Catechol Estrogens Are More Potent Antioxidants than Estrogens for the Cu^{2+}-Catalyzed Oxidation of Low or High Density Lipoprotein: Antioxidative Effects of Steroids on Lipoproteins

Susumu TANIGUCHI, Toshihiko YANASE, Kunihisa KOBAYASHI, Ryouichi TAKAYANAGI, Masafumi HAJI, Fumio UMEDA, and Hajime NAWATA

The Third Department of Internal Medicine, Faculty of Medicine, Kyusyu University, Fukuoka 812, Japan

Abstract. In order to clarify the mechanism of antiatherogenic action of several steroids such as estrogens, dehydroepiandrosterone (DHEA) and dexamethasone, we investigated the effects of various steroids on the copper (Cu^{2+})-catalyzed oxidation of low density lipoprotein (LDL) or high density lipoprotein (HDL) in 0.15 M NaCl by measuring thiobarbituric acid-reactive substances (TBARS). At a concentration of 10^{-5} M, estrogens strongly protected against LDL oxidation by 0.5 µM Cu^{2+} in the following order of inhibition: estradiol (E_2) (75%), estrone (E_1) (35%) and estriol (E_3) (30%). However, the corresponding metabolites of these estrogens, the catechol estrogens, had an even more protective effect on LDL oxidation by 0.5 µM Cu^{2+} in the following order of inhibition: 2-hydroxyestradiol (2-OHE_2) (98%), 2-OHE_1 (97%) and 2-OHE_3 (96%). E_2 and 2-OHE_2 from 10^{-7} M to 10^{-5} M inhibited LDL oxidation in a dose-dependent manner, with a more marked effect for oxidation by 0.1 µM Cu^{2+} than by 0.5 µM Cu^{2+}. 10^{-5} M dexamethasone produced a slight (10%) but significant inhibition of LDL oxidation by 0.5 µM Cu^{2+}. In addition, the estrogens and catechol estrogens were also effective in protecting against HDL oxidation by 0.5 µM Cu^{2+}. Other steroids including DHEA and DHEA-sulfate had no antioxidative effects on either LDL or HDL in this system. These results indicate that estrogens and their metabolites, the catechol estrogens, exert antioxidative effects on both LDL and HDL. The catechol estrogens may be more important antioxidants than estrogens for both LDL and HDL. Dexamethasone may exert its antiatherogenic effect partly by inhibiting the oxidation of lipoproteins, but this may not be the case for DHEA and DHEA-sulfate.

Key words: Estrogen, Catechol estrogen, Oxidation, LDL, HDL

THE DEPOSITION of macrophage-derived cholesterol ester-laden foam cells in the subendothelial region is a characteristic feature of the initial events of atherosclerotic plaque formation [1]. Low density lipoprotein (LDL) is believed to be the source of the cholesterol in foam cells, and LDL levels in serum are positively correlated with the risk of atherosclerosis [1]. It is widely accepted that modified LDL, including oxidized LDL, is taken up by macrophages, at least in part, via the scavenger receptors, leading to their transformation to foam cells [1, 2]. Indeed, the existence in vivo of oxidatively modified LDL has recently been reported in atherosclerotic lesions [3-6]. Probucol, an agent originally developed as an antioxidant, has been shown to prevent the progression of atherosclerotic-
sis in hypercholesterolemic rabbits [7]. Although the in vivo mechanism of LDL oxidation is not clear, LDL can be oxidatively modified in vitro by certain cultured cells [8-10] or by copper ions [11]. Conversely, it is postulated that high density lipoprotein (HDL) mediates its antiatherogenic effect by transporting cholesterol from foam cells back to the liver for excretion [1]. Interestingly, it has been reported that oxidatively modified HDL loses its ability to stimulate the efflux of cholesterol from foam cells [12].

The reduced incidence of coronary artery disease in premenopausal women as compared to men and postmenopausal women suggests that estrogens protect against atherogenesis [13]. While estrogens have favorable effects on serum lipoprotein metabolism, namely the reduction of LDL and increase of HDL [14], this alone does not adequately explain its protective effect against atherosclerosis. A previous study in cholesterol-fed rabbits has shown that 17-β estradiol reduced atherosclerotic plaque formation without any change in cholesterol levels [15]. An antioxidative effect of estrogens on LDL was recently demonstrated in vitro [16-19]. Furthermore, it has also been shown that the degradation by macrophages of oxidized LDL was markedly reduced by treatment with estrogens [17]. These results suggest that an inhibitory effect of estrogens on foam cell formation may be another mechanism for its antiatherogenic action.

Dehydroepiandrosterone (DHEA) and dexamethasone have also been shown to be antiatherogenic in cholesterol-fed rabbits [20-23], independent of changes in plasma total and LDL cholesterol levels, although the precise mechanism of their antiatherogenic action has not been clarified.

Since previous studies have mainly focused on the antioxidative effects of estrogens on LDL oxidation and have not thoroughly examined the effects of steroids on HDL oxidation, we undertook the present study to test various steroids including catechol estrogens, DHEA and dexamethasone with respect to their ability to inhibit LDL or HDL oxidation catalyzed by copper ions (Cu²⁺) in vitro.

Materials and Methods

Lipoprotein preparation

LDL (d=1.019-1.063 g/ml) and HDL (d=1.063-1.21 g/ml) were isolated from the mixture of the plasmas of more than three normolipidemic male volunteers by density gradient ultracentrifugation and dialyzed against saline-EDTA as described [24]. Each fraction was passed through a 0.45-μM filter after dialysis. The protein concentration of LDL was determined with a BCA kit, with bovine serum albumin as a standard (Pierce, Rockford, IL, USA).

Lipoprotein oxidation

Lipoproteins were dialyzed overnight against a 5000 fold volume of 0.15 M NaCl at 4°C, which was changed at least twice. For Cu²⁺ oxidation, the lipoproteins were diluted in 0.15 M NaCl (pH 7.4) to 200 μg of protein/ml. The lipoproteins were then incubated in the presence of 0-0.5 μM CuSO₄ at 37°C for 12 h. Lipoprotein oxidation was performed in the absence or presence of a steroid (0-10⁻⁵ M in ethanol). All of the steroids and α-tocopherol (Vitamine E, Vit. E) were purchased from Sigma (St. Louis, MO, USA). Ethanol was always used in a final concentration of 1%. Control experiments included an equivalent volume of ethanol. Lipoprotein oxidation was terminated by refrigeration and the addition of 0.1 mM EDTA and determined by the thiobarbituric acid-reactive substances (TBARS) assay [10]. TBARS are expressed as nanomoles of malondialdehyde (MDA) equivalents per milligram lipoprotein protein compared with tetraethoxypropane standards.

Statistics

Statistical evaluation was performed by Student's t-test.

Results

Oxidative modification of LDL or HDL in 0.15 M NaCl as measured by TBARS production at 0 to 0.5 μM Cu²⁺ was observed to occur in a dose-de-
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The degree of Cu²⁺-catalyzed oxidation of HDL was less than that of LDL at the same protein concentration.

Fig. 2A shows the effects of various steroids at a concentration of 10⁻⁵ M on the oxidative modification of LDL by 0.5 µM Cu²⁺ as measured by TBARS production. 10⁻⁵ M estrogens strongly protected against LDL oxidation by 0.5 µM Cu²⁺ in the following order of inhibition: estradiol (E₂) (75%), estrone (E₁) (35%) and estriol (E₃) (30%). However, the corresponding metabolites of these estrogens, the catechol estrogens, had an even more protective effect against LDL oxidation by 0.5 µM Cu²⁺ in the following order of inhibition: 2-hydroxyestradiol (2-OHE₂) (98%), 2-OHE₁ (97%) and 2-OHE₃ (96%). 10⁻⁵ M dexamethasone protected a slight but significant inhibition of LDL oxidation by 10%, whereas other steroids including DHEA, DHEA-sulfate (DHEA-S), testosterone (T) and dihydrotestosterone (DHT) did not inhibit LDL oxidation by 0.5 µM Cu²⁺. E₂ and 2-OHE₂ 10⁻⁷ M to 10⁻⁵ M significantly inhibited LDL oxidation in a dose-dependent manner, with a more marked effect for oxidation by 0.1 µM Cu²⁺ than by 0.5 µM Cu²⁺ (Fig. 3A and 3B). The more protective effect of 2-OHE₂ than E₂ against LDL oxidation by 0.1 µM Cu²⁺ was observed in a dose-dependent manner (Fig. 2A, B).

10⁻⁵ M estrogens and catechol estrogens were also effective in protecting against HDL oxidation by 0.5 µM Cu²⁺ in the following order of inhibition: 2-OHE₂ (98%), 2-OHE₁ (97%), 2-OHE₃ (93%), E₂ (86%), E₁ (81%) and E₃ (78%) (Fig. 2B). DHEA, DHEA-S, testosterone (T) and dexamethasone 10⁻⁵ M were ineffective in inhibiting HDL oxidation by 0.5 µM Cu²⁺. The effect of various concentrations

![Fig. 1](image1.png)  
*Fig. 1. Effect of copper concentration on oxidation of LDL or HDL. LDL or HDL (200 µg protein/ml) were incubated with Cu²⁺ for 12 h at 37°C in 0.15 M NaCl. TBARS, thiobarbituric acid-reactive substances; MDA, malondialdehyde. Each bar represents the mean ± SD for three tubes of pooled LDL and HDL.*

![Fig. 2](image2.png)  
*Fig. 2. Effect of various steroids on copper-catalyzed LDL and HDL oxidation. A, LDL oxidation; B, HDL oxidation. LDL or HDL (200 µg protein/ml) were incubated with 0.5 µM Cu²⁺ for 12 h at 37°C in 0.15 M NaCl. The concentration of all steroids was 10⁻⁵ M. As a positive control, the antioxidative effect of 20 µM Vitamin E is shown in Fig. 2A. Each bar represents the mean ± SD for three tubes of pooled LDL and HDL. Means of control values: (A) 371.28 ± 4.02 (B) 157.96 ± 2.48 nmol equivalent malondialdehyde/mg LDL protein. *, P<0.05; **, P<0.01 vs. control. Dex, dexamethasone; Δ⁴-A, Δ⁴-androstenedione.*
Fig. 3. Effect of estradiol (E2) or 2-hydroxyestradiol (2-OHE2) on copper-catalyzed LDL oxidation. A, E2; B, 2-OHE2. LDL (200 μg protein/ml) was incubated with 0.1 μM or 0.5 μM Cu²⁺ for 12 h at 37°C in 0.15 M NaCl in the presence or absence of E2 or 2-OHE2. Each point represents the mean ± SD for three tubes of pooled LDL. Means of control values: (A) Cu²⁺ 0.5 μM, 417.06 ± 2.87; 0.1 μM, 74.61 ± 5.14; (B) Cu²⁺ 0.5 μM, 398.56 ± 4.41; 0.1 μM, 141.91 ± 18.11 nmol equivalent malondialdehyde/mg LDL protein. *, P<0.01 vs. values in the absence of steroid.

Fig. 4. Effect of estradiol (E2) or 2-hydroxyestradiol (2-OHE2) on copper-catalyzed HDL oxidation. HDL (200 μg protein/ml) was incubated with 0.5 μM Cu²⁺ for 12 h at 37°C in 0.15 M NaCl in the presence or absence of steroids. Each point represents the mean ± SD for three tubes of pooled HDL. Means of control values: 162.12 ± 7.40 nmol equivalent malondialdehyde/mg LDL protein. *, P<0.01 vs. values in the absence of steroid.

Discussion

The oxidative modification of LDL by Cu²⁺ was significantly protected by E₂, E₁ and E₃, as was recently reported in two other studies [17, 18]. Although E₂ was consistently the most potent estrogen acting against LDL oxidation, no protective effect of E₁ against Cu²⁺-catalyzed LDL oxidation was observed by Rifici et al. [18] but was seen by us and by Maziere et al. [17]. This discrepancy is probably due to differences in the Cu²⁺ concentration used for LDL oxidation. The 0.1–0.5 μM Cu²⁺ concentrations in 0.15 M NaCl in our study were rather low compared with the 5 μM Cu²⁺ in PBS usually used in other studies [17, 18]. More importantly, catechol estrogens, the main metabolites of estrogens, had an even more potent inhibitory effect on LDL oxidation catalyzed by Cu²⁺. The potent antioxidative effects of catechol estrogens on membrane phospholipid oxidation have also been demonstrated by measuring the oxygen consumption of phospholipid liposome-induced iron-ADP-adriamycin complex [25]. The catechol estrogens, 2-OHE₁ and 2-OHE₂, could be formed from their corresponding estrogens by estrogen 2-
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hydroxylase [26] in microsomes in the brain [27] or liver and other tissues [28]. The presence of 2-OHE$_1$ in human plasma [29] and in urine [30] has been reported. Although the biological significance of catechol estrogens is unclear, they possess the phinolic structure and catechols, both of which are known to be strongly antioxidative. Estrogens may therefore exert their antiatherogenic action alone or through their metabolites, the catechol estrogens.

In the present study, the degree of oxidative modification of HDL was relatively weaker than that of LDL at the same protein concentration. Interestingly, an antioxidative effect of lipoprotein containing apoprotein A-I on Cu$^{2+}$-catalyzed oxidation of LDL was recently reported [31]. While admittedly such as comparison is not easy because of the big difference between LDL and HDL particle size, the decreased susceptibility of HDL to Cu$^{2+}$-catalyzed oxidation could partly result from the antioxidative effects of apoprotein A-I, a major apoprotein in HDL. Estrogens and catechol estrogens were similarly effective in inhibiting HDL oxidation by Cu$^{2+}$, suggesting another important mechanism of the action of estrogens against atherosclerosis, since oxidized HDL has been reported to be less effective if accelerating cholesterol efflux from cholesterol ester-laden cells (foam cells) [12]. Although the concentration of estrogens and catechol estrogens required to inhibit LDL or HDL oxidation in these in vitro experiments was supraphysiological as compared to the physiological concentrations in human (<1.5 nM), we also noted that estrogens were more effective when LDL was subjected to less extensive oxidation by a lower Cu$^{2+}$ concentration. Therefore, a low but constant level of estrogens may exert its antioxidative effect against minimal or slight oxidation of LDL in vivo. Since esterified estrogens have been found in serum [32], the integration of the esterified estrogens into lipoprotein particles could stabilize and inhibit against LDL oxidation [18]. Indeed, an increased concentration of serum lipid peroxide after ovalectomies has been reported in mice [33], suggesting the physiological significance of estrogen action on lipid peroxidation in vivo.

DHEA and its sulfate ester, DHEA-S, are weak androgens that are produced primarily by the adrenal gland. DHEA is thought to be antiatherogenic in humans since the administration of DHEA to normal men reduces their body fat and total and LDL cholesterol levels [34] and since circulating levels of DHEA-S are inversely related to death from cardiovascular disease in men over 50 years of age [35]. Indeed, the administration of DHEA to cholesterol-fed rabbits reduced aortic involvement by fatty streaks, independent of changes in serum lipids [20, 21]. The present study indicates that DHEA or DHEA-S is not antioxidative against LDL or HDL, suggesting a different mechanism for its antiatherogenicity.

A glucocorticoid, dexamethasone, is another steroid that may be antiatherogenic. While glucocorticoids tend to be atherogenic with respect to lipid metabolism in humans [36], it has been reported that dietary supplementation with dexamethasone significantly reduces the atherosclerotic changes in cholesterol-fed rabbits despite aggravation of lipid metabolism [23]. The effect of dexamethasone in protecting against LDL oxidation by Cu$^{2+}$ was slight but significant, suggesting some contribution to its antiatherogenic effect.

In summary, we surmise that catechol estrogens may be more important antioxidants than estrogens for both LDL and HDL. The antioxidative effects of estrogens, including catechol estrogens and dexamethasone, on lipoprotein oxidation may be a mechanism of the antiatherogenic action of these steroids.

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References


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