Free Rad. Res. Comms., Vol. 8, No. 3, pp. 167-173
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Printed in the United Kingdom

17 BETA-ESTRADIOL INHIBITS LDL OXIDATION AND CHOLESTERYL ESTER FORMATION IN CULTURED MACROPHAGES

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(Received August 10, 1989; in revised form September 25, 1989)

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The effects of 17 beta estradiol, testosterone, the estradiol benzoate, and probucol on the oxidation kinetics of low density lipoprotein (LDL) *in vitro* in absorption presence of 10 μM Cu (II) are examined. Changes in the absorption at 234 nm (A 234) and fluorescence (Ex340/Em420) are monitored. The kinetics of the changes observed let us suggest a precursor-product relationship between dienes and fluorochromes in the oxidized LDL. The addition of 17 beta estradiol and probucol to LDL results in a prolongation of the lag phase characterized by only insignificant formation of dienes and fluorochromes. The addition of testosterone and estradiol benzoate used as control compounds has no effect on the lag phase and thus no LDL stabilizing effect.

Conditioned LDL which was incubated in F-10 medium before exposure to cultured P388D.1 macrophages increases the formation of cytoplasmic lipid droplets and of cellular cholesteryl esters. The LDL stabilizing compounds beta estradiol and probucol (but not testosterone) cause a reduction of the cholesteryl ester content of the cultured macrophages. Protection of LDL particles against oxidative damage apparently results also in lowering of cytoplasmic cholesteryl ester in cultured P388D.1 cells. We conclude that the known antiatherosclerotic potency of 17 beta estradiol may in part result from its LDL stabilizing activity.

KEY WORDS: Low density lipoprotein, atherosclerosis, macrophages, estradiol, probucol, antioxidants.

ABBREVIATIONS: CE, cholesteryl ester; P, protein; LDL, low density lipoprotein.

INTRODUCTION

Oxidative modification of low density lipoprotein (LDL) by aortic wall cells and subsequent uptake of oxidized LDL by intimal macrophages was suggested to be involved in the conversion of macrophages to cholesteryl ester rich foam cells in the early atherosclerotic lesion.[1]

It was reported that probucol, a highly lipophilic antioxidant transported in plasma

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lipoproteins increases the resistance of LDL against reactive oxygen and blocks foam cell formation by its antioxidant activity in the hyperlipidemic WHHL rabbit.[2,3] Based on these observations, a search for other compounds increasing the stability of LDL could be attractive.

Interestingly, protection of the aortic wall was reported without any alteration in plasma lipoprotein levels under treatment with high doses of 17 beta estradiol in cholesterol fed rabbits. It was shown that arterial influx of cholesteryl esters per cm^2 of aorta per hour was significantly lower in estradiol treated than in the untreated rabbit although the disappearance of labelled lipoproteins from plasma was similar.[4]

It is not known whether sex hormones are stabilizers of low density lipoprotein and whether such an activity could explain some of the antiatherosclerotic activity of 17 beta estradiol in cholesterol fed rabbits. The present studies were undertaken to test for this possibility by utilizing the *in vitro* LDL oxidation system with copper as redox metal as described by Steinbrecher *et al.*[5] and Esterbauer *et al.*[6] to analyse the influence of sex hormones on the LDL oxidation and on LDL oxidation-dependent cholesteryl ester deposition in a macrophagocytic cell line. In using these techniques, we have obtained information about an LDL stabilizing effect and cholesteryl ester reducing activity in macrophages by 17 beta estradiol but not by testosterone (used as a control compound).

METHODS

LDL and LDL Oxidation

Human plasma LDL was prepared by sequential ultracentrifugation at preselected densities as described.[7] The LDL preparation was from a plasma pool obtained from four healthy normolipidemic blood donors. LDL was dialysed against 50 mM Tris/HCl, pH 8, 0.15 M NaCl, 0.1 mM EDTA and stored under nitrogen. The concentration of LDL protein was determined according to Lowry with bovine serum albumin as standard.[8] Before use in oxidation assays LDL was dialysed repetitively against EDTA-free buffer under nitrogen. The oxidation of LDL was followed in a 2 ml assay at 37 °C containing 125 μg/ml (= 0.25 μM) LDL, 10 μM copper chloride in 50 mM Tris/HCl, 150 mM NaCl in plastic tubes. The appearance of dienes was followed as A 234; fluorochromic lipid peroxidation products were detected at Ex 340/Em 420 as described by Esterbauer.[6]

Macrophage Culture

P388 D.1 macrophagocytic cell line was obtained from Dr. Via, Baylor College of Medicine, Houston, Texas. P388 D.1 cells were grown in RPMI-1640 with glutamine, penicillin, streptomycin, and 5 % fetal calf serum (FCS) as described.[9] To test for LDL oxidation dependent formation of cholesteryl esters the P388 D.1 cells were incubated for 18 hours in F-10 medium (containing 2.2 μM Cu^{2+}) + 5 % FCS + 50 μg/ml conditioned LDL and RPMI 1640 + 5 % FCS + 50 μg/ml conditioned LDl as control. LDL was conditioned by 5 hour preincubation in F-10 medium at 37 °C.

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Cholesterylester Determination

The neutral lipids were extracted from the cultured P388 D.1 cells as described and determined according to Schmitz *et al.* by thin layer chromatography and fluorescence detection.[10] Cells were washed three times with F-10 medium and sonicated. One aliquot was used for protein determination according to Lowry, another aliquot containing 500 μg protein was delipidated by the modified Folch procedure. The evaporated samples were dissolved in chloroform, applied to HPTLC plates and separated in n-hexane-n-heptane-diethylether-acetic acid 63:18,5:18:5,1 (v/v). The lipid spots were detected by a manganese chloride sulphuric acid reagent and quantified by a CAMAG-TLC scanner.

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RESULTS

Kinetics of LDL Oxidation as Determined by Spectroscopy

LDL oxidation was studied by following the appearance of conjugated dienes at 234 nm and the formation of fluorochromic lipid-protein conjugates as described previously.[6] LDL was incubated in presence of Cu (II) as pro-oxidant. 17 beta estradiol, estradiol benzoate, testosterone, and probucol as a control antioxidant were added to test the LDL stabilizing activity of the compounds.

Incubation of LDL with Cu (II) results in formation of detectable amounts of lipid-peroxidation products. Figure 1 shows the changes observed at 234 nm and subsequent generation of fluorochromes. Within 60 minutes of the incubation there was a slight linear increase of A 234 nm. After 105 minutes a propagation reaction began leading to a rapid 3.5-fold increase of absorbance and a plateau at A 234 = 1.05 within 80 minutes. Fluorochromes in the LDL oxidation assay were very low at the beginning of the incubation. Shortly after the addition of Cu (II) only a slight but constant increase of fluorescence was recorded. However after 135

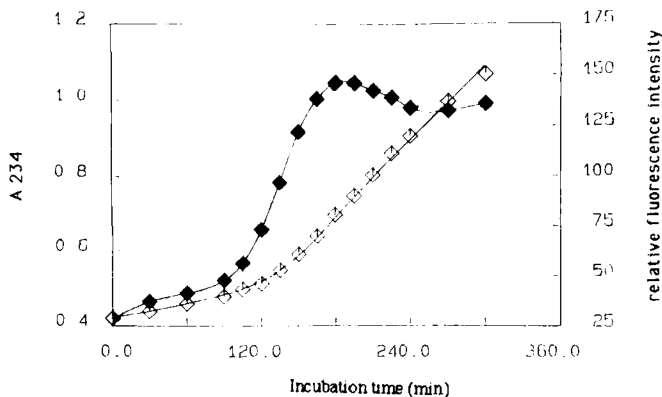

FIGURE 1 Kinetics of LDL oxidation in presence of Cu^{++} as determined by formation of dienes and fluorochromic lipid peroxidation products. 0.25 μM LDL in 50 mM Tris/Cl, pH 8, 150 mM NaCl, 10 μM Cu Cl_2 was incubated at 37 °C. ◆ Observed A 234 kinetics, curve interpolated by a cubical spline function. ◇ Observed E 340/Em 420 kinetics and curve constructed by formal integration of A 234's interpolating spline function.

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minutes, 20 minutes after the beginning of the A 234 propagation reaction, the rate of the fluorochrome formation increased. A cubical spline function was generated to interpolate the A 234 data obtained by LDL oxidation.[11] To investigate a possible precoursor-product relationship between the formation of A 234 nm oxidation products and the formation of the Ex 340/Em 420 fluorescent products we integrated the spline function containing the A 234 data. The integral of the A 234 profile describes accurately the kinetics of the fluorescent products and is providing direct evidence for the precursor-product relationship as expected.

LDL Stabilizing Activity of 17 Beta Estradiol, Estradiol Benzoate, Testosterone, and probucol

Figure 2 characterizes LDL that is oxidized in the presence of test compounds and tested for the kinetics of oxidation of lipids by following the absorbance at 234 nm. Four compounds are compared: testosterone, probucol, 17 beta estradiol, and estradiol benzoate. The compounds were added to the LDL at the beginning of the incubation at a final concentration of 0.25 μM–7.5 μM to test their antioxidant activities at molar ratios between 1:1 and 30:1 molecules per LDL particle. The addition of testosterone (0.25 μM–2.5 μM) did not alter the characteristic biphasic oxidation of 0.25 μM LDL solution. No effect on the lag phase (90 minutes) and the slope of the curve during the rapid propagation reaction could be demonstrated

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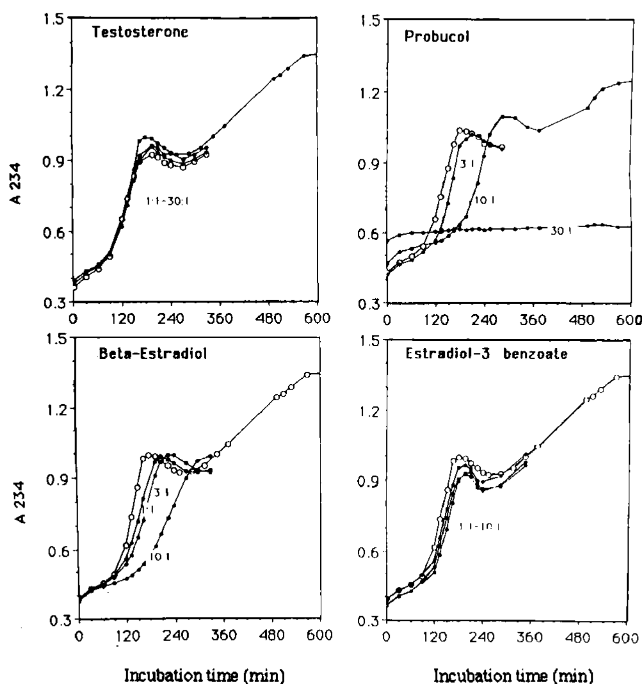

FIGURE 2 LDL oxidation and influence of testosterone, probucol, beta-estradiol, and estradiol-3 benzoate on the kinetics of LDL-oxidation in presence of copper. Assay conditions were as in Figure 1, except that testosterone, probucol, beta-estradiol, and estradiol-3 benzoate were supplemented: ○, 0.25 μM LDL as control; ●, 0.25 μM (1:1), 0.75 μM (3:1), 2.5 μM (10:1), 7.5 μM (30:1) of test compount present.

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(Figure 2A). Probucol, while completely eliminating oxidation of LDL at a concentration of 7.5 μM (30 molecules per LDL particle), did not prolong the lag phase at 0.25 μM at lower concentrations (1 molecule per LDL particle). In presence of 0.75 μM and 2.5 μM probucol the lag phase was prolonged (+ 24 min. and + 80 min.), (Figure 2B). Figure 2C shows the apparent LDL stabilizing activity of 17 beta estradiol. The addition of 0.25 μM 17 beta estradiol (1 molecule per LDL particle) results in an increase of the lag phase (+ 16 min). Furthermore, a concentration dependent prolongation of the lag phase could be demonstrated with 0.25 μM, 0.75 μM, 1.25 μM, and 2.5 μM 17 beta estradiol. The lag time increased up to 80 minutes in the presence of 2.5 μM 17 beta estradiol. The addition of the same concentrations of estradiol benzoate did not change the lag phase of the LDL significantly (Figure 2D). The fluorochrome formation as a parameter for lipid-protein crosslinks had the same changes in the lag phases as the A 234 profiles for all the test compounds. However, none of the test compounds affected the slope of fluorescence curves during the propagation reaction (data not shown). Thus, we are able to demonstrate a specific antioxidant effect of 1 molecule of 17 beta estradiol. 17 beta estradiol has a higher LDL stabilizing potency than probucol, since it causes a more pronounced prolongation of the lag phase at lower concentration.

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LDL Oxidation-Dependent Formation of Cholesteryl Esters in Cultured Macrophages

A final query concerned the ability of estradiol to inhibit LDL oxidation-dependent formation of cholesteryl esters in macrophages. Formation of cholesteryl esters in the macrophages is increased when modified forms of LDL are taken up avidly via the scavenger receptor.[12] The scavenger receptor recognizes oxidized LDL with high affinity.[13] To test whether antioxidant activity would decrease the formation of cholesteryl esters in macrophages, control LDL, LDL in presence of 10 μM estradiol, and 10 μM testosterone were conditioned by preincubation in F-10 medium to exhaust the endogenous antioxidants. Then conditioned LDL was incubated with P388 D.1 murine macrophages in F-10 medium supplemented with 5 % FCS to measure the cholesteryl ester formation in the cells. To control for the oxidative condition, preincubation of LDL and the subsequent incubation with the macrophages were performed also in RPMI 1640 medium. RPMI 1640 medium does not contain redox-active metals and would therefore not allow oxidation of LDL.[14]

Typically, 100 μg/ml LDL was incubated in the absence and presence of test compounds in F-10 medium and RPMI 1640 medium for 5 hrs at 37 °C. During the 5 hr incubation a slight increase of dienes could be observed, as at the end of the lag phase in the LDL oxidation assay above. To evaluate the potential of the conditioned LDL to stimulate the formation of cholesteryl ester, P388 D.1 cells were plated at 10×10^5 cells per ml in F-10 medium + 10 % FCS and RPMI 1640 + 10 % FCS. LDL was added to achieve a final concentration of 50 μg/ml conditioned LDL, 5 % FCS, and 5×10^5 cells. The assays lasted 18 hrs. As shown in Figure 3 there are apparent differences in the formation of intracellular cholesteryl ester between the macrophages in F-10 medium and the cells in RPMI 1640 medium. Cholesteryl ester formation per cellular protein (CE/P) in F-10 medium was 2.8-fold above control in RPMI 1640 medium in absence of a test compound. The difference was even more pronounced when 10 μM testosterone was present, in which case CE/P was about 4.5 times higher in the F-10 medium as in the RPMI 1640 medium. Since these incuba-

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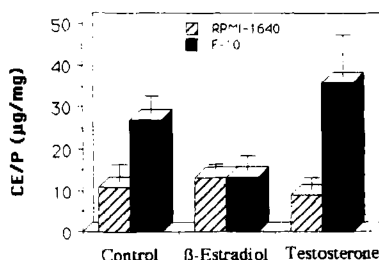

FIGURE 3 Influence of medium and test compounds on cholesteryl ester/protein ratio of P388 D.1 cells. 0.25 μM LDL was incubated for 5 hours in F-10 medium plus or minus 10 μM testosterone or, beta-estradiol. The conditioned LDL was then added to the P388 D.1 cells (3×10^6 cells in 5 ml either F-10 medium or RPMI-1640 medium + 5 % FCS) resulting in a final concentration of 0.1 μM LDL and 10 μM test compound. The cells were incubated for 18 hours, harvested, and extracted for cholesteryl ester and protein determination as described in Material and Methods. Values are means ± standard deviations (SD) from triplicates.

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tions in RPMI 1640 medium appeared to prevent enhanced formation of cholesteryl esters, at least part of the formation of cholesteryl esters may have been due to the oxidative conditioning of LDL in the presence of Cu^{++} ions in the F-10 medium. Indeed, addition of 17 beta estradiol at a concentration of 10 μM to the incubation assays in F-10 medium abolished the enhancement of cholesteryl ester formation in F-10 medium, suggesting that at least part of the estradiol effect observed under our experimental condition may be due to its antioxidant activity. For comparison, probucol in the assay system was less effective than 17 beta estradiol. 10 μM probucol blocked just 50 % of the increase in cholesteryl ester per unit cellular protein in this assay (data not shown).

DISCUSSION

Incubation of LDL with 17 beta estradiol at low molar ratios leads to a modification of LDL resulting in increased stability of LDL particles against reactive oxygen and less cholesteryl ester formation when LDL are subsequently added to culture of macrophages. Testosterone and estradiol benzoate used as control compounds do not have such activity. Probucol, a highly lipophilic antioxidant for LDL particles,[2,3] is less effective than 17 beta estradiol at low molar ratios (1 and 3 molecules per LDL particle). The striking effect of 17 beta estradiol on the stabilizing of LDL at low molar ratios is surprising, since the content of polyunsaturated fatty acids of LDL particles is rather high and the antioxidants are quite low. One LDL particle contains an average 959 molecules 18:2 and 89 molecules 20:4 but only 5–6 molecules vitamin E as major antioxidant and it was demonstrated that only vitamin E depleted LDL is able to undergo lipid peroxidation.[15] Indeed, the quantities of 17 beta estradiol and probucol required to yield increased protection of LDL against oxidation are, like vitamin E, orders of magnitude lower than those of the polyunsaturated fatty acids. It is intriguing to speculate about the higher LDL protective activity of 17 beta estradiol than probucol at the lower molar ratios per LDL particle tested in our assay systems. It could be that 17 beta estradiol is integrated in the LDL at sites where the LDL particles are most sensitive against reactive oxygen species. Currently, it is not known whether such sites with increased susceptibility for reactive oxygen species do

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exist. However, for example Cu^{++} ions may bind to specific sites. Beta estradiol could preferentially interfere with the Cu^{++} dependent initiation of the oxidation process at these sites and act as a "site-specific" antioxidant. It seems also possible that such an "endogenous" oxidase could accelerate the LDL oxidation process when activated. 17 beta estradiol might have a higher inhibitory activity for such a "cryptic" oxidase in the LDL particles than probucol. However, at this point we can just speculate about the mechanisms and further studies about the antioxidant activity of beta estradiol and probucol in LDL will be needed.

P388 D.1 macrophages were reported to possess high numbers of scavenger receptors.[9] Uptake of modified forms of LDL in these cells was reported to cause massive accumulation of cholesteryl esters in cytoplasmic lipid droplets. In the 18 hours macrophage assay, increased cholesteryl ester formation could be prevented by beta estradiol and to a lesser extent also by probucol compared to controls in absence and presence of testosterone. For the evaluation of the maximal compound effect in the macrophage system we preincubated LDL with the antioxidant before the addition to the cells. Under these assay conditions the observed reduction of the LDL-dependent increased cholesteryl ester formation should have been due at least partially to the antioxidant activity of 17 beta estradiol.

We speculate that, at least partially, the observed antiatherosclerotic effects of 17 beta estradiol reported by Hough & Zilversmith[4] could have resulted from its antioxidant activity. Their rabbits were injected with 17 beta estradiol at the rather high dose of 0.5 mg/kg per week. Unfortunately plasma concentrations were not determined in their study. Nevertheless, their high dosages could have given high plasma levels sufficient to stabilize LDL to some extent and reducing its atherogenity.

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References

1. Steinberg, D., Parthasarathy, S., Carew, T.E., Khoo, J.C. and Witztum, J.L., *New Engl. J. Med.*, **320**, 915–924, (1989).
2. Parthasarathy, S., Young, S.G., Witztum, J., Pittman, R.C. and Steinberg, D., *J. Clin. Invest.*, **77**, 641–644, (1986).
3. Carew, T.E., Schwenke, D.C. and Steinberg, D., *Proc. Natl. Acad. Sci. USA*, **84**, 7725–7729, (1987).
4. Hough, J.L. and Zilversmith, D.B. *Arteriosclerosis*, **6**, 57–63, (1986).
5. Steinbrecher, U.P., Witztum, J.L., Parthasarathy, S. and Steinberg, D., *Arteriosclerosis*, **7**, 135–143, (1987).
6. Esterbauer, H., Striegl, G., Puhl, H. and Rotheneder, M. *Free Rad. Res. Comms.***6**, 67–75.
7. Havel, R.J., Eder, H.A. and Bragdon, J.H., *J. Clin. Invest.*, **34**, 1345–1353, (1955).
8. Lowry, O.H., Rosenbrough, N.J., Farr, A.L. and Randall, R.J., *J. Biol. Chem.*, **193**, 265–275, (1951).
9. Via, D.P., Plant, A.L., Craig, I.F., Gotto, A.M., jr. and Smith, L.C., *Biochim. Biophys. Acta.*, **833**, 417–428, (1985).
10. Schmitz, G., Assmann, G. and Bowyer, D.E., *J. Chromatrogr.*, **307**, 81–89, (1984).
11. Späth, H., Spline-Algorithmen, Oldenburg-Verlag, München, Wien (1983).
12. Goldstein, J.L., Ho, Y.K., Basu, S.K. and Brown, M.S., *Proc. Natl. Acad. Sci. USA*, **76**, 333–337, (1979).
13. Parthasarathy, S., *Biochim. Biophys. Acta*, **917**, 337–340 (1987).
14. Steinbrecher, U.P., *Biochim. Biophys. Acta*, **959**, 20–30, (1988).
15. Esterbauer, H., Jürgens, G., Quehenberger, O. and Koller, O., *J. Lipid Res.*, **28**, 495–509, (1987).

Accepted by Prof. H. Esterbauer

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