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Cardiovascular disease-related risk factors in perimenopausal women

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Cardiovascular disease-related risk factors in perimenopausal women

by

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This is to certify that the Master's thesis of

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Signatures have been redacted for privacy

DEDICATION

I would like to dedicate this thesis to my parents, Joe and Patricia Dent, for their love and support throughout my college career. Their encouragement and nurturing has enabled me to appreciate the value of education and has brought out in me a passion for excellence in everything I pursue.

I would also like to dedicate this thesis to Christopher Joseph Hencyk since it was through his everyday understanding and encouragement that I have been able to successfully finish my degree.

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LIST OF ABBREVIATIONS

CAD	Coronary Artery Disease
CHD	Coronary Heart Disease
CVD	Cardiovascular Disease
ERT	Estrogen Replacement Therapy
FVIIa	Factor VII Activity
GSH	Glutathione
GS-SG	Glutathione Disulfide
HDL	High Density Lipoprotein
HRT	Hormone Replacement Therapy
IDL	Intermediate Density Lipoprotein
LDL	Low Density Lipoprotein
Lp(a)	Lipoprotein(a)
NO	Nitric Oxide
PAI-1	Plasminogen Activator Inhibitor-1
ONOO	Peroxynitrite
TC	Total Cholesterol
TG	Triacylglycerol
VLDL	Very Low Density Lipoprotein

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GENERAL INTRODUCTION

Thesis Organization

This thesis begins with a general introduction including a statement of the research problem, followed by a review of literature, a manuscript, general conclusions, and a list of references for the introduction and review of literature.

Objectives, Hypotheses, Specific Aims, Limitations, and Significance of Proposed Study

Objectives

The purpose of this 24-week study was to determine whether isoflavone-rich soy protein reduces the risk of cardiovascular disease (CVD) by favorably altering plasma lipid and lipoprotein concentrations, coagulation and fibrinolytic factors, and oxidative balance in perimenopausal women.

Hypotheses

Due to the estrogen-like characteristics of isoflavones, isoflavone-rich soy protein will reduce CVD risk factors by:

1. Favorably altering lipid/lipoprotein concentrations
   a. by lowering total cholesterol (TC), low density lipoprotein cholesterol (LDL-C), triacylglycerol (TG), TC/ high density lipoprotein cholesterol (HDL-C) ratio and
   b. by increasing HDL-C.
2. Improving total antioxidant status.
3. Favorably altering coagulation/fibrinolytic factors by lowering lipoprotein(a) [Lp(a)], fibrinogen, factor VII antigen, and plasminogen activator inhibitor–1 (PAI-1).

Specific aims

1. To determine whether 24 weeks of isoflavone-rich soy protein intake exerts an estrogen-like effect on CVD risk factors in perimenopausal women by assessing changes in:
   a. Lipid and lipoprotein concentrations,
   b. Total antioxidant status, and
   c. Coagulation and fibrinolytic factors.

2. To determine the collective contribution of circulating coagulation/fibrinolytic and other factors (such as dietary intake of selected nutrients; serum estrogens, ferritin, iron, liver enzymes; body size/composition; blood pressure; and physical activity) to plasma lipids and lipoproteins at baseline in these perimenopausal women.

Limitations

Perimenopause is a transition period during which hormones may dramatically fluctuate. Thus, one limitation of our study may have been that the hormonal changes during the menopausal transition could have had an overriding effect on some of the cardiovascular end points. Alternatively, the stage at which we obtained blood samples may have been too early in the menopausal transition to reveal marked adverse effects on circulating lipids/lipoproteins or coagulation/fibrinolytic factors, thus making it more difficult to demonstrate an effect of treatment. Matthews et al. (1994) reported that perimenopausal women did not have more adverse lipid/lipoprotein profiles when compared with premenopausal controls. Significant changes have been noted once perimenopausal women become postmenopausal (Matthews et al. 1994), suggesting that our six-month study may have been too short to have fully captured the potential treatment-induced changes in the lipid/lipoprotein profile. Also, differences among individuals in absorption (Xu et al. 1995) and metabolism (Kelly et al. 1995) of isoflavones may influence the response to treatment.

Significance of proposed study

Despite the uncertainty of the exact time point at which lipid-related CVD risk factors increase, perimenopause has been identified as a critical period in which to initiate preventive efforts (Matthews et al. 1994). The effect of soy with isoflavones on the lipid/lipoprotein profile of postmenopausal women has been examined (Potter et al. 1998), but we know very little about the effect during the perimenopausal period. Also, soy isoflavone treatment in relation to the antioxidant activity in perimenopausal women has not been documented. Information is available on the effects of soy isoflavones on coagulation/fibrinolytic factors *in vitro* (Wilcox and Blumenthal 1995), but there are no published data on the *in vivo* effects. If this study indicates that soy protein with isoflavones improves the lipid profile, improves and/or maintains antioxidant status, and/or favors fibrinolysis, this could serve as an alternative or adjuvant treatment during menopause. This may be most important for women

who are poor candidates for, or chose not to take, estrogen replacement therapy (ERT) or hormone replacement therapy (HRT).

REVIEW OF LITERATURE

Overview of Cardiovascular Disease Risk Factors in Women

All forms of cardiovascular disease lead to more than 50 percent of all deaths among women each year, accounting for over 500,000 deaths in the United States (Giardina 1998). Atherosclerotic CVD is a major contributor to this mortality statistic. Atherosclerosis is the underlying process leading to the clinical sequelae of coronary heart disease (CHD), cerebrovascular disease, and peripheral vascular disease. Recent investigations have identified both non-modifiable and modifiable CVD risk factors that more directly relate to women's health.

Non-modifiable risk factors

Non-modifiable risk factors include sex, age, race, and family history. Premenopausal women have one-third the incidence of CHD than men of the same age. Although overall morbidity and mortality for women is less than that of males for CHD, the morbidity rate is greater for postmenopausal women than for males of similar age (Lerner and Kannel 1986). Age is considered a major risk factor for CVD in women, since mortality from CVD increases approximately 50 percent for each succeeding five-year age group (Vital Statistics of the United States 1987). Race also influences CVD as illustrated by young adult and mid-life African-Americans having nearly twice the number of deaths compared with their Caucasian counterparts. In contrast, Asian and Hispanic adults have nearly half the number of CVD-related deaths compared with similarly aged Caucasians. However, after 65 years of age, mortality among Caucasians matches that of their African-American counterparts. After age 65, Asian and non-black Hispanics have the lowest CVD mortality rates, approximately half that of Caucasians (National Center for Health Statistics 1991). Family history is a strong risk factor for young women; increased death due to CHD occurs in women whose first-degree female relatives have been diagnosed with CVD before 55 years of age (Slack and Evans 1966). If the onset of CVD is after the age of 55, there is no clear association with family history (Higgins et al. 1987).

Modifiable risk factors

Modifiable risk factors include elevated blood pressure, smoking, dyslipidemia, obesity, diabetes, and physical inactivity. As a woman ages, she actually has a greater chance

of developing high blood pressure than a man (Legato 1991). Cigarette smoking is considered the single most preventable cause of heart disease in this country. Smoking has a dose-dependent effect on the number of deaths due to CVD, with 10 and 20 cigarettes per day increasing the risk of CVD-related death by 50 and 100 percent, respectively (Bush and Comstock 1983). Quitting smoking helps to reduce the risk of death by CVD, perhaps to that of a nonsmoker (Redberg 2000). Plasma lipoprotein levels are useful for diagnosing CVD, with lipid risk factors being elevated TC, LDL-C (particularly oxidized LDL), TG, and Lp(a), and low HDL-C. Age, hormonal status, body composition and weight, physical activity, and diet influence these lipid risk factors. Dietary intake, through excessive energy, influences CVD risk by contributing to dyslipidemia, obesity, and perhaps diabetes. Also, diet influences CVD risk via fat (total amount and type) consumption that alters lipid/lipoprotein profiles. The inclusion of supplements and/or foods containing antioxidants has been shown to prevent the harmful oxidation of lipids/lipoproteins (Jialal and Fuller 1995; Chopra and Thurnham 1999; Weisburger 1999). Physical inactivity is a common finding among CVD patients. More than 50 percent of women do not exercise at all, placing them at an increased risk for developing CVD (Redberg 2000). Researches have concluded that physical inactivity is an independent risk factor for CVD by demonstrating the protective effect of leisure time physical activity on coronary death in men (Rosengren and Wilhelmsen 1997).

Recently identified risk factors

Two more recently identified risk factors that are directly associated with CVD – coagulation/fibrinolytic factors and total antioxidant status – are currently under investigation. Coagulation factors include fibrinogen and factor VII. The factors that influence the fibrinolytic pathway are Lp(a) and PAI-1. Fibrinogen is a plasma protein responsible for inducing the formation of blood clots. Factor VII is involved in inducing clot formation and increases with age and during the menopausal transition. Lipoprotein(a) is considered both a thrombotic risk marker and an atherogenic lipoprotein. If elevated, Lp(a) is associated with an increased risk of CVD since Lp(a) interferes with plasmin production. Genetics is the primary determinant of Lp(a) concentrations, although hormonal changes that occur during menopause may elevate Lp(a) (Mijatovic et al. 1997). Plasminogen activator

inhibitor-1 also interferes with the formation of plasmin. The appropriate balance between coagulation and fibrinolytic factors is critical in atherogenesis. Response to injury involves the coagulation pathway and may lead to thrombi formation and hence blockage of an artery. Fibrinolytic factors are involved in countering this thrombotic event.

Oxidative stress plays a role in the development of CVD since oxidized LDL particles induce atherogenesis. Supplemental antioxidants have been shown to reduce the level of oxidative stress, thus reducing the clinical manifestations of atherosclerosis (Diaz et al. 1997). The antioxidant defense system may be compromised after menopause, with an increase in lipid peroxidation. This places postmenopausal women at higher risk for CVD than their premenopausal counterparts.

Effect of menopause

The several years during which ovarian function begins to decline is defined as perimenopause (Klein and Soules 1998). The perimenopausal period is associated with irregular menses or cessation of menses for less than one year and in some women vasomotor symptoms (such as hot flushes and/or night sweats) (Greendale et al. 1999) due to the reduction of 17 β-estradiol. Postmenopause is defined as cessation of menstruation for greater than 12 months (WHO 1981). During the postmenopausal period, the primary source of estrogen is androstenedione secreted from the adrenal cortex, which is converted to estrone by adipose tissue, skeletal muscle, liver, brain, kidney, and hair follicles (Lievertz 1987). These hormonal changes during menopause predispose women to CVD, as evidenced by the higher incidence for postmenopausal than premenopausal women. Hormone replacement therapy after menopause is protective against CHD and stroke (NCEP 1993). Women with intact uteri must be protected from endometrial hyperplasia with ERT use by the addition of progestins. Estrogen and progestogen combined has a less well-understood effect on CVD risk. Hormone replacement therapy has been shown to favorably alter LDL-C and HDL-C/LDL-C ratios (Speroff et al. 1996; PEPI 1995), decrease fibrinogen (Vorster 1999), and enhance fibrinolytic activity (Lijnen and Collen 1996). The antioxidant effect of 17 β-estradiol has been shown *in vitro* to inhibit LDL oxidation (Huber et al. 1990). Although HRT is beneficial in reducing CVD risk, HRT has some adverse side effects and

may induce non-compliance due to continued bleeding in many women, as well as the fear of cancer (Samsioe 1996).

Soy protein with isoflavones

A potential alternative to HRT may be a diet high in plant-based foods that have been linked epidemiologically to lower occurrence of hormone-dependent diseases, such as CVD and some types of cancer (Setchell and Cassidy 1999). Soybeans contain a class of phytoestrogens called isoflavones. The major dietary isoflavones are the glycosides of genistein and daidzein. It has been shown *in vitro* and *in vivo* that isoflavones, particularly genistein, exert a weak estrogenic effect (Mayr et al. 1992). Numerous studies have documented that soy protein, particularly in conjunction with isoflavones, exert a hypocholesterolemic effect (Sirtori 1995; Anthony et al. 1996; Balmir et al. 1996). Potter et al. (1998) demonstrated a positive effect, whereas three studies (Hodgson et al. 1998; Simons et al. 2000; Nestel et al. 1999) have reported no effect of isoflavones alone on lipid profiles of postmenopausal women. Whether a beneficial effect can be replicated in perimenopausal women (Washburn et al. 1999) will require further research. The examination of the effect of soy protein with isoflavones on coagulation factors is limited, with a couple of studies showing no effect on Lp(a) in normocholesterolemic women (Hodgson et al. 1998; Yamashita et al. 1998) and one *in vitro* study indicating that genistein induces fibrinolytic enzyme activity (Sitter et al. 1996). Like estrogen, isoflavones exert antioxidant properties (Naim et al. 1976); however, the ability of isoflavones *in vivo* to affect total antioxidant status during menopause has not been documented.

Characteristics, Metabolism, and Factors Affecting Lipids/Lipoproteins

Plasma lipoproteins: Characteristics; menopause, estrogen/hormone replacement therapy, and soy

This section examines plasma lipoproteins and their associated apolipoproteins in relation to CVD risk during menopause. Lipoproteins have similarities in structure, which is described as a whole, along with their interrelated function, formation, and metabolism. Clinical manifestations of impaired lipid/lipoprotein metabolism and its involvement in the development of CVD are reviewed next. The relationship between plasma lipoproteins and

estrogen is addressed in the context of usual changes experienced during menopause. The effects of ERT/HRT and soy protein containing isoflavones on the lipid profile throughout menopause conclude this section.

Lipoproteins and their associated apolipoproteins

Plasma lipoproteins and apolipoproteins are synthesized in the liver and the intestinal mucosal cells. Lipoproteins are circulating complexes of lipid packaged with apolipoproteins, the latter allowing them to be water-soluble and readily transported. Apolipoproteins not only facilitate transport, but also serve a structural role in maintaining the integrity of lipoproteins, and as recognition sites for receptors, thereby directing the metabolic fate of lipoproteins (Davis 1997; Olson 1998).

Classification of lipoproteins is based upon two methods: electrophoresis and ultracentrifugation. Classes defined by electrophoresis are chylomicrons, prebeta, broad beta, and alpha lipoproteins. Using ultracentrifugation, five classes analogous to the previous method are defined as chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), LDL-C, and HDL-C (Olson 1998; Alaupovic 1996). The latter nomenclature is most commonly used today.

Apolipoproteins are grouped according to their function. They are classified into groups A, B, C, D, and E. Each class contains several subclasses of proteins; for example, class A is comprised of A-I, II, III, and IV (Chappell and Spector 1991). Apolipoproteins in class A are associated with HDL and nascent chylomicrons, whereas those in class D are found only with HDL. Class B apolipoproteins are associated with chylomicrons, VLDL, IDL, and LDL, whereas those in class C are predominately associated with VLDL and HDL. Apolipoproteins in class E are associated with VLDL, IDL, and chylomicron remnants (Alaupovic 1996).

Structure. Lipoproteins have a similar structure upon maturation in the circulation, with various proportions of TG and cholesteryl esters in the core, as well as different proportions of apolipoproteins, phospholipids, and free cholesterol in the hydrophilic outer shell (Basile-Borgia and Abel 1996). Phospholipids and cholesterol are important in cell membrane synthesis and integrity, whereas TG are the main source of energy for peripheral tissue. Chylomicrons are the least dense of the lipoproteins with a density of 0.920 to 0.960

g/ml. The composition of this lipoprotein by weight is as follows: 87% TG, 8% phospholipid, 1.5% free cholesterol, 2% cholesteryl esters, and 1.7% protein. The diameter of chylomicrons ranges from 75 to 600 nanometers (nm). The major apolipoproteins imbedded in the phospholipid-cholesterol surface are apolipoproteins B-48, A-I, and A-IV. Very low density lipoproteins are structurally similar to chylomicrons, but are denser and smaller; their density ranges from 0.950 to 1.006 g/ml and their diameter from 28 to 75 nm. The particle content of VLDL by weight is 55% TG, 20% phospholipid, 10% free cholesterol, 5% cholesteryl esters, and 9% protein. The major apolipoproteins imbedded in the phospholipid-cholesterol surface of VLDL are apolipoprotein B-100, C-I, II and III. Intermediate density lipoproteins are smaller and denser than VLDL, with a diameter of approximately 21 nm and a density ranging from 1.006 to 1.019 g/ml. The particle content of IDL by weight is 20% TG, 18% phospholipid, 25% cholesteryl esters, 20% free cholesterol, and 18% protein. The apolipoproteins B-100 and E are associated with the IDL particle. The particle content of LDL is 8% TG, 24% phospholipid, 8% free cholesterol, 35% cholesteryl esters, and 25% protein. The density of LDL ranges from 1.019 to 1.063 g/ml and these particles have a diameter of 17 to 26 nm. The core of LDL is primarily cholesteryl esters and a phospholipid-cholesterol shell containing apolipoprotein B-100 derived from VLDL metabolism. Due to the heterogeneity of LDL, these particles can be classified according to two different phenotypes, A and B. Low density lipoproteins in phenotype A are large and buoyant, whereas those in phenotype B are small and dense (Austin 1992). The composition of HDL is 0.6% TG, 21% phospholipid, 5% free cholesterol, 15% cholesteryl esters, and 55% protein. The density of HDL ranges from 1.063 to 1.210 g/ml and HDL particles have a diameter of about 10.8 nm. Newly synthesized HDL contain apolipoproteins E, A-I, II, III, and a phospholipid-cholesterol bilayer (Olson 1998; Ginsberg 1998). Subclasses of HDL exist, differing by which apolipoprotein(s) they contain, which in turn determines the unique cholesteryl ester content of each subclass (Olson 1998). The subclass HDL_3 accepts free cholesterol from peripheral tissues. After accumulating cholesterol, HDL_3 enlarges and gains additional apolipoproteins. Enzymatic reactions then convert HDL_3 into HDL_2 (Basile-Borgia and Abel 1996).

Function. Lipoproteins function primarily to mediate the flux of lipid and cholesterol among the liver, intestine, adipose, muscle, and other tissues (Alaupovic 1996). Lipoproteins are also recognized as playing a role in immune function and coagulation (Chambaud et al. 1999; Rosenson and Lowe 1998). The individual lipoproteins are discussed with regard to their unique involvement in lipid transport. Chylomicrons package dietary fatty acids from the intestine and transport TG to tissues, whereas VLDL transport TG derived from the liver to extrahepatic tissues. Triacylglycerol from VLDL may contain fatty acids synthesized by the liver from glucose (or other sources of acetyl coenzyme A) or fatty acids extracted from the blood by the liver and serve as an initial product formed in VLDL catabolism. Intermediate density lipoproteins serve as an initial product formed during VLDL catabolism. Cholesterol transport is the main function of LDL (Griffin 1999). During their residence time in circulation, LDL engulf cholesterol and then deliver it to extrahepatic tissues. In contrast, HDL transport lipids and apolipoproteins during the metabolism of TG-rich lipoproteins. Specifically, HDL are key in reverse cholesterol transport since these particles take up cholesterol from extrahepatic tissues and carry cholesterol to the liver for degradation and eventual removal (Basile-Borgia and Abel 1996).

Formation and metabolism. Chylomicrons are synthesized in the intestine after the ingestion of dietary fat. Bile acids and pancreatic enzymes in the intestine emulsify dietary TG and cholestryl esters into fatty acids, 2-monoacylglycerides, and cholesterol. The amount of dietary fat consumed dictates the size of the chylomicron. Cholesterol needed to form chylomicrons may also arise from *de novo* synthesis or from cholesterol transferred from other lipoproteins present in the lymph and plasma. These products are passively absorbed into the mucosal cell; TG are resynthesized in the smooth endoplasmic reticulum. Micelles are formed in the intestine after the addition of cholesterol and phospholipids. Apolipoproteins B-48, A-I, A-II, and A-IV synthesized in the rough endoplasmic reticulum are combined with lipid to make a newly synthesized chylomicron. Apolipoprotein B-48 is required for chylomicron synthesis and contributes to the stability of the lipoprotein. After formation, chylomicrons enter the lymph system and enter the general circulation via the thoracic duct. As chylomicron metabolism begins, modifications to apolipoproteins are made, such as loss of apolipoproteins A-I and IV to HDL and the acquisition of

apolipoprotein E. Complete chylomicron metabolism yields a chylomicron remnant, which has lost TG, apolipoprotein C, some apolipoprotein E, but has retained apolipoprotein B and acquired some cholesteryl esters. Retention of apolipoproteins B and E is crucial for the hepatic uptake and degradation of the remnant via phagocytosis (Ginsberg 1998). Specific remnant receptors in the liver recognize apolipoprotein B-48- and E-containing lipoproteins and are termed 'apolipoprotein E receptors'. Circulating lipoprotein remnants can bind to the LDL receptor via apolipoprotein E (Olson 1998). The next step is the uptake of the remnant by LDL receptors and/or LDL receptor-related proteins by classical receptor-mediated endocytosis (Chappell and Medh 1998; Havel 1998).

The origin of VLDL and chylomicron synthesis differs; chylomicrons are produced in the intestine, whereas VLDL are synthesized in the liver. The TG core of VLDL is derived from the liver rather than dietary fat. Triacylglycerol from VLDL may contain fatty acids synthesized by the liver from glucose (or other sources of acetyl coenzyme A) or fatty acids extracted from the blood by the liver. Metabolism of VLDL is similar to chylomicron metabolism with the result being TG removal. After production in the liver, the newly synthesized nascent VLDL particle enters the circulation. Newly synthesized VLDL have two fates: 1) removal from circulation into the liver by specific receptors on hepatocyte cell membranes, or 2) conversion into smaller, denser, cholesterol-rich LDL (Young and Parthasarathy 1994). Metabolism causes changes such as the addition of apolipoproteins C and E, with retention of apolipoprotein B-100. After further metabolism, fatty acids are released, the TG core is reduced, cholesteryl esters are gained, and a fraction of apolipoproteins E and C are lost. The initial product of VLDL catabolism is IDL, but not all VLDL particles experience complete catabolism past this intermediate 'remnant' form. About half of the VLDL particles are cleared from the circulation by specific hepatic receptors. The early phase of VLDL remnant clearance may be due to hepatic sequestering and/or apolipoprotein E-mediated binding (Eisenberg and Sehayek 1995). The remaining IDL particles undergo rapid turnover to LDL; within two to six hours from the time they first appear, IDL are no longer present in plasma.

After the final processing of VLDL remnants, LDL particles evolve from enzymatic action in the plasma. Compared with TG-rich lipoproteins, LDL have a longer residence time

of two to three days. Small VLDL and LDL bind to hepatic LDL receptors via apolipoprotein B-100 and are thus removed from the circulation. The presence of other apolipoproteins, C-III and E, has been shown to impair the interaction of apolipoprotein B with the LDL receptor, suggesting that apolipoprotein E participates instead in the LDL receptor binding (Clavey et al. 1991). After binding, LDL are removed from the circulation by incorporation into intracellular clathrin-coated vesicles with subsequent digestion in lysosomes. The liver removes 60 to 70 percent of LDL, whereas other tissues expressing LDL receptors remove the remaining LDL particles. Overall, LDL-C concentrations depend on the rates of conversion of VLDL to LDL and subsequent LDL clearance (Grundy 1995).

High density lipoproteins are synthesized from protein and phospholipids secreted by the intestine and liver. The small particle size of HDL allows it to easily cross the vascular endothelium. After entering the circulation, HDL lose some of their apolipoproteins C and E, gain apolipoprotein A, and free cholesterol is converted to cholesteryl esters. During TG uptake, apolipoprotein C is acquired along with phospholipid and cholesterol (Ginsberg 1998). Through the metabolic process of reverse cholesterol transport, HDL accumulates cholesterol from extrahepatic tissues. The HDL particle is capable of reverse cholesterol transport as described by an aqueous diffusion model and/or an HDL receptor. Through aqueous diffusion, free cholesterol from HDL and cell membranes form between them an aqueous phase. Either the cell or the HDL particle then resorbs the cholesterol. The net movement of cholesterol to either the cell or HDL depends on the phospholipid content of HDL; phospholipid-rich HDL readily accepts free cholesterol. The HDL receptor model proposes that cells having HDL receptors, thus permitting the transfer of cholesterol out of the cell, accomplish cholesterol removal. The final step in the reverse cholesterol transport pathway is the removal of cholesteryl esters contained in VLDL, IDL, and LDL through LDL receptors in various tissues (Olson 1998). The cholesterol is then either: 1) excreted in bile or 2) secreted back into circulation via nascent lipoproteins. The HDL particle is cleared by LDL receptors if it is enriched with apolipoprotein E, or the particle may be removed by a distinct HDL receptor (Kwiterovich, Jr. 1998). Thus, HDL-C concentrations may be indicative of the reverse cholesterol transport activity. The concentration of HDL-C depends on: 1) apolipoprotein rate of synthesis, 2) rates of free cholesterol esterification to

cholesteryl ester, 3) concentrations of TG-rich lipoproteins and transfer of cholesteryl esters from HDL, and 4) clearance of HDL lipids and apolipoproteins from plasma (Ginsberg 1998). Table 1 lists the reference ranges for lipid/lipoprotein concentrations.

Table 1. Reference ranges for lipids/lipoproteins for women over 40 years of age

Lipid/lipoprotein	Present reference range and units	SI reference range and units
Total cholesterol[1]	<265 mg/dL	<6.85 mmol/L
Low density lipoprotein cholesterol[1]	50 – 190 mg/dL	1.30 – 4.90 mmol/L
High density lipoprotein cholesterol[1]	30 – 90 mg/dL	0.80 – 2.35 mmol/L
Triacylglycerol[1]	<160 mg/dL	<1.80 mmol/L
Total cholesterol/high density lipoprotein cholesterol[2]	<5.60	<5.60

[1] From Young 1998
[2] From Kinosian et al. 1994

Enzymes and related proteins. Several enzymes are involved in lipoprotein metabolism including lipoprotein lipase, hepatic lipase, lecithin-cholesterol acyltransferase, and cholesteryl ester transfer protein. Lipoprotein lipase is the key enzyme involved in the delivery of fatty acids (Fielding and Frayn 1998). The synthesis of lipoprotein lipase occurs in the interior of either the capillary endothelial cells or the parenchymal cells in various tissues (Beisiegel 1996). Lipoprotein lipase then migrates into the vasculature, where it adheres tightly to heparan-like polysaccharide chains on endothelial cell surfaces (Ginsberg 1998). Concentrations of lipoprotein lipase may vary due to changes in physiological and nutritional states, such as pregnancy and fasting (Fielding and Frayn 1998). Lipoprotein lipase is activated as it comes in contact with apolipoprotein C-II, which is donated by either VLDL or HDL. The hydrolysis of TG and phospholipid is catalyzed by lipoprotein lipase (Jansen et al. 1998). This enzyme immediately hydrolyzes the TG in chylomicrons and

VLDL and transforms the lipoproteins into remnants. For example, the action of lipoprotein lipase on VLDL induces the loss of TG resulting in LDL; this conversion takes place within a few hours of VLDL appearance. Fatty acids are released from TG after transport through capillaries coated with lipoprotein lipase. This enzyme hydrolyzes either the one- or three-ester bond of a TG and the one-ester bond of a diacylglyceride, releasing free fatty acid anions, which are carried through the plasma membrane of the recipient cell by a carrier protein. Once fatty acids are in the cell, they are converted to acyl coenzyme A and then further converted to TG for storage. Lipoprotein lipase is just one in a cascade of enzymes necessary for the deposition of fat into adipose tissue (Fielding and Frayn 1998). Besides lipoprotein lipase's role in lipid metabolism, a recent study has discovered that this enzyme, when expressed by cells such as macrophages in the vascular wall, could contribute to the formation of foam cells and atherosclerosis (Mead et al. 1999).

Hepatic lipase is similar to lipoprotein lipase in that it also catalyzes the hydrolysis of TG and phospholipids (Ginsberg 1998). Crawford and Borensztajn (1999) have found that hepatic lipase, through non-receptor mediated uptake, can mediate endocytosis of chylomicron remnants independent of apolipoprotein E; thus hepatic lipase helps to regulate plasma clearance and liver uptake of chylomicron remnants. Hepatic lipase may also mediate the conversion of HDL particles HDL_2 to HDL_3 (Beisiegel 1996). Concentration of hepatic lipase is increased by androgens and decreased by estrogens (Chappell and Spector 1991).

The catalysis of cholesteryl esters is mediated by lecithin-cholesterol acyltransferase. The metabolic role of lecithin-cholesterol acyltransferase involves reverse cholesterol transport, in which the substrates are cholesterol and phosphatidyl-choline (Dobiasova and Frohlich 1998). Synthesized in the liver, lecithin-cholesterol acyltransferase binds to nascent HDL and esterifies cholesterol from a fatty acid donated by a phospholipid. Activation of lecithin-cholesterol acyltransferase does not occur until apolipoprotein A-I is transferred from a chylomicron to HDL. As the esters accumulate, they are transferred to other lipoproteins, such as delipidated chylomicrons and VLDL. These TG-rich lipoproteins in return donate TG to HDL through the action of a lipid transfer protein (Rueckert and Schmidt 1990). A recent study shows that lecithin-cholesterol acyltransferase works in this protective manner only when the HDL_2 fraction is present in high concentration. If HDL_2 concentration

is low, lecithin-cholesterol acyltransferase aids in the esterification of other lipoproteins, such as LDL, and may serve an atherogenic role in this instance (Dobiasova and Frohlich 1998).

Cholesteryl ester transfer protein is another player in lipid metabolism. This protein transfers lipid among lipoproteins, but also takes up cholesterol directly from tissues. Depending on the situation, this enzyme can also have both proatherogenic and antiatherogenic roles (Ginsberg 1998). Genetic mutations leading to cholesteryl ester transfer protein deficiency, overexpression of cholesteryl ester transfer protein, or pre-existing disease, such as coronary artery disease (CAD), will determine whether this enzyme positively or negatively influences the atherosclerotic process (Stevenson 1998).

Clinical manifestations of impaired metabolism

Modification of lipoproteins and apolipoproteins occurs continuously. Metabolism of lipoproteins involves complex modifications, with abnormal synthesis and metabolism leading to the development of atherosclerotic CVD. The atherogenicity of LDL is partially conferred by small dense LDL particles and not due to the transport of cholesterol *per se*. Small, dense LDL are significantly associated with increased coronary risk (Keys 1970; Kannel et al. 1971), likely due to increased cholesterol influx into the arterial wall because of its small, dense structure. The extracellular matrix attached to arteries binds small dense LDL with high affinity, sequestering these lipoproteins in a prooxidative environment, allowing cholesterol deposition. Low density lipoproteins have been found to be more susceptible to oxidative stress than the larger, less dense lipoproteins (Griffin 1999). Within the arterial wall, LDL particles are oxidized, with these particles then being taken up by macrophages, implicating oxidized LDL's involvement in the development of foam cells and eventual lesions (Young and Parthasarathy 1994).

Increased levels of both VLDL and subsequently IDL are associated with the clinical features of premature atherosclerosis, glucose intolerance, and eruptive xanthomas. Plasma concentrations of IDL are not normally identified due to rapid turnover, but IDL concentrations are used to define Type III hyperlipidemia. Overproduction and increased residence time allows more cholesterol deposition in the arterial wall (Griffin 1999). The atherogenic nature of these particles is thought to be due to their TG-rich core. Alterations in enzymes involved in lipid metabolism are related to impaired metabolism of atherogenic

lipoproteins; individuals with CHD have decreased lipoprotein lipase activity (Henderson et al. 1999).

Overall, the greater the HDL-C concentration, the lower the CVD risk. This association of HDL with antiatherogenicity is supported by a mechanism other than reverse cholesterol transport. This mechanism involves apolipoproteins A-I and two enzymes associated with HDL, paraoxanase and platelet-activating factor acetylhydrolase, which appear to reduce the formation of oxidized LDL (Kwiterovich, Jr. 1998).

Menopause, estrogen/hormone replacement therapy, and soy

Endogenous sex hormones during menopause. Due to the hormonal changes during menopause, women typically lose their resistance to CVD. Declining estrogen concentrations and increasing androgenic effects of progesterone have been implicated in altering lipid profiles. The net result of these modifications in lipid concentrations for postmenopausal women is an adverse change in the ratio of HDL-C/LDL-C and an associated increase of risk of CAD (Khaw 1992; Tremollieres et al. 1999). Throughout menopause, 17 β-estradiol, the predominant form of estrogen in premenopausal women, decreases considerably; the major drop occurs in the perimenopausal and immediate postmenopausal periods (Cauley et al. 1989). The major source of estrogens in postmenopausal women is the conversion of androgen precursors (Khaw 1992) to estrone. Lipid changes closely correlate with perimenopausal changes of decreased estrogen and increased gonadotropins (Gaspard et al. 1995).

Before menopause, estrogen is present in high enough concentrations to exert a protective role against atherogenic lipoproteins. The antiatherogenic effects of estrogen include increasing/maintaining HDL-C and apolipoproteins A-I and A-II, but decreasing TC, LDL-C, and apolipoprotein B (Bonithon-Kopp et al. 1990). Premenopausal endogenous estrogen also maintains low TG concentrations; lack of estrogen during menopause is associated with an increase in TG (Jensen et al. 1990).

The mechanism of estrogen's protective effect is uncertain, but may be at the level of regulating enzyme concentrations relevant to lipid metabolism. Estrogen reduces hepatic lipase, which reduces TG and phospholipid hydrolysis and inhibits HDL-C catabolism, thereby increasing HDL-C concentrations. However, declining estrogen status due to natural

menopause has not been shown necessarily to promote the usual decrease in HDL-C (Lobo 1990). Some researchers have reported that HDL-C concentrations will plateau and/or decrease after menopause due to decreased hepatic production (Sullivan 1996; Nasr and Breckwoldt 1998). However, other researches have found that overall concentration of HDL-C may not change because a decline in HDL_2 is followed by an increase in HDL_3 (Matthews et al. 1994). Estrogen also affects lipid metabolism by influencing the concentration of lipoprotein lipase. As estrogen decreases during menopause, lipoprotein lipase activity is enhanced, thereby increasing the plasma concentration of LDL-C (Wakatsuki and Sagara 1995). Estrogen also has a cardioprotective effect by stimulating the synthesis of LDL receptors *in vitro* (Streicher et al. 1998), which would result in increased uptake of LDL-C and chylomicron remnants. Thus, if estrogen is deficient, as occurs during menopause, LDL-C rises presumably due to decreased LDL receptor activity (Abbey et al. 1999).

Estrogen affects all stages of the atherosclerotic process. In the early phases, estrogen attenuates the buildup of atheromatous material in arterial walls and prevents the oxidation of LDL, a critical step in atherogenesis (Sullivan 1996; Young and Parthasarathy 1994). The antioxidant effect of estrogen will be discussed in a subsequent section. The antiatherogenic effect of estrogen has been documented at the molecular, cellular, tissue, and organ level.

Exogenous sex hormones during menopause. The previous section illustrates the cardioprotective effects of endogenous estrogen. Estrogen replacement therapy has been shown to prevent the increased risk in CVD during menopause due to declining endogenous estrogen. A meta-analysis of observational studies using ERT shows a 44 to 50 percent reduction in overall risk for CVD, thus having a significant benefit in reducing total CVD mortality (Sullivan 1996). The role of estrogen in reducing CVD risk has been attributed to its effect on lipids, but lipid-related changes account for only 25 to 50 percent of CVD risk reduction (Nasr and Breckwoldt 1998). Additional mechanisms are likely related to estrogen as an antioxidant and anticoagulant.

Overall, studies show that estrogens favorably alter the lipid profile, with effects being both dose- and structure-dependent (Nasr and Breckwoldt 1998). With estrogen use, HDL-C concentrations may increase by 20 percent and LDL-C may decrease similarly (Samaan and Crawford 1995). Synthetic estrogens, such as ethinyl estradiol and

diethylstilbestrol, have the most potent effects, whereas natural estrogens, such as estradiol, estrone, and conjugated equine estrogens, have less marked effects. Routes of estrogen delivery, such as oral, transdermal, or subdermal, do not seem to change the lipid-lowering ability of estrogen, if sufficient dosages and time are allowed (Lobo 1990). The administration of estrogen decreases the deposition of cholesterol and the overall size of the plaque in the arterial wall, as evidenced by animal studies (Adams et al. 1990; Hanke et al. 1996). More LDL are converted into large VLDL during oral estrogen administration due to its stimulatory effect on hepatic LDL receptors. A significant increase in TG, HDL-C, and apolipoprotein A-I concentrations occurs along with a significant decrease in LDL-C. These changes are beneficial except for the increase in TG; however, the significance of this increase is questionable, since an increased output of large, TG-rich VLDL also occurs with ERT (Nasr and Breckwoldt 1998). In contrast, transdermal ERT does not increase hepatic production of TG or VLDL, but actually lowers TG and VLDL concentrations while favorably altering the lipid profile, although to a lesser degree (Crook and Stevenson 1996).

The combination of estrogen and progestins is commonly recommended for postmenopausal women with intact uteri. However, the positive alteration in the lipid profile due to estrogen alone cannot be considered without recognizing the risks of this drug therapy. The risk of endometrial cancer is attenuated by the addition of progestins, whereas the risk of gallbladder disease and venous thromboembolic disease remains elevated (Barrett-Connor 1999). Progestins counter the effects of estrogen due to their androgenic action, thus having an adverse effect on the lipid profile by increasing LDL-C and attenuating the increase in HDL-C (Goh et al. 1995). Depending upon the androgenic strength of the progestin, the positive effects of estrogen may not be completely abolished (Samaan and Crawford 1995). Several studies conclude, that despite the addition of progestins, HRT maintains most of the beneficial effects on lipids/lipoproteins, but attenuates the unfavorable effect of oral estrogen on total TG (Speroff et al. 1996; PEPI 1995). Translating this to disease risk, women at risk of developing CAD are more protected by ERT than HRT, assuming that progestins lessen estrogen's cardioprotective effect (Chow 1995).

Soy protein with isoflavones. A meta-analysis documents the association of soy protein intake and lower risk for CHD in humans (Anderson et al. 1995). However, soy

protein has been known to retard the progression of atherosclerosis for more than 50 years (Clarkson et al. 1998). The cardioprotective effect of soy protein is evident when comparing data from Asian countries, where soy consumption is high and CHD rates are lower than in Western countries, where soy consumption is low and CHD rates are higher (Coward et al. 1993; Terpstra et al. 1983). Many mechanisms have been proposed to account for the cholesterol-lowering effect of soy protein. One mechanism may be that soy up-regulates the LDL receptor, thereby increasing LDL turnover (Samman et al. 1990). Another proposed mechanism for the cholesterol-lowering effect is that soy protein inhibits the absorption of dietary cholesterol and the intestinal reabsorption of bile acids, thereby lowering circulating cholesterol. An additional purported mechanism is that soy protein influences various aspects of the endocrine system (Potter 1998). For example, current evidence suggests that dietary soy protein increases plasma thyroxine concentrations; therefore, increased thryoxine concentrations result in increased bile acid synthesis, increased fecal bile acid excretion, and increased hepatic clearance of LDL and VLDL (Forsythe III 1995). Soy protein is being investigated to identify the fraction primarily responsible for its cholesterol-lowering effect. Isoflavones, particularly genistein and daidzein, are hypothesized to lower TC and LDL-C because of their structural similarity to estradiol (Markiewicz et al. 1993). Isoflavones bind to and activate estrogen receptors, particularly the type 2, in target tissues. However, isoflavones exert a much less potent estrogenic effect due to their lower relative molar binding affinities than estrogens (Samman et al. 1990). In women after menopause, LDL-C concentrations are reduced by up to 19 percent in estrogen users (Samaan and Crawford 1995) compared to a reduction of up to 7 percent with dietary intake of soy (Potter et al. 1998; Washburn et al. 1999).

Characteristics and Factors Affecting Coagulation/Fibrinolytic Factors and Oxidative Balance

Coagulation and fibrinolytic factors: Interrelationships; menopause, estrogen/hormone replacement therapy, and soy

Kelleher (1992) concludes that several coagulation factors are directly associated with the incidence of CVD. Thrombotic response after an atherosclerotic plaque rupture and/or erosion is influenced by many factors, most notably the reactivity of coagulation factors and

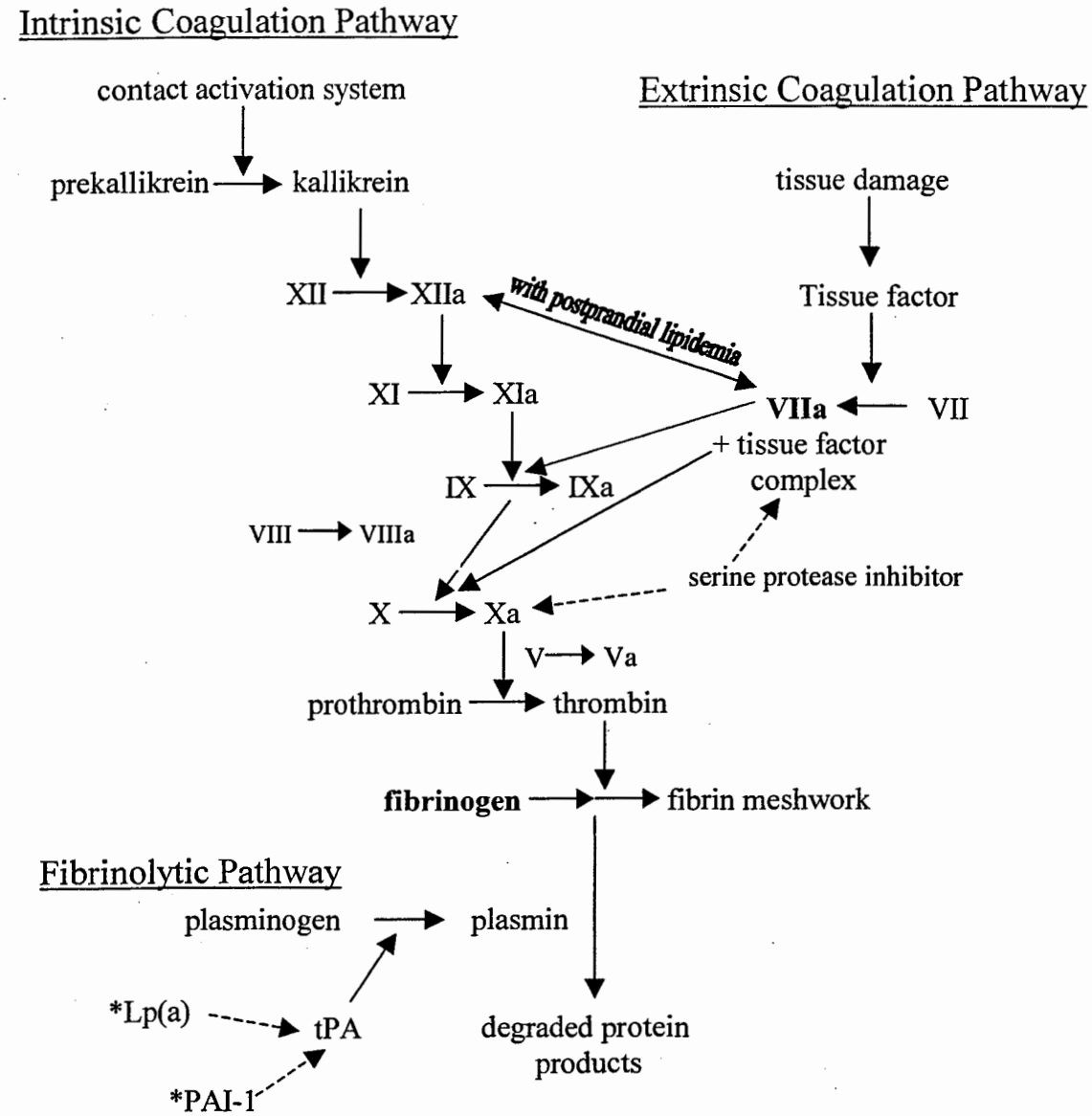
the effectiveness of protective fibrinolysis (Rosenson and Lowe 1998). Figure 1 displays the interrelationship between coagulation and fibrinolysis. To understand how coagulation mechanisms work, both extrinsic and intrinsic pathways will be closely examined with an emphasis on factor VII and fibrinogen; fibrinolysis will focus on PAI-1 and Lp(a). Table 2 lists the reference ranges for the coagulation/fibrinolytic factors in this thesis. The influence of menopause, ERT/HRT, and soy with isoflavones on both coagulation and fibrinolytic factors will also be discussed.

Coagulation/fibrinolysis

The enzymatic products of the coagulation cascade from one reaction initiate the subsequent reaction. The cascade reactions are ordered and controlled due to the high sensitivity of the coagulation enzymes and by positive/negative feedback systems. This process is thought to be autocatalytic and self-limiting (van Dam-Mioras and Muller 1986).

Extrinsic coagulation pathway. The extrinsic coagulation pathway is triggered by tissue damage or injury to the vasculature. Factor VII is the first enzyme in the extrinsic coagulation pathway; however, factor VII alone has limited ability to induce clotting. To exert its clotting activity, factor VII must bind with tissue factor, a cell surface glycoprotein expressed by endothelial cells and macrophages in atherosclerotic plaque. Tissue factor is the primary initiator of this pathway by serving as the cellular receptor for factor VII, as well as the cofactor for factor VII activation. Activated factor VII then forms a complex with tissue factor to initiate the blood coagulation process (Kalafatis et al. 1997). From this point, the extrinsic pathway merges with the intrinsic pathway to complete the final processes of coagulation. Tissue factor pathway inhibitor, a serine protease inhibitor, counterbalances the extrinsic coagulation activation pathway and is transported by LDL-C, Lp(a), and HDL-C. This inhibitor binds to factor VII activity-tissue factor complex and to activated factor X in the intrinsic pathway, preventing the coagulation cascade from continuing (Rosenson and Lowe 1998).

Factor VII is one of the major vitamin K-dependent prothrombic factors, but primarily exists as a zymogen, an inert form of the enzyme. The zymogen form can be quantified by measuring the antigen concentration. The antigen of factor VII will reflect the zymogen's physical presence, but does not determine its functional ability. Once activated,



Key

a	Active form of factor
Bold	Coagulation factors reviewed in text
*	Fibrinolytic factors reviewed in text
----▶	Inhibiting pathway
——▶	Inducing pathway
tPA	Tissue plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
Lp(a)	Lipoprotein(a)

Figure 1. Interrelationship between the coagulation and fibrinolytic pathways. (Adapted from Rosenson and Lowe 1998; van Dam-Mieras and Muller 1986)

Table 2. Reference ranges for coagulation/fibrinolytic factors

	Present reference range and units	SI reference range and units
Coagulation factors		
Factor VII[1]	50 – 150 %	50 – 150 %
Fibrinogen[2]	200 – 400 mg/dL	2.0 – 4.0 g/L
Fibrinolytic factors		
Plasminogen activator inhibitor –1[1]	0 – 15,000 units/L	0 – 250 μkat/L
Lipoprotein(a)[1]	10 – 30 mg/dL	17.2 – 51.7 nmol/L

[1] From DeMott 1996
[2] From Young 1998

this factor is referred to as factor VIIa and is a plasma serine proteinase responsible for initiating clotting. Only one percent of factor VII is activated and factor VIIa concentrations can be assessed separately from the zymogen form. A combined measure of the zymogen and preformed activity is termed factor VII coagulant activity (Morrissey 1996). Factor VII coagulant activity was identified in 1986 as a powerful predictor of ischemic events (Meade et al. 1986). Increased factor VII coagulant activity and factor VII antigen concentrations are considered CHD risk factors due to their correlation with major lipid/lipoprotein fractions, most notably with plasma TG since factor VII binds to apolipoprotein B containing lipoproteins (Rosenson and Lowe 1998). However, factor VIIa concentrations have not been found to be significantly related to fasting serum lipid levels, but assessment of this activated factor may be important in predicting risk of occlusive thrombi (Morrissey 1996). Factor VII concentrations have been found to be associated with other CHD risk factors, such as hypertriglyceridemia, hyperinsulinemia, diabetes, and high body mass index (Pearson et al 1997). Total dietary fat intake, rather than the type of fatty acid, influences factor VII clotting activity by mediating the activation of the intrinsic coagulation pathway via the lipolysis of VLDL and chylomicrons (Kelleher 1992; Rosenson and Lowe 1998; Miller 1998). Factor

VII coagulant activity is also related to age; it continually increases throughout the lifespan, with a predominant rise in women after their fifties (Balleisen et al. 1985).

Intrinsic coagulation pathway. In nonendothelial tissue, the glycoproteins prekallikrein and kallikrein initiate the intrinsic pathway by activating factor XII. Factor XII may activate factor VII during postprandial lipidemia. The proteolysis of factor XII activity results in the activation of factor XI. Factor IX is activated by both factor XI and factor VII. Like factor VII, factor IX is a vitamin K-dependent coagulation factor thought to be related to nonfasting TG and TC concentrations (Rosenson and Lowe 1998). Through the key formation of a complex, factor IX activity and factor VIII together activate factor X. Factor VII may also activate factor X. Factor X, coupled with factor V, then activates the reaction of prothrombin to thrombin. Thrombin, a serine protease, is then responsible for the conversion of fibrinogen to fibrin, promoting formation of the fibrin meshwork.

Fibrinogen is synthesized by hepatocytes and is an acute phase protein triggered by interleukin-6 during inflammation (Herrick et al. 1999). This protein functions to clot the blood and thereby forms a barrier against blood leakage through the damaged blood vessel wall. A fibrin clot acts as a network retaining blood cells and certain plasma proteins. Fibrinogen also serves to support fibroblast proliferation during wound healing and may be involved in defense mechanisms. Fibrinogen is a major determinant of blood and plasma viscosity; concentrations greater than 400 mg/dl have been linked to cerebrovascular disease (Kelleher 1992). Elevated concentrations of fibrinogen are also used to independently predict an increased risk of atherosclerotic CVD, since fibrinogen exists as a surface component of both fatty streaks and atherosclerotic plaque. Once in plaque, fibrinogen may be converted to fibrin by cells within the arterial wall. Fibrin then provides an absorptive surface area for the atherogenic lipoproteins LDL and Lp(a) to localize within the plaque. Degradation of fibrinogen occurs as well during the process of plaque formation, and evokes cell disorganization and migration within the endothelium (Rabbani and Loscalzo 1994; Warkentin 1995). Factors directly associated with higher fibrinogen concentrations are female sex, black race, low socioeconomic status, age, menopause, obesity, a carbohydrate-rich diet, diabetes, cigarette smoking, inflammation, infection, elevated systolic blood pressure, TC, LDL-C, Lp(a), and/or serum insulin. In regions with high CVD rates and cold

ambient temperatures, fibrinogen concentrations are also higher. Lower fibrinogen concentrations are associated with male sex, adult height, HDL-C, testosterone concentration, postmenopausal estrogens, physical activity, moderate alcohol consumption, and/or a diet rich in polyunsaturated fat (Pearson et al. 1997).

Fibrinolytic pathway. This pathway is capable of dissolving blood clots and includes the components plasminogen activators, plasminogen/plasmin, and fibrinolytic inhibitors. Plasminogen activators, primarily tissue plasminogen activator, convert the liver-derived inactive plasminogen to plasmin. Plasmin functions to degrade fibrinogen/fibrin by proteolysis. Fibrinolytic inhibitors include PAI-1 and Lp(a) (Dobroski and Loscalzo 1996). The fibrinolytic pathway is regulated by the balance between tissue plasminogen activator and its main inhibitor PAI-1.

Plasminogen activator inhibitor-1, a serine protease inhibitor, acts specifically on tissue plasminogen activator to indirectly impair fibrinolysis through the conversion of plasminogen to plasmin. Endothelial cells produce PAI-1 in response to the daily circadian fluctuation of net fibrinolytic activity (Kadish 1995). Elevated concentrations of PAI-1 are associated with increased VLDL and TG concentrations, possibly linking PAI-1 to atherogenesis (Dobroski and Loscalzo 1996). Elevated PAI-1 concentrations have been noted in patients with preexisting CAD and in survivors of myocardial infarction who are less than 45 years of age (Rosenson and Lowe 1998). Although concentrations of PAI-1 are directly related to the extensiveness of atherosclerotic plaque, it is difficult to determine which is the predisposing factor. Obesity and associated metabolic conditions, such as hyperglycemia and hyperinsulinemia, also seem to be associated with elevated PAI-1 concentrations (Pearson et al. 1997). Additional factors that influence PAI-1 concentrations include gender and smoking, but body mass index along with TG concentrations are the strongest predictors of PAI-1 (DeLoughery 1999).

Lipoprotein(a) is a macromolecular complex having a cholesteryl-ester rich LDL-like structure. Lipoprotein(a) contains a protein moiety, which is similar to plasminogen in structure (Scanu 1992). Low density lipoproteins and Lp(a) have several similar physical and chemical properties, including a high content of cholesteryl ester, ~30 to 45% by weight. The properties that distinguish LDL from Lp(a) are its larger particle size (28 nm), higher

molecular weight, more buoyant density (1.050-1.100 g/ml), higher content of protein (30% by weight), and higher carbohydrate content (Chapman et al. 1994). Lipoprotein(a) synthesis occurs in the liver at a rate speculated to be dependent upon the flux of cholesterol and TG into the hepatocyte (Scanu 1992). Clearance of Lp(a) from the circulation depends upon whether the particle exhibits low or high molecular weight. Low molecular weight Lp(a) have been shown to be preferentially taken up by the LDL receptor, whereas the high molecular weight Lp(a) are thought to be taken up by the LDL receptor-related protein/alpha 2-macroglobulin receptor (März et al. 1993). However, Lp(a) catabolism using both cell culture systems and *in vivo* turnover studies has not invariably implicated the LDL receptor in Lp(a) degradation (Scanu 1992).

Although "normal" concentrations of Lp(a) have yet to be established, a typical upper limit in the literature is 30 mg/dl (Bartens and Wanner 1994). Circulating concentrations are influenced largely by heritability (Dahlén 1994), but also by sex hormones, resulting in decreases with androgen and estrogen administration and increases with growth hormone administration (Berglund 1995). Disease states, such as diabetes, hyperlipidemia, and kidney disease, typically increase Lp(a) concentrations (Bartens and Wanner 1994). Tamoxifen, niacin, gemfibrozil, omega-3 fatty acids, *N*-acetylation, prednisone, and neomycin are considered Lp(a) lowering agents (Hajjar and Nachman 1996). A physiological function of Lp(a) has not been defined, although evidence indicates that Lp(a) is atherosclerotic and thrombotic in nature. Lipoprotein(a) transverses the endothelium, permitting the particle to accumulate in the extracellular space.

The following series of events may be responsible for the atherosclerotic potential of Lp(a). Lipoprotein(a) leaves the bloodstream and accumulates in the intimal space of the arterial wall. Chemical modification of Lp(a) occurs due to interaction with oxygen-free radicals, proteoglycans, glycosaminoglycans, or other matrix components, such as fibrin. The oxidative behavior of Lp(a) is determined by the nature and proportion of fatty acids associated with cholesteryl esters, phospholipids, and TG, in addition to the antioxidant content. Chemically modified Lp(a) is taken up by the scavenger receptor of resident macrophages which is then transformed into foam cells (Scanu 1992). This last event is an initiating step in the atherosclerotic process. Elevated LDL-C may in fact be obligatory for

the atherogenicity of Lp(a). However, the relative risk of elevated Lp(a) is significantly increased in patients who also have high LDL-C (Bartens and Wanner 1994) concentrations. Overall, Lp(a) favors cholesterol deposition in the developing plaque (Chapman et al. 1994).

Lipoprotein(a) exerts a prothrombotic effect through its role in the fibrinolytic system. As previously mentioned, Lp(a) is similar in structure to plasminogen and interacts with cellular components and protein cofactors of fibrinolysis (Scanu 1992). Specifically, Lp(a) inhibits tissue plasminogen activator activity, the primary activator of plasmin formation. Lipoprotein(a) also prevents plasminogen from binding to the endothelium, thus impairing endothelial cell-surface-mediated fibrinolysis (Dobroski and Loscalzo 1996). Overall, Lp(a) favors the development of blood clots.

Menopause, estrogen/hormone replacement therapy, and soy

Endogenous sex hormones during menopause. Variations of circulating endogenous estrogen in premenopausal women during the follicular and luteal phases of menstruation have not been associated with significant fluctuations in Lp(a) concentrations (Haines et al. 1996), although fibrinolysis may be reduced during the luteal phase. Pregnancy has been associated with an increase in Lp(a), fibrinogen, and factor VII concentrations (Meilhan et al. 1992). Endogenous androgens are also known to favorably affect fibrinolytic activity (Winkler 1996).

Research suggests that hemostatic changes contribute to the increased risk of CVD after menopause (Meilhan et al.1992). Loss of estrogen is accompanied by large increases in factor VII coagulant activity, plasma fibrinogen, and in PAI-1 (Winkler 1996). Postmenopausal women tend to have a 6 to 10 percent higher concentration of factor VII coagulant activity and fibrinogen when compared to premenopausal women of the same age (Meade et al. 1983). The type of menopause also influences the extent of the increase in these factors, with greater increases in factor VII coagulant activity and fibrinogen occurring in women who have experienced natural versus artificial menopause (Meade et al. 1990).

Exogenous sex hormones during menopause. The effect of exogenous sex hormones on coagulation/fibrinolysis is dose- and structure-dependent. Synthetic estrogen in the form of oral contraceptives has been associated with an increase in factor VII concentrations, but consistent effects have not been reported for Lp(a) (Meilhan et al. 1992).

Estrogen in the form of 17 β-estradiol or ERT has been shown to decrease Lp(a) concentrations by 24 percent, but this drop is not maintained after 12 weeks of treatment (Shewmon et al. 1994). Factor VII has also been shown to increase primarily with synthetic oral estrogens, whereas transdermal estrogens have not been shown to significantly alter coagulation/fibrinolytic factors, likely due to their lack of first pass through the liver. Overall, chronic ERT has not been shown to improve blood flow through a main coronary artery, suggesting a possible lack of effect of treatment on coagulation/fibrinolytic functioning (Guzzo et al. 1999). With the addition of progesterone, HRT has some positive effects in improving fibrinolysis (Lijen and Collen 1996). After one year of cyclic or continuous HRT administration, Andersen et al. (1999) found a decrease in fibrinogen, tissue plasminogen activator, PAI-1, and Lp(a), but an increase in factor VII coagulant activity. However, this increase in factor VII with HRT is not as dramatic as the increase noted with ERT. Wright et al. (1997) reported significantly higher mean factor VII coagulant activity concentrations in ERT (144%) compared with HRT users (116%). Those postmenopausal women not taking any form of replacement hormones had an intermediate increase in factor VII coagulant activity (130%). It is hypothesized that estrogen may increase factor VII concentrations through increased activation while the addition of progesterone abolishes this rise (Wright et al. 1999). Overall, the implications of increases in factor VII with ERT or decreases with HRT are unclear. Estelles et al. (1999) found decreases in PAI-1 along with decreases in the inhibition of plasmin generation due to a decrease in Lp(a) concentrations with HRT administration. However, based on all available studies to date, it seems that there is an overall shift toward a procoagulant state during ERT/HRT administration. This may explain the increased risk of thromboembolism (van Baal et al. 2000) with hormone therapy.

Soy protein with isoflavones. Soy protein, particularly the isoflavone genistein, has been shown to interfere with platelet aggregation and thrombin action (Wilcox and Blumenthal 1995). Therefore, genistein may reduce thrombosis associated with atherosclerosis. Studies have shown that genistein hinders thrombosis by inhibiting tyrosine kinase activity (Ozaki et al. 1993), thus preventing thrombin-induced platelet activation and aggregation (Sargeant et al. 1993; Asahi et al. 1992). These studies focused on *in vitro*

mechanisms, whereas further studies to examine the *in vivo* affects of soy with isoflavones on the coagulation system should be conducted.

Oxidative balance: Mechanisms; menopause, estrogen/hormone replacement therapy, and soy

Oxidative stress may be defined as a disturbance in the equilibrium status of prooxidant/antioxidant systems in intact cells. A balance between the generation of prooxidant and the detoxification by antioxidants occurs during normal production of radical species. If dietary antioxidants are depleted or excess radicals are formed due to disease, oxidative stress may occur causing damage to carbohydrates, nucleic acids, proteins, and lipids. Oxidative stress is currently being studied for its role in the development and progression of human disease, including atherosclerotic CVD. This section will discuss the types of radical species and their actions on macromolecules. The antioxidant defense systems that include enzymes and vitamins are reviewed next. The antioxidant properties of estrogen and the changes during menopause are reviewed in relation to CVD risk. Soy and their isoflavones with their antioxidant effect in preventing lipid peroxidation conclude this section.

Radical species

A radical species is defined as any atom capable of independent existence that contains one or more orbital electrons with unpaired spin states. Radicals may be very reactive with other biomolecules or may be relatively inert. Radicals may be composed of part of a large biomolecule, such as carbohydrate or lipid, or a small molecule, such as oxygen. Reactive molecules that contribute to the oxidative stress of aerobic cells include oxygen and its radical derivatives, transition metals such as iron and copper, and nitrogen radicals (Cheeseman and Slater 1993). Table 3 lists reactive species as discussed in the text.

Radicals of oxygen. Radicals of oxygen are formed by one-electron reductions starting with atmospheric oxygen. Atmospheric oxygen has orbital electrons with the same spin states; thus, atmospheric oxygen is not very reactive with biomolecules. Oxidation of atmospheric oxygen by cellular processes, including mitochondrial electron transport systems, produces the superoxide anion. Additional sources of the superoxide anion may be from the action of phagocytic cells as they destroy foreign organisms or xanthine

dehydrogenase/oxidase reactions. The superoxide anion is not very reactive alone, but may combine with other reactive species to yield a highly reactive radical. The superoxide ion is involved in both the initiation and termination of lipid peroxidation (McCord 1993). The fate of the superoxide anion involves the rapid conversion to hydrogen peroxide, the next one-electron reduction in line for the oxygen species. Hydrogen peroxide is considered a nonradical oxygen species since it has no unpaired electrons. Sources of hydrogen peroxide are similar to that of the superoxide anion. Metabolic roles of hydrogen peroxide include the iodination of thyroid hormones via the peroxidase enzyme in the thyroid gland and the expression of certain genes that play a role in immune function. Further reduction of hydrogen peroxide yields the hydroxyl radical. This radical is highly reactive with all biomolecules and is considered the most toxic of the partially reduced oxygen species, causing structural damage to macromolecules and breakage of DNA strands (Santanam et al. 1998). Singlet oxygen, a second form of oxygen, is the final result of further oxidation of other partially reduced oxygen species. Singlet oxygen differs from atmospheric oxygen since its pair of electrons is not in the same spin state and therefore is more reactive (Cheeseman and Slater 1993).

Table 3. Reactive species[1]

Name	Symbol
Singlet oxygen	O_2
Superoxide radical	$O_2^{\cdot -}$
Hydrogen peroxide	H_2O_2
Hydroxyl radical	·OH
Nitric oxide	NO·
Peroxyl radical	ROO·
Perhydroxyl radical	HOO·

[1] From Rice-Evans and Burdon 1993

Transition metals. Transition metals interact with oxygen and its derivatives in reversible redox reactions to generate free radicals (Halliwell 1993). Pathological production of superoxide liberates iron from a bound state; unbound iron is then available to catalyze the Haber-Weiss reaction, which produces the hydroxyl radical. Free iron may also serve to initiate lipid peroxidation (McCord 1993). Reduced iron and copper are more reactive with hydrogen peroxide than the oxidized forms of these metals (Cheeseman and Slater 1993).

$$Fe^{3+} + O_2^{\cdot -} \rightarrow Fe^{2+} + O_2$$

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \rightarrow {}^{\cdot}OH + OH^- + Fe^{3+}$$

Nitric oxide radicals. Nitrogen radicals are in the form of nitric oxide (NO) and are generated from the oxidation of arginine via nitric oxide synthase. Several compounds, such as calcium and calmodulin, along with the process of phosphorylation, regulate NO synthesis (Bredt and Snyder 1994). Nitric oxide functions both as a prooxidant and antioxidant. Nitric oxide serves as a prooxidant by inhibiting peroxidase enzymes, which serve as potential initiators of lipid peroxidation. Nitric oxide also serves as an antioxidant by inhibiting lipid peroxidation through its action as a scavenger for lipid peroxyl radicals (Hogg and Kalyanaraman 1999). Another role of NO that may attenuate CVD risk is due to inhibition of platelet aggregation and hence regulation of the vascular system (Bredt and Snyder 1994). Nitric oxide functions as a biological signal in smooth muscle relaxation, neurotransmission, and immune regulation. Some of the prooxidant biological effects may be due to NO combining with another reactive molecule, such as superoxide anion, to produce significant amounts of peroxynitrite (ONOO), which in turn mediates the oxidation of both non-protein and protein sulfhydryls (Radi et al. 1991).

$$O_2^{\cdot -} + NO \rightarrow ONOO^-$$

$$ONOO^- + H \leftrightarrow ONOOH$$

$$ONOOH \rightarrow OH + NO_2$$

$$\updownarrow$$

$$NO_3^- + H^+$$

Radical species and macronutrients. The overall reactivity of free radicals depends on the type of radical and what challenge is presented to the radical. Two radical species may react to join their unpaired electrons to form a covalent bond, the result of which is a more reactive molecule. When a free radical reacts with a nonradical, a new free radical forms, causing the start of a chain reaction. Most biological reactions are nonradical, so interaction with a radical such as the hydroxyl radical can result in much damage if antioxidant defense systems are suppressed (Halliwell 1999). Radical species especially may have detrimental effects on macromolecules, possibly leading to disease states.

When carbohydrates react with free radicals, a hydrogen atom is removed from one carbon atom leaving a carbon-centered radical. The loss of hydrogen atoms causes molecules to lose their structure and function. Hyaluronic acid, a polysaccharide, is an example of such a molecule affected by radical action on a carbohydrate. Hyaluronic acid is degraded by the superoxide anion-generating systems resulting in free radical production. Changes in the viscosity of hyaluronic acid and the build-up of radicals in the synovial fluid surrounding the joints can induce inflammation (Strukov 1983). This process accounts for a decrease in synovial fluid and may be linked to rheumatoid arthritis.

Nucleic acids undergo base modifications when reacting with oxidizing radical species near their vicinity. Free radical species produced from the reaction of free radicals with amino acids or other organic molecules can damage DNA. Hydroxyl radicals in particular react with DNA to produce carbon-centered radicals that can then add oxygen to form peroxyl radicals. These modifications can lead to cell death, reproductive death, mutation, and carcinogenic transformation (Bohr et al. 1998).

Proteins are not as prone to damage as are lipids. Protein reacting with a radical species causes fragmentation due to many reactive sites on the protein molecule. Damage to a cell is only significant if the fragments are allowed to accumulate in the cell or if certain sites are affected (Cheeseman and Slater 1993). Proteins with metal-binding sites are especially susceptible to oxidative stress, as these sites interact with other metals. Modification of proteins can signal them to be degraded or possibly protect them, depending on which amino acid is affected. Degradation by the oxidation of a thiol group can lead to the loss of enzyme function, of ion transport, and/or of contractile function of proteins. The

protective nature of the amino acids cysteine and methionine is due to reverse modification to different forms of amino acids, which eventually causes the detoxification of oxidant species (Thomas 1999).

Lipids are the most susceptible class of biomolecules to attack by free radicals. The chain reaction process caused by the oxidizing radicals is called lipid peroxidation, or the oxidative destruction of polyunsaturated fatty acids. Table 4 delineates the steps of lipid peroxidation. The types of lipids and lipoproteins most affected in cells are those involved in membrane structure. Once these lipids and lipoproteins are damaged, serious impairment of cell function occurs. A more detailed list of consequences of lipid peroxidation is summarized in Table 5.

Table 4. Steps of lipid peroxidation[1]

Step	Reaction
Initiation	lipid + R/OH · $\rightarrow$ lipid ·
Propagation	lipid · + O_2 $\rightarrow$ lipid-OO ·
	lipid-OO · + lipid $\rightarrow$ lipid-OOH + lipid ·
Termination	lipid · + lipid · $\rightarrow$ lipid-lipid
	lipid-OO · + lipid · $\rightarrow$ lipid-OO-lipid
Scavenging	lipid · + vitamin E $\rightarrow$ lipid + vitamin E ·

[1] From Thomas 1999

Table 5. Consequences of lipid peroxidation[1]

Loss/degradation of polyunsaturated fatty acids
Altered membrane permeability and membrane-associated enzymes
Altered ion transport
Generation of cytotoxic metabolites of lipid hydroperoxides
Release of material from subcellular compartments (e.g. lysosomal enzymes)

[1] From Rice-Evans and Burdon 1993

Oxidized LDL is considered a risk factor for CVD (Berliner and Heinecke 1996; Regnström et al. 1994), and is a result of lipid peroxidation via free radicals. The first reaction of lipid peroxidation is termed initiation, involving the removal of a hydrogen atom from a polyunsaturated fatty acid by a free radical. A carbon-centered lipid radical results, reacting with oxygen in the next reaction to yield a lipid peroxyl radical. Chain propagation begins by the peroxyl radical extracting a hydrogen atom from a nearby lipid, resulting in a lipid hydroperoxide and a new lipid radical. The rate of lipid peroxidation is dependent upon the presence of antioxidants and the rate of termination. Antioxidants, mainly phenolic, scavenge the lipid peroxyl radical to yield a phenoxyl radical with low reactivity that will not propagate the lipid peroxidation chain. Natural termination occurs when two lipid peroxyl radicals combine to form a nonradical product and oxygen (Cheeseman and Slater 1993).

Once the oxidized LDL is formed by lipid peroxidation, it is not taken up by classical LDL receptors, but is instead taken up by macrophages via a scavenger receptor (Steinberg 1996). After being deposited in arterial walls, oxidized LDL releases cytotoxic lipid peroxidation products resulting in damage to the endothelial cell layer. This damage invokes endothelial cell death, platelet aggregation, accumulation of inflammatory cells, and increased infiltration of LDL. T-lymphocytes are oxidized by oxidized LDL, which in turn stimulates the proliferation of smooth muscle cells. Thus, oxidized LDL plays a central role in atherogenesis (Jialal and Devaraj 1996).

Lipid peroxidation also impairs the function of HDL-C, causing it to lose its protective effect against elevated serum cholesterol. Oxidized HDL particles are not taken up by macrophages and do not lead to foam cell formation, unlike oxidized LDL. Instead, once HDL is modified by oxidation, this lipoprotein has an impaired ability to stimulate efflux of cholesterol from foam cells (Nagano et al. 1991) and extrahepatic tissues (Wakatsuki et al. 1998).

Antioxidant defense systems

Exposure to hydroxyl radicals is unavoidable since they are naturally made during the splitting of oxygen and hydrogen bonds in water due to ionizing radiation from the environment (Santanam et al. 1998). The highly reactive hydroxyl radical interacts with all biological molecules and thus damage is unavoidable. Antioxidant defense systems minimize

the production of the hydroxyl radical and subsequent damage. Examples of antioxidant defense systems include enzymes, plasma proteins, and dietary intake of antioxidants (Thomas 1999). Total plasma or serum antioxidant levels in normal human plasma are within the range of 1.30 to 1.77 mM (Miller et al. 1993). Components of the various antioxidant defense systems are specified in Table 6.

Table 6. Antioxidant defense systems[1]

Enzymes:
(i) **superoxide dismutase** – converts radicals to hydrogen peroxide
(ii) **catalase** – detoxifies hydrogen peroxide
(iii) **glutathione peroxidase** – detoxifies hydrogen peroxide and lipid peroxidases in the presence of reduced glutathione
Plasma proteins:
(i) **ceruloplasmin** – acts as an antioxidant by virtue of its ferroxidase activity
(ii) **albumin** – binds metals, especially copper but also iron weakly
(iii) **transferrin** – sequesters Fe^{3+} rendering it unavailable for catalyzing the Haber--Weiss reaction, initiating lipid peroxidation
(iv) **heptoglobin/hemopexin** – binds free heme
Dietary intake:
(i) **alpha tocopherol** – scavenges peroxyl radical intermediates
(ii) **ascorbic acid** – water soluble antioxidant; acts synergistically with tocopherol
(iii) **beta-carotene** – mechanism undefined

[1] From Rice-Evans and Burdon 1993

Enzymes. *Superoxide dismutase* functions as an enzymatic antioxidant defense system. Superoxide dismutase is present in human cells as a copper-zinc-containing enzyme in the cytoplasm and as a manganese-containing enzyme in the mitochondria. Superoxide dismutase is responsible for minimizing intracellular superoxide radicals by converting the radical to hydrogen peroxide (Fridovich 1989):

$$2O_2^{\cdot -} + 2H^+ \rightarrow H_2O_2 + O_2$$

The concentration of intracellular superoxide dismutase must be kept in balance since too little may be lethal while excess may result in hydrogen peroxide toxicity. The genetic overexpression or therapeutic administration of superoxide dismutase may cause metabolic problems or exacerbate an injury, which illustrates the importance of maintaining optimal concentrations of this enzyme (McCord 1993). Once hydrogen peroxide is formed, catalase converts this toxic nonradical to water and oxygen. Catalase is a heme protein formed in peroxisomes of most cells or in the cytoplasm of the erythrocytes (Thomas 1999).

Glutathione and reactive sulfur-containing proteins are also participants in the enzymatic antioxidant defense system. Glutathione detoxifies by reacting with harmful by-products of aerobic life, hydrogen peroxide, and organic peroxides. Glutathione is the major intracellular soluble antioxidant in the cytoplasm, nuclei, and mitochondria. Enzymes mediate the glutathione redox cycle, which cause glutathione to cycle between a thiol form and an oxidized form. Glutathione peroxidase, a selenium-containing enzyme, catalyzes the oxidation of glutathione (GSH) by hydrogen peroxide to glutathione disulfide (GS-SG):

$$2GSH + H_2O_2 \longrightarrow GS\text{-}SG + 2H_2O$$

Other enzymes using lipid peroxides may also catalyze this oxidative reaction rather than using hydrogen peroxide as the oxidant, indicating that glutathione detoxifies both soluble and lipid peroxides:

$$2GSH + LOOH \longrightarrow GS\text{-}SG + LOH + H_2O$$

To complete the cycle, glutathione disulfide is reduced to glutathione via glutathione reductase using NADPH as the reductant (Thomas 1999):

$$GS\text{-}SG + NADPH + H^+ \longrightarrow 2GSH + NADP$$

Glutathione's antioxidant effect depends upon its cellular concentration, which may vary due to the extent of nutrient limitations, exercise, and oxidative stress (Meister 1988).

Reactive protein sulfhydryls participate in detoxification via the protein S-thiolation cycle. Protein sulfhydryls are found in soluble and membrane-bound proteins. Oxidative stress modifies these proteins to create mixed disulfides with attached glutathione (S-thiolation). The sulfur atom easily loses a single electron, resulting in a sulfur radical species with a longer lifespan, compared to other radical species resulting from oxidative stress (Thomas 1999).

Plasma proteins. Human cells have specific enzyme antioxidant defense systems, whereas plasma contains little of these enzymes; thus, oxidative stress affects mostly lipoproteins, such as LDL. Protein constituents and other small molecules exert protective antioxidant properties. Important proteins that serve to sequester potentially damaging transition metals, such as iron and copper, are *ceruloplasmin*, *albumin*, and *transferrin*. If unbound to proteins, iron and copper can participate in generating the most reactive radical species, the hydroxyl radical. *Heptoglobin* and *hemopexin* proteins bind the source of iron that is involved in lipid peroxidation, free heme. However, it is unlikely that extracellular free metal ions exist under normal arterial conditions (Thomas 1999). The proteins may have a protective effect, particularly during pathological conditions when metal ions might be available (Berliner and Heinecke 1996).

Dietary intake. Vitamins also serve as free radical scavengers within membranes and lipoproteins. *Vitamin E* (α-tocopherol) slows the chain reaction of lipid peroxidation by scavenging the peroxyl radical intermediates. The resulting α-tocopherol radical is less reactive than peroxyl radicals and may be recycled to α-tocopherol by the action of ascorbic acid (Diaz et al. 1997). The antioxidant effect of *ascorbic acid* is also demonstrated by its detoxification of inhaled air pollutants (i.e., ozone, nitrogen dioxide). Ascorbic acid also has prooxidant effects in the presence of transition metal ions by reducing them and subsequently generating free radicals. However, metal ions are not readily available *in vivo*, and thus ascorbic acid exerts an overall antioxidant effect (Jacob 1999). High plasma levels of *β-carotene* have been linked to a decrease in cancer and CVD. The mechanism of β-carotene as an antioxidant is in question, since antioxidant effects have been documented *in vitro*, whereas the mechanism of β-carotene's effect *in vivo* remains unknown. All of these dietary constituents have antioxidant characteristics *in vitro* and *in vivo* by reducing the oxidative susceptibility of LDL (Diaz et al. 1997; Jialal and Fuller 1995).

Menopause, estrogen/hormone replacement therapy, and soy

Endogenous sex hormones during menopause. Estrogens produce both prooxidant and antioxidant effects, with potency being structure-dependent (Klein and Berlin 1997). The prooxidant effects of estrogens occur when they are converted to catecholestrogens upon oxidation during the redox cycle. Reactive oxygen species result from this cycle, which may

induce damage to DNA, proteins, and lipids. One condition when an antioxidant, such as estrogen, acts as a prooxidant is during peroxidase-catalyzed oxidation reactions. During this type of reaction, antioxidants form phenoxy radicals to promote oxidation by interaction with either apolipoprotein molecules of LDL or directly with lipids. Once oxidized, LDL may be rapidly cleared from the circulation by the liver. This process may aid in the clearance of LDL by the liver. Hence, estrogens, in the case of peroxidase-catalyzed oxidation reactions, may be antiatherogenic since the oxidation of LDL is promoted (Nathan and Chaudhuri 1998).

Estrogens may exert antioxidant effects by participating in the iron redox cycle. Estrogens alter iron chelation and prevent iron from inducing lipid peroxidation (Schwenke 1998). Estrogens also act on genes with response elements for antioxidants. By this mechanism, estrogens may inhibit expression of proteins involved in atherogenesis (Nathan and Chaudhuri 1998). Overall, *in vivo* physiological concentrations of estrogen may have modest antioxidant activity, whereas prooxidant activity is unlikely (Schwenke 1998). Shwaery et al. (1998) speculate that 17 β-estradiol in particular is responsible for the majority of the antioxidant protective effect in premenopausal women.

Exogenous sex hormones during menopause. The oxidation of HDL particles has been shown to decrease with estrogen *in vitro* (Wakatsuki et al. 1997); however, the cardioprotective effect of exogenous administration of ERT/HRT has been mainly attributed to the inhibition of peroxidation, which slows the uptake of oxidized LDL by macrophages (Mazière et al. 1991). Endogenous metabolites of estradiol have been found to be more potent inhibitors of LDL oxidation than both exogenous estradiol and vitamin E combined *in vitro* (Seeger et al. 1998). However, equine estrogens are found to be more potent antioxidants than human estrogens and effectively inhibit fatty acid and cholesterol oxidation *in vitro* (Nathan and Chaudhuri 1998).

In vitro administration of supraphysiological concentrations of 17 β-estradiol have an inhibitory effect on both copper and cell-mediated oxidation of LDL (Mazière et al. 1991; Huber et al. 1990). *In vivo* studies with postmenopausal women involving various types of estrogen treatment have shown mixed results. Sack et al. (1994) found that administration of 17 β-estradiol at physiological levels has an antioxidant effect of inhibiting the oxidation of

LDL. However, McManus et al. (1997) found that transdermal estradiol, oral estradiol valerate, and conjugated equine estrogen all failed to support the antioxidant effect of estrogens. Explanations for this lack of effect *in vivo* include: 1) concentrations of estrogens necessary for reduced lipoprotein oxidation must be 10^4 to 10^6 times greater than that found in the plasma of premenopausal women, or as a result of HRT in postmenopausal women and/or 2) estrogens also increase the proportion of small, dense LDL particles, which would increase their susceptibility to oxidation and increase their rate of uptake, with no net change in oxidized LDL concentration. It is unclear whether estrogens are in fact incorporated into LDL particles deposited in the arterial wall and in turn affect production of free radicals and oxidative modification of LDL. More studies are needed to determine the dose of estrogen needed to exert an antioxidant effect and the mechanism of estrogen's purported antioxidant activity (McManus et al. 1997).

Soy protein with isoflavones. Flavonoids, particularly isoflavones, exert antioxidant properties through reversible and irreversible electron transfer reactions. The antioxidant capacity of isoflavones is due to their ability to donate hydrogen atoms and/or electrons from their hydroxyl groups to free radicals (Mitchell et al. 1998). The extent of flavonoid antioxidant activity depends on the reduction potentials of their radicals and their availability at the site of pathological oxidative processes. Flavonoids have been specifically studied in relation to their inactivation of the superoxide radical. Through this mechanism, flavonoids inhibit lipid peroxidation and singlet oxygen quenching that in turn may cause damage to DNA, alter respiration, and damage the endothelium (Jovanovic et al.1998). The major aglycone forms of isoflavones, genistein, daidzein, and glycitein, are known to exert antioxidant effects due to their unique structures. However, the antioxidant activity does not seem to be dictated by form, since activity of the aglycone forms of the isoflavones are comparable or superior to their parent forms (genistin, daidzin, and glycitin) (Arora et al. 1998). Genistein is the most potent antioxidant among the isoflavones and is approximately 40% more powerful than daidzein due to structural differences (Rice-Evans and Miller 1998). Genistein exhibits antioxidant properties by preventing lipid peroxidation induced by the iron redox cycle (Cai and Wei 1996). Kapiotis et al. (1997) found human endothelial cell-mediated LDL oxidation *in vitro* to be inhibited upon incubation with genistein.

Summary

In summary, dyslipidemia, altered coagulation/fibrinolysis, and oxidative stress are viewed as CVD risk factors in mid-life women. Estrogen concentrations in premenopausal women serve to: 1) exert a beneficial effect on circulating lipids, 2) favorably alter coagulation/fibrinolysis, and 3) maintain oxidative balance. Declining 17 β-estradiol concentrations during menopause are related to adverse changes in the lipid profile, increased coagulation activity, and increased oxidative stress. Hormone therapy favorably alters the lipid profile and the balance between coagulation and fibrinolysis. However, based on available studies to date, there is an overall shift towards a procoagulant state with ERT, likely explaining the increased risk of thromboembolism. Studies have produced mixed results as to whether or not hormone therapy exerts an antioxidant effect on circulating lipids/lipoproteins. Alternatives for hormone therapy that reduce CVD risk but do not impose further risk, as is associated with HRT/ERT, are currently being investigated. Soy protein, particularly in conjunction with its isoflavones (estrogen-like compounds), has been documented to exert a hypocholesterolemic as well as an antioxidant effect. Genistein in particular induces fibrinolytic enzyme activity, thus favoring fibrinolysis. Researchers are currently investigating whether consumption of soy protein with isoflavones or isoflavones alone in peri- and postmenopausal women will have beneficial effects similar to HRT/ERT. More research is needed to determine if soy protein, whether or not due to its isoflavone content, consistently favorably alters the lipid profile, coagulation/fibrinolysis, and oxidative balance in normo- and hypercholesterolemic peri- and postmenopausal women.

CIRCULATING LIPIDS/LIPOPROTEINS AND COAGULATION/FIBRINOLYTIC FACTORS IN PERIMENOPAUSAL WOMEN TREATED WITH SOY PROTEIN

A paper to be submitted to American Journal of Clinical Nutrition.

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ABSTRACT

Background: Soy protein has been shown to favorably alter serum lipids/lipoproteins, thereby reducing cardiovascular disease (CVD) risk.

Objective: The primary purpose was to determine the effect of soy protein (40 g/d) on circulating lipids/lipoproteins and coagulation/fibrinolytic factors in perimenopausal women. The secondary purpose was to determine the contribution of coagulation/fibrinolytic and other factors (e.g., body size/composition; physical activity; blood pressure; serum estrogens, ferritin, iron; and dietary intake) to lipids/lipoproteins.

Design: Subjects were randomly assigned, double-blind, to treatment: isoflavone-rich soy protein (n=24), isoflavone-poor protein (n=24), or whey protein control (n=21). We measured circulating lipids/lipoproteins at baseline, wk 12, and wk 24, and coagulation/fibrinolytic factors at baseline and wk 24.

Results: Treatment did not alter lipids/lipoproteins or coagulation/fibrinolytic factors. Time had an effect on HDL cholesterol ($P \leq 0.0001$) and triacylglycerol ($P=0.024$). Approximately 43% of the variability in total cholesterol, 34% in triacylglycerol, 57% in HDL cholesterol,

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and 35% in lipoprotein(a) was accounted for by the factors examined. Lean body mass, serum 17 β-estradiol, and dietary factors (vitamin E, selenium, fiber, and folate) had beneficial effects ($P<0.05$), whereas plasma fibrinogen and plasminogen activator inhibitor-1, diastolic blood pressure, body weight, and serum liver enzymes and ferritin had detrimental effects on lipid/lipoprotein concentrations.

Conclusions: Isoflavone-rich or –poor soy protein had no effect on lipid/lipoprotein or coagulation/fibrinolytic factors, whereas the effect of time suggested that the hormonal milieu during the menopausal transition may have overridden any detectable treatment effect. The unconventional contributors to the lipid profiles identified in this study should be further examined as indices of CVD risk in mid-life women.

KEY WORDS Cardiovascular disease risk, Factor VII, Fibrinogen, Isoflavones, Lipoprotein(a), Plasminogen activator inhibitor-1, Soy protein

INTRODUCTION

Perimenopause is considered a critical period in which preventive efforts to reduce the menopause-associated increase in cardiovascular disease (CVD) risk should be initiated (Matthews et al. 1994). Soy isoflavones, estrogen-like substances structurally similar to 17 β-estradiol, have been reported to favorably alter lipid profiles in perimenopausal women (Washburn et al. 1999) and postmenopausal (Potter et al. 1998) women. In contrast, other studies have not shown a beneficial effect of isoflavones (Hodgson et al. 1998; Simons et al. 2000; Nestel et al. 1999) on circulating lipids and lipoproteins. Isoflavones, specifically genistein, also have been reported to favorably alter fibrinolysis by interfering with platelet aggregation and thrombin action *in vitro* (Wilcox and Blumenthal 1995). Yet, there are no published data on the effect of soy protein or its isoflavones on coagulation/fibrinolytic activity in mid-life women. Lipids and lipoproteins modulate the expression or function of coagulation and fibrinolytic factors, establishing their role in atherogenesis (Rosenson and Lowe 1998). Several coagulation factors are directly associated with CVD (Kelleher 1992), as evidenced by the reactivity of coagulation factors and the effectiveness of protective fibrinolysis (Rosenson and Lowe 1998) following the thrombotic response to an

atherosclerotic plaque rupture and/or erosion. Although studies have examined the relationship between lipids/lipoproteins and coagulation/fibrinolytic factors in atherogenesis, data are lacking in women during the very early stages of menopause. Thus, we determined the contribution of coagulation/fibrinolytic factors (plasma fibrinogen, plasminogen activator inhibitor-1 [PAI-1], and factor VII antigen), along with other pertinent factors (such as body size and composition; physical activity; blood pressure; serum estrogens, ferritin, and iron; dietary intake of fat, alcohol, fiber, and antioxidants), to total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol, total/HDL cholesterol, and lipoprotein(a) [Lp(a)] concentrations in perimenopausal women at baseline.

SUBJECTS AND METHODS

Research design and treatment

This 24-wk double-blind study examined the effects of SPI+ (Protein Technologies International; St. Louis, MO), isoflavone-poor protein (SPI -; Protein Technologies International; St. Louis, MO), and whey protein (control; Ross Laboratories; Columbus, OH) on circulating lipids/lipoproteins and coagulation/fibrinolytic factors in perimenopausal women. Each perimenopausal woman was randomly assigned to one of three treatment groups: SPI+ (80.4 mg/d aglycone components; n=24), SPI - (4.4 mg/d aglycone components; n=24), or control (n=21). The whey protein served as a control for protein, whereas the SPI - served as a control for soy protein. As free-living subjects, the subjects were asked to consume a total of 40 g protein/d provided in a jumbo muffin and as protein powder. The muffins were baked in the Human Metabolic Unit of the Center for Designing Foods to Improve Nutrition at Iowa State University. The subjects were instructed to consume the muffin and protein powder as a meal replacement and not as a supplement, since they were provided an additional ~2.09 MJ (500 kcal)/d. The subjects also received instruction on how to avoid other isoflavone-containing foods. A daily over-the-counter vitamin and mineral supplement was provided; the women were instructed to stop taking any of their own supplements before the study began. An informational meeting was held prior to baseline testing where the women were informed of their rights as volunteers in this study, signed consent forms, and were given a medical release form to be signed by their

physicians. The Iowa State University Human Subjects Review Committee (IRB# 01; Assurance ID# M1361) approved this project's protocol and consent forms. Every six wks, the women were required to visit the Human Metabolic Unit for testing, replenishment of supplies, and for submitting 24-hr urine samples.

Subject screening, selection, and characteristics

Subjects were recruited throughout the state of Iowa through newspaper and bulletin board advertisements, local television news stories, and newspaper articles. Telephone interviews were conducted to screen potential perimenopausal women to ensure they met our inclusion/exclusion criteria: experiencing $\geq$10 hot flushes and/or night sweats per wk, had irregular menses or cessation of menses for less than one y, had one or both ovaries remaining, had a body mass index (BMI) (kg/m^2) between 19 and 31, were willing to be randomly assigned to treatment, and able to participate for 24 wks. Women were excluded if they had a chronic disease (i.e., heart disease or osteoporosis), were taking medications chronically, had taken hormone replacement therapy or estrogen replacement therapy during the past 12 months, had a history of an eating disorder or menstrual disorder, and/or were excessive exercisers (>10.46 MJ [>2500 kcal] expenditure/wk). Once potential subjects qualified after the initial screening, blood was drawn to ensure that follicle stimulating hormone concentrations were $\geq$30 IU/L (Wilson et al. 1998); 22 were excluded on this basis. Women discontinued participation due to inability to tolerate treatment (n=6), medical conditions preventing continuance (n=2), death (n=1), or death in the family (n=1); we dropped one woman due to noncompliance. Because of our strict inclusion/exclusion criteria, we recruited women in four waves or cohorts, with subjects randomly assigned to treatment within each cohort. The cohorts began in January 1997, May 1997, September 1997, and March 1998.

Data collection and measurement

Information on health and medical history and physical activity was obtained using interviewer-administered questionnaires. The health and medical history was used to rule out women with chronic or acute conditions or diseases and those who chronically used various drugs (nonprescription, prescription, or illegal). The Paffenbarger Physical Activity Recall (Paffenbarger et al. 1978) was used to obtain information on weekly physical activity during

the previous year. Each recreational activity was summed to provide an estimate of weekly energy expenditure. Dietary intake was assessed at baseline, wk 12, and wk 24 using five-d food records. To assist the subjects in quantifying portion sizes, two-dimensional food portion visual aids (Nutritional Consulting Enterprises, Morgan/Posner, 1981; Framingham, MA) were provided. The food records were analyzed by trained nutrition students using the Nutritionist IV computerized nutrient database program (Version 4.1, 1995; First DataBank, Inc., San Bruno, CA). The vitamin and mineral supplement provided to the subjects was not included in these analyses.

Anthropometry data included measurement of height (using a stadiometer) and weight (using a balance beam scale [Health-o-meter, Inc.; Bridgeview, Illinois]). Dual-energy x-ray absorptiometry via QDR-2000+ (Hologic, Inc.; Waltham, MA) was used to assess total body composition (lean and fat mass). Two researchers trained by Hologic, Inc. performed all scans; one of these researchers analyzed all total body scans with software provided by the manufacturer (Version 7.10, 1992; Waltham, MA). Trained personnel used a random-digit sphygmomanometer (Marshall Electronics, Inc.; Skokie, IL) to measure resting blood pressure.

Each subject collected a 24-hr urine sample in polyethylene containers the day prior to each visit to the Human Metabolic Unit. After the first morning's void, all urine was collected, including the next morning's void. Total volume was measured and recorded to the nearest 10 ml. Aliquots in 5 ml vials were frozen at -80°C for shipment to Fujicco Co. Ltd. (Kobe, Japan), where urinary isoflavones were analyzed by reversed-phase HPLC to monitor compliance.

Fasted blood was drawn in the early morning from each subject at baseline, wk 12, and wk 24 and serum (1000 x *g* for 15 min at 4°C) or plasma (1200 x *g* for 10 min at 4°C) was separated for measurement for the following blood analytes. Quest Diagnostics (St. Louis, MO), a certified clinical laboratory, performed the serum total cholesterol, LDL cholesterol, HDL cholesterol, and triacylglycerol measurements at each time point. LDL cholesterol concentrations were calculated using the Friedewald equation: (total cholesterol) – (HDL cholesterol) – (triacylglycerol/5) (Friedewald et al. 1972). Plasma Lp(a), factor VII antigen, and fibrinogen were analyzed under the supervision of Dr. Larry Brace in the

Hematology and Coagulation Laboratory at the University of Illinois at Chicago. Plasma Lp(a) [TintElize®Lp(a)] concentrations were measured at baseline, wk 12, and wk 24, using an immunoenzymetric method with affinity purified polyclonal antibodies against Lp(a), according to the manufacturer's (Biopool, International; Ventura, CA) guidelines. Lp(a) concentrations were read using an automated microtiter plate reader (EL311sx, Bio-Tech Instruments, Inc.; Winooski, VT). The remaining coagulation/fibrinolytic factors were measured from baseline and wk 24 plasma samples. Factor VII antigen (Asserachrom®VII:Ag) concentrations were measured by an enzyme immunoassay procedure for the quantitative determination of factor VII by the sandwich technique according to the manufacturer's (Diagnostica Stago; Asinieres-Sur-Seine, France) guidelines. Factor VII antigen concentrations were read with the automated plate reader. A certified technician from Diagnostica Stago performed the analyses on factor VII coagulant activity, PAI-1, and fibrinogen. Plasma factor VII coagulant activity (Staclot®VIIa-rTF) was quantitatively determined by measuring the clotting time of plasma after exposure to recombinant soluble tissue factor according to the manufacturer's (Diagnostica Stago; Asinieres-Sur-Seine, France) guidelines. We were not able to measure factor VII coagulant activity in samples from the last cohort because of technical difficulties. Because PAI-1 is cold temperature-sensitive, the plasma samples for PAI-1 were not placed on ice, but were centrifuged and kept at room temperature until frozen. PAI-1 (Stachrom®PAI) was quantitatively determined by the synthetic chromogenic substrate method according to the manufacturer's (Diagnostica Stago; Asinieres-Sur-Seine, France) guidelines. Fibrinogen was measured by a Clauss method using Diagnostica Stago equipment and reagent according to the manufacturer's (Diagnostica Stago; Asinieres-Sur-Seine, France) guidelines. Factor VII coagulant activity, PAI-1, and fibrinogen were analyzed using the STA-R automated coagulation analyzer (Diagnostica Stago; Asinieres-Sur-Seine, France).

Serum ferritin was determined using an enzyme-linked immunoassay obtained from RAMCO Labs (Houston, TX). Quest Diagnositics (St. Louis, MO) measured serum concentrations of liver enzymes (aspartate aminotransferase and alanine aminotransferase), iron, 17 β-estradiol, and estrone.

Statistical analyses

Statistical analyses were performed with PC SAS (Version 8, 1999; Cary, NC); results were considered statistically significant at $P<0.05$. Descriptive statistics included means for normally distributed data (total cholesterol, LDL cholesterol, and total/HDL cholesterol; age; body size and composition; blood pressure) and medians for data that were not normally distributed (triacylglycerol, HDL cholesterol, and Lp(a); physical activity; dietary intake of nutrients; coagulation/fibrinolytic factors). Repeated measures ANOVA was employed to determine the effect of treatment on total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol, total/HDL cholesterol, and Lp(a). Cohort was included in the analyses as a covariate to account for random assignment of subjects to treatment within each cohort. Residual analysis indicated non-constancy of error variance (it increased with greater $\hat{Y}_i$ values) for the triacylglycerol, HDL cholesterol, and Lp(a) regression models. Thus, these values were log-transformed for the repeated measures ANOVA and regression analyses. Log-transforming triacylglycerol, HDL cholesterol, and Lp(a) for the regression models markedly improved the residual plots. To determine the effect of treatment on fibrinogen, factor VII antigen, and PAI-1, ANOVA was used, with cohort as a covariate. Since fibrinogen and PAI-1 were not normally distributed, these values were log-transformed for the ANOVA.

Stepwise multiple regression was used to determine the effect of contributors for each outcome at baseline: total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol, total/HDL cholesterol, and Lp(a) concentrations. Classes of variables in modeling the outcomes of lipids/lipoproteins included baseline values for age, body size/composition (weight, lean mass, fat mass, percentage body fat), resting blood pressure (diastolic, systolic), dietary factors (fat [total g and as percentage of total energy], polyunsaturated fat, fiber, alcohol, vitamin A, vitamin C, vitamin E, alpha-tocopherol, iron, selenium), physical activity, and circulating analytes (serum iron, ferritin, aspartate aminotransferase, alanine aminotransferase, 17 β-estradiol, estrone, plasma PAI-1, fibrinogen, factor VII antigen). Factor VII coagulant activity was not included in the regression models due to the reduced sample size. Serum liver enzymes emerged as significant contributors and hence were included post-hoc. Violation of model assumptions was not evident, since residual analyses

indicated that the model assumptions of independence of residuals, normality of error terms, and homogeneity of residual variance were satisfied for these regression models. No notable multicollinearities emerged among the independent variables, as indicated by the low variance inflation factors in the regression analyses.

RESULTS

Compliance to dietary treatment

The urinary excretion of isoflavones (genistein and daidzein) and self-reported consumption of muffins (87% of subjects consumed 100%) and powder (84% of subjects consumed 100%) in each group reflected excellent adherence to the dietary treatment. However, one control subject's data was removed from all subsequent analyses due to her urinary excretion of isoflavones during treatment being similar to women in SPI+ group. Detailed information on compliance is described elsewhere (Alekel et al. 2000).

Lipid/lipoprotein and coagulation/fibrinolytic factor concentrations

Baseline characteristics of these perimenopausal women are presented in **Table 1**. The expected potential contributors to lipid/lipoprotein concentrations included age, body size and composition, physical activity, resting blood pressure, dietary intake of selected nutrients, plasma coagulation/fibrinolytic factors, and serum estrogens, ferritin, and iron. Since serum liver enzymes emerged as contributors to total cholesterol, triacylglycerol, and LDL cholesterol, values for these analytes at baseline are also presented. None of these characteristics at baseline were significantly different among treatment groups.

Treatment had no significant effect on circulating lipids/lipoproteins (**Figure 1**) or coagulation/fibrinolytic factors as shown in **Table 2**. However, time had a significant effect on HDL cholesterol, triacylglycerol, and total/HDL cholesterol. At wk 24, HDL cholesterol decreased in all groups, triacylglycerol increased in the SPI+ and control groups, and total/HDL cholesterol increased in all groups. Changes in triacylglycerol, HDL cholesterol, and Lp(a) concentrations over the 24 weeks are shown in Figure 1. Although Lp(a) concentrations nearly doubled from baseline to wk 24 in the control group, this increase was not significant because of the large variability in these values, particularly for this group. The

increase in factor VII coagulant activity was significant ($P \leq 0.0001$) from baseline to week 24 (Table 2). Cohort ($P=0.042$) had a significant effect on factor VII coagulant activity.

Since we did not demonstrate a treatment effect, we performed further analyses to examine the influence of purported CVD-related factors on baseline circulating lipids/lipoproteins in these perimenopausal women. Coagulation factors were included as predictors of circulating lipid/lipoprotein models due to their established relationship in acute cardiovascular syndromes (Rosenson and Lowe 1998). Factors that contributed to the variability of total cholesterol, LDL cholesterol, HDL cholesterol, triacylglycerol, total/HDL cholesterol, and Lp(a) at baseline are presented in **Table 3**. After variable elimination was completed, 43% of the variability in serum total cholesterol ($P \leq 0.0001$) was accounted for by serum aspartate aminotransferase, lean mass, resting diastolic blood pressure, dietary folate, and serum 17 β-estradiol. Plasma PAI-1, dietary vitamin E, and serum alanine aminotransferase accounted for greater than 33% of the variability in serum triacylglycerol ($P \leq 0.0001$). Plasma fibrinogen, serum iron, lean mass, dietary fat (g), and serum aspartate aminotransferase (albeit NS) accounted for more than 32% of the variability in serum LDL cholesterol ($P=0.0003$). Dietary fat (% of total energy), dietary fiber, cohort 2, dietary selenium, and plasma fibrinogen accounted for greater than 34% of the variability in plasma Lp(a) ($P \leq 0.0001$). Fifty-seven percent of the variability in HDL cholesterol ($P \leq 0.0001$) was accounted for by dietary vitamin E, plasma PAI-1, dietary fat (% of total energy), body weight, and serum ferritin. Almost 50% of the variability in serum total/HDL cholesterol ($P \leq 0.0001$) was accounted for by plasma PAI-1, dietary vitamin E, and plasma fibrinogen.

DISCUSSION

Dietary supplementation with SPI+ or SPI - for 24 wks did not significantly alter circulating lipid/lipoprotein and coagulation/fibrinolytic factor concentrations. Nonetheless, we identified a number of known and unconventional factors that contributed to the variability in lipid and lipoprotein concentrations at baseline in these perimenopausal women. Since postmenopausal women have increased CVD risk when compared to their premenopausal counterparts (Peters et al. 1999), the identification of specific risk factors is important in initiating preventive efforts early during the menopausal transition. To our

knowledge, this is the only published study examining the relationship between lipids/lipoproteins and coagulation/fibrinolytic factors in perimenopausal women.

Significant changes in HDL cholesterol, triacylglycerol, and total/HDL cholesterol during the 24-wk period were attributed to a time effect. Thus, any effect of treatment may have been overshadowed by hormonal changes during the menopausal transition, as evidenced by the effect of time (Table 2). Indeed, time had a significant effect ($P \leq 0.0001$) on serum 17 β-estradiol and estrone concentrations during the course of treatment. Although cohort did not reach statistical significance ($P=0.060$) for fibrinogen, it is noteworthy that season (perhaps reflected by cohort) has been shown to influence fibrinogen concentrations (Pearson et al. 1997). A significant effect of cohort emerged for factor VII coagulant activity. Cohorts 1 and 2 began in January and May, respectively, and both experienced an increase in factor VII coagulant activity; however, seasonal variations have not been reported to date. Our finding of a cohort effect may be simply due to the small sample size (N=46) for this particular measure.

Plasma PAI-1 was a strong contributor to triacylglycerol, HDL cholesterol, and total/HDL cholesterol concentrations at baseline. Similar to the findings in our study, the European Concerted Action on Thrombosis (ECAT) Angina Pectoris Study has also reported a positive correlation between plasma triacylglycerol and PAI- activity (Juhan-Vague et al. 1996). The underlying mechanism of the direct relationship between PAI-1 and triacylglycerol is unknown. However, triacylglycerol-rich lipoproteins are known to stimulate PAI-1 release from hepatic cells (Mussoni et al. 1992), perhaps corroborating our findings. The negative relationship between PAI-1 and HDL cholesterol in our regression model is similar to the negative correlation ($P \leq 0.0001$) reported in the ECAT Angina Pectoris study (Juhan-Vague et al. 1996). HDL cholesterol exhibits antioxidant properties through HDL-associated hydrolase, paraoxonase (Paragh et al. 1999), which may inhibit lipid peroxidation of LDL particles. It is through this mechanism that HDL cholesterol may attenuate LDL cholesterol-induced changes in the generation of fibrinolytic regulators, such as PAI-1, from vascular endothelial cells (Ren and Shen 2000). Our finding of a positive relationship between plasma fibrinogen and LDL cholesterol has also been reported in population (Cremer et al. 1994; Koenig et al. 1992) and cross-sectional (Møller and

Kristensen 1991; Halle et al. 1996) studies. These same population studies have reported a positive association between fibrinogen concentrations and Lp(a), possibly explaining in part the relationship of coagulation and fibrinolytic factors to atherogenesis. Fibrin, the end-product of fibrinogen conversion, provides an absorptive surface area for Lp(a) and LDL cholesterol to localize within plaque (Rabbani and Loscalzo 1994), perhaps illustrating Lp(a) and fibrinogen's involvement in atherogenesis. Researchers (Møller and Kristensen 1991) have also reported a negative correlation between fibrinogen and HDL cholesterol, possibly corroborating our finding that fibrinogen was positively related to the total/HDL cholesterol ratio. The serum liver enzymes, aspartate aminotransferase and alanine aminotransferase, were important positive contributors to total cholesterol, triacylglycerol, and LDL cholesterol, suggesting that as hepatic protein synthesis increased, lipoprotein synthesis likewise increased (Gopal and Rosen 2000). The positive relationship of the serum liver enzymes coincide with the positive association of the liver-derived coagulation/fibrinolytic factors with the atherogenic lipids/lipoproteins, emphasizing the role of the liver in lipoprotein metabolism.

Our study supports the idea that dietary factors in general were important contributors to circulating lipids/lipoproteins. It is well accepted that a high fat intake increases serum cholesterol (Goldberg and Schonfeld 1985), particularly LDL and HDL. The influence of dietary fat intake on Lp(a) is less well established, but we found that fat intake (as a percentage of total energy) was negatively related to Lp(a). It has been reported that omega-3 fatty acids, rather than total fat as in our study, reduce Lp(a) concentrations (Hajjar and Nachman 1996). It may be that as percentage of total energy from fat increases, the total amount contributed by omega-3 fatty acids likewise increases. However, we could not determine whether or not this was the case in our study since Nutritionist IV did not provide estimates of omega-3 fatty acid intake. Although dietary selenium was also negatively related to Lp(a), selenium has not been shown to be related to or reduce Lp(a). However, a parallel effect of low selenium and LDL cholesterol has been reported in the development of CVD (Salonen et al. 1992). Perhaps since Lp(a) and LDL are similar in structure, this parallel effect may also have been seen with low selenium and Lp(a). Interestingly, dietary fiber was also negatively related to Lp(a), whereas its effect on Lp(a) has produced mixed results.

Supplementation with pectin has been shown to decrease Lp(a) (Veldman et al. 1997) concentrations, but oat bran (Kelley et al. 1994) or oat husk fiber (Sundell and Ranby 1993) does not. These dietary influences on Lp(a) should be further explored. Vitamin E had a positive impact on triacylglycerol, HDL cholesterol, and total/HDL cholesterol concentrations, perhaps signifying the role of vitamin E in quenching lipid peroxidation (Henning and Alvarado 1993). Vitamin E administration has been documented to reduce lipid peroxide within cells, which leads to increased hepatic uptake of triacylglycerol-rich lipoproteins (Staprans et al. 1993). Finally, the negative relationship between total cholesterol and dietary folate may reflect its role in lowering homocysteine concentrations (Fanapour et al. 1999), although the direct connection between dietary folate and risk of coronary artery disease is debated (Siri et al. 1998; Boushey et al. 1995). Nonetheless, elevated homocysteine concentrations may damage vascular endothelial cells, thereby allowing damage and lipid accumulation (Fanapour et al. 1999), paralleling circulating lipid concentrations.

Most studies have reported the positive relationship between total cholesterol and fat mass (Rosenfalck et al. 1996), but our study demonstrated the negative relationship between lean mass and total cholesterol or LDL cholesterol, suggesting that lean mass *per se* may exert protective effects. Perhaps the effect of lean mass on lipids has not been reported because lean mass is typically not assessed, but rather fat mass (or percentage body fat) or another index of body mass is usually reported. In our study, BMI was negatively related ($r=-0.38$; $P=0.001$) to HDL cholesterol, but did not remain as a significant contributor to overall HDL cholesterol once additional factors were considered. Simple correlations between BMI or fat mass and other circulating lipids were less impressive. However, body weight was a significant contributor to HDL cholesterol; the inverse relationship in our study has been previously reported (Glueck et al. 1980; Hayashi et al. 1987; Chen et al. 1983). It is not surprising that physical activity did not emerge as a contributor to any lipid/lipoprotein, since this was not an exercise intervention study. The finding that resting blood pressure was directly related ($r=0.32$; $P=0.0008$) to total cholesterol may be corroborated by the association of reduced LDL cholesterol with the improvement in endothelium-dependent vasoreactivity in response to cholesterol-lowering drugs (Cohen et al. 2000). Elevated blood pressure can cause extravasations of plasma into the arterial wall, thus contributing to

cholesterol accumulation and the progression of atherosclerosis. Since the arterial wall cannot fully degrade and metabolize the build-up (Chappell and Spector 1991), cholesterol accumulates. However, we did not examine arterial cholesterol concentrations or vasoreactivity in this study.

The negative relationship between serum ferritin and HDL cholesterol has been previously reported (Salonen et al. 1992). Our results indicate that with higher iron stores (as reflected by circulating ferritin), HDL cholesterol was negatively affected, perhaps an indication of greater lipid peroxidation and hence greater removal of oxidized HDL particles via scavenger-receptor BI (Van Berkel et al. 2000). Although the iron bound to ferritin does not dissociate readily, perhaps it is a source of iron for redox reactions (Salonen 1993). Likewise, the negative relationship between serum iron and LDL cholesterol may be explained by the effect of iron on cholesterol oxidation. As serum iron increases, LDL cholesterol oxidation may also increase, thus leading to greater uptake of modified LDL particles by scavenger receptor pathways (Regnström et al. 1994). This in turn might have a cholesterol-lowering effect, explaining the negative relationship of serum iron and LDL cholesterol. However, since we did not document this same relationship for total cholesterol and LDL cholesterol is a calculated value, one must view these results with caution.

Our finding that serum 17 β-estradiol was negatively associated with total cholesterol (Pearson correlation: $r = -0.25$; $P=0.038$) at baseline was shown in other studies (Vagenakis 1989; Bonithon-Kopp et al. 1990) and illustrates the antiatherogenic role of estrogen. Throughout menopause, 17 β-estradiol concentrations decline (Cauley et al. 1989), thereby reducing the cardioprotective effect of estrogen in the peri- and postmenopausal years.

In hindsight, the stage at which we obtained blood samples may be too early in the menopausal transition to show marked adverse effects on circulating lipids/lipoproteins or coagulation/fibrinolytic factors, thus making it more difficult to demonstrate an effect of treatment. Matthews et al. (1994) reported that perimenopausal women did not have more adverse lipid/lipoprotein profiles when compared with premenopausal controls. However, significant changes have been noted once perimenopausal women become postmenopausal (Matthews et al. 1994), suggesting that our six-month study did not continue long enough

into the early postmenopausal stage to have captured potential treatment-induced changes in the lipid/lipoprotein profile.

In conclusion, treatment for 24 wks with SPI+ or SPI - did not alter lipid/lipoprotein or coagulation/fibrinolytic factor concentrations in these perimenopausal women. However, lean body mass, serum 17 β-estradiol, and dietary factors (vitamin E, selenium, fiber, and folate) favorably contributed to lipid profiles, whereas plasma fibrinogen and PAI-1, diastolic blood pressure, body weight, serum liver enzymes, and serum ferritin adversely affected circulating lipid and lipoprotein concentrations.

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TABLE 1

Baseline characteristics in perimenopausal women

Measurement (n=69)	**Mean±SD** [min-max]
Age (years)	50.2±3.6 [41.9-61.6]
Height (cm)	164.8±5.0 [151.6-177.5]
Weight (kg)	65.3±9.0 [48.5-98.1]
BMI (kg/m^2)	24.1±3.2 [17.4-33.7]
Lean mass (kg)[1]	39.8±6.8 [31.4-48.6]
Body fat (%)[1]	37.2±6.3 [18.4-53.1]
Physical activity (MJ; median [min-max])	4.33 [0.17-17.14]
Resting blood pressure	
Systolic (mmHg)	119±11 [100-144]
Diastolic (mmHg)	71±10 [54-98]

TABLE 1 (continued)

Dietary intake[2,3]	**Median** [min-max]
Energy (MJ)	7.46 [4.07-10.64]
Fat (g)	59.4 [26.8-99.8]
Fat (% of total energy)	30.2 [14.8-45.8]
Saturated fat (g)	18.7 [7.6-48.4]
Polyunsaturated fat (g)	10.5 [2.8-20.6]
Dietary fiber (g)	18.7 [4.1-51.2]
Vitamin C (mg)	116.8 [27.8-586.2]
Vitamin E (mg)	10.7 [1.5-29.7]
Vitamin A (RE)	1015.5 [195.1-4732.0]
Folate (µg)	246 [74-725]
Selenium (mg)	0.045 [0.004-0.097]

TABLE 1 (continued)

Alcohol intake (g)[4]	162.1
	[0.8-1131.3]
Serum	
Aspartate aminotransferase (μkat/L)	0.35
	[0.23-1.08]
Alanine aminotransferase (μkat/L)	0.22
	[0.05-1.68]
Estrone (pmol/L)	122.1
	[37.0-447.6]
17 ß-estradiol (pmol/L)	150.5
	[73.4-785.6]
Ferritin (μg/L)	17.0
	[0.4-210.0]
Iron (μmol/L)	15.9
	[1.4-29.7]

[1] Assessed by dual energy x-ray absorptiometry

[2] Median values are reported for serum analytes and dietary intake, since most were not normally distributed

[3] Dietary intake was assessed from 5-d food diaries collected prior to baseline testing

[4] Refers to 33 women who reported that they consumed alcohol during the recording of the 5-d food diary

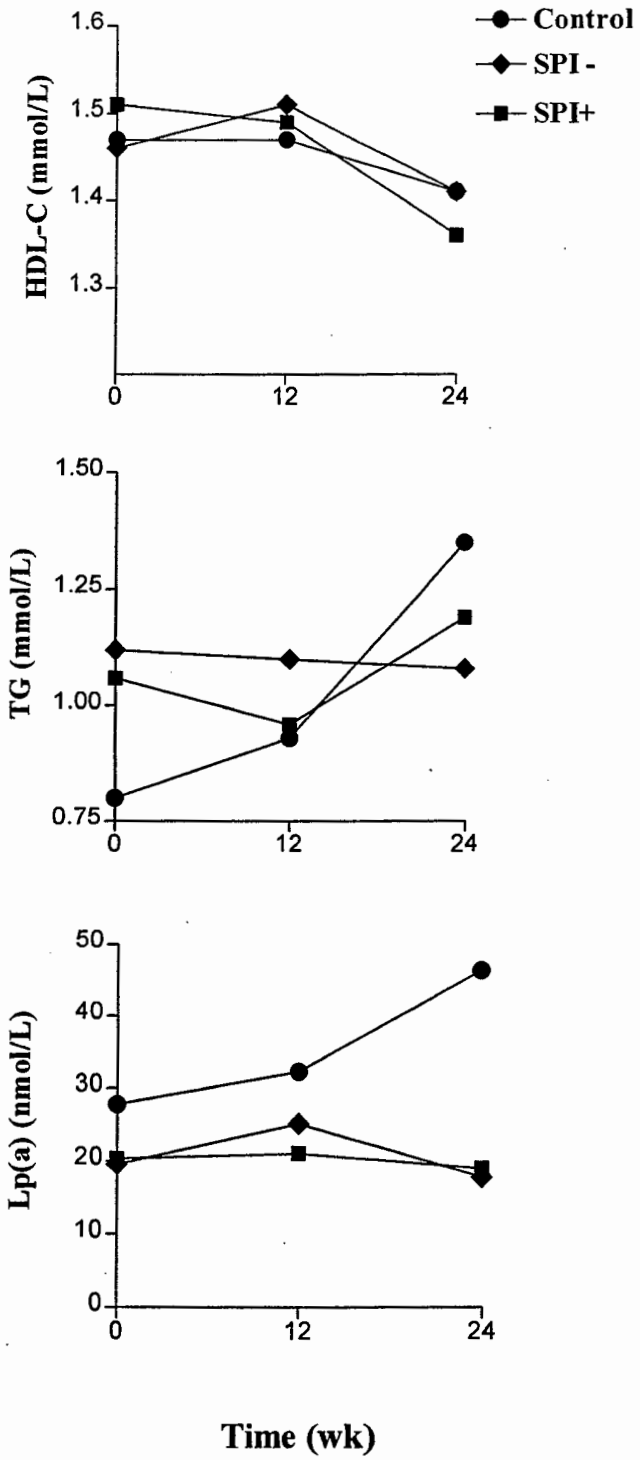

FIGURE 1. Change in HDL cholesterol (HDL-C), triacylglycerol (TG), and lipoprotein(a) [Lp(a)] concentrations (median) in perimenopausal women from baseline to week 24 according to treatment: isoflavone-rich soy protein isolate (SPI+ = ■; n = 24), isoflavone-poor soy protein isolate (SPI - = ◆; n = 24), and whey protein (control = ●; n = 21).

TABLE 2

Lipid/lipoprotein concentrations at baseline, wk 12, and wk 24 and and coagulation/fibrinolytic concentrations at baseline and wk 24 in perimenopausal women

Measure (mean±SD or median [min-max])	Isoflavone-rich soy protein (SPI+)			Isoflavone-poor soy protein (SPI -)			Whey protein (control)			ANOVA[1] (*P* values)		
	Base	Wk12	Wk24	Base	Wk12	Wk24	Base	Wk12	Wk24	Tx[3]	Time	Cohort
Total cholesterol (mmol/L)[2]	5.42 ± 0.65	5.36 ± 0.69	5.43 ± 0.66	5.62 ± 1.03	5.55 ±0.92	5.47 ± 1.08	5.49 ± 0.87	5.28 ± 0.81	5.47 ± 1.04	0.62	0.60	0.19
LDL cholesterol (mmol/L)[2]	3.27 ± 0.54	3.27 ± 0.50	3.43 ± 0.54	3.49 ± 0.89	3.43 ± 0.76	3.51 ± 0.97	3.48 ± 0.83	3.30 ± 0.82	3.52 ± 1.01	0.63	0.12	0.26
Total / HDL cholesterol[2]	3.62 ± 0.90	3.54 ± 0.76	3.86 ± 0.94	3.83 ± 1.16	3.60 ± 0.78	3.95 ± 0.99	3.76 ± 1.08	3.51 ± 0.72	3.94 ± 1.02	0.91	≤0.0001	0.17

	Base	Wk24	Base	Wk24	Base	Wk24	Tx[3]	Cohort
Fibrinogen (g/L)[4,6]	3.3 [2.5-4.6]	3.5 [2.6-4.4]	3.2 [1.4-4.5]	3.2 [2.7-4.2]	3.3 [2.5-4.1]	3.1 [2.5-4.8]	0.29	0.060
Factor VII antigen (%)[6]	79.4 ± 12.1	76.8 ± 12.3	74.1 ± 12.0	75.0 ± 15.8	79.4 ± 11.6	77.4 ± 10.9	0.54	0.61

TABLE 2 (continued)

	Base	Wk24	Base	Wk24	Base	Wk24	Tx[3]	Cohort
Factor VII coagulant activity (%)[7]	46.4 ± 32.5	76.7 ± 33.4	42.4 ± 30.6	73.4 ± 39.6	58.3 ± 40.3	101.8 ± 54.7	0.60	0.042
Plasminogen activator inhibitor-1 (μkat/L)[4,8]	283.4 [66.7-550]	283.4 [16.7-1234]	333.4 [150.0-734]	300.1 [183.4-700]	216.7 [100.0-684]	216.7 [100.0-517]	0.80	0.80

[1] Repeated measures ANOVA performed on lipids/lipoproteins; ANOVA performed on coagulation/fibrinolytic factors

[2] SPI+ (n = 24); SPI - (n = 24); control (n = 21); total N = 69

[3] Tx = Treatment

[4] Median values are reported for fibrinogen, and plasminogen activator inhibitor-1 because they were not normally distributed; log-transformed values of these same variables were then used for the ANOVA

[5] SPI+ (n = 23); SPI - (n = 24); control (n = 20); total N = 67

[6] SPI+ (n = 20); SPI - (n = 23); control (n = 21); total N = 64

[7] SPI+ (n = 16); SPI - (n = 15); control (n = 15); total N = 46; factor VII coagulant activity increased ($P \leq 0.0001$) from baseline to wk 24

[8] SPI+ (n = 21); SPI - (n = 23); control (n = 21); total N = 65

TABLE 3

Regression analyses: Contributors to lipid/lipoprotein concentrations at baseline

Total Cholesterol: Overall Model R^2=43.3% (Adj R^2=38.8%); F (5, 63)=9.61; ($p \leq 0.0001$)

Independent Variable	Parameter Estimate	Percentage Variance[1]	*P* value[2]	Variance Inflation[3]
Intercept	6.3666		≤0.0001	
Serum aspartate aminotransferase	2.5454	10.33	0.0012	1.03
Lean mass	-0.0652	7.67	0.0049	1.03
Resting diastolic blood pressure	0.0220	6.28	0.010	1.02
Dietary folate	-0.0020	6.21	0.011	1.02
Serum 17 ß-estradiol	-0.0010	4.04	0.038	1.03

Triacylglycerol[4]: Overall Model R^2=33.8% (Adj R^2=30.6%); F (3, 61)=10.39; ($p \leq 0.0001$)

Intercept	-0.2257		0.18	
Plasma plasminogen inhibitor activator-1	0.0013	13.38	0.0008	1.14
Dietary vitamin E	-0.0231	7.36	0.012	1.23
Serum alanine aminotransferase	0.5964	4.42	0.048	1.20

TABLE 3 (continued)

LDL cholesterol: Overall Model R^2=32.3% (Adj R^2=26.5%); F (5, 58)=5.54; (p=0.0003)

Independent Variable	Parameter Estimate	Percentage Variance	*P* value	Variance Inflation
Intercept	3.7097		0.0056	
Plasma fibrinogen	0.4246	8.70	0.0084	1.11
Serum iron	-0.0383	7.22	0.016	1.08
Lean mass	-0.0576	7.11	0.017	1.04
Dietary fat (g)	0.0106	4.95	0.050	1.15
Serum aspartate aminotransferase	1.4595	4.29	0.060	1.02

Lipoprotein(a)[4]: Overall Model R^2=34.8% (Adj R^2=29.2%); F (5, 58)=6.20; ($p \leq 0.0001$)

Intercept	5.2256		$\leq$0.0001	
Dietary fat (% of total energy)	-0.0711	12.39	0.0016	1.44
Dietary fiber	-0.0434	8.41	0.0082	1.41
Cohort 2	0.6061	6.11	0.023	1.09
Dietary selenium	-12.8457	5.91	0.026	1.12
Plasma fibrinogen	0.4487	5.38	0.033	1.09

TABLE 3 (continued)

HDL cholesterol[4]: Overall Model R^2=56.5% (Adj R^2=52.9%); F (5, 59)=15.35; ($p \leq 0.0001$)

Independent Variable	Parameter Estimate	Percentage Variance	*P* value	Variance Inflation
Intercept	0.4779		0.0207	
Dietary vitamin E	0.0142	13.62	≤0.0001	1.10
Plasma plasminogen activator inhibitor-1	-0.0005	7.77	0.0019	1.17
Dietary fat (% of total energy)	0.0108	7.01	0.0031	1.05
Body weight (kg)	-0.0056	3.76	0.028	1.11
Serum ferritin	-0.0014	3.50	0.033	1.04

Total Cholesterol/HDL cholesterol: Overall Model R^2=49.9% (Adj R^2=47.4%); F (3, 60)=19.92; ($p \leq 0.0001$)

Intercept	1.4014		0.023	
Plasma plasminogen activator inhibitor-1	0.0028	16.56	≤0.0001	1.09
Dietary vitamin E	-0.0522	10.72	0.0007	1.06
Plasma fibrinogen	0.6112	10.61	0.0007	1.04

[1] Squared semi-partial Type II correlation coefficient; accounts for shared variance among variables

TABLE 3 (continued)

[2] Variables left in models are significant at $P \leq 0.10$ level

[3] Measures inflation in the variances of parameter estimates due to multicollinearities among regressors

[4] Models were log-transformed since HDL cholesterol, triacylglyceridol, and lipoprotein(a) were not normally distributed

GENERAL CONCLUSIONS

In summary, the results from the primary objective of this study suggest that isoflavone-rich or isoflavone-poor soy protein do not favorably alter circulating lipids and lipoproteins or coagulation and fibrinolytic factors in perimenopausal women. Thus, the inclusion of soy products to the diets of perimenopausal women is not likely an appropriate alternative to hormone replacement therapy for women at-risk for CVD. To verify this finding, the influence of isoflavone-rich soy protein on other CVD risk factors needs to be examined. The results from the secondary objective determined that lean body mass, serum 17 β-estradiol, and dietary factors (vitamin E, selenium, fiber, and folate) favorably contributed to lipid profiles, whereas plasma fibrinogen and PAI-1, diastolic blood pressure, body weight, serum liver enzymes, and serum ferritin adversely affected circulating lipid and lipoprotein concentrations at baseline.

Future data analysis will be conducted to determine the relationship between isoflavone-rich and isoflavone-poor soy protein and antioxidant status, since isoflavones have antioxidant properties. We will also account for the contributors of dietary (fat, antioxidants, fiber, alcohol) and serum (estrogen, iron, ferritin) factors in determining total antioxidant status in these perimenopausal women.

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