17β-Estradiol acts separately on the LDL particle and artery wall to reduce LDL accumulation

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Abstract **Estrogen replacement therapy has been shown to attenuate atherogenesis, although the mechanisms for this effect are incompletely defined. Previously, we showed that 17-β estradiol (estradiol) attenuated oxidant stress-induced increases in vascular low density lipoprotein (LDL) accumulation. It was unclear whether estradiol's effect was imparted on the lipoprotein particle or the artery wall. To examine this, we chronically treated rats with the following sex hormones: low estradiol, high estradiol, progesterone, low estradiol + progesterone, placebo, or control. Carotid arteries (n = 8/group) were isolated and perfused with fluorescently labeled LDL. Rates of LDL accumulation were measured before and after treatment with 10 ng/ml tumor necrosis factor-α (TNF) using quantitative fluorescence microscopy. We observed a 50% decrease in basal LDL accumulation rates ($P < 0.01$) and a 25% decrease in endothelial layer permeability ($P < 0.01$) in arteries from estradiol-treated animals. There was no effect of hormone replacement on rate of TNF-induced LDL accumulation ($P = 0.451$), while incubation of LDL with 65 pg/ml estradiol attenuated the TNF effect ($P < 0.01$). These experiments suggest two independent mechanisms of anti-atherogenic protection by estradiol: *1*) decreased endothelial layer permeability; and *2*) incorporation of estradiol into the LDL particle and prevention of LDL binding to the artery wall.**—Walsh, B. A., A. E. Mullick, C. E. Banka, and J. C. Rutledge. **17β-Estradiol acts separately on the LDL particle and artery wall to reduce LDL accumulation.** ***J. Lipid Res.*** **2000.** 41: **134–141.**

Supplementary key words lipoprotein flux • estradiol • carotid arteries • antioxidant • atherosclerosis

One of the initial steps in the development of atherosclerosis is the accumulation of low density lipoprotein (LDL) in the artery wall (1). Furthermore, LDL accumulates more readily in the artery wall under oxidant stress conditions (2). Oxidant stresses cause increased modification of LDL (1), as well as modification of vascular wall matrix components. This modification results in increased binding and accumulation of LDL in the vasculature (3). Antioxidant protection of plasma lipids and vascular wall tissue against oxidative damage could attenuate the development of atherosclerosis.

Estrogens protect against the development and progression of atherosclerosis (4–6). Some of estrogen's protective role is attributed to its antioxidant activity. Because of its phenolic structure, estrogen has been compared to potent antioxidants such as α-tocopherol and shown to be at least as effective, it not more so, in protecting LDL from oxidation (7). For example, estrogen protected LDL against 2,2′-azobis (2-amindino-propane) dihydrochloride- and copper-mediated in vitro LDL modification (8). Furthermore, studies of cell culture showed that estrogen protects LDL from cell-mediated LDL modification (9). Thus, in vitro and ex vivo work supports an antioxidant role for estradiol.

Studies examining the role of estrogen at the level of the artery wall have yielded similar results. Recent findings in nonhuman primates (10, 11) indicate decreased arterial metabolism and lipid peroxidation in LDL during estrogen replacement therapy. Previous work from our laboratory (12) showed that acute exposure to physiological levels of estradiol attenuated both cell-mediated and xanthine/xanthine oxidase-mediated increases in LDL modification and accumulation in perfused rat arteries. Whether estradiol's antioxidant protection was solely attributed to protection of the LDL particle itself and/or to a more widespread protection of vascular tissue against oxidant stress was unclear.

Some studies performed thus far suggest that estrogen must be associated with LDL in order to confer its antioxidant protection and prevent the progression of atherosclerosis (13, 14). Conversely, findings from Alzheimer's disease work, another clinical disease in which oxidative damage plays a major role, indicate that estrogen provides a far-reaching protective tissue effect against oxidant stress (15). It is possible that estradiol provides more widespread antioxidant protection of the vascular wall in the prevention of cardiovascular disease as well. We undertook this study to: *1*) discriminate between estrogenic

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Abbreviations: LDL, low density lipoprotein; TNF, tumor necrosis factor-α; DiI, 1,1-dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine.

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effects exerted on LDL versus vascular wall tissue; and *2*) determine whether estrogen, present in the artery wall, would alter basal levels of LDL accumulation and protect LDL from cell-mediated modification and subsequent increased accumulation.

METHODS

Chemicals and materials

Kreb's-Henseleit buffer consisted of (in mm): NaCl 116, KCl 5, $CaCl_2*H_2O$ 2.4, $MgCl_2$ 1.2, NH_2PO_4 1.2, and glucose 11. All 90-day hormone pellets were obtained from Innovative Research of America. Tumor necrosis factor-α (TNF; used to induce cell-mediated oxidant stress) and ReagentSet (used to precipitate apoprotein B-containing lipoproteins for estradiol analysis) were purchased by Boehringer Mannheim. The fluorophores 1,1-dioctadecyl-3,3,3′,3-tetramethyl-indocarbocyanine (DiI) and tetramethylrhodamine isothiocyanate dextran (TRITC-dextran) were obtained from Molecular Probes and Sigma Chemicals, respectively.

Fluorescent solutes

LDL was isolated and labeled as described by Pitas et al. (16). Briefly, blood from fasting human males was obtained in Vacutainers containing ethylenediaminetetraacetic acid and centrifuged for 10 min at 2800 rpm at a temperature of 4°C. The plasma was recovered and LDL (d 1.01–1.06 g/ml) and lipoprotein-deficient plasma were obtained by sequential density gradient ultracentrifugation. LDL was labeled with the fluorescent hydrocarbon probe 1,1-dioctadecyl-3,3,3′,3′-tetramethylindocarbocyanine (DiI) and dialyzed in PBS at 4°C for 48 h. The spectral properties of DiI are the following: excitation maximum 540 nm and emission maximum 556 nm. The labeled LDL (~1 mg protein/ml) was diluted 1:20 in Kreb's-Henseleit solution (final concentration ~50 μg/ml) for use in these experiments.

Some arteries were perfused with dextran 76,000 MW labeled with tetramethylrhodamine isothiocyanate (TRITC). TRITC-dextran was dissolved in H_2O to a concentration of 75 mg/ml and diluted 1:1200 in Kreb's-Henseleit solution (final concentration = 63 μg/ml).

Animal care and hormone supplementation

Ovariectomized female Crl:CD®(SD)BR rats (5 weeks old) were purchased from Charles River Laboratories and maintained in a facility approved by the Animal Use Committee at the University of California, Davis. Animals received rat chow and water ad libitum and were kept on a 12:12 h light/dark cycle.

Pellets containing ovarian sex hormones were implanted in these rats 3–4 weeks after their arrival. The animals were anesthetized intraperitoneally with xylazine (10 mg/kg) and ketamine (80 mg/kg). A posterior longitudinal 1.0-cm length skin incision was made in the neck starting from the base of the skull. Underlying muscle tissue was separated from the skin, being careful not to tear the muscle or surrounding fascia. Pellets contained either low estradiol (2.5 mg estradiol), high estradiol (25 mg estradiol), progesterone (200 mg), low estradiol + progesterone, placebo (no hormone), or control (no implant), and were placed in the small lateral subcutaneous spaces posterior to the ears. The dorsal skin incision was sealed with Nexaband™ tissue glue and the animals were given an intramuscular injection of the antibiotic enrofloxine (10 mg/kg) and placed on a heating pad for recovery. This procedure was repeated after 90 days (total of 180 days of treatment).

Perfusion experiments

The procedures for perfusions of rat carotid arteries with fluorescently labeled LDL were identical to those reported previously (12, 17). In brief, all rats were fasted for 12 h prior to the experiment. Rats were anesthetized intraperitoneally with 65 mg/100 mg body weight sodium pentobarbitol. Both common carotid arteries were exposed and cannulated as follows. An anterior midline skin incision was made from the mandible to the sternum. The superficial neck muscles were retracted and the carotid arteries were carefully dissected free from surrounding tissue. An incision was made in the proximal artery and a cannula (polyethylene-60 tubing) was inserted and tied into place with 4-0 silk. A second incision was made in the distal portion of the artery, just proximal to the bifurcation of the common carotid artery, and another cannula was inserted and tied into place. Throughout the surgery, the artery was superfused with Kreb's-Henseleit solution. Upon completion of the cannulations, the artery was perfused with Kreb's-Henseleit solution plus 1% bovine serum albumin gassed with 95% compressed air and 5% CO_2 until the start of the experiment. The artery was excised and taken to the experimental rig for the perfusion experiment.

Experimental rig

The cannulated artery was placed in a clear fluid-filled superfusate chamber and mounted on a Nikon MM-11 upright microscope stage for viewing. The artery was continuously bathed in a Kreb's-Henseleit solution that was maintained at 37°C. This was achieved by directing the buffer solution from a reservoir through a heating coil to the superfusate chamber. A thermometer placed in the superfusate chamber monitored the temperature. The temperature of the superfusate solution was adjusted by changing the temperature of the heating coil.

Perfusate flowed through the artery at a physiological flow rate (7 ml/min) and hydrostatic pressure (90 cm H_2O). The perfusate consisted of either *1*) a clear, non-fluorescent solution of Kreb's-Henseleit solution + 1% bovine serum albumin or *2*) a fluorescent solution containing the same components as *1*) and 50 μg/ml fluorescently labeled LDL molecules. These perfusate solutions were kept in separate reservoirs and were alternately perfused into the artery via a series of tubing and 2- and 3-way stopcocks. Both solutions were maintained at a pH of 7.35. A 10-cm length of perfusate tubing attached to the proximal cannula was submersed in a 38°C water bath to ensure physiological temperature of the perfusate as it entered the vessel.

The portion of the artery in the superfusate chamber to be examined was brought into focus using a Nikon Plan X4 objective (NA 0.1) mounted on the microscope head. Light from a mercury bulb passed through a filter specific for the fluorescent molecules to be excited causing photons to be emitted from the fluorophore. A dual optical path tube transmitted the fluorescent image to a Hamamatsu CCD television camera and Nikon P1 photometer and controller. The photons emitted from the fluorophore were captured and quantified by the photometer. Changes in fluorescence intensity were measured by the photometer and input to a chart recorder and computer. A videocassette recorder and high resolution monitor received the output from the television camera.

Measurement of LDL accumulation

The photometric measuring window was centered on a segment of the vessel positioned on the microscope stage and fluorescence was captured from this same segment for the duration of the experiment. Also, the same segment of the artery was imaged using the optical system described above. Initially, the artery was perfused with the nonfluorescent solution to determine a baseline level of fluorescence intensity (I_f). Then, the artery was perfused with the buffer solution containing the fluorescently labeled DiI-LDL for 10 min, immediately followed by wash out (10-min) of the fluorescent solution with the clear nonfluorescent

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solution. This sequence of a 10-min perfusion of the fluorescent solution followed by a 10-min perfusion of the nonfluorescent solution will subsequently be referred to as a "run" (Fig. 1A).

Perfusion of the artery with the fluorescent solution resulted in a step increase in the fluorescence intensity (I_{f0}, Fig. 1A). I_{f0} represented the initial fluorescence intensity of the solution filling the artery lumen. During perfusion of fluorescent solution, some fluorescent molecules crossed into the artery wall or bound to the endothelium. When the fluorescent solution was washed out by the nonfluorescent solution, there was a rapid drop in fluorescence intensity and I_f approached the previously determined baseline. Fluorescently labeled LDL molecules remaining in/on the artery wall were designated I_f accumulation (Fig. 1B) and were measured in millivolts (mV).

We were able to quantify the rate of LDL accumulation in the artery wall by converting the photometric units (mV) to the physiological units of ng LDL protein/min per cm^2 by knowing the following parameters: concentration of DiI-LDL protein in the perfusate solution, I_{fo}, or initial fluorescence of DiI-LDL solution filling the artery lumen in the photometric viewing window, time of DiI-LDL perfusion, and radius and length of the artery in the photometric viewing window. The artery was assumed to be a standard cylinder where volume = $\pi r^2 l$. We first determined the number of photons measured by the photometer (mV) corresponding to the concentration of LDL (μg protein). From this, we calculated a rate of LDL accumulation. To illustrate, a typical carotid artery has a radius (r) of 0.05 cm. The length (l) of the artery is fixed (0.12 cm) and determined by the physical limitations of the photometric measuring window. Here, the artery lumen volume within the measuring window was 0.000942 ml. If I_{fo} equals 38 mV and the concentration of DiI-LDL protein is 0.050 mg/ml, we know that the 0.000942 ml volume of LDL solution filling the artery contains 0.0471 μg DiI-LDL. Hence, a photometric reading of 38 mV represents 0.0471 μg DiI-LDL. If, after a 10-min perfusion, it was observed that the fluorescent intensity had increased by 1 mV, this corresponded to an accumulation rate of 3.3 ng LDL/min per cm^2.

Measurement of dextran accumulation (estimates of permeability)

Measurements of dextran accumulation were performed exactly as described above for LDL accumulation. As LDL binds to the artery wall, thus confounding any direct permeability measurements, we used rate of accumulation of this water-soluble non-lipid reference molecule as an estimate of arterial permeability.

Estradiol and progesterone quantification

After removal of both carotid arteries, blood was collected from each animal through the right atrium using a 22 g needle and a heparanized syringe. Blood was transferred to sterile vacutainers and centrifuged (2800 rpm for 10 min). Plasma samples were separated from blood cells and kept at −20°C. These samples were sent to the UC Davis Endocrinology Laboratory for determination of estradiol (18) and progesterone (19) concentrations.

Statistical analysis

For each artery, the rate of LDL accumulation was measured for each run. A control rate of LDL accumulation was determined by averaging the rate of accumulation for the three runs of the experiment performed under control conditions. Then, 10 mg/ml TNF was added directly to the perfusate solutions and the rate of LDL accumulation was determined for each subsequent run. The rate of LDL accumulation after treatment with TNF was determined by averaging the rate of accumulation for the four runs of the experiment performed under TNF treatment conditions. The mean and standard error of the mean were determined for each treatment group.

All statistical analyses utilized SigmaStat 2.0 software by Jandel Scientific Software. Differences between two treatment effects and across multiple hormone treatment groups were analyzed using *t*-test and one-way ANOVA, respectively. Student-Newman-Keuls post hoc test was used to analyze for differences among the groups when a significant effect was found. Tests of significance were applied at $P < 0.05$.

RESULTS

Hormone effects on baseline rate of LDL accumulation

To determine whether chronic sex hormone administration altered LDL flux in the artery wall, we examined the rate of LDL accumulation under control conditions in vessels from animals undergoing 6 months of hormone treatment. LDL accumulated less in vessels from estrogen-supplemented groups (**Fig. 1C**). The rates of LDL accumulation (ng LDL protein/minute/cm^2) in ascending order were: low estradiol = 1.24 ± 0.16; high estradiol = 1.43 ± 0.24; placebo = 2.32 ± 0.42; control = 2.42 ± 0.53, progesterone = 2.59 ± 0.78; and progesterone + low estradiol = 2.78 ± 0.63 ($P = 0.150$; **Fig. 2**). When the two estrogen-only vessel groups were compared to all other treatment groups, a significant difference was seen (1.33 ± 0.13 ng/min/cm^2 versus 2.51 ± 0.27 ng/min/cm^2, $P = 0.006$). Similar findings were observed in ovariectomized female 3-month-old rats receiving 21-day subcutaneous hormone treatment (data not shown).

Hormone effects on estimated arterial permeability

Next we sought to determine whether the estrogen effect on LDL accumulation rate was due to alterations in vessel permeability or reduction in LDL binding to the artery wall. To test the former possibility, we perfused each artery with the nonlipid reference macromolecule TRITC-dextran 76,000 MW (dextran) and compared the baseline rate of accumulation in the various hormone-treated vessels. There was a tendency for decreased permeability in estradiol-treated vessels when compared to non-estradiol-treated vessels. Rate of dextran accumulation values in ng/minute per cm^2 were: high estradiol = 4.26 ± 0.22; progesterone + low estradiol = 4.32 ± 0.08; low estradiol = 4.33 ± 0.41; progesterone = 5.49 ± 0.60; placebo = 5.73 ± 0.78; and control = 5.79 ± 0.50 ($P = 0.150$; **Fig. 3**). When all estrogen-treated vessels (n = 10) were compared to all other non-estrogen-treated vessels (n = 11), a significant difference was seen (4.30 ± 0.17 mV/min versus 5.66 ± 0.32 ng/minute/cm^2, $P = 0.006$). Thus, 6 months of chronic estradiol treatment resulted in decreased estimated arterial permeability.

Effect of chronic ovarian sex hormone treatment on TNF-induced increases in LDL accumulation rate

Tumor necrosis factor-α (TNF) promotes oxidant stress through cellular production of superoxide anions in both endothelial cells (20, 21) and smooth muscle cells (22). In one study, superoxide anions secreted by cultured monocytes and endothelial cells in response to TNF resulted in oxidation of LDL (23). Earlier work from our lab found

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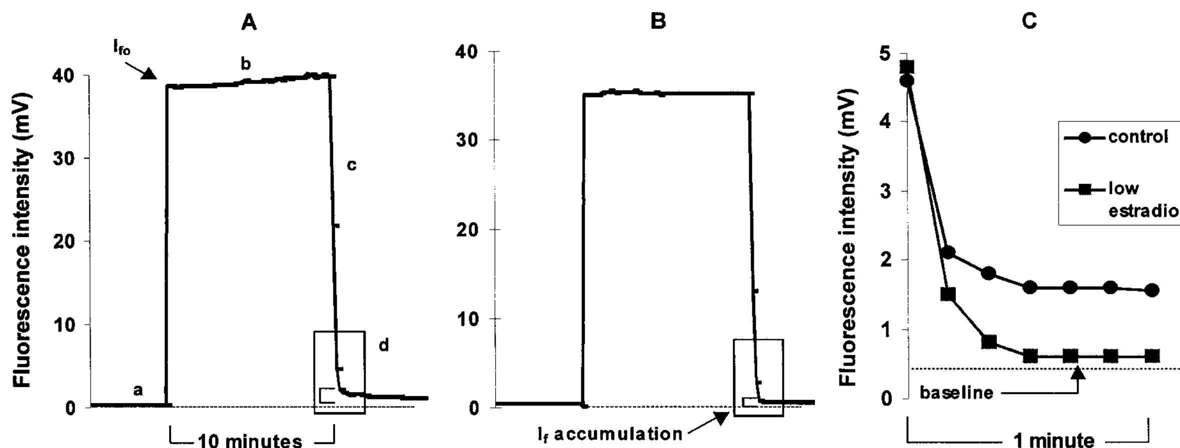

Fig. 1. A: Measurement of fluorescence intensity in an artery from a control rat during perfusion of DiI-LDL. (a) Baseline fluorescence intensity (I_f) obtained during perfusion of the nonfluorescent buffer solution. (b) Perfusion of the buffer solution containing DiI-LDL where I_{fo} represents the fluorescence intensity of the DiI-LDL perfusate initially filling the artery lumen. (c) Washout of the fluorescent LDL solution with nonfluorescent solution. (d) LDL efflux from the artery wall (see Fig. 1C). B: Assessment of LDL accumulation in an estradiol-treated artery. Note that the slope of (b) is decreased, suggesting decreased LDL accumulation in the artery wall over the course of arterial exposure to LDL. I_f accumulation is the amount of DiI-LDL remaining in the artery wall after lumen washout. C: Examination of LDL washout from the artery wall after LDL perfusion under control conditions in arteries extracted from control and estradiol-treated rats ("d" inserts from Figs. 1A and 1B). Note the change in the fluorescence scale. Estradiol treatment results in decreased accumulation of LDL, indicating decreased LDL retention in the artery wall.

that TNF caused profound increases in rate of LDL accumulation in carotid arteries from ovariectomized rats, independent of permeability changes (12). Addition of physiological levels of estradiol or α-tocopherol directly to the perfusate solutions attenuated this effect. It was not determined whether the protective effect of estradiol was due to direct antioxidant protection of the LDL particle itself and/or a tissue effect. In this study, we tested whether estradiol, present only in the artery wall, would prevent increased LDL accumulation due to this cell-mediated oxidant stress. After determination of baseline LDL accumulation rates in each artery, TNF (10 ng/ml) was added to both perfusate solutions and the change in LDL accumulation was measured. The percent change in LDL accumulation was calculated for each individual artery using the following calculation: [(accumulation rate with TNF − baseline accumulation rate)/baseline accumulation rate)]*100. Chronic sex hormone treatment had no effect on the rate of LDL accumulation induced by TNF ($P = 0.453$). Percent increases in TNF-induced rates of accumulation across hormone treatment groups ranged from 90 to 180% and the mean percent increase in all estradiol-treated vessels was 131 ± 24% (**Table 1**).

Estradiol protects LDL from modification by direct association with the lipid particle

We next tested whether estradiol, when directly associated with the LDL particle, protects LDL from modifica-

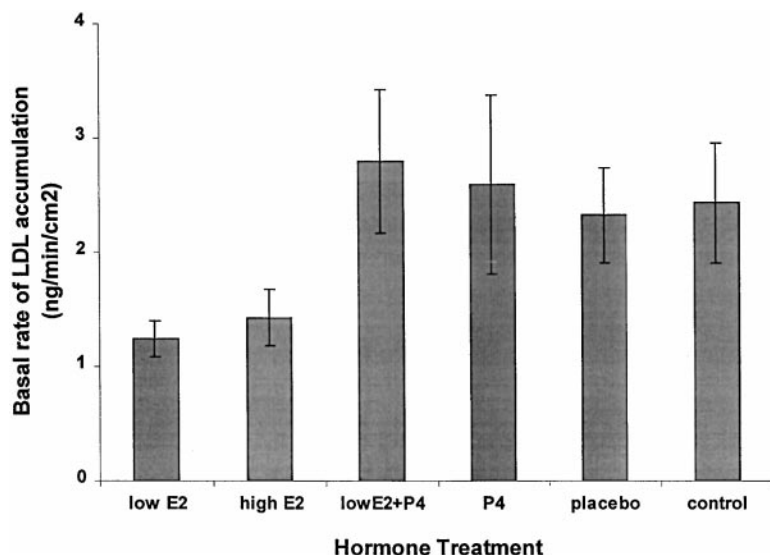

Fig. 2. Basal rates of LDL accumulation were measured in vessels from rats receiving either low estradiol (E_2), high E_2, low E_2 + progesterone, progesterone, placebo, or control (no implant). Arteries from rats receiving high and low doses of unopposed estradiol exhibited baseline rates of LDL accumulation 50% lower than those observed in all other treatment groups.

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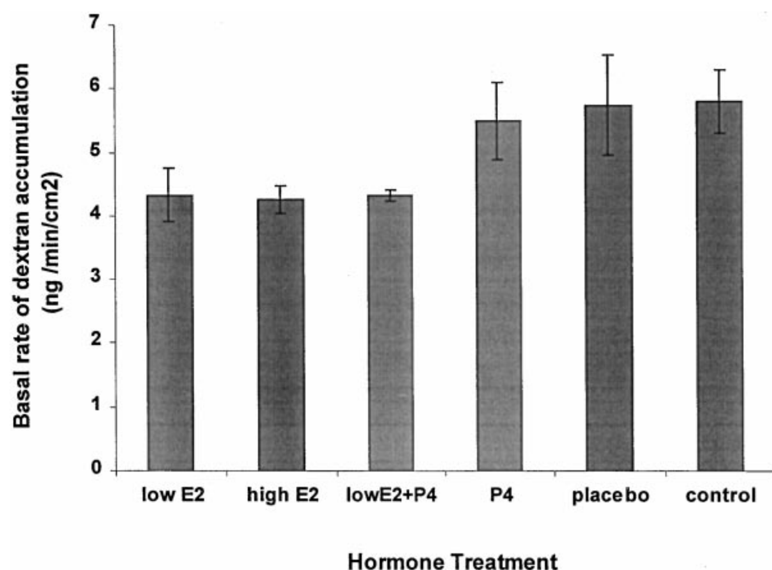

Fig. 3. Arterial permeability was estimated in vessels from hormone-treated rats by perfusion with 76,000 MW TRITC-dextran. Vessels from rats receiving high estradiol (E_2), low E_2, or low E_2+ progesterone (estradiol) displayed permeability values 25% lower than vessels from control, placebo, and progesterone treatment groups as estimated by rate of dextran accumulation measurements.

tion and subsequent accumulation due to an oxidant stress. Four vessels from untreated ovariectomized rats were perfused with LDL that had been incubated with 65 pg/ml estradiol (estradiol-treated LDL) for 48 h at 4°C. Rates of LDL accumulation under control and oxidant stress (TNF) conditions were determined and compared to the rates of LDL accumulation determined for four vessels (from untreated ovariectomized rats) perfused with control LDL. We found that incubation of LDL with physiological levels of estradiol greatly diminished the TNF-induced response (Table 1). The % increase in LDL accumulation due to TNF treatment was 5.5-fold higher in control LDL compared to estradiol-treated LDL (144 ± 16% versus 26 ± 15%; $P < 0.01$). These findings, in combination with the results of arteries from estradiol-implanted rats (above), indicate that estradiol's antioxidant protection of LDL from cell-mediated modification is due to a direct effect on the LDL particle itself. There was no effect of estrogen association with the LDL particle on baseline LDL accumulation (2.21 ± 0.47 ng LDL protein/minute/cm^2 versus 2.18 ± 0.58 ng LDL protein/minute/cm^2 for estradiol-treated and control LDL accumulation, respectively, $P = 0.571$).

TABLE 1. Comparison of estradiol's effect against TNF-induced (10 ng/ml) LDL accumulation when incorporated into the LDL particle versus in the artery wall

Treatment Group	n	Lipoprotein	Artery	% Change in Rate of LDL Accumulation with TNF Treatment
1	4	LDL	Ovariectomized	144 ± 16
2	4	LDL+estradiol	Ovariectomized	26 ± 15[a]
3	15	LDL	Ovariectomized + estradiol	131 ± 23

Vessels from ovariectomized rats were perfused with either LDL (group 1) or LDL exposed to 65 pg/ml estradiol (group 2) and compared to LDL-perfused vessels from ovariectomized rats chronically implanted with either high estradiol, low estradiol or low estradiol + progesterone (group 3). Baseline and treatment (TNF) rates of LDL accumulation were measured in each artery, each artery serving as its own control. The % change in rate of LDL accumulation with TNF treatment was calculated for each artery as follows: [(accumulation rate with TNF − baseline accumulation rate)/baseline accumulation rate)] × 100. The % changes of all vessels in each group were averaged and the results listed. Data listed for treatment group 1 have been published previously (12).

[a] Corresponds to significant difference in % change from other treatment groups, $P < 0.01$.

To determine whether estradiol treatment of DiI-labeled, isolated LDL resulted in association of estradiol with the LDL in concentrations parallel to or greater than the association with LDL in whole plasma, we quantitated the association of estradiol and LDL. DiI-LDL was incubated with 65 pg of estradiol as was plasma from the same donor (both at 1.0 mg cholesterol/ml). [^{3}H]estradiol was added as a tracer. After 48 h of incubation at 4°C, the DiI-LDL was diluted to 50 μg cholesterol/ml with Kreb's-Henseleit buffer containing 1% bovine serum albumin and the plasma was diluted with saline. The apolipoprotein B-containing lipoproteins were then precipitated with phosphotungstic acid, magnesium chloride (ReagentSet, Boehringer Mannheim). The [^{3}H]estradiol precipitated with the lipoproteins was determined by counting the supernatant and the mass of estradiol precipitated was calculated. Isolated LDL contained 4.71 ± 0.26 pg estradiol/mg cholesterol. Precipitated lipoproteins from plasma contained 10.38 ± 0.28 pg estradiol/mg cholesterol. Based on the LDL and VLDL cholesterol content of the plasma sample, LDL in plasma was calculated to contain 7.11 pg estradiol/mg cholesterol. These results suggest that estrogen associated with LDL in plasma to a greater extent than with isolated LDL confirming the results of Shwaery, Vita, and Keaney (14). Thus, association of a small quantity of estradiol with LDL was sufficient to reduce LDL accumulation in the artery wall.

Plasma hormone levels resulting from various hormone implants

The high estradiol group obtained plasma levels of estradiol 5- to 10-fold higher than the two groups receiving

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TABLE 2. Plasma estradiol and progesterone levels resulting from subcutaneous hormone implants

Treatment Group	n	Plasma Estradiol	Plasma Progesterone
		pg/ml	*ng/ml*
Low E2	6	62.6 ± 18.6[b]	25.2 ± 6.1
High E2	6	343.0 ± 77.5[c]	25.2 ± 6.8
Low E2 + progesterone	6	18.6 ± 7.22[b]	28.5 ± 7.6
Progesterone	6	7.44 ± 3.4[a]	17.2 ± 4.0
Placebo	6	6.76 ± 3.0[a]	15.5 ± 6.0
Control	6	6.2 ± 2.5[a]	22.2 ± 5.2

Ovariectomized female rats were implanted for 6 months with pellets containing either low E2 (2.5 mg estradiol), high E2 (25 mg estradiol), progesterone (200 mg), low E2 + progesterone, placebo (no hormone), or control (no implant).

[a,b,c] Correspond to significant differences, $P < 0.05$.

the low dose of estradiol (**Table 2**). All animals receiving estrogen implants obtained higher plasma levels of estradiol compared to the groups receiving placebo, progesterone, or no implant ($P = 0.05$). Plasma progesterone levels in ng/ml were: progesterone = 17 ± 4, placebo = 15 ± 6, control = 22 ± 5, high estradiol = 25 ± 7, P_4 + low estradiol = 28 ± 7, and low estradiol = 25 ± 6. All values were in the normal range as average values in cycling rats range between 5 and 30 ng/ml (24). Statistically, there was no difference among the groups. These progesterone findings were unexpected and will be explained further in the discussion.

DISCUSSION

Our studies demonstrate two possible mechanisms for the atheroprotective effects of estradiol. One is the antioxidant protection of LDL. This is an action that appears to require incorporation of estradiol into the lipid particle itself. The second, a decrease in estimated arterial permeability, is due to a chronic estrogenic effect on the vascular wall. These findings are important in that they elucidate the diverse roles involved in estradiol's protection against the arterial accumulation of lipid.

We utilized rat carotid arteries in these studies in order to examine lipoprotein interactions with the vascular wall uncomplicated by confounding factors such as the presence and/or progression of vascular disease. Previous work from our laboratory (25) and others (26) indicates that macromolecule flux in the artery wall is sensitive to physical parameters (for example, hydrostatic pressure). Our experimental preparation allows us to make serial real time measurements of LDL accumulation in individually perfused arteries under controlled conditions. Perfusate flow, hydrostatic pressure, pH, and temperature, as well as the perfusate and superfusate composition are precisely defined and can be manipulated. Thus, the effects of estradiol on the artery wall could be studied in these isolated arteries independent of other systemic and local alterations that may occur with chronic hormone treatment.

We were able to obtain physiological levels of plasma estradiol in our experimental animals using subcutaneous hormone implants. The high estradiol group achieved plasma estradiol levels seen in pregnant rats (27) and both of the low estradiol groups (low E_2 and low E_2 + progesterone) obtained plasma estradiol levels seen in intact cycling rats (24). Our plasma progesterone levels resulting from the implants were less dependable. Although all in the normal range (15–28 ng/ml), values were higher than would be expected with ovariectomy and there was no difference in plasma progesterone values between the progesterone-implanted rats and the other groups in this study. We believe that the progesterone values obtained here were the result of an acute stress-induced adrenal response to the handling and anesthetizing of the rats on the experimental day. The concept that the adrenal glands are capable of producing significant amounts of progesterone is well documented (28, 29). Resko (28) and others have described a direct relationship between increased stress and ACTH levels with increased levels of adrenal progesterone production in rats. Therefore, the acute elevation in plasma progesterone and the lack of parity between progesterone supplementation and plasma progesterone levels seen here does not accurately represent the actual 6-month hormone treatment. More importantly, this phenomenon does not alter levels of plasma estradiol and so does not compromise the reliability of these hormone measurements. As such, this issue does not negatively impact our findings regarding estradiol's effects on the vascular wall.

Our observations support previous work in primates (10, 11) describing an anti-atherogenic role for estrogen in the artery wall. Wagner and colleagues (10, 11) found that hormone replacement therapy reduced LDL uptake and accumulation in the artery wall independent of plasma lipid levels. We showed that, at least in part, estrogen decreased basal LDL accumulation by decreasing permeability. Conversely, Haarbo et al. (30) found that estrogen did not affect aortic permeability to LDL in rabbits. Although the importance of arterial permeability in the development of atherosclerosis has been demonstrated (31), there is very little research available regarding factors controlling permeability in arteries.

Estrogen's effect on arterial permeability may occur through genomic and/or nongenomic mechanisms. Estrogen receptors are found in the vessel walls of many species, including humans (32) and rats (33). Through these receptors, estrogen may act to alter the transcription of permeability factors or basement membrane proteoglycans, such as vascular endothelial growth factor and perlecan, which could affect permeability. An alternative mechanism of estrogenic action may be through estrogen's incorporation into, and stabilization of, the endothelial cell membrane. Synthetic estrogens (34) and other lipophilic antioxidants, such as α-tocopherol (35), have been shown to have membrane stabilizing effects. Last, the existence of cell surface estrogen receptors has been described for several cell types (15, 36–38). There is mounting evidence that estrogen can modulate intracellular second messenger systems possibly through action at the cell surface, although to date there has not been a specific

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non-nuclear estrogen receptor identified in the vasculature. However, these data (39) suggest that this is another potential mechanism by which estradiol exerts its effects.

It is difficult to determine whether the effect of estradiol on permeability is due to genomic or nongenomic mechanisms. Acute studies (12) involving exposure of perfused arteries from ovariectomized rats to physiological levels of estradiol resulted in no change, or insignificant increases, in LDL accumulation, supporting a genomic mechanism. In other work, 1-year-old rats that had been supplemented with hormone for 9 months exhibited permeability responses similar to those reported here, even when the experiments were performed after the hormone implants expired and the plasma estradiol levels were diminished (unpublished). These results do not discern between estrogenic genomic and nongenomic mechanisms. Further work is needed to elucidate the mechanisms involved in estradiol's persistent long-term effects.

Our finding that estradiol serves to protect LDL from modification and accumulation supports recent work showing that estradiol is a potent biological antioxidant. The exact mechanism involved in the antioxidant protection of LDL from modification is not clear. However, many biochemical and cell culture studies suggest that estrogen acts to scavenge oxygen radicals, and thus protects LDL from modification (8, 9). Niki and Nakano (40) demonstrated that estradiol acts as a classic chain-breaking antioxidant having free radical scavenging activity. Recent studies by Tang, Abplanalp, and Subbiah (13) found that estradiol, or its derivatives, directly associate with LDL and confer antioxidant protection. The major criticism of these studies was the use of supraphysiological doses of estradiol.

Studies utilizing physiological doses of estradiol are conflicting. Recent work by Santanam et al. (41) suggested that at physiological concentrations, estradiol did not function as an antioxidant and protect LDL from Cu^{+2}-mediated oxidation. Conversely, work by Shwaery and colleagues (14) showed that physiological levels of estradiol associated with LDL and also, incubation of estradiol with LDL in the presence of plasma protected against aqueous peroxyl radical-mediated oxidation. Our studies using a different method to modify LDL, showed that physiological levels of estradiol associated with LDL and protected against cell-mediated LDL modification and accumulation in the artery wall. Thus, studies of the antioxidant properties of estradiol are conflicting, possibly related to differences in estradiol concentration, procedures utilized to induce oxidation of lipids and tissue-specific effects.

We cannot rule out an antioxidant role for estradiol in the artery wall even though we found that estradiol alone present in the artery wall did not protect against an acute TNF-induced LDL accumulation. Many studies describe the membrane antioxidant protection of estradiol (15, 42, 43), which could be a factor in protecting endothelial and vascular smooth muscle cells from oxidative damage and subsequent lipid accumulation. Also, estrogen could protect the vascular wall from oxidative damage by altering levels of antioxidant enzymes (44, 45).

The studies presented in this paper examine the chronic effects of estradiol in the artery wall. We performed these studies in order to identify and isolate estrogenic mechanisms affecting LDL interaction with the artery wall and vascular wall function. These studies form the basis for further studies that examine biochemical and molecular vascular wall changes responsible for the physiological changes described here.

In conclusion, physiological levels of estradiol protect against LDL accumulation through two distinct mechanisms: *1*) a lipid particle effect whereby estrogen provides antioxidant protection to the LDL particle against a cytokine-induced oxidant stress, and *2*) a vascular wall effect whereby estrogen causes decreased vascular permeability. Thus, our studies support an anti-atherogenic role for estradiol. Further studies are required to elucidate the cellular mechanisms involved in the vascular wall effects.

The authors wish to thank Kristine Lewis, Patricia Alegria, and Dominic Dawson for their technical assistance. This research was supported by a grant from the National Institutes of Health Heart, Lung, and Blood Institute (RO1 HL 55667).

Manuscript received 11 February 1999 and in revised form 15 October 1999.

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