Postprandial effects of soy isoflavones on low-density lipoprotein oxidative resistance with a high carbohydrate meal
by Bobbi Jo Miller

A thesis submitted in partial fulfillment of the requirements for the degree of Master of Science in Health and Human Development
Montana State University

Abstract:
Cardiovascular disease (CVD) encompasses a wide array of health problems including atherosclerosis which results in 50% of all cardiac deaths. Currently the American Heart Association developed the Step I guidelines for reducing the risk of CVD limits total fat to ≤30% of total energy, saturated fat to <10% of total energy, and cholesterol to < 300 mg/day. Reducing dietary fat generally decreases plasma cholesterol however carbohydrate (CHO) content typically rises accompanied by increasing plasma triacylglycerol (TC). Elevated TG may possibly be a risk factor for CVD, referred to as "CHO-induced hypertriacylglycerolemia" (HPTG). The disease process of atherogenesis has been hypothesized by Zilversmit as a postprandial phenomenon based on the formation of chylomicron remnants, low-density lipoproteins (LDL), and the uptake of these cholesterol and TG rich molecules by arterial cells. Oxidation of LDL and phagocytic immune system cells have been implicated in the mechanism involving fatty streaks and occlusion of the arterial lumen. The isoflavones diadzein and genistein in soy-protein have been associated with oxidative resistance of LDL due to their antioxidant activity. The purpose of this study was to determine if the oxidative resistance of postprandial LDL is enhanced with the consumption of a meal containing 39.0 g of soy protein (80 mg aglycone isoflavones) vs 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal. Fifteen healthy male subjects participated in a double-blind, crossover feeding study in the Nutrition Research Lab (NRL) at Montana State University. Subject's height, weight, and baseline blood draw were completed before consuming the challenge meal consisting of 2 high carbohydrate muffins and a soy or milk protein shake (899 calories, 22% fat, 58.6% CHO, 19.4%). Blood samples were collected by venipuncture postprandially at hours 2, 4, and 6.

Isolated LDL was subjected to ex vivo copper-induced oxidation. Initial absorbance, lag time, and propagation rate were calculated for each time point. Results indicated no significant difference ($p>0.05$) between the protein treatments or their interaction on LDL oxidation parameters. Additional research is needed to ascertain the function of soy in prevention of CVD, specifically in the postprandial state.

POSTPRANDIAL EFFECTS OF SOY ISOFLAVONES ON LOW-DENSITY LIPOPROTEIN OXIDATIVE RESISTANCE WITH A HIGH CARBOHYDRATE MEAL

by

Bobbi Jo Miller

A thesis submitted in partial fulfillment
of the requirements for the degree

of

Master of Science

in

Health and Human Development

MONTANA STATE UNIVERSITY
Bozeman, Montana

August, 2003

APPROVAL

Of a thesis submitted by

Bobbi Jo Miller

This thesis has been read by each member of the thesis committee and has been found to be satisfactory regarding content, English usage, format, citations, bibliographic style, and consistency, and is ready for submission to the College of Graduate Studies.

Christina Gayer Campbell, Ph.D., R.D. Christina Gayer Campbell 8/19/03
(Signature) Date

Approved for the Department of Health and Human Development

Craig Stewart, Ed.D. _____ 8/22/03
(Signature) Date

Approved for the College of Graduate Studies

Bruce R McLeod, Ph.D. Bruce R. McLeod 9-15-03
(Signature) Date

STATEMENT OF PERMISSION TO USE

In presenting this thesis in partial fulfillment of the requirements for a master's degree at Montana State University, I agree that the Library shall make it available to borrowers under rules of the Library.

If I have indicated my intention to copyright this thesis by including a copyright notice page, copying is allowable only for scholarly purposes, consistent with "fair use" as prescribed in the U.S. Copyright Law. Requests for permission for extended quotation from or reproduction of this thesis in whole or in parts may be granted only by the copyright holder.

Signature Bobbi Jo Miller

Date 8/18/03

ACKNOWLEDGMENTS

I would like to recognize Dr. Christina Gayer Campbell, my committee chair and advisor, for instilling in me perseverance and commitment, the requirements for success.

Appreciation also goes to those who served on my graduate committee, notably Dr. Mary Miles, who provided ongoing encouragement and support; Bettie Stanislao for checking in on me regularly; and Dr. Blakely Brown, who, although many miles away, was gracious enough to join my committee and to travel to Bozeman frequently on my behalf.

To Danielle Dufner, I am especially grateful for her guidance, encouragement, friendship and belief in me, even when I questioned myself. A sincere *thank you* goes to Dr. Jeff Banfield and Dr. Betsy Palmer for directing me through the mire of statistics.

There are no two people in the world who have believed in, and given me the strength to accomplish my goals, more than my parents, Bob and Linda Miller. Thank you for your love and support.

I am indebted to all my friends for their patience, understanding and support. They made me laugh when I wanted to cry, reminded me that play is a requirement, and help was only a phone call away. Special thanks go to Adrian Winn and Chris Grinnell, my on-call computer wizards, for their technical expertise, and Missy Grinnell for the stress-relieving trips to the gym, providing a listening ear, and always being there for me.

And finally, many thanks to all of the participants who made this study possible.

TABLE OF CONTENTS

TABLE OF CONTENTS – CONTINUED

LIST OF TABLES

LIST OF FIGURES

Figure Page

LIST OF FIGURES – CONTINUED

ABSTRACT

Cardiovascular disease (CVD) encompasses a wide array of health problems including atherosclerosis which results in 50% of all cardiac deaths. Currently the American Heart Association developed the Step I guidelines for reducing the risk of CVD limits total fat to ≤30% of total energy, saturated fat to <10% of total energy, and cholesterol to < 300 mg/day. Reducing dietary fat generally decreases plasma cholesterol however carbohydrate (CHO) content typically rises accompanied by increasing plasma triacylglycerol (TG). Elevated TG may possibly be a risk factor for CVD, referred to as "CHO-induced hypertriacylglycerolemia" (HPTG). The disease process of atherogenesis has been hypothesized by Zilversmit as a postprandial phenomenon based on the formation of chylomicron remnants, low-density lipoproteins (LDL), and the uptake of these cholesterol and TG rich molecules by arterial cells. Oxidation of LDL and phagocytic immune system cells have been implicated in the mechanism involving fatty streaks and occlusion of the arterial lumen. The isoflavones diadzein and genistein in soy-protein have been associated with oxidative resistance of LDL due to their antioxidant activity. The purpose of this study was to determine if the oxidative resistance of postprandial LDL is enhanced with the consumption of a meal containing 39.0 g of soy protein (80 mg aglycone isoflavones) vs 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal. Fifteen healthy male subjects participated in a double-blind, crossover feeding study in the Nutrition Research Lab (NRL) at Montana State University. Subject's height, weight, and baseline blood draw were completed before consuming the challenge meal consisting of 2 high carbohydrate muffins and a soy or milk protein shake (899 calories, 22% fat, 58.6% CHO, 19.4%). Blood samples were collected by venipuncture postprandially at hours 2, 4, and 6. Isolated LDL was subjected to *ex vivo* copper-induced oxidation. Initial absorbance, lag time, and propagation rate were calculated for each time point. Results indicated no significant difference ($p>0.05$) between the protein treatments or their interaction on LDL oxidation parameters. Additional research is needed to ascertain the function of soy in prevention of CVD, specifically in the postprandial state.

CHAPTER 1

INTRODUCTION

Cardiovascular disease (CVD) is currently the leading cause of death (1) encompassing a wide array of health problems including atherosclerosis, hypertension, stroke, and hyperlipidemia (2). Atherosclerosis is a degenerative disease of the vascular endothelium that is associated with vessel damage and impeded blood flow (3). It is the most deadly CVD resulting in 50% of all cardiac deaths (2). Due to the morbidity and mortality rates, CVD has become a major health problem (2). More than 58 million Americans have at least one form of CVD, with associated costs exceeding $274 billion yearly (2), therefore, research is directed towards lowering associated risk factors that could potentially lead to the formation of CVD. Epidemiological studies have repeatedly shown high degrees of correlation between the intake of dietary fats and cholesterol with the prevalence of CVD (4).

Health promotion efforts now emphasize the importance of reducing risk factors such as high dietary fat and cholesterol intake. Currently the American Heart Association's Step I guidelines for reducing the risk of CVD limits total fat to $\leq$30% of total energy, saturated fat to <10% of total energy, and cholesterol to <300 mg/day. As individuals reduce their dietary fat, dietary carbohydrate (CHO) content typically rises to replace lost calories. The desired reduction in total cholesterol is frequently reached, but is typically accompanied by an elevation in triacylglycerol (TG) level (5). For the purpose of research, a high CHO diet has been termed "low-fat, high-carbohydrate" if $\leq$30% of the

total energy is derived from fat and ≥55% of energy is derived from CHO (5). Concern now stems from a possible increase in CVD occurrence when TG levels are elevated as a result of lowered dietary fat and increased dietary CHO, a phenomenon referred to as CHO-induced hypertriacylglycerolemia (HPTG) (5). If this phenomenon shares a physiological foundation with endogenous HPTG, commonly observed in high-fat diets, a similar atherogenic risk may ensue following high CHO diets.

Triglyceride-rich lipoproteins are derived through processes involving the intestine (chylomicrons) and the liver (very low-density lipoproteins [VLDL]). TheseTG -rich lipoproteins have been determined to come from four distinct sources; *de novo* lipogenesis, lipolysis, chylomicron remnant formation, and TG droplets stored in the liver (6). The chylomicron and VLDL are metabolized in the blood stream by the action of lipoprotein lipase on the surface of endothelial cells in the muscle and adipose tissue (7, 8). Multiple interactions lead to the hydrolysis of TG-rich molecules and to the formation of low-density lipoproteins (LDL) (7, 8). Low-density lipoprotein becomes the major cholesterol carrying lipoprotein and resides much longer in the blood than do chylomicron or VLDL remnants (days for LDL vs. minutes to hours for VLDL) (7). This increased exposure time in the blood allows free radicals a greater chance to manipulate the native LDL into an oxidized form (3). The associated risk of CVD has been related to the persistence in the circulation of the remnant particles that carry the dietary TG in the plasma (7, 9).

Atherogenesis begins with phagocytic immune system cells, primarily monocytes and T lymphocytes, responding to endothelial cell injury caused by high levels of oxidized

LDL (3). The oxidized LDL penetrates the endothelium into the arterial intima causing adherence of monocytes and platelets (3). The perpetuation of atherogenesis occurs by two routes. First, monocytes, activated by the presence of oxidized LDL, release chemoattractants which draw additional monocytes to the endothelium (3). Phagocytosis of oxidized LDL leads to the transformation of monocytes to macrophages, inhibiting motility and trapping the macrophages in the endothelial space (3). At this point, the lipid-engorged macrophages become foam cells. A second, proposed mechanism is through the release of growth factors stimulating the proliferation of smooth muscle cells in the arterial media. Oxidized LDL rapidly accumulates within the smooth muscle cells leading to the accumulation of foam cells, which contributes to fatty streaks and occlusion of the arterial lumen compromising blood flow (3).

Zilversmit (4) hypothesized that atherosclerosis was a postprandial phenomenon based on the formation of chylomicron remnants, LDL, and the uptake of these cholesterol and TG rich molecules by arterial cells after a meal (4, 10). Fasting blood cholesterol concentrations are used clinically to determine if an individual is at elevated risk for the development of CVD, however most individuals exist in a postprandial state 16-18 hours per day (6). Disease risk may be better predicted by a postprandial test than a fasting test due to the amount of time an individual spends in a postprandial state (6). The measurement of blood TG has also been shown to be elevated in the postprandial state in patients with documented CVD (11). A high CHO diet increases postprandial TG, which can further contribute to increased atherogenic risk (6).

The cholesterol lowering effects of soy protein as compared to animal protein on lowering CVD risk has been recognized in animal studies for more than 80 years (12). In 1995, Anderson et al. (12) conducted a meta-analysis to determine the effect of soy protein intake on serum lipids. Beneficial effects were associated with the substitution of soy protein for animal protein foods in diets already low in saturated fat (<10% of total energy) and dietary cholesterol (<200 mg/d) (13). Significant reductions in serum concentrations of total cholesterol (23.2 mg/dL), LDL cholesterol (21.7 mg/dL), and TG (13.3 mg/dL) without significantly influencing high-density lipoprotein (HDL) cholesterol (2.4 mg/dL increase) were observed (12, 14). These lipid lowering effects have generally not been seen in humans fed isoflavones separated from the soy protein (13), suggesting there may be additional factors within the intact soy protein that contributes to the hypocholesterolemic effects.

Isoflavones are one of the three main categories of phytoestrogens (15), a plant derived estrogen analog (16). Soy products are rich in isoflavones, daidzin and genistin, that possess antioxidant activity. These isoflavones are both hydrolyzed from the β-glycoside form to the highly bioavailable aglycone form, diadzein and genistein, by bacterial glycosidases in the large intestine (15). Several studies suggest that soy isoflavone consumption may protect LDL cholesterol from oxidative damage due to its antioxidant properties (12, 16, 17, 18).

Although there have been advances in research regarding the consumption of soy products for beneficial effects related to CVD, much is still unknown. Whether isoflavones are effective in reducing atherogenesis needs to be studied further.

Understanding the mechanism behind LDL oxidation and the beneficial components of soy products, researchers can begin to examine how soy can protect against a variety of diets. For example, soy may offer protection against HPTG observed after ingesting a high CHO diet. This will enable researchers to gain further knowledge of the possible anti-atherosclerotic effects of soy.

Purpose

The purpose of this study was to determine if the oxidative resistance of postprandial LDL was enhanced with the consumption of a shake containing 39.0 g soy protein (85 mg aglycone isoflavones) vs. 39.9 g milk protein (0 mg aglycone isoflavones) in combination with a high carbohydrate meal.

Hypothesis

Postprandial oxidative stress, as measured by copper-induced LDL oxidation following the consumption of a high carbohydrate meal, will be reduced following the 39.0g of soy (85mg aglycone isoflavones) as compared to the same meal with 39.9g of milk protein (0mg aglycone isoflavone) due to the isoflavone content of the soy protein.

CHAPTER 2

REVIEW OF LITERATURE

The randomized clinical trial has the potential to provide a compelling rationale for accepting or rejecting a treatment. One drawback of clinical trials of diet in CVD is their high cost and impracticality for testing many nutrients and foods. Also, clinical trials may not last long enough to detect such effects that take more than a few years to make them seen. An alternative to using CVD as an outcome in a dietary trial is to select surrogate end points that are in the causal path between a food or nutrient and CVD. Plasma lipoproteins such as total and LDL cholesterol are an important surrogate end point because of their strong link to the pathogenesis of atherosclerosis and their strong predictive association with CVD which has been supported strongly by a consistent body of evidence from clinical trials and epidemiological studies (19). National health organizations advocate dietary changes that decrease intake of saturated and trans-unsaturated fat and cholesterol to prevent CVD. The rationale is to reduce LDL concentration; however, diets affect not only LDL but also HDL and TG, which are also independent lipid risk factors.

Postprandial Metabolism

In 1910, the presence of cholesterol in lesions of diseased arteries was described by Windaus (4). Since that time, epidemiological studies have repeatedly confirmed a high degree of correlation between the intake of cholesterol and other lipids with the

occurrence of CVD (4). Over 20 years ago, Zilversmit hypothesized that atherosclerosis was a postprandial phenomenon (10). This hypothesis was based on the formation of chylomicron remnants, LDL, and the normal process of lipid absorption by arterial cells after a meal (4, 10).

Many studies of lipoprotein metabolism have been carried out in the fasted state, because this was thought to be more reproducible. However, most individuals exist in the postprandial state 16-18 hours per day and subside in a fasted state for approximately 6 hours per day, typically during sleep (6). Therefore, the assessment of disease risk could be improved by a postprandial test of lipoproteins, rather than a fasted test. Indeed, studies have shown blood TG levels are elevated in the postprandial state in patients with documented CVD (11).

The metabolism of chylomicrons and VLDL molecules are similar in many respects. Absorption, synthesis, and secretion by the enterocyte and transportation of dietary lipids are functions of the intestine dependent on chylomicron formation. The chylomicron transports dietary fatty acids in the form of TG through the lymph to the liver. In addition to apolipoprotein (apo) B-48, the chylomicron acquires apo E and apo C from HDL in circulation (20). Once in the blood, chylomicrons interact with lipoprotein lipase on the surface of mainly muscle and adipose endothelial cells, catalyzing the hydrolysis of TG molecules into monoglycerides and fatty acids (21). The hydrolytic products are then transported across the endothelium where they are oxidized by the muscles or reesterified for storage in the adipose tissue. Some of the free fatty acids from the chylomicron escape the uptake and become bound to albumin and are transported to the liver for

reesterification or inclusion into native VLDL. The apo C of the chylomicron is then transferred back to the HDL as cholesterol esters are gained (21).

The remaining chylomicron particle is termed a "chylomicron remnant". The remnant interacts with the hepatic lipase to hydrolyze any remaining TG and expose the apo E, which is essential for recognition and clearance of the chylomicron remnant by the liver (10). Postprandial uptake of chylomicron remnants and TG stimulates the hepatocytes to produce native VLDL.

Once the apoB-100 containing VLDL particle is in circulation, apo E and C are transferred to the VLDL particle from HDL. Lipoprotein lipase hydrolyzes large VLDL-TG similar to chylomicrons, but smaller VLDL are hydrolyzed more slowly because of limited interaction with lipoprotein lipase due to their size and the increased concentration of lipoproteins postprandially. The large VLDL remnants are then taken up rapidly by the liver's LDL receptors and degraded, while the small VLDL interact with hepatic lipase, eventually losing their apo E and C protein, the majority of their TG, and ultimately become LDL particles. The apo E receptor is essential for binding with the liver and ultimate clearance of the particles from circulation. The number of LDL particles in the blood significantly surpasses that of their precursors due to the lack of apo E receptor-binding domain. They are eventually taken up by the liver via interactions with a receptor-binding domain on the apo B-100 with the LDL receptor (7).

The rapid increase in chylomicrons and VLDL-TG in the postprandial phase saturates the liver's capacity by increasing competition for the common removal mechanism (apo E receptor) by the hepatocytes, leading to a longer duration that the TG-rich lipoprotein

remnants remain in circulation (4, 10). This increased circulation time enhances the possibility that the lipoprotein particles may become oxidized and enter into the endothelial space (10, 22). This progression further increases the risk for atherosclerosis and CVD.

Oxidative Hypothesis of Atherosclerosis

In the 1970's, two groups of researchers simultaneously proposed the oxidative modification hypothesis of atherogenesis. Chisolm et al. (23) witnessed that the injury of endothelial cells *in vitro* was dependent on oxidatively modified LDL, while Steinberg et al. (23), determined that foam cell formation could not be induced by native LDL. Both Chisolm and Steinberg later demonstrated that modification of LDL was possible *in vivo* and that the modified LDL was recognized by savenger receptors on macrophages due to oxidative modification. Oxidized LDL causes endothelial injury initiating an immune system response drawing phagocytic monocytes and T lymphocytes to the arterial site (3). Macrophages rapidly up-take oxidized LDL due to the abundance of binding sites for LDL modified by oxidation (7, 24). These engorged macrophages become trapped in the endothelial spaces and become lipid-laden foam cells (25). As the foam cells increase in size, their lipid contents form fatty streaks in the intima of the arterial wall, enlarging and occluding the arterial lumen and impeding blood flow (3). In more recent years oxidative stress has become the term used to define the imbalance between free-radical producing pro-oxidants (e.g. copper, iron) and antioxidant defenses (e.g. soy isoflavones, vitamin E)

that contributes to LDL oxidation and the pathogenesis of vascular complications, such as athrosclerosis.

The field of research regarding the oxidation of LDL has rapidly developed over the last 20 years beginning with only a few published articles, manifesting into hundreds. The widely accepted process of oxidative stress leading to the pathogenesis of atherosclerosis involves a cascade of cellular events in the endothelium of the arterial wall contributing to plaque formation (26, 27). The formation of atherosclerotic lesions in the arterial intima is believed to be caused by a number of factors including, endothelial injury brought about by hyperlipidemia or toxic agents (28). The reactions causing oxidation of LDL vary; the presence of metal ions *in vitro* (e.g. copper and iron), superoxide radicals and heme-containing compounds *in vivo*, have all been suspected of modifying LDL (3).

The initial stage of LDL oxidation involves the free radical peroxidation of predominately polyunsaturated fatty acids (PUFA) and to a lesser extent monounsaturated fatty acids (MUFA) in LDL, due to the double bonds possessed by these fatty acids (29). The process occurs such that hydrogen is removed by a free radical and a molecular rearrangement occurs, forming a conjugated diene (29). Oxygen is then taken up and a peroxy radical is formed initiating the removal of hydrogen from another fatty acid. It is important to remember that these processes are occurring simultaneously and are at different phases *in vivo*. In a controlled environment *in vitro*, this phase is known as the propagation stage of the oxidation process and represents a chain reaction

leading to the formation of lipid hydroperoxides (30). Likewise, the decomposition stage refers to the conversion of lipid hydroperoxides into reactive aldehydes and ketones and is the final phase of LDL oxidation *in vitro* (30).

A study conducted by Lechleitner et al. (31), examined the hypothesis that modified LDL but not native LDL are capable of leading to foam cell formation in the course of postprandial lipemia. Macrophages were incubated with fasted and postprandial native LDL from 17 healthy volunteers. After the LDL was isolated, postprandial LDL was found to be more susceptible to *in vitro* oxidation than fasting LDL. A significantly higher cellular cholesterol ester accumulation (postprandial: 477 ± 286%; fasted: 212 ± 173% respectively; $p<0.003$) was induced by postprandial LDL than fasting LDL (31). The increase in cellular cholesterol ester synthesis is evidence that oxidized postprandial LDL but not native LDL leads to foam cell formation in macrophages.

Regnstrom et al. (32) investigated the relationship between the ability of LDL to resist oxidation *in vitro* and the severity of CVD. Low-density lipoprotein was isolated from 35 young male survivors of myocardial infarction. The LDL was subjected to copper induced oxidation and the lag time was measured. Oxidative modification of LDL cholesterol correlated independently with the severity level of CVD ($r = -0.45$, $p<0.02$). The lag time was also related to the TG content of the LDL fraction ($r = -0.55$; $p<.002$). The finding that the susceptibility of LDL to oxidation is positively associated with the severity of atherosclerosis indicates that lipid oxidation promotes premature CVD and that individuals with LDL rich in TG are at particularly high risk. This experiment is

beneficial in directing further research toward the cause of elevated TG levels and possible mechanisms to increase oxidative resistance of LDL.

The expanded research surrounding the oxidative modification hypothesis of atherogenesis proposed over 20 years ago has provided a strong body of evidence to suggest that oxidized LDL has an important pathological role in the development of atherosclerosis. Further research must now be conducted to determine if oxidative resistance of LDL can aid in the prevention of CVD. One potential mechanism may be antioxidants, such as soy isoflavones, which may lend protection to LDL, thus increasing resistance and reducing the risk of atherosclerosis.

Benefits of Soy in CVD

In 1999, the Food and Drug Administration authorized the use of a health claim for soy protein based on data reviewed from 38 clinical studies in a meta-analysis, conducted by Anderson et al. (13). The study concluded that 25 g of soy protein included in a diet low in total fat (<30% of total daily calories), saturated fat (<10% of total daily calories), and cholesterol (<200 mg of total daily calories) may reduce the risk of CVD by significantly lowering blood cholesterol levels (33). The claim indicated that four daily soy servings of 6.25 g/serving can reduce levels of LDL by as much as 10% (33). The scientific community generally agrees that a 1% drop in total cholesterol can equal a 2% drop in heart disease risk (33). Dietary interventions that can lower cholesterol are important tools in the fight against CVD.

The combined LDL-lowering and antioxidant effects of soy protein and their isoflavones may contribute to the lower rates of CVD among the Asian population, who consume 30 to 50 times (34) more soy protein in their diet as compared to the intakes of Americans (14). Whole soy foods are a good source of fiber, B vitamins, calcium, and omega-3 essential fatty acids, as well as offering a complete protein profile and soy isoflavones. Soy contains many other potentially active components, including saponins, plant sterols, and PUFAs, all of which may contribute to plasma cholesterol reduction (16). The amino acid content in soy protein is different from animal and most other vegetable proteins, and appears to alter the synthesis and metabolism of cholesterol in the liver (33).

The isoflavones in soy are phytoestrogens, a plant derived estrogen analog (16). Isoflavones in soy have several features in common with β-estradiol including an aromatic ring with a hydroxyl group substitution and a second hydroxyl group in the same plane, which enable isoflavones to bind to estrogen receptors (35) (see Figure 1). The isoflavones in soy may have effects that are similar to those of estradiol, which increases LDL receptor expression and decreases hepatic lipase activity, thus having a favorable effect on plasma LDL concentrations (36).

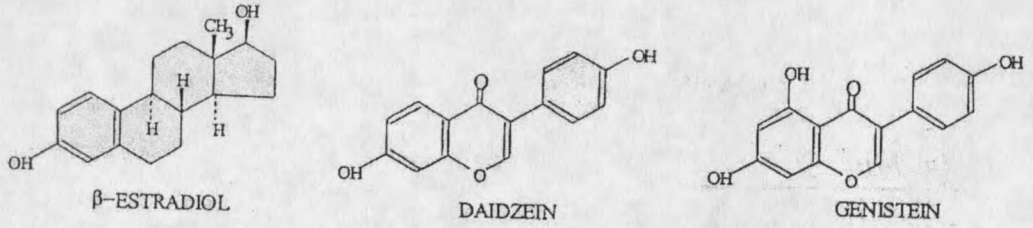

Figure 1. Structural Comparison of Estrogen to Isofalvones (35).

Isolates of soy protein (ISP) are commonly used products in soy research and are beneficial because of the control they provide. The content of isoflavones and the ratio of individual isoflavones can be manipulated enabling researchers to prepare isocaloric meals thatcan be produced and easily compared to placebos (37). These benefits are also due to the negligible content of CHO, fiber, and fat in ISP.

The major isoflavones, daidzein and genistein, exist in four chemical forms: aglycone, glycoside, acetylglucoside and malonylglucoside. After ingestion of the isoflavones, daidzin and genistin are hydrolyzed from the β-glycoside form to the bioavailable aglycone form, daidzein and genistein, by bacterial glycosidases in the large intestine (15, 38, 39). The metabolism of daidzein *in vivo* yields an isoflavone, equol. The isoflavone equol, is a more potent antioxidant *in vitro* than either daidzein or genistein and has shown the greatest antioxidant activity (15). Some individuals however, may differ in their ability to metabolize this isoflavone. Only 30% of the population are capable of complete metabolism of daidzin to equol (40, 41), therefore 70% of individuals may not reap the antioxidant health benefit associated with the consumption of soy (42).

The scientific research investigating the effects of soy on CVD have examined the results of ISP, purified isoflavones, and whole soy foods on plasma lipids, all of which provide differing results (37). Simons et al. (42) conducted a randomized 16 week cross-over study with 20 postmenopausal women to evaluate the effects of a diet containing purified soy isoflavones as compared to a placebo. The soy isoflavone extract was provided daily in a tablet containing 80 mg of isoflavones. An isocaloric fat-restricted diet (30% energy from fat, <10% energy from saturated fat, cholesterol intake <300

mg/day) was consumed for 21 days prior to treatment. Fasted blood sample analysis showed no significant differences in plasma lipid or lipoprotein levels for total, LDL, and HDL cholesterol although isoflavone levels were significantly higher during treatment. The link between consumption of purified soy isoflavone and cholesterol-lowering effects was not observed. These results strongly suggest there is an interaction between the soy isoflavones or an additional component such as soy protein which must also be present for the cholesterol-lowing effects.

In contrast to the previous study, Crouse et al. (34) investigated the use of ISP in reducing total and LDL cholesterol in 156 moderately hypercholesterolemic (LDL cholesterol levels of 140-200 mg/dL) men and women. They compared 25 g/d of casein protein to 25 g/d of ISP containing 3, 27, 37, and 62 mg of isoflavones. The ISP containing 62 mg of isoflavones revealed the greatest reduction in plasma concentration of total and LDL cholesterol of 4% (243 ± 26 mg/dL vs. follow-up: 233 ± 20 mg/dL; p=0.04) and 6% (baseline: 166 ± 25 mg/dL vs. follow-up: 156 ± 17 mg/dL; p=0.01) respectively. For further analysis, subjects were divided into high and low LDL cholesterol groups based on their baseline values. The high LDL cholesterol group who received isolated soy protein containing 62 mg of isoflavones had a reduced total cholesterol of 9% (baseline: 261 ± 23 mg/dL vs. follow-up: 237 ± 21 mg/dL; p<0.001) and LDL cholesterol of 10% (baseline: 185 ± 21 mg/dL vs. follow-up: 163 ± 18 mg/dL; p=0.001). The 37 mg of isoflavones also lowered both the total (baseline: 260 ± 16 mg/dL vs. follow-up: 240 ± 25 mg/dL; p=0.007) and LDL (baseline: 182 ± 16 mg/dL vs. follow-up: 165 ± 22 mg/dL; p=0.02) cholesterol by 8% in the high LDL cholesterol

group. The plasma concentration of TG and HDL cholesterol was not adversely influenced for all isoflavone levels. This study exemplifies the potent interaction isoflavones possessin conjunction with the isolated soy protein to reduce total and LDL cholesterol, with the greatest effect on subjects with above average LDL levels and greater amounts of isoflavone provided.

Soy and Oxidation

The hypocholesterolemic effects of soy have been studied for years, more recently, the antioxidant potential of soy and its isoflavones has been examined. Products containing soy may protect against atherosclerosis not only by increasing antioxidants to plasma and lipoproteins, but also minimizing the postprandial increase in lipid hydroperoxides. It has been purposed thatthe Cu^{2+} metal ion acts as pro-oxidants and bind to the apo B on LDL, triggering lipid peroxidation (43). Therefore, it is conceivable that soy isoflavones could act as an antioxidant defense and become bound to apo B or in some way cause a steric hindrance blocking the Cu^{2+} from binding to the apo B site, thus inhibiting lipid peroxidation (43).

Preliminary works have studied vitamin E, soy protein isoflavones, plant phenols using green tea, and β-carotene *in vitro* and revealed the administration of antioxidants, significantly reduces LDL oxidation in humans (14). The antioxidant effect of genistein against LDL oxidation *in vitro* was investigated by Kerry et al. (44). The LDL was isolated from a single healthy volunteer, incubated with 0, 25, 50, or 100 μmol/L of genistein, and subjected to both copper-mediated and radical-mediated LDL oxidation.

Genistein inhibited LDL oxidation in a concentration-dependent manner by increasing the lag time (control: 54.1 ± 5.1 min vs. 5 μmol/l genistein: 107.1 ± 1.8 min; $p<0.001$) and decreased the propagation rate (control: 14.4 ± 1.9 nmol/mg/min vs. 5 μmol/l: 7.4 ± 1.1 nmol/mg/min; $p<0.001$). Approximately 3-4% of genistein present in the plasma was incorporated into LDL. This study demonstrates that isoflavones can inhibit LDL oxidation *in vitro.*

Tikkanen et al. (18) examined the effect of soy as a possible strategy for preventing oxidation through incorporation of isoflavones into LDL particles. Six healthy young subjects consumed one soy bar containing 12 mg of genistein and 7 mg of daidzein and 7.1 g of protein 3 times daily for 2 weeks. Fasting blood was drawn at baseline, at the end of the treatment period, and 12 days after discontinuation of soy. The proportion of genistein and daidzein in purified LDL was less than 1% of total plasma content of these substances, yet a significant mean prolongation of the lag phase by more than 20 minutes (baseline:147 ± 9 min vs. day 14: 173 ± 19 min, $p<0.02$) was observed after 2 weeks of soy treatment (18).

In support of Tikkanen, Meng et al. (43) also reported that dietary intake of soybean isoflavones resulted in increased oxidative resistance of isolated LDL *in vitro.* Following reports that human estrogen could be incorporated into lipoproteins *in vitro*, they hypothesized that isoflavones could become esterified by a similar mechanism and lead to oxidative resistance. Diadzein and genistein were esterified into several different isoflavones fatty acid esters to increase their lipid solubility. The incorporation of these isoflavones into LDL and the ability to protect isoflavone-containing LDL from copper-

mediated oxidation was analyzed. Relatively small amount (0.33 molecules of isoflavone per LDL particle, or less) of the unesterified isoflavones were incorporated into LDL particles. The esterified esters of daidzein and genistein were incorporated more effectively (2.19 molecules of isoflavone per LDL particle) and prolonged the lag times by 46% ($p<0.05$) and 202% ($p<0.01$), respectively. Both of these studies illustrate the antioxidant capabilities of soy isoflavones and their ability to aid in the protection of LDL from oxidation.

Jenkins et al. (16) assessed the effects of soy isoflavones on LDL oxidation using 31 hyperlipidemic subjects in a 2 month randomized crossover study. All subjects had an elevated serum LDL cholesterol of >158 mg/dL and a triglyceride level <154 mg/dL at recruitment. Subjects consumed a test meal providing 33 g/d of soy protein (86 mg isoflavones/ 2000 kcal/d) and a lacto-ovovegetarian control diet with low-fat milk products. A fasted blood sample at baseline showed no significant differences in pretreatment values for blood lipids between the test and control diets. However, fasted blood samples obtained following week 2 and 4 of treatment showed lower mean test values for oxidized LDL assessed by conjugated dienes formation (test: 56 ± 3 μmol/L vs. control: 63 ± 3, $p<0.001$). The ratio of conjugated dienes to LDL cholesterol was also reduced (15.0 ± 1.0 test vs. 15.7 ± 0.9 control, $p=0.032$) between the two dietary treatments. These findings demonstrate soy isoflavone consumption may protect LDL cholesterol from oxidative damage *in vitro* and reduce the concentration of oxidized LDL cholesterol as expressed by conjugated diene formation (16). This study also demonstrated the consumption of high isoflavones foods appears to be associated with

reduced levels of circulating oxidized LDL. The effect of dietary antioxidants in reducing the risk of atherosclerosis has potential value in CVD risk reduction, possibly without adverse side effects.

Further research by Jenkins et al. (17) assessed the effects of a soy-based breakfast cereal on serum lipids and oxidized LDL in a randomized crossover design with two three-week ad libitum diets. Twenty-five hyperlipidemic subjects with elevated serum LDL cholesterol concentrations of >74 mg/dL and triglyceride levels <72 mg/dL were placed on a NCEP Step 2 diet (<30% energy as total fat, <7% saturated fat, and <200 mg/d dietary cholesterol) 1 month prior to the study to control the subject's background diet. Following the run-in period, subjects consumed 36 g/d soy protein (168 mg/100 g isoflavones) daily for 3-weeks. Low-density lipoprotein was reduced compared with the control both as total dienes in LDL and as the ratio of conjugated dienes to cholesterol in the LDL fraction by 9.2 ± 4.3%, p=0.042 and 8.7 ± 4.2%, p=0.050. This study further demonstrates that daily consumption of soy can reduce the concentration of oxidized LDL cholesterol. These results may be attributed to the increased isoflavone intake in conjunction with soy protein (17).

In addition to the work conducted by Tikkanen and Jenkins, Weisman et al. (38) also examined the effects of soy protein on *in vivo* biomarkers of lipid peroxidation and oxidative resistance. Twenty-four healthy subjects participated in a randomized crossover design and consumed a high isoflavone (HI) burger (21.2 mg daidzein, 34.8 mg genistein) and a low isoflavone (LI) burger (0.9 mg daidzein, 1.0 mg genistein) once a day for 17 days along with an ad libitum dietseparated by a 25 day washout period.