Opposing Effects of Estrogen and Progestins on LDL Oxidation and Vascular Wall Cytotoxicity: Implications for Atherogenesis (44446)

XIAODONG ZHU,* BARTOLOME BONET,† HIEDI GILLENWATER,‡ AND ROBERT H. KNOPP*,[1]

Department of Medicine, Northwest Lipid Research Clinic, University of Washington, Seattle, Washington 98104; †Department of Internal Medicine, Universidad de San Pablo-CEU, Centro de CC Experimentales y Técnicas, 28668 Madrid, Spain; and ‡School of Medicine, University of North Carolina, Chapel Hill, North Carolina 27514

Abstract. **Estrogens are widely regarded as beneficial to arterial wall health. Among the mechanisms of this benefit are antioxidant effects on LDL and the arterial wall. Because progestins oppose the effect of estrogen in several systems, we asked if progestins oppose the antioxidant effect of estrogen. To study this question, LDL and various female sex hormones were incubated alone and combined in the absence or presence of bovine aortic endothelial cells, placental trophoblast, or macrophages, and LDL oxidation and cytotoxicity were quantitated. In the absence of cells, LDL incubated with copper in phosphate-buffered saline enhanced the oxidation of LDL. When 17β-estradiol was added to this system, an antioxidant effect was observed. Progestins inhibited this protective estrogenic effect. In endothelial cell culture, progestins also opposed the antioxidant effect of estrogen, with the strongest antiestrogenic effect seen with the synthetic progestins, levonorgestrel and medroxyprogesterone acetate (MPA). Endothelial cell cytotoxicity was proportional to the enhanced lipid peroxide formation observed with progestins or estrogen. Similar opposing effects were seen when estrogen and progesterone were added to primary cultures of placental trophoblast or macrophages. Thus, three cell culture systems modeling circulating arterial blood contact with cell surfaces demonstrated opposing effects of estrogens and progestins on LDL oxidation and cell cytotoxicity. These studies are in keeping with published reports that female sex steroids influence LDL oxidation *in vivo* and consequent arterial wall injury.** [P.S.E.B.M. 1999, Vol 222]

Beneficial Effects of Estrogen

Estrogen is widely regarded as beneficial to arterial wall function. As shown in Figure 1, the beneficial effects include changes in the circulating blood, the physiology of the endothelium, and the intima-media of the arterial wall. The estrogen-induced changes in the bloodstream are well described. In plasma, these changes include a reduction in LDL, an increase in HDL, a reduction in Lp(a), and an increase in plasma triglyceride levels (1–5). Also described in the literature are reductions in plasma fibrinogen, PAI-1 activity, diminished LDL oxidation in plasma, enhanced glucose metabolism, and enhanced insulin sensitivity (1, 2, 6–8).

In the arterial endothelium, estrogen increases nitric oxide synthase activity and the secretion of nitric oxide (9–11). Nitric oxide has favorable effects on arterial vasomotion in a number of models, including women who have angina pectoris due to vasospasm, so-called cardiovascular syndrome X (12, 13). In the intima-media of the arterial wall, estrogen diminishes calcification and diminishes the secretion of a number of inflammatory cytokines, including FGF, ICAM-1, VCAM-1, the nuclear receptor NFκB, and atherosclerosis itself in several animal models (1, 2, 12–16).

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[1] To whom requests for reprints should be addressed at Northwest Lipid Research Clinic, 325 Ninth Avenue, #359720, Seattle, WA 98104.

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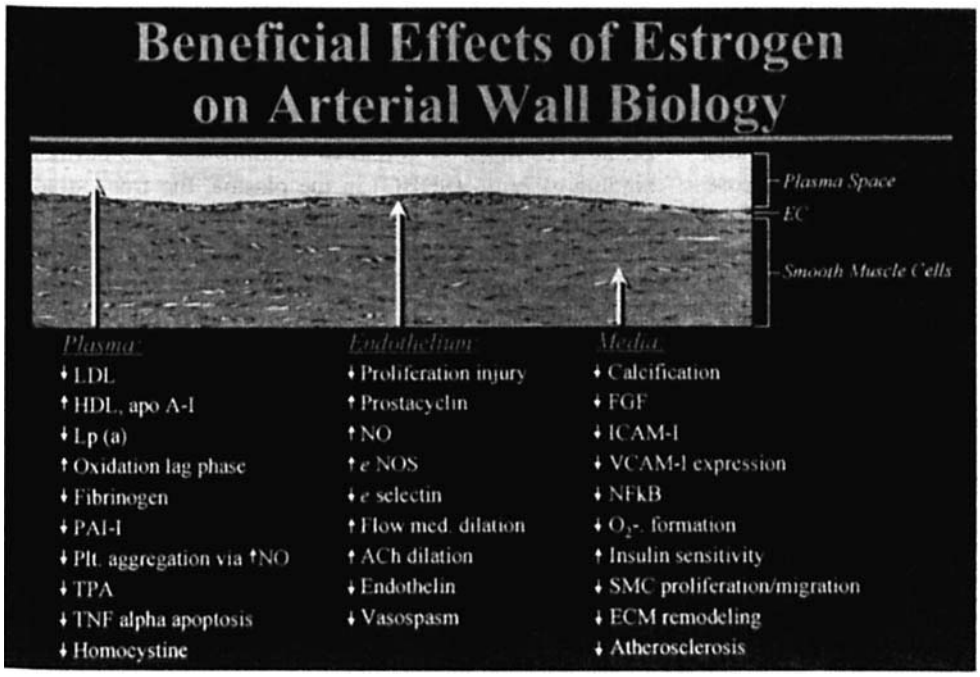

Figure 1. Beneficial effects of estrogen on arterial wall biology. Beneficial effects are listed as occurring in the vascular space, the arterial endothelium, and the arterial wall itself.

Opposing Effects of Progestins on Estrogen Action

The opposing effects of estrogen and progestins are seen in their divergent effects on lipoprotein transport. As shown in Figure 2, estrogens enhance the flow of cholesterol from the diet through chylomicrons and chylomicron remnants to the liver, subsequently through VLDL to LDL to cells, through reverse cholesterol transport from cells *via* HDL to the liver and then the bile (1–3). Under the influence of estrogen, plasma triglyceride levels rise because of increased VLDL production; LDL levels are reduced due to the upregulation of the LDL receptor; and HDL is increased due to increased secretion of apoprotein A-I and diminished removal of HDL lipid due to a reduction in hepatic lipase activity (1–4). On the other hand, progestins appear to slow the stimulatory effect of estrogens on lipoprotein transport in the bloodstream. Specifically, VLDL secretion is reduced, remnant removal appears to be impaired, LDL receptor activity is downregulated with a rise in LDL-C in some settings, and HDL levels fall in association with an increase in hepatic lipase activity. Antiestrogenic effects of the progestins are also described for plasma levels of glucose, insulin, and fibrinogen, all of which tend to increase (1, 2, 7).

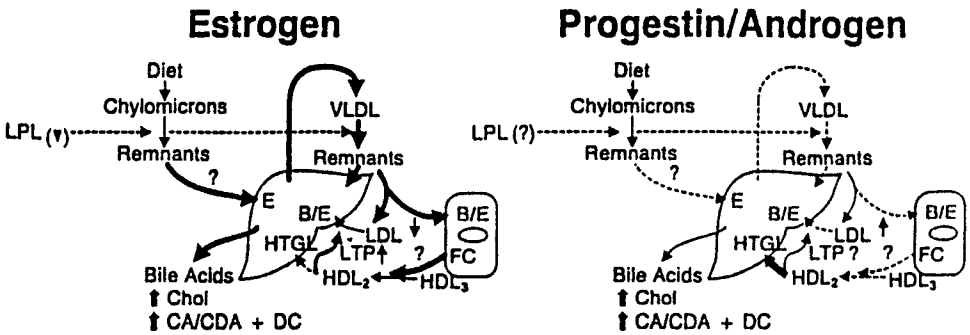

Figure 2. The effect of estrogen and estrogen plus progestin on lipoprotein transport. The broad lines in the left illustration depict an increased rate of cholesterol flow from the intestine to the liver and then from the liver to peripheral tissues and then back to the liver. All steps are believed to be increased by the effect of estrogen. These effects include the enhanced secretion of VLDL by the liver, enhanced removal of VLDL remnants by the upregulated LDL receptor, and increased removal of the LDL also by the LDL receptor. HDL levels are increased, and transport may be increased due to the increased production of apoprotein A-I and the reduction of hepatic lipase (HTGL), which shunts HDL cholesterol from the liver back to LDL, facilitated by cholesterol ester transport protein (LTP). Shown on the right is the effect of progestins added to estrogen, which inhibits all of the steps upregulated by estrogen. (Reproduced by permission from Ref. 39.)

The effects of progestins on atherosclerosis are controversial. Several studies have reported no adverse effect, including natural progesterone in the subhuman primate (16) as well as synthetic progestins in the cholesterol-fed rabbit model (14, 15). On the other hand, Hanke and associates have shown that high doses of natural progesterone oppose the antiatherosclerotic effect of estrogen (17, 18). Others have reported a proatherosclerotic/antiestrogenic effect of synthetic progestins, including medroxyprogesterone acetate (MPA) (19) and levonorgestrel (20) as well as testosterone (21). In addition, an antivasodilatory effect of MPA has been described by Miyagawa *et al.* (22), associated with an alteration in arterial wall calcium signaling (23). Finally, Imthurn *et al.* (24) have found that two progestins, MPA and cyproterone acetate, oppose the estrogen-induced increase of plasma nitric oxide metabolites (nitrate and nitrite). This observation links progestin administration to an antiestrogenic effect on nitric oxide synthesis at the endothelial surface or possibly other sites in the body and is in keeping with the reports of a proatherosclerotic effect of progestin in the presence of estrogen.

The potential clinical significance of the antagonism of estrogen by progestin on the arterial wall has recently been underscored by the surprising results of the Heart and Estrogen/progestin Replacement Study (HERS) (25), which showed that postmenopausal women with the diagnosis of coronary artery disease given an equine estrogen–MPA combination had no better outcome after a 4–5 year follow-up than women given a placebo. In fact, in the first year of the study, an increase was observed in cardiovascular morbidity and mortality, which tended to decline in the subsequent years. It is possible that an enhanced sensitivity to clotting from estrogen among some of the women might explain the excess mortality in the first year. Another possibility is that the progestin administered with estrogen might have attenuated the ability of estrogen to benefit the arterial wall over the course of the study.

Estrogen and Progestin Effects on LDL Oxidation

A potential mechanism whereby estrogen benefits the arterial wall is by its function as an antioxidant (1, 2, 8, 12, 13, 26, 27). As shown in Figure 3, the susceptibility of LDL to copper-mediated oxidation in the formation of conjugated dienes is inhibited in a dose-dependent manner by increasing concentrations of estrogen. The effect in these studies is discernible at a plasma concentration of 1 n*M*, which is equivalent to the circulating plasma estrogen concentration. Because estrogen is bound to albumin and sex-hormone-binding-globulin (SHBG) in the plasma, the free estradiol concentration in the plasma is even lower. However, the relevance of free vs. plasma-bound estradiol to oxidation is uncertain. In addition, it is possible that estrogen may be concentrated in certain microenvironments, including the surface of LDL, the ovary and placenta where estrogen is secreted, or areas where estrogen receptors are abundant, which could include the arterial wall.

Since progestins oppose the effects of estrogen in so many systems, as described above, we reasoned that progestins may have a pro-oxidant effect that opposes the antioxidant effect of estrogen. As shown in Figure 4, progestins and androgens accelerated the rate of LDL oxidation in the conjugated dienes assay compared to the inhibitory effect of 17β-estradiol.

We further investigated the pro-oxidant effect of progestins in the presence of cultured bovine aortic endothelial cells (28). As shown in Figure 5, four different progestins, at a medium concentration of 5 μ*M*, increased lipid peroxide formation after 24 hr of incubation. When estrogen was added alone at a concentration of 1 μ*M*, lipid peroxide formation was inhibited. When estrogen was combined with progestin, the estrogen effect was opposed by the addition of progestins, most strongly by levonorgestrel and medroxyprogesterone acetate (28).

Shown in the bottom of Figure 5 is the effect of LDL oxidation on endothelial cell cytotoxicity (28). When progestins were added alone, levonorgestrel and MPA diminished conversion of the tetrazolium reagent (MTT) to a colored formazan, indicating diminished cell viability. On the other hand, when 1 μ*M* 17β-estradiol was added alone, MTT conversion was greatly enhanced. This estrogen effect was diminished in each of the cultures where progestin was added in combination. The greatest antiestrogenic effect was seen with levonorgestrel and MPA, statistically significantly in the case of MPA versus norgestimate. As shown in Figure 6, a curvilinear relationship describes the association between the formation of lipid peroxides and reduction in

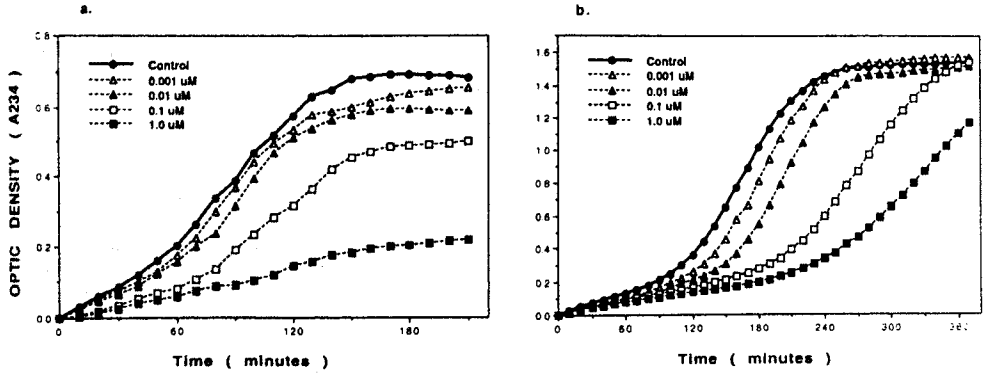

Figure 3. Effect of increasing concentrations of 17β-estradiol on LDL oxidation expressed as conjugated diene formation in the presence of 2 μ*M* copper and 0.1 mg/ml LDL protein. Inhibition of oxidative stress by estrogen is dose-dependent and is detectable down to an estrogen concentration of 1 n*M*.

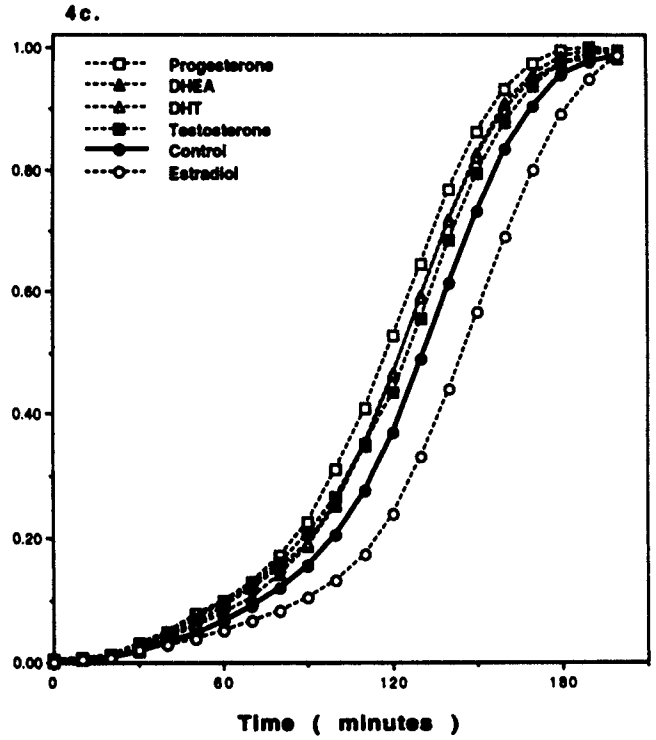

Figure 4. Effect of 0.1 μ*M* concentrations of progesterone and several androgens on LDL oxidation. Conditions are the same as in Figure 3.

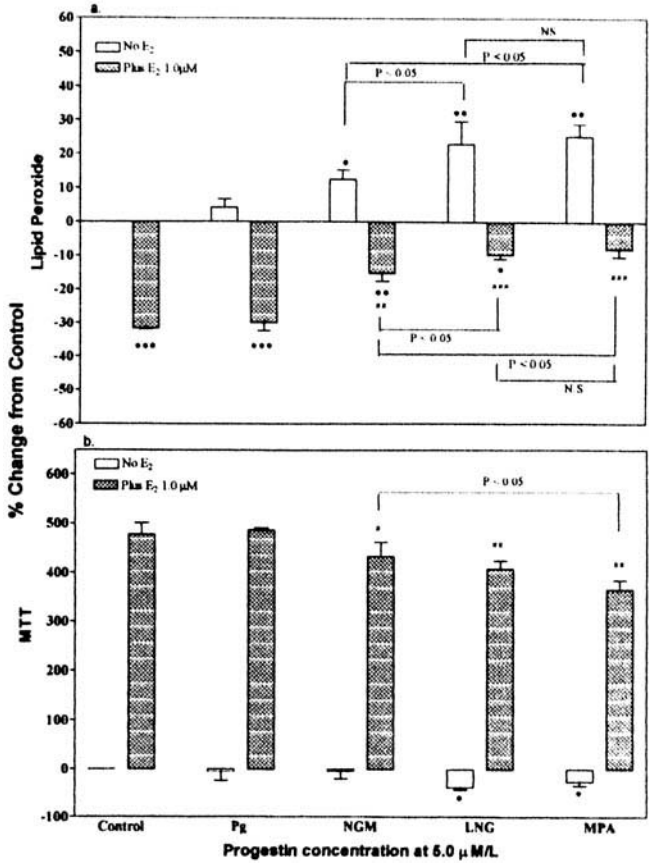

Figure 5. Effect of progestins at 5 μ*M* concentrations and on LDL oxidation. Copper is present at 0.5 μ*M*. Pro-oxidant effects of progestins are greatest for levonorgestrel, and MPA and these two progestins have the maximum antiestrogenic effect in this system. Similarly, in the bottom of Figure 5, levonorgestrel and MPA inhibit the conversion of MTT to formazan. The effect of estrogen is also opposed to the greatest extent by these two progestins. *, **, ***, or #, ##, ### denote significant differences from control without or with estrogen respectively at $p < 0.05$, 0.01, or 0.001. (From Ref. 28 with permission.)

MTT conversion to formazan, based upon the aggregate of the cell culture studies performed (28).

In summary, these studies suggest that the opposing effects of estrogen and progestin on plasma and arterial endothelial cell redox state can modulate the oxidizability of LDL, the viability of the adjacent cells in the arterial wall, and the consequent progression of atherogenesis.

Effects of Estrogen, Progesterone, and Testosterone on Placental Cell-Mediated LDL Oxidation and Cytotoxicity

Another model of the interaction of LDL with vascular cells is the interface of blood and trophoblast in the intervillous space. In a cell culture model of this system, we have again observed opposing effects of estrogen and progestin in placental trophoblast and macrophage primary cell culture (29). Placental cells were isolated from healthy term elective cesarean section pregnancies. Placentas were dissected free of membrane, cut into small pieces, and subjected to enzymatic digestion. Trophoblast and macrophages were then isolated on a 40% Percoll gradient (29). As shown in Figure 7, increased LDL oxidation increased the release of Cr^{51} from prelabeled cells. These results indicate a cytotoxic effect of LDL oxidation in this placental cell culture system (29). The effects of adding metal ions or inhibiting LDL oxidation with antioxidants had the expected pro- and anticytotoxic effects on these cells, as previously described in the arterial wall (30). For this reason, we believe that primary cultures of trophoblast and macrophages from healthy human placenta are another model of the interaction between oxidative stress in the circulating blood stream and the cells with which it comes in contact.

The influence of sex hormones on these processes is shown in Figures 8 and 9. In Figure 8, the antagonistic effect of estrogen on cytotoxicity is illustrated in cultured placental macrophages. In contrast, natural progesterone and testosterone increase Cr^{51} release from macrophages in the presence of LDL and 0.5 μ*M* copper. Linear relationships are described in the inset between lipid peroxide formation or TBARS formation and Cr^{51} release, as observed in the aggregate of the experiments performed.

Similarly, in the cultured trophoblast (Fig. 9), increasing concentrations of estrogen again inhibit cytotoxicity whereas progesterone and testosterone enhance cytotoxicity. A linear relationship is again observed between lipid peroxide or TBARS formation and Cr^{51} release, as shown in the inset.

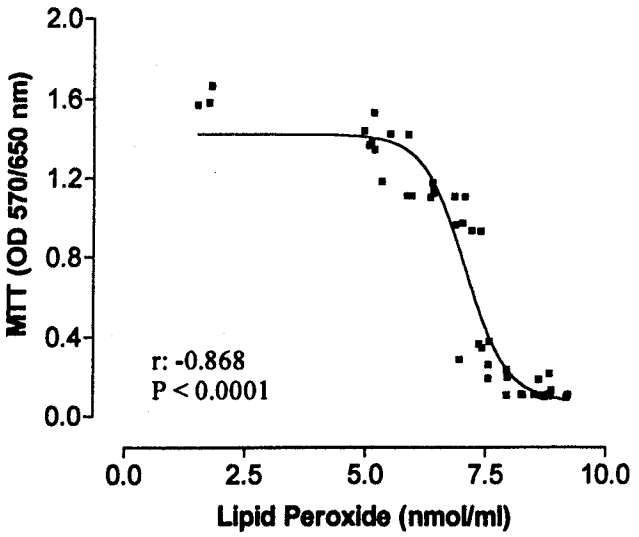

Figure 6. A curvilinear relationship exists between lipid peroxide formation and MTT conversion to formazan. (From Ref. 28 with permission.)

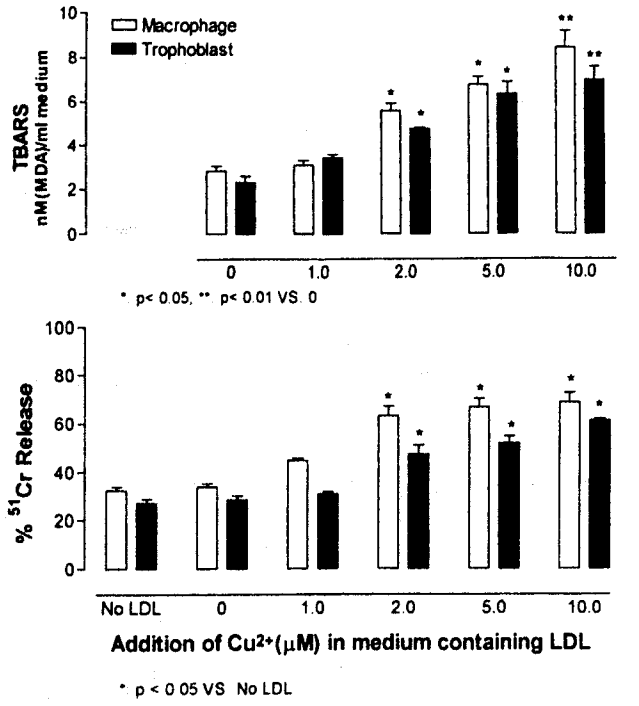

Figure 7. Effect of increasing concentrations of copper on LDL oxidation and release of Cr^{51} from prelabeled placental trophoblast and macrophages. Cr^{51} release is enhanced as is TBARS formation after cells are exposed to increasing oxidative stress. (From Ref. 29 with permission.)

A depiction of the circulation of the blood in the placental bed is shown in Figure 10. The illustration shows how maternal arterial blood flows into the intervillous space and percolates over the placental villi, which are covered by trophoblast. Fixed macrophages (Hofbauer cells) are present in the interior of the placental villi. A similarity exists

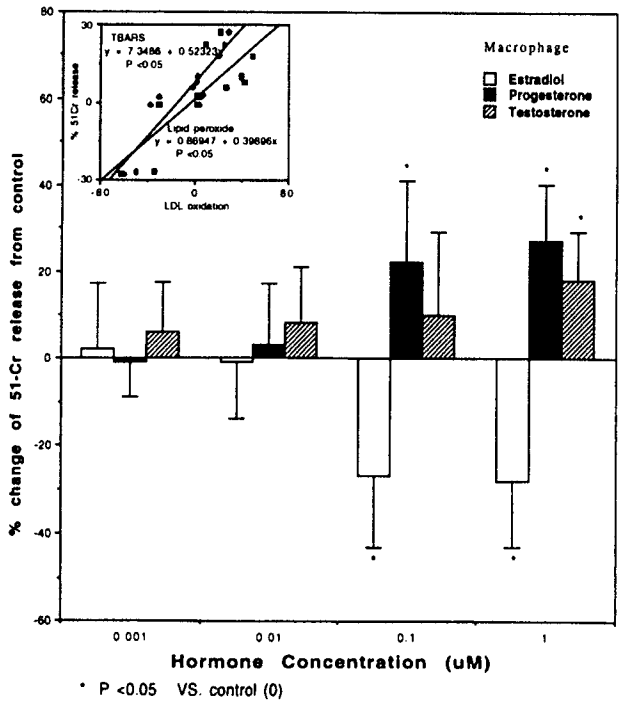

Figure 8. Effect of increasing concentrations of estrogen, progesterone, or testosterone on placental macrophage Cr^{51} release. In this system containing LDL and 0.5 μ*M* copper, Cr^{51} release is inhibited by increasing concentrations of estrogen and enhanced by increasing concentrations of progesterone and testosterone. The inset shows a parallel association of lipid peroxide and TBARS formation on Cr^{51} release. (From Ref. 29 with permission.)

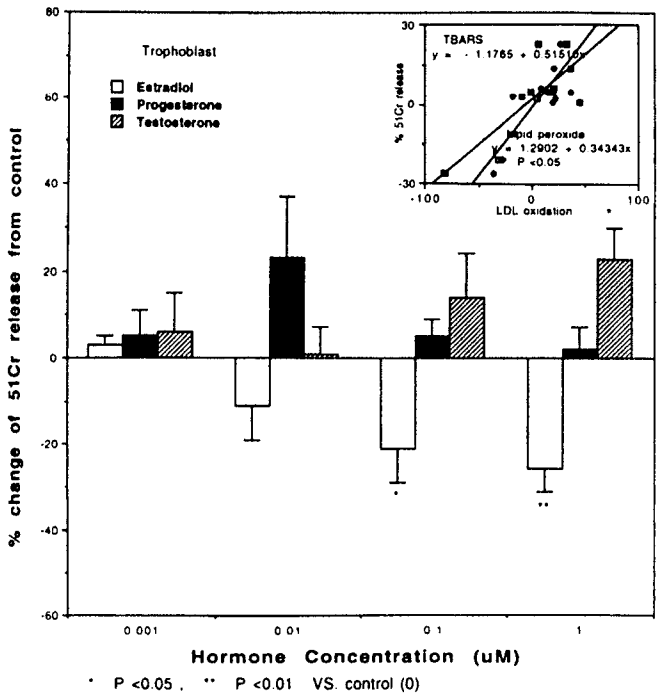

Figure 9. Effect of sex hormones on placental trophoblast cytotoxicity. Incubation conditions are the same as in Figure 7. Increasing concentrations of the 17β-estradiol again inhibit Cr^{51} release whereas progesterone and testosterone increase this release. The inset shows a parallel association of lipid peroxide and TBARS formation on Cr^{51} release. (From Ref. 29 with permission.)

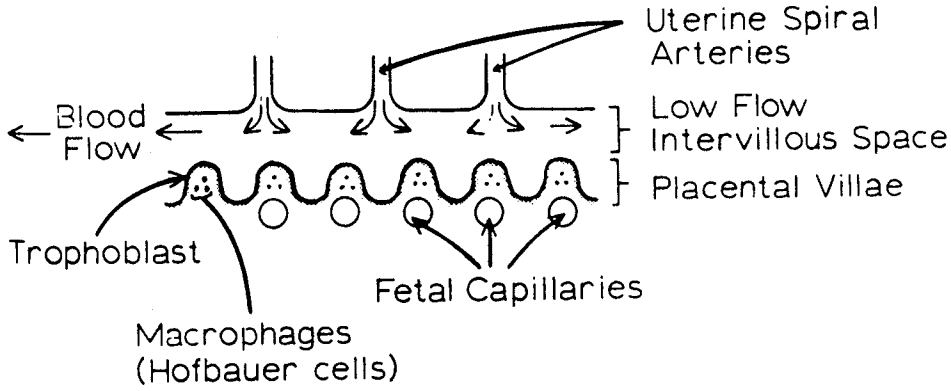

Figure 10. Schematic of placental blood flow. Maternal blood passes over placental villae, which are covered with a layer of trophoblast, and tissue macrophages that are present in the subtrophoblast area of the placental villae. As in the arterial wall, enhanced oxidative stress may have an injurious effect on placental health, as is seen in toxemia or diabetes. (From Ref. 30 with permission.)

Table I. Effects of Sex Hormones on LDL Oxidation *in vivo*

Authors	Journal	Year	Results
Sack *et al.* (8)	Lancet	1993	Patch: lag phase ↑ 36 min E_2 infusion: lag phase ↑ 45 min
Keaney *et al.* (32)	Circulation	1994	Swine: lag phase ↑ 50 min (endogenous E_2)[a] lag phase ↑ 70 min (exogenous E_2)[a]
Guetta V et al. (33)	Am J Cardiol	1995	Patch: lag phase ↑ 18 min + vitamin E
Wilcox *et al.* (34)	Fertil Steril	1997	Equine E_2: lag phase ↑ 24.5–87.8 min
Wagner *et al.* (35)	Arterioscler	1996	Estx. E_2: aortic MDA ↓ 50% (ovariectomized monkeys)
McKinney *et al.* (36)	Fertil Steril	1997	E_2: lag phase ↑ 53 min (postmenopausal monkeys) E_2 + Pg: lag phase ↑ 27 min
McManus *et al.* (37)	Atheroscler	1997	CEE: lag phase ∅ Implant: LOOH ↓ 12%
Brussard *et al.* (38)	Arterioscler	1997	Estrace 2 mg: lag phase ∅ -Diabetics

[a] vs ovariectomy.

between arterial endothelium and trophoblast, both of which are exposed to LDL and to potential arterial injury. Macrophages may then be recruited, either from the blood stream, as in the case of the arterial wall (31), or from the interior of the placenta itself in response to the inflammatory injury of oxidative stress, such as is seen in diabetes and preeclampsia or eclampsia.

Regarding the related question of whether testosterone is atherogenic, the studies of Adams *et al.* (21) in 1995 indicated that testosterone administration to female monkeys can induce atherosclerosis without any influence of changes in plasma lipoproteins.

The above observations raise the question of whether the changes in plasma hormone levels or the presence of estrogen and progestin *in vivo* can influence LDL susceptibility to oxidation *in vitro*. Table I summarizes the eight publications of which we are aware (8, 32–38). All but one show an antioxidant effect of estrogen in some parameter, including prolongation of lag phase, reduction of arterial malonyldialdehyde (MDA) content, and reduction in formation of plasma lipid peroxides. The only study that did not show an effect of estrogen on lag phase or other oxidative parameter is that of Brussard *et al.* (38) who found no benefit of estrogen treatment on the LDL of diabetic postmenopausal hormone-treated women. This result may be related to the oxidative stress associated with diabetes. Conversely, McKenny *et al.* (36) found that the addition of natural progesterone to estrogen in postmenopausal monkeys was associated with an enhanced susceptibility of LDL to oxidation.

Summary

The opposing effects of estrogen and progestin on multiple physiological systems in the body have been reviewed. Estrogens and progestins oppose each other in many ways: at the receptor level, on lipoprotein physiology, in carbohydrate metabolism, on insulin sensitivity, and on the clotting system. Regarding arterial wall biology, progestins oppose the effects of estrogen in atherogenesis itself, nitric oxide formation, arterial vasomotion, and, in our studies, LDL oxidation and cultured vascular cell cytotoxicity. In addition, we found some distinctions among the progestins, with the greatest pro-oxidant effect associated with MPA and the androgenic progestin, levonorgestrel. In light of the surprising lack of benefit of an estrogen/progestin combination in preventing recurrent coronary artery disease in the HERS study, some consideration should be given to the use

of lower doses of progestin, alternative progestins, or non-oral progestins. Overall, our results point to the possibility that progestins as well as estrogens may be involved in the health of cells exposed to arterial blood flow, including the arterial endothelium and the placental trophoblast covering the placental villi. The results also suggest that common pathophysiological mechanisms may be involved in atherosclerosis and the placental injury seen in maternal diabetes and preeclampsia.

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