

# Estradiol as an antioxidant: incompatible with its physiological concentrations and function

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**Abstract** Estradiol has been documented to inhibit the oxidation of low density lipoprotein (LDL). We show that physiological concentrations of estradiol do not inhibit the oxidation of LDL by copper. LDL samples isolated from *a*) premenopausal and postmenopausal women and from *b*) women at different time periods during their menstrual cycle, who differ vastly in plasma estradiol levels, were also oxidized at the same rates by copper. In contrast, LDL samples isolated from *c*) women who were hyperstimulated during in vitro fertilization (IVF), with estradiol concentrations above 2000 pg/ml, were resistant to oxidation by copper. However, these LDL samples were also oxidized at a higher rate by peroxidases. More importantly, subjects with high estradiol levels also showed an increase in myeloperoxidase (MPO) protein in the plasma. Based on these results, we conclude that at physiologic concentrations, it is unlikely that estradiol could act as an antioxidant. In fact, the ability of estradiol to induce MPO and become a prooxidant might instead suggest that MPO-mediated oxidative clearance of LDL from plasma by liver might favorably influence the outcome of atherosclerosis.—Santanam, N., R. Shern-Brewer, R. McClatchey, P. Z. Castellano, A. A. Murphy, S. Voelkel, and S. Parthasarathy. **Estradiol as an antioxidant: incompatible with its physiological concentrations and function.** *J. Lipid Res.* 1998. 39: 2111–2118.

**Supplementary key words** atherosclerosis • menopause • coronary artery disease • lipid peroxidation • myeloperoxidase • oxidized low-density lipoprotein

Although the overall rate of death due to coronary artery disease (CAD) does not differ greatly between genders, the disease typically develops 10 to 15 years later in women, with a woman's risk rising exponentially after menopause. Estradiol, which is produced in women at relatively high levels before menopause, is thought to offer some protection against heart disease (1, 2). A large body of epidemiological and experimental data supports the idea that estrogens are able to slow the progress of atherosclerosis and to reduce the risk of subsequent ischemic heart disease (2)

Three different mechanisms are suggested for the protective effect of estrogen or 17 $\beta$ -estradiol. Estradiol may

have a direct effect on genes, which are involved in lipid or lipoprotein metabolism, such as the LDL receptor (3–6). Estradiol may also have an effect on several antiatherogenic genes, such as the nitric oxide synthase (7) thereby increasing the levels of nitric oxide (NO), an antioxidant (8–10). Similarly, it may affect the synthesis of monocyte chemotactic protein-1 (MCP-1) (11–13) thus limiting the chemotactic recruitment of monocytes into the artery.

The third mechanism may involve its role as an antioxidant. Estrogens, especially estradiol and 17 $\beta$ -estradiol, which possess a phenolic hydroxyl group, have an effective antioxidant action and inhibit lipid peroxidation in model membranes (14–19). Antioxidant properties of estrogen have been proposed for the inhibition of oxidation of LDL, a key step in atherogenesis (20). Estradiol, at supraphysiological concentrations (10<sup>-6</sup> mol/L), protected LDL from both cellular and copper-mediated oxidation in vitro (21–27). Recently Shwaery, Vita, and Keaney (28) showed an inhibition of in vitro oxidation of LDL in the whole serum, by lower concentrations (0.1 to 100 nmol/L) of estradiol. Sack, Rader, and Cannon (29) demonstrated that estradiol treatment of postmenopausal women is associated with increased LDL resistance to ex vivo copper-mediated oxidation.

Even though all the above studies do show an antioxidant role for estradiol, there are several major inconsistencies with these findings. The concentration of estradiol in the plasma of normal women is less than 0.5 ng/ml (less than 2 nm). In most of the studies estradiol has been used in concentrations ( $\mu$ m levels) hundreds of fold higher than normal plasma levels (24–27). Many of these studies also used copper as an in vitro oxidant for studying the oxidation of LDL, a condition that may not be physiologically relevant (24, 25, 28).

Abbreviations: LDL-low density lipoprotein; IVF- In vitro fertilization; MPO-myeloperoxidase; CAD-coronary artery disease; NO-nitric oxide; MCP-1 monocyte chemotactic protein-1; HRP, horse-radish peroxidase; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; 3-ME, 3-methoxyestradiol; HDL, high density lipoprotein; ELISA, enzyme linked immunosorbant assay; PBS, phosphate-buffered saline; HPLC, high performance liquid chromatography; MAb, monoclonal antibody.

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The concentration of plasma estradiol depends on the age and time of menstrual cycle in women (30). Younger women tend to have higher levels of estradiol, with plasma concentrations ranging from 50 to 500 pg/ml whereas postmenopausal women would be expected to have estradiol values of approximately 10–20 pg/ml. Estradiol levels vary during the normal menstrual cycle of women reaching peak levels during mid-cycle. Tissue estradiol levels also vary depending on the target organ. The uterine endometrium is exposed to follicular fluid in which the concentrations of estradiol reach values as high as 5,000 pg/ml (31).

Peroxidase-mediated oxidation of LDL has recently been considered to be an important mechanism by which LDL undergoes oxidation *in vivo* (32, 33). Myeloperoxidase (MPO) has been shown to be present in high levels in atherosclerotic artery (34). Peroxidase-like compounds such as heme and hemin have also been shown to promote the oxidation of LDL (35). While substituted phenols such as vitamin E, butylated hydroxy toluene, and probucol have been shown to have potent antioxidant activity *in vitro*, recent studies by Bowry, Ingold, and Stocker (36) have provided a new insight into the mechanism of action of these compounds. These studies suggest that in a lipid-rich environment vitamin E could become a prooxidant. Studies by Yamamoto and Niki (37) also suggested a delicate balance between prooxidant and antioxidant effects of vitamin E in the presence of iron. We have shown that, in the presence of peroxidases, simple phenols such as vitamin E and tyrosine readily form phenoxy radicals and promote the oxidation of lipids and lipoproteins (38).

In the present study, we explore the role of estradiol in the oxidation of LDL by peroxidases and compare it with its already established antioxidant role in copper-mediated oxidation of LDL in subjects with varying levels of plasma estradiol. These subjects include *a*) postmenopausal women, *b*) premenopausal women at different phases of the menstrual cycle, and *c*) women undergoing ovarian hyperstimulation for *in vitro* fertilization (IVF). LDL isolated from plasma collected from these subjects was used for oxidation studies using both copper and peroxidase systems.

## MATERIALS AND METHODS

Horseradish peroxidase (HRP) (P6140, lot 103H9558), MPO (M6908, Lot 126H9408), 17 $\beta$ -estradiol,  $\alpha$ -tocopherol, and other reagents were obtained from Sigma Chemical Co. (St. Louis, MO). Potassium bromide (KBr) and other solvents for HPLC were purchased from Fisher Chemicals (Pittsburgh, PA).

### Subjects recruitment

Subjects were recruited through the Emory GYN/OB department and advertisements and flyers posted at the Emory Campus and in the Atlanta Journal/Constitution. The Human Investigation Committee of the Emory University (Atlanta, GA) approved the human subject protocol. Criteria for participation in the study included: 1) non-smokers, 2) non-vitamin/mineral supplement users for at least previous 6 months, 3) aerobically active less than 3 h each week for at least previous 6 months, 4) no known heart disease, 5) no estrogen therapy within previous 1

year, 6) non-diabetic, and 7) non-hypertensive. These criteria were needed to control for possible confounding factors, which may effect oxidative stress. Additional exclusion criteria included severe menopausal symptoms, abnormal thyroid-stimulating hormone levels, and contraindications to estrogen. The subjects were selected from the general population and were not restricted depending on race or socioeconomic status. Subjects were asked to complete 1) an approved human consent form for participation in the study, 2) a medical history questionnaire, 3) a food frequency questionnaire, 4) a physical activity history, and 5) gynecological history. All subjects were asked to participate in an initial fasting blood draw and a final fasting blood draw.

### Blood samples

Blood (15 ml) was drawn from subject's forearm vein after an overnight fast. Sodium heparin vacutainer tubes were used and immediately placed on ice. The blood was centrifuged at 2000 rpm for 20 min and the plasma was separated. An aliquot of the plasma was frozen at  $-80^{\circ}\text{C}$  and another aliquot was kept on ice until further assays were performed that day. Assays performed on the same day included isolation of LDL, lipid analysis, protein estimation, and *in vitro* oxidation of LDL using copper and peroxidase systems. The frozen samples were used for vitamin E analysis, estradiol analysis, and MPO protein analysis.

### Lipid analysis

Fasting plasma total cholesterol, triglycerides, high density lipoprotein (HDL)- and LDL-cholesterol measurements were determined using the Cholestech L\*D\*X analyzer (Cholestech Corporation, Hayward, CA).

### LDL isolation

LDL was isolated from heparinized plasma using a Beckman TL-100 tabletop ultracentrifuge. Single-spin gradient isolation was done adjusting 2 ml of plasma to a d 1.31 g/ml with KBr layered with saline and spun at 100,000 rpm for 1 h. The isolated samples were respun at d 1.21 g/ml to concentrate and purify the LDL from any albumin contamination. The isolation was carried out without any EDTA and was completed in less than 3 h. The isolated LDL was dialyzed against phosphate buffered saline (PBS) at  $4^{\circ}\text{C}$  for 4–6 h (38). The purity of the isolated LDL samples was established by agarose and acrylamide gel electrophoresis (38, 39).

### Protein determination

Protein was determined by the method of Lowry et al. (40) using bovine serum albumin as the standard.

### Formation of conjugated diene

LDL samples were subjected to oxidation immediately after isolation and each experiment consisted of 4 samples, which included 2 controls and 2 test subjects (38). Typically, 100  $\mu\text{g/ml}$  of LDL was incubated in PBS with 0.5  $\mu\text{M}$  copper or 1 U HRP/0.1 U MPO with 50  $\mu\text{M}$   $\text{H}_2\text{O}_2$ . The oxidation of LDL was followed continuously by measuring the formation of conjugated dienes at OD 234 nm in an SLM-Aminco DB-3500 spectrophotometer equipped with a 12-chamber cuvette changer. Samples and references were measured continuously for periods of up to 6–12 h.

### Vitamin E analysis

Vitamin E was extracted using n-hexane from 100  $\mu\text{g}$  protein of total plasma or isolated LDL by the method described by Cheeseman et al. (41) and quantitated using HPLC. Samples were injected into a C18 Microsorb reverse phase column and separated using a Rainin high performance liquid chromatography (HPLC) (Varian Chromatography Systems, Walnut Creek, CA) (42). Methanol was used as the solvent and the eluant was mon-

itored on an UV detector at 297 nm.  $\alpha$ -Tocopherol acetate (Sigma Chemical, St. Louis, MO) was used as an internal standard.

### Estradiol analysis

Plasma or isolated LDL estradiol levels were measured using an automated DPC-Immulate analyzer (DPC Cirrus, Diagnostic Products Corporation, Randolph, NJ). The Immulate System utilizes assay-specific antibody-coated plastic beads as the solid phase test unit. The Test Unit serves as the reaction vessel for the immune reaction, incubation, wash, and signal development. Light emission from the chemiluminescent substrate reacting with enzyme conjugate bound to the bead is proportional to the amount of analyte originally present in the patient sample. A commercial control serum pool (Con 6, DPC, Los Angeles, CA) with three estradiol concentrations (low, medium, and high) was used as quality control with each run. The Immulate Estradiol assay has a broad working range of 20 to 2,000 pg/ml. Specimens with estradiol concentrations greater than 2,000 pg/ml were diluted with commercial diluent (DPC, Los Angeles, CA) and the analysis was repeated.

### Determination of plasma myeloperoxidase (MPO) protein

An ELISA method was used to measure plasma MPO protein levels (kit #21013; Bioxytech Oxis International, Inc., Portland, OR). One hundred  $\mu$ l of the total plasma from different subjects was incubated in the wells of a sectionable microplate, which had been coated with a first monoclonal antibody (MAb) to MPO (MPO-MAb). The MPO-MAb was labeled with a biotin-linked polyclonal antibody prepared from goat MPO-antisera. A biotin-avidin coupling occurs when avidin is covalently linked to alkaline phosphatase. The amount of MPO in each sample was enzymatically measured upon addition of 4-nitrophenyl-phosphate, by reading the microplate at 405 nm. Standards of known MPO concentration were quantified using a standard curve plotted against which samples were determined.

### Preparation of 3-methoxyestradiol (3-ME)

3-Methoxyestradiol was prepared from estradiol by the method of Johnstone and Rose (43). Briefly, to 1 ml dimethyl sulfoxide, 112 mg powdered potassium hydroxide was added and mixed well. After 5 min incubation, 136 mg estradiol was added along with 142 mg methyl iodide. The mixture was stirred for 30 min and extracted with dichloromethane. The final extract was evaporated under nitrogen, recrystallized in acetone and the final pellet was suspended in ethanol at a final concentration of 69 mg/ml. The 3-ME was used at different concentrations in both copper- and peroxidase-mediated oxidation of LDL.

### Statistics

Student's *t* test was used to analyze any differences between the subject groups, including age, LDL and plasma vitamin E levels, LDL and plasma estradiol levels and lipid levels. The results are given as mean  $\pm$  standard deviation. Statistical analysis was performed using GB stat software (Dynamic Microsystems, Inc., Silver Spring, MD).

## RESULTS

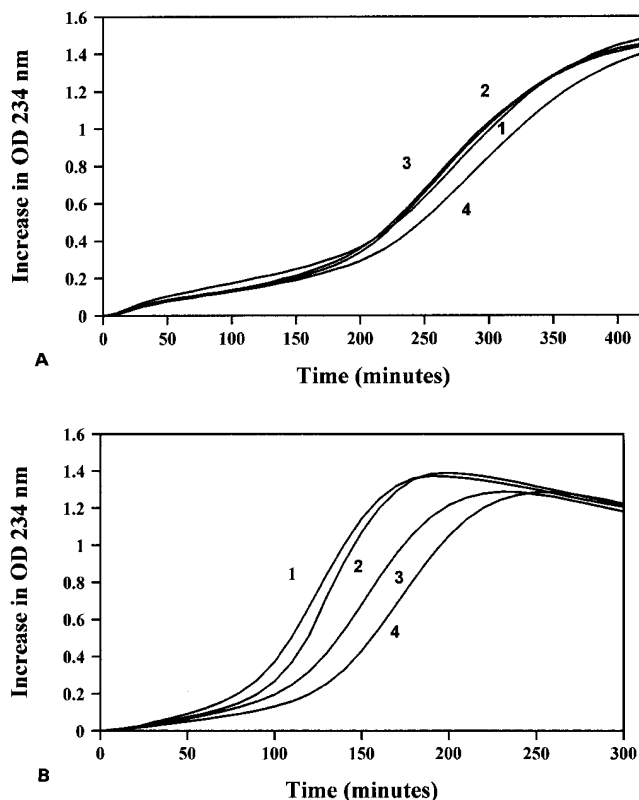
### Estradiol inhibits copper-mediated oxidation of LDL at higher concentrations but has no effect at physiologic concentrations

Increasing concentrations of an ethanolic solution of estradiol were incubated with 100  $\mu$ g/ml of LDL in the presence of 0.5  $\mu$ M copper. A lower concentration of cop-

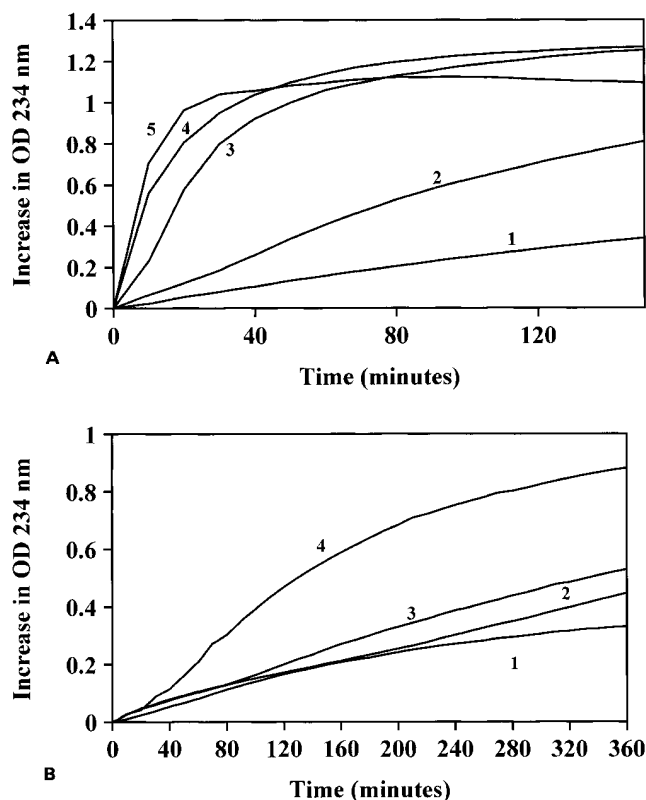
per (0.5  $\mu$ M) was used in our study in order to keep a lower rate of oxidation, to increase detection of small differences in the rate of oxidation. As shown in Fig. 1A, the addition of estradiol at lower concentrations from 136 pg/ml to 1360 pg/ml (0.5 nm to 5 nm) (which reflect physiologic concentrations) had no effect on the formation of conjugated dienes. However, when higher concentrations of estradiol from 272 pg/ml to  $27.2 \times 10^3$  pg/ml (1 nm to 100 nm) were added to the incubations, there was an increase in lag time, suggesting an apparent antioxidant effect (Fig. 1B).

### Estradiol promotes oxidation of LDL by peroxidases

This study provides evidence for the pro-oxidant activity of supraphysiologic concentrations of estradiol in the presence of peroxidase- (HRP 1 U/MPO 0.1 U with 50  $\mu$ M  $H_2O_2$ ) mediated oxidation of LDL. As seen in Fig. 2A and B, the addition of estradiol (in ethanol) at micromolar concentrations decreases lag time, suggesting that in a



**Fig. 1.** A: Effect of physiologic concentration of estradiol on oxidation of LDL by copper. One hundred  $\mu$ g of LDL was incubated with 0.5  $\mu$ M copper sulfate solution in a total volume of 1 ml PBS. The formation of conjugated diene was measured continuously at OD 234 nm in a spectrophotometer. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL + 136 pg/ml (0.5 nm) estradiol; 3: LDL + 272 pg/ml (1 nm) estradiol; 4: LDL + 1360 pg/ml (5 nm) estradiol. B: Effect of non-physiologic concentrations of estradiol on oxidation of LDL by copper. The conditions were as described in Fig. 1A. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL + 272 pg/ml (1 nm) estradiol; 3: LDL + 2720 pg/ml (10 nm) estradiol; 4: LDL +  $27.2 \times 10^3$  pg/ml (100 nm) estradiol.



**Fig. 2.** A: Effect of estradiol on oxidation of LDL by H<sub>2</sub>O<sub>2</sub>/HRP. One hundred  $\mu$ g of LDL was incubated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 1 U of HRP in the presence of various concentrations of estradiol in a total volume of 1 ml PBS. The formation of conjugated diene was measured continuously at optical density 234 nm in spectrophotometer. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL +  $27.2 \times 10^3$  pg/ml (0.1  $\mu$ M) estradiol; 3: LDL +  $68.1 \times 10^3$  pg/ml (0.25  $\mu$ M) estradiol; 4: LDL +  $136 \times 10^3$  pg/ml (0.5  $\mu$ M) estradiol; 5: LDL +  $272 \times 10^3$  pg/ml (1  $\mu$ M) estradiol. B: Effect of estradiol on oxidation of LDL by H<sub>2</sub>O<sub>2</sub>/MPO. One hundred  $\mu$ g of LDL was incubated with 50  $\mu$ M H<sub>2</sub>O<sub>2</sub> and 0.1 U of MPO in the presence of various concentrations of estradiol in a total volume of 1 ml PBS. The formation of conjugated diene was measured continuously at optical density 234 nm in spectrophotometer. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL +  $27.2 \times 10^3$  pg/ml (0.1  $\mu$ M) estradiol; 3: LDL +  $68.1 \times 10^3$  pg/ml (0.25  $\mu$ M) estradiol; 4: LDL +  $136 \times 10^3$  pg/ml (0.5  $\mu$ M) estradiol.

peroxidase system, like other phenolic antioxidants (vitamin E), estradiol can become pro-oxidant. This observation is very novel in that this is the first study to show that estradiol, in the presence of peroxidases, promotes the oxidation of LDL.

#### Susceptibility of LDL isolated from pre/postmenopausal women to in vitro copper oxidation

We compared the ease of in vitro oxidation of LDL isolated from a group of premenopausal ( $n = 10$ ) and postmenopausal ( $n = 10$ ) women. The two groups were significantly different at a  $P < 0.05$  level with regard to plasma estradiol, plasma cholesterol, and LDL levels (Table 1). The premenopausal group had increased levels of estradiol but, as expected, had significantly lower levels of cho-

lesterol, LDL, and triglycerides. There were no differences in the levels of HDL. Isolated LDL (100  $\mu$ g/ml) was incubated with 0.5  $\mu$ M copper in 1 ml PBS and the oxidation was followed by the increase in the formation of conjugated diene at OD 234 nm in spectrophotometer. There was no statistically significant difference in the oxidation of LDL between the two groups using the in vitro copper system (lag time;  $91.3 \pm 18.6$  minutes versus  $96.5 \pm 20.0$  min, respectively). Nor were there significant differences in vitamin E concentrations.

#### Susceptibility of LDL isolated from plasma of women during the follicular phase of the menstrual cycle

During the menstrual cycle, the estradiol levels slowly increase during the follicular phase until ovulation, after which the levels go down. Blood was collected from normal women ( $n = 5$ ) on day 1, day 7, day 9, and day 12 (during the follicular phase). LDL was isolated from plasma and subjected to in vitro oxidation by copper (0.5  $\mu$ M). There were no statistically significant differences in plasma cholesterol, triglycerides, HDL, and LDL levels during the follicular phase (Table 2). Plasma estradiol levels peaked around the 7th day ( $\approx 4$ -fold) and remained elevated over the 9th and 12th day ( $\approx 2.5$ -fold). No statistically significant difference in the susceptibility to in vitro copper-mediated oxidation was observed (lag time:  $89.72 \pm 9.91$ , day 1;  $99 \pm 1.75$ , day 7;  $84 \pm 8.12$ , day 9;  $100 \pm 2.21$ , day 12; min, respectively). Our data demonstrate that even at peak concentrations of estradiol (178 pg/ml, day 7) there is no significant inhibition of oxidation of LDL.

#### 3-Methoxyestradiol does not inhibit copper- or peroxidase-mediated oxidation of LDL

The phenolic hydroxyl group at the 3 position in the estradiol is responsible for its antioxidant and pro-oxidant activity. When this hydroxyl group is blocked as in 3-ME by a methoxy group, the estradiol loses its ability to inhibit or promote the oxidation of LDL as seen in Fig. 3A and 3B.

#### Susceptibility of LDL isolated from plasma of women or subjects undergoing ovarian hyperstimulation for IVF

Blood was obtained from patients undergoing IVF. Subjects hyperstimulated for IVF often have estrogen levels above 2000 pg/ml. This is an order of magnitude higher than the concentration observed in normal women. LDL was isolated and subjected to both copper- and peroxidase-mediated oxidation. As seen in Table 3, at high concentrations, estradiol inhibited copper-mediated oxidation and promoted peroxidase-mediated oxidation. Our results suggest that even under such high levels of estradiol concentrations, if peroxidase-mediated oxidation of LDL is atherogenic, then the prooxidant effects are likely to prevail over the antioxidant effects.

#### Increased MPO protein in the plasma of subjects with high estradiol levels

The administration of estradiol in animals has been shown to result in an induction of peroxidase activity in

TABLE 1. Analysis results of premenopausal and postmenopausal groups

	Pre-menopausal Women (n = 10)	Post-menopausal Women (n = 10)	P values
Age (yr)	35 ± 6	53 ± 5	<0.05
Plasma estradiol (pg/ml)	119 ± 28	49 ± 28	<0.05
Total cholesterol (mg/dl)	176 ± 37	226 ± 50	<0.05
LDL (mg/dl)	97 ± 19	131 ± 40	<0.05
HDL (mg/dl)	69 ± 21	64 ± 13	0.60
Triglyceride (mg/dl)	67 ± 23	156 ± 93	<0.05
Vitamin E-plasma (nmol/ml plasma)	10.6 ± 5.8	13.9 ± 13.5	0.30
Vitamin E-LDL (nmol/mg LDL protein)	5.1 ± 3.2	7.2 ± 4.9	0.10
Lag time-copper (min)	91.3 ± 18.6	96.5 ± 20	0.30

Values given as mean ± SD.

target tissue (44, 45). We present evidence using IVF hyperstimulated subjects that increased plasma estradiol levels are associated with an increase in plasma MPO protein levels as determined using a specific ELISA assay for MPO protein. As can be seen in Table 3, a significant increase in plasma estradiol is associated with a significant increase in plasma MPO protein levels. This is the first report to show such an increase in MPO protein levels in the plasma of women with increased estradiol concentrations.

## DISCUSSION

The antioxidant effects of estradiol have been proposed as a potential mechanism by which the hormone may protect against the development of CAD. This proposed mechanism originated from *in vitro* studies describing its ability to inhibit the oxidation of LDL under conditions using copper as the oxidant and nonphysiological levels of estradiol. In this study, we demonstrate that physiologic levels of estradiol (136 pg/ml to 1360 pg/ml) failed to inhibit the oxidation of LDL even when lower concentrations of copper were used to slow down the rate of oxidation. However, at concentrations ranging above the physiological concentrations ( $1.36 \times 10^5$  pg/ml to  $1.36 \times 10^6$  pg/ml) estradiol promoted peroxidase-mediated oxidation of LDL.

We provide support for the above conclusion (that estradiol at physiologic concentrations is not an antioxi-

dant) using LDL samples isolated from women with vastly different plasma estradiol levels. An attractive feature of this study is that in two of our three experimental setups, the same subjects provided baseline control values. First, the postmenopausal group had significantly lower estradiol levels as compared to premenopausal group; however, there was no difference in the rate of *in vitro* oxidation of LDL using a standard copper system between the two groups. Brussaard et al. (46) reported a very similar observation, where there was no effect of estradiol supplementation on the LDL oxidizability in postmenopausal women. Guetta et al. (47) also observed that supplementation of postmenopausal women with vitamin E and estradiol had no effect on its *ex vivo* oxidizability.

Second, when LDL isolated from plasma obtained from women during their follicular phase of the menstrual cycle was subjected to oxidation, there was no significant reduction in oxidation by copper, despite differences in plasma- and LDL-associated estradiol levels. We document that plasma estradiol is indeed associated with isolated LDL (Table 2) providing a strong basis for studying the effects of estradiol on the oxidation of LDL. To our knowledge, such information is unavailable in the literature. However, there have been studies by Tang, Abplanalp, and Subbiah (48) and Abplanalp et al. (49) showing that upon *in vitro* incubation of plasma with estradiol, at least 10% of the estradiol was associated with lipoproteins.

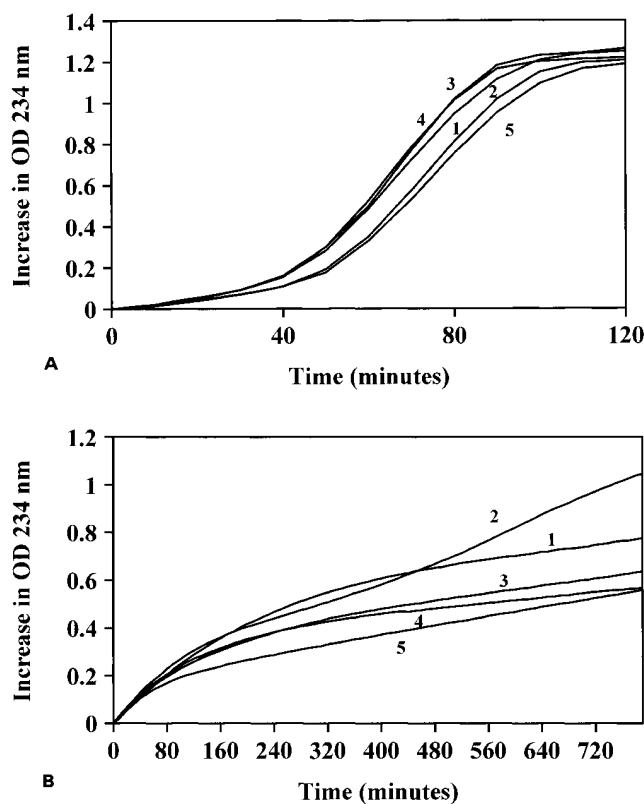
Peroxidase-mediated oxidation of LDL has recently been considered to be an important mechanism by

TABLE 2. Analysis results of samples from premenopausal women (n = 5) during the follicular phase of the menstrual cycle

	Day 1	Day 7 <sup>a</sup>	Day 9	Day 12
Plasma estradiol (pg/ml)	48.88 ± 16.35	178.18 ± 74.79	116.44 ± 89.05	112.56 ± 36.64
LDL estradiol (pg/mg protein)	124.4 ± 9.45	100.1 ± 5.28	128.8 ± 43.2	117.48 ± 27.88
Total cholesterol (mg/dl)	187 ± 38.77	189.6 ± 32.59	194.2 ± 48.86	188.8 ± 36.07
LDL (mg/dl)	98.75 ± 31.62	105.8 ± 24.39	120.4 ± 40.18	108.2 ± 26.77
HDL (mg/dl)	68.5 ± 9.98	64.2 ± 10.03	57.6 ± 18.68	64.2 ± 9.65
Triglyceride (mg/dl)	100 ± 51.62	98.6 ± 45.16	80.2 ± 24.87	83.2 ± 43.37
Lag time-copper (min)	89.72 ± 9.91	99 ± 1.75	84 ± 8.12	100 ± 2.21
Age (yr)		26.8 ± 2.28		

Values given as mean ± SD.

<sup>a</sup>Results of oxidation and lipid parameters on day 7 (with highest estradiol levels) were not significantly different from those on days 1, 9, and 12.



**Fig. 3.** A: Effect of 3-methoxyestradiol (3-ME) on oxidation of isolated LDL by copper. One hundred  $\mu\text{g}$  of LDL was incubated with  $0.5 \mu\text{M}$  copper sulfate solution in a total volume of 1 ml PBS. Conjugated diene formed was measured continuously at OD 234 nm in a spectrophotometer. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL +  $136 \times 10^3 \text{ pg/ml}$  ( $0.5 \mu\text{M}$ ) 3-ME; 3: LDL +  $272 \times 10^3 \text{ pg/ml}$  ( $1 \mu\text{M}$ ) 3-ME; 4: LDL +  $680 \times 10^3 \text{ pg/ml}$  ( $2.5 \mu\text{M}$ ) 3-ME; 5: LDL +  $1360 \times 10^3 \text{ pg/ml}$  ( $5 \mu\text{M}$ ) 3-ME. B: Effect of 3-methoxyestradiol (3-ME) on oxidation of isolated LDL by  $\text{H}_2\text{O}_2/\text{HRP}$ . One hundred  $\mu\text{g}$  of LDL was incubated with  $50 \mu\text{M}$   $\text{H}_2\text{O}_2$  and 1 U of HRP in a total volume of 1 ml PBS. Formation of conjugated diene was measured continuously at optical density 234 nm in spectrophotometer. The figure represents a typical experiment from over 4 individual experiments. 1: Control LDL; 2: LDL +  $136 \times 10^3 \text{ pg/ml}$  ( $0.5 \mu\text{M}$ ) 3-ME; 3: LDL +  $272 \times 10^3 \text{ pg/ml}$  ( $1 \mu\text{M}$ ) 3-ME; 4: LDL +  $680 \times 10^3 \text{ pg/ml}$  ( $2.5 \mu\text{M}$ ) 3-ME; 5: LDL +  $1360 \times 10^3 \text{ pg/ml}$  ( $5 \mu\text{M}$ ) 3-ME.

which LDL becomes proatherogenic. MPO levels have recently been shown to be increased in the atherosclerotic artery (34). Our earlier study (38) showed that in a peroxidase-mediated oxidation of LDL, phenols such as vitamin E act as prooxidants and increase oxidation. In the present study we show evidence that in peroxidase-mediated oxidation of LDL, estradiol at supraphysiologic concentrations becomes a prooxidant and activates the oxidation.

How do we reconcile the prooxidant nature of estradiol in the presence of peroxidases with its role as a protector against CAD? First, there are other mechanisms by which estradiol protects against CAD that far outweigh its role as a prooxidant. For example, if estradiol inhibits the generation of MCP-1 (12) and thus decreases the presence of arterial monocytes, its prooxidant role would be insignifi-

**TABLE 3.** Results of LDL oxidation, plasma estradiol and plasma MPO protein values obtained from women undergoing hyperstimulation for IVF ( $n = 15$ )

	Before Hyperstimulation	After Hyperstimulation	P Value
Plasma estradiol (pg/ml)	$32.95 \pm 13.8$	$2676.4 \pm 860.77$	0.00005
Lag time-copper (min)	$159 \pm 3$	$191 \pm 19$	0.025
Lag time-peroxidase (min)	$268 \pm 33$	$195 \pm 17$	0.014
Plasma MPO protein (ng/ml)	$0.86 \pm 0.83$	$1.84 \pm 1.6$	0.024

MPO protein in the plasma was estimated using an ELISA protocol as explained in Materials and Methods.

cant due to the reduced availability of MPO. Second, the ability of estradiol to channel leukocytes into other tissues and to activate MPO in tissues other than the artery might also channel the oxidation of LDL away from the artery. In fact, the results presented in this study are the first evidence in humans for an increase in plasma MPO protein in association with elevated levels of estradiol. Our preliminary studies show that when younger women were given vitamin E (800 IU) supplementation for 8 weeks, there was no significant increase in plasma MPO protein levels even though there was a significant increase in vitamin E levels (unpublished observations). This is an important observation because it is well established that estradiol activates several peroxidases in vivo such as the estradiol peroxidase, uterine peroxidase, and MPO (50). Estradiol induces peroxidases in uterine endometrium. However, the estrogen-induced increase in uterine peroxidase activity is not derived from resident uterine cells, but results primarily from infiltration of eosinophils, rich in this enzyme, into the uterus. Detailed studies show that the peroxidases of uterine endometrium are analogous to the MPO enzyme of the neutrophils (44, 45, 50, 51). Thus uterine peroxidase is analogous to neutrophil MPO and represents a constitutive peroxidase specific to uterine tissue. Estradiol has also been shown to increase neutrophil activation during phagocytosis, thereby increasing MPO release (50, 52). Estradiol undergoes a number of reactions in the presence of peroxidases and these include the formation of estradiol radical, which results in dimerization, crosslinking to proteins, and the ability to induce lipid peroxidations (53–55).

Our study, therefore, indicates that, under conditions in which there is an increase in peroxidase activity, estradiol may act as a prooxidant and promote the oxidation of LDL. The increase in plasma MPO protein levels when estradiol levels are high could be taken to indicate rapid oxidation of LDL in the plasma and the clearance of oxidized LDL by the liver. This, in turn, would generate an anti-atherogenic lipid and lipoprotein profile analogous to the condition that prevails during strenuous exercise (56, 57). ■

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