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Effects of 17 beta-estradiol on cellular and molecular atherogenic events in animal models

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EFFECTS OF 17 BETA-ESTRADIOL ON CELLULAR AND MOLECULAR ATHEROGENIC EVENTS IN ANIMAL MODELS

BY

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DISSERTATION

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This dissertation has been examined and approved.

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ABSTRACT

EFFECTS OF 17 BETA-ESTRADIOL ON CELLULAR AND MOLECULAR ATHEROGENIC EVENTS IN ANIMAL MODELS

by

Glenn T. Shwaery
University of New Hampshire, September 1994

Estrogens may protect against coronary artery disease (CAD) as women have less CAD than age-matched men, and postmenopausal women on estrogen have less CAD than those without replacement therapy. To elucidate potential benefits of 17 beta-estradiol on atherogenesis, female swine and hamsters were treated to yield three groups: 1) ovariectomized (ovex), 2)ovariectomized with estradiol replacement (ovex + estradiol), and 3) sexually intact. Postoperatively, animals were switched from a low fat to a high fat diet for measurement of acute estradiol effects in swine and chronic effects in hamsters. Swine monocyte and platelet adhesion to porcine aortic endothelial cells (PAEC) were measured as well as mononuclear cell degradation of low density lipoprotein (LDL) during the low fat diet and after 4, 8, and 12 weeks of high fat feeding. Antioxidant content, fatty acid composition, and resistance to oxidation of swine LDL were measured after 14 weeks of experimental diet. In hamsters, aortic lesion area was assessed histologically and ultrastructurally after 4, 9, and 18 months of hormonal treatment. Both animal models became hyperlipidemic on high fat diets. Estradiol did not significantly alter circulating lipid levels. Monocyte adhesion to PAEC was elevated only at 4 weeks of high fat feeding in the ovex +

estradiol group while platelet adhesion was unaltered throughout the study. Mononuclear cell degradation of LDL was primarily receptor-mediated and not associated with plasma estradiol levels. Estradiol increased resistance of LDL to oxidation by increasing lag time and decreasing conjugated diene propagation rate. Oxidative resistance was associated with conservation of alpha-tocopherol in LDL from animals with estradiol. Hamster aortic lesion area was less in ovex + estradiol than in ovex animals, although statistically significant only at 4 months of high fat feeding. Lesions were primarily lipid-enriched with no involvement of smooth muscle cells. Older animals, independent of estradiol status, developed lesions characterized by extracellular matrix proliferation punctuated by lipid. Although all animals became hyperlipidemic, total cholesterol to high density cholesterol ratios were approximately 2.0 in all treatment groups throughout the study. Estradiol directly or indirectly altered some of the determinants of atherogenesis potentially reducing the risk for CAD.

INTRODUCTION

Cardiovascular disease remains the major cause of morbidity and mortality in the United States and Western Europe (1,2). In the majority of cases, death results from myocardial or cerebral infarction, the principal clinical consequences of atherosclerosis. The common endpoint in atherosclerotic lesion development is most often a thickened, fibrous plaque which protrudes into the lumen of the artery occluding the flow of blood to varying degrees. This progressive intrusion results in complete blockage in some instances but alters the rheological pattern of flow in every instance. The pathologic progression to this common endpoint has been the focus of intense research not only to gain an understanding of the natural history of the atherosclerotic plaque, but to identify possible points of intervention to prevent or retard the progression of lesion development or, more recently, to regress the disease (3-5).

Although much fruitful research has led to a wealth of information regarding the genesis and progression of arterial lesions, and preventative and therapeutic advances have been at the forefront of medical research for the past decade, the opening statement remains valid. This is most likely a result of the complexity of atherosclerotic lesion development. The disease is multifactorial and includes components such as genetic susceptibility, hemodynamic forces, and potential risk factors including but not limited to hypercholesterolemia, hypertension, diabetes, obesity, viral infection, immune responses, smoking, homocyst(e)inemia, hypercoagulability, and others (6-13). In symptomless patients, earlier stages of lesion development go undetected, as reliable, non-invasive imaging techniques have yet to be developed. Progression occurs slowly in humans over

the course of years or decades and thus, is difficult to follow in detail in an individual patient. This fact also impinges upon the interpretation of data from animal models in which atherosclerosis has been induced through genetic or dietary manipulation over a relatively short period of time. Since lesions in these animals develop in an abbreviated fashion, they can only approximate the natural history of cellular dynamics and biochemical events of lesion progression in humans. Nevertheless, the use of animal models and recent large scale epidemiologic and clinical studies have further clarified the role of many risk factors listed above in the development of these debilitating and potentially fatal lesions.

Little doubt remains regarding the association between hypercholesterolemia and the risk of coronary artery disease (CAD). As early as the 1950's, it became evident that high concentrations of low density lipoprotein (LDL) cholesterol were positively associated with an increased incidence of the disease (14), while it was not recognized until the 1970's that high density lipoprotein (HDL) concentrations, especially HDL_2, were inversely associated with incidence of the disease (15,16). In many patients, hypercholesterolemia is diet-induced and can be treated by dietary modification. Thus, researchers seized upon the opportunity to study the genesis, progression, and regression of the arterial lesions induced by diet in animal models. Observations in these models led to the so-called lipid hypothesis of atherosclerosis (17).

The lipid hypothesis of atherosclerosis suggests that high concentrations of the more atherogenic apolipoprotein (apo) B-100 containing lipoproteins (primarily LDL) penetrate the vascular endothelial barrier and become trapped in the intimal, subendothelial space primarily occupied by extracellular matrix proteins and

proteoglycans. The earlier "response to injury" hypothesis of Ross and colleagues (18) and its subsequent modifications (8,19) state that endothelial dysfunction is an initiating event in atherosclerosis. This modification of endothelial cells may be as harsh as cell death or as subtle as biochemical alterations which change cell permeability to selected plasma components. However, it is presently unclear whether endothelial dysfunction precedes enhanced lipid infiltration into the vessel wall or whether the accumulation of subendothelial lipids over months, years, or decades is the causative factor of subsequent endothelial injury in these selected regions of the vasculature. Sequestered from the antioxidant rich plasma, the unsaturated fatty acid moieties of intimal LDL become oxidized to form lipid hydroperoxides. These oxidized lipids have been shown to be cytotoxic to endothelial cells *in vitro* (20,21), chemotactic to circulating monocytes (22,23), inhibitory to tissue macrophage motility (23,24), and to induce the production of chemotactic and mitogenic cytokines and growth factors by endothelial cells (25-28). This interaction further promotes the recruitment of monocytes into the vascular wall, and once established, their emigration is inhibited by oxidized LDL components such as lysolecithin (24). These responses are reminiscent of events associated with focal inflammation, including the recruitment of T-lymphocytes (29-31). As the endothelium becomes further "insulted", metabolic alteration may lead to exacerbated permeability to the atherogenic lipoproteins and even endothelial cell death. The recruited monocytes undergo limited replication, become activated in the subendothelial space, and begin to secrete their own chemotactic and proliferative cytokines (19,32-34). End products of their metabolism further oxidize LDL to the extent that it is no longer recognized by the native LDL receptor, but is instead recognized by the macrophage scavenger receptor whose

expression and rate of endocytosis is not sensitive to regulation by intracellular stores of cholesterol, as is the case with the native LDL receptor (35,36). These macrophages become filled with droplets of neutral lipid (cholesterol esters) and develop into foam cells, so-named due to their foamy appearance when examined in ultrastructural sections (37,38). Collections of these intimal macrophage-derived foam cells form the first identifiable lesion in the arterial wall and are referred to as the fatty streak. Although yet to be established with certainty, most investigations suggest that these fatty streaks are the initial lesions which progress to the early atherosclerotic fibro-fatty lesion (39-42).

The fibro-fatty lesion is characterized by the introduction of large numbers of vascular smooth muscle cells (SMC) into the milieu of macrophage-derived foam cells and accumulating modified lipoproteins (31,41-43). The macrophages, T-lymphocytes, and metabolically altered endothelium produce and release cytokines that are chemotactic and mitogenic for SMC (see 19 for review). SMC migrate through the intercalated, partially degraded internal elastic lamina into the intimal space of the arterial wall. Here they express scavenger receptors (44) and may take on a foam cell appearance, although in most cases they accumulate the modified lipoproteins to a lesser extent than the macrophages. However, their primary pathologic impact is not as a player in LDL oxidation or accumulation, but instead they are the dominant proliferative cell type in the lesion (31,33,45,46). During their migration from the media, their metabolic state changes from one of a contractile to a synthetic phenotype, producing both paracrine and autocrine growth factors which in turn lead to lesion progression and expansion (19,47-49). SMC continue to proliferate and migrate within the lesion resulting in an overgrowth of the fatty lesion with a less fragile, fibrous cap consisting primarily of SMC and their extracellular

matrix products, collagen, proteoglycans, and elastin (50-52). As this cap thickens and the core of the lesion becomes dissociated from a vascular supply, necrosis may occur resulting in release of cellular lipid-rich contents into the center of the lesion. Often, cholesterol crystals precipitate in this region of low oxygen tension and macrophage-derived foam cells. Thus, the lesion core tends to become less cellular and the periphery more fibrous as progression occurs. Eventually, the lesion thickens to a state in which it protrudes into the lumen of the vessel and partially occludes the flow of blood.

The fatal or non-fatal but debilitating coronary event in the majority of cases is the result of thrombus development on the normally nonthrombogenic endothelial surface. This phenomenon may be a result of changes in the balance between procoagulant and antithrombotic/fibrinolytic activities at the vessel wall. Alterations in endothelial expression of local antithrombotic prostanoids or thrombolytic molecules, vasodilators, or adhesion molecules may lead to activation and adhesion of platelets to the lumen wall. However, a majority of the cases are believed to be precipitated by plaque rupture or fissure, exposing the thrombogenic molecules normally covered by the intact endothelial cells which, although spread thinly over many areas of the lesion, still provide an intact covering (53-56). In the case of non-fatal events, the adhesion and subsequent aggregation of platelets to the altered endothelium through contact with specific adhesion molecules or subendothelial aspects of a lesion not only alter the flow of blood in the local environment, but activated platelets release platelet derived growth factor (PDGF), a potent mitogen for SMC, resulting in further growth and fibrotic elaboration of the lesion (57,58). The frequency of such thrombotic events over a developing lesion has yet to be determined. Microthrombi may be formed quite frequently providing a nearly continuous

source of PDGF resulting in further SMC involvement within the body of the lesion. Less frequent thrombotic events may result in more episodic growth of the lesion in response to PDGF from outside of the vessel wall. In a fatal myocardial infarct, platelet activation and the resulting aggregate, simply by nature of its size, may be sufficient to completely occlude the vessel lumen or may result in severe ischemia and persistent vasospasm preventing blood flow.

Atherosclerotic lesions develop in both humans and animal models at predictable sites within the vasculature. These susceptible sites are at areas of blood flow disturbance such as branch points in the vascular tree or areas of distinct curvature such as the inner, more tightly curved portion of the aortic arch, where shear forces are low and "dwell time" of plasma components and blood cells are increased (6,59-62). These observations suggest that in these areas of site-specific susceptibility, altered rheology may play a role in modifying the functional state of endothelium and/or underlying cells involved in the initial processes of atherogenesis. Furthermore, as lesion growth continues and the lumen is compromised by partial occlusion, local rheology is altered leading to expansion of the atherosclerotic lesion. The margins of the lesion in these turbulent regions are characterized by altered, dysfunctional endothelium which is permeated by LDL and invaded by monocytes, the cycle of events leading to an ever increasing area of involvement (41,62,63).

As stated above, endothelial cells respond to the pathologic stimuli associated with atherosclerosis with increased permeability to lipoproteins and expression and secretion of cytokines and growth factors which stimulate advancement and expansion of the lesion (30,64-67). It has been recently demonstrated as well that in areas with a predilection for

lesion development, endothelial cells express molecules on their lumenal surface which result in hyperadhesiveness for leukocytes and platelets (68-71). Although in some reports expression of adhesion molecules appears to be constitutive in sites where rheological influences are associated with susceptibility to lesion development, risk factors lead to an induction and overexpression of these binding sites. These inducible surface proteins (endothelium-leukocyte adhesion molecules, ELAMs, vascular cell adhesion molecules, VCAMs, intercellular adhesion molecules, ICAMs, and platelet-endothelial cell adhesion molecules, PECAMs) consist of a series of immunoglobulin-like domains that are the counterreceptors of a number of different binding sites on mononuclear cells and platelets termed integrins. Both molecular species must be induced or constitutively expressed for these selective adhesions to occur (72-74). This expression leads to enhanced adherence of leukocytes to the lumenal surface of the endothelium, followed by extravasation of cells by diapedesis if chemotactic molecules are present in the subendothelial space. Hence, early in the lesion one finds an increased population of monocytes which differentiate into antigen presenting resident macrophages under the influence of molecules within the intimal space. Either concomitantly or subsequently, an enhanced population of T-lymphocytes is involved in local immune function thought to be a response, at least in part, to the immunogenic epitopes found on oxidized LDL (31,75,76).

This functional change in adhesiveness in "activated endothelium" is the primary mechanism by which endothelial cells recruit monocytes into the subendothelial space (77). This series of phenomena is strikingly similar to those characteristic of endothelial-dependent inflammatory responses . As is the case for a number of atherogenic processes,

the initiating event which stimulates expression of adhesion molecules and early monocyte recruitment is unknown. However, transcriptional upregulation of these molecules is mediated by a variety of cytokines produced in the developing atherosclerotic lesion (58,66). These cytokines can thus be responsible for amplification of mononuclear cell populations in the lesion, but not for initiation of monocyte invasion. It is reasonable to hypothesize that oxidized LDL may again be the culprit. Expression of adhesion molecules on endothelial cells is site-specific and is limited to focal areas of recruitment (73). LDL accumulates focally in areas where foam cells later develop (78), and this LDL may be oxidized once trapped in the subendothelial space (79). Endothelial cells in culture have shown an increased adhesion for monocytes when incubated in the presence of oxidized LDL or lysolecithin (26,80). Thus oxidized LDL may induce hyperadhesiveness prior to the accumulation of the primary cell types that enhance adhesion molecule expression by production of cytokines.

CAD mortality in the United States has become nearly independent of gender. Of the 500,000 deaths annually attributed to the disease, 49% occur in women (81). In women, cardiovascular deaths (CAD and stroke) exceed those caused by all cancers combined (82). Of significant interest is the fact that women remain at lower risk for CAD with the rate of disease per 100,000 population remaining significantly higher in men than in age-matched women (83,84). These figures reveal that the initial diagnosis of CAD in women is more serious and the prognosis for recovery less promising resulting in higher morbidity and mortality rates. In oophorectomized or postmenopausal women, the incidence of CAD and clinical events is more prevalent compared to premenopausal women of the same age (85,86). Furthermore, treatment of postmenopausal women with

estrogen replacement therapy (ERT) reduces the incidence of coronary events by as much as 45% in this population, even in those with established disease (87-89). These studies suggest that estrogen plays a significant role in reduction of established risk factors for CAD, including putative protective mechanisms altering lipoprotein metabolism, blood pressure, coagulation factors, vasoreactivity, and oxidative stress (84,90,91).

The decrease in coronary risk may in part be explained by favorable changes in lipoprotein profiles as a result of exposure to endogenous or exogenous estrogen. It has been well documented that women of child-bearing age have a lipoprotein profile thought to be less atherogenic than that of age matched men (83,92). These gender differences emerge at puberty and persist in women throughout premenopausal life (93,94, Figure 1). Women have lower plasma concentrations of total cholesterol (TC), LDL-C, and triglycerides (TG), while having higher levels of HDL-C, especially HDL_2 subfractions. After menopause (approximately 50 years of age), these concentration differences narrow and with increasing age, LDL levels in women equal or surpass those of men. These changes have been shown to be significantly associated with an increased incidence of CAD (92,95,96).

Oral ERT or combined hormone replacement therapy (HRT, estrogen and progestin) reverse these trends both in lipoprotein concentrations and CAD risk (97-100). Estrogen administration decreases LDL-C concentrations while increasing total HDL-C, HDL_3-C and HDL_2-C. These changes are considered to be antiatherogenic. Less clear are the changes in VLDL-C and TG, primarily due to differing effects dependent upon both dose and route of administration of the estrogens (100-102). Higher doses of orally

administered estrogen such as those encountered in oral contraceptives (OC) raise VLDL-C and TG.

Recently, innovations have expanded the methods of drug delivery, including continuous, transdermal administration of hormones to postmenopausal women (101,102). Transdermal delivery of estrogen in the doses presently administered demonstrates little effect on plasma VLDL-C, LDL-C, and TG concentrations in short term studies, while effects may be noted after longer exposures to the lower levels of estrogens delivered in this manner (103).

Oral estrogens are absorbed by the intestine and expose the liver to supraphysiological concentrations via the portal circulation (104,105). Thus, the effects of estrogen by oral administration may be quite different from those of parenteral exposure although the peripheral circulating concentrations may be similar. Oral administration results in a large proportion of the hormone being removed from the circulation by the liver in the "first pass" through the portal circulation. Lievertz (105) reports that up to 30% of orally administered estrogen may be removed and/or metabolized to inactive forms before reaching the peripheral circulation where estrogen may have more direct actions on the vascular wall and circulating cells. This exposure of the liver to such high doses of the hormone when compared to peripheral levels explains, in part, the differences that routes of administration of the hormones have on lipoprotein metabolism described below.

Although results from large, controlled clinical studies are as yet unavailable, observational data suggest that estrogens reduce cardiovascular disease and its clinical manifestations by 50% (89,106). Observations linking estrogen administration with retarded progression of atherosclerosis were made as early as 1942 in rabbits (107), and in

the 1950's estrogen treatment decreased the incidence of CAD in patients with reproductive hormone metabolic disorders (108). In animal models, estrogen has been shown to attenuate the development of atherosclerosis (109-112). However, there has been a great deal of uncertainty concerning the effect of added progestins to replacement formulations (111-114). Their effect is in large part dependent upon their androgenic activity, but the majority of studies concur that the progestins do not significantly antagonize the beneficial effects of estrogen administration. Early studies focused primarily on lesion size and development as much of this work preceded theories of specific lipoprotein involvement in atherogenesis and were compromised by a lack of quantitative, reproducible, accurate assays for lipoprotein cholesterols. More recently, lipoprotein levels and composition have been shown to be influenced by gender and by physiologic conditions such as puberty, pregnancy and menopause, conditions during which dramatic changes in sex hormones occur (93,115). The general trend of these observations suggests that estrogen in women provides an antiatherogenic lipoprotein profile with minimal adverse effects on TG levels.

These favorable changes in lipid profiles have been shown to be brought about at least in part by changes in hepatic metabolism of lipoproteins. Pharmacological doses of estrogens in animal models and the higher doses in OC formulations (especially early forms) reduce plasma LDL-C by increasing receptor-mediated clearance by the hepatic LDL receptor (116-118). Both *in vitro* and *in vivo* studies have demonstrated an increase in LDL receptor mRNA and protein in response to high doses of estrogens (118,119). More controversial are reports that physiologic doses of exogenous or endogenous estrogens may not increase LDL receptor activity directly but reduce cholesterol levels by

increasing its secretion into bile in a dose dependent manner (120,121). This decrease in the hepatic regulatory pool may secondarily enhance LDL receptor activity, but this has not been demonstrated in every case.

Sex differences in LDL particle size have been demonstrated with men having significantly smaller LDL than women (122). Postmenopausal women experience a slight decrease in LDL size related to decreases in circulating E_2, but the change is not so dramatic that the density profile equals that of males (123). Campos *et al.* (124) have shown that oral doses of estrogens increase levels of VLDL and TG, decrease LDL-C, while maintaining levels of apo B, suggesting an increase in density of LDL. Interestingly, higher doses of estrogens in OC's decrease LDL particle size to a pattern similar to postmenopausal women. Again, the effect of estrogen on particle size seems to vary with dose and route of administration.

The impact of estrogens on HDL-C concentrations is thought to be mediated by changes in hepatic metabolism as well. At puberty, HDL-C concentrations in boys fall by about 10 mg/dL while those of girls remain stable (93,94). Epidemiologic studies have shown that estrogen users have higher HDL_2, HDL_3, and apo A-I levels than non-users (99,100,125). Clinical studies have suggested that the regulation of HDL-C is mediated in part by a decrease in the activity of hepatic lipase with exposure to estrogen (126). This suppression of hepatic lipase activity leads to a decreased catabolism of HDL, especially HDL_2, the concentration of which has been shown to be inversely associated with hepatic lipase activity. These changes in HDL metabolism seem to be more sensitive to estrogen than those of LDL since even the low levels of estrogen yield these changes in HDL while having little effect on LDL concentrations.

Effects of estrogen on VLDL-C and TG concentrations have varied in animal, epidemiologic and clinical studies, again, primarily dependent on dose and route of administration. Premenopausal women have lower VLDL-C and TG concentrations than age-matched men, and these concentrations increase disproportionately in women after menopause (94,100,125, Figure 1). Doses of estrogen in OC and oral replacement formulations increase VLDL-C and TG levels while percutaneous administration of ERT or HRT lower these lipid values (100,103). The increases in VLDL are a result of increased synthesis of nascent VLDL particles by the liver. Little of this VLDL is converted to LDL in circulation since lipoprotein lipase activity is suppressed by these doses of estrogen. Instead, the VLDL particles are cleared by the hepatic B,E receptor and thus have little influence on other circulating lipoprotein levels through their delipidation in plasma.

Effects of estrogen on lipoprotein(a) (Lp [a]) have not demonstrated any clear pattern although the base of experimental knowledge is narrow compared to that of other more widely known atherogenic lipoproteins. Lp(a) concentrations are thought to be independent of gender (127), but studies with high doses of estrogens for long periods of time showed significant decreases in Lp(a) levels (128-130). These effects were observed with oral administration of the hormones and were associated with increased VLDL and TG concentrations. Transdermal routes of estrogen exposure or low doses of oral estradiol valerate had no effect on Lp(a) levels, at least not in the short term (131). These observations may reflect short-term absence of effect as is seen for most lipoprotein concentrations when the dose of estrogen is at the low end of the physiologic spectrum. However, caution must be taken before one discounts any estrogenic effects as plasma

lipid profiles do not change in the short term with transdermal patch use in ERT, but over longer periods of time, gradual low dose estrogenic changes in lipoprotein levels are seen.

In normally cycling women, changes in concentrations of lipoproteins have been described during different phases of the menstrual cycle most often associated with changes in estrogen levels in plasma (132-134). VLDL-C and TG peak near ovulation, while LDL-C is suppressed during the luteal phase. HDL-C levels are higher at midcycle and during the luteal phase when E_2 and progesterone are concurrently elevated.

Although there is substantial evidence that estrogens beneficially alter the plasma lipid profile, a number of animal studies have demonstrated that changes in plasma lipids are not a prerequisite for atherosclerotic risk reduction in individuals exposed to endogenous or exogenous estrogens (110-112,117,118). Furthermore, evidence for protection by mechanisms other than effects on lipid profiles comes from a number of studies in which protection against CAD has been provided by parenteral estrogen without changes in plasma lipids (102,135). Women using transdermal estrogen patches show substantial protection from development of coronary lesions or clinical events with little change in lipoprotein concentrations. In monkeys and rabbits, estrogen has been shown to attenuate the development of atherosclerosis without changes in LDL-C concentrations (110,111,136). In fact, regression analysis of large-scale studies has shown that only 25-50% of the protective effects of estrogen are contributed by changes in lipoprotein levels (84,89,97,137). Other potential benefits of estrogen on CAD include antioxidant functions protecting LDL from oxidative damage, and changes in vasoreactivity, hemostatic factors, and blood pressure (91,138-140).

Studies demonstrating the antioxidant properties of estrogens support the hypothesis that these hormones protect against CAD by means other than lipid-lowering. Certainly, estrogens reduce the plasma concentrations of LDL-C and thus decrease risk factors and consequences associated with elevated levels of this lipoprotein. These pathologic consequences may include but are not limited to increased insudation of lipid into the vascular wall, increased expression of adhesion molecules, and alterations in hemostasis at the endothelial surface. Estrogens and the structurally related antiestrogens, tamoxifen and 4-hydroxytamoxifen, have been shown to inhibit membrane phospholipid peroxidation in a dose dependent manner (141). All molecules did not protect equally, but in each case the degree of oxidative protection was associated with the degree of incorporation into the membrane itself. Estrogens with phenolic structure have been shown to protect LDL from modification *in vitro* by copper ions, monocytes and endothelial cells (142-145) and to reduce serum and liver peroxide levels *in vivo* (146). In all studies to date, E_2 has demonstrated the most potent antioxidant activity of the natural estrogens examined, including estrone, estriol and 17 α-estradiol. However, these studies have demonstrated a protective effect only with supraphysiologic concentrations of E_2, with the minimal effective doses in the micromolar concentration range, at least 10^3 times the levels observed in cycling women and exceeding those observed even during pregnancy. However, these observations that protection occurs only at pharmacologic doses of E_2 *in vitro* do not preclude the potential beneficial effects at physiologic concentrations on LDL oxidative susceptibility *in vivo*.

In vitro experiments have been conducted under conditions which limited the impact of estrogens upon the composition of LDL particles which may affect its oxidative

susceptibility. Estrogens may be carried in LDL *in vivo*, and these experiments did not attempt to incorporate estrogens into LDL and measure subsequent oxidative resistance as has been done with other antioxidants (142-144,147,148). Additionally, the impact of circulating estrogens on the antioxidant and fatty acid composition of LDL could not be ascertained in these *in vitro* studies. Esterbauer and colleagues (149,150) have shown that the initial resistance to oxidation is provided by the antioxidant content of the LDL particle. Upon consumption of the antioxidants, further resistance to oxidation is determined in part by the polyunsaturated fatty acid (PUFA) content of the particle (151-154). Thus, in vivo experiments need to be designed to investigate the effects of circulating estrogens on LDL particle composition. As stated previously, sex differences in lipoprotein metabolism and LDL particle size profiles have been demonstrated, but to date no definitive studies of LDL antioxidants, fatty acid composition, and oxidative resistance by sex have been carried out, and only one brief, preliminary report has described a beneficial effect of HRT on LDL oxidative resistance in postmenopausal women (155).

Estrogens may also alter risk factors associated with thrombotic events. High doses of estrogens in OC and estrogen-only replacement therapy are associated with increases in coagulation. Epidemiologic and clinical studies have demonstrated an increase in Factor VII and protein C in estrogen users compared to nonusers or combined estrogen and progestin users (99,140). Although estrogen use is also associated with decreases in other clotting factors such as fibrinogen and antithrombin III concentrations (99,102,140,156), combined hormonal therapy may provide a better physiologic profile than estrogen use alone (157). Since estrogen concentrations have declined dramatically

in OC formulations, the thrombogenicity of these combinations has been dramatically reduced. These hypercoagulation profiles previously seen in the high oral doses of estrogens are not present in women taking the lower oral doses or transdermal applications presently prescribed.

Although the liver is the primary target tissue of E_2 in terms of lipid metabolism (158,159), the blood vessel wall remains a potentially important site of E_2 action in response to risk factors for atherosclerosis. E_2 has been shown to lower blood pressure in spontaneously hypertensive rats (160), and systolic pressure in women increases significantly after menopause (161). Receptors for sex hormones have been identified in the vasculature of several animals and the distribution of receptor densities have been identified in some models (162-166). Interactions between progesterone and E_2 action and their receptors have been studied in the rat revealing translocation of receptors from cytosol to nucleus, and subsequent regulation of receptor number within the tissue (167). More recently, the nuclear response elements for E_2 have been identified and characterized in the human aorta (168). These studies suggest the possibility for receptor mediated steroidal action on cells of the arterial wall which may alter the susceptibility of the tissue to atherosclerotic risk factors or modulation of the phenotypic expression of cells in response to these stimuli.

The endothelium participates in the regulation of arterial tone and platelet reactivity through the activity of endothelium-dependent relaxation factor (EDRF) (169,170). Endothelial dysfunction in atherosclerotic arteries may be related to oxidative modification of LDL in the vessel wall. Endothelium dependent relaxation is impaired in atherosclerosis and in normal arteries exposed to oxidized LDL (171,172). Oxidized LDL

may directly inactivate EDRF and/or may alter the ability of endothelial cells to respond to vasoactive stimuli (172,173).

Estrogens may serve as cardioprotective agents of vasomotor responses. Sex differences in response to vasoactive substances have been observed in rabbits (174), while estrogen replacement in ovariectomized rabbits, dogs and monkeys restores vasorelaxation to acetylcholine stimulation which is ablated in the absence of estrogen (91,175,176). Acute exposure to pharmacological doses of ethinyl estradiol has a short lived vasodilatory effect on precontracted vessels (177). Additionally, the administration of E_2 to atherosclerotic animals lessens the extent of vasoconstriction in these pathologic states. Estrogens may also have direct effects on SMC function as Jiang *et al.* (90) have shown that estrogens decrease the contractile response to endothelin-1 exposure. Thus, estrogens modulate the activity of endothelium dependent vasomotor responses and attenuate the vasospasm and contractility of pathologic vessels by a direct effect on smooth muscle.

There is evidence to support the hypothesis that E_2 may improve vasomotor activity by virtue of its antioxidant properties. Other antioxidants, most notably probucol and α-tocopherol, preserve endothelial function under hypercholesterolemic and prooxidative conditions compared to control vessels (178,179). Since exposure to oxidized LDL has been shown to interfere with endothelium dependent vasodilation, antioxidant properties of estrogens may preserve the relaxation responses to vasoactive stimuli (138).

Although the impact of estrogen exposure on lipoprotein metabolism has been elucidated in some detail, effects on both physical and chemical cellular interactions and

subsequent behavior and metabolism of the cells involved in lesion initiation are relatively unexplored. Since effects of estrogen on plasma lipid concentrations may only account for 25-50% of the risk reduction observed in women (84,97), cellular components of the vessel wall are likely targets of additional hormone action in light of constitutive expression of estrogen receptors by arterial cells (162-166). A more detailed description of these effects may lead to a better understanding of the disease process itself, as well as a more complete appreciation of the potential uses of these hormones or their analogues as sources of preventative and/or therapeutic care for CAD. For example, the debate over widespread recommendations for HRT in menopausal women presently rages due to a lack of well-defined, controlled studies of the widespread metabolic and cellular consequences of hormonal administration. To this end, experiments in animal models were designed in our laboratory to investigate the effects of a specific natural estrogen, E_2, on cellular and biochemical events deemed critical to the initiation and progression of atherosclerotic lesion development, i.e., atherogenesis.

Since lipoprotein metabolism and atherosclerotic development are both complex and dynamic processes, the selection of animal models that approximate human regulation and responses to perturbations such as diet-induced hypercholesterolemia is a challenging task. Characteristics such as lipoprotein cholesterol distribution, genetic variability, dietary responsiveness and atherosclerotic resistance are important considerations when selecting an appropriate model for the phenomenon to be studied. Recent use of genetic variants (180,181) and the proliferation of transgenic animal models (182) has allowed researchers to probe with greater detail into the regulation of lipoprotein metabolism and

the development of atherosclerotic lesions under a variety of pathologic conditions including hypertension, diabetes, and hypercholesterolemia to name only a few.

The primary focus of this study, however, is the impact of E_2 on diet-induced hypercholesterolemia, cellular events of atherogenesis, and subsequent development of atherosclerotic lesions in female animal models. Therefore, it is of some importance that the animal of choice respond to a dietary challenge in a manner similar to that of humans. The Syrian golden hamster was selected as such a model for diet-induced atherosclerosis since its lipoprotein metabolism is similar to that of humans (183-185), and arterial lesions with similar pathology develop in the thoracic aorta at predictable sites comparable to those in humans (7,61,183,186). Hamsters have plasma LDL-C levels higher than most other animal models which primarily carry cholesterol in HDL. Their synthesis of cholesterol and regulation of LDL receptor activity is sensitive to dietary fat, and they respond to modest levels of dietary fat and cholesterol typical of American diets with increases in LDL-C and TC/HDL-C ratios in a manner similar to humans (187,188). Additionally, they respond to antiatherosclerotic drugs, which makes them a useful model to examine the efficacy of interventions on lesion progression and/or regression (61,186).

Hamsters have been studied for cholesterol gallstones (189,190) and a variety of diet and drug intervention trials to assess the susceptibility of vascular sites to atherosclerosis (7,61,183,186). In response to dietary cholesterol and/or fat-enriched diets, they develop atherosclerosis and related pathological cholesterol metabolism diseases and conditions driven by elevated plasma VLDL-C and LDL-C concentrations. Their relatively inexpensive cost allows for larger numbers to be used for studies in which variability of response may be expected, and their small size affords ease in handling for

hormonal treatments, collection of blood samples, and preparation of the vasculature for assessment of lesion development. Thus, the hamster is a relevant model for relatively rapid or preliminary studies in which the time of lesion formation is compressed, and the degree of atherosclerosis can be used as an endpoint in a logistically efficient and cost-effective manner.

Anatomic and physiologic similarities between swine and humans have led to the former's wide use in the study of disease states affecting humans. As a result, there exists a large, well-documented body of literature describing these similarities, further supporting the continued use of swine as a model for many pathogenic studies. Yucatan miniature swine were selected as the animal model for studies of cellular aspects of atherogenesis which required large volumes of plasma or large numbers of circulating cells from multiple bleedings. The pig has become an important model for the study of lipoprotein metabolism and atherosclerosis for its similarities to humans in the pathophysiologic events which lead to lesion formation in the vasculature (191-194), and the close resemblance of the platelet-coagulation systems (195). Like the hamster, pigs' lipoprotein profiles are altered by dietary cholesterol and fatty acids in a manner similar to humans', and the distribution and cellular composition of lesions are parallel as well (43,191,193,196). Swine readily develop atherosclerotic lesions in a relatively short time period when challenged with a high fat, high cholesterol diet which provides a cost-effective model for studying cellular aspects and lesion formation in a larger animal model whose coronary arteries are of manageable diameter and thus, suitable for vasoresponse studies (138,191,197).

Since there is no acceptable animal model for natural menopause, alterations in plasma E_2 concentrations were brought about by surgical menopause in both of the animal models employed in these studies. Three hormonal conditions were generated in female pigs and hamsters, reproductively intact, sham-treated females, and ovariectomized females with and without estrogen replacement. Using these three hormonal conditions in dietarily-induced hypercholesterolemic swine, the objectives of these experiments were to study the effects of E_2 exposure on cellular and biochemical interactions believed to be critical to initiation and development of atherosclerotic lesions. In swine, effects of the varied concentrations of plasma E_2 on the interaction of monocytes and platelets isolated from whole blood with swine endothelial monolayers *in vitro* were measured at three time points during dietarily induced hypercholesterolemia. Additionally, swine mononuclear cell binding and degradation of LDL were measured *ex vivo* as an estimation of changes in LDL catabolism that may have occurred *in vivo* as a result of E_2 exposure and dietary treatment. Hormonal effects on isolated LDL oxidation *ex vivo* were examined after 14 weeks of high fat feeding. In hamster studies, the objectives were to investigate the effects of E_2 on high fat, high cholesterol dietarily induced hypercholesterolemia and the subsequent initiation and progression of lesion development in the aortic arch, and to identify any differences in cellular composition of lesions as a result of exposure to E_2.

MATERIALS AND METHODS

Materials

Commercial low fat swine diet and hamster RMH 3000 chow were purchased from Agway Feeds (Syracuse, NY). Purified cholesterol, coconut oil, and sodium cholate were from Research Diets (New Brunswick, NJ). Crystiben™ was purchased from Solvay Veterinary, Inc. (Princeton, NJ). Isoflurane was obtained from J.A. Webster (Sterling, MA) and ketamine HCl and xylazine from Aveco Co., Inc. (Fort Dodge, IA). All laparoscopic instruments including trocars, graspers, scissors, and staplers were purchased from or donated by U.S. Surgical Corp. (Norwalk, CT). Polyglycolate suture material was from Davis & Geck, Inc. (Manati, P.R.) and 9 mm autoclips were from Clay Adams (Parsippany, NJ). Estrogen implants were prepared using Silastic™ tubing from Dow Corning (Midland, MI) and 17 β -estradiol from Steraloids (Wilton, NH). Antibody to 17 β-estradiol was provided as a gift by Dr. G. D. Niswender of Colorado State University. Estradiol[2,4,6,7,16,17-3H(N)] and ^{125}iodine were purchased from Dupont NEN Research Products (Boston, MA). Indium-111-oxyquinoline was purchased from Medi-Physics, Inc. (Arlington Heights, IL). Chemicals for lipoprotein cholesterol and triglyceride measurements were obtained from Abbott Laboratories (N. Chicago, IL). Sodium EDTA and heparin tubes were obtained from Becton, Dickinson and Co. (Lincoln Park, NJ). Nycodenz solutions and dextran 500 for separation of blood cells were purchased from Accurate Chemical and Scientific Corp. (Westbury, NY).

All cell culture buffers, media and media supplements were from Sigma Chemical Co. (St. Louis, MO) unless noted otherwise. Phosphate buffered saline (PBS) consisted

of 12.3 mM NaCl, 0.24 mM KCl, 0.075 mM $NaHCO_3$, and 0.13mM KH_2PO_4 in double distilled, deionized, 18 megohm water. Fetal bovine serum (FBS) was obtained from Hyclone, Inc. (Logan, UT). Bovine hypothalami were purchased from Pel-Freez Biologicals (Rogers, AR). All plastic cultureware was purchased from Corning Inc. (Corning, NY). Aprotenin, sodium azide, BHT and chemicals used in conjugated diene assays were of high purity and were purchased from Sigma. Dialysis tubing was from Spectrum (Houston, TX) and PD-10 columns were purchased from Pharmacia (Uppsala, Sweden). 2, 2'-azobis (2-amidinopropane) dihydrochloride (AAPH) was obtained from Polysciences Inc. (Warrington, PA). High performance liquid chromatography (HPLC)-grade ethanol, methanol, hexane, tetrahydrofuran, methylene chloride and ammonium acetate used in the analysis of antioxidants, and high purity-grade chloroform, methanol, sulfuric acid, hexane, and methylene chloride used in fatty acid analysis were purchased from Burdick and Johnson (Noskegon, MI). Fatty acid standards were from Nu-Check Prep (Elysian, MN) and the 17:0 internal standard was purchased from Matreya, Inc. (Chalfont, PA). Reacti-Therm vials and caps were obtained from Pierce Chemical Co. (Rockford, IL). Ultrapure gases, N_2, He, H_2, and O_2 were from Northeast Airgas (Manchester, NH). Dil-Ac-LDL was from Biomedical Technologies, Inc. (Stoughton, MA) and Factor VIII related antibodies were purchased from Sigma.

Glutaraldehyde, osmium tetroxide, and Epon 812 used in preparation of tissues for electron microscopy were from Polysciences, Inc. (Warrington, PA). Aqueous mounting medium was from Signet Laboratories, Inc. (Dedham, MA). All other reagent grade chemicals were from Sigma.

Experimental Design

Experiments were designed to examine the effects of E_2 on selected cellular and biochemical aspects of atherogenesis in swine and on aortic lesion development in hamsters. In both models, animals were surgically altered to yield three hormonal treatment groups: (1), animals received sham surgery and remained sexually intact or (2), were ovariectomized and provided with a subcutaneous placebo or (3) E_2-filled Silastic™ implants, the latter providing physiologic concentrations of hormone. After one week of recovery, animals were fed a high fat, high cholesterol diet to induce hypercholesterolemia and subsequent development of atherosclerosis.

Blood samples from adult swine (n = 5/group) were drawn at 2 week intervals twice before surgery and at 4, 8, and 12 weeks after high fat feeding to measure plasma lipids and circulating E_2. Monocytes and platelets were isolated from these samples and their degree of adhesion to porcine aortic endothelial cells (PAEC) *in vitro* was assayed. The rate of LDL internalization and degradation by mononuclear cells was also measured *ex vivo* at these time points. In each of these assays, differences were statistically analyzed not only among treatment groups at each time point but also within treatment groups over the course of the experiment, including samples during both low fat and high fat feedings. After 14 weeks on the high fat diet, LDL was isolated from each animal and its lipophilic antioxidant content, fatty acid composition, and resistance to oxidation were determined.

Three hormonal treatment groups of hamsters (n = 30/group) were surgically established to determine the effects of E_2 on plasma lipids and aortic atherosclerotic lesion development over time. Animals were treated by sham surgery or by ovariectomy with placebo or E_2 subcutaneous implants. Following surgical treatment, hamsters were fed

an atherogenic high fat, high cholesterol purified diet for up to 18 months. Effects of E_2 on aortic lesion development were determined by histological and ultrastructural methods at 4, 9, and 18 months of dietary and hormonal treatment. Blood samples were taken at two and four months, and thereafter at four month intervals and at the time of sacrifice to determine plasma lipid and E_2 concentrations. Plasma lipid and lesion area data were analyzed among groups and over time within groups.

Swine Studies: Animals and Diet

Fifteen female Yucatan miniature swine (Sus scrofa) were randomly assigned to three groups of five and reared to sexual maturity on a low fat, low cholesterol commercial swine diet at the University of New Hampshire Miniature Swine Faculty in Lee, New Hampshire When animals reached sexual maturity at 6 months of age, blood was drawn from each fasted (18 hr) animal from the anterior vena cava into EDTA (1.5 mg/ml) for measurement of parameters described above during low fat feeding and prior to surgical treatment.

Three hormonal treatment groups were established by laparoscopic ovariectomy or sham surgery. Animals were premedicated with 40,000 units/kg of benzithine penicillin, induced with a mixture of 15 mg/kg ketamine HCl and 3 mg/kg xylazine intramuscularly, and maintained under general inhalation anesthesia with a nose cone using 1.5-2.0% isoflurane and oxygen. The abdomen was shaved and scrubbed using aseptic technique with betadine scrub-alcohol-final spritz of betadine solution. After draping, the abdomen was punctured at the umbilicus with a pneumoperitoneum needle through which the abdomen was distended to 13 mm Hg pressure with CO_2. Pressure was maintained throughout the procedure by continuous insufflation of CO_2 by a high flow Stryker CO_2

insufflator (Stryker Endoscopy, San Jose, CA). A 10 mm trocar and sleeve were inserted into the abdomen through a 1 cm skin incision over the umbilicus. A laparoscope was placed in the sleeve and used to view the abdominal viscera on a color monitor. Five mm trocars and sleeves were inserted 5 cm lateral to each of the third nipples. The ovaries were identified and stabilized by endograspers and endoscissors were placed through the lateral sleeves.

A 12 mm trocar and sleeve were inserted through a 1.5 cm skin incision midline caudal to the last pair of nipples. An endo-GIA-stapler was inserted into this sleeve and placed across the ovarian pedicle and fired. The incised ovaries were removed through the lateral skin incision. Electrocautery was used if hemorrhage or incomplete ovarian separation was encountered.

The abdomen was deflated and incisions were closed in two layers with absorbable monofilament polyglyconate. Prior to final closure, a placebo or E_2 implant was positioned subcutaneously at each lateral incision site in the ovariectomized animals. E_2 implants were prepared from Silastic™ tubing (1.25 in. x 0.132 in. ID x 0.183 in. OD) packed with 200 mg of E_2, and sealed with silicon rubber. Placebo implants were left empty. Implants were gas sterilized with ethylene oxide and soaked in sterile PBS at 37°C for 24 h just prior to use. Sham treated animals underwent the same surgical procedure except the ovaries were simply identified, then the instruments were withdrawn, and the incisions closed as described above without any subcutaneous implant. Triple antibiotic ointment was applied over all incisions. Animals were allowed to recover from anesthesia and were returned to the University's Miniature Swine Facility where their recovery was monitored daily. These procedures yielded three treatment groups (n=5/group) designated

as: a) intact (sham-treated), b) ovex (ovariectomized with placebo implants), and c) ovex + E_2 (ovariectomized with E_2 implants). One week after surgery, the diet was changed to a high fat, high cholesterol diet consisting of standard chow supplemented with 40% of calories as coconut oil, 1% (w/w) purified cholesterol, and 0.7% (w/w) sodium cholate for the duration of the study. Animals were individually fed two 450 g meals/day and allowed water *ad libidum*. All animal procedures were approved by the Animal Care and Use Committee of the University of New Hampshire, Durham, NH.

Monocyte and Platelet Adhesion

Monocyte and platelet adhesion to porcine aortic endothelial cells (PAEC) *in vitro* was measured twice at 2-week intervals using blood cells from each animal to establish baseline adhesion values prior to surgical treatment and high fat feeding. Adhesion assays were repeated after 4, 8, and 12 weeks of high fat feeding by a modification of methods previously described (198).

PAEC were obtained from female Yucatan miniature swine fed the maintenance diet by a modification of the procedure of Taylor *et al.* (199). PAEC were removed from thoracic aortas by gentle scraping with a moistened cotton swab after a 30-min. Type I collagenase (0.1% in medium M199 w:v with 1% FBS v:v) digestion at 37°C. Endothelial cell pellets were resuspended in medium M199 containing 10% FBS, 100 μg/ml endothelial cell growth supplement prepared in our laboratory from bovine hypothalami (200), 100 μg/ml heparin, 5 μg/ml insulin and transferrin, 5 ng/ml sodium selenite, 100 U/ml penicillin, and 100 μg/ml streptomycin (complete medium) and seeded into human fibronectin (FN) coated T-25 flasks. Complete medium was changed every 3 days until the cells attained confluency. PAEC were passaged into FN coated T-75 flasks using

0.05% trypsin and 0.02% EDTA. Cells were subsequently passaged into FN coated 12-well plates. The PAEC were examined to monitor growth and the time at which the cultures became confluent. Cells were characterized by phase contrast cell morphology, the ability to take up Dil-Ac-LDL (201) and the presence of Factor VIII related antigen as shown by immunofluorescence (202). All experiments were conducted with PAEC cultures that were 24-30 h post-confluent in passage 4 or less.

Twelve ml of venous blood were collected into EDTA (1.5 mg/ml) from each fasted animal at each time point indicated above. Leukocyte-rich plasma (LRP) was prepared by mixing whole blood with dextran 500 (6% in 0.15 M NaCl) at a volume ratio of 10:1 (blood: dextran) for 30-45 minutes. LRP was layered over Nycoprep™ 1.068 and centrifuged at room temperature at 600 x g for 15 min. in a Beckman TJ-6 centrifuge (Beckman Instruments, Fullerton, CA) with a swinging bucket rotor to isolate monocytes and platelets from lymphocytes. Monocytes and platelets were collected from the density interface and washed in PBS containing 0.35 mM EDTA and 1% BSA (w:v) at 400 x g.

Cells were resuspended in buffer and layered over Nycoprep 1.063 and centrifuged at 350 x g for 15 min. to isolate platelets from monocytes. Platelets were collected in the supernatant and pelleted at 500 x g. The isolated monocytes and platelets were then washed in buffer, resuspended in serum-free medium M199 at 37°C, and an aliquot was examined by both phase-contrast microscopy and Wright's stained smears to confirm purity of these samples. Monocyte purity was also assessed by a non-specific esterase staining using Sigma kit #91-A. All monocyte and platelet preparations used were ≥90% purity. Cells were radiolabeled by cellular uptake during incubation with 20 μCi indium-111-oxyquinoline in serum-free medium for 15 minutes at 37° C with gentle rotation. The

radiolabeled monocytes and platelets were centrifuged at 150 x g and each cell type was resuspended in 5 ml of medium M199. Aliquots from each sample were counted for 5 minutes with correction for dead time and half-life decay using a LKB CompuGamma (Model 1282, Pharmacia LKB Nuclear, Inc., Gaithersburg, MD) to determine the total counts added. PAEC monolayers were washed twice with 2 ml serum-free M199. One ml of labeled platelet or monocyte suspension was added to each of 4 wells for 1 h at 37° C in humidified 5% CO_2/95% air. Medium was then removed and each well was washed twice with Hank's balanced salt solution (HBSS) to remove any free or loosely attached cells.

The PAEC with any adhering monocytes or platelets were solubilized with 1 ml 0.1 M NaOH/0.1% SDS for 5 min. and each well was washed twice with 1 ml 0.1% SDS. Gamma counts from the solubilized cells and SDS washes were pooled and measured as above. Percentage of cells bound was determined by:

$$\% \text{ adhesion} = \frac{\text{no. counts adhered}}{\text{total counts added}} \times 100\%$$

The adhesion values from all 5 animals from each treatment group were used to determine a mean ± SD for platelet and monocyte adhesion to endothelial cells at each time point of the dietary challenge.

Binding and Degradation of LDL by MNC

The effect of E_2 on the rate of ^{125}I -LDL binding and degradation by MNC *ex vivo* was determined by a modification of methods previously described (203). Donor LDL was prepared from two females in the sexually intact group which provided LDL that was metabolically generated in the presence of endogenous female hormones. MNC

degradation of LDL was assayed twice during the low fat feeding and 4, 8, and 12 weeks after high fat feeding. Donor blood from 2 randomly selected intact females was collected into anticoagulation cocktail (final concentration/ml whole blood: 10 KI aprotenin, 0.1% (w:v) sodium azide and EDTA). Plasma was prepared as above and one µl of 20 mM BHT in 100% ethanol was added per ml of plasma. Samples were pooled for LDL isolation by preparative ultracentrifugation (204). Briefly, plasma density was increased to 1.210 g/ml with KBr. Samples were then centrifuged in a fixed angle TI-70 rotor (Beckman) at 65,000 rpm (350,000 x g) for 24 h at 10°C. Lipoproteins floated to the top of the tube and were collected by aspiration. The density of the lipoprotein solution was adjusted to 1.250 g/ml and was overlaid with equal volumes of KBr solution (d = 1.100) and distilled deionized water. Samples were centrifuged in a SW 41.0 swinging bucket rotor (Beckman) at 39,000 rpm (200,000 x g) for 22 h at 15°C. The LDL band was collected and kept at 4°C at all subsequent preparative steps. The LDL was then dialyzed (Spectra/Por tubing, molecular weight cut off, 12-14 Kd) overnight against 250 volumes of normal saline containing EDTA (0.01% w:v, pH 7.0). Protein was determined by the procedure of Markwell *et al.* (205). 2.5 mg LDL protein was radioiodinated by the iodine monochloride method of MacFarlane (206) as modified by Bilheimer *et al.* (207). Free iodine was removed by gel filtration through a PD-10 Sephadex G-25 M column by eluting 2.5 ml of sample with 3.5 ml of PBS. An aliquot was counted to determine specific activity.

MNC were isolated from swine whole blood collected in EDTA. Whole blood was diluted 1:1 by volume with normal saline, and 6 ml of this mixture was overlaid on 3 ml of Nycoprep™ 1.077 and centrifuged at 600 x g for 15 min. at room temperature.

Isolated MNC were collected from the density interface and washed twice at 350 x g for 15 min. in PBS containing 1% BSA (w:v) and 0.35 mM EDTA, then resuspended in 2.25 ml of HBSS supplemented with 15 mM HEPES, 5mM $CaCl_2$ and 0.1% glucose (w:v). The cells were then divided into 8 microcentrifuge tubes at 250 µl/tube. Cells were incubated with ^{125}I -LDL (15 ug/ml) in the presence or absence of a 30-fold excess of unlabelled LDL in a final volume of 500 µl. A "no cell" set of tubes served as a control to measure spontaneous LDL degradation or deiodination under culture conditions at 37°C. Caps were loosely placed on the tubes and the samples were incubated at 37°C in a 0.5% CO_2/99.5% air atmosphere to maintain pH. MNC were kept in suspension on a rotary shaker at 200 rpm. An aliquot of the remaining cell suspension was diluted with a vital stain and counted on a hemocytometer to determine cell density and cell viability. From these determinations, the number of viable cells added to each tube at the beginning of the incubation was calculated. After a 4 hour incubation, the cell suspension was quickly cooled to 4°C on ice, then centrifuged at 150 x g to pellet MNC. An aliquot of super-natant was treated with an equal volume of 20% trichloroacetic acid on ice for 30 min. to precipitate undegraded labeled LDL. The precipitate was centrifuged at 5000 x g for 5 min. at 4°C. The acid soluble supernatant was counted in a LKB gamma counter to determine the amount of iodotyrosine released as a result of LDL degradation. After subtracting the appropriate dpm's attributable to non-cellular degradation or deiodination as measured in the "no cell" tubes, specific binding and degradation was determined by subtracting the amount of catabolized ^{125}I -LDL in the presence of unlabelled LDL from that in its absence. Rates of LDL degradation were expressed on a per 10^6 cell basis per 4 hours based on the number of viable cells in the tubes at the start of the incubation.

Radiolabel uptake and degradation by MNC from the three hormonal treatment groups were compared to one another at each time point of the assay as well as changes within a treatment group over time.

Plasma lipid and E_2 measurements

Venous blood samples were drawn from fasted (18 h) animals at the previously described time points before and after administration of the high fat diet. Plasma was separated at 1,500 x g for 20 min. at 4°C using EDTA as an anticoagulant. TC and TG were quantified enzymatically as previously described (208,209). HDL-C was measured after phosphotungstic acid-$MgCl_2$ precipitation of apo B containing lipoprotein fractions (210). The difference between TC and HDL-C was calculated as the combined cholesterol in VLDL and LDL fractions. Plasma lipid assays were standardized by participation in the Centers for Disease Control - National Heart, Lung and Blood Institutes Standardization Program in the laboratory of Dr. Robert Nicolosi at the University of Massachusetts at Lowell, MA.

Plasma concentrations of E_2 were measured by standard radioimmunoassay (RIA). Two hundred ml of plasma were extracted twice with 10 volumes of ethyl ether (211). The extract was dried under N_2, then reconstituted in 200 µl of PBS (pH 7.0) containing 0.1% gelatin (w:v). The sample was divided into two 100 µl aliquots and assayed in duplicate by incubation overnight at 4°C in the presence of ^{3}H- E_2 and antibody to E_2. Standards were run in triplicate with a concentration range of 5-320 pg/ml. Sample concentrations of E_2 were measured by competitive assay with ^{3}H- E_2 using scintillation counting. The antibody demonstrated approximately 40% binding to E_2 in standards. All unknown samples were reported as the mean of the duplicate values. Recovery of ^{3}H- E_2

in the extraction procedure was 90-95%. Aliquots from a pool of pig plasma were analyzed in each assay in replicates of 4 to serve as an internal control. A plasma pool was also assayed after being twice stripped of steroid hormones with dextran-coated charcoal, yielding a plasma blank ≤ 5 pg/ml. The average intraassay and interassay coefficients of variation were 4.2 and 8.6% respectively.

Data Analysis

Unless otherwise specified, all data are presented as mean ± SEM. Plasma lipid levels, E_2 levels, monocyte and platelet adhesion to PAEC, and MNC binding and degradation of LDL were compared among treatment groups and over time within treatment groups using multivariate analysis of variance (MANOVA) with post hoc Neuman-Keuls comparison. The relationship between plasma lipids or the logarithm of plasma E_2 levels and cell adhesion or LDL degradation rates were examined by relating these data using linear regression (Pearson). Statistical significance was accepted if the null hypothesis was rejected at the 0.05 level.

Resistance of LDL to Oxidation *Ex Vivo*

After 14 weeks of dietary and hormonal treatment, serum from each animal was prepared at 4°C in the absence of anticoagulants and exogenous antioxidants. LDL was isolated from serum by a single spin density gradient ultracentrifugation as previously described (204). The fraction collected for oxidation assay was in the density range of 1.019-1.040 g/ml in order to avoid the apoprotein E-rich HDL_C found in the fraction d > 1.040 in hypercholesterolemic pigs (212). Samples were dialyzed against 250 volumes of buffer for 6 h, protected from light and under an atmosphere of nitrogen. Purity of the LDL fractions collected was assessed by agarose gel electrophoresis (213) which

demonstrated that the sample was free of any contaminants with alpha-mobility. Samples were stored in the dark under nitrogen at 4°C until use and were assayed within 12 h of isolation.

Susceptibility of LDL to oxidation was measured by conjugated diene formation using a modification of Esterbauer *et al.* (149) employing AAPH as an aqueous peroxyl radical initiator. LDL protein was determined as described by Markwell *et al.* (205) using bovine serum albumin as a standard. The final incubation conditions for measurement of LDL oxidation were: 25 μg LDL, 5 mmol/L Hepes and 2.5 μmol/L AAPH in a final volume of 1.0 ml. Absorbance at 234 nm at 37°C was monitored and recorded at 5 min. intervals for 400 min. using a Lambda 4B, UV/VIS spectrophotometer (Perkin-Elmer, Norwalk, CT). Six samples were assayed simultaneously including two samples from each treatment group in each assay. Duration of the lag phase, rate of diene formation (propagation phase), and total dienes formed were calculated from the oxidation curves as previously described (149).

LDL Antioxidant Vitamin Content

Aliquots of the above samples were used to measure antioxidant content by HPLC using a modification of the method described by Bieri and colleagues (214). Three hundred μl of the LDL samples above were precipitated with an equal volume of ethanol, containing echininone as an internal standard, and extracted twice into equal volumes of hexane. The hexane layer was removed into an amber glass vial and dried under N_2. The sample was reconstituted in 100 μl of ethanol for analysis by reverse-phase HPLC using a C18 Baker Bond wide-pore (5 μm) column (J.T. Baker, Phillipsburg, NJ) and a variable wavelength spectrophotometer (Beckman Model 167, Beckman Instruments, Inc.,

Fullerton, CA). The isocratic mobile phase was methanol: tetrahydrofuran:water:methylene chloride (70:18:7:5 v:v:v:v) with 1% ammonium acetate (w:v). Retinol, α-tocopherol and ß-carotene were quantified at wavelengths of 325, 286 and 452 nm, respectively. Injection volume was 20 µl, with all three analytes being quantitated in one run. Concentration calculations were based on external standards with the internal standard, echininone, used to correct for volume loss during sample preparation. This lab, under the direction of Dr. Joanne Curran-Celentano, is part of the National Institute of Standards & Technology program for analytical assurance. All procedures were done under yellow lights to protect photosensitive pigments.

Isolated LDL was extracted with ethyl ether for measurement of E_2 concentrations. Concentrations were determined by RIA as described above for plasma. Limits of detection for both plasma and LDL E_2 levels were 5 pg/ml.

Fatty Acid Analysis

Lipids were extracted from LDL samples using a modification of the procedure described by Folch *et al.* (215). Total lipids were extracted with chloroform:methanol (2:1, v:v) containing 0.06% BHT (w:v). The solvent was washed twice by thorough vortexing with an equal volume of 0.9% saline. The chloroform, lipid-containing fractions were evaporated under N_2, reconstituted in methanol containing 2% sulfuric acid under N_2, tightly capped and incubated in a 70°C water bath with gentle stirring for one hour in Reacti-Therm vials. After cooling, the solution of fatty acid methyl esters was twice extracted with equal volumes of hexane. The hexane layers were pooled, evaporated under N_2 and reconstituted in methylene chloride containing C17:0 methyl ester as an internal standard just prior to analysis using a Hewlett Packard 5890A gas chromatograph

(Hewlett Packard, Avondale, PA) equipped with a flame ionization detector (FID) and a DB-23 fused silica capillary column, 25 m x 0.25 mm ID, 0.25 mm film thickness (J&W Scientific, Folsom, CA) in the laboratory of Dr. R. Nicolosi. One ml of the reconstituted samples was introduced into the gas chromatograph via a split/splitless injection system. Ultra high purity helium was used as the carrier gas at a flow rate of 1.5 mL/min. Injector and detector temperatures were set at 250°C. Following injection, the column temperature was programmed to increase from 160°C to 200°C at a rate of 1°/min and held at maximum temperature for five minutes prior to termination.

LDL fatty acids were identified by comparison of relative retention times to that of the internal standard (C17:0 methyl ester) and those of the Fatty Acid Methyl Ester Combination Standard (GLC-85). Relative percentages from the fatty acid profile were calculated as the individual peak areas divided by the sum of all peak areas x 100%.

Data Analysis

All data are reported as mean ± SEM, unless indicated otherwise. The resistance of LDL to oxidation *ex vivo* and concentrations of plasma lipids, E_2, and LDL antioxidants were compared among treatment groups by analysis of variance using Neuman-Keuls means separation test. Logarithmic transformation was performed for plasma E_2 values to stabilize variance for these data. The associations between LDL resistance to oxidation and concentrations of plasma LDL, E_2, and LDL α-tocopherol were determined using linear regression and Pearson product-moment correlation coefficients for these variables. Similar relationships were examined between plasma E_2 concentrations and the variables above. A probability value less than 0.05 was considered significant.

Hamster Studies: Animals and Diet

As described above for pigs, three hormonal treatment groups were established for hamsters by sham surgery or ovariectomy with E_2 or placebo implants (n=30/group). Ninety female Syrian golden hamsters (*Mesocricetus auratus*, 14 weeks old, BioBreeders Inc., Watertown, MA) were used in this study. Animals were housed in pairs in clear polycarbonate box-style cages (inner dimensions, 15 cm x 21 cm x 14 cm, W x L x H) with stainless steel tops. Animals were maintained on RMH 3000 rodent diet. Water and food were provided *ad libitum*. Animals were maintained under identical controlled environmental conditions with a 14 h/10 h light/dark cycle. All conditions and procedures were in compliance with the guidelines set by the Animal Care and Use Committee of the University of New Hampshire.

After two weeks of acclimatization, animals were fasted for 20 hours and blood was collected under CO_2 anesthesia via the retroorbital sinus into heparinized capillary tubes. Plasma was harvested by low-speed centrifugation (2000 x g) for 30 minutes at 4° C and used for determination of plasma lipids and lipoprotein cholesterol as described above. One week later, hamsters were anesthetized with ketamine HCl (11 mg/100 body wt.) and xylazine (3.5 mg/100 g). Dorsolateral surfaces were shaved and prepared for surgery with betadine/alcohol/betadine as described above. Using sterile technique, bilateral skin incisions were made 5 mm posterior to the last pair of ribs. The underlying muscle was also cut with scissors to expose the abdominal pad of fat which surrounds the ovary. This fat pad was withdrawn from the abdominal cavity and the ovary and oviduct were isolated by a loop of 4-0 surgical suture material which was then tied in a triple knot ligating these organs and their vascular supply. The ovary and oviduct were cut above the

ligature and the incision was observed to be certain that hemorrhage did not occur. The fat pad was then placed back inside the abdominal cavity and the overlying muscle was closed with two stitches of 4.0 absorbable polyglyconate. Half of the ovariectomized animals received one Silastic™ E_2 subcutaneous implant and half received a placebo implant. E_2 implants were prepared by filling Silastic™ tubing (9 mm length x 0.062 in. ID and 0.095 in. OD) with 10 mg of purified E_2 or leaving them empty and sealing the ends with silicon rubber sealer. Sterile implants were soaked in PBS for 24 h at 37°C just prior to use. The skin incisions were then closed using 9 mm Autoclip surgical staples, and the incision was treated with triple antibiotic ointment. A third treatment group of sexually intact animals was established by sham surgery in which the ovaries were exposed and identified but not ligated or excised. Animals were allowed to recover from anesthesia and returned to their cages where they were observed for one week, after which skin staples were removed under CO_2 anesthesia. Animals were taken off the low fat low cholesterol maintenance diet and fed *ad libitum* a high fat high cholesterol purified diet commercially prepared by Research Diets (New Brunswick, NJ, Tables 1,2). All animals were weighed and blood samples were taken from the retroorbital sinus every two months to measure changes in plasma lipids and to assess by RIA the continuous release of E_2 from the Silastic™ implants. Plasma lipids and E_2 concentrations were measured as described above for swine. E_2 implants were replaced in any animals if consecutive plasma samples showed a 50% or greater decrease in circulating E_2.

Quantitating Fatty Streaks

Animals were sacrificed at 4, 9, and 18 months after high fat feeding to determine the extent of atherosclerotic lesion development in the aortic arch by a modification of

methods previously described (61). At the time of sacrifice, animals were fasted for 20 h, anesthetized with ketamine HCl: xylazine (15 mg:5 mg per 100 g body wt.), incised midventrally and bled from the left ventricle into heparinized tubes for lipid and E_2 analyses. The right atrium was incised for outflow and the circulatory system was flushed via the left ventricle for 1 minute with 4% phosphate buffered paraformaldehyde (pH 7.0) at room temperature and a pressure of 110 mm Hg. The right atrium was clamped shut and the flow rate of paraformaldehyde was reduced to a level that would maintain pressure for an additional 25 minutes. Tissue was dissected away from the thoracic aorta, the heart and aorta were removed, pinned at in situ length, and fixed overnight in 3% glutaraldehyde in 0.1M cacodylate buffer (pH 7.3) with $CaCl_2$ (1 drop of 1% per 10 ml fixative) at 4°C. The heart was removed and the aorta was stored at 4°C in cacodylate buffer with 6.8% sucrose (w:w) until processing. A 1 cm length of aortic arch was subdivided and processed for analyses as shown in Figure 2. For each *en face* preparation the segments were cleaned of adventitia, rinsed in 60% isopropanol, and the inner aortic surface stained for 25 min. with oil red O (ORO) at 60% of saturation in 60% isopropanol. The aorta was rinsed with distilled water and the lumenal surface stained with Gill's type hematoxylin (1:20 dilution) for 1 minute. The tissue was rinsed, opened longitudinally, and mounted on a glass slide with a glass coverslip and aqueous mounting medium. All segments were photographed using bright field microscopy (Olympus BH-2, Olympus America, Inc., Lake Success, NY).

The area of ORO staining in each aorta was quantified using an image analysis hardware and software package developed by Image Technologies, Inc. (New York, NY). The image from the *en face* preparation was projected through a video camera onto a

black and white monitor. After calibrating pixels to μm^2 and setting threshold to identify ORO stained lipid, the area of the lesion was analyzed directly from the *en face* slides. All *en face* lesion area measurements are expressed in square microns.

Statistical Analyses

Data analyses of plasma lipid profiles, E_2 levels, and aortic lesion area of animals in each hormonal treatment group were performed by analysis of variance with a post hoc Newman-Keuls comparison. In addition, to normalize the hormonal and lesion area data and to stabilize the variance, these raw data were transformed to $\log_{10}$ values before any analyses were performed. Correlation coefficients and regression analyses (Pearson) were used to measure the statistical associations among plasma lipids, E_2, and lesion area.

Preparation and Examination by Histology and Electron Microscopy

One mm segments from the proximal, mid, and distal aortic arch were dehydrated and processed into paraffin by standard histologic procedures. Sections were stained with hematoxylin and eosin, Weigert's resorcin fuchsin (for elastin) or Masson's trichrome for histologic examination. Samples for transmission electron microscopy (TEM) were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer (pH 7.3), dehydrated in a graded series of ethanol, embedded in Epon 812, thin sectioned on a Reichert Ultracut E (Reichert-Jung, Vienna), mounted on copper grids and stained with uranyl acetate and lead citrate. Sections were examined in a Hitachi H600 electron microscope (Hitachi Scientific Instruments, Mountain View, CA) and images were recorded on photographic film. Prints were prepared from representative negatives.

RESULTS

Effects of hormonal treatment on swine plasma lipid and E_2 concentrations

Prior to surgery and hormonal treatment, female miniature swine had fasting (18 h) plasma cholesterol and TG concentrations characteristic of those associated with a low fat, chow diet, with a mean TC and TG of 98 mg/dL and 63 mg/dL, respectively for all animals (n=15). There were no statistically significant differences among pretreatment concentrations of any plasma lipid fractions when data were assigned to and analyzed by subsequent hormonal treatment groups (Table 3). During the low fat feeding, approximately 44% of the plasma cholesterol was carried in the HDL fraction, yielding TC/HDL-C ratios indicative of low cardiovascular risk.

Animals responded to the high fat, high cholesterol diet with significant increases in plasma TC, LDL-C, and HDL-C, while TG remained at concentrations characteristic of low fat feeding (Table 4). Although both the HDL and the VLDL + LDL fractions were significantly elevated after 4 weeks of high fat feeding, the increase in TC was primarily in the VLDL + LDL fraction, resulting in significant changes in the TC/HDL-C ratios indicative of greater cardiovascular risk profiles than those established during the low fat diet. Thus, the early response to the high fat, high cholesterol diet was characterized by a disproportionate rise in the more atherogenic lipoproteins compared to the rise in HDL fractions in all treatment groups. Cholesterol values in all lipoprotein fractions were significantly elevated after 4 weeks of cholesterol feeding. However, the trend in all animals was for TC and LDL-C to decrease from these elevated levels after 12 weeks on the diet, while HDL-C remained elevated within a very narrow range (Table 4). Thus, the

TC/HDL-C ratio and overall lipoprotein risk profile gradually improved over time. There were no significant differences among hormonal treatment groups in any of the lipid parameters measured within each time point, and the changes described above during the 12 week high fat dietary treatment period were not statistically significant. Plasma cholesterol concentrations were increased by dietary treatment but unaffected by hormonal treatment, while plasma TG appeared to be unaltered by either. All animals increased their body weight by approximately 40% independent of hormonal treatment while on the high fat diet, and there were no significant differences in weight among groups.

Plasma E_2 concentrations in female swine normally range from 20-220 pmol/L . Prior to surgical treatment, plasma concentrations of E_2 in the 15 female swine ranged from 22-195 pmol/L (6-53 pg/ml) and reflected the physiologic variability seen in sexually intact, cycling animals. After surgical treatment and 4 weeks of high fat feeding, circulating E_2 concentrations reflected hormonal treatment with ovex animals demonstrating significantly reduced plasma levels, while those with E_2 implants had physiologically high levels (274 ± 38 pmol/L, $x \pm$ SEM) which were significantly different from both ovex and intact groups ($p \leq 0.001$, Table 5). Intact animals continued to cycle asynchronously, and thus, plasma was obtained at different times during the estrous cycle as demonstrated by a high degree of variability in plasma E_2 levels (62 ± 15 pmol/L, $x \pm$ SEM, $p \leq 0.005$ vs. ovex, range 18-178 pmol/L). The plasma concentrations in the ovex and ovex + E_2 groups were relatively stable throughout the 12 week high fat period, indicating successful ovariectomy and continuous release of hormone from the E_2-filled Silastic implants.

Uptake and degradation of ^{125}I-LDL by isolated MNC *ex vivo*

During the low fat feeding period prior to surgical alteration of the swine, isolated MNC from the 15 animals bound and degraded 63 ± 17 ng LDL protein/10^6 cells/4 h (x ± SEM) at 37° C. When data were assigned to and analyzed by subsequent treatment groups, there were no significant differences in rates of LDL degradation during this low fat feeding period (Table 6). Approximately 68% of the binding and internalization activity in these cells was receptor-mediated, as coincubation with excess, unlabelled competitor (LDL) resulted in only 32% of the activity observed in the absence of competitor.

After 4 weeks of hormonal and dietary treatment, MNC responded with nearly a 4-fold increase in the total amount of LDL catabolized in the intact group, while ovex and ovex + E_2 animals showed approximately a 2.5-fold increase compared to rates during the low fat diet (Table 6). Although these changes were statistically significant only in the intact group ($p \leq 0.001$), probabilities for the ovex and ovex + E_2 groups were 0.065 and 0.070, respectively. These substantial increases in total uptake and degradation were nearly entirely accounted for by receptor-mediated binding that was significantly increased for all groups ($p \leq 0.05$ vs. low fat feeding). There were no significant changes in nonspecific, receptor independent activities in MNC in any hormonal treatment group. Although intact animals demonstrated the greatest rate of LDL catabolic activity, differences did not reach statistical significance among treatment groups.

At 8 and 12 weeks of hormonal treatment, there were no statistical differences among treatment groups in total, receptor, or non-receptor activity (Table 6). However, after 8 weeks of high fat feeding, the pattern was similar to that at 4 weeks with the intact

group demonstrating significantly increased total catabolism of LDL ($p \leq .05$ vs. low fat rates) while ovex and ovex + E_2 increases equated with probabilities of $p = 0.085$ and 0.077, respectively. Additionally, non-receptor mediated degradation was elevated over values at 4 weeks in the intact and ovex animals ($p \leq 0.05$). Receptor mediated activity had decreased from rates at 4 weeks such that they were no longer significantly elevated above low fat levels, although probability values were 0.077, 0.067, and 0.091 for intact, ovex, and ovex + E_2 groups, respectively (8 wks vs. low fat). By 12 weeks on the high fat diet, total, receptor, and non-receptor mediated catabolism of LDL continued to decrease from the previous 8 week values to the point whereby intact animals had receptor mediated degradation rates that were significantly decreased from their maximum values measured at 4 weeks ($p \leq 0.05$, Table 6).

Throughout the course of the study, MNC demonstrated a predominance of receptor-mediated catabolism of LDL independent of the dietary or hormonal treatment. Receptor-mediated uptake accounted for at least 75% of all activity during the 12 weeks of high fat feeding. Changes in total and receptor-mediated LDL degradation rates paralleled the trend of decreasing plasma LDL-C concentrations, and degradation rates by MNC *ex vivo* were strongly associated with plasma LDL-C and TC levels when analyzed by regression analysis (Pearson, Table 7). Catabolic rates of LDL were not associated with plasma concentrations of E_2 throughout the study.

Monocyte and platelet adhesion to PAEC *in vitro*

Multivariate analysis of variance of data collected during the low fat diet showed there to be a significant effect of the day of assay on monocyte and platelet adhesion to PAEC *in vitro*. Since monocytes and platelets from all 15 animals could not be assayed on

the same day, values at each time point were normalized as percent change before comparing effects by treatment groups. Due to this effect on the adhesion assay, treatment effects could not be analyzed over the time course of the experiment, but only within individual time points.

There were no significant differences in monocyte adhesion to PAEC among groups during the pretreatment period of low fat feeding. After 4 weeks on the atherogenic diet, monocyte adhesion in the ovex + E_2 group (3.95 ± .41%, x ± SEM) was elevated by 30% and 45% compared to ovex and intact groups respectively ($p \leq 0.01$, Fig.3). Although monocyte adhesion values from ovex + E_2 animals remained higher than those of ovex and intact animals at 8 and 12 weeks of the high fat diet, differences did not attain statistical significance. Monocyte adhesion was not significantly associated with either plasma lipid or E_2 concentrations at any time point (Table 8).

Platelet adhesion to PAEC was similarly assayed and revealed no significant effect of hormonal treatment during either low fat or high fat feeding. During the high fat diet, little variability was evident among animals from all treatments with a mean value of 2.0% adhesion to PAEC for the entire high fat period (Figure 4).

LDL oxidation

All 12 animals sampled for LDL oxidation *ex vivo* were hypercholesterolemic at 14 weeks of high-fat feeding and plasma E_2 concentrations were comparable to those at earlier time points of high fat feeding (Tables 9,10).

LDL from female swine exposed to either endogenous or exogenous E_2 showed dramatically increased resistance to oxidation *ex vivo* as measured by conjugated diene formation when compared to LDL from ovex animals (Fig. 5). The curves generated for

each sample were characterized by three distinct phases including an initial lag phase in which little change in absorbance occurred, a propagation phase where the rate of diene formation rapidly increased, and a final phase in which diene formation plateaued at a maximum absorbance value. The duration of the lag phase preceding the propagation of conjugated dienes is directly proportional to oxidative resistance (149). Lag time and rate of diene propagation were significantly altered in ovex animals (Table 11). Lag times for individual samples ranged from least resistant in the ovex group (15 min) to most resistant in the intact group (160 min). Estrogen replacement in ovex swine yielded oxidation profiles similar to those observed for intact females with the propagation rate and maximum diene formation nearly identical in the two groups (Fig. 5, Table 11). Exposure to estrogen increased lag times by approximately 240-300% and decreased both the propagation rate and total diene formation by 38% and 18%, respectively.

When lipid soluble antioxidants were extracted from LDL samples and quantified by HPLC, significantly increased α-tocopherol levels were measured in LDL from E_2-exposed animals ($p \leq 0.05$ vs. ovex, Table 12). Intact females had higher levels of α-tocopherol in LDL than animals with E_2 implants (1.96 ± 0.13 vs. 1.59 ± 0.09 nmol/mg LDL protein), but the difference did not reach statistical significance. Levels of ß-carotene and retinol extracted from LDL were below the limits of detection in this assay (0.017 nmol for both). Concentrations of E_2 carried in the LDL particles were below the limits of detection as measured by RIA. Thus, α-tocopherol appears to be the primary dietarily-derived lipid soluble antioxidant found in swine LDL under these dietary and hormonal conditions, and its concentration was significantly decreased with ovariectomy.

In order to determine if E_2 replacement altered LDL composition, total fatty acids in the LDL samples were extracted and analyzed by gas chromatography. Fatty acid composition profiles are shown in Table 13. Although the coconut oil in this study was primarily composed of medium chain saturated fatty acids 12:0 and 14:0 (48.7% and 18.2%, w/w, respectively, analysis by supplier), only a small percentage of these fatty acids were found in the LDL fraction. In all animals, the principal LDL fatty acid was 18:2, with lesser amounts of 16:0 and 18:0 and no significant differences in any individual fatty acid among treatment groups (Table 13). However, there was a small but statistically significant increase in the percentage of total polyunsaturated fatty acids (PUFA) and in the polyunsaturated/saturated (P/S) ratio in animals that received E_2 implants ($p \leq 0.05$). Although endogenous concentrations of E_2 in the intact group did not alter total PUFA content of LDL compared to ovex treatment, intact animals demonstrated a significantly lower ratio of linoleic acid to oleic acid compared to both ovex and ovex + E_2 animals ($p \leq 0.05$).

Regression analysis and Pearson product-moment correlation coefficients were employed to examine the relationship between E_2 and susceptibility to oxidation, plasma LDL concentrations, and LDL α-tocopherol content. Similarly, LDL oxidation was examined in relation to α-tocopherol levels, plasma LDL concentrations, and LDL fatty acid composition. Table 14 shows that duration of the lag phase was significantly associated with LDL α-tocopherol content ($r = 0.604$, $p = 0.037$), and a highly significant inverse association was demonstrated between duration of lag phase and maximum propagation rate of diene formation for the 12 LDL samples examined in this study ($r = -0.818$, $p \leq 0.001$). There was no significant association between α-tocopherol levels and

plasma E_2 or LDL concentrations. The logarithm of plasma E_2 was not associated with duration of the lag phase nor LDL levels, but was significantly associated with the total PUFA content and the P/S ratio of LDL ($r = 0.627$ and 0.642, $p = 0.029$ and 0.024, respectively). No significant association was observed between oxidative resistance and the PUFA content or linoleic acid/oleic acid ratio in LDL.

Effect of hormonal treatment on hamster plasma lipid and E_2 concentrations

During the 18 month high fat feeding trial, several animals died of undetermined causes, and thus, only 8 animals in each treatment group were analyzed at each of the three time points. There were no significant differences among treatment groups in the amount of weight gained with a mean weight at sacrifice of 154, 151, and 154 grams at 4, 9, and 18 months, respectively. Plasma lipid values for all animals ($n = 72$) prior to surgical treatment were indicative of a low fat diet with values of 155 ± 11 mg/dl, 72 ± 9 mg/dl, 83 ± 9 mg/dl and 299 ± 65 mg/dl for TC, HDL-C, LDL-C, and TG, respectively ($x \pm SD$). As in swine, approximately 46% of the TC was carried in the HDL fraction, indicative of a low risk lipoprotein profile for cardiovascular disease. The purified high fat, high cholesterol diet produced moderate hypercholesterolemia and more severe hypertriglyceridemia in the hamsters throughout the study (Table 15). All lipoprotein cholesterol fractions were significantly increased compared to concentrations during the low fat diet. Although ovex animals during high fat feeding had higher cholesterol levels than the two groups exposed to either endogenous (intact) or exogenous (ovex + E_2) E_2, differences were statistically different only at the earliest time point (4 mo., $p \leq 0.01$, ovex vs. intact and ovex + E_2, Table 15). HDL-C in the ovex + E_2 group was also significantly elevated compared to the intact group at this point. TC/HDL-C ratios did not significantly

change over time despite dietary and hormonal treatments (Table 15). Unlike the response in swine (Table 4), hamsters responded to the purified high fat diet with equivalent, proportional increases in plasma cholesterol in both the VLDL + LDL and the HDL fractions. Thus, although there were significant increases in the masses of these lipoproteins carried in plasma, their relative amounts were altered neither by diet nor by hormonal status. Therefore, TC/HDL-C ratios were not increased, and this predictor of relative risk for CAD remained stable and extremely low (Table 15).

Plasma TG were relatively high during the low fat diet compared to those of swine and humans, and all animals became hypertriglyceridemic during high fat feeding, although responses within treatment groups were widely variable (Table 15). Differences among treatment groups were statistically different only at 4 months when values for ovex animals were higher than those for animals exposed to either endogenous or exogenous E_2 ($p \leq 0.05$), and at 18 months when TG in ovex + E_2 animals were less than those of ovex animals ($p \leq 0.01$). TG in the ovex + E_2 group never increased much above values measured during the low fat feeding period.

Prior to surgery and hormonal treatment, all female hamsters appeared to be cycling normally as demonstrated by the appearance of post-estrus vaginal discharge. Subsequently, plasma E_2 concentrations reflected the surgical and hormonal treatment with ovex animals demonstrating significantly reduced levels compared to intact and ovex + E_2 groups at all time points (Table 16). Ovex females had a mean plasma E_2 level of 19 pg/ml over the course of the experiment, while intact animals demonstrated the variability observed in the four day estrous cycle ranging from 25 - 287 pg/ml. Ovex + E_2 animals had plasma hormone levels which were at the high end of the physiologic range and

decreased over time. E_2 implants were replaced after 8 months as circulating levels had decreased by approximately 50% in most of these animals. Both intact and ovex + E_2 animals maintained physiologic levels of E_2 throughout the study, although concentrations had decreased by 20% in the intact group and 30% in the ovex + E_2 group by the end of the study. At the time of sacrifice, E_2 implants were removed, and in all cases at least 3/4 of the crystalline hormone remained as assessed by visual inspection.

Effect of E_2 on hamster lesion formation

Although female hamsters were fed a high fat, high cholesterol diet for up to 18 months, only the earliest recognizable form of the atherosclerotic lesion, the fatty streak, developed within the aortic arch of any individual regardless of hormonal status. The area of fatty streak involvement was restricted to the innermost curvature of the arch, and in no case was the lesion continuous in either of the two segments of vessel examined (see Fig. 2 for area analyzed). Hematoxylin staining revealed that the number of MNC adherent to the endothelial surface was extremely low. In the majority of cases, ORO positive material appeared to be intracellular, accumulated within intimal, subendothelial cells which appeared to be macrophage-derived foam cells as assessed by their histological and ultrastructural morphology (Figs.6-8). These simple lesions were characterized most often by neutral lipid-laden foam cells and extracellular liposomes with occasional intimal matrix proliferation. There was rarely evidence of focal fragmentation of the internal elastic lamina or involvement of SMC at any time point in the study.

Vessels from a smaller number of individuals were characterized by a significant amount of extracellular lipid droplets concentrated along this lesion-prone area of the aorta. These ORO positive lipid deposits appeared as individual, small spheres of uniform

size and distribution within the inner curvature of the aortic wall, but they had not been concentrated within subendothelial MNC (Fig. 6B). This lipid material was not confined to the intimal space, as droplets could be observed scattered throughout the connective tissue matrix and SMC of the media, coincident with a complete absence of macrophage-derived foam cells.

In histologically prepared cross-sections of tissue adjacent to the *en face* segments, lesions appeared to be confined to small areas of the aortic circumference that were characterized by focal, intimal thickenings composed of macrophage foam cells on top of or embedded in a matrix of dense granular material which resulted in slight protrusions into the lumen of the vessel (Fig. 7). Foam cells in these lesions were stacked only one to three layers deep in the thickest region of the lesion beneath an apparently morphologically intact endothelium (Fig. 7), features indicative of early fatty streak lesions.

Ultrastructural examination revealed that the lumenal endothelial surface was intact with most cells expressing thin extensions of cytoplasm to cover the lumenal surface (Fig. 8). Subendothelial lipid-rich foam cells and extracellular matrix material appeared to impinge upon the endothelial cells, attenuating their cytoplasm in these lesion-prone areas of the aorta. In these early lesions, foam cells, presumably monocyte-derived macrophages, were characterized by large, convoluted nuclei and numerous cytoplasmic lipid droplets (Figs. 7B,8). Occasional MNC were observed adherent to the endothelium and were presumably migrating by diapedesis into the subendothelial space (Fig. 9). MNC appeared to have active surface micropodia on their exposed lumenal surface while demonstrating a relatively large number of contact points with the endothelial cell(s).

These lesion-prone areas were often characterized by endothelial cells and SMC demonstrating a high degree of vesicular activity at the membrane surface (Fig. 10). The contents of these vesicles or the direction of their movement were not determined in this study, but this activity was not readily apparent in areas outside the lesion.

In other vessels or in localized sites apart from fatty streaks, subendothelial proliferation of extracellular matrix was the dominant feature of the vascular pathology (Figs. 11,12). Although macrophage-derived foam cells were rare in these thickened, intimal areas, endothelial cells with large vacuoles or lipid droplets were often observed (Fig. 10A). The greatly thickened extracellular matrix was primarily amorphous, non-fibrillar, and acellular with occasional elaboration of matrix fibers and small lipid droplets. These areas were occasionally associated with foci of cellular degeneration and debris that were acellular, characterized by spherical lamellar bodies and various inclusions of material that appeared to include myelin figures, secondary lysosomes, and extracellular debris and/or necrotic tissue (Fig.12). These areas of cellular degeneration and debris were often found in deeper layers of the media surrounded by SMC with no appearance of altered morphology. The integrity of the internal elastic lamina did not appear to be compromised in these vessels, and in most samples, SMC were usually confined within the lamellae of elastin and collagen fibers and basement membrane, characteristic of control, lesion-free vessels. These observations were not restricted to a particular hormonal treatment group, and several animals were characterized by intimal thickening which appeared to be sparsely populated by foam cells or fibrillar connective tissue.

Variability in the area of lipid accumulation was extensive in all treatment groups throughout the study. At each time point, there was at least one animal in each treatment

group in which there was little or no ORO positive material detected. Only at the earliest time point was there a statistical difference in lesion area as defined by ORO staining, as ovex + E_2 animals had significantly less ORO positive area than ovex animals at 4 months of high fat feeding (Fig. 13). However, the trend was for animals exposed to either endogenous or exogenous E_2 to have smaller areas of lesion involvement than those of ovex animals at each time point. Only the ovex + E_2 animals showed any increase in aortic lesion area over time, but this area remained less than that of the other treatment groups (Fig. 13). The area of ORO positive lipid in the ovex + E_2 group at 4 months of high fat feeding was significantly lower than that in ovex animals at 9 and 18 months ($p \leq 0.05$). Regression analyses demonstrated that the logarithm of lesion area was significantly associated with plasma TG at the 4 month time point and with plasma TC, LDL-C, and HDL-C at 9 and 18 months of high fat feeding (Table 17). However, lesion area was not associated with the logarithm of plasma E_2 levels at any time in the study. Plasma lipids were not significantly associated with hormone concentrations.

DISCUSSION

In this study, the hypercholesterolemia induced in swine during the high fat, high cholesterol diet period was not significantly altered by treatment of ovex swine with high levels of exogenous E_2. The plasma concentrations of E_2 in both the sexually intact animals and the E_2 replaced animals were apparently ineffective in modifying plasma lipoprotein cholesterol distribution in contrast to observations in other animal and human studies (109,112,124,125). In this short-term study, circulating E_2 levels may have been insufficient to precipitate the changes previously credited to E_2's impact on lipoprotein profiles. In women, changes in plasma lipoprotein levels were generally not observed if E_2 was administered parenterally and plasma levels did not exceed 200 pg/ml (102,103). Additionally, when maximal E_2 concentrations remain consistently below 200 pg/ml in postmenopausal women, adverse changes in plasma lipid concentrations are observed (85,95,97,103). In this study, none of the female swine demonstrated peripheral plasma E_2 concentrations greater than 100 pg/ml, and thus, changes in plasma lipid profiles were not expected. Additionally, the diet was pharmacologic in terms of its cholesterol and cholate concentrations. Thus, any effects of E_2 may have been overwhelmed by the mass of cholesterol and additional lipids in the diet. Nevertheless, TC and VLDL-C + LDL-C concentrations decreased in all treatment groups independent of hormone levels over time, an observation previously made in swine in response to a high fat dietary challenge (216).

Since no attempt was made to sample plasma lipids or E_2 concentrations on the same day of the estrous cycle in intact animals, a high degree of variability resulted in the measurement of estrogen at each time point of the high fat diet during which other

parameters (cell adhesion and LDL catabolism) were assayed. Thus, if endogenous hormone levels acutely alter these parameters, effects of widely fluctuating (although relatively low concentrations compared to premenopausal women) reproductive hormoneobtained in this group of animals. Although no clear trend of E_2 action could be characterized in either monocyte and platelet adhesion or MNC catabolism of LDL, this possible confounding factor should be noted. Ovex and ovex + E_2 animals maintained narrow ranges of circulating E_2, minimizing any effects of such variability in these two groups.

Swine in this study responded to the dietary challenge with an increase in all plasma lipoprotein fractions, with the greatest increase occurring in the VLDL-C + LDL-C fraction. Concentrations of cholesterol in this fraction tended to decrease over time independent of hormonal treatment. These trends in apo B100-containing lipoproteins were paralleled by rates of ^{125}I-LDL binding and degradation by isolated MNC. The primary increase in these rates was accounted for by changes in receptor-mediated activity. Roach and colleagues (217) have shown in the cholesterol-fed rabbit that LDL receptor activity of circulating MNC reflected changes observed in hepatic activity in response to cholesterol-lowering pharmaceuticals. Both pravastatin and simvastatin decreased hepatic and plasma cholesterol levels and increased both liver and MNC LDL receptor activity, although in all cases liver was more responsive than MNC. Liver and mononuclear LDL receptor activities were directly correlated, and both activities were inversely correlated with plasma cholesterol levels. Although a similar association was observed in human subjects in response to simvastatin, a reduction of dietary fat intake from 38 to 20% of energy and cholesterol from 239 to 96 mg/day reduced plasma

cholesterol without any change in LDL receptor activity (218). Thus, regulation of the LDL receptor expression may be under different control mechanisms mediated by cholesterol-lowering drugs compared to cholesterol-lowering diets. Conversely, the introduction of a cholesterol-raising diet in this study of hormonally treated swine did not alter LDL receptor activity of MNC in the expected fashion. Since dietary cholesterol downregulates the hepatic LDL receptor(118,219), one would expect a similar response in MNC receptor activity if they are truly hepatocyte equivalents and reflect these receptor changes in response to diet (217,220,221). However, LDL receptor activity in MNC from swine increased in all treatment groups with increased plasma cholesterol concentrations. The gradual decrease in plasma cholesterol levels was reflected by similar decreases in receptor-mediated LDL catabolism by isolated MNC. Thus, receptor activity in these cells did not respond in a fashion predicted by the established hepatic response to changes in dietary cholesterol. Just as MNC did not respond to reductions in dietary fat and cholesterol in hypercholesterolemic patients (218), swine MNC did not respond to dramatic dietary changes in this study. Instead, receptor activity seemed to be driven by plasma TC and/or LDL-C levels, as changes in these concentrations over time were mirrored by changes in LDL catabolic rates independent of E_2 status. Thus, in this model under the prescribed conditions, MNC LDL receptor activity does not appear to respond to fluctuations in dietary and plasma cholesterol concentrations in a manner which parallels the established changes observed in hepatic receptor activity.

The degree to which circulating monocytes participate in lipoprotein metabolism is presently undefined. Previous studies indicated that severe hypercholesterolemia may result in monocytosis and an accumulation of lipid in these cells (222, 223). However, a

more recent investigation has shown little difference in MNC intracellular lipid content between hyperlipidemic patients and normocholesterolemic individuals (224), further supporting the observation that diet-induced plasma lipid changes have no significant influence on uptake of LDL in isolated MNC (218).

The rate of binding and degradation of LDL by MNC may be partially regulated by E_2 as estrogen receptors have been identified in circulating MNC (225,226). However, there were no significant differences in LDL catabolic rates among hormonal treatment groups, and these rates were not significantly associated with plasma E_2 when subjected to regression analysis. Although pharmacologic doses of E_2 have been shown to upregulate the production of LDL receptor mRNA and LDL receptor protein (116-118), more recent studies have shown that physiologic doses of oral contraceptive estrogens in sexually intact non-human primates have no apparent effect on mRNA for the LDL receptor protein (121). It is possible that E_2 may have no receptor-dependent or receptor-independent influence on MNC LDL degradation rates. Alternatively, the lack of regulation by E_2 in these cells may have been a function of the aforementioned low levels of plasma E_2 in swine compared to the primate female, as well as the relative insensitivity of MNC LDL receptor activity to dietary changes in fat and cholesterol as demonstrated by the study of Roach *et al.* (218).

The adhesion of monocytes to arterial endothelium and their migration into the subendothelial space are important early features of fatty streak formation. Since circulating monocytes are the precursors of early lesion foam cells, their degree of adherence to endothelium is an important factor in determining the rate of lesion development. Since E_2 has been shown to be associated with protection from

atherosclerotic development (88,99,110,157), E_2 concentrations may impact the degree of adhesion to EC. Monocytes isolated from the three treatment groups of hypercholesterolemic swine showed no significant difference in adhesion to PAEC *in vitro* except at the first high fat time point of 4 weeks. In this assay, monocytes from ovex + E_2 animals showed significantly increased binding to PAEC *in vitro* when compared to cells from ovex and intact animals. Both animal and human studies have shown that hypercholesterolemia and hypertriglyceridemia increased the adhesion of monocytes but not polymorphonuclear leukocytes in both *in vivo* and *in vitro* assays (6,227,228). Elevated levels of plasma lipids, especially native (227,229,230) and oxidized LDL (228), activated monocytes and enhanced their adherence to normal EC. It is clear that activation of the monocyte, or the EC, or both can lead to increased monocyte adhesion to EC. These *in vitro* assays assume that confluent PAEC monolayers behave as normal EC and play a static role in the potential variability expressed in the assay. However, this may be an oversimplification of the metabolic state of the EC, as previous studies have shown that EC in culture express some characteristics of activated endothelium (27,231). Additionally, there was such variability in results from day to day that MANOVA revealed a significant effect of day of assay that prevented the statistical comparison of results from one time point to another. One can assume that, other than differences in the state of monocyte activation, the largest variable between day to day assays is the metabolic condition of the different EC monolayers to which monocytes were exposed in each assay. Thus, if the EC monolayers did not express the same level of adhesion molecules or other active components which may influence cell to cell adhesion, the variability may have been a response to differences in EC, not isolated monocytes.

Although E_2 has been shown to enhance tumor necrosis factor (TNF)-induced binding of leukocytes and expression of EC adhesion molecules, Cid and colleagues (232) showed that E_2 did not increase binding of isolated mononuclear cells without prior activation by PMA. E_2 alone did not increase adhesion of monocytes to EC in the absence of TNF. Only after activation of both EC by TNF and activation of monocytes by PMA did E_2 enhance this cell interaction. Although there was no specific attempt in the present study to characterize the state of differentiation or activation of either the isolated swine monocytes from the three hormonal treatment groups or the confluent PAEC monolayers, the increase in adhesion of monocytes from ovex + E_2 swine at 4 weeks of high fat feeding is consistent with the result of Cid *et al.* (232) if one assumes that the hypercholesterolemic state in swine led to increased activation of circulating monocytes, and that EC in culture express some properties of activated endothelium. As stated above, cells of both types are likely to be in an activated state under the conditions in which the assays are performed in these studies (27,227). At the later time points, there were no such effects of E_2 on monocyte adhesion to PAEC. As plasma lipids, especially VLDL + LDL, decreased over time, the degree of circulating monocyte activation may have been attenuated, thus reducing the probability that E_2 concentrations would affect monocyte adhesion.

Since platelets are nucleus-deficient cells, the classic genomic effect of E_2 in these cells is not a possibility, but non-genomic, receptor-independent effects may modulate platelet function. Atherogenic plasma lipid profiles consisting of elevated beta lipoproteins and reduced levels of alpha lipoproteins are associated with increased platelet activity (233-235). Improvement in the lipoprotein profiles reduces platelet activity as well. *In*

vitro incubation of isolated platelets with LDL or VLDL led to platelet activation, a response driven by the binding of LDL to a specific, high-affinity receptor (233). Thus, a greater percentage of circulating platelets in hypercholesterolemic swine exposed to elevated levels of LDL-C and/or VLDL-C may be expected to undergo activation upon isolation and adhere to PAEC when co-cultured *in vitro*. Additionally, changes in platelet activation should parallel changes in plasma lipid profiles, especially indicators such as TC/HDL-C which incorporate both HDL and VLDL + LDL into the ratio. However, in this study, all animals in all treatment groups demonstrated a small percentage adherence to PAEC *in vitro* independent of hormonal treatment. Since EC in culture have been observed to produce EDRF *in vitro* (170,236), an antithrombotic environment may have been the predominant influence in determining the degree of platelet adhesion as opposed to any direct hormonal effect on platelet reactivity in the three treatment groups. Since hormonal treatment had no significant effect upon lipoprotein concentrations at any time point, the absence of differences in platelet activity as mediated by LDL exposure was expected. *In vivo*, the more significant effect of E_2 on platelet adhesion to endothelium may be mediated through changes in EC metabolism, since E_2 may modify EDRF production (175,176), act as an antioxidant protecting EC from oxidative injury (138), and lower atherogenic lipoprotein concentrations (100,125), all changes leading to a decreased thrombogenic environment within the blood.

The data presented here clearly show that the removal of endogenous E_2 by ovariectomy of hypercholesterolemic swine rendered LDL susceptible to oxidation *ex vivo*, and hormonal replacement with high physiologic concentrations of E_2 restored LDL oxidative resistance to levels characteristic of LDL from sexually intact females. The

protective effect of E_2 was in part mediated by changes in the LDL particle content of α-tocopherol, the sole lipid soluble antioxidant detected in these samples. Additionally, E_2 replacement significantly increased the LDL PUFA content while sexually intact animals showed a significantly lower linoleic acid to oleic acid ratio. As previously described in postmenopausal women (100), the parenteral delivery of low doses of E_2 (≤ 200 pg/ml of plasma) did not alter total plasma lipid profiles and thus, oxidative resistance was not associated with changes in LDL-C concentrations. However, the oxidative resistance of LDL was significantly correlated with LDL α-tocopherol levels and weakly associated with plasma E_2 concentrations.

Previous *in vitro* studies of LDL oxidation have demonstrated antioxidant effects of several estrogens (142-145) which act as chain breaking antioxidants by scavenging free radicals (237). However, micromolar concentrations of estrogens were required to inhibit metal ion- and cellular-induced oxidation of LDL from normocholesterolemic subjects as compared to the picomolar concentrations found in circulation. Additionally, protocols called for coincubation or relatively brief preincubations of isolated LDL with estrogens prior to the onset of the assay. These conditions did not allow for assessment of effects that estrogens may have *in vivo* on LDL particle composition that may alter its susceptibility to oxidation. In contrast, it was clearly demonstrated in this *ex vivo* study that altering the estrogenic condition of swine by only picomolar concentrations significantly impacted upon LDL oxidative resistance as measured by lag time and propagation rate. Furthermore, this enhanced resistance was mediated *in vivo* by compositional changes in LDL antioxidants.

Although E_2 may be carried in the LDL particle and act as an antioxidant in situ, plasma concentrations are so low *in vivo* that it is unlikely that E_2 itself plays a major role as a direct chain breaking antioxidant in LDL. E_2 was undetected by radioimmunoassay in isolated LDL. Although Huber *et al.* (142) demonstrated that E_2 was a more potent antioxidant than probucol and was effective in molar ratios as small as 1:1 (1 E_2 molecule:1 LDL particle), it is estimated on a protein basis from isolated LDL (as did Huber *et al.*) that sexually intact hypercholesterolemic swine in this study had LDL particle concentrations ranging from approximately 1.4 to 2.0 μmol/L while plasma E_2 concentrations ranged from 18-88 pmol/L. These concentrations correspond to E_2:LDL ratios far below those used to demonstrate antioxidant effects of E_2 *in vitro*. During the menstrual cycle, plasma levels of E_2 range from approximately 150 pmol/L in early follicular stages to a short-term preovulatory spike of approximately 1800 pmol/L including a fraction bound to either steroid hormone binding globulin (SHBG) or albumin, and thus unavailable to act directly as an antioxidant in LDL. Although these E_2 concentrations are higher than those observed in swine, and LDL concentrations in normocholesterolemic, premenopausal women are considerably lower than those above, ratios of E_2:LDL in women remain several orders of magnitude less than those used *in vitro*. Therefore, E_2 in circulation may not be available in sufficient quantity to associate with LDL and act as a primary component of oxidative resistance. Duration of the lag phase was not significantly correlated with plasma E_2 concentrations, suggesting the presence of some additional antioxidant molecule(s) or alternative mechanism(s) of protection by E_2. However, it is important to note that plasma concentrations of these substances may not reflect their ratios in the relatively antioxidant-poor environment of the

arterial wall, where LDL oxidation is believed to play a major role in atherogenesis. Arterial concentrations of E_2 and LDL may provide a more favorable ratio of antioxidant to substrate such that E_2 plays a significant role in oxidative resistance at the site which is most pathologically pertinent.

Female swine exposed to endogenous or exogenous E_2 had significantly greater concentrations of α-tocopherol in their LDL compared to ovex animals (Table 12). Alpha-tocopherol has been shown to be the primary lipid soluble antioxidant in animal and human studies (147,238) and supplementation with high doses of vitamin E has been correlated with decreased incidence of pathophysiological coronary events in human epidemiologic reports (239,240). In swine, α-tocopherol may be the only LDL-associated antioxidant in significant quantities as our analyses showed β-carotene and retinol to be below detectable levels, data which support the findings of Knipping *et al.* (238). We observed approximately twice the concentrations of LDL α-tocopherol as those previously reported. Enhanced absorption of dietary vitamin E as a result of high dietary fat content may partially account for this difference. However, E_2 has been shown to regenerate tocopherols (239) and may thus contribute to oxidative resistance by maintaining endogenous antioxidants in a manner similar to ascorbate (240). This potential for antioxidant regeneration may account for the increased α-tocopherol observed in our swine with higher plasma E_2 concentrations, as well as differences between these data and previous observations (238). Additionally, estradiol replacement in castrated female rats prevented the decrease in hepatic α-tocopherol levels observed in those without estradiol (241). Thus, E_2 may alter α-tocopherol levels incorporated into nascent LDL by modifying the association of α-tocopherol and lipoproteins in the liver.

In studies of lipid soluble antioxidants such as vitamin E and probucol, lag time has been associated with these molecules in a concentration-dependent manner (142,147,148, 238). In this study, the association between LDL α-tocopherol content and duration of the lag phase (Table 14) confirms previous observations in swine (238). However, the relationship is not as clear in human studies (242,243). Since the antioxidant profile in swine is likely to be less complex than that of human LDL (238), oxidative resistance is more likely associated with LDL α-tocopherol content in swine than in humans. However, the association does not preclude the presence of other molecules which delay peroxidation of the lipid core.

Since PUFA are the primary substrate for oxidation during the propagation phase, one could speculate that their concentration and availability in LDL may be associated with both the rate of oxidation and total amount of dienes formed in the assay. In human and rabbit studies, dietary supplementation with linoleate led to its enrichment in LDL and an increased rate of Cu^{2+}-mediated oxidation and total diene production compared to LDL enriched with dietary oleate, and oxidized LDL from PUFA supplemented animals showed greater rates of degradation by macrophages (151-154). However, no such relationship was observed between fatty acid composition and oxidation profiles in swine LDL. None of the treatment groups in this study differed in enrichment of any individual LDL fatty acid, yet LDL from ovex animals had propagation rates nearly twice those of samples exposed to endogenous or exogenous E_2. PUFA as a percent of total were significantly greater in ovex + E_2 animals and intact animals had a lower 18:2/18:1 ratio (Table 5). Despite these opposing changes in substrate proportions, LDL from these treatment groups demonstrated rates of propagation and total diene formation that were nearly

identical, and both were significantly lower than those for LDL from ovex animals (Table 11). Unopposed estrogen treatment has previously been shown to alter plasma PUFA concentrations (244,245) and a similar effect was observed in this study. Thus, striking differences in susceptibility to oxidation were observed in our treatment groups independent of the hormonally altered, relative amounts of PUFA, linoleic/oleic acid ratios, and P/S ratios.

The mechanisms and/or target tissues through which E_2 alters LDL antioxidant and fatty acid composition may be fundamentally different from those underlying dietary interventions. Animals were individually fed the same diet, and thus, differences in LDL composition were likely a result of sex hormone alterations in the metabolism and/or distribution of the substrates of LDL production. The data suggest that these alterations, in addition to the presence of different circulating levels of E_2, may shift the complex balance between lag phase and propagation rate such that lag phase determinants (e.g. α-tocopherol content), rather than fatty acid composition of the LDL, have a greater impact on propagation rate.

A growing body of evidence suggests that the determinants of the lag phase are complex, as components of LDL other than antioxidants have been shown to be associated with oxidative resistance. Recent studies have demonstrated a significant association between the primary determinants of propagation rate, namely PUFA, and lag phase duration. Vitamin E-deficient patients without any dietary controls had vitamin E-poor LDL that was more resistant to oxidation than normal controls due to a dramatic decrease in PUFA (151). In rats and humans, PUFA-enriched LDL showed an increased conjugated diene propagation rate accompanied by significant shortening of the lag phase

even though the vitamin E content of the diets was matched and plasma levels were similar (152,246). Likewise, in normocholesterolemic men, the addition of vitamin E to a fish oil supplemented diet not only increased the lag phase but decreased the propagation rate of LDL oxidation (247). Chait *et al.* (248) observed that a decrease in the lag phase in LDL subfractions was accompanied by an increase in rate of oxidation. In the present study, a similar pattern was observed in which the rate of oxidation was clearly inversely correlated with lag phase in the 12 samples examined. Therefore, the oxidation rate may well be influenced by duration of the lag phase and by both the quantity and type of antioxidants and fatty acids in swine LDL.

Replenishing the E_2 in ovex swine at high physiologic concentrations was adequate to restore fully the capacity of LDL particles to resist oxidation in these assays. Although the animals with intact ovaries have additional steroidal hormones, including other estrogens as well as progestins, it is apparent from the data presented here that E_2 alone can act to alter the α-tocopherol content of the LDL particle and subsequent oxidation kinetics *ex vivo*. Steroid sex hormones significantly affect α-tocopherol, lipoprotein, and fatty acid metabolism (241,245,249). These alterations may significantly impact parameters which determine LDL composition and oxidative resistance. For example, the presence of estrogen receptors in the intestinal epithelium (250) provides a mechanism whereby E_2 may alter antioxidant vitamin absorption increasing the pool of antioxidants available for incorporation into nascent lipoproteins in the liver. Since a significant inverse correlation between lag time and propagation rate, and no significant correlation between PUFA composition and either propagation rate or total diene formation was observed, it is possible that E_2 action (genomic or non-genomic) may be modifying the LDL particle in

its antioxidant and/or lipid composition, concentration, distribution, or accessibility to free radical or peroxyl radical initiation in a manner that protects the lipid core from propagation during the oxidative process. This may be yet another protective mechanism of E_2 which lowers the incidence of cardiovascular disease in premenopausal women and postmenopausal women on hormone replacement therapy.

The hamster has been shown to be an easy to handle, reliable animal model that is susceptible to diet induced hypercholesterolemia and atherosclerosis (7,183,185). They carry the bulk of their plasma cholesterol in LDL and respond to dietary fat and cholesterol in a manner similar to humans (184,185). However, when hamsters in this study were fed a cholesterol- and coconut-rich diet for up to 18 months, changes in plasma lipid concentrations did not reflect those generally considered to be atherogenic, i.e., the relative increase in HDL-C was generally equal to the relative increase in VLDL-C + LDL-C resulting in TC/HDL-C ratios that were not significantly different from those during low fat feeding. In fact, these TC/HDL-C ratios reflected a relatively low risk profile for plasma lipids.

Although the animals became hyperlipidemic on the high fat diet, the significant increase in HDL-C accounted for approximately 57% of the increase in TC. Plasma HDL-C concentrations have been shown to be powerful predictors of CAD risk and are strongly inversely correlated with the incidence of disease and coronary events (96). HDL administration to atherosclerotic rabbits induced significant lesion regression (251) and decreased the rate of lipid deposition in the liver and arterial wall without lowering plasma lipid concentrations (252). Thus, the relatively high concentrations of HDL-C carried in the plasma of these hypercholesterolemic females were likely to inhibit significantly the

initiation and progression of arterial lesions as described above in rabbits. The few lesions that developed in these hamsters were characteristic of the earliest stages of atherosclerosis. Only localized fatty streaks were found in the area of the aortic arch which has been shown to be highly susceptible to lesion development in previous studies in this strain of hamster (61,186).

In this study hamsters demonstrated high concentrations of fasting TG independent of either hormonal treatment or dietary fat and cholesterol levels. Ontko *et al.* (253) have shown that hamsters are characterized by higher fasting TG than most rodents. In this species there is elevated hepatic production of VLDL associated with increased incorporation of non-esterified fatty acids into triacylglycerol. Hypercholesterolemia and hypertriglyceridemia have also been linked in this species as increased levels of dietary cholesterol and fat induced significant increases in cholesterol and TG in VLDL fractions (254).

There has been considerable debate over the atherogenicity of TG. The most prevalent posture states that TG are atherogenic in and of themselves, even under conditions of normocholesterolemia, and this effect is enhanced by hypercholesterolemia (96,255). VLDL-C concentrations and associated TG are normally higher in women than age-matched men due to increased hepatic production thought to be an effect of higher estrogen levels in premenopausal women (98,100,124). However, the elevated TG in these animals did not induce any significant lesion development independent of hormonal treatment, and E_2 exposure in intact or ovex + E_2 females did not appear to enhance plasma VLDL-C or TG levels.

It is of significant interest to note that previous studies of aortic lesion development in the hamster have been conducted exclusively in males. In fact, in a recent article by Sessions and Salter, they specifically cite that the "male hamster" has become an important model of human lipoprotein metabolism (256). Sex differences in lipoprotein metabolism and lesion development have not been characterized in this species. In all mammalian species studied to date, females were more resistant to atherosclerosis than males. In this study of sexually intact and ovex female hamsters, one would have expected ovex hamsters to be more susceptible to atherosclerosis than the hamsters that were exposed to physiologic concentrations of estrogens, the putative protective steroids in trials of hormone replacement in both animals (110,112) and humans (97,99). However, there was no evidence of any E_2 effect on plasma lipoproteins while animals were fed the purified high fat diet. Circulating E_2 levels were within the physiologic range and tended to decrease in concentration over time in both the intact and the ovex + E_2 groups (Table 16). It is likely that the modest levels of circulating estrogens were not great enough to modulate the extreme hyperlipidemic effects of the high fat and cholesterol content of this diet. Additionally, the regulatory limits of HDL and LDL metabolism may have been approached as the proportional concentration of HDL-C was extraordinarily high in this state of hypercholesterolemia. In cases of such elevated TC, it is extraordinary to encounter TC/HDL-C ratios such as those measured in this study (Table 15). Thus, the reproductive hormones may not have been able to enhance any further the mass of circulating HDL-C under these hormonal and dietary conditions. Additionally, since apo B-containing lipoproteins were not separated before being quantified, there was no ability to detect equal but opposite changes in VLDL-C and LDL-C levels that normally result

from estrogen administration (98,124,125). Thus, increases in VLDL production may have been offset by increases in LDL clearance, resulting in no significant net change in the total apo B cholesterol-carrying lipoprotein concentrations. Regardless of hormonal treatment, hamsters showed no evidence of accommodation in their lipoprotein levels even after 18 months, as plasma lipid levels were not significantly reduced over time (Table 15), as was observed in swine (Table 4).

As stated above, the natural history of the atherosclerotic lesion in female hamsters has not been described. The unusual features of early arterial lesions and acellular areas of intimal swelling in some animals may have been a result characteristic of their gender or the high fat diet or a combination of the two. Since the range of response to diet and hormonal treatment was extremely variable as measured by ORO positive material in the aortic arch, one cannot conclude that these observations in a small number of hamsters are characteristic of the early fatty lesion development in females of this strain. This type of pathology is atypical of the early atherosclerotic lesion or the processes which lead to fatty streak formation described in other animal models and humans (6-8,37,257,258). Of significant potential importance may be the consideration that many of these fatty streaks were examined in older animals that had been fed the high fat diet for 18 months. There was little change in ORO positive area of the aortic arch between 4 and 18 months on this diet (Fig. 11). Thus, these scattered lesions at 18 months may be "old" fatty streaks, perhaps areas that have not progressed and have undergone some cellular turnover, regression, or degeneration. This possibility is consistent with the deposition of cellular debris, lysosomes, and myelin bodies, perhaps indicative of macrophage foam cell and/or SMC death. These areas were characterized by pools of extracellular debris and necrotic

tissue, as if the area had been invaded by macrophages which had subsequently undergone necrosis, leaving myelin figures, lysosomes and residual bodies in the subendothelial space. Little fibrous material was present in these areas minimizing the possibility of previous fibroproliferative involvement. This process of accumulating the infiltrated lipid to the point of cell death may be a normal, protective, inflammatory response mediated by circulating mononuclear cells (259). Females of this strain of hamster may have been protected from further lesion progression by a mechanism other than those previously proscribed for E_2.

It should be noted that the characterization of risk factors and calculation of relative risk serve to predict a population's relative and statistical probability of developing a pathologic condition, in this case CAD. Certainly there are examples in both the human population and animal models in which atherosclerosis is not the inevitable consequence of hyperlipidemia. The hamsters in this study, even though hyperlipidemic, never developed cholesterol profiles typical of those that are atherogenic. VLDL-C + LDL-C were not the predominant fractions which increased in response to the high fat diet, and the significant increases in the HDL fraction more than likely proved to be antiatherogenic, even in ovex animals in which endogenous sources of E_2 had been removed. The predominant impact on lesion initiation appeared to be dietary or some yet undescribed feature of female anatomy and physiology, which may or may not include reproductive hormonal influence.

In this study, female hamsters fed the purified high fat, high cholesterol diet did not respond with increases in plasma lipids or develop arterial lesions to the same extent as those seen in previous studies in male hamsters fed equivalent amounts of cholesterol and fat (coconut oil) for much shorter periods of time (61,186). However, the diets fed to

males were not purified, and perhaps the most significant difference between the diets was the source of protein. The studies in males have incorporated animal protein (casein) into the diet while in this study, females were fed plant proteins (soybean, Table 2). Previous studies in animal models have demonstrated that plant protein is less atherogenic than animal protein when combined with dietary cholesterol (260,261). This decreased atherogenic property is reflected in the lipoprotein profile established on the plant protein diet. In general, TC, VLDL-C, LDL-C, and TG are decreased when plant proteins are substituted for animal proteins in diets containing cholesterol and a modest amount of fat (262-264). Bakhit and colleagues (265) observed that the degree of cholesterol lowering by dietary plant protein was correlated with the initial plasma cholesterol concentration. Females and growing juveniles were found to be more sensitive to these effects of dietary protein than males and full-grown adult counterparts (266).

These effects on the lipoprotein profiles may be mediated by the differences in the types of fiber between plant and animal proteins. Although the mechanism for this effect on lipoprotein metabolism is not known, a number of studies suggest the alteration of enterohepatic circulation of bile may play a significant role. Casein-fed animals excreted less fecal steroids and showed an enhanced intestinal absorption or reabsorption of cholesterol and bile acids than animals fed soy protein (267-269). Monkeys and pigs demonstrated a dramatic decrease in plasma TC while the latter showed striking increases in bile acid and neutral steroid excretion on a diet including soy protein compared to a casein-containing diet (264,270). Similarly, psyllium decreased plasma cholesterol to baseline levels and increased bile acid excretion fourfold when added to a cholestatic diet in hamsters (271). This increase in bile acid removal most likely increases hepatic

cholesterol turnover by affecting transhepatic cholesterol flux into bile acids and biliary cholesterol, and resulting increases in LDL receptor activity. Since functional estrogen receptors have been identified in the small intestinal epithelium of both males and females (250), the potential for sex differences in intestinal absorption of cholesterol and its metabolites in response to dietary protein exists. However, the physiologic significance of these receptors is unclear at this point.

The proposed active component in the plant proteins which improves plasma lipoprotein profiles and enhances bile acid and cholesterol excretion is thought to be a high molecular weight component exerting a resin-like effect which serves to decrease the hepatic cholesterol pools and increase the receptor-dependent clearance of LDL-C (272,273). These plant proteins contain the type of nonabsorbable soluble fiber that bind cholesterol and its metabolites and block their absorption in the intestine, similar to the mechanism of cholesterol-lowering of the synthetic resins such as cholestyramine (272,273). This hypocholesterolemic effect is likely a result of changes in the net sterol balance across the liver.

These components of plant proteins not only block the absorption or reabsorption of cholesterol and bile acids, but of structurally related compounds that are metabolites of cholesterol. High fiber, vegetarian diets in women have been shown to reduce the levels of circulating estrogens (274). Similarly, dietary intervention with a high carbohydrate, low fat, high fiber diet lowered E_2 concentrations in men by 50% within three weeks (275). Studies relating levels of E_2 and the incidence of breast cancer have been concerned primarily with dietary fat, but some have additionally demonstrated a negative correlation between dietary fiber and plasma E_2 levels (274,276). After 2 months of dietary treatment

in which the levels of fat were not altered, but dietary fiber intake was doubled, estrone and E_2 concentrations in plasma were significantly reduced (274). This effect was observed only in those patients consuming wheat bran as the source of dietary fiber, as oat and corn bran had no effect on levels of these estrogens.

A significant proportion of estrogens are conjugated with glucuronides and sulfoglucuronides by the liver which renders them inactive in terms of receptor-mediated activity (277). These conjugated estrogens are excreted with bile and cholesterol into the intestine, where glucuronidases and sulfatases formed by the intestinal, bacterial flora cleave the conjugated side chains converting the estrogens to their biologically active forms. Approximately 50-80% of these forms are generally reabsorbed within the gut and returned to the circulation (277,278). However, the nonabsorbable fibers bind these conjugated and free estrogens and block their reabsorption, as is the case for the bile acids described above, altering the enterohepatic circulation of estrogens (274,279). These dietary fibers also resulted in the reduction of β-glucuronidase activity, thus blocking conversion of conjugated estrogens into a form that can be reabsorbed by the intestinal epithelium. An additional mechanism has been proposed by Whitten and Shultz (280) suggesting that fiber in the gut binds and blocks the reabsorption of unconjugated estrogens that have escaped metabolism in the liver or have been acted upon by the intestinal enzymes which cleave the glucuronides. This mechanism is further supported by *in vitro* experiments in which water-insoluble fiber, such as that found in wheat bran, avidly bound E_2 and estrone (280). Again, the presence of functional estrogen receptors in the intestinal epithelium provides a potential mechanism for estrogens to influence their own reabsorption either directly or by altering the intestinal impact of dietary fiber in the

gut. With varying levels of estrogens and their receptors in the intestine, the machinery for sex differences in these reabsorptive functions is in place.

Alterations in sex hormone metabolism may be mediated by other dietary components as well. Approximately half of plasma E_2 is bound to SHBG and is therefore considered biologically unavailable to most tissues. The remainder is loosely associated with albumin and a small percentage remains free in the plasma (281). Jacques and colleagues have shown that the substitution of lean white fish protein for vegetable protein negated the beneficial effects of the n-6 PUFA's ingested as fish oil (282). Although the fish protein did not alter the relatively low levels of plasma E_2 in these postmenopausal women, the concentration of SHBG was significantly increased in the women fed fish protein, effectively decreasing the amount of biologically available E_2 as described above.

Although the fat and cholesterol quantity and quality affect both the lipoprotein response in animals and humans and the atherosclerotic risk and development of lesions, other dietary components such as protein may dramatically alter the end-result homeostatic condition both by altering apoprotein and lipoprotein concentrations as well as circulating steroid hormones including the estrogens measured in this study. It is therefore speculated that the dominant effect of the soy protein in this experimental, purified, high fat, high cholesterol diet overrode any hormonal influence established by the surgical procedures carried out on these female hamsters. This diet was not atherogenic in female hamsters and could not be used to distinguish between animals with physiologic circulating levels of E_2 and those with significantly reduced levels after ovariectomy.

In addition to the secretion of mitogenic and chemotactic PDGF by adherent platelets during thrombotic episodes encountered at sites of lesion formation, adherent

platelets and coagulation molecules may alter endothelial permeability at any stage of lesion development including possible initiating events. Platelet adherence to the endothelium has been described in non-diseased, prelesional vessels (283). These adherent platelets or microthrombi may alter endothelial permeability in these otherwise normally functioning vessels. Conditions which precipitate platelet adhesion and plasminogen activation mediate retraction of endothelial monolayers *in vitro* which subsequently may initiate or exacerbate the influx of atherogenic lipoproteins and mononuclear cells into the subendothelial compartment of the arterial wall (283-285). Honn *et al.* have recently observed that an arachidonic acid metabolite of platelets (12[S]-HETE, 12[S]-hydroxyeicosatetraenoic acid) mediates endothelial cell retraction of *in vitro* monolayers in a concentration dependent manner (284). Since activated platelets produce significant quantities of 12[S]-HETE, their adherence to the endothelium may lead to the extravasation of plasma and cellular components that are thought to contribute to lesion initiation or lesion progression and expansion. Additionally, other mediators of inflammation elicit similar response in endothelial cells, including thrombin and bradykinin that disrupt endothelial integrity in a non-denuding fashion (see 286 for review). 12[S]-HETE may have additional biological effects pertinent to atherogenesis as it has been shown to regulate cytoskeletal organization and integrin receptor expression (284).

Cellular retraction of the endothelium requires loosening or destruction of cell-cell and cell-basement membrane connections. The most thoroughly known cell surface proteolytic system is the plasminogen cascade (285,287) associated with inflammation and plasmin activity (288). Thus, plasminogen activation and initiation of the clotting cascade may retract endothelial cells resulting in local infiltration of plasma lipids and attachment

of mononuclear cells to the subendothelial matrices. Additionally, the local production of mitogenic and chemotactic compounds such as PDGF would further contribute to the atherosclerotic process.

Conforti *et al.* (285) have shown that the urokinase-like plasminogen activator receptor colocalizes with PECAM at cell-cell contacts and junctional structures. Thus, platelet adhesion and a proteolytic mechanism combine to loosen cell-cell contacts and elicit EC retraction. This activity of platelets may in part explain enhanced permeability to atherogenic lipoproteins during hypercholesterolemia. With the increased expression of adhesion molecules at susceptible sites of the vasculature, platelet adherence and activation may occur more frequently in the hyperlipidemic state with episodic, if not continuous enhancement of extravasation of lipoproteins and circulating cells. Therefore, the relative procoagulant nature of hyperestrogenic OC's in the past may have led to an increased incidence of CAD both through thrombotic emboli which eventually occluded arteries and by enhanced lesion progression through increased permeability to components in the blood.

However, in the present study, endogenous E_2 and the low concentration of exogenous E_2 did not alter the reactivity of platelets above a basal level of adherence as assessed by observing their interactions with EC *in vitro* in a static assay. Somewhat obvious is the notion that *in vivo*, both EC and platelets are exposed to the same plasma components including E_2, and the enhanced reactivity may be mediated by effects on EC *in vivo*. Nevertheless, the results in swine demonstrate that any alterations in platelets that may have occurred as a result of changes in circulating E_2 neither enhanced nor diminished

their reactivity to EC under standardized, uniform conditions during the 12 weeks of high fat feeding.

CONCLUSIONS

In this study, surgical ovariectomy and replacement of E_2 by subcutaneous implants allowed for the study of E_2 effects on cellular and molecular events associated with atherogenesis and lesion progression in female swine and hamsters. This surgical treatment followed by dietary manipulation to a high fat, high cholesterol diet in both animal models led to elevated plasma lipid levels that were potential targets of E_2 action. The circulating levels of plasma E_2 in the treatment groups varied to the extent that all were significantly different from one another, although the levels in females with replacement implants did not approach the pharmacological levels that have been used in other studies. However, no effects of E_2 were observed on plasma lipid profiles among treatment groups at any time point in either animal model. It is likely that the E_2 concentrations in these animals were not sufficient to impact lipoprotein metabolism in a significant manner. The changes in plasma lipids observed in other species or under other experimental conditions were a result of higher concentrations of estrogens than those observed in animals in the present study. Thus, binding and degradation of LDL by swine MNC was not altered by these concentrations of E_2 in plasma. Additionally, these data question the equivalency of circulating mononuclear cells and hepatocytes under hyperlipidemic conditions in this model, since LDL receptor mediated catabolism by MNC was not downregulated by high plasma cholesterol levels, but instead appeared to be driven by these levels.

Monocyte and platelet adhesion to PAEC *in vitro* was not significantly altered at any time point by E_2 treatment with the exception of an increase in monocyte adhesion at

4 weeks of high fat feeding in ovex + E_2 animals. Neither monocyte nor platelet adhesion were associated with plasma E_2 or lipid concentrations.

After 14 weeks of high feeding and hormonal treatment, LDL from ovex animals showed significantly enhanced susceptibility to oxidation *ex vivo* compared to LDL from animals exposed to physiologic levels of E_2. The greater oxidative resistance in E_2-exposed animals appeared to be mediated by enhanced levels of α-tocopherol carried in the LDL particle. This conservation of antioxidant in LDL may have been a function of circulating E_2, as physiologic concentrations of this hormone are not likely to be great enough to act directly as an antioxidant *in vivo*. Increases in PUFA and the P/S ratio in swine LDL was also associated with plasma E_2 concentrations. Nevertheless, propagation rate was inversely associated with plasma E_2 levels, leading to the conclusion that the association between lag time and propagation rate is a complex one, most likely involving more than a single common variable.

Although E_2 had no significant effect on plasma lipid values in hyperlipidemic hamsters, the trend at each time point in this study was for ovex animals to have the greatest area of aortic lesion involvement and E_2 replaced animals to have the least area of involvement, although statistical significance was observed only at the earliest time point (4 mo.). Lesion area was increased with time in all treatment groups reaching statistical significance only in the ovex + E_2 group at 18 months. Early lesions were typical of the fatty streaks observed in other animal models and humans, characterized by intact endothelium and internal elastic lamina which bracketed foam cells apparently derived from circulating monocytes. Later lesions at 18 months were atypical of previously described lesions with thickened intimal material which appeared to be proteoglycan and

elaboration of basement membrane. Areas of degenerative tissue existed in both the intima and media. These areas were characterized by remnants of lipid which was both membrane bound and free as droplets. Peripheral laminated membrane and secondary lysosomes were also present in many of these areas. Some of the degenerative regions were in the deeper layers of the media, surrounded by SMC without any sign of morphological perturbation or pathology. The lack of lesion progression in these animals may have been a function of the soy protein in the diet which has been shown to alter lipoprotein and sterol metabolism.

The study of these two animal models provided both cellular and molecular observations of effects of E_2 which further elucidated some of its potential protective mechanisms against CAD in women. These experiments were by no means comprehensive and gaps of knowledge must be filled in order to provide a clearer picture of E_2's protective role in retarding the development of atherogenic conditions in the plasma or in the arterial wall. Although accumulating evidence suggests a significant role for E_2 in atherosclerotic susceptibility, much of the knowledge of estrogens' mechanisms of action has been gained from studies using pharmacological concentrations of hormones in order to demonstrate an effect. Physiologic concentrations over the reproductive lifetime of a female may provide unique and/or more subtle, but sustained, protective effects. Additionally, relatively low level, chronic exposure to E_2 may have as yet undiscovered effects on atherogenic events, especially within the arterial wall, and other female anatomical, physiological, and biochemical factors may similarly impact upon the development of this multifactorial disease process. Clearly, a large body of research has

yet to be completed if one is to understand the potential uses of E_2 and related compounds in a preventative and/or therapeutic role in the management of vascular disease.

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APPENDIX

Table 1. Hamster Diet with 10% Coconut Oil and 0.1% Cholesterol.

Ingredient	gm	kcal
Soy Protein	200	800
DL-Methionine	3	0
Corn Starch	405	1,620
Maltodextrin 10*	100	400
Cellulose	80	0
Guar Gum	20	0
Corn Oil	20	180
Coconut Oil	100	900
Salt Mix S30003	60	0
Vitamin Mix V30002	10	40
Choline Bitartrate	2	0
Cholesterol	1	0
Total	1,001	3,940

*Maltodextrin 10 is enzyme digested corn starch with a dextrose equivalence of 10%. Assume physiologic fuel values for protein, carbohydrate, fat and fiber are 4, 4, 9 and 0 kcal/gm, respectively. On this basis, this diet has about 3.9 kcal/gm, with protein, carbohydrate and fat contributing 20, 52 and 28% of calories, respectively.

Table 2. Vitamin Mix for Purified Hamster Diet Use at 10 gm/kg Diet.

Ingredient	gm/kg	Amount in 10 gm of Vitamin Mix kcal
Vitamin A Palmitate 500,000 IU/gm	1.0	5,000 IU
Vitamin D_3 100,000 IU/gm	1.0	1,000 IU
Vitamin E Acetate 500 IU/gm	10.0	50 IU
Menadione Sodium Bisulfite 62.5% Menadione	0.3	2 mg
Biotin, 1.0%	2.0	0.2 mg
Cyanocobalamin, 0.1%	1.0	10 μg
Folic Acid	0.2	2 mg
Nicotinic Acid	2.0	20 mg
Calcium Pantothenate	2.0	20 mg
Pyridoxine HCl	1.0	10 mg
Riboflavin	0.5	5 mg
Thiamin HCl	0.5	5 mg
Sucrose	978.5	
Total	1,000.0	

Formulated by E.A. Ulman, Ph.D., Research Diets, Inc., October 1987.

Table 3. Plasma Lipids of Yucatan Miniature Swine During Pretreatment, Low Fat Diet Period

Hormonal Treatment		Pretreatment Low Fat
INTACT	TC(mg/dl)*	100 ± 5
	HDL-C	46 ± 4
	VLDL-C + LDL-C	54 ± 4
	TG	65 ± 6
	TC/HDL-C	2.20 ± .11
OVEX	TC	96 ± 4
	HDL-C	44 ± 4
	VLDL-C + LDL-C	52 ± 2
	TG	63 ± 11
	TC/HDL-C	2.21 ± .12
OVEX + E_2	TC	98 ± 9
	HDL-C	39 ± 5
	VLDL-C + LDL-C	58 ± 4
	TG	62 ± 5
	TC/HDL-C	2.48 ± .08

* Plasma was prepared from fasted animals during the low fat feeding period. Total cholesterol (TC), VLDL and LDL cholesterol (VLDL-C + LDL-C) HDL cholesterol (HDL-C) and triglycerides (TG) were determined as described in Methods. Subsequently, animals would undergo sham ovariectomy (intact), ovariectomy (ovex), or ovariectomy with 17 β-estradiol implants (ovex + E_2) and consume an atherogenic diet for 12 weeks. Values represent the mean ± SEM with 5 animals/group. There were no statistically significant differences in pretreatment plasma lipid concentrations when data were assigned to and analyzed by subsequent treatment groups.

Table 4. Plasma Lipids of Yucatan Miniature Swine During Experimental Dietary and Hormonal Treatments.

		Time		
Hormonal Treatment		4 wks High Fat	8 wks High Fat	12 wks High Fat
INTACT	TC(mg/dl)	474 ± 90	388 ± 44	384 ± 82
	HDL-C	120 ± 11	123 ± 11	123 ± 13
	VLDL-C + LDL-C	354 ± 80	261 ± 39	264 ± 73
	TG	46 ± 6	51 ± 4	40 ± 3
	TC/HDL-C	3.85 ± .36	3.14 ± .36	3.11 ± .42
OVEX	TC	546 ± 91	486 ± 88	405 ± 57
	HDL-C	143 ± 11	132 ± 20	133 ± 8
	VLDL-C + LDL-C	402 ± 96	354 ± 90	271 ± 51
	TG	48 ± 5	42 ± 7	41 ± 3
	TC/HDL-C	3.93 ± .79	4.03 ± 1.05	3.06 ± .29
OVEX + E_2	TC	459 ± 99	452 ± 106	418 ± 89
	HDL-C	127 ± 14	123 ± 15	122 ± 14
	VLDL-C + LDL-C	332 ± 89	328 ± 99	296 ± 80
	TG	42 ± 4	48 ± 4	58 ± 10
	TC/HDL-C	3.55 ± .54	3.64 ± .75	3.37 ± .52

Plasma was prepared from fasted animals that had undergone sham ovariectomy (intact), ovariectomy (ovex), or ovariectomy with 17 β-estradiol implants (ovex + E_2) and had consumed a low fat diet, then an atherogenic diet for 12 weeks. Total cholesterol (TC), VLDL and LDL cholesterol (VLDL-C + LDL-C) HDL cholesterol (HDL-C) and triglycerides (TG) were determined as described in Methods. Values represent the mean ± SEM with 5 animals/group. There were no statistically significant differences among treatment groups at any time point or within a treatment group over time.

Table 5. Plasma 17 β-Estradiol in Swine During the High Fat Diet.

Group	17 β-estradiol (pmol/L)* 4 wk	8 wk	12 wk
Intact	62 ± 17^{a}	68 ± 20^{a}	54 ± 19^{a}
Ovex	12 ± 7	10 ± 8	15 ± 7
Ovex + E_2	274 ± 42^{b}	289 ± 47^{b}	250 ± 32^{b}

*Plasma was prepared from animals which had undergone sham surgery (intact), ovariectomy (ovex), or ovariectomy with 17 β-estradiol implants (ovex + E_2) and had been fed an atherogenic diet for 12 weeks. Plasma was extracted twice with diethyl ether and 17 β-estradiol was measured by radioimmunoassay as previously described by Korenman *et al.* (212). Values represent the mean ± SEM with n = 5 per group. [a]Significantly different from ovex ($p \leq 0.05$); [b]Significantly different from intact and ovex ($p \leq 0.001$).

Table 6. **Uptake and Degradation of ^{125}I-LDL by Swine Mononuclear Cells *Ex Vivo***

Time	Hormonal Treatment	Total	Receptor-mediated	Non - receptor-mediated
Pretreatment	INTACT	56.0 ± 13.1	39.2 ± 13.9	6.9 ± 7.4
Low fat diet	OVEX	68.7 ± 14.7	45.0 ± 10.4	23.7 ± 9.6
	$OVEX_2$	64.3 ± 8.9	44.1 ± 12.5	20.3 ± 7.7
	INTACT	214.4 ± 34.0[a]	201.8 ± 32.1[b]	12.6 ± 3.6
4 wk high fat	OVEX	156.5 ± 33.1	151.7 ± 32.4[b]	5.1 ± 0.8
	OVEX + E_2	164.6 ± 30.5	159.2 ± 30.4[b]	5.4 ± 1.5
	INTACT	172.7 ± 25.9[b]	131.5 ± 29.0	41.2 ± 7.7[c]
8 wk high fat	OVEX	160.7 ± 40.6	128.7 ± 34.8	32.1 ± 9.0[c]
	OVEX + E_2	157.8 ± 28.6	134.3 ± 28.8	23.5 ± 7.8
	INTACT	120.7 ± 12.8	100.6 ± 13.0[c]	20.1 ± 7.8
12 wk high fat	OVEX	110.3 ± 25.8	94.6 ± 22.5	15.7 ± 6.5
	OVEX + E_2	100.3 ± 16.2	79.4 ± 13.2	21.0 ± 5.9

MNC were isolated from fasted animals that had consumed a low fat diet, then had undergone hormonal treatment described in Methods and were switched to an atherogenic diet for 12 weeks. Cells were incubated with ^{125}I-LDL from sexually intact females and total, receptor-mediated, and non-receptor-mediated uptake and degradation were determined. Values represent the mean ± SEM of ng LDL protein degraded/10^6 cells/4h with 5 animals/group. [a]Significantly different from low fat, $p \leq 0.001$. [b]Significantly different from low fat, $p \leq 0.05$. [c]Significantly different from 4 wk value, $p \leq 0.05$.

Table 7. Correlations Between LDL Uptake and Degradation, Plasma LDL-C, and Plasma E_2 Concentrations.

Analysis	r	P
Total x receptor	.972	≤.001
Total x non-receptor	.147	.262
Total x LDL	.540	≤.001
Total x E_2	-.026	.842
Receptor x LDL	.528	≤.001
Receptor x E_2	-.013	.919
Non-receptor x LDL	.064	.627
Non-receptor x E_2	-.057	.668

Pearson product moment correlations were calculated between LDL catabolic rates and plasma lipid and E_2 concentrations. Data from all four time points (one low fat and three high fat) were pooled to yield n = 60.

Table 8. Correlations Between Monocyte and Platelet Adhesion, Plasma LDL-C, HDL-C, and E_2 Concentrations.

Analysis	r	P
Monocyte x LDL-C	-.179	.171
Monocyte x HDL-C	.110	.381
Monocyte x E_2	-.204	.098
Platelet x LDL-C	.088	.504
Platelet x HDL-C	-.048	.714
Platelet x E_2	-.064	.625

Pearson product moment correlations were calculated between monocyte or platelet adhesion to PAEC and plasma lipid or E_2 concentrations. Data from low fat and high fat time points were pooled to yield n = 60.

Table 9. Plasma Lipid Profiles in Hypercholesterolemic Swine After 14 Weeks of High Fat Feeding.

Group	TC (mg/dl)	VLDL-C + LDL-C (mg/dl)	HDL-C (mg/dl)	TG (mg/dl)
Intact	395 ± 93	275 ± 83	120 ± 14	40 ± 4
Ovex	380 ± 57	248 ± 51	132 ± 8	43 ± 3
Ovex + E_2	353 ± 58	234 ± 45	119 ± 16	56 ± 11

Plasma was prepared from hormonally treated animals that had consumed an atherogenic diet for 14 weeks. Plasma lipids were determined as described in Methods. Values represent the mean ± SEM with 4 animals/group. There were no statistically significant differences in any of the plasma lipids among groups.

Table 10. Plasma 17 β-Estradiol Levels After 14 Weeks of Hormonal and Dietary Treatment.

Group	17 β-estradiol (pmol/L)
Intact	60 ± 15*a*
Ovex	14 ± 6
Ovex + E_2	264 ± 35*b*

Plasma was prepared from experimentally treated animals that had been fed an atherogenic diet for 14 weeks. Plasma was extracted twice with diethyl ether and 17 β-estradiol was measured by radioimmunoassay as previously described by Korenman *et al.* (212). Values represent the mean ± SEM with n = 4 per group. *a*Significantly different from ovex ($p \leq 0.05$); *b*Significantly different from intact and ovex ($p \leq 0.001$).

Table 11. Effects of Hormonal Treatment on Susceptibility of Isolated LDL to Oxidation.

Group	Lag time (min)	Maximum propagation rate (nmol conjugated dienes/ mg LDL protein/min)	Total dienes (nmol/mg LDL protein)
Intact	85 ± 15*a*	8.11 ± 1.55*a*	681 ± 46
Ovex	35 ± 12	13.53 ± 0.02	827 ± 142
Ovex + E_2	105 ± 20*a*	8.79 ± 1.27*a*	681 ± 38

LDL was isolated from hormonally treated animals fed an atherogenic diet for 14 weeks. The LDL fraction d = 1.019-1.040 was collected and assayed for susceptibility to oxidation *ex vivo* by aqueous peroxyl radicals as described in Methods. Lag phase duration, rate of conjugated diene formation and total dienes formed were measured from absorbance plots generated for each sample. Values represent the mean ± SEM of data from 4 animals/group. *a*Significantly different from ovex, $p \leq 0.05$.

Table 12. Effects of Hormonal Treatment on α-Tocopherol Content in LDL.

Group	α-tocopherol (nmol/mg LDL protein)
Intact	1.96 ± 0.13^a
Ovex	1.11 ± 0.15
Ovex + E_2	1.59 ± 0.09^a

Alpha-tocopherol was extracted from LDL isolated from experimental animals on an atherogenic diet for 14 weeks. Antioxidant levels were measured by reverse-phase HPLC as described in Methods. Values represent the mean ± SEM with n = 4/group.
^{a}Significantly different from ovex $p \leq 0.05$.

Table 13. Fatty Acid Composition of LDL from Hypercholesterolemic Swine (Expressed as Percentage of Total Fatty Acids).

Fatty Acid	Hormonal Treatment		
	Intact	Ovex	Ovex + E_2
12:0	0.45 ± 0.05	0.34 ± 0.03	0.37 ± 0.06
14:0	2.30 ± 0.18	2.14 ± 0.11	2.09 ± 0.10
16:0	16.69 ± 0.41	17.00 ± 0.27	15.91 ± 0.28
16:1 n-7	1.35 ± 0.14	1.26 ± 0.07	1.18 ± 0.08
18:0	23.44 ± 1.04	23.46 ± 0.35	22.24 ± 0.67
18:1 n-9	11.20 ± 0.31	10.86 ± 0.15	10.83 ± 0.22
18:2 n-6	29.84 ± 1.35	31.71 ± 0.69	33.04 ± 1.00
18:3 n-6	0.89 ± 0.10	0.81 ± 0.14	1.14 ± 0.06
18:3 n-3	0.87 ± 0.24	0.57 ± 0.10	0.85 ± 0.06
20:3 n-6	1.23 ± 0.19	1.33 ± 0.15	1.32 ± 0.04
20:4 n-6	9.23 ± 0.64	8.54 ± 0.40	8.26 ± 0.27
20:5 n-3	0.69 ± 0.10	0.53 ± 0.14	0.72 ± 0.19
22:6 n-3	1.83 ± 0.35	1.47 ± 0.22	2.07 ± 0.28
Total saturates (S)	42.88 ± 0.69	42.94 ± 0.46	40.61 ± 0.94
Total polyunsaturates (P)	44.58 ± 0.29	44.95 ± 0.28	47.38 ± 0.91*a*
P/S	1.04 ± 0.02	1.05 ± 0.02	1.17 ± 0.05*a*
18:2/18:1	2.66 ± 0.06*b*	2.92 ± 0.05	3.05 ± 0.07

Total fatty acids were extracted from LDL from swine fed an atherogenic diet for 14 weeks. Swine had been surgically treated to provide 3 hormonal groups: sham ovariectomy (intact), ovariectomy (ovex), and ovariectomy with 17 β-estradiol implants (ovex + E_2). Fatty acids were converted to their methyl esters and analyzed by gas chromatography as described in Methods. Values represent the mean ± SEM with an n = 4 for each hormonal treatment. *a*Significantly different from intact and ovex, $p \leq 0.05$. *b*Significantly different from ovex and ovex + E_2, $p \leq 0.05$.

Table 14. Correlations Between LDL Oxidation *Ex Vivo* and Plasma LDL-C, E_2, and LDL α-Tocopherol Levels and Between Plasma E_2 and LDL Oxidation and α-Tocopherol Levels.

Analysis	r	P
lag phase x α-tocopherol	.604[a]	.037
lag phase x E_2	.474	.119
lag phase x propagation rate	- .818[a]	.001
a-tocopherol x propagation rate	- .556	.061
E_2 x α-tocopherol	.458	.134
E_2 x propagation rate	- .528	.077
E_2 x PUFA	.627[a]	.029
E_2 x P/S	.642[a]	.024
lag phase x PUFA	.189	.557
propagation rate x PUFA	- .150	.641
total dienes x PUFA	- .162	.616

Pearson product-moment correlation coefficients were calculated to measure the association between susceptibility of LDL to oxidation (lag phase, propagation rate, and total dienes formed) and the plasma and LDL constituents measured in this study. Data from the three treatment groups were pooled to yield n = 12. [a]Statistical significance was accepted at $p \leq 0.05$.

Table 15. Plasma Lipids of Female Hamsters During Experimental Dietary and Hormonal Treatment

Hormonal Treatment		Time		
		4 months High Fat	9 months High Fat	18 months High Fat
INTACT	TC(mg/dl)	228 ± 12	279 ± 25	266 ± 23
	HDL-C	117 ± 3	131 ± 10	139 ± 9
	VLDL-C + LDL-C	111 ± 10	147 ± 17	128 ± 14
	TG	321 ± 45	492 ± 75	407 ± 66
	TC/HDL-C	1.95 ± .08	2.15 ± .09	1.90 ± .05
OVEX	TC	334 ± 15[a]	293 ± 15	300 ± 19
	HDL-C	167 ± 5[a]	145 ± 5	155 ± 8
	VLDL-C + LDL-C	162 ± 12[a]	149 ± 12	146 ± 14
	TG	517 ± 51[b]	392 ± 63	497 ± 45
	TC/HDL-C	2.00 ± .08	2.02 ± .06	1.94 ± .08
OVEX + E_2	TC	246 ± 16	261 ± 19	259 ± 22
	HDL-C	138 ± 5[c]	124 ± 9	141 ± 6
	VLDL-C + LDL-C	115 ± 12	137 ± 12	119 ± 17
	TG	349 ± 60	300 ± 54	279 ± 21[e]
	TC/HDL-C	1.78 ± .02	2.11 ± .09[d]	1.83 ± .08

Plasma was prepared from fasted animals that had undergone hormonal treatments described in Methods and had consumed a high fat, high cholesterol diet for up to 18 months. Plasma lipids were determined as described in Methods. Values represent the mean ± SEM with 8 animals/group at each point. [a]Significantly different from intact and ovex + E_2 at 4 mo., $p \leq 0.01$. [b]Significantly different from intact and ovex + E_2 at 4 mo., $p \leq 0.05$. [c]Significantly different from intact at 4 mo., $p \leq 0.01$. [d]Significantly different from ovex + E_2 at 4 mo., $p \leq 0.05$. [e]Significantly different from ovex at 18 mo., $p \leq 0.01$.

Table 16. Plasma 17 β-Estradiol in Hamsters During the High Fat Diet.

Group	17 β-estradiol (pg/ml) 4 mo	9 mo	18 mo
Intact	121 ± 37^a	114 ± 36^a	97 ± 22^a
Ovex	18 ± 3	20 ± 3	18 ± 3
Ovex + E_2	424 ± 29^b	284 ± 28^b	302 ± 29^b

Plasma was prepared from animals which had undergone sham surgery (intact), ovariectomy (ovex), or ovariectomy with 17 β-estradiol implants (ovex + E_2) and had been fed an atherogenic diet for up to 18 months. Plasma was extracted twice with diethyl ether and 17 β-estradiol was measured by radioimmunoassay as previously described by Korenman *et al.* (212). Values represent the mean ± SEM with n = 8 per group for each time point. [a]Significantly different from ovex ($p \leq 0.05$); [b]Significantly different from intact and ovex ($p \leq 0.001$).

Table 17. Pearson Correlations Between Hamster Aortic Lesion Area, Plasma Lipids, and Plasma E_2 Concentrations.

Analysis	4 months r	4 months P	9 months r	9 months P	18 months r	18 months P
AREA x TC	.175	.413	.486[a]	.016	.451[a]	.027
AREA x LDL-C	.218	.306	.398	.051	.437[a]	.033
AREA x HDL-C	.120	.577	.531[a]	.008	.405[a]	.050
AREA x TG	.416[a]	.043	.199	.351	.355	.089
AREA x TC/HDL-C	.200	.348	-.054	.804	.359	.085
AREA x E_2	-.115	.337	-.055	.801	.123	.567
TC x E_2	-.292	.174	.220	.297	.199	.351
LDL-C x E_2	-.204	.186	.341	.103	.206	.183
HDL-C x E_2	-.326	.105	.302	.139	.316	.114
TG x E_2	.204	.186	.216	.304	.344	.099

For each time point, the mean plasma lipid and E_2 concentrations over the course of the study were used in analysis for each animal. Lesion area was statistically associated with plasma TG concentrations after 4 months of consuming a high fat, high cholesterol diet, with HDL-C after 9 months, and with TC, LDL-C, and HDL-C after 18 months.
[a]Designates significant association between variables (n = 8/time point).

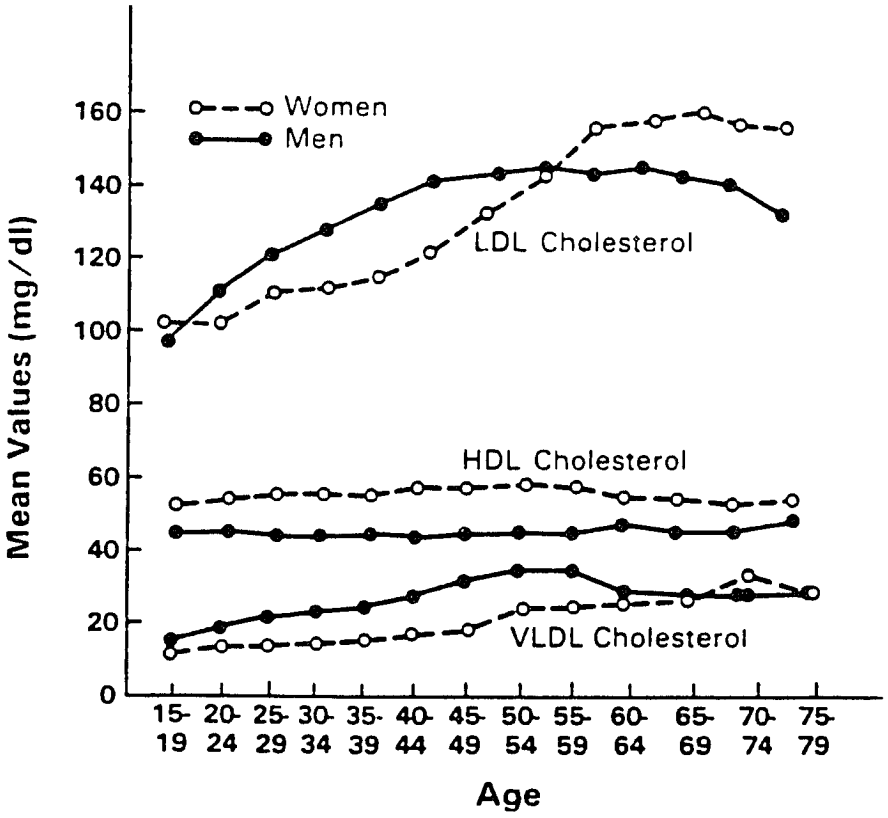

Figure 1. Age trends in lipoprotein-cholesterol fractions. Framingham Study. Reprinted with permission from Nutrition Reviews, February, 1988 copyright (c) 1988 Springer-Verlag New York Inc., New York, NY. All rights reserved.

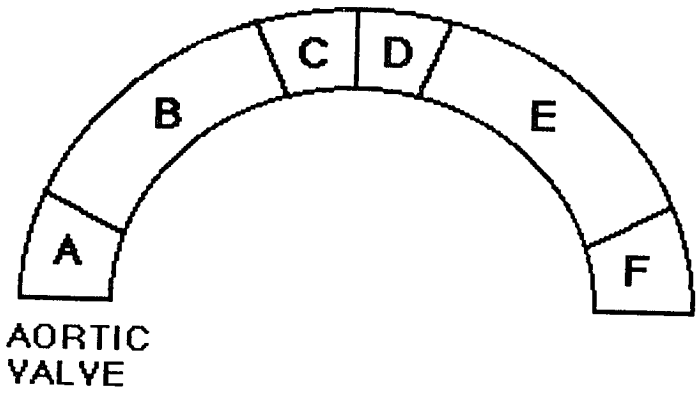

Figure 2. Area of hamster aortic arch analyzed for lesion development. A 1 cm length of aortic arch beginning just distal to the aortic valve was subdivided for analysis. A, C, and F were 1 mm segments prepared for histological examination. B and E were 3 mm segments prepared for *en face* measurement of ORO lesion area. Segment D was a 1 mm segment analyzed by TEM.

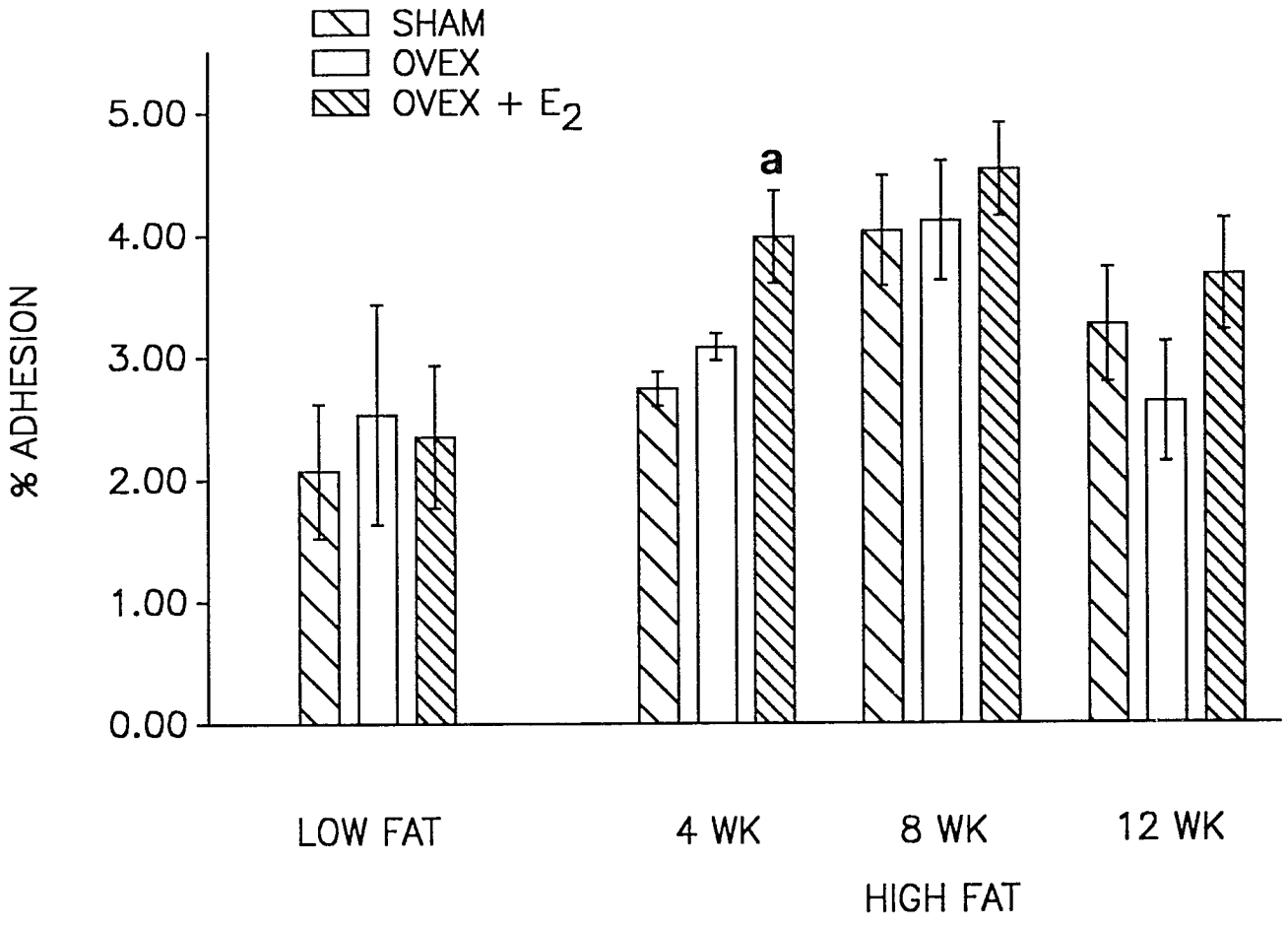

Figure 3. Effect of E_2 on swine monocyte adhesion to PAEC *in vitro* during low fat and high fat diets. These hormonal treatment groups of swine were generated as described in Methods. Monocytes were isolated during the low fat feeding and at 4, 8, and 12 weeks after high fat feeding and hormonal treatment. Values represent the mean ± SEM with 5 animals/treatment group at each time point. The value for each animal was calculated from 4 replicates from each assay. [a]Significantly different from sham and ovex values at 4 weeks ($p \leq 0.05$).

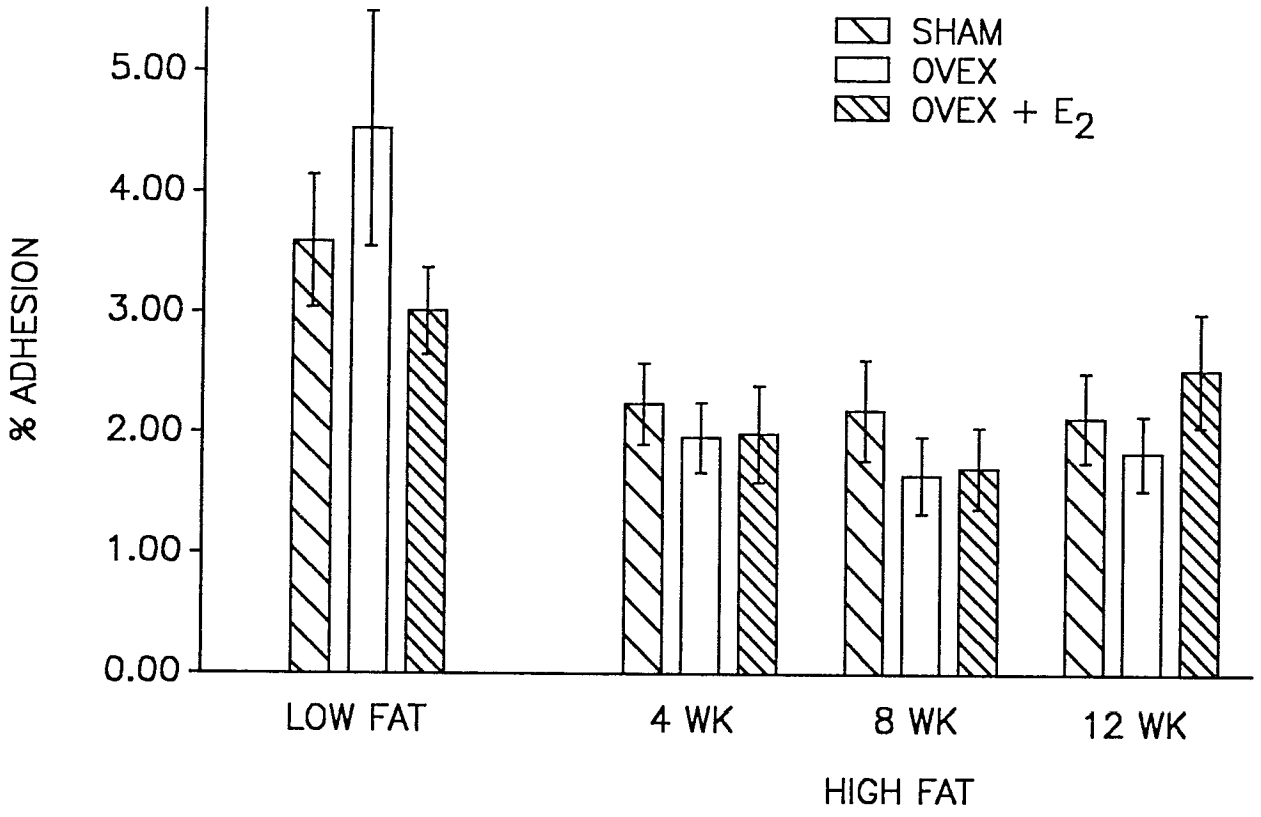

Figure 4. Effect of E_2 on swine platelet adhesion to PAEC *in vitro* during low fat and high fat diets. These hormonal treatment groups of swine were generated as described in Methods. Platelets were isolated during the low fat feeding and at 4, 8, and 12 weeks after high fat feeding and hormonal treatment. Values represent the mean ± SEM with 5 animals/treatment group at each time point. The value for each animal was calculated from 4 replicates from each assay. There were no significant differences among treatment groups at any time point.

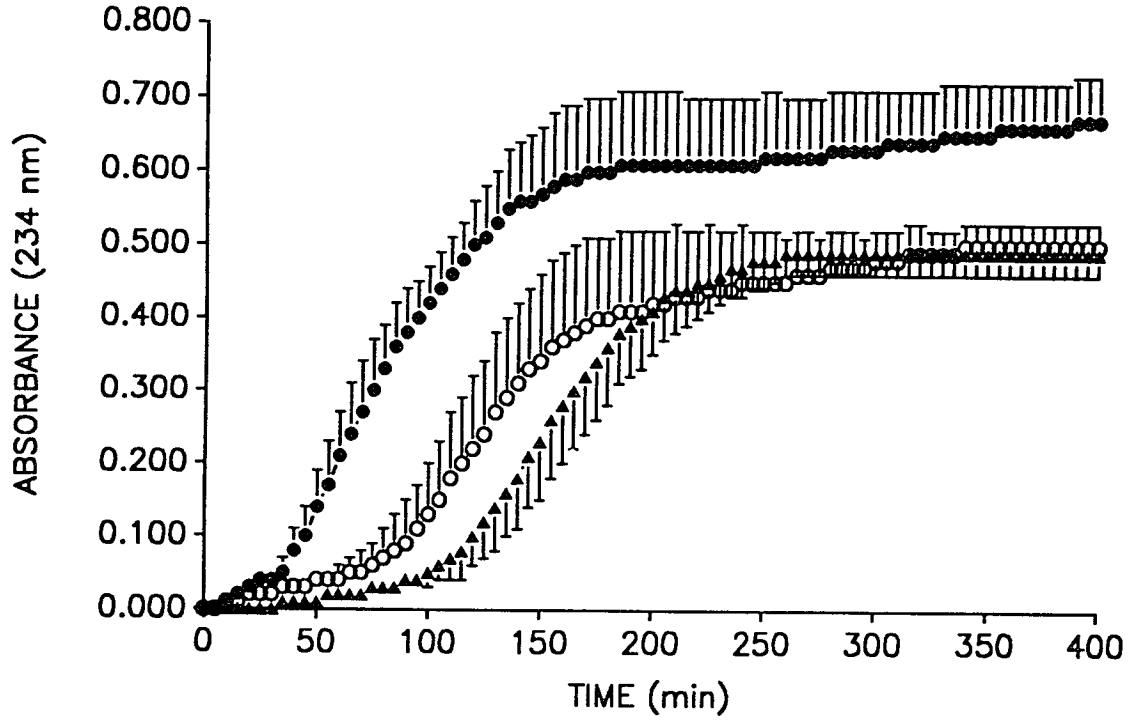

Figure 5. Effect of 17 beta-estradiol on the oxidative resistance of LDL. Oxidation curves were generated by continuous monitoring of conjugated diene formation at 234 nm in LDL samples isolated from female swine which had undergone sham surgery (O), ovariectomy (●), or ovariectomy with 17 β-estradiol implants (▲). Ovariectomized animals without estrogen replacement demonstrated a significantly greater susceptibility to oxidation characterized by a shorter lag phase, a greater rate of diene propagation, and a greater amount of total dienes formed (all parameters $p \leq 0.05$). Data points represent the mean absorbance of four samples per treatment. Error bars indicate SEM.

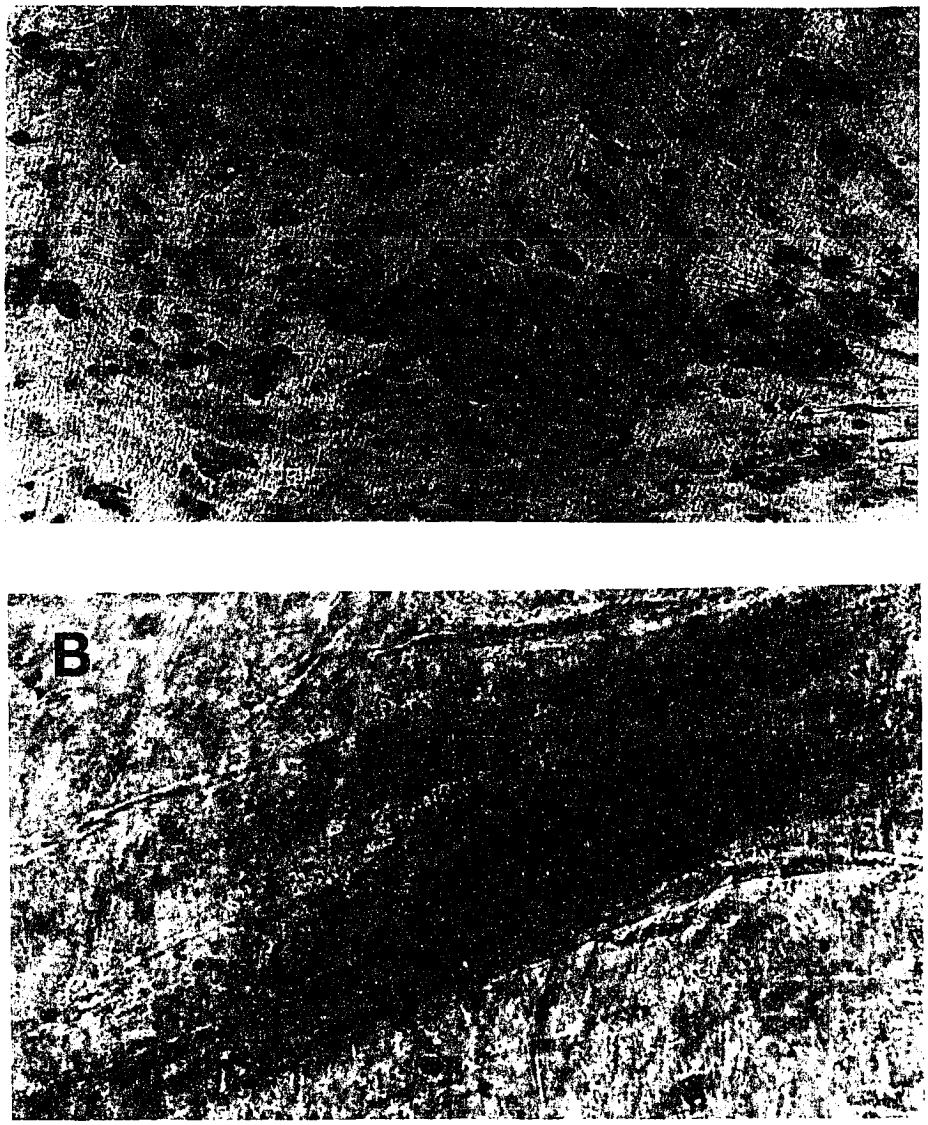

Figure 6. *En face* preparation of hamster aortic fatty streak lesion. After nine months of high fat feeding, hamster aortic arch was perfusion fixed, then stained with ORO and counterstained with hematoxylin. *A.* A fatty streak developed along, and was restricted to, the inner curvature of the aortic arch. ORO-positive material wsa primarily intracellular and concentrated within subendothelial, intimal foam cells (arrowheads). Photographed at 200 x. *B.* Aortas also demonstrated focal ORO-positive droplets of smaller diameter than those in typical fatty streaks. These smaller droplets appeared to be extracellular and scattered throughout the intima of the inner curvature, as well as deeper medial layers of the vessel wall. Photographed at 400 x.

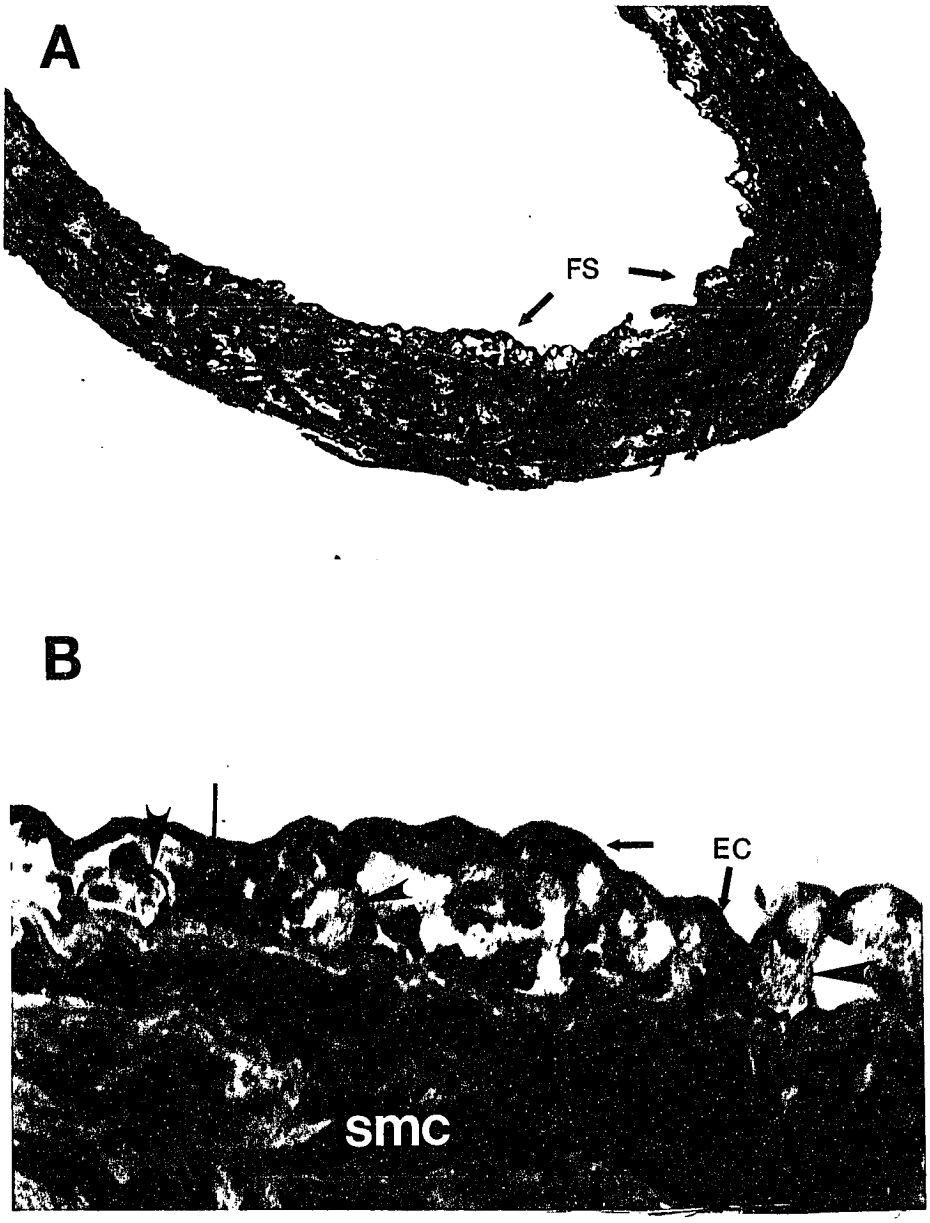

Figure 7. Histological cross-section of hamster aortic lesion. *A.* At nine months of high fat feeding, fatty streak (FS) development was restricted to the inner curvature of the aortic arch and protruded slightly into the vessel lumen. Six micron section stained with Masson's trichrome. Cytoplasm-red, connective tissue-blue, nuclei-brown/black. Photographed at 100 x. *B.* Higher magnification (400 x) of fatty streak. Macrophage-derived foam cells (arrowheads) are stacked 3-4 cells deep between an intact endothelium and internal elastic lamina (long arrow), separating the intima from alternating bands of SMC and connective tissue (stained blue). Masson's trichrome. smc, smooth muscle cells; EC, endothelial cells.

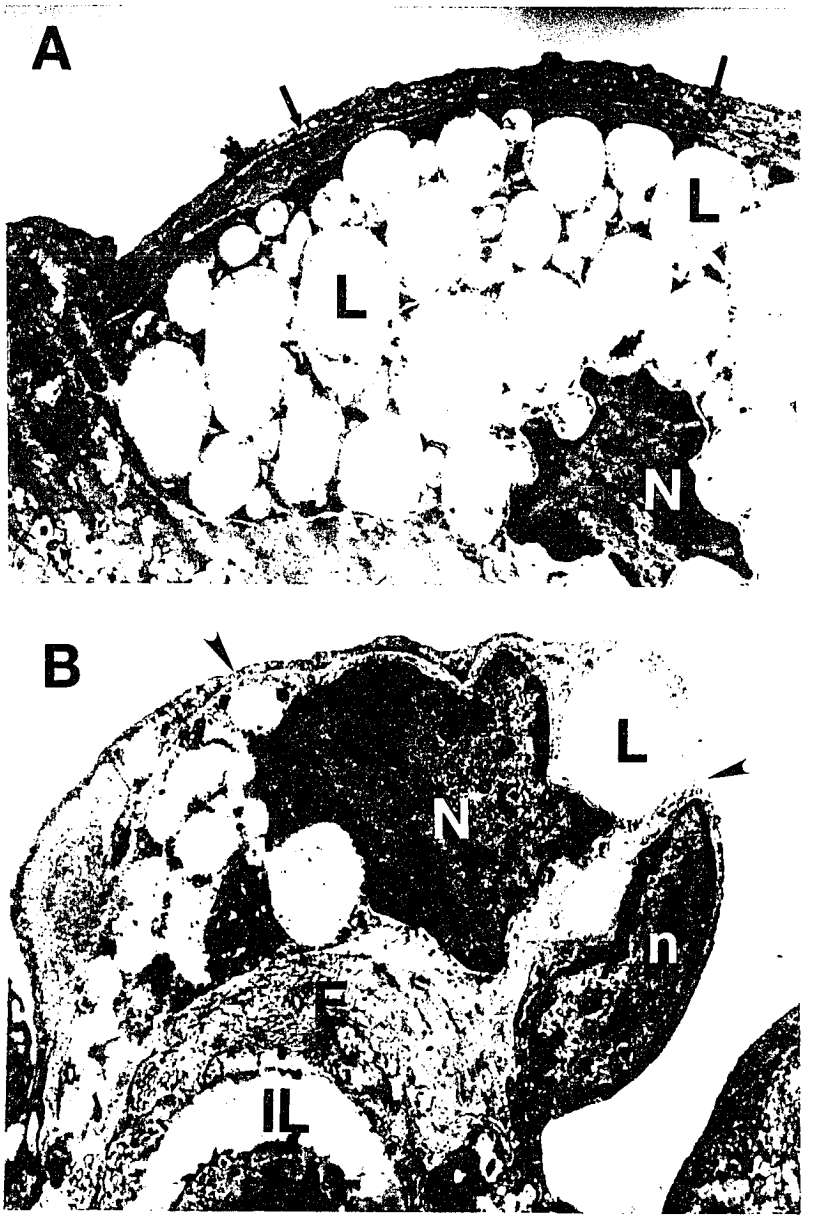

Figure 8. TEM of macrophage foam cells in hamster fatty streak lesion. *A.* Lipid deposits (L) were the dominant cytoplasmic feature surrounding the macrophage nucleus (N). A thin extension of endothelial cytoplasm (arrows) stretched over the foam cell maintaining an intact endothelium. Magnification 10,000 x. *B.* Cytoplasmic extensions (arrowheads) of an endothelial cell covered a lipid filled foam cell in a shallow portion of intima. N, foam cell nucleus; n, endothelial cell nucleus; L, lipid deposits; E, extracellular matrix; IL, internal elastic lamina. Magnification 10,000 x.

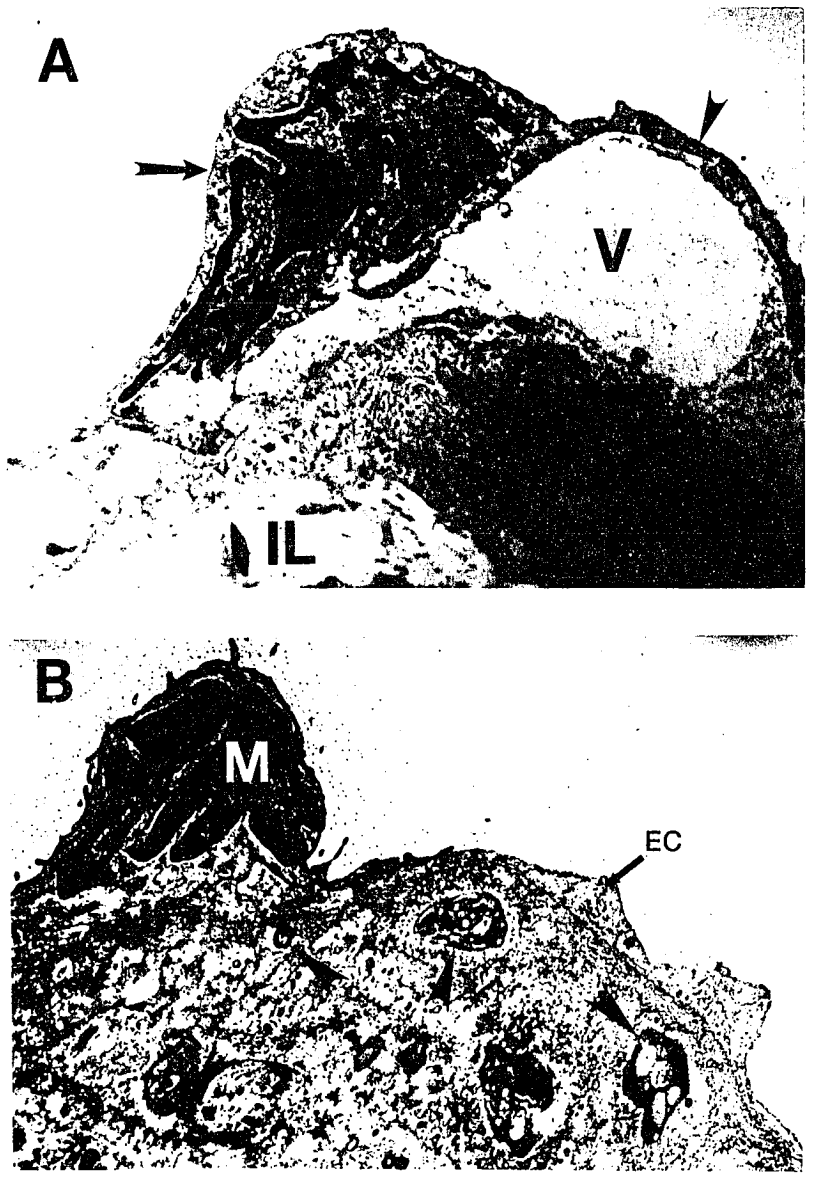

Figure 9. TEM of mononuclear cells adherent to hamster aortic endothelium. *A.* After 4 months of high fat feeding, mononuclear cells (arrow) were observed attached to endothelium (arrowhead). Attached cell was slightly flattened, perhaps migrating by diapedesis to the left in this micrograph. The intimal region appeared to be expanded with extracellular matrix protein (E) and a large extracellular vacuole (V) beneath the endothelium (see Figure 11A, also). IL, internal elastic lamina. Magnification 9,000 x. *B.* At 18 months of high fat feeding, a mononuclear cell was adherent to the endothelium which covered an area of cell debris and necrosis. Numerous vacuoles and fragments of cells appear in the subendothelial space (arrowheads). M, mononuclear cell; EC, endothelial cell cytoplasm. Magnification 6,000 x.

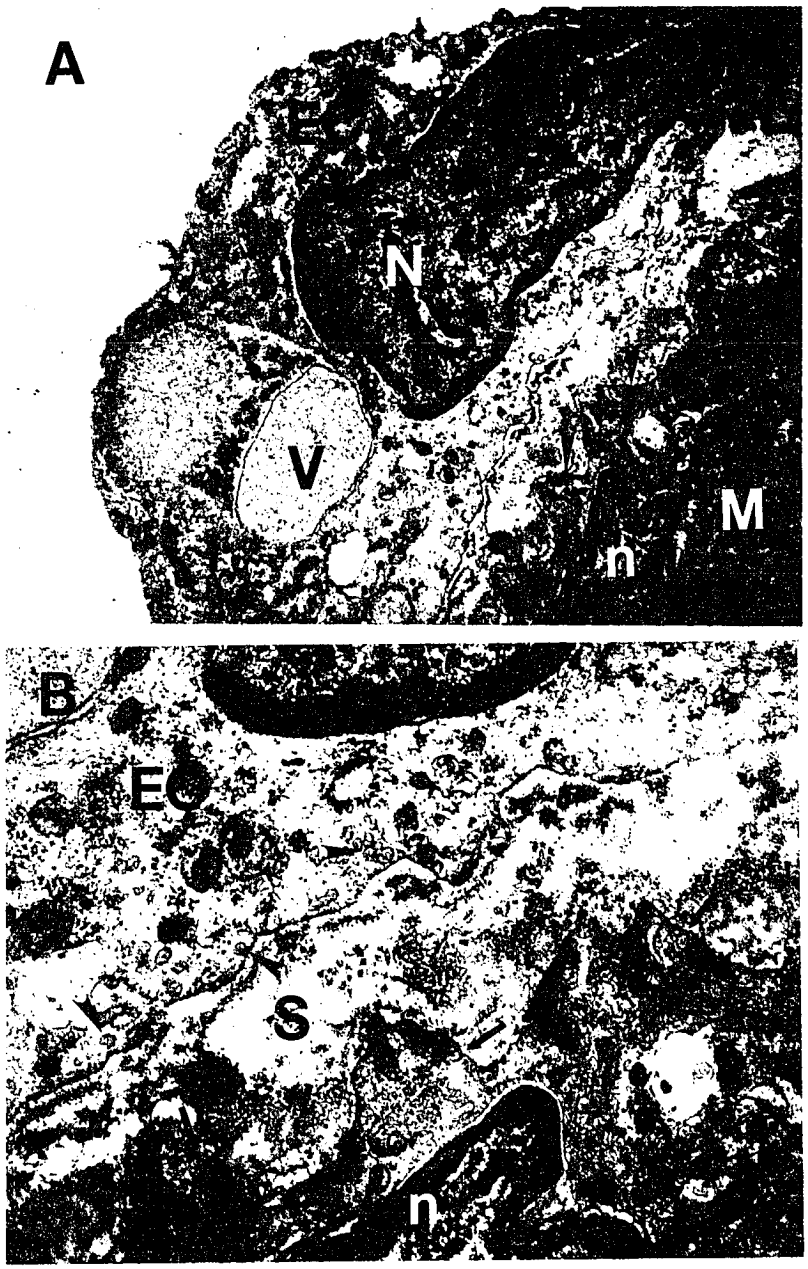

Figure 10. Intimal smooth muscle in aortic lesion. *A.* TEM of hamster lesion area after 9 months on high fat diet shows an intimal smooth muscle cell (M) adjacent to the endothelium (EC). SMC is characterized by dense bodies (arrowheads) and narrow, elongated nucleus (n). EC contains two large vacuoles (V), which appear to contain material proteinaceous in nature and be membrane bound. N, endothelial nucleus. Magnification 9,500 x. *B.* Detail of *A.* above. Ablumenal surface of endothelial cell (EC) contains numerous pinocytotic vesicles (arrowheads) while opposing membrane of smooth muscle cell shows fewer vesicles (arrows). Extracellular space (S) is characterized by the presence of proteinaceous material. n, smooth muscle nucleus. Magnification 20,000 x.

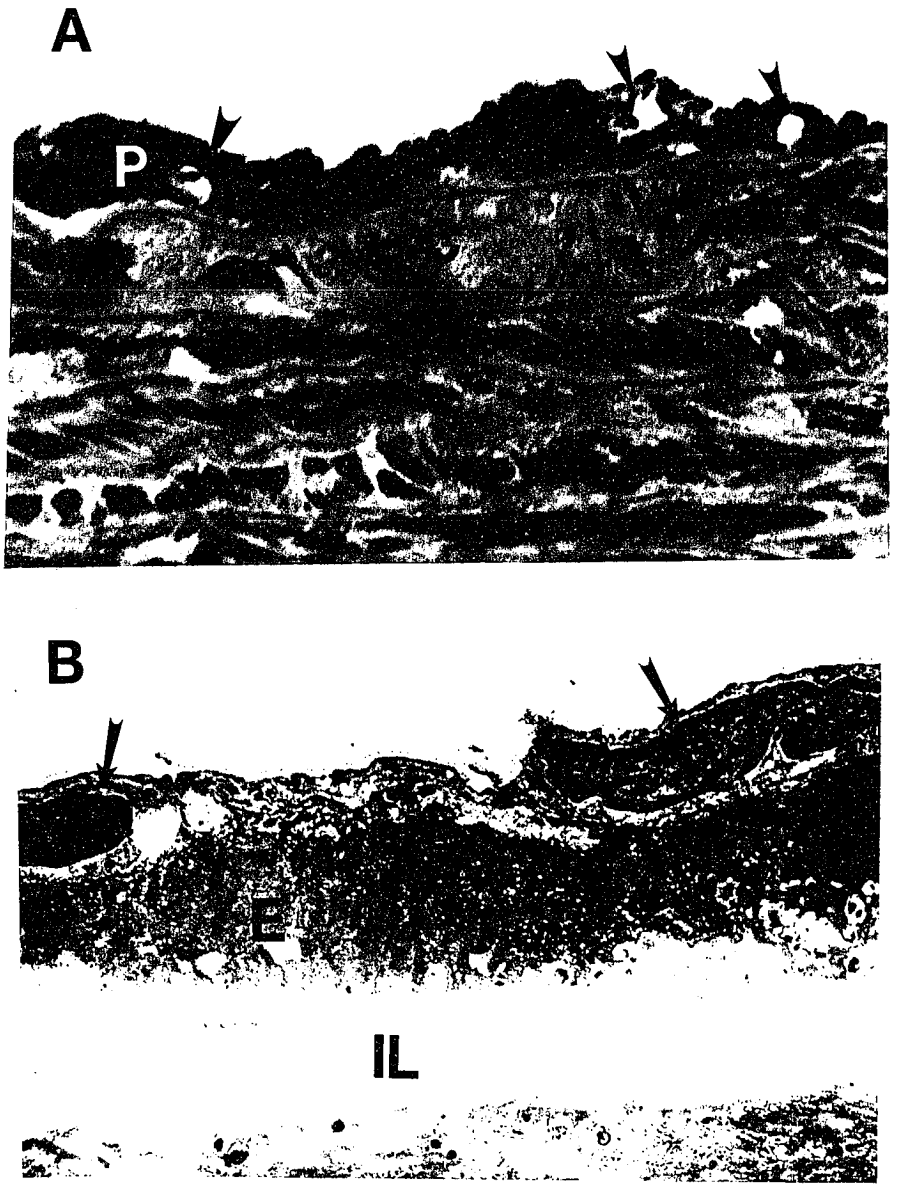

Figure 11. Lipid-poor lesions in hamster aortic arch. *A.* In contrast to fatty streak lesions, some vessels showed an intimal thickening of proteinaceous material (P) that stained red with Masson's trichrome. Occasional lipid droplets or foam cells (arrowheads) punctuate the thickened, intimal material. The internal elastic lamina (arrow) appears to be narrowed in some areas, primarily those adjacent to expansion of connective tissue (C) which has replaced smooth muscle in the lamellae of the arterial media. Photographed at 400 x. *B.* TEM of arterial lesion similar to that above. Endothelial nuclei (arrows) are above an acellular area of thickened, extracellular matrix (E) resting on the internal elastic lamina (IL). Magnification 5,000 x.

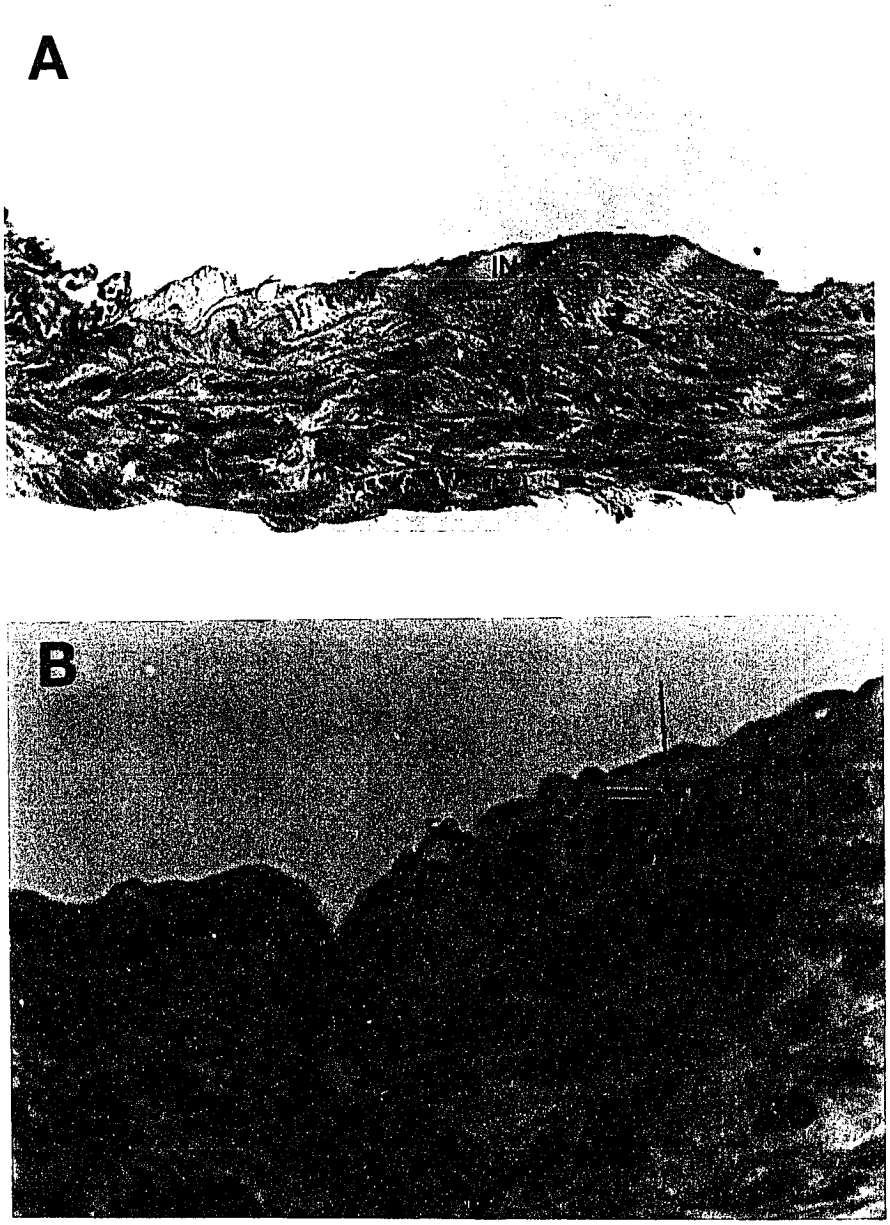

Figure 12. Medial necrosis associated with intimal expansion. *A.* A large region of necrotic debris (arrow) exists in medial layer of vessel wall. This region is located directly beneath an area of intima (IN) which appears thickened and acellular in nature. Hematoxylin and eosin staining. Photographed at 100 x. *B.* Necrotic debris (arrowheads) again appear immediately beneath the internal elastic lamina (long arrow) in an area of expanded extracellular material in the subendothelial space. Occasional foam cells (FC) appear near the lumenal surface of the vessel. Hematoxylin and eosin staining. Photographed at 400 x.

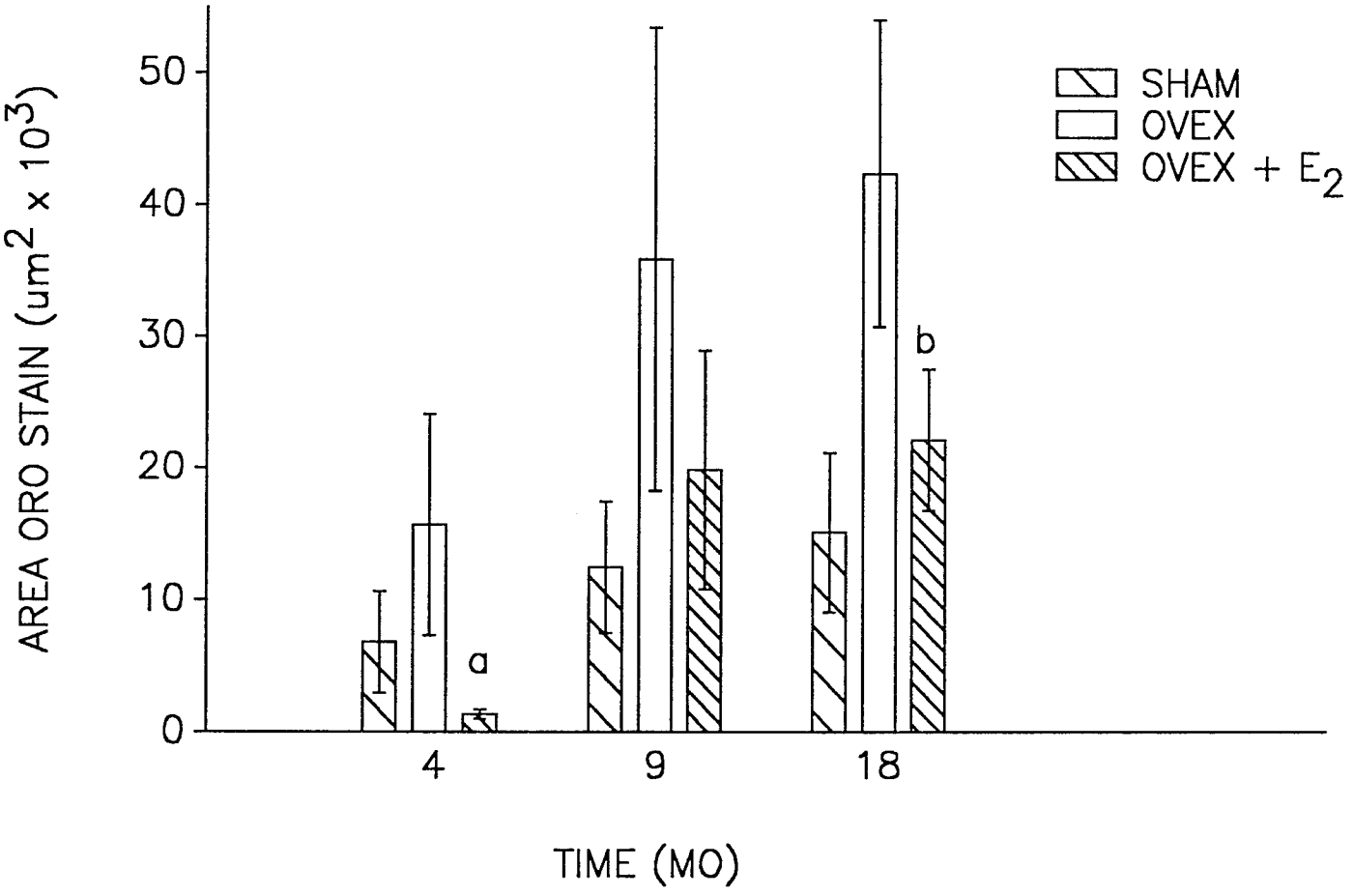

Figure 13. Effect of E_2 on hamster aortic fatty lesion areas over 18 months. Hamsters were surgically treated to yield the three hormonal conditions described in Methods and were fed a high fat, high cholesterol purified diet for up to 18 months. *En face* preparations were stained with ORO and quantified by image analysis. Values represent mean SEM with n = 8/treatment group at each time point. [a]Statistically different from ovex at 4 months ($p \leq 0.05$). [b]Statistically different from ovex + E_2 at 4 months ($p \leq 0.05$).