RESEARCH COMMUNICATION

Protective actions of tamoxifen and 4-hydroxytamoxifen against oxidative damage to human low-density lipoproteins: a mechanism accounting for the cardioprotective action of tamoxifen?

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Tamoxifen and 4-hydroxytamoxifen protect isolated human low-density lipoproteins (LDLs) against copper-ion-dependent lipid peroxidation: 4-hydroxytamoxifen is more protective than tamoxifen or 17 β -oestradiol. 4-Hydroxytamoxifen and 17 β -oestradiol also prevent the increase in the electrophoretic mobility of

LDL caused by exposure to copper ions, presumably by protection of the apoprotein B of LDL against oxidative modification. Our observations may help to account for the cardioprotective benefits reported to be associated with tamoxifen therapy and prophylaxis in breast cancer.

INTRODUCTION

Tamoxifen is an anti-cancer drug that is widely used in the treatment of breast cancer, with considerable success [1–4]. Indeed, it is now being assessed in clinical trials as a prophylactic agent against this disease [5–9]. It is also being used to treat cancers of the liver [10] and brain [11], and may have general application in the prevention and treatment of cancer [12]. Cardiovascular benefits have also been reported in clinical studies [9,13–16]. In breast cancer patients who are treated with tamoxifen the incidence of fatal myocardial infarction was significantly decreased [13].

Both tamoxifen and its metabolite 4-hydroxytamoxifen inhibit lipid peroxidation in rat liver microsomes and ox brain phospholipid liposomes [17–20], and recently they have been shown to protect cardiac microsomes against lipid peroxidation [21]. It is possible that the beneficial cardiovascular effects of tamoxifen are related to its anti-oxidant ability, since the role of lipid peroxidation in cardiovascular injury [22] and in the development of atherosclerosis [23–27] is well documented. Recently, 17 β -oestradiol, which has anti-atherogenic effects in rabbits [28], has been reported to inhibit the peroxidation of low-density lipoproteins (LDLs) [29,30], which is an important atherogenic event [26,27]. We report herewith a comparative investigation of the abilities of tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol to protect LDL against oxidation. Isolated LDL was stimulated to undergo peroxidation by addition of copper ions. This is a widely used experimental system [25–27] that is relevant to events occurring within atherosclerotic lesions [31].

MATERIALS AND METHODS

Materials

Tamoxifen and 17 β -oestradiol were obtained from the Sigma Chemical Co. (Poole, Dorset, U.K.). 4-Hydroxytamoxifen was kindly donated by ICI Pharmaceuticals (Macclesfield, Cheshire, U.K.). All other reagents were of the highest quality and obtained

from the Sigma Chemical Co. (Poole, Dorset, U.K.) or from BDH Ltd. (Dagenham, Essex, U.K.).

Preparation and oxidation of LDL

LDL was isolated from human plasma in a Beckman L-70 ultracentrifuge using a fixed-angle rotor according to the method of Chung et al. [32]. The plasma was centrifuged for 1.75 h at 100000 *g* and then the crude LDL obtained was re-centrifuged for 14 h at 100000 *g* to remove minor protein contaminants. LDL was dialysed against 10 mM PBS, pH 7.4 (containing 10 μ M EDTA), for 4 h. The concentration of protein was determined by a modification of the procedure of Markwell et al. [33] and LDL was used at a final protein concentration of 0.2 mg/ml. The LDL was filtered through a 0.45 μ m-pore-diam. filter after dialysis and stored under N₂ for up to 10 days.

LDL (0.2 mg/ml) in a final volume of 0.5 ml of 5 mM PBS, pH 7.4 (containing 10 μ M EDTA), was incubated at 37 °C with CuCl₂ (final concentration 15 μ M) for 2 h: this incubation time was found to give maximal levels of thiobarbituric acid-reactive substances (TBARS). Tamoxifen, 4-hydroxytamoxifen or 17 β -oestradiol was added in ethanol to give a final concentration of ethanol in the reaction mixture of 0.5% (v/v) and this concentration of ethanol was always used in controls. The reaction was terminated by the addition of 15 μ l of 10 mM butylated hydroxytoluene dissolved in ethanol, which was added at the end of the incubations to suppress peroxidation during the thiobarbituric acid (TBA) test itself. The extent of lipid peroxidation was determined by the TBA assay [34]. Trichloroacetic acid [0.5 ml of 10% (w/v)] was added to the samples on ice, followed by 0.5 ml of TBA [0.75% (w/v) in 0.1 M HCl]. The A₅₃₂ value of the supernatant was measured after heating at 95 °C for 20 min and centrifugation.

Samples (15 μ l) were removed from the incubation mixture before the addition of the TBA assay reagents for the determination of the relative electrophoretic mobility of the LDL. The samples were applied to agarose gels (Beckman Paragon Lipo Gel electrophoresis system) to assess any change in the

Figure 1 Concentration-dependent inhibition of CuCl_2 -dependent LDL peroxidation (measured as TBARS)

LDL (0.2 mg/ml) at pH 7.4 was incubated at 37 °C with 15 μM CuCl_2 for 2 h and peroxidation was measured using the TBA assay [34] as described in the Materials and methods section. The results are expressed as percentage inhibition of peroxidation in the presence of tamoxifen (■), 4-hydroxytamoxifen (●) or 17 β -oestradiol (▲), all in the range 0–30 μM . Results are means $\pm$ S.D., $n = 3$.

Figure 2 Time course of CuCl_2 -induced conjugated-diene formation

Conjugated-diene formation in LDL (0.1 mg/ml) at pH 7.4, incubated at 37 °C with 15 μM CuCl_2 for 1 h, was measured by difference spectroscopy [35] as described in the Materials and methods section and is expressed as A_{234} in the control [containing 0.5% (v/v) ethanol] (●) or in the presence of tamoxifen (■), 4-hydroxytamoxifen (○) or 17 β -oestradiol (▲), all at a concentration of 15 μM . Results are means $\pm$ S.D., $n = 3$.

Table 1 IC_{50} values for inhibition of copper-dependent LDL lipid peroxidation by tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol

Values are deduced from the graphs shown in Figure 1, which show the inhibition of copper-dependent LDL peroxidation, measured as TBARS, by the compounds and in which each point represents the mean $\pm$ S.D. of three separate assays.

Compound	IC_{50} (μM)
Tamoxifen	29.3
4-Hydroxytamoxifen	1.9
17 β -Oestradiol	3.1

Figure 3 Action of tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol against changes in the electrophoretic mobility of LDL which result from peroxidation

LDL (0.2 mg/ml) at pH 7.4 was incubated at 37 °C with 15 μM CuCl_2 for 2 h and samples were applied to agarose gels as described in the Materials and methods section. Lanes 2 and 7, native LDL; lane 3, Cu^{2+} and 0.5% (v/v) ethanol added; lane 4, Cu^{2+} and 30 μM tamoxifen added; lane 5, Cu^{2+} and 30 μM 17 β -oestradiol added; lane 6, Cu^{2+} and 30 μM 4-hydroxytamoxifen added.

surface charge as a result of oxidation. Lipoproteins were visualized by staining with Sudan Black B stain.

In addition, the effects of the compounds on the oxidation of the polyunsaturated fatty acid side-chains of the LDL were followed by continuous monitoring of conjugated-diene formation by difference spectroscopy, assessing the increase in the absorbance between 200 nm and 270 nm with time [35]. Due to the high absorption coefficient of conjugated-diene hydroperoxides in the region of 230–245 nm, 0.1 mg/ml concentrations of LDL were used here. It should be noted that compound:LDL concentration ratios are thus double those used in the other assays. The formation of conjugated dienes was recorded on a Beckman DU65 spectrophotometer fitted with Quant 1 software and linked to an IBM PC/2.

RESULTS

Protection of LDL against copper-dependent lipid peroxidation and changes in electrophoretic mobility by tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol

Exposure of human LDL to copper(II) ions causes rapid peroxidation, as measured by TBARS formation (Figure 1) and diene conjugation (Figure 2). Figure 1 also shows that tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol were all capable of

Table 2 Relative electrophoretic mobility values for LDL oxidized by copper in the absence and presence of tamoxifen, 4-hydroxytamoxifen and 17 β -oestradiol

Relative electrophoretic mobility is shown relative to the mobility of the untreated LDL. Values are deduced from gels such as that shown in Figure 3 and are given as mean $\pm$ S.D. of three separate gels.

Compound added	Relative electrophoretic mobility
None (LDL)	1 $\pm$ 0.00
None [LDL + 15 μ M Cu ²⁺ + 0.5% (v/v) ethanol]	2.38 $\pm$ 0.26
Tamoxifen (30 μ M)	2.38 $\pm$ 0.26
17 β -Oestradiol (30 μ M)	1.38 $\pm$ 0.19
4-Hydroxytamoxifen (30 μ M)	1.13 $\pm$ 0.13

protecting LDL against copper-dependent lipid peroxidation, measured by the formation of TBARS. 4-Hydroxytamoxifen and 17 β -oestradiol were much more effective at protecting LDL against peroxidation than tamoxifen over the concentration range used (0–30 μ M), and 4-hydroxytamoxifen was more effective than 17 β -oestradiol. These differences are reflected in the IC₅₀ values (Table 1).

Figure 2 shows that copper-ion-dependent conjugated-diene formation in the LDL [0.1 mg/ml LDL + Cu(II) + ethanol] was complete after 40 min (compared with 2 h for TBARS) under our experimental conditions. All three compounds, at 15 μ M, protected LDL against peroxidation. The order of effectiveness was again 4-hydroxytamoxifen > 17 β -oestradiol > tamoxifen (see Figure 2).

Peroxidation of LDL can result in changes in electrophoretic mobility due to oxidative modification of apoprotein B by products of peroxide decomposition [27]. 4-Hydroxytamoxifen and 17 β -oestradiol at 30 μ M almost completely protected LDL against these changes (Figure 3 and Table 2). Tamoxifen at this concentration was a much less effective inhibitor of lipid peroxidation (Table 1) and did not prevent the change in mobility (Figure 3 and Table 2).

DISCUSSION

Our results show that tamoxifen itself can protect LDL against copper-ion-dependent lipid peroxidation. Its metabolite 4-hydroxytamoxifen was more effective, more so than 17 β -oestradiol, which itself has been reported previously to be superior to the anti-atherogenic anti-oxidant probucol as an anti-oxidant in LDL [29]. 17 β -Oestradiol has been suggested to stabilize the LDL particle, which comprises a large central core of cholesteryl ester and triacylglycerols surrounded by a monolayer of phospholipid molecules and free cholesterol with the apoprotein B embedded in this outer layer [25], against oxidative damage [29]. It is possible that tamoxifen and 4-hydroxytamoxifen, which are structural mimics of 17 β -oestradiol (this is the basis of their oestrogen antagonism) and of membrane-stabilizing sterols [20], may act in a similar way and thus stabilize LDL against peroxidation by interaction between their hydrophobic rings and the polyunsaturated residues of the phospholipid layer of LDL. Nevertheless, other mechanisms cannot be ruled out.

4-Hydroxytamoxifen also prevented peroxidation-induced alterations in the surface charge of LDL. Since these changes are

associated with recognition and uptake of LDL by macrophages in atherosclerotic lesions [27], our observations could explain the beneficial cardiovascular effects of tamoxifen therapy. It is likely that tamoxifen did not prevent alteration to the surface charge of LDL in this study, because it is a much less effective inhibitor of lipid peroxidation than 4-hydroxytamoxifen. Tamoxifen maximally inhibited lipid peroxidation by only approx. 50% (see Figure 1); thus presumably sufficient aldehydic breakdown products (such as malondialdehyde and 4-hydroxynonenal) are still formed to alter the surface charge of LDL via modification of the ϵ -amino groups of the lysine residues [27], despite the presence of tamoxifen.

The prophylactic use of tamoxifen to prevent breast cancer might thus additionally provide long-term cardiovascular benefits. These include the prevention of heart disease by mechanisms which include the lowering of plasma LDL levels [15,16] and also the protection of LDL against oxidative damage by tamoxifen and its 4-hydroxy metabolite, which are highly lipophilic compounds and thus may accumulate in the lipophilic atheromal plaques associated with the arterial wall, to achieve the protective concentrations reported here.

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