Ascorbic acid enhances 17 β-estradiol-mediated inhibition of oxidized low density lipoprotein formation

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Abstract

Postmenopausal women who use estrogen appear to be protected from coronary heart disease (CHD). Studies have demonstrated that estrogen can lower low-density lipoprotein (LDL) levels and the antioxidant activity of 17 β-estradiol can prevent the oxidation of this LDL. Ascorbic acid is regarded as a major hydrophylic antioxidant, however, its impact on the prevention of CHD has yet to be clearly demonstrated. Modified low density lipoprotein (LDL\textsuperscript{−}) is an important marker of LDL oxidation in vivo, since it contributes to the oxidative susceptibility of low density lipoprotein, and at physiological levels displays pro-inflammatory and cytotoxic properties. Previously we showed that women taking estrogen replacement therapy have lower LDL\textsuperscript{−} levels along with lower predisposition of the LDL to oxidize. In this study, we evaluated the potential action of 17 β-estradiol (E\textsubscript{2}) in combination with ascorbic acid (AA) measured on the basis of LDL oxidative susceptibility in vitro and in the presence of cultured cells. High concentrations of E\textsubscript{2} were able to inhibit LDL oxidation, whereas in the presence of ascorbic acid nano- to picomolar levels of E\textsubscript{2} were sufficient to suppress LDL oxidation (P < 0.05). Preconditioning male aortic endothelial cells (RAEC) with 5 ng/ml of E\textsubscript{2} (E\textsubscript{2}RAEC) reduced the formation of LDL\textsuperscript{−} (P < 0.005), and a more extensive inhibition was found in the presence of AA (P < 0.0001). Interestingly, E\textsubscript{2} enhanced the uptake of LDL in the absence or presence of AA, however, this was not seen for the uptake of LDL\textsuperscript{−}. These results provide the first evidence that ascorbic acid can enhance the antioxidant effect of E\textsubscript{2} by preventing LDL oxidation by copper ions or cells. The cytoprotective and antiatherogenic effect of E\textsubscript{2} appears to involve a reduction in the extent of oxidized LDL formation and uptake. The enhanced activity of E\textsubscript{2} in the presence of ascorbate indicates that the antioxidant and antiatherosclerosis activity of E\textsubscript{2} may occur at concentrations within the physiological range.

Keywords: Estrogen; Coronary heart disease; Low-density lipoprotein; 17 β-estradiol; Male aortic endothelial cells; Ascorbic acid

1. Introduction

Coronary heart disease (CHD) is the leading cause of death in men and women in the United States and in developing countries. The progression of CHD is determined by several factors among which gender is a striking example. The risk for CHD is less in premenopausal women versus age-matched men, however, this ‘protective’ effect is lost with menopause and potentially regained with estrogen replacement therapy (ERT). The potentially protective effect of estrogen has been described in animal and epidemiological studies and is now being investigated by several randomized controlled clinical trials.

The mechanisms by which estrogen inhibits atherogenesis are not known, but part of this beneficial effect may be explained by alteration of the plasma lipoprotein profile [1–4]. Changes in the lipid profile do not fully account for the cardioprotective effect afforded by estrogen, indicating that other mechanisms are likely to be involved [5,6]. One mechanism may include estrogen’s antioxidant activity [7]. Estrogen exhibits natural

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antioxidant activity during membrane phospholipid peroxidation [8], influences agonist-mediated calcium signaling responses [9], potentiates endothelium-derived relaxing factor in coronary arteries [10], and inhibits LDL modification [11,12]. Estrogen also facilitates prostacyclin production [13,14], limits cholesterol ester influx [15], reduces apoptosis of human endothelial cells [16], and suppresses TNF-α, IL-1 and NF-κB activation as induced by lipopolysacharides [17]. Many of these processes are linked to the production of reactive oxygen species (ROS) and hence the modulation of ROS production and consequent effects on cell redox status may be a mechanism by which estrogen exerts its ‘antioxidant/atheroprotective’ activity.

It is widely held that oxidative modification of low density lipoprotein (LDL) contributes to the development of atherosclerosis [18,19]. Oxidized LDL has been found in human plasma as a more electronegatively charged lipoprotein (LDL−) that contains a number of oxidized components [12,20–22] similar to other forms of oxidized LDL produced in vitro [11,23,24]. LDL− exists mainly in the denser LDL subclass (δ > 1.055 g/ml), is more susceptible to oxidation and is capable of facilitating the oxidation of other lipoproteins [25]. Individuals with higher levels of small dense LDL (1.050–1.063 g/ml) are more prone to CHD [26], suggesting that the risk factor(s) may reside in an increased proportion of oxidatively modified LDL subclasses.

Reports have been inconsistent in postmenopausal women taking ERT in regards to the susceptibility of LDL to oxidize in vitro. Some reports have shown that ERT can inhibit LDL and HDL oxidation in the presence of copper [27,28] whereas others have shown that there was no effect on LDL oxidative susceptibility in postmenopausal women receiving ERT [2,29].

Inhibition of LDL oxidative modification in vivo has been approached in large measure through the use of dietary antioxidants. Studies have often included ascorbic acid, a central component of the antioxidant defense system [30]. Ascorbate intake and dietary demand varies with lifestyle and age, and in the latter instance plasma levels were shown to decrease during aging at a rate of approximately 0.06 mg/dl per decade [31]. Men tend to have lower levels of ascorbic acid than women [32] and require larger doses of ascorbic acid to reach the same serum concentration [33]. This gender related effect seems to begin in adolescence and is thought to be hormonally mediated [34].

Epidemiological studies suggest that increased ascorbic acid intake may afford protection against cardiovascular disease based on the postulated inhibition of lipoprotein oxidation, regulation of cholesterol homeostasis, and lowering of blood pressure [34]. Martin et al. [35] reported recently that both intracellular and extracellular ascorbic acid inhibited endothelial cell-mediated LDL oxidative modification. Surprisingly, there is very little information concerning the interaction between estradiol and ascorbic acid with respect to their effects on oxidatively modified LDL formation. In this report, we describe an antioxidant action of estrogen that is facilitated by ascorbic acid.

2. Methods

2.1. Chemical and reagents

The following reagents were obtained from Sigma Chemical Co. (St. Louis, MO): EDTA, BHT, 17β-estradiol (E2), progesterone, L-ascorbic acid (Vitamin C), 2,4,6, tripyridyl-s-triazine, ferric chloride, sodium acetate, NaBr, NaCl, TRIS, trichloroacetic acid, metaphosphoric acid, triethanolamine, 2-vinylpyridine, 1,1′-dioctadecyl-3,3,3′,3′-tetramethyldiuroncarbocyanine perchlorate (DiI), was purchased from Molecular Probes (Eugene, OR). Buffers, media, and cell culture supplies were obtained from Life Science (New York, NY) and male serum from Omega Scientific (Van Nuys, CA). All organic solvents and copper sulfate were HPLC grade and purchased from T.J. Baker Chemical Co. (Phillipsburg, NJ).

2.2. Lipoprotein separation

Venous blood was obtained from fasting adult human volunteers and plasma was immediately separated by centrifugation at 1500 × g for 10 min at 4°C. LDL (δ = 1.019–1.063 g/ml) was isolated from freshly separated plasma by preparative ultracentrifugation using a Beckman L8-55 ultracentrifuge and a SW-41 rotor. The technique used for separating LDL was similar to that described previously [20]. The isolated LDL was extensively dialyzed against argon-sparged 0.01 mol/l TRIS buffer, pH 7.2, containing 10 μmol/l EDTA, sterilized by filtration (0.2 μm Millipore membrane) and stored at 4°C under nitrogen.

2.3. LDL− preparation

Separation of LDL− from native LDL (n-LDL) was accomplished by subjecting the isolated LDL fraction to anion exchange high pressure liquid chromatography (Perkin-Elmer Series 4 HPLC) as described previously [20]. The eluent was monitored at 280 nm, and the peaks corresponding to the LDL− fraction collected using a fraction collector, (Frac-100, Pharmacia Biotech). The pooled LDL− was concentrated and desalted using Centricon 30 000 molecular-weight cut-off microconcentrators. Before storage, the LDL− was diluted in PBS containing 10 μmol/l EDTA to a final concentration of 500 μg/ml protein, sterilized by ultra-filtration (0.2 μm Millipore membrane) and kept at 4°C under nitrogen.
2.4. *In vitro* oxidation of LDL

The kinetics of LDL oxidation were analyzed by adding 10 μmol/l CuSO₄ to 200 μg/ml LDL protein. Formation of conjugated dienes was monitored continuously at 234 nm for up to 5 h using a Beckman DU-650 spectrophotometer equipped with a six position automated sample changer. Oxidation kinetics were analyzed in phosphate buffer on the basis of: (1) the oxidation lag time which was defined as the interval between initiation of oxidation and the intercept of the tangent for the slope of the absorbance curve during the propagation phase; (2) the rate of oxidation during the lag time, defined as the initial oxidation rate before the onset of the propagation phase; (3) the rate of oxidation during the propagation phase, i.e. the log rate which was defined as the maximal rate of oxidation calculated from the slope of the absorbance curve during the propagation phase.

The kinetics of LDL oxidation were analyzed in the presence or absence of E₂, progesterone, α-tocopherol (added in ethanol), and ascorbic acid. The amounts of ethanol vehicle were the same for all the experiments (0.2% v/v).

2.5. Vitamin E measurements

LDL fractions were collected during the kinetic assay and extracted for vitamin E analysis. A modified method of Bui [36] was used to measure vitamin E levels. 500 μl of LDL was mixed with 50 μl of internal standard (α-tocopherol acetate) and extracted twice with 500 μl of hexane containing BHT. The pooled phases were evaporated under nitrogen, the residue dissolved in 200 μl of ethanol, and injected into a Perkin Elmer Series 4 HPLC equipped with a BioRad reverse-phase column Bio-Sil ODS-5S, 250 × 4 mm (BioRad Instruments, CA). Samples were eluted with acetonitrile-tetrahydrofuran-H₂O at 0.9 ml/min, the eluent monitored using a UV/Vis monitor model 1706 (BioRad instruments, CA), and the peak areas integrated with Axxi-chrom 747 analytical chromatography software.

2.6. Ascorbic acid measurement

Total ascorbic acid levels were determined by a modified colorimetric method of Day et al. [37] based on the reduction of ferric chloride by ascorbic acid. Samples were added to 0.615 M of trichloroacetic acid, mixed, and centrifuged at 1500 rpm for 10 min and the supernatant mixed with the color reagent consisting of 2.4,6 tripyridyl-s-triazine and ferric chloride in sodium acetate buffer.

2.7. Cell culture

Male New Zealand White rabbit aortic endothelial cells (RAEC) were used between passages 19 and 21. Cells were passaged using a 1:2 split ratio, allowed to grow to confluency, and transferred by trypsinization. The doubling time of the cells was approximately 38 h in complete media (DMEM without phenol red containing 15% male serum, 20 μg/ml ECGS, 50 μg/ml gentamicin and 20 μg/ml heparin). This is referred to as control medium. Prior (5 days) to the experiments, cells were split in complete media in the presence (E₂RAEC) or absence (RAEC) of 5 ng/ml of E₂.

2.8. Cytotoxicity assays

Cells were seeded into 24-well dishes 1 day prior to measurements of cell survival and plating efficiency which were used as estimates of cytotoxicity following various treatments. The cytotoxicity produced by LDL and LDL⁻ was determined using nearly confluent cultures grown in the presence of 2% male serum and cell numbers measured after 24 h incubation using a Coulter counter (Model ZB). Treatments typically used 1 × 10⁴ cells/cm². The parameters related to the measurement of cytotoxicity included: plating efficiency (PE) and growth curves based on the surviving fraction (SF) measured at 24 h after treatment with lipoprotein.

2.9. Measurement of cell-mediated oxidation

Formation of LDL⁻ by endothelial cells was used as a measure of cell-mediated LDL oxidation. LDL⁻ formation was determined after adding 100 μg/ml of fresh LDL protein and 0.5 μM CuSO₄ to endothelial cell cultures comprised of 2 × 10⁵ cells/10 cm². Ratios of LDL⁻ formation relative to total LDL were measured over a 24 h interval for control cells with and without 100 μM of ascorbic acid, and E₂RAEC cultures with and without ascorbic acid in serum-free control media. At selected time points, aliquots of the media were removed and centrifuged at 800 rpm for 5 min to remove dead cells and cell debris. The supernatant was mixed with 15 ml PBS and concentrated using a Centricron 30 000 molecular weight cut-off microconcentrator to adjust LDL concentrations and remove low molecular weight components from the medium. These brief reconstituting procedures and adjustments of LDL concentrations did not affect the amounts of LDL⁻ formation [25] [20]. Samples were concentrated to the volume of the initial aliquot removed from the cell culture medium and then analyzed by HLPC for LDL⁻ content. LDL⁻ levels were calculated on the basis of the cell number and corrected for LDL⁻ formation in a cell-free system.
2.10. DiI LDL uptake

Sub-confluent cultures (4 × 10^4 cells) were treated with DiI-labeled lipoproteins at 10 µg/5 × 10^4 cells for 2 h in complete medium in the presence of 2% male serum. The incorporated DiI was measured fluorometrically (523 nm Excitation/563 nm Emission) after extraction with isopropanol. Cells were stained with Giemsa and protein concentrations determined on the basis of absorbance at 590 nm with parallel measurements of cell number using a Coulter counter. Confocal microscopy (Zeiss LSM-1) was used to confirm the internalization of LDL particles [38] relative to the amount of fluorescence associated with the cell membrane. Measurements of relative electrophoretic mobility (REM) of the LDL samples after labeling with DiI indicated that the lipoproteins were unaltered by this labeling procedure. The extent of uptake and concentration of lipoprotein fluorescence was estimated on the basis of relative fluorescence intensity per mg LDL protein. The assumption was made that the amount of cell-associated fluorescence represented the same fluorescence concentration as in the original LDL. Results were expressed as µg uptake of LDL as mg cell protein in 2 h.

2.11. 17 β-estradiol measurement

Estradiol levels were measured using a Pantex RIA assay (Santa Monica, CA), where estradiol calibrators and fixed amounts of radiolabeled estradiol (tracer) compete for binding sites in fixed volumes of antiserum. Binding values are plotted versus concentrations, and levels of estradiol in samples determined from the calibration curve.

2.12. Glutathione measurements

Total glutathione (GSH) levels were measured using a standard kit (Cayman, MI), Briefly, cells were removed by scraping in cold PBS, the pellets were washed once with PBS and deproteinized with 10% of metaphosphoric acid. The assay utilizes glutathione reductase for the quantification of GSSG and GSH as total GSH. Concentrations of samples were determined using established calibration curves that were run with each batch of samples.

2.13. Statistics

All results are expressed as mean and standard errors determined from at least five independent experiments with all measurements performed in duplicate. Determinations of statistical significance between various treatment groups were made using the paired two-tailed student t-test.

3. Results

3.1. In vitro oxidation of LDL is inhibited by E₂ and ascorbic acid

Fig. 1 shows an antioxidant effect of E₂ that is typical of the suppression of radical chain reactions in LDL lipids by various antioxidants. The inhibitory effect of E₂ was manifested by a prolongation of the oxidation lag time Fig. 1A, and inhibition of peroxide accumulation during the lag phase (a decreased lag rate) with increasing concentration of E₂. In Fig. 1, E₂
Fig. 2. Kinetics of LDL oxidation measured by the formation of conjugated dienes (OD234) in the presence of 17β-estradiol. Freshly isolated human LDL (200 μg protein/ml) was incubated with 10 μmol/l of CuSO4 in the absence and presence (50 ng/ml) of 17β-estradiol as described in Section 2. Symbols are for LDL ( ); LDL in the presence 50 ng/ml of 17β-estradiol (○); LDL in the presence of 100 μM of ascorbic acid (▲); and LDL in the presence of 100 μM of ascorbic acid and 50 ng/ml of 17β-estradiol (▼). Vitamin E consumption under the same treatment conditions is shown as open symbols.

was added to cuvettes containing 200 μg/ml of LDL protein over a concentration range 1–200 ng/ml. The inhibitory effect of E2 was significant (P < 0.05) at concentrations greater than 5 ng/ml. Fig. 1B shows that the oxidation lag rates were also inhibited in the presence of E2 with significant inhibition occurring at concentrations > 10 ng/ml.

The rate of vitamin E consumption did not change during the oxidation lag period for LDL samples in the presence of E2 as compared to LDL alone (Fig. 2). This suggested that the antioxidant effect of E2 was not due to direct interaction with vitamin E, and that the presence or content of vitamin E in the LDL samples had little effect on the oxidation kinetics as influenced by E2. Also shown in Fig. 2 are the effects of ascorbic acid on LDL vitamin E levels during oxidation. The levels of vitamin E were determined during the oxidation of LDL alone, LDL in the presence of 50 ng/ml of E2, LDL in the presence of 100 μM of ascorbic acid and LDL in the presence of 100 μM of ascorbic acid and 50 ng/ml of E2. Relatively small decreases in vitamin E levels were found when 100 μM of ascorbic acid or 100 μM of ascorbic acid and 50 ng/ml of E2 were added to LDL, indicating a preservation of vitamin E by ascorbate but not by E2 alone. When 100 μM ascorbic acid was added to the LDL samples after addition of E2, a strong antioxidant effect was evident and the amounts of added E2 required to significantly inhibit LDL oxidation was reduced to the range of 1–5 ng/ml (Fig. 3). The combination of ascorbic acid and E2 was 4 times more potent than E2 alone and more than twice as potent as ascorbic acid alone (Fig. 4). Vitamin E did not show the same effect as ascorbic acid (Fig. 4) when 200 μg/ml of LDL protein was incubated with either E2 and ascorbic acid or E2 and vitamin E.

Notably, progesterone added at 200 μg/ml, had no significant effect on LDL oxidation (data not shown), indicating that the observed antioxidant activity was not a general property of steroid hormones.
are expressed as a percent of the rate of LDL. 

Table 1
Determinations of cytotoxicity after 24 h treatments *

<table>
<thead>
<tr>
<th>Treatment</th>
<th>RAEC</th>
<th>RAEC + AA</th>
<th>E2RAEC</th>
<th>E2RAEC + AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control media</td>
<td>1</td>
<td>0.943 ± 0.071</td>
<td>0.992 ± 0.044</td>
<td>0.980 ± 0.046</td>
</tr>
<tr>
<td>10 μg/ml of LDL</td>
<td>0.768 ± 0.081</td>
<td>0.706 ± 0.080</td>
<td>0.808 ± 0.051</td>
<td>0.792 ± 0.034</td>
</tr>
<tr>
<td>20 μg/ml of LDL</td>
<td>0.744 ± 0.076*</td>
<td>0.674 ± 0.088</td>
<td>0.969 ± 0.058*</td>
<td>0.825 ± 0.093</td>
</tr>
<tr>
<td>500 μg/ml of LDL</td>
<td>0.725 ± 0.070</td>
<td>0.609 ± 0.095</td>
<td>0.795 ± 0.067</td>
<td>0.721 ± 0.067</td>
</tr>
<tr>
<td>1000 μg/ml of LDL</td>
<td>0.551 ± 0.041**</td>
<td>0.607 ± 0.049**</td>
<td>0.810 ± 0.056**</td>
<td>0.689 ± 0.049**</td>
</tr>
</tbody>
</table>

* P < 0.001.
** P < 0.0005.
+ P < 0.0001.
++ P < 0.05.

3.2. E2 cells are more resistant to LDL and LDL−

Male rabbit endothelial cells pre-incubated with 5 ng/ml of E2 (E2RAEC) for 5 days were more resistant to the cytotoxic effects of high concentrations of LDL or LDL−, as shown in Table 1. Treatments with 10 and 20 μg/ml of LDL− produced significant toxicity (P < 0.01) to control cells but no toxicity to E2RAEC under the same treatment conditions. Treatment of E2RAEC with 500 μg/ml LDL also produced no increase in cytotoxicity whereas these high levels of LDL were toxic to RAEC (P < 0.001). Pre-incubation with E2 was sufficient to confer protection, and the presence of E2 was not required during the treatment periods with LDL or LDL−. This indicates that protection afforded by E2 did not require interaction with lipoproteins in the cell culture medium but was related to a conferred protection or induction of resistance of the cells to normal or modified LDL.

Table 1 also shows the effects of LDL and LDL− treatments in the presence of 100 μM of ascorbic acid. There was no cytoprotective effect of ascorbic acid on LDL-mediated toxicity in RAEC or E2RAEC, but a protective effect was found when the cells were treated with 500 μg/ml of LDL (P < 0.05).

3.3. Cell-mediated LDL− formation

Control RAEC and E2RAEC were incubated with 100 μg/ml of LDL protein for 24 h and the amount of LDL− formation determined by HPLC analysis of total LDL recovered from the medium. Fig. 5 shows that less LDL was converted to LDL− in the presence of E2 (E2RAEC) than RAEC (P < 0.005). The results are expressed as a percent of the rate of LDL− formation by RAEC under the standard culture conditions. The SF following 24 h incubation with 100 μg/ml LDL was not significantly different from untreated (control) cultures. Moreover, the presence of ascorbic acid in E2RAEC cultures further inhibited LDL− formation (P < 0.005) as compared to E2RAEC without ascorbate. By contrast, addition of ascorbic acid to RAEC tended to increase the rate of LDL− formation. The effect of ascorbic acid was clearly related to increased cell-mediated activity on LDL since no effects of ascorbic acid were found under cell-free conditions (data not shown). To confirm the oxidation status of LDL and LDL− before addition to RAEC and E2RAEC, agarose gel electrophoresis was performed on the samples, and REM determined in the preparations (data not shown).
3.4. LDL but not LDL<sup>-</sup> uptake is enhanced in E<sub>2</sub>RAEC

As shown in Fig. 6, uptake of LDL was greater in E<sub>2</sub>RAEC and E<sub>2</sub>RAEC in the presence of ascorbic acid than in RAEC or RAEC in the presence of ascorbic acid when cells were grown in standard medium containing 15% of male serum, P < 0.05. The extent of uptake is shown relative to LDL or LDL<sup>-</sup> uptake in control cultures (RAEC), which was set as 100%. DiI LDL uptake in RAEC was 0.743 ± 0.169 μg/10<sup>5</sup> cells while DiI-LDL<sup>-</sup> uptake in RAEC was 0.304 ± 0.034 μg/10<sup>5</sup> cells. Although there was apparently greater LDL<sup>-</sup> uptake in E<sub>2</sub>RAEC treated with ascorbic acid, the effect was not significant compared to E<sub>2</sub> alone or to control RAEC. The tendency for E<sub>2</sub>RAEC to incorporate more LDL<sup>-</sup> in the presence of ascorbic acid may be due to an antioxidant effect such that less LDL is degraded to a form that is not assimilated by LDL receptors.

3.5. 17β-estradiol levels are increased in E<sub>2</sub>RAEC

Levels of 17β-estradiol were measured in the RAEC and E<sub>2</sub>RAEC incubated in the presence and absence of ascorbic acid. The measurements were performed in media and cells. Table 2 shows that E<sub>2</sub>RAEC cells and E<sub>2</sub>RAEC incubated with ascorbic acid have increased 17β-estradiol levels relative to RAEC or RAEC treated with ascorbic acid. Incubation of E<sub>2</sub>RAEC with ascorbic acid did not increase the levels of 17β-estradiol in cells as compared to E<sub>2</sub>RAEC. The levels of 17β-estradiol in the medium from E<sub>2</sub>RAEC or E<sub>2</sub>RAEC incubated with ascorbic acid were not different.

3.6. Total glutathione levels are increased in E<sub>2</sub>RAEC

Table 2 shows that RAEC and RAEC in the presence of ascorbic acid have similar levels of total GSH while E<sub>2</sub>RAEC and E<sub>2</sub>RAEC in the presence of ascorbic acid have increased GSH levels as compared to RAEC. The increase in GSH in E<sub>2</sub>RAEC is essentially attributable to the effects of E<sub>2</sub> since ascorbate addition had little effect on GSH levels in E<sub>2</sub>RAEC.

4. Discussion

A novel antioxidant interaction between estradiol and ascorbic acid has been found that is highly effective in preventing LDL oxidation. The marked reduction of the rates of conjugated diene formation during the lag phase (i.e. lag rate) in the presence of E<sub>2</sub> indicates a suppression of lipid peroxide formation, typical of the antioxidant protected phase for radical chain propagation during lipid peroxidation. The enhanced protection in the presence of ascorbic acid suggests an interaction between E<sub>2</sub> and ascorbic acid and/or other LDL antioxidants. This interaction may be similar to that described for vitamin E and ascorbate where the former is reduced by the latter and its antioxidant activity preserved [39]. Conservation of vitamin E in LDL is

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**Table 2**

<table>
<thead>
<tr>
<th>Estradiol levels</th>
<th>RAEC</th>
<th>RAEC + AA</th>
<th>E&lt;sub&gt;2&lt;/sub&gt;RAEC</th>
<th>E&lt;sub&gt;2&lt;/sub&gt;RAEC + AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Media (pg/ml)</td>
<td>14.7 ± 0.33</td>
<td>17.0 ± 1.53</td>
<td>4200 ± 925</td>
<td>5700 ± 706</td>
</tr>
<tr>
<td>Cell (pg/mg protein)</td>
<td>12.1 ± 1.00***</td>
<td>11.2 ± 0.97*++,+++</td>
<td>23.8 ± 3.26*+</td>
<td>25.3 ± 4.66*++,+++</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Glutathione levels</th>
<th>RAEC</th>
<th>RAEC + AA</th>
<th>E&lt;sub&gt;2&lt;/sub&gt;RAEC</th>
<th>E&lt;sub&gt;2&lt;/sub&gt;RAEC + AA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell (N/mg/cell protein)</td>
<td>1.45 ± 0.032**</td>
<td>1.44 ± 0.048*++,+++</td>
<td>1.72 ± 0.118*+</td>
<td>1.81 ± 0.071*++,+++</td>
</tr>
</tbody>
</table>

* RAEC (control cell), RAEC + AA (control cells in the presence of 100 μM ascorbic acid), E<sub>2</sub>RAEC (cells incubated with 17 β-estradiol for 5 days) and E<sub>2</sub>RAEC + AA (E<sub>2</sub>RAEC in the presence of 100 μM of ascorbic acid).

** Control cells in the presence of 100 μM of ascorbic acid (RAEC + AA), 17 β-estradiol pre-conditioned cells (E<sub>2</sub>RAEC) and 17 β-estradiol pre-conditioned cells in the presence of 100 μM of ascorbic acid (E<sub>2</sub>RAEC + AA). Significant differences over control values are indicated by *, **, *+, ++P < 0.005.
considered as a primary antioxidant action for ascorbate in terms of suppressing LDL oxidation [40]. It can not be determined at this time whether the protective effect of E2 was related to the amount of incorporation or association with LDL. It is possible that the physicochemical characteristics of LDL could be altered by E2 to a form more resistant to oxidation. Moreover, incubating LDL with E2 for up to 4 h did not confer more resistance to oxidation (data not shown), indicating that the extent of uptake or association with LDL is not influenced by the time of incubation but is related to E2 treatment concentrations. It should be noted that Shwaery et al. [11] reported that an antioxidant effect of E2 at low concentrations was only seen when E2 and LDL were incubated in plasma. The extent of E2 incorporation into LDL may be facilitated by plasma enzymes or factors, however, the extent of incorporation is difficult to measure given the amounts of LDL available for analysis of E2 content. The apparent synergistic interaction between ascorbic acid and E2 may also be related to the recently described inhibition by ascorbic acid of a-tocopherol oxidation by copper [41]. The ability of ascorbic acid to inhibit copper binding via oxidation of histidine to oxohistidine may reduce the extent of LDL oxidation, thus decreasing the demand for antioxidant activity from E2 and other antioxidants.

The antioxidant activity of E2 is in one aspect atypical of other LDL antioxidants since there is no inhibition of vitamin E consumption during LDL oxidation. This suggests that E2 is not interacting directly with vitamin E or preventing its reaction with peroxyl radicals, as described for other antioxidants [42]. Estrogen is a weak chain breaking antioxidant based on the rate constant for reactions with peroxyl radicals and high concentrations are typically required to effectively inhibit LDL lipid peroxidation [43]. The antioxidant protective action may be due to its phenolic structure, which can donate hydrogens after E2 is incorporated into the lipid core of LDL. Direct reactions with peroxyl radicals are possible, as described thermodynamically for reactions between phenolic compounds (including estrogen) and peroxyl radicals [43]. This is supported by the clear suppression of conjugated diene formation during the prolonged lag phase. The consumption of vitamin E during this phase may reflect the facile reaction of a-tocopherol with copper [44]. The kinetic profiles suggest, but do not prove, that E2 may be inhibiting tocopherol-mediated peroxidation [45], and one may speculate that E2 suppresses interactions between tocopheroxyl radical and LDL core lipids. Since the levels of vitamin E and rates of vitamin E consumption are unaffected by E2, it is likely that peroxidation reactions subsequent to initiation of tocopheroxyl radical are being inhibited by E2. Although a direct antioxidant activity for E2 is not found using concentrations comparable to those found in human blood, (which in pre-menopausal women can reach 443 pg/ml and is < 59 pg/ml in postmenopausal women), inhibition of LDL oxidation was found at concentrations as low as 5 ng/ml. This represents levels that are more than 10 times lower than reported previously [11]. More importantly, our findings show that significant antioxidant activity can be demonstrated by E2 in the presence of physiological concentrations of ascorbic acid and under these conditions marked antioxidant activity for E2 is found even at physiological concentrations.

Since LDL is less oxidized in the presence of E2, it is possible that suppressed formation of LDL-\(\cdot\), or other modified forms of LDL, may contribute to E2-mediated protection. This may also account for the cytoprotection afforded after treatments with high levels of LDL. Treatments with 500 \(\mu\)g/ml of LDL are likely to be accompanied by substantial amounts of contaminating LDL, present either in the isolated LDL or formed by cell-mediated oxidation. In either case, if the levels of LDL-\(\cdot\) were comparable to that reported previously for LDL from human plasma (i.e. 2–6%) [25], then LDL-\(\cdot\) levels could be as high as 20 \(\mu\)g/ml which are sufficient to produce cytotoxicity [25]. Surprisingly, the presence of ascorbic acid did not afford a protection against LDL-mediated toxicity to RAEC or E2RAEC. The protective effect E2 appears to be due to altered cell properties (conferring resistance) elicited only after pre-treatment or pre-conditioning of cells. Since cytotoxicity resulting from high doses of LDL or LDL-\(\cdot\) may be due to factors other than oxidative stress, ascorbic acid could alter the response of cells to E2 even though there were no apparent effects on uptake (Table 2). However, the combination of E2 and ascorbic acid may increase the amount of LDL-\(\cdot\) uptake as compared to E2 alone (Fig. 6) producing the greater cytotoxicity described in Table 1.

During cell-mediated oxidation a variety of events contribute to the formation of LDL-\(\cdot\). Two important processes include: (1) extracellular modification of LDL, and (2) uptake of LDL and/or LDL-\(\cdot\). Inhibition of extracellular modification of LDL by E2 could be explained by the increased levels of GSH found in E2 pre treated cells, either in the presence or absence of ascorbic acid. The latter could plausibly influence the degree of LDL modification. Although E2RAEC assimilated LDL more readily than RAEC, E2RAEC was less susceptible to LDL or LDL-\(\cdot\)-induced toxicity. This suggests that E2 protects endothelial cells by assimilating LDL more readily, but not LDL-\(\cdot\).

During atherogenesis, a stimulated adherence of monocytes to the endothelium marks an early response to hyperlipidemia and is associated with the formation of foam cells. In this respect, modifications to LDL have been shown to facilitate foam cell formation by
stimulating monocyte/macrophage adherence to endothelium and influx into the intima [46] followed by uptake of oxidized LDL-cholesterol [18]. E₂ may not only inhibit LDL accumulation by vascular cells but also suppress LDL modification and, in turn, the consequent pro-inflammatory effects of modified LDL [47]. Estrogens have been shown to inhibit LDL oxidation as catalyzed by cupric ions, vascular smooth muscle cells, and endothelial cells [24,48–51]. E₂ and ascorbic acid may prevent this process by reducing LDL oxidation formation and its potential vascular cell effects. Increased uptake of LDL may contribute to the lower levels of plasma LDL in women taking estrogen replacement therapy. Our findings suggest three possible properties of E₂ that can contribute to its anti-atherogenic effect: (1) reduced formation of LDL⁻; (2) less uptake of LDL⁻; (3) higher uptake and recycling of LDL, making it less prone to oxidation.

These results provide the first evidence that ascorbic acid can enhance the antioxidant effect of E₂ by preventing LDL oxidation by copper ions or cells. The cytoprotective and possibly anti-atherogenic effect appears to be manifested by reducing the extent of oxidized LDL formation and uptake. The enhanced activity of E₂ in the presence of ascorbate indicates that the antioxidant and anti-atherosclerosis activity of E₂ may occur at concentrations within the physiological range in the presence of ascorbate.

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References


