 5 and 10%).

Discussion

According to Brown and Goldstein (1979), Esterbauer *et al.* (1992b), Reid and Michinson (1993) and Steinbrecher *et al.* (1989, 1990), the following properties of oxidized LDL and thus consequences for atherogenesis have to be envisaged:

- binding to scavenger receptor and consequently formation of fatty streaks, foam cells, liberation of tissue factors and increased thrombocyte aggregation and increase of LDL infiltration;
- inhibition of the action of EDRF (NO) and thus contraction of blood vessels;

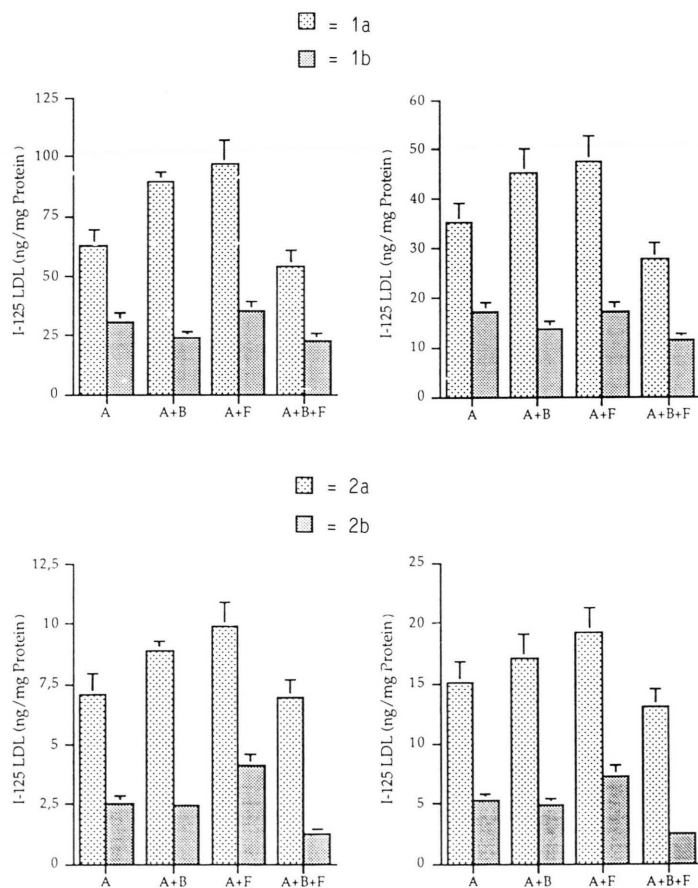


Fig. 2. Influence of probucol on binding and internalization of Cu-oxidized ^{125}I -LDL by macrophages at 37 °C (left side) or 4 °C (right side). (for details see legend of Fig. 1).

- c) stimulation of the membrane-expression of ELAMs in endothelial cells and liberation of MCP-1 and MCSF; as a consequence differentiation and adhesion of macrophages to endothelial cells followed by their immigration into the intima is observed;
- d) inhibition of the mobility of macrophages and thus diminished transport of cholesterol from the intima by emigrating cells;
- e) stimulation of monocyte-derived Interleukin-1 and thus multiplication and activation of both B- and T-lymphocytes and thus increase of immune reactions;
- f) oxidized LDL is immunogenic and thus provokes formation of antibodies and immune complexes supporting foam cell formation.

Inhibitor of LDL oxidation and/or unspecific binding and internalization in macrophages is thus supposed to decrease atherogenic properties. Inhibitory properties have been reported for α -Toc

Table IV. Comparism of the effects of (100 μ M each) MPPG, PP, α -Toc and Prob on binding and internalization of Cu-oxidized LDL by macrophages at 37 °C and 4 °C.

a) Total binding and internalization (% of control):				
Effector	Binding		Internalization	
	4 °C	37 °C	4 °C	37 °C
MPPG	53	54	76	87
PP	51	50	52	74
α -Toc	118	106	128	105
Prob	76	78	62	61

b) Specific binding and internalization (% of control):				
Effector	Binding at		Internalization at	
	4 °C	37 °C	4 °C	37 °C
MPPG	141	142	195	96
PP	80	95	136	55
α -Toc	51	70	213	208
Prob	51	50	82	96

(Esterbauer *et al.*, 1991a), Prob (Kuzuya *et al.*, 1991, 1993) and MPPG (Kögl *et al.*, 1994) where the protective effects most probably are based on both different mechanisms and localization within the LDL molecule.

The adherent macrophage cell line RAW 264,7 in most experiments binds ca. 50% more LDL at 4 °C as compared to 37 °C. This effect is presumably brought about by an increased number of accessible receptors at low temperature. In contrast, internalization of LDL is about double as fast at 37 °C as compared to 4 °C. This effect is due to the increased metabolic activity at the higher temperature.

In the following we should like to compare the properties of MPPG, PP, α -Toc and Prob at both 37 °C and 4 °C. Although three different concentrations (10 μ M, 100 μ M and 500 μ M) have been tested (c.f. Table III) only the results obtained with 100 μ M concentration are shown. In order to render the data comparable, per cent data are given instead of the absolute, molar concentrations of LDL bound or internalized.

As can be seen from Table IV α -Toc supports both total binding and internalization of LDL as well as specific internalization. MPPG, PP and Prob reduce total and thus unspecific binding and internalization of LDL. Reduction of unspecific binding together with an increase of specific internalization, according to the present hypothesis, is supposed to represent a desirable effect since this combination may warrant controlled cholesterol transport from the blood vessels and suppressed import of LDL-laden macrophages into the intima. These desirable effects are especially represented by the antioxidative Schiff-base MPPG (Meyer *et al.*, 1992) and in part also by the other tested substances, pyridoxalphosphate and probucol.

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