

ABSTRACT

Title of Dissertation: LOX-1 GENOTYPE, DIETARY FAT INTAKE,
AND AEROBIC EXERCISE TRAINING:
INFLUENCE ON ENDOTHELIAL FUNCTION,
OXIDATIVE STRESS, LIPOPROTEIN-LIPIDS,
AND SOLUBLE LOX-1

Tina Janelle Ellis, Doctor of Philosophy, 2006

Dissertation directed by: Professor James M. Hagberg
Department of Kinesiology

The lectin-like oxidized LDL receptor (LOX-1) is the major receptor for oxidized LDL (ox-LDL) in endothelial cells and plays a major role in the initiation and progression of atherosclerosis. Ox-LDL via LOX-1 causes endothelial activation and injury, induces lipid peroxidation, and alters inflammatory gene expression, and variation in the LOX-1 gene has been associated with cardiovascular disease risk. In addition, a soluble form of LOX-1 has been identified in plasma and may predict atherosclerotic disease progression. Thus, the purpose of this study was to investigate the effect of the LOX-1 3'UTR C/T and G501C polymorphisms on endothelial function, oxidative stress, plasma lipoprotein-lipids, and soluble LOX-1. The effect of these polymorphisms on the responses to dietary fat intake and aerobic exercise training was also examined. Forearm blood flow was measured using venous occlusion plethysmography at rest and during reactive hyperemia, and plasma levels of nitrates/nitrites, nitrotyrosine, ox-LDL, total

antioxidant capacity, lipoprotein-lipids, and soluble LOX-1 were measured before and after six months of aerobic exercise training. The dietary ratio of polyunsaturated fat to saturated fat (P:S ratio) was determined using 7-day food records. The 3'UTR/CC and 501GC+CC groups had significantly higher baseline soluble LOX-1 levels than the CT/TT and GG groups, respectively. The G501C polymorphism was a significant predictor of baseline soluble LOX-1 levels, even after accounting for age, gender, race, BMI, and the 3'UTR polymorphism ($p=0.024$). There was a significant interaction between the 3'UTR polymorphism and dietary fat intake for plasma ox-LDL levels ($p=0.011$). At a high P:S ratio, the 3'UTR/CC group had significantly higher ox-LDL levels than the TT group ($p=0.025$). The opposite relationship was found at a low P:S ratio ($p=0.044$). The 3'UTR polymorphism also influenced changes in plasma TG levels with exercise training ($p=0.036$), while the G501C polymorphism influenced changes in soluble LOX-1 levels ($p=0.012$). In conclusion, variation in the LOX-1 gene does not appear to be associated with endothelial function, oxidative stress, or plasma lipid levels, but may influence changes in these variables in response to dietary fat intake and exercise training. Moreover, polymorphisms in LOX-1, especially the G501C polymorphism, may regulate circulating levels of soluble LOX-1.

LOX-1 GENOTYPE, DIETARY FAT INTAKE, AND AEROBIC EXERCISE
TRAINING: INFLUENCE ON ENDOTHELIAL FUNCTION, OXIDATIVE
STRESS, LIPOPROTEIN-LIPIDS, AND SOLUBLE LOX-1

by

Tina Janelle Ellis

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Advisory Committee:

Professor James M. Hagberg, Chair

Professor Thomas W. Castonguay

Associate Professor Michael D. Brown

Research Assistant Professor Dana A. Phares

Assistant Professor Stephen M. Roth

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LIST OF ABBREVIATIONS

AHA – American Heart Association

BH₄ – tetrahydrobiopterin

BMI – body mass index

CAD – coronary artery disease

ELISA – enzyme-linked immunosorbent assay

eNOS – endothelial nitric oxide synthase

FBF – forearm blood flow

HDL – high-density lipoprotein

HRT – hormone replacement therapy

LDL – low-density lipoproteins

LOX-1 – lectin-like oxidized LDL receptor

NCEP – National Cholesterol Education Program

NO – nitric oxide

NO_x – nitrates/nitrites

Ox-LDL – oxidized LDL

PUFA – polyunsaturated fatty acids

ROS – reactive oxygen species

SFA – saturated fatty acids

TAC – total antioxidant capacity

TG – triglycerides

UTR – untranslated region

VO₂ max – maximum volume of oxygen consumption

INTRODUCTION

The oxidative modification of low-density lipoproteins (LDL) plays a key role in the initiation and progression of atherosclerosis. (1) Oxidized LDL (ox-LDL) promotes an inflammatory response in the vascular wall by inducing foam cell formation, apoptosis and necrosis of vascular cells, leukocyte adhesion/infiltration, smooth muscle cell migration and proliferation, and platelet aggregation. (1-5) The lectin-like ox-LDL receptor (LOX-1) is the major receptor for ox-LDL on endothelial cells and mediates many of the atherogenic effects of ox-LDL. (6) LOX-1 expression is upregulated *in vitro* by various pro-inflammatory, pro-oxidant, vasoactive, and hemodynamic stimuli, and is upregulated *in vivo* in pathological settings such as hypercholesterolemia, hypertension, diabetes, and myocardial ischemia. (6,7) Ox-LDL-mediated activation of LOX-1 has been shown to cause endothelial dysfunction and oxidative stress in cultured endothelial cells. (8-12) Recently, it has been demonstrated that LOX-1 activation also downregulates LDL receptor expression in endothelial cells. (13) Taken together, these findings suggest that LOX-1 may promote atherosclerosis via its effects on endothelial function, oxidative stress, and plasma lipoprotein-lipid levels. However, no studies have examined these associations in humans.

The endothelium plays a major role in vascular homeostasis through the release of various vasoactive and thromboregulatory substances, signaling molecules, and growth factors. (14) One of the most important endothelium-derived substances is nitric oxide (NO), and this free radical signaling molecule mediates many of the vasoprotective effects of the vascular wall. (15) Reduced NO bioactivity is a major mechanism for endothelial dysfunction and commonly results from decreased NO production and/or

increased NO degradation by superoxide. (15,16) Endothelial dysfunction is considered an early marker of atherosclerosis, often preceding clinical evidence of atherosclerotic plaques. (15) In many cases, endothelial dysfunction is characterized by reduced plasma concentrations of the major NO metabolites, nitrate and nitrite (NO_x), or impaired endothelium-dependent vasodilation in response to mechanical (i.e. blood flow) or pharmacological stimuli. (14,17)

Oxidative stress, or the imbalance in oxidants and antioxidants, in favor of the former, has been implicated in vascular dysfunction and the pathogenesis of many cardiovascular diseases. (1,18) Oxidative stress reduces NO bioactivity by promoting the reaction between NO and superoxide to form the strong oxidant peroxynitrite. (19) Peroxynitrite may subsequently react with the aromatic ring of tyrosine to form nitrotyrosine, a stable marker of protein modification by NO-derived oxidants. (20) Oxidative stress may also contribute to the formation of ox-LDL through both direct, enzymatic and indirect, non-enzymatic oxidation reactions. (1) Furthermore, in the presence of oxidative stress increased reactive oxygen species (ROS) and other oxidants can impair antioxidant activities and overwhelm the body's antioxidant defense system. (1) Consistent with the role of oxidative stress in atherogenesis, increased plasma levels of nitrotyrosine and ox-LDL and decreased antioxidant defenses have been found in patients with vascular disease. (1,21,22)

While endothelial dysfunction and oxidative stress may be considered emerging cardiovascular risk factors, it is well established that lipoproteins play a major role in the development of atherosclerosis. (23) The relative abundance of the different plasma lipoproteins appears to be of primary importance, as elevated levels of atherogenic

lipoproteins and/or reduced levels of atheroprotective lipoproteins may contribute to atherogenesis and increase risk for cardiovascular disease. (24) Moreover, abnormal plasma lipoprotein levels (i.e. dyslipidemia) increase cardiovascular disease risk by promoting LDL oxidation, vascular dysfunction, plaque formation, and thrombosis. (25,26)

The presence of LOX-1 in the vascular wall has important implications for the development of atherosclerosis; however, circulating LOX-1 may have a very different role in pathophysiology. Like many cell-surface receptors with a single transmembrane domain, LOX-1 can be cleaved at the juxtamembrane region and secreted in a soluble form. (27) Studies have shown that soluble membrane receptors can modulate disease activity by binding to its ligand and preventing ligand uptake by the receptor at the cell surface. In addition, soluble membrane proteins in plasma may reflect expression of membrane-bound proteins and disease activity. Although the role of soluble LOX-1 is unclear, elevated levels have been found to predict atherosclerotic disease progression in humans. (28)

Over the last decade, LOX-1 has emerged as a promising candidate gene for the development of cardiovascular disease, as the chromosomal region encoding the LOX-1 gene is associated with both atherosclerosis and hypertension. (29,30) Seven polymorphisms have been identified in the LOX-1 gene, including a C-to-T substitution in the 3'-untranslated region (UTR). (31) This polymorphism affects the binding of nuclear proteins in an allele-specific manner, and the 3'UTR/T allele has been associated with an increased risk for coronary artery disease (CAD) and acute myocardial infarction. (31-33) In addition, the 3'UTR polymorphism is in complete linkage disequilibrium with

5 intronic polymorphisms that regulate the splicing of exon 5 and the subsequent generation of a truncated LOX-1 protein. (31,34) A second functional polymorphism has been identified in exon 4 which involves a G-to-C substitution at nucleotide 501, leading to a nonconservative amino acid change (Lys to Asn) in codon 167. (35) Due to its location in the ligand-binding domain, this polymorphism may be protective against cardiovascular disease by reducing the binding and uptake of ox-LDL and the subsequent activation of LOX-1. (36) However, not all studies have supported a protective role for the 501C allele. (35,37) Thus, the association of this polymorphism with cardiovascular disease remains to be elucidated.

Atherosclerosis is a multifactorial disease that involves complex interactions between genes and environmental factors. (38) Dietary fat intake and aerobic exercise training are important environmental factors that can significantly impact cardiovascular risk. (24) In general, polyunsaturated fatty acids (PUFAs) improve vascular function, reduce inflammation, and lower plasma lipid levels, while saturated fatty acids (SFAs) impair vascular function, promote thrombosis, and increase plasma lipid levels. (39,40) Thus, the relative amount of PUFAs and SFAs in the diet (i.e. the P:S ratio) is an important determinant of disease risk and may be inversely associated with atherosclerosis. (41) Similarly, exercise training reduces cardiovascular risk by improving endothelial function, reducing oxidative stress, and improving the plasma lipoprotein-lipid profile. (42-44) However, the inter-individual responses to dietary fat intake and aerobic exercise training are highly variable and may be influenced by genetic factors. (45,46) Given that LOX-1 expression is regulated by linoleic acid and shear stress, two stimuli that are important components of dietary fat and exercise, respectively,

it is possible that genetic variation in LOX-1 may influence responses to these environmental factors. (47,48) Thus, the purpose of this study was to 1) investigate the influence of the LOX-1 3'UTR and G501C polymorphisms on endothelial dysfunction (as assessed by forearm blood flow and plasma NOx levels), oxidative stress (as assessed by plasma nitrotyrosine levels, ox-LDL levels, and total antioxidant capacity), dyslipidemia, and soluble LOX-1 levels; and 2) determine if these polymorphisms influence the responses in these variables to dietary fat intake and aerobic exercise training.

HYPOTHESES

- 1) The LOX-1 3'UTR C/T and G501C polymorphisms will be associated with endothelial function, oxidative stress, plasma lipoprotein-lipid levels, and soluble LOX-1 levels.

- 2) The LOX-1 3'UTR C/T and G501C polymorphisms will interact with the dietary P:S ratio to influence endothelial function, oxidative stress, plasma lipoprotein-lipid levels, and soluble LOX-1 levels.

- 3) The LOX-1 3'UTR C/T and G501C polymorphisms will influence the exercise training-induced changes in endothelial function, oxidative stress, plasma lipoprotein-lipid levels, and soluble LOX-1 levels.

METHODS

Subjects and Screening

Men and women 50 – 75 years old were recruited to participate in this study and screened to ensure eligibility. All subjects were sedentary, non-smokers, non-diabetic, and free of heart, liver, kidney, and lung disease. Subjects were either a) normotensive with at least one National Cholesterol Education Program (NCEP) lipid abnormality (total cholesterol >200 mg/dL, LDL-C >130 mg/dL, HDL-C <40 mg/dL, or TG >200 mg/dL) or b) hypertensive (BP <160/90 mmHg), but controlled by medications not affecting lipid metabolism, and with or without at least one NCEP lipid abnormality. All females were postmenopausal and agreed to maintain their hormone replacement therapy (HRT) regimen for the duration of the study. Individuals with a body mass index (BMI) >37 kg/m², fasting TG >400 mg/dL, and fasting or postprandial glucose levels >126 mg/dL and >200 mg/dL, respectively, were excluded from the study. Subjects also completed a maximal graded exercise test to detect any cardiovascular, pulmonary, or other chronic diseases that would preclude exercise testing or training. (49) Individuals with tests indicating signs or symptoms of cardiovascular disease were excluded from the study. Subjects were informed of the study requirements and provided their written consent. This study was approved by the University of Maryland at College Park Institutional Review Board.

Dietary Stabilization

Subjects were stabilized for 6 weeks on an American Heart Association (AHA) Step I diet, consisting of ≤30% of daily calories from fat. Midway through dietary stabilization, subjects completed a 3-day food record to determine their average energy

and nutrient intake over the 3-day period. Food records were analyzed using Computrition dietary software (Computrition Inc., Chatsworth, CA), and the amount of fat that constituted ~30% of their total caloric intake was determined. Subjects were encouraged to maintain this fat intake for the duration of the study.

Baseline Testing

After the dietary stabilization period, subjects underwent baseline testing to assess the main outcome variables before exercise training. Ten milliliters of blood was drawn into EDTA tubes in the morning after a 12-hour overnight fast on at least 2 separate days and averaged to determine baseline lipid levels. Fifteen milliliters of blood was drawn into EDTA tubes to measure plasma NO_x, ox-LDL, and nitrotyrosine levels, and five milliliters of blood was drawn into heparin tubes to measure plasma total antioxidant capacity (TAC) and soluble LOX-1 levels. Blood samples were centrifuged at 3000 rpm at 4°C for 20 min, and frozen aliquots were stored at -80°C until further analysis. Total body fat was measured by dual energy x-ray absorptiometry, and intra-abdominal fat was quantified at lumbar vertebrae L4-L5 using computerized tomography, as described previously. (50,51) Maximum volume of oxygen consumption (VO₂ max) was determined during a graded exercise treadmill test, and standard criteria were used to verify that VO₂ max was achieved. (52) At the conclusion of baseline testing, subjects completed a 7-day food record to assess compliance with the AHA Step I diet. The proportion of PUFAs and SFAs in the diet were used to calculate the P:S ratio. Subjects were divided into 2 diet groups based on the median P:S ratio: high P:S ratio and low P:S ratio.

Forearm Blood Flow

Forearm blood flow (FBF), in mL per 100 mL of forearm volume per minute (mL/100 mL/min), was measured by venous occlusion plethysmography using the EC5R Strain Gauge Plethysmograph (D.E. Hokanson Inc., Bellevue, WA). A mercury-in-silastic strain gauge was placed at the widest part of the forearm, and subjects lay supine for 15 minutes with the forearm placed above heart level. An arm cuff was placed proximal to the strain gauge to control blood flow to the forearm, while a wrist cuff was placed distally to eliminate hand flow. To determine FBF at rest, the wrist and arm cuffs were inflated to >180 mmHg and 55 mmHg, respectively, and FBF was measured for 7 cardiac cycles. This procedure was repeated two more times and the 3 measurements were used to determine the average resting FBF. To determine FBF during reactive hyperemia, ischemia was induced for 5 minutes by inflating the arm cuff to 50 mmHg above resting systolic blood pressure and FBF was measured every 15 seconds for 3 minutes. Given that peak blood flow has been shown to occur at ~1 minute following ischemia, reactive hyperemia was calculated as the percent change in FBF from resting to 1 min post-ischemia. (53,54)

Nitrates/Nitrites

Plasma NO_x levels were determined using a modified Griess assay. In brief, plasma samples were first deproteinated using centrifugal filter units (Ultrafree-MC, Millipore Corporation, Billerica, MA). After centrifugation at 9000 rpm at 4°C for 40-50 min, 40 µL of the filtered sample was incubated with 20 µL of nitrate reductase (1050 U/L) and 10 µL of NADPH (175 µM) for 1 hour at room temperature to convert nitrate to nitrite. To remove excess NADPH, 20 µL of NH₄Cl (250 mM), 10 µL of glutamate

dehydrogenase (2500 U/L), and 20 μ L of α -ketoglutarate (10 mM) were added and the samples were incubated for 1 hour at room temperature. To start the Griess reaction, 40 μ L of a sulfanilamide solution (1% sulfanilamide in 5% phosphoric acid) was added to the mixture and incubated for 30 minutes at room temperature. The mixture was then incubated with 40 μ L of a 0.1% N-1-naphthylethylenediamine dihydrochloride solution for 30 minutes at room temperature, and the absorbance was measured on a microplate reader at a wavelength of 541 nm. All samples were assayed in duplicate. The intra-assay and inter-assay coefficients of variation (CVs) were 3.3% and 4.9%, respectively.

Oxidized LDL

Plasma ox-LDL levels were measured using a competitive ELISA (Merckodia, Uppsala, Sweden) that utilizes the murine monoclonal antibody 4E6. This antibody is directed against a conformational epitope in LDL that is generated from substitution of at least 60 lysine residues in apo B with aldehydes. (55) This number corresponds to the minimal number of substituted lysines required for scavenger receptor-mediated uptake of ox-LDL. The amount of ox-LDL in plasma was determined by reading the absorbance at 450 nm. All samples were assayed in duplicate. The intra-assay and inter-assay CVs were 18.9% and 15.6%, respectively.

Nitrotyrosine

Plasma nitrotyrosine levels were measured using a sandwich ELISA (Cell Sciences, Canton, MA) that detects nitrosylated protein adducts. The amount of nitrotyrosine in plasma was determined by measuring the absorbance at 450 nm. All samples were assayed in duplicate. The intra-assay and inter-assay CVs were 39.6% and 12.46%, respectively.

Total Antioxidant Capacity

TAC was measured using a commercially available kit (Cayman Chemical, Ann Arbor, MI) that measures the combined antioxidant activities of all aqueous and lipid-soluble antioxidants in plasma, including vitamins, proteins, lipids, and enzymes. This assay relies on the ability of antioxidants in plasma to inhibit the oxidation of ABTS[®] (2,2'-Azino-di-[3-ethylbenzthiazoline sulphonate) to ABTS[®].⁺ by metmyoglobin. The capacity of the antioxidants in plasma to prevent ABTS[®] oxidation is compared with that of Trolox, a water-soluble vitamin E analogue, and is quantified as millimolar Trolox equivalents. The amount of ABTS[®].⁺ produced was measured by reading the absorbance at 750 nm. All samples were assayed in duplicate. The intra-assay and inter-assay CVs were 10.5% and 4.4%, respectively.

Lipoprotein-Lipid Profile

Plasma TG and total cholesterol levels were measured enzymatically, and LDL-C was calculated using the Friedewald equation. (56-58) HDL-C was measured after precipitation with dextran sulfate, and HDL₂ and HDL₃ were separated and quantified using a second precipitation with dextran sulfate. (59,60) HDL₂-C was calculated as the difference between total HDL-C and HDL₃-C.

Soluble LOX-1

Plasma levels of soluble LOX-1 were measured by a sandwich ELISA using two different human LOX-1-specific antibodies, as described previously. (28) These antibodies were obtained after purification of serum from rabbits that were immunized with a recombinant protein corresponding to the extracellular domain of human LOX-1.

Exercise Training Intervention

Following the baseline tests, subjects completed 24 weeks of aerobic exercise training, as described previously. (61) Briefly, in the initial sessions, subjects exercised for 20 minutes at 50% VO₂ max, 3 days/week. Exercise duration and intensity were gradually increased such that by week 10, subjects were exercising for 40 min/day at 70% VO₂ max. A fourth unsupervised exercise session was added during the last 14 weeks of training. Subjects completed food frequency checklists every 2 months to monitor dietary intake during the exercise intervention.

Final Testing

At the completion of the exercise training intervention, subjects provided a final 7-day food record to ensure dietary compliance prior to reassessment of the outcome variables. Subjects continued exercise training until all final tests were complete. Blood samples were drawn 24-36 hours after an exercise session.

Genotyping

A separate blood sample (10 mL) was drawn to genotype for the LOX-1 3'UTR C/T and G501C polymorphisms. DNA was isolated from peripheral lymphocytes and genotyped using fluorescence polarization. Primers for the 3'UTR polymorphism were: forward, 5'-AGCTATTCTTTGTCACCTTGGGTG-3'; reverse, 5'-CTGAGTTCAGAGGG TTTTCAAGC-3'; and internal reverse, 5'-GGGAAGCTTGGGACAAGCTAGGTGAAA TAATACAG-3'. Primers for the G501C polymorphism were: forward, 5'-CAGCTCCTT GTCCGCAAGACTGGAT-3'; reverse, 5'-GAACACTCACCCAGATCAGCTGTGCT-3'; and internal reverse, 5'-CTTGGCATCCAAGACAAGCACTTCTCTTGGCT-3'. PCR amplification for both polymorphisms was carried out in a reaction volume of 10 µL

containing 1 μL of PCR Buffer (10X), 0.3 μL of MgCl_2 (50 mM), 0.2 μL dNTP (1.25 mM), 0.05 μL of each primer (20 μM), 0.04 μL of Taq DNA polymerase (5 U/ μL), 7.36 μL of deionized water, and 1 μL of DNA. PCR was performed using an initial denaturation at 95°C for 5 min, followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 46°C for 15 sec, and extension at 72°C for 30 sec. At the end of the PCR assay, 10 μL of an enzymatic cocktail containing 1 μL of SAP Buffer (10X), 1 μL of shrimp alkaline phosphatase (1 U/ μL), 0.1 μL of exonuclease I (10 U/ μL), and 7.9 μL of deionized water was added to the PCR product. The mixture was then incubated at 37°C for 90 min and the enzymes were heat inactivated at 95°C for 15 min. After purification of the PCR products, single base extension was carried out in a reaction volume of 10 μL containing 1 μL of thermosequenase buffer (10X), 1 μL of the internal primer (10 μM), 0.05 μL of dye-labeled ddNTP mix (25 μM), 0.1 μL of thermosequenase (4 U/ μL), and 7.85 μL of deionized water. Single base extension was performed using an initial denaturation at 94°C for 1 min, followed by 40 cycles of denaturation at 94°C for 10 sec and annealing at 52°C for 30 sec. The fluorescence polarization measurement and genotype assignments were performed as described previously. (62)

Statistical Analyses

All statistical analyses were performed using SAS version 9.1. Plasma nitrotyrosine levels were skewed and had to be log transformed for subsequent analysis. Chi square frequency tests were used to compare differences in categorical variables between genotype groups. Pearson correlation coefficients were used to examine relationships between outcome variables. Paired t-tests were used to determine the changes in outcome variables with exercise training. Analysis of covariance was used to

compare differences between genotype groups at baseline and in response to training. Age, gender, race, HRT status, and intra-abdominal fat were used as covariates based on previous data supporting their influence on endothelial function, oxidative stress, and plasma lipid levels. Given that very limited data is available on the relationship between soluble LOX-1 and cardiovascular risk factors, correlations with age and body composition were examined. To assess the use of gender, HRT, and race as potential covariates for soluble LOX-1, interactions between the LOX-1 polymorphisms and these variables were tested for significance. Based on these analyses, differences in soluble LOX-1 levels between genotype groups were adjusted for BMI. Changes in the outcome variables with training were also adjusted for the baseline value. Linear regression was used to determine the proportion of variation in soluble LOX-1 levels that is accounted for by the LOX-1 polymorphisms. A 2x2 factorial analysis was used to test for gene-diet interactions after adjusting for confounding variables. Statistical significance was set at $p \leq 0.05$.

RESULTS

Overall Subject Characteristics

The total study population consisted of 233 subjects, including 104 men and 129 women. The mean age was 58 yrs (range 50 – 75 yrs) and the mean BMI was 28.4 ± 0.3 kg/m² (range 19.5 – 36.9 kg/m²). Sixty-six percent of the subjects were Caucasian, 25% were African-American, and the remaining 9% were Asian/Pacific Islander, American Indian, Hispanic, or Other. Fifty-eight percent of the women were on HRT. Genotype data and data for the outcome variables before and/or after exercise training were not available for all 233 subjects. Thus, genotype comparisons included only those individuals with available data for a given outcome variable. Among the 226 subjects with available genotype data for the LOX-1 3'UTR polymorphism, the frequencies of the CC, CT, and TT genotype groups were 31%, 46%, and 23%, respectively, resulting in a T-allele frequency of 46%. Among the 227 subjects with available genotype data for the LOX-1 G501C polymorphism, the frequencies of the GG, GC and CC genotype groups were 83%, 16%, and 1%, respectively, resulting in a C-allele frequency of 9%. Given the low frequency of the 501C allele, the GC and CC groups were combined for all of the analyses. Endothelial function, oxidative stress, plasma lipid levels, and soluble LOX-1 levels in the overall group at baseline are presented in Table 1. There were no significant correlations among endothelial function, oxidative stress, and plasma lipid levels at baseline. Soluble LOX-1 levels were inversely associated with TAC (Figure 1), but not any other outcome variables.

Baseline Genotype Differences

Age, weight, body composition, and VO₂ max were similar between genotype groups for both LOX-1 polymorphisms. There were no significant baseline differences among 3'UTR/CC, CT, and TT genotype groups or between 501GG and GC+CC genotype groups for any of the endothelial function variables, oxidative stress markers, or plasma lipid levels (Tables 2 and 3). The 3'UTR/CC group had significantly higher soluble LOX-1 levels than both the CT and TT groups (p=0.038) (Figure 2). Similarly, the 501GC+CC group had significantly higher soluble LOX-1 levels than the GG group (p=0.011) (Figure 3). In univariate analysis, both the 3'UTR and G501C polymorphisms were significant predictors of soluble LOX-1, explaining 9.18% and 4.33%, respectively, of the variation in soluble LOX-1 levels (Table 4). BMI was also a significant predictor of soluble LOX-1 levels in univariate analysis (p=0.018). Only the G501C polymorphism remained a significant predictor of soluble LOX-1 after accounting for age, gender, race, and BMI. In multivariate analysis, the LOX-1 G501C polymorphism explained 5.13% of the variation in soluble LOX-1 levels (Table 4).

Gene-Diet Interactions

The dietary fat intake of the overall group is shown in Table 5. Based on the median P:S ratio = 0.55, subjects were categorized into two diet groups: high P:S ratio and low P:S ratio. Subjects in the high P:S ratio group had a significantly lower dietary intake of total fat and SFA, and a higher PUFA intake compared to subjects in the low P:S ratio group (Table 6). There was no difference in genotype frequencies between the two diet groups. Total fat, SFA, and P:S ratio were similar in the 3'UTR CC, CT, and TT genotype groups, although the CC group had a slightly higher PUFA intake than the TT

group (0.050 ± 0.002 vs. 0.041 ± 0.003 , respectively, $p=0.038$). There were no differences in dietary fat intake between 501GG and GC+CC genotype groups.

There were no significant interactions between the LOX-1 3'UTR polymorphism and diet for any of the endothelial function variables, plasma nitrotyrosine levels, TAC, plasma lipid levels, or soluble LOX-1 levels after adjusting for confounding factors. There were also no significant main effects for diet or genotype. There was a significant interaction between the LOX-1 3'UTR polymorphism and diet for plasma ox-LDL levels ($p=0.011$ for interaction) (Figure 4). At a high P:S ratio, the 3'UTR/CC group had higher ox-LDL levels than the TT group ($p=0.025$), but at a low P:S ratio, the CC group had lower ox-LDL levels compared to the TT group ($p=0.044$). In addition, 3'UTR/CC genotype individuals with a high dietary P:S ratio had significantly higher plasma ox-LDL levels than those with a low P:S ratio ($p=0.029$). In contrast, 3'UTR/TT genotype individuals with a high dietary P:S ratio had significantly lower ox-LDL levels than those with a low dietary P:S ratio ($p=0.036$). There were no significant interactions between the LOX-1 G501C polymorphism and dietary P:S ratio for plasma NO_x levels, oxidative stress markers, lipid levels, or soluble LOX-1. Interactions for resting FBF and reactive hyperemia could not be tested due to insufficient sample sizes. None of the main effects were significant for either diet or genotype.

Training Changes in the Overall Group

Six months of exercise training increased VO₂ max by 14%, indicating a significant training effect (baseline: 25.3 ± 0.3 mL/kg/min vs. final: 29.2 ± 0.5 mL/kg/min, $p<0.0001$). As expected, the subjects remained within $\pm 5\%$ of their baseline weight, with the average change in weight being -1.1 ± 0.1 kg ($-1.3 \pm 0.2\%$, range -5.0% to $+4.7\%$).

Total body fat decreased from $36.5 \pm 0.6\%$ to $34.1 \pm 0.8\%$ with training ($p=0.019$), and intra-abdominal fat decreased from $129.4 \pm 3.7 \text{ cm}^2$ to $118.4 \pm 4.3 \text{ cm}^2$ ($p<0.0001$). There were no significant changes in resting FBF, reactive hyperemia, or plasma NOx levels with training (Table 7). Plasma nitrotyrosine levels increased by 12% ($p=0.025$), while ox-LDL levels decreased by 11% ($p=0.032$). There was a significant decrease in plasma TG levels and significant increases in HDL-C, HDL₂-C, and HDL₃-C. There were no significant changes in TAC, total cholesterol, LDL-C, or soluble LOX-1 levels with training. Changes in ox-LDL levels with training were negatively correlated with changes in reactive hyperemia (Figure 5). There were no other significant correlations between changes in outcome variables with exercise training.

Genotype Differences in the Training-induced Responses

There were no significant differences in the changes in endothelial function or oxidative stress among 3'UTR/CC, CT, and TT genotype groups (Table 8). Changes in plasma TG levels with training were significantly different between genotype groups ($p=0.036$), with the 3'UTR/CT group having significantly greater reductions compared to the TT group ($p=0.010$). Changes in soluble LOX-1 levels were not different between the 3'UTR genotype groups ($p=0.33$) (Figure 6). There were no significant differences in the changes in endothelial function, oxidative stress, or plasma lipid levels between the G501C genotype groups (Table 9). The GG and GC+CC groups tended to have different responses in TAC with training, although this only approached significance ($p=0.068$). Changes in soluble LOX-1 levels were significantly different between the 501GG and GC+CC genotype groups ($p=0.012$). (Figure 7)

Table 1. Endothelial function, oxidative stress, plasma lipid levels, and soluble LOX-1 levels in the overall group at baseline

| Characteristic | N | Baseline | Range |
|-----------------------------|-----|------------|---------------|
| Total Cholesterol (mg/dL) | 196 | 200±2 | 119 – 279 |
| Triglycerides (mg/dL) | 196 | 135±5 | 42 – 384 |
| HDL-C (mg/dL) | 196 | 48±1 | 20 – 112 |
| HDL ₂ -C (mg/dL) | 195 | 6.6±0.6 | 0 – 46.1 |
| HDL ₃ -C (mg/dL) | 195 | 41.4±0.7 | 20.0 – 74.0 |
| LDL-C (mg/dL) | 196 | 124±2 | 52 – 201 |
| Resting FBF (mL/100mL/min) | 54 | 2.11±0.12 | 0.84 – 5.61 |
| Reactive hyperemia (%) | 54 | 117.3±12.0 | -5.3 – 387.7 |
| NOx (µmol/L) | 106 | 18.68±0.51 | 9.24 – 33.86 |
| Log Nitrotyrosine (nmol/L) | 72 | 1.01±0.09 | 0 – 2.86 |
| Ox-LDL (U/L) | 82 | 96.3±6.3 | 14.7 – 262.4 |
| TAC (mmol/L) | 100 | 2.80±0.10 | 0.36 – 5.80 |
| Soluble LOX-1 (pg/mL) | 99 | 42.79±2.45 | 4.28 – 125.05 |

Table values are expressed as means ± SE.

Figure 1. Correlation between soluble LOX-1 levels and total antioxidant capacity in the overall group at baseline

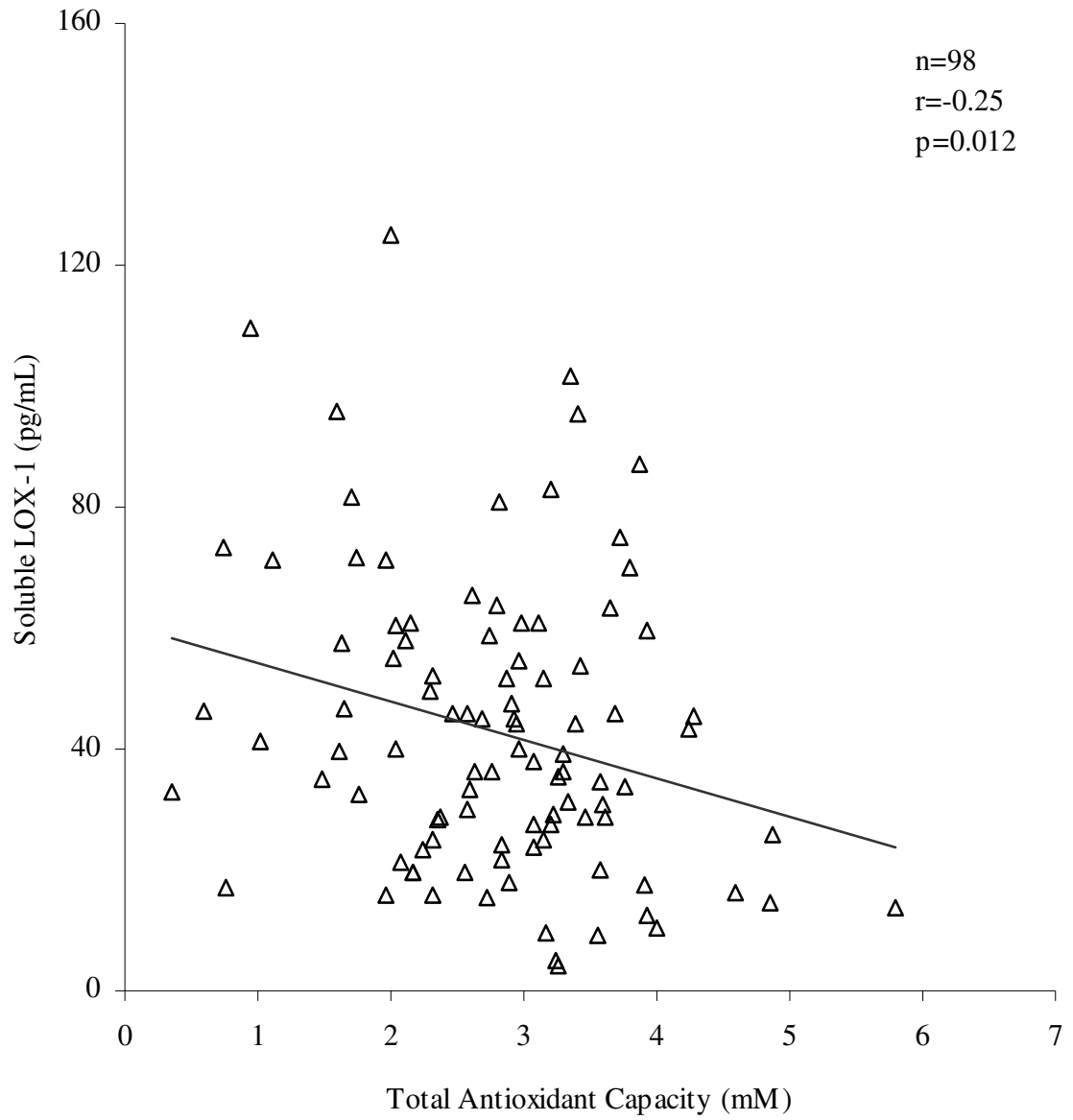


Table 2. Baseline differences in endothelial function, oxidative stress, and plasma lipid levels by the LOX-1 3'UTR polymorphism

| | Genotype | | | P-value |
|-----------------------------|--------------------|--------------------|--------------------|---------|
| | CC | CT | TT | |
| Resting FBF (mL/100mL/min) | 2.18±0.31 (10) | 1.78±0.34 (7) | 2.07±0.28 (12) | 0.67 |
| Reactive hyperemia (%) | 125.3±28.1 (10) | 109.8±30.8 (7) | 94.1±25.9 (12) | 0.77 |
| NOx (μmol/L) | 18.99±1.05 (28) | 18.37±0.89 (38) | 18.47±1.07 (27) | 0.90 |
| Log Nitrotyrosine (nmol/L) | 0.86±0.17 (20) | 1.26±0.15 (24) | 0.91±0.21 (14) | 0.17 |
| Ox-LDL (U/L) | 99.7±12.9 (21) | 100.8±11.8 (25) | 103.1±14.6 (17) | 0.98 |
| TAC (mmol/L) | 2.63±0.18 (22) | 2.58±0.15 (31) | 2.73±0.21 (17) | 0.85 |
| Total Cholesterol (mg/dL) | 200±5 (44) | 205±4 (76) | 205±5 (46) | 0.66 |
| Triglycerides (mg/dL) | 137±10 (44) | 141±7 (76) | 136±9 (46) | 0.88 |
| HDL-C (mg/dL) | 48±2 (44) | 48±2 (76) | 48±2 (46) | 0.95 |
| HDL ₂ -C (mg/dL) | 6.3±1.2 (43) | 6.5±0.90 (76) | 5.8±1.2 (46) | 0.89 |
| HDL ₃ -C (mg/dL) | 41.8±1.2 (43) | 41.7±0.92 (76) | 42.1±1.2 (46) | 0.97 |
| LDL-C (mg/dL) | 123±4 (44) | 128±3 (76) | 129±4 (46) | 0.50 |

Table values are expressed as adjusted means ± SE. Numbers in parentheses represent sample sizes.

Table 3. Baseline differences in endothelial function, oxidative stress, and plasma lipid levels by the LOX-1 G501C polymorphism

| Characteristic | Genotype | | P-value |
|-----------------------------|--------------------|--------------------|---------|
| | GG | GC+CC | |
| Resting FBF (mL/100mL/min) | 2.06±0.16 (26) | 1.81±0.48 (3) | 0.62 |
| Reactive hyperemia (%) | 101.8±14.5 (26) | 166.6±43.5 (3) | 0.17 |
| NOx (µmol/L) | 18.43±0.61 (80) | 20.22±1.38 (16) | 0.24 |
| Log Nitrotyrosine (nmol/L) | 1.11±0.22 (45) | 1.04±0.11 (12) | 0.77 |
| Ox-LDL (U/L) | 98.8±7.9 (54) | 121.5±18.4 (10) | 0.26 |
| TAC (mmol/L) | 2.67±0.11 (57) | 2.52±0.24 (12) | 0.57 |
| Total Cholesterol (mg/dL) | 203±3 (140) | 206±6 (27) | 0.62 |
| Triglycerides (mg/dL) | 139±5 (113) | 143±12 (24) | 0.78 |
| HDL-C (mg/dL) | 48±1 (140) | 48±3 (27) | 0.77 |
| HDL ₂ -C (mg/dL) | 6.0±0.7 (139) | 7.1±1.5 (27) | 0.47 |
| HDL ₃ -C (mg/dL) | 41.8±0.7 (139) | 41.1±1.6 (27) | 0.71 |
| LDL-C (mg/dL) | 127±2 (140) | 127±5 (27) | 0.99 |

Table values are expressed as adjusted means ± SE. Numbers in parentheses represent sample sizes.

Figure 2. Baseline differences in soluble LOX-1 levels by the LOX-1 3'UTR polymorphism

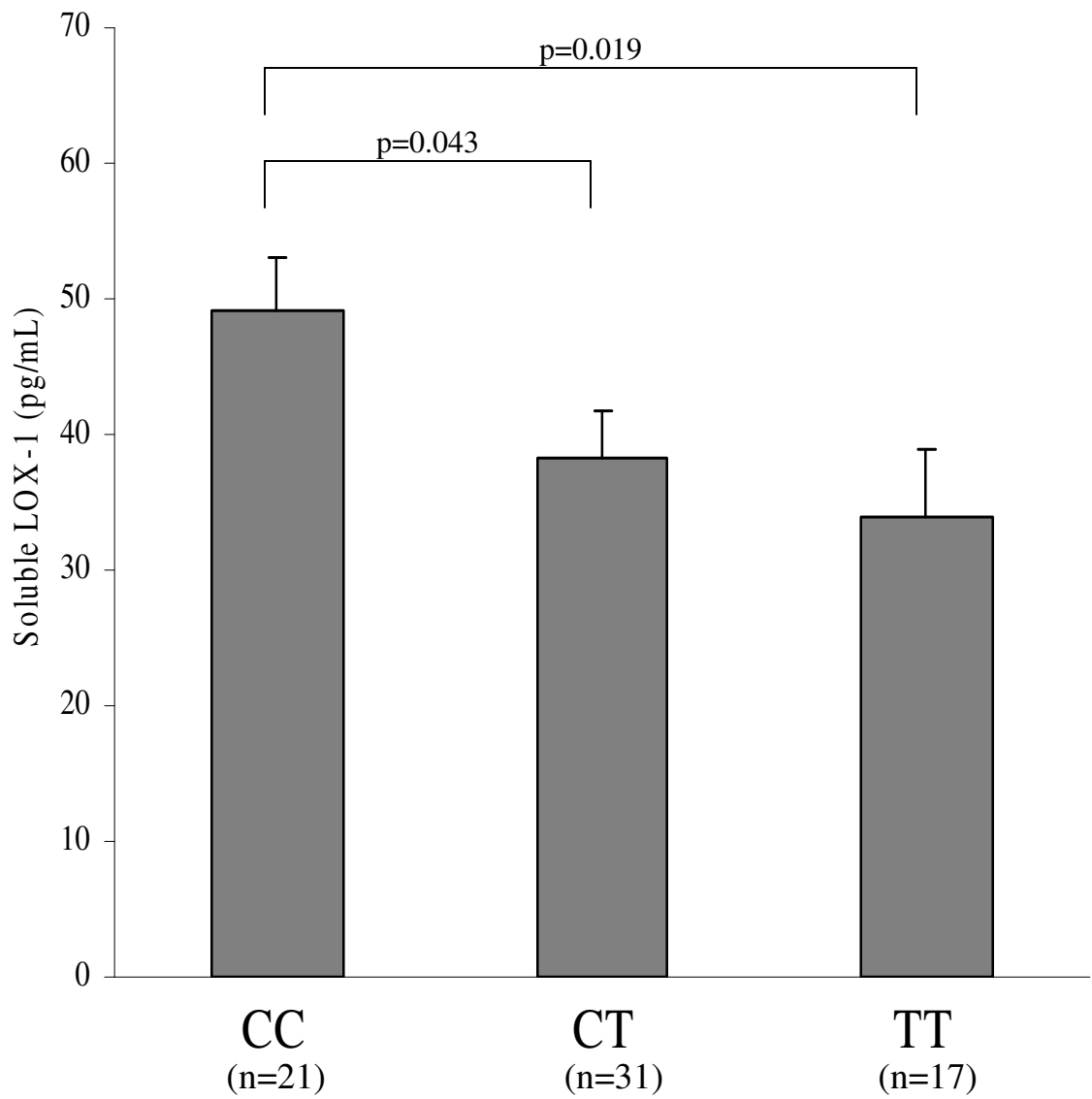


Figure 3. Baseline differences in soluble LOX-1 levels by the LOX-1 G501C polymorphism

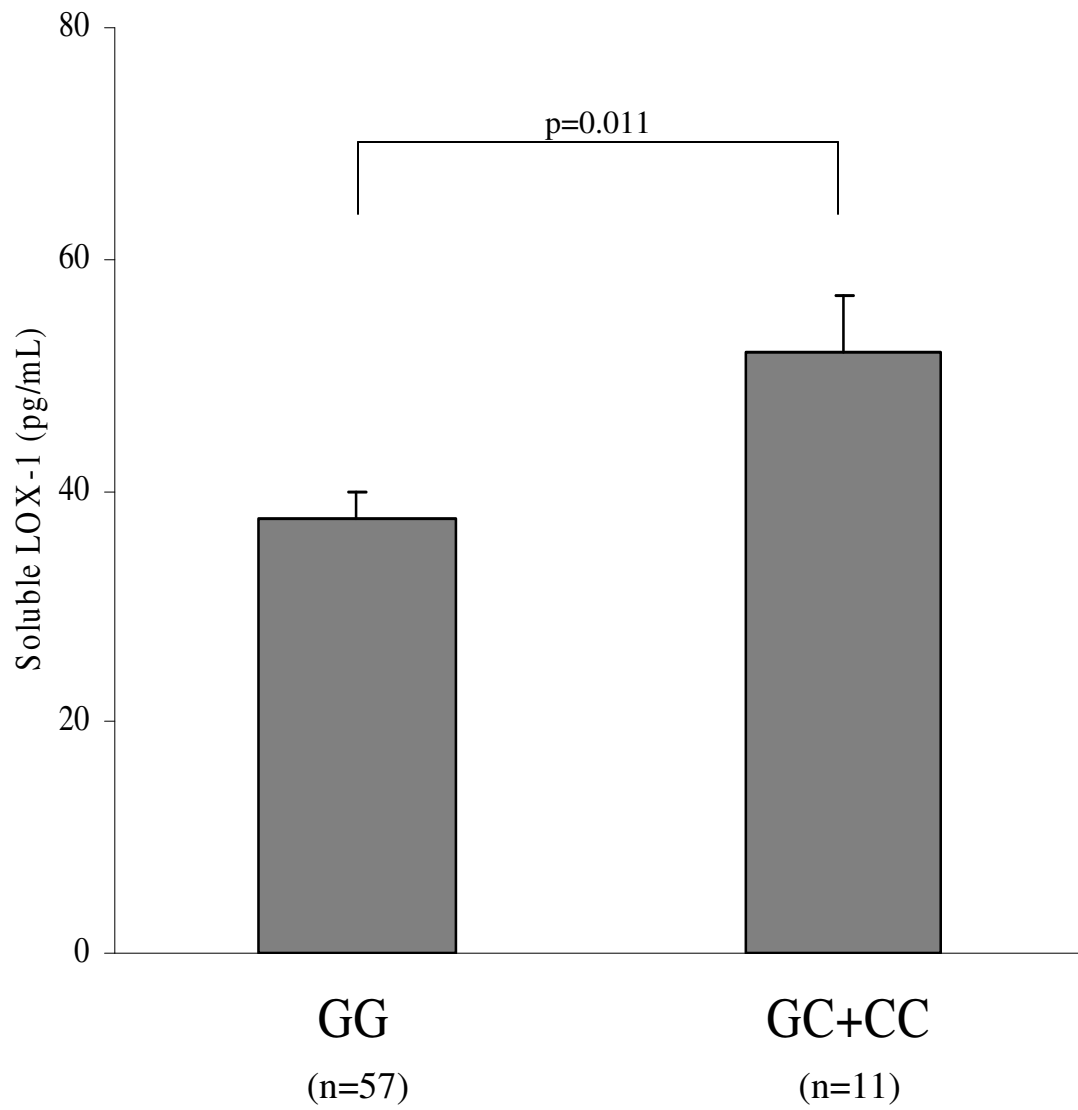


Table 4. Linear regression models with soluble LOX-1 as the dependent variable and LOX-1 polymorphisms, age, gender, race, and BMI as independent variables. #Indicates the probability that the given independent variable is a predictor of soluble LOX-1 levels.

| | Univariate | | Multivariate | |
|-----------|------------|----------|--------------|----------|
| | β | P-value# | β | P-value# |
| 3'UTR C/T | -10.41 | 0.0017 | -5.60 | 0.096 |
| G501C | 13.69 | 0.025 | 13.17 | 0.024 |
| Age | 0.28 | 0.54 | 0.62 | 0.15 |
| Gender | 6.91 | 0.16 | 2.81 | 0.56 |
| Race | 2.15 | 0.31 | 0.62 | 0.75 |
| BMI | 1.54 | 0.018 | 1.02 | 0.11 |

Table 5. Dietary fat intake in overall group (n=162)

| | Mean \pm SE | Median |
|---------------|-----------------|--------|
| Total Fat (%) | 27.2 \pm 0.4 | 27.1 |
| PUFA (%) | 4.6 \pm 0.1 | 4.6 |
| SFA (%) | 8.2 \pm 0.2 | 8.4 |
| P:S Ratio | 0.60 \pm 0.02 | 0.55 |

Table 6. Dietary fat intake by diet group

| | Diet Group | | P-value |
|---------------|-------------|------------|---------|
| | High (n=81) | Low (n=81) | |
| Total Fat (%) | 26.3±0.7 | 28.2±0.6 | 0.032 |
| PUFA (%) | 5.4±0.2 | 3.9±0.1 | <0.0001 |
| SFA (%) | 7.1±0.3 | 9.2±0.3 | <0.0001 |
| P:S Ratio | 0.76±0.02 | 0.43±0.02 | <0.0001 |

Figure 4. Plasma ox-LDL levels by the LOX-1 3'UTR polymorphism and dietary fat intake. Numbers in parentheses represent sample sizes at a high and low P:S ratio, respectively. #Statistically significant difference compared to CC genotype group at a high P:S ratio, $p < 0.03$. †Statistically significant difference compared to TT genotype group at a low P:S ratio, $p < 0.05$.

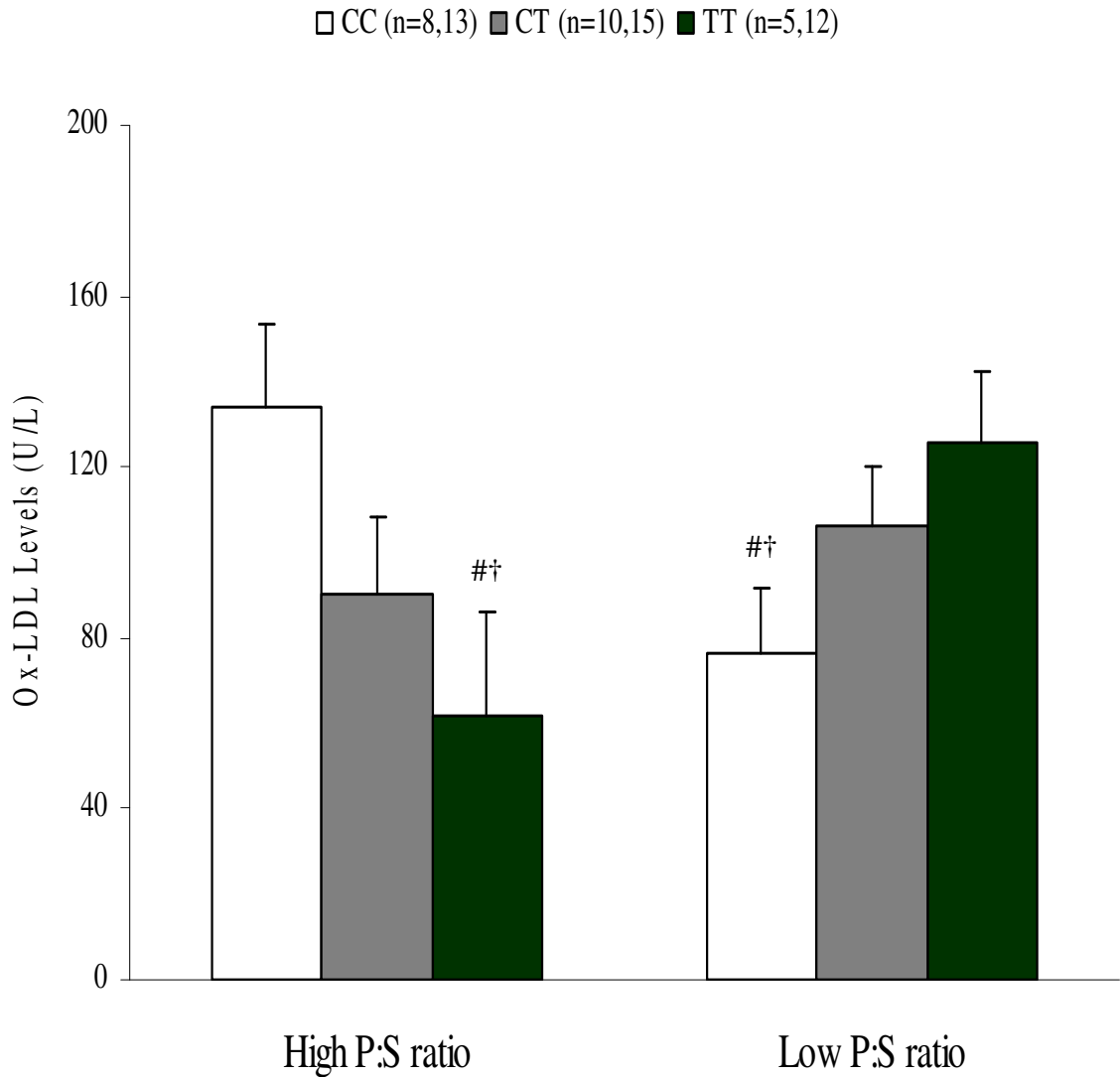


Table 7. Changes in endothelial function, oxidative stress, plasma lipid levels, and soluble LOX-1 levels with exercise training in the overall group

| | N | Change | P-value |
|-----------------------------|-----|-------------|---------|
| Resting FBF (mL/100mL/min) | 33 | 0.045±0.16 | 0.79 |
| Reactive hyperemia (%) | 33 | 2.5±19.2 | 0.90 |
| NOx (µmol/L) | 99 | -0.15±0.61 | 0.81 |
| Log Nitrotyrosine (nmol/L) | 43 | 0.12±0.05 | 0.025 |
| Ox-LDL (U/L) | 55 | -11.2±5.1 | 0.032 |
| TAC (mmol/L) | 57 | 0.070±0.079 | 0.38 |
| Total Cholesterol (mg/dL) | 146 | -3±2 | 0.09 |
| Triglycerides (mg/dL) | 145 | -15±3 | <0.0001 |
| HDL-C (mg/dL) | 145 | 3±0 | <0.0001 |
| HDL ₂ -C (mg/dL) | 144 | 1.7±0.5 | 0.0004 |
| HDL ₃ -C (mg/dL) | 144 | 1.6±0.5 | 0.002 |
| LDL-C (mg/dL) | 145 | -2±1 | 0.17 |
| Soluble LOX-1 (pg/mL) | 54 | -0.025±2.67 | 0.99 |

Table values are expressed as means ± SE.

Figure 5. Correlation between changes in plasma ox-LDL levels and changes in reactive hyperemia with exercise training

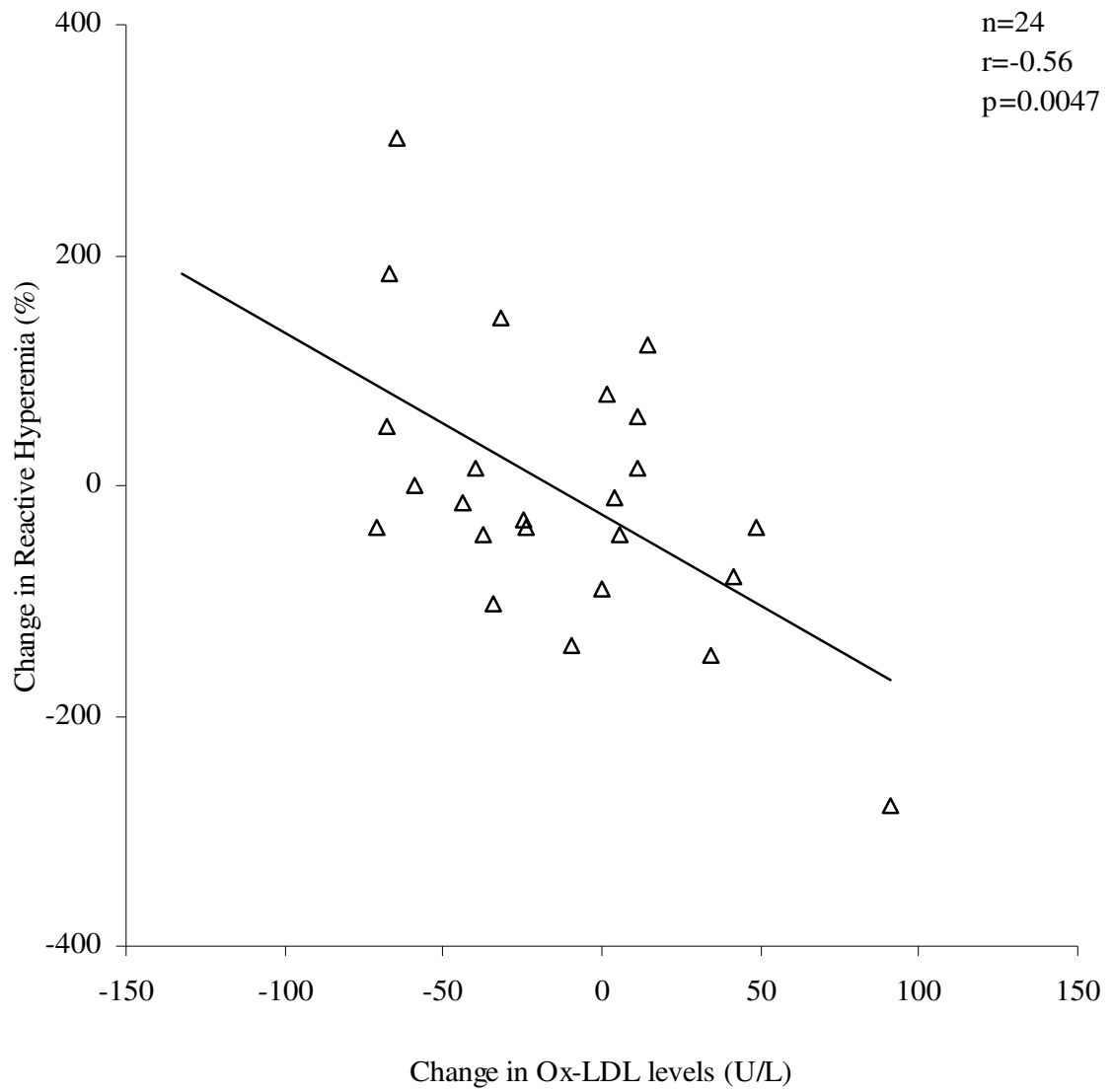


Table 8. Changes in endothelial function, oxidative stress, and plasma lipid levels with exercise training by the LOX-1 3'UTR polymorphism

| | Genotype | | | P-value |
|-----------------------------|---------------------|--------------------|--------------------|---------|
| | CC | CT | TT | |
| Resting FBF (mL/100mL/min) | -0.51±0.26 (3) | -0.075±0.23 (3) | 0.010±0.20 (4) | 0.51 |
| Reactive hyperemia (%) | 4.6±134.3 (3) | 136.9±103.1 (3) | -28.4±122.7 (4) | 0.60 |
| NOx (μmol/L) | -0.19±1.02 (26) | 0.30±0.90 (32) | -1.02±1.09 (22) | 0.65 |
| Log Nitrotyrosine (nmol/L) | 0.24±0.10* (12) | 0.12±0.08 (16) | -0.016±0.15 (6) | 0.33 |
| Ox-LDL (U/L) | -12.2±10.2 (14) | -16.5±10.0 (16) | -9.5±14.2* (8) | 0.92 |
| TAC (mmol/L) | -0.021±0.17 (12) | 0.095±0.16 (15) | 0.40±0.22 (8) | 0.32 |
| Total Cholesterol (mg/dL) | -1±3 (31) | -3±2 (60) | -5±3 (36) | 0.71 |
| Triglycerides (mg/dL) | -13±6* (31) | -21±4* (59) | -2±6 (36) | 0.036 |
| HDL-C (mg/dL) | 4±1* (31) | 3±1* (59) | 2±1* (36) | 0.52 |
| HDL ₂ -C (mg/dL) | 2.3±1.1 (30) | 1.7±0.77* (59) | 1.7±0.99* (36) | 0.88 |
| HDL ₃ -C (mg/dL) | 1.4±1.1 (30) | 1.1±0.76* (59) | 1.1±0.99 (36) | 0.98 |
| LDL-C (mg/dL) | 0±3 (31) | -2±2 (59) | -4±3 (36) | 0.48 |

Table values are expressed as adjusted means ± SE. Numbers in parentheses represent sample sizes. *Indicates significant change with training within genotype group, p≤0.05 based on paired t-test

Table 9. Changes in endothelial function, oxidative stress, and plasma lipid levels with exercise training by the LOX-1 G501C polymorphism

| | Genotype | | P-value |
|---|---------------------|---------------------|---------|
| | GG | GC+CC | |
| Resting FBF (mL/100mL/min) [#] | -0.023±0.19 (19) | 0.44±0.49 (4) | 0.38 |
| Reactive hyperemia (%) [#] | 19.0±58.7 (19) | 2.62±23.0 (4) | 0.80 |
| NOx (μmol/L) | -0.38±0.62 (67) | -0.047±1.38 (14) | 0.78 |
| Log Nitrotyrosine (nmol/L) | 0.097±0.07 (25) | 0.26±0.12* (9) | 0.30 |
| Ox-LDL (U/L) | -10.2±6.5 (31) | -27.6±14.1 (7) | 0.28 |
| TAC (mmol/L) | 0.22±0.10 (28) | -0.24±0.21 (7) | 0.068 |
| Total Cholesterol (mg/dL) | -2±2 (106) | -7±4 (22) | 0.28 |
| Triglycerides (mg/dL) | -12±4* (105) | -22±8* (22) | 0.27 |
| HDL-C (mg/dL) | 3±1* (105) | 3±1* (22) | 0.98 |
| HDL ₂ -C (mg/dL) | 1.6±0.6* (104) | 2.7±1.3 (22) | 0.41 |
| HDL ₃ -C (mg/dL) | 1.3±0.6* (104) | 0.8±1.3 (22) | 0.74 |
| LDL-C (mg/dL) | -2±2 (105) | -3±4 (22) | 0.75 |

Table values are expressed as adjusted means ± SE. #Table values are unadjusted due to small sample sizes. Numbers in parentheses represent sample sizes. *Indicates significant change with training within genotype group, p≤0.05 based on paired t-test.

Figure 6. Changes in soluble LOX-1 levels with exercise training by the LOX-1 3'UTR polymorphism

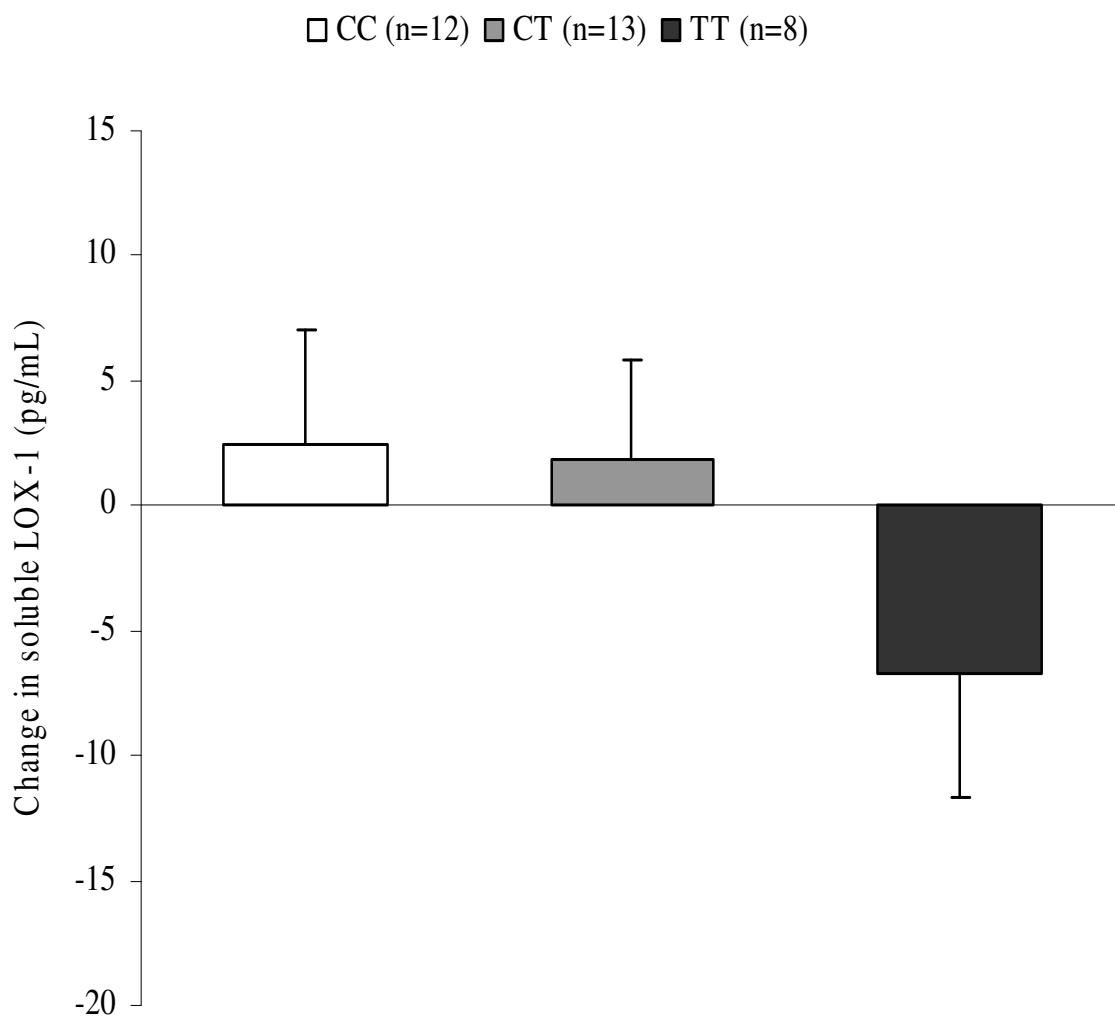
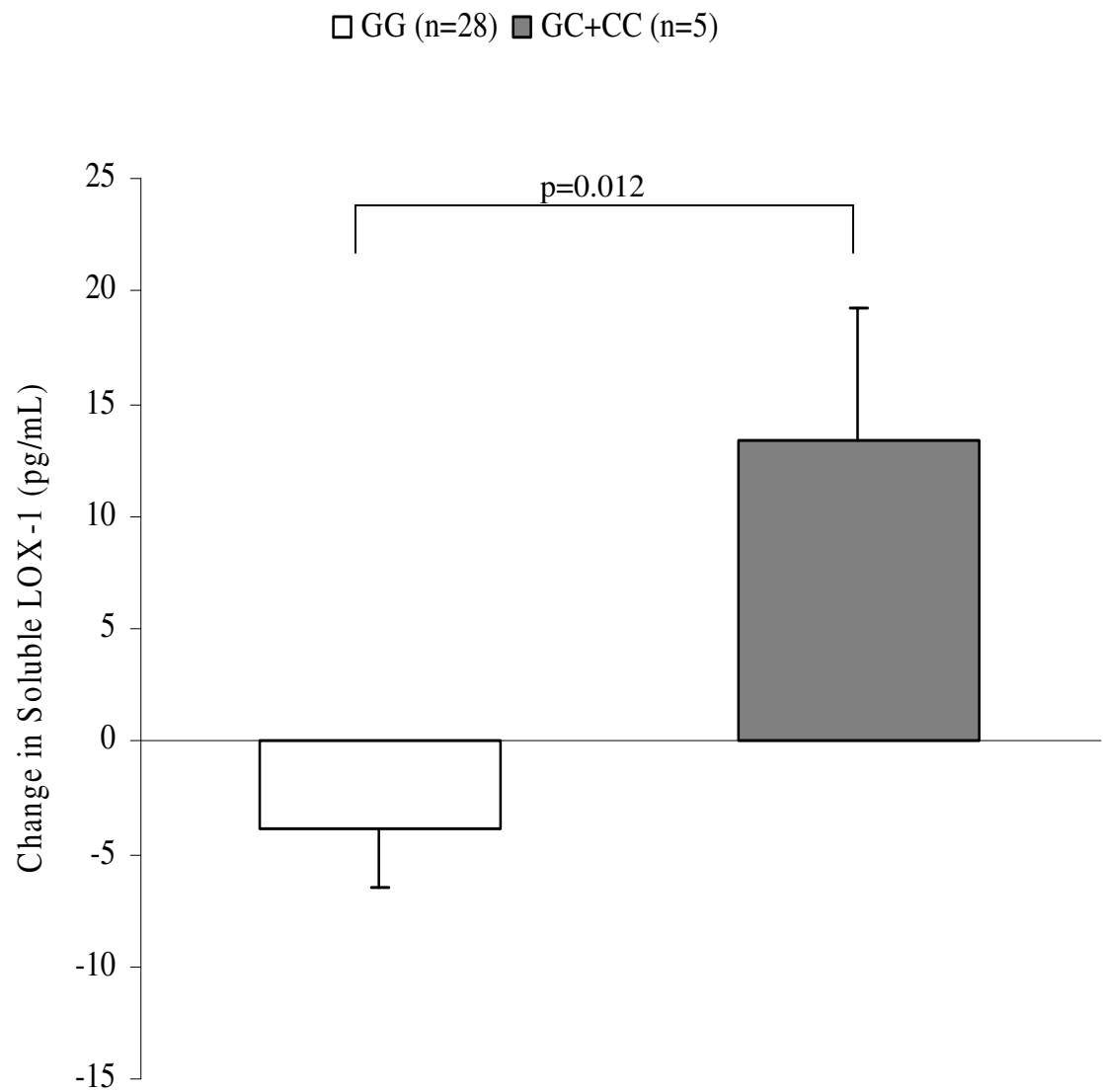


Figure 7. Changes in soluble LOX-1 levels with exercise training by the LOX-1 G501C polymorphism



DISCUSSION

Effects of LOX-1 Gene Polymorphisms

LOX-1 plays a major role in the development of atherosclerosis via the binding and uptake of ox-LDL in endothelial cells, macrophages, and smooth muscle cells. (6) Previous studies have shown that variation in the LOX-1 gene, including the 3'UTR C/T and G501C polymorphisms, is associated with acute myocardial infarction and CAD severity. (31-33,35,37) In the present study, we hypothesized that these polymorphisms would be associated with cardiovascular risk factors in a middle-to-older-aged population. A previous study in hypercholesterolemic subjects showed that plasma ox-LDL levels were significantly higher in carriers of the 3'UTR/T allele compared to the C allele. (63) However, using a similar commercial assay, we were not able to confirm these findings. We also found no associations between LOX-1 polymorphisms and other markers of oxidative stress or endothelial function. Ox-LDL-induced LOX-1 activation has been shown to reduce eNOS expression and NO production, and may be involved in impaired endothelium-dependent vasodilation in hypertensives. (9-11) LOX-1 activation also contributes to the formation of ROS, induces lipid peroxidation, and alters the activity of antioxidant enzymes. (8,64) However, it is not known if variation in the LOX-1 gene influences these LOX-1 mediated effects. In addition, while LOX-1 causes endothelial injury/dysfunction and oxidative stress *in vitro*, it is possible that these intracellular events are not reflected by circulating markers in plasma.

In this study, LOX-1 polymorphisms were also not associated with the plasma lipoprotein-lipid profile. Puccetti et al. found similar results with the 3'UTR polymorphism. (63) Although ox-LDL via LOX-1 downregulates LDL receptor

expression in endothelial cells, this may not have significant effects on plasma cholesterol levels. (13) The uptake of LDL and other apolipoprotein B/E-containing lipoproteins by vascular endothelial cells is not important for cholesterol removal, but rather serves as a means to supply these cells with lipids necessary for normal cell function. Thus, a decrease in LDL receptor expression in endothelial cells may simply be a compensatory response to prevent further uptake and intracellular accumulation of cholesterol. On the other hand, the hepatic LDL receptor removes up to 70% of circulating LDL, and thus is the major clearance pathway for plasma cholesterol. Small amounts of LOX-1 have been found in liver macrophages and endothelial cells, and ox-LDL uptake may alter LDL receptor expression in these cells. (65) However, it is unlikely that this would significantly increase hepatic LDL uptake.

We found that both the 3'UTR/T allele and the 501GG genotype were associated with lower soluble LOX-1 levels at baseline. Soluble LOX-1 is produced by the cleavage of membrane-bound LOX-1 via serine proteases. (27) Hayashida et al. found that soluble LOX-1 levels are significantly elevated in patients with acute coronary syndromes and may reflect plaque instability. (28) They suggested that in vulnerable plaques, enhanced protease activities may cleave LOX-1 from the cell surface, thereby increasing the circulating levels of soluble LOX-1. However, the role of soluble LOX-1 in disease-free populations has not been studied. Mango et al. recently demonstrated that intronic polymorphisms in LOX-1, which are in complete linkage disequilibrium with the 3'UTR polymorphism, regulate the expression of a functional splicing isoform of LOX-1 known as LOXIN. (34) They demonstrated that LOXIN accumulates intracellularly in the perinuclear region and displays no surface expression in 90% of transfected cells and

lower expression in 10% of the cells. Macrophages from subjects homozygous for the “risk” haplotype (i.e. TT genotype) had a significantly higher ratio of LOX-1 to LOXIN than subjects homozygous for the “non-risk” haplotype (i.e. CC genotype). Moreover, LOXIN was able to “rescue” macrophages from ox-LDL-induced apoptosis when co-expressed with LOX-1, reducing the number of apoptotic cells by 72% when expressed at a ratio of 4:1 (LOX-1 to LOXIN) and completely preventing apoptosis when expressed at a ratio of 1:1. Based on these findings, they suggested that LOXIN exerts its protective effect by downregulating the membrane expression of LOX-1 receptors or by forming inactive heterodimers with LOX-1 at the cell surface, significantly reducing the binding and uptake of ox-LDL by vascular endothelial cells. (34) An increased expression of membrane-bound LOX-1 in TT homozygotes would be expected to lead to higher soluble LOX-1 levels in these individuals. Surprisingly, we found that soluble LOX-1 levels were lower in the 3’UTR/CT and TT groups compared to the CC group. However, it is not clear if soluble LOX-1 is a marker or a mediator of cardiovascular disease, and thus the significance of elevated soluble LOX-1 levels in disease-free populations remains to be elucidated.

Trabetti et al. reported the following haplotype estimates for the 3’UTR and G501C polymorphisms: 53% G-T, 39% G-C, 7.5% C-C, and 0.5% C-T. (37) Although we were not able to perform a haplotype study due to small sample sizes, our results are consistent with these estimates in that the 3’UTR/CT and TT groups and the 501GG group had similar soluble LOX-1 levels at baseline compared to the 3’UTR/CC and 501GC+CC groups. In other words, it appears that the 3’UTR/T allele and the 501G allele are inherited together in most cases, as these two polymorphisms are highly linked

($D'=0.87$). (37) There is strong evidence that the 3'UTR polymorphism alters the binding of a nuclear protein and is associated with the generation of a splicing isoform. (32,34) In contrast, the effect of the G501C polymorphism on the binding and uptake of ox-LDL has not been proven directly. (35,36) Consistent with this, haplotype analyses using these two polymorphisms revealed that addition of the G501C polymorphism does not improve cardiovascular risk prediction compared to the 3'UTR polymorphism alone. (31) These findings suggest that the 3'UTR polymorphism is driving the association with cardiovascular disease. (37) However, we found that the G501C polymorphism was a significant independent predictor of soluble LOX-1 levels after accounting for the 3'UTR polymorphism, but not vice versa. Our results showed that the G501C polymorphism explains ~5% of the variation in soluble LOX-1 levels. These disparate findings are most likely due to the different phenotypes observed. In addition, there may be other unidentified polymorphisms in LOX-1 that may regulate LOX-1 expression and/or soluble LOX-1 levels.

Effects of Dietary Fat Intake

According to the NCEP Adult Treatment Panel III and the 2000 AHA dietary guidelines, a PUFA intake of up to 10% of total calories and a SFA intake of <7% or <10% of total calories (depending on the risk category) is recommended to lower cardiovascular risk. (66,67) This corresponds to a P:S ratio >1. In this study, there was no significant effect of diet (P:S ratio) on any of the outcome variables measured. This may be due to the fact that both dietary groups had average P:S ratios well below 1 (0.43 and 0.76). In addition, PUFAs can be categorized into n-3 and n-6 PUFAs, and these fatty acids have very different cardiovascular health effects. (41) It has been suggested that the

balance between dietary n-6 and n-3 fatty acids is an important factor influencing cardiovascular risk. (68) Thus, by not examining these two fatty acid categories separately, we may not have been able to fully realize the effects of dietary PUFA intake. It is also possible that changes may have occurred in the main outcome variables as a result of dietary stabilization. Dietary records were not taken at the beginning of the 6-week dietary stabilization phase. However, if dietary fat intake was compared before and after the dietary intervention, it is possible that improvements would be seen in endothelial function, oxidative stress, plasma lipoprotein-lipid levels, and soluble LOX-1 levels.

Monounsaturated fat is another major component of dietary fat intake that has significant cardiovascular effects, and the NCEP recommends a monounsaturated fat intake of up to 20% of total calories. (66) Ecological studies show an inverse association between monounsaturated fat intake and the risk of CAD, although the association is weaker than that for PUFAs. (41) The protective effect of monounsaturated fat is largely due to the ability of these fatty acids to enhance endothelium-dependent vasodilation and maintain endothelial integrity. (69,70) Monounsaturated fat is also resistant to oxidative modification, inhibits inflammatory gene expression in endothelial cells, and decreases plasma levels of total cholesterol, LDL-C, and TG. (40,41,71) Thus, examining the amount of monounsaturated fat in the diet may have provided more information about the effects of dietary fat intake. Furthermore, given the importance of specific fatty acids, such as palmitic acid (16:0), stearic acid (18:0), oleic acid (18:1), linoleic acid (18:2n-6), linolenic acid (18:3n-3), eicosapentaenoic acid (20:5n-3), and docosahexaenoic acid

(22:6n-3), it would have been interesting to analyze the intake of individual fatty acids and assess how they influence the outcome variables. (41)

Effects of Aerobic Exercise Training

Exercise training has been shown to improve endothelial function, especially in individuals with *a priori* endothelial dysfunction. (43) Studies using venous occlusion plethysmography have shown increases in endothelium-dependent vasodilation with exercise training in response to both pharmacological agents and increased blood flow. Plasma NO_x levels have also been shown to increase after training. (17,72,73) These changes are largely due to shear stress-induced increases in eNOS expression, phosphorylation status, and enzyme activity. (74,75) However, there is strong evidence to suggest that improvements in endothelial function may not only depend on the initial level of endothelial function, but also the duration of the training program. (43) Training duration in studies reporting improvements in endothelium-dependent vasodilation or plasma NO_x levels generally range from 1 to 12 weeks. To our knowledge, only one study has examined endothelial function after six months of exercise training. Hambrecht et al. found that in chronic heart failure patients, exercise training significantly increased basal NO production and acetylcholine-induced blood flow in the femoral artery. (76) In the present study, six months of exercise training had no significant affect on endothelial function at rest or during reactive hyperemia. Differences in the study population, the vascular bed examined, and the measurement of endothelial function may account for the dissimilar results. However, a lack of significant improvement in endothelial function with prolonged exercise training is consistent with the findings that long term training

normalizes shear stress via increases in arterial diameter, and this returns eNOS expression, NO bioactivity, and NO vasodilator function to baseline levels. (43,77)

Oxidative stress generally appears to be reduced after aerobic exercise training. (44) This may be due to a decrease in ROS production and the oxidation of lipids and proteins, or an increase in antioxidant defenses. We found a significant reduction in plasma ox-LDL levels with exercise training (11%). Using a similar assay to the one used in this study, Elosua et al. reported a 16% reduction in ox-LDL levels after 16 weeks of aerobic exercise training in young healthy men and women. (78) We also found a small, but significant increase in plasma nitrotyrosine levels. Goto et al. reported that 12 weeks of high-intensity exercise training (75% VO_2 max) increases oxidative stress in young healthy men. (79) It is possible that the exercise intensity used in our study (70% VO_2 max) leads to small increases in nitrotyrosine. However, previous studies have shown that plasma nitrotyrosine levels in older men (walking/jogging at 50-80% heart rate max for 12-42 min/day, 3 days/week, for 16 weeks) and the nitrotyrosine content in porcine aortic endothelial cells (isolated from pigs who ran 4-6 mph for 60 min/day, 5 days/week, for 16-19 weeks) are unchanged by exercise training. (80,81) While the effect of training on lipid peroxidation has been widely studied, its effects on protein modification, nitrotyrosine in particular, is still largely unknown. This may be partly due to the difficulty in measuring nitrotyrosine in plasma and the need for sensitive and specific detection methods that are time-consuming and require expensive equipment and expertise. (82,83)

Fatouros et al. found that 16 weeks of aerobic exercise training at 50-80% VO_2 max increased TAC by 6% in older men. (80) Similarly, in the present study, TAC

increased by 8%, although it did not reach statistical significance. TAC is a complex trait that is affected by the relative contributions of each oxidant and antioxidant species in plasma. (84) While it provides a cumulative measure of the ability of plasma antioxidants to scavenge free radicals, it gives no information about the individual antioxidants involved. Moreover, it has been estimated that ~25-35% of the plasma TAC is provided by uncharacterized antioxidants. Studies show that the activities of antioxidant enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase may be increased with exercise training. (73,75,78,85,86) The plasma levels of other antioxidants, such as vitamins C and E, glutathione, and uric acid, may also be altered by training. Taken together, our findings suggest that six months of exercise training may have resulted in increased antioxidant scavenging, less free radical formation, and reduced lipid peroxidation. ROS-mediated signaling appears to be an important mechanism behind these training-induced reductions in oxidative stress. (86) Repeated bouts of exercise are thought to stimulate antioxidant gene expression through the increased production of ROS. (86,87) In addition, exercise-induced shear stress may upregulate antioxidant gene expression and downregulate enzymes involved in ROS production. (26,88)

Interestingly, we found that changes in ox-LDL with training were inversely correlated with changes in reactive hyperemia. A previous study showed that ox-LDL specifically inhibits NO-mediated vasomotor function, as vasodilation was almost completely restored by incubation of ox-LDL-treated vessels with the NO precursor L-arginine. (89) Ox-LDL also increases superoxide production and reduces intracellular L-arginine concentrations. (89-91) A reduction in the availability of L-arginine may cause

eNOS uncoupling, and this could further decrease NO bioactivity through direct inactivation of NO by superoxide and peroxynitrite formation. These findings suggest that reductions in ox-LDL with training may contribute to improvements in endothelial function.

As expected, the plasma lipoprotein-lipid profile significantly improved with exercise training. Consistent with previous studies, we found significant increases in plasma HDL-C, HDL₂-C, and HDL₃-C levels, and significant reductions in TG levels. (42) These changes are largely due to enhanced lipoprotein metabolism, which is mediated by the activities of various lipolytic enzymes and lipid transport proteins. (42) We found no changes in soluble LOX-1 levels with training. Given that the role of soluble LOX-1 is still unclear, the importance of changes in soluble LOX-1 with training, or the lack thereof, remains to be elucidated.

Gene-Diet Interactions

Fatty acids are important regulators of gene expression, particularly genes related to endothelial activation, inflammation, oxidative stress, and lipid metabolism. (68,92-94) Recently, Maingrette et al. showed that linoleic acid, the major n-6 PUFA in the American diet, upregulates LOX-1 mRNA and protein expression via increased superoxide production. (47) In addition, incubation of endothelial cells with linoleic acid resulted in a significant increase in the binding of nuclear proteins to the regulatory NF- κ B sequence in the LOX-1 gene promoter. Activation of the transcription factor NF- κ B is critical for the expression of inflammatory genes, and multiple NF- κ B binding sites have been identified in the LOX-1 promoter. (65) Based on these findings, we hypothesized that variation in the LOX-1 gene would influence the response to dietary fat intake. We

found a significant gene-diet interaction with the 3'UTR polymorphism, but only for plasma ox-LDL levels. The 3'UTR/CC group had higher ox-LDL levels at a high P:S ratio, but lower ox-LDL levels at a low P:S ratio when compared with the TT group. These results suggest that CC homozygotes may be more susceptible to diet-induced increases in lipid peroxidation in the presence of higher PUFA intakes, while TT homozygotes may be more adversely affected by increased SFA intake. On the other hand, CT heterozygotes appear to be unaffected by changes in dietary fat intake. This interaction between the LOX-1 3'UTR C/T polymorphism and dietary fat intake suggests that the induction of LOX-1 expression by linoleic acid, and the subsequent uptake of ox-LDL, may differ in individuals with the CC and TT genotypes. However the mechanisms involved remain to be elucidated.

Gene-Exercise Interactions

Shear stress is a major component of exercise, as increased blood flow results in increased frictional force along the vessel wall. (88) Shear stress modulates a variety of endothelial functions, and these appear to be mediated by intracellular signal transduction cascades and subsequent activation of transcription factors. (88,95) Thus, chronic exercise training may have a significant impact on gene expression, and ultimately, reduce risk for cardiovascular disease. Murase et al. reported that physiological levels of shear stress upregulate LOX-1 mRNA and protein expression in cultured endothelial cells. (48) They also found that the increase in LOX-1 expression was transient and early signal transduction events were most important in gene expression. However, the effect of chronic, repeated bouts of exercise-induced shear stress on LOX-1 expression is not known. We hypothesized that variation in the LOX-1 gene would influence the response

to exercise training. We found that the 3'UTR polymorphism was associated with changes in plasma TG levels with training. As mentioned above, TG levels are generally decreased with training, but how the LOX-1 3'UTR influences changes in plasma TG levels with training is unclear. We also found that the G501C polymorphism was associated with changes in soluble LOX-1 levels with exercise training. Circulating levels of serine proteases have been shown to increase after training. (96) This suggests that the cleavage of membrane-bound LOX-1 may be increased during exercise training due to increased activities of serine proteases. Although neither group had significant changes in soluble LOX-1 with training in this study, the levels of circulating LOX-1 increased by 15% in the 501GC+CC group, but decreased by 4% in the GG group. While the mechanism behind this gene-exercise effect is not clear, it is possible that the induction of LOX-1 expression by exercise, and hence shear stress, differs between carriers and non-carriers of the 501C allele.

Limitations

There were a few limitations in this study. First, sample sizes were small and we did not have adequate statistical power to detect differences in the outcome variables, especially for the FBF data (power for all comparisons was $\leq 37\%$). In addition, power was particularly low for the G501C polymorphism given the C-allele frequency of only 9%. Second, reactive hyperemia was calculated as the percent change in FBF from resting to 1 min post-ischemia based on studies reporting that peak blood flow occurs ~1 min after release of the occluding cuff. (53,54) However, other studies demonstrate that peak blood flow may occur anywhere between 5-45 seconds following ischemia. (97-99) Thus, using the FBF at 1 minute may not represent the true hyperemic response. Third,

nitrotyrosine was difficult to measure with the commercially available sandwich ELISA. Other methods, such as gas chromatography/mass spectrometry or high-performance liquid chromatography, have also been used to detect nitrotyrosine *in vivo*. (82,83) Although these methods can offer greater specificity and sensitivity, they require expensive equipment and expertise and can be quite tedious and time-consuming.

Conclusions

LOX-1 is a critical player in the development of atherosclerosis, and this is the first study to investigate the effects of the LOX-1 3'UTR C/T and G501C polymorphisms on endothelial function, oxidative stress, plasma lipid levels, and soluble LOX-1. These polymorphisms appear to be important in the regulation of soluble LOX-1 levels in plasma and may also influence the responses of cardiovascular risk factors to dietary fat intake and aerobic exercise. However, future studies need to resolve the association of the G501C polymorphism with cardiovascular disease, verify the function of the G501C polymorphism, and determine the significance of soluble LOX-1 in (patho)physiology.

REVIEW OF LITERATURE

Atherosclerosis, a chronic inflammatory disease characterized by lipid accumulation and intimal thickening in the arteries, is the major cause of morbidity and mortality in the developed world. (1,100) This disease involves complex interactions between inflammatory cells (i.e. monocytes/macrophages) and vascular cells (i.e. endothelial cells and smooth muscle cells), which ultimately lead to the formation of the atherosclerotic plaque. (100,101) Over the past century, three major hypotheses have emerged in the effort to explain atherogenesis, including the response-to-injury, the response-to-retention, and the oxidative-modification hypotheses. (1) To some degree, all three hypotheses involve the accumulation of aggregated and/or oxidized low-density lipoproteins (ox-LDL), injury, activation, and dysfunction of the vascular endothelium, and induction of inflammatory processes. Recently, Stocker and Keaney proposed the oxidative-response-to-inflammation hypothesis. (1) According to this hypothesis, cardiovascular risk factors induce an inflammatory response in the vascular wall, subsequent to lipoprotein retention and vascular injury. This hypothesis also recognizes that reactive oxygen species (ROS) are important by-products of the inflammatory process and can promote oxidative stress and damage healthy tissue. Taken together, it appears that lipoproteins (particularly LDL), vascular dysfunction, inflammation, and oxidative events all contribute to the pathogenesis of atherosclerosis.

It is well established that age, elevated levels of LDL, reduced levels of high-density lipoproteins (HDL), hypertension, diabetes, obesity, family history of premature coronary artery disease (CAD), cigarette smoking, male gender, high-fat diet, and physical inactivity can all increase the risk of atherosclerosis. (1,24) However, a number

of other novel risk factors have also been found to be associated with atherosclerosis. The following sections will review the role of these so-called emerging risk factors (endothelial dysfunction and oxidative stress), as well as the role of traditional risk factors (plasma lipid levels, dietary fat intake, and aerobic exercise training) in the development of atherosclerosis. This review will also discuss the importance of genetic susceptibility to cardiovascular disease and the role of the lectin-like oxidized LDL receptor (LOX-1), a promising candidate gene that is involved in endothelial dysfunction and plaque development. In addition, there will be a brief discussion on the interactions between genetic and environmental factors and their impact on cardiovascular disease.

Endothelial Dysfunction

The endothelium is the monolayer of thin, flat cells that lines the interior surface of all blood vessels and forms an interface between circulating blood and the rest of the vascular wall. In addition to acting as a selectively permeable barrier, the endothelium is a dynamic organ that responds to physical and chemical stimuli by synthesis and/or release of a variety of vasoactive and thromboregulatory substances, signaling molecules, and growth factors. (14,102) As such, the endothelium plays a major role in the regulation of vascular tone, inflammation, lipid metabolism, vessel growth and remodeling, thrombogenesis, platelet activation, and monocyte adhesion. (26) Under normal conditions, the endothelium has anticoagulant and fibrinolytic functions, vasodilatory actions, anti-adhesive effects against leukocytes and platelets, and growth-inhibitory actions on underlying smooth muscle cells. (102)

Given its unique anatomical position, the endothelium is a primary target for mechanical and biochemical injuries that can lead to endothelial dysfunction, or the loss

of vascular homeostasis. (103) Endothelial dysfunction is characterized by impaired vasodilation and increased adhesion of monocytes and platelets, endothelial permeability to lipoproteins and monocytes/macrophages, and smooth muscle cell migration and proliferation. (14) Endothelial dysfunction is considered an early marker for atherosclerosis, often preceding clinical evidence of atherosclerotic plaques. (15) Consistent with this, studies show that endothelial dysfunction can be detected in individuals with cardiovascular risk factors, but normal arteries. (15,26) In the presence of atherosclerosis, endothelial dysfunction plays a key role in the development and growth of atherosclerotic lesions in the early stages of the disease and contributes to plaque progression and destabilization, thrombus generation, and pathological arterial wall remodeling in the later stages of the disease. (103)

Nitric Oxide

One of the most important endothelium-derived substances is nitric oxide (NO), a free radical signaling molecule that mediates many of the vasoprotective effects of the endothelium. (15) NO is produced from the guanidino terminal nitrogen atom of the amino acid L-arginine by the constitutively expressed enzyme endothelial NO synthase (eNOS). (102) eNOS functions as a homodimer with each monomer consisting of a C-terminal reductase domain and an N-terminal oxygenase domain that are linked by a regulatory hinge region. (1) Upon calcium/calmodulin binding, eNOS transfers electrons from NADPH, via the flavins FAD and FMN in the reductase domain, to the heme iron in the oxygenase domain, where the substrate L-arginine is oxidized to L-citrulline and NO via the intermediate N^o-hydroxy-L-arginine. (102,104) As the most potent endogenous vasodilator, NO mediates endothelium-dependent vasodilation by binding to soluble

guanylyl cyclase located in adjacent smooth muscle cells. (105) This leads to increased cyclic GMP production, reduced intracellular calcium levels, relaxation of vascular smooth muscle (vasodilation), and increased flow through the vessel. NO also inhibits a number of atherogenic processes, including platelet adherence and aggregation, leukocyte adhesion and infiltration, smooth muscle cell proliferation, and LDL oxidation. (15)

Assessment of Endothelial Function

Endothelial function is commonly assessed as the vasodilatory response to pharmacologic or mechanical stimuli. (26) Evaluating the responses to endothelium-dependent and endothelium-independent vasodilators provides important information about the contribution of different components of the NO-dilator system to blood flow control. (43) Clinically, vasoregulation has been measured invasively and non-invasively in both the coronary and peripheral circulations using changes in vessel diameter as an index of conduit vessel endothelial function and changes in blood flow as an index of endothelial function in resistance vessels. (26)

One of the most common non-invasive methods used to assess resistance vessel endothelial function in the peripheral circulation is forearm venous occlusion plethysmography. (97) This technique measures reactive hyperemia, or the rapid increase in blood flow following a brief period of limb ischemia. The underlying principle for this technique is that when venous drainage from the arm is briefly interrupted, arterial inflow is unaltered and blood can enter the forearm but cannot escape. (99) This results in a linear increase in forearm volume over time, which is proportional to arterial blood inflow. Changes in forearm volume lead to a corresponding change in arm circumference, and these changes can be detected by a plethysmograph. Using this method, it has been

shown that NO plays a significant role in regulating blood flow at rest. (98) During reactive hyperemia, NO plays a minimal role in peak blood flow, but plays a modest, but significant, role in the mid-to-late phase of reactive hyperemia.

There is increasing emphasis on supplementing functional assessments with blood markers that may be indicative of vascular dysfunction. (14,77) For example, endothelial injury may result in the release of various endothelium-derived factors that can be detected in the circulation. (77) These markers have important functions in coagulation, fibrinolysis, and leukocyte adhesion, processes that are altered in the presence of endothelial dysfunction. Other circulating markers that have been used to assess endothelial function reflect alterations in NO production. Direct measurement of NO is extremely difficult because its biological half-life is very short and its concentration *in vivo* is very low (i.e. in the nanomolar range). (106) In the presence of oxygen, NO is rapidly oxidized to nitrite, which is then taken up by red blood cells and oxidized to nitrate. (107) Thus, NO production can be estimated by measuring the major NO metabolites nitrate and nitrite, which are collectively known as NO_x. (14,108) Additionally, NO production can be estimated by measuring the NO-related amino acids, L-arginine and L-citrulline, the stable intermediate compound N^ω-hydroxy-L-arginine, or the second messenger cyclic GMP. (107,109)

Plasma NO_x Levels

A number of studies have examined the effect of cardiovascular disease on NO production, as assessed by the plasma level of the major NO metabolites. In these studies, higher, lower, and similar plasma NO_x levels have been reported in CAD patients compared to controls. (17,110,111) Although the results have been fairly inconsistent,

alterations in plasma NO_x levels may have important clinical significance in the pathophysiology of vascular disease. (17) However, interpretation of these results should be made with caution as plasma NO_x levels do not reflect biologically active NO. (108) A better assessment of NO bioactivity can be made when measures of plasma NO_x are examined as part of a panel of functional and biochemical measurements. (108)

NO Bioactivity and Endothelial Dysfunction

Although no single event can fully explain the loss of vascular homeostasis, impaired NO bioactivity has been proposed as a major mechanism of endothelial dysfunction and contributor to atherosclerosis. (15,16) In general, endothelial NO bioactivity can be regulated at three different levels: 1) eNOS gene expression, 2) eNOS enzymatic activity, and 3) NO degradation or inactivation. (16) Dysfunction of any of these mechanisms can cause endothelial dysfunction.

The expression pattern of eNOS in diseases such as hypercholesterolemia, hypertension, and atherosclerosis appears to depend on the stage, severity, and duration of the disease. (104) Studies show normal or even upregulated eNOS expression in the early stages of disease, and this can occur despite the presence of endothelial dysfunction. (16,104) However, in advanced stages, vascular eNOS expression is usually diminished and NO production reduced, resulting in reduced NO bioactivity. (104) The enzymatic activity of eNOS may also be altered, either constitutively (i.e. as a result of mutations in the eNOS gene) or reversibly (i.e. by post-translational modifications). (16) For example, several clinical genetic studies have identified common eNOS gene polymorphisms that influence eNOS enzymatic activity and may be associated with atherosclerotic CAD. (16) On the other hand, the post-translational regulation of eNOS activity is highly complex

and depends on multiple factors including subcellular localization, protein interactions, and phosphorylation state. (16)

The availability of the eNOS substrate L-arginine and the eNOS co-factor BH₄ is also important in the regulation of eNOS activity. (16) In the absence of either molecule, eNOS may become uncoupled and generate the toxic free radical superoxide instead of NO. Similarly, vascular disease is associated with increased production of free radicals and other oxidants, and convincing evidence suggests that impaired NO bioactivity is largely due to the increased occurrence of pathological oxidative events. (91) Free radicals such as superoxide can react with NO and form highly reactive oxidants, thereby impairing NO-mediated functions and endothelium-dependent vasodilation. Additionally, the oxidation of LDL can impair NO bioactivity by altering eNOS expression, enzyme activity, or signal transduction.

Summary

Endothelial dysfunction plays a significant role in the pathogenesis of atherosclerosis, largely due to a reduction in NO bioactivity. In pathological conditions, an upregulation of eNOS often represents a futile compensatory mechanism that goes along with reduced amounts of bioactive NO due to dysfunctional enzymatic activity, rapid inactivation of NO by superoxide, and/or uncoupling of eNOS. In many cases, this results in impaired endothelium-dependent vasodilation and/or reduced plasma NO_x levels.

Oxidative Stress

Mammalian cells produce energy by reducing molecular oxygen to water during aerobic respiration. (112) During this process, metabolic intermediates known as reactive

oxygen species (ROS) are generated, including superoxide, hydrogen peroxide, and hydroxyl radical. However, ROS are not simply by-products of normal oxidative metabolism, but rather are important mediators of vascular cell function in both normal and pathological conditions. (1,113) In this respect, studies have shown that ROS play a role in signal transduction, cellular adhesion, vascular cell proliferation and growth, cellular necrosis and apoptosis, and platelet aggregation.

Owing to the presence of unpaired electrons, ROS are highly reactive and have a strong tendency to extract electrons to reach a more stable structure. (114) As such, ROS are capable of eliciting oxidative damage to various cellular components, including proteins, lipids, and DNA. (112) The susceptibility of vascular cells to oxidative damage is a function of the overall balance between the concentration of ROS and other oxidants and the antioxidant defense capability. (75) Under normal conditions, antioxidant defenses are able to modulate a low steady state balance of ROS. (112) However, excessive ROS production overwhelms the endogenous antioxidant defenses and causes oxidative stress, or an imbalance in oxidants and antioxidants in favor of the former. (1,112) There is growing evidence that oxidative stress contributes to mechanisms of vascular dysfunction. (112) These observations are consistent with the findings that oxidative stress is involved in the pathogenesis of many cardiovascular diseases, including hypercholesterolemia, atherosclerosis, hypertension, diabetes, and heart failure. (18)

Sources of ROS

Under normal physiological conditions, the majority of ROS are produced in the mitochondrial electron transport chain. (114) However, in disease states, other sources

may contribute to the formation of ROS and other oxidants in the vascular wall, including NADPH oxidase, xanthine oxidase, eNOS, myeloperoxidase, and lipoxygenase. (1) NADPH oxidase is a membrane-associated multicomponent enzyme complex that utilizes electrons derived from NADPH to reduce molecular oxygen to superoxide. Xanthine oxidase catalyzes the oxidation of xanthine to uric acid with concomitant reduction of molecular oxygen to superoxide and hydrogen peroxide. (113) As mentioned earlier, uncoupled eNOS generates superoxide instead of NO, and thus may be a major contributor to oxidative stress. (1) Myeloperoxidase is a heme-containing enzyme that uses hydrogen peroxide and chloride ions to catalyze the formation of the non-radical oxidant hypochlorous acid and a number of other reactive species. Lipoxygenases are enzymes that catalyze the stereospecific oxidization of polyunsaturated fatty acids. These enzymes all contribute to oxidative stress by generating powerful oxidants that are capable of oxidizing lipids, modifying proteins, facilitating foam cell formation, and impairing NO bioactivity. (1,91)

Peroxynitrite

A number of oxidants may be formed from the reaction of NO with ROS. (1) For example, because superoxide and NO both contain unpaired electrons in their outer orbitals, they undergo an extremely rapid, diffusion-limited radical/radical reaction, leading to formation of peroxynitrite, a strong oxidant with potent biological effects. (85) Thus, controlling the amount of superoxide is critically important for preserving NO bioactivity in the vessel wall. Under physiological conditions, endogenous antioxidant defenses minimize the interaction between superoxide and NO. (18) However, under pathological conditions, such as hypercholesterolemia, hypertension, or atherosclerosis,

superoxide generation is increased through multiple pathways, and NO generation may also be markedly increased. (19) eNOS can also become partially uncoupled, such that both superoxide and NO are produced simultaneously, and in these settings, peroxynitrite formation is very likely to be enhanced. (18,19) Peroxynitrite is capable of inducing cellular injury via lipid peroxidation, DNA fragmentation, apoptosis, antioxidant depletion, and protein nitration. (115) Additionally, peroxynitrite can convert active eNOS dimers into inactive eNOS monomers, reduce L-arginine substrate availability, and promote eNOS uncoupling, ultimately leading to impaired NO production, NO bioactivity, and endothelium-dependent vasodilation. (1,16)

Nitrotyrosine

In vitro studies demonstrate that peroxynitrite spontaneously reacts with the aromatic ring of tyrosine to yield nitrotyrosine. (20) Accordingly, nitrotyrosine is a stable marker of protein modification by NO-derived oxidants. Extensive nitration of tyrosine is observed in atherosclerotic lesions from human coronary arteries, particularly in the vascular endothelium, lipid-laden macrophages, and inflammatory cells. (115) Nitrotyrosine levels have been found to be ~3.5-fold higher in atherosclerotic vessels compared to normal vessels and are significantly elevated in LDL isolated from atherosclerotic tissue. In addition, studies show that nitrotyrosine levels are very low or even undetectable in the plasma of healthy individuals. (82,83,116-118) In contrast, elevated plasma nitrotyrosine levels have been found in patients with chronic inflammation, renal failure, septic shock, and celiac disease. (116-118) Similarly, Shishehbor et al. found that plasma nitrotyrosine levels were higher in patients with CAD compared with controls (median values, 9.1 $\mu\text{mol/mol}$ tyrosine vs. 5.2 $\mu\text{mol/mol}$

tyrosine). (22) Moreover, patients in the highest quartile of nitrotyrosine levels (≥ 10.05 $\mu\text{mol/mol}$ tyrosine) had a 4- to 6-fold greater risk of CAD compared with patients in the lowest quartile (< 3 $\mu\text{mol/mol}$ tyrosine). In several of these studies, elevated nitrotyrosine levels were paralleled by an increase in plasma NO_x levels, suggesting that nitrotyrosine formation is enhanced in conditions where NO production is also enhanced. (116,118)

Oxidized LDL

The oxidative modification of LDL is a key step in the initiation and progression of atherosclerosis. (1) Much of our knowledge of how native LDL is converted to ox-LDL originates from *in vitro* studies exposing LDL to copper ions or to cells cultured in transition metal-containing medium. (3) Under these experimental conditions, the oxidation of LDL is a free-radical-driven chain reaction that occurs in three phases: an initial lag phase (i.e. consumption of endogenous antioxidants), a propagation phase (i.e. rapid oxidation of unsaturated fatty acids to conjugated dienes and lipid hydroperoxides), and a decomposition phase (i.e. conversion of hydroperoxides to reactive aldehydes). These reactive aldehydes may in turn bind to N^ε-amino groups of lysine residues in apolipoprotein B-100, giving the lipoprotein an increased net negative charge. This ultimately results in increased recognition by scavenger receptors and formation of foam cells.

It must be noted that many changes may have occurred in the LDL particle before reaching the state in which it is taken up by macrophages, and these changes may have important biological consequences. (2) Many of the atherogenic effects of ox-LDL are exerted by modulation of gene expression in vascular cells, including the expression of cellular adhesion molecules, scavenger receptors, oxidant and antioxidant enzymes,

growth factors, cytokines, chemokines, vasoactive mediators, and hemostasis proteins.

(4) More specifically, ox-LDL facilitates the adhesion of circulating monocytes to the endothelium and their subsequent migration into the intimal space; stimulates ROS production; induces smooth muscle cell migration and proliferation; promotes the oxidation of cellular lipids and proteins; induces apoptosis and necrosis of vascular cells; promotes the differentiation of monocytes into tissue macrophages; inhibits the motility of resident macrophages; increases the expression of macrophage scavenger receptors; stimulates platelet adhesion/aggregation; inhibits fibrinolysis; and increases the expression of extracellular matrix-digesting enzymes (i.e. metalloproteinases). (1,3-5,119) As such, ox-LDL contributes to foam cell formation, thrombosis, plaque disruption, and vascular remodeling. Ox-LDL also impairs NO bioactivity and endothelium-dependent vasodilation via increased uncoupling of eNOS, impaired signal transduction, reduced eNOS expression and activity, altered phosphorylation status, displacement of eNOS from the cell membrane, and reduction in L-arginine availability. (89-91)

There is convincing evidence that ox-LDL exists *in vivo*, however where, how, and to what extent LDL becomes oxidized during atherogenesis remains to be elucidated.

(1) It has been speculated that in the vascular wall LDL oxidation occurs in “microenvironments” where oxidants are produced excessively and/or where the antioxidant protection is not longer intact. In addition, the same enzymes and oxidants that contribute to oxidative stress in general are also thought to contribute to the oxidation of LDL. As LDL circulates in plasma, a portion enters the subendothelial space only to arrive back in the circulation. The transit of LDL across this space would likely generate

a small amount of LDL that is oxidized, and chemical analysis of circulating LDL has been reported to yield a minor fraction that exhibits an enhanced content of oxidized lipids. Consistent with these findings, human plasma contains immunoreactivity towards epitopes generated from ox-LDL. (1)

Plasma Ox-LDL Levels

Itabe et al. estimated that one ox-LDL particle is present in every 10,000 LDL particles in normal human plasma, a very low concentration that is maintained by effective clearance mechanisms. (21) Consistent with this, radio-labeled ox-LDL is rapidly and almost completely cleared from the circulation within 10 minutes after intravenous injection into rats. This is because specialized macrophages present in the liver are capable of removing ox-LDL from the circulation. However, plasma ox-LDL levels are increased in vascular disease, and this may represent a major change in the body's oxidative status. Elevated levels of circulating ox-LDL have been associated with CAD, acute coronary syndromes, angina, intima-media thickness and plaque occurrence in the carotid and femoral arteries, inflammation, and CAD risk factors. (55,120-122) Moreover, elevated concentrations of ox-LDL are predictive of future CAD events in apparently healthy men, independent of other traditional and non-traditional risk factors. (123)

Antioxidants

Antioxidants are substances that can prevent or significantly delay the oxidation of other substrates. (1) The classic endogenous antioxidant systems are largely cell-associated enzymes such as superoxide dismutase, catalase, glutathione peroxidase, and glutathione reductase. Superoxide dismutase catalyzes the conversion of superoxide to

hydrogen peroxide and molecular oxygen. There are three forms of superoxide dismutase in mammalian systems: cytosolic, mitochondrial, and extracellular, in which the latter is localized in high concentrations between the vascular endothelium and the smooth muscle layer. Catalase is found predominately in peroxisomes and decomposes hydrogen peroxide to water and molecular oxygen. Glutathione peroxidase cooperates with catalase in the removal of hydrogen peroxide by using glutathione (GSH) to reduce hydrogen peroxide to water and glutathione disulfide (GSSG). Glutathione peroxidase not only detoxifies hydrogen peroxide, but also converts lipid hydroperoxides to non-toxic alcohols, thus acting as a chain-breaking antioxidant of lipid peroxidation. Glutathione reductase, which co-localizes with glutathione peroxidase, functions in the regeneration of glutathione from glutathione disulfide at the expense of NADPH.

In addition to protein antioxidants, there are also several low-molecular-weight compounds that are thought to contribute to the body's antioxidant defense capacity. (1) For example, a number of water-soluble compounds have been found to have antioxidant properties, including vitamin C, flavanoids, and polyphenols from the diet, as well as uric acid and bilirubin from xanthine oxidation and heme degradation, respectively. Lipid-soluble antioxidants, such as vitamin E and ubiquinol found in lipoproteins and cell membranes, also play an important role in preventing oxidative damage. Overall, these antioxidants help prevent oxidation reactions induced by both radical and non-radical oxidants. Besides oxidant scavenging, antioxidants also help maintain eNOS activity and inhibit LDL oxidation.

Total Antioxidant Capacity

Total antioxidant capacity (TAC) is a complex trait that is affected by the relative contributions of each oxidant and antioxidant species in biological specimens. (84) The measurement of TAC in plasma reflects residual antioxidant capacity after the consumption of free radicals. For a given level of antioxidants in plasma, increased production of ROS will result in a reduced TAC level. On the other hand, increased antioxidant availability in plasma will enhance TAC levels for a given amount of ROS. Thus, TAC is a continuous measure of oxidative stress in plasma that is relevant to oxidation-induced pathological processes. Moreover, the measurement of TAC is indicative of the ability of circulating antioxidants to scavenge free radicals present in plasma before they can injure the vascular wall.

Summary

The balance between oxidants and antioxidants has important implications for cardiovascular diseases. In pathological conditions, there is often increased production of oxidants accompanied by a decreased antioxidant capacity, which causes oxidative stress. An overwhelming amount of evidence supports oxidative stress-induced destruction of NO as a major mechanism for reduced NO bioactivity and vascular dysfunction in cardiovascular disease. Furthermore, oxidative stress leads to pathological oxidative damage to proteins and lipids, as evidenced by increased levels of nitrotyrosine and ox-LDL.

Dyslipidemia

Endothelial cells are constantly exposed to circulating lipids, and in some cases to lipids that have accumulated in subendothelial regions. (124) Interactions between the

endothelium and lipids have direct relevance to atherogenesis and thrombosis. (119) Epidemiologic, clinical, genetic, experimental, and pathological studies have all clearly established the major role of lipoproteins in atherogenesis. (23) The relative abundance of the different plasma lipoproteins appears to be of primary importance, as elevated levels of atherogenic lipoproteins are a prerequisite for most forms of the disease. (24) Dyslipidemia is a disorder of lipoprotein metabolism, including lipoprotein overproduction or deficiency, and is generally manifested by elevated serum total cholesterol, LDL-C, and TG levels, and/or decreased HDL-C levels. (125) There are several mechanisms by which dyslipidemia may influence the risk of clinical cardiovascular events, including effects on endothelial function, atherosclerotic plaque development, and thrombosis. (25)

Hypercholesterolemia

The National Cholesterol Education Program (NCEP) classifies total cholesterol >240 mg/dL and LDL-C >160 mg/dL as elevated, and total cholesterol between 200-239 mg/dL and LDL-C levels >130 mg/dL as borderline high. (126) Hypercholesterolemia, or elevated levels of total cholesterol and/or LDL-C, is strongly associated with increased cardiovascular disease risk. (25) This is because the increased availability of lipids in hypercholesterolemia promotes atherosclerosis by increasing endothelial permeability to LDL, the accumulation of cholesterol in the subendothelial space, and the subsequent oxidation of LDL and uptake by macrophages. (127-129) Furthermore, hypercholesterolemia promotes functional and structural vascular wall injury via activation of oxidation-sensitive mechanisms. (26) Accordingly, a 1% decrease in total cholesterol has been associated with a 1-2% reduction in risk, while a 1% decrease in

LDL cholesterol has been estimated to reduce the risk of major coronary events by 1.7%.
(40)

Considerable evidence shows that high levels of total cholesterol and LDL-C are associated with endothelial dysfunction, largely due to impaired NO bioactivity. (26) High levels of LDL-C decrease eNOS activity by promoting the interaction between eNOS and inhibitory proteins and reducing the availability of L-arginine and BH₄. (26,130) At the same time, hypercholesterolemia is associated with increased NO inactivation, which is partly due to enhanced superoxide production via NADPH oxidase, xanthine oxidase, and uncoupled eNOS. (127,130) Peroxynitrite formation is also increased in the presence of high cholesterol levels, and this serves to further impair NO bioactivity. (127) Additionally, elevated levels of lipid peroxidation products, including ox-LDL, have been found in patients with hypercholesterolemia, and this can enhance the deleterious effects on NO bioactivity and vascular function. (131)

Low HDL-C Levels

HDL-C levels are independently and inversely related to the severity of atherosclerosis and the risk of CAD with levels ≤ 40 mg/dL classified as “low” and levels ≥ 60 mg/dL as “desirable/optimal.” (126) In the Framingham Heart Study HDL-C level was the strongest predictor of CAD risk in both men and women, even after adjusting for other lipids and traditional risk factors for CAD. (132) Moreover, data from four large prospective studies showed that for every 1 mg/dL increment in HDL-C, there is an associated 2% decreased risk of CAD in men and a 3% decreased risk in women, independent of age, blood pressure, smoking status, BMI, and LDL-C level. (3) There are two major subclasses of HDL based on size and density: HDL₂ is the larger, less dense

particle whereas HDL₃ is the smaller, denser particle. (132) These subclasses seem to differ in their cardioprotective effects, with HDL₂ particles appearing to be more cardioprotective than HDL₃ particles. (132)

The inverse relationship between HDL and CAD is due to the fact that HDL is associated with a variety of anti-inflammatory, antioxidant, anticoagulant, and profibrinolytic actions that contribute to its ability to inhibit atherosclerotic plaque formation. (132) For example, HDL promotes cholesterol efflux (reverse cholesterol transport), reduces the expression of cellular adhesion molecules, inhibits lipid peroxidation, including that in LDL, inhibits the expression of inflammatory molecules, and prevents apoptosis. (119) Additionally, HDL inhibits coagulation, platelet aggregation and adhesion, and vascular smooth muscle cell growth and migration. (103,133) Recent data also indicate that HDL can help to prevent or correct endothelial dysfunction by enhancing eNOS enzymatic activity and augmenting eNOS expression. (132,133)

Hypertriglyceridemia

Compared to plasma cholesterol, the potential independent relationship between plasma TG and CAD is more complex and controversial because elevated (>400 mg/dL) or borderline high levels of TG (200-400 mg/dL) generally do not occur as isolated entities. (126) More commonly, hypertriglyceridemia, or high plasma TG levels, is associated with other metabolic disturbances and risk factors, particularly low HDL-C. Several studies indicate that while elevated TG levels have been linked to increased cardiovascular risk, when HDL-C is included in the analysis, the effect of TG on CAD risk is significantly weakened or even disappears. (134) Nevertheless, a meta-analysis of

17 studies found that after adjusting for other factors, including HDL-C, an 88 mg/dL increase in TG was associated with a 14% increase in CAD risk among men and a 37% increase among women, supporting the independent effect of TG levels on CAD risk.

(42) Consistent with this, the accumulation of TG-rich lipoproteins in the arterial wall can induce the synthesis of factors that promote inflammatory responses. (127) High TG levels have been linked to a shift in the prothrombotic-fibrinolytic balance toward coagulation. (25) In addition, high fasting and post-prandial TG levels have been associated with endothelial dysfunction, as evidenced by impaired endothelium-dependent and elevated plasma levels of soluble cell adhesion molecules.

Summary

Dyslipidemia is a common abnormality that typically results from increased levels of atherogenic lipoproteins and/or decreased levels of atheroprotective lipoproteins. In this setting, vascular function is compromised due to increased oxidation of LDL, inflammation, and plaque formation, and decreased NO bioactivity.

Genetic and Environmental Factors

Given that atherosclerosis is a multifactorial disease, it is not surprising that the pathogenesis of atherosclerosis involves complex interactions between multiple genes and environmental factors. (38) Epidemiological studies over the past 50 years have identified numerous risk factors for atherosclerosis, including age, elevated levels of LDL, reduced levels of HDL, hypertension, diabetes, obesity, family history of premature CAD, cigarette smoking, male gender, high-fat diet, and physical inactivity. (1,24) More recently, emerging risk factors such as endothelial dysfunction and oxidative stress have also been found to contribute to the development of atherosclerosis. Many of these risk

factors have a strong genetic component. (38) Additionally, genetic factors may partly explain the effect of environmental factors on atherogenesis (i.e. gene-environment interactions).

The importance of genetic and environmental factors in cardiovascular disease has been examined in many family and twin studies, which show that the heritability of cardiovascular disease (i.e. the fraction of disease explained by genetics) in most populations is between 40% and 60%. (1,24,38) The genetic basis of atherosclerosis has been supported by linkage analyses which have revealed several genomic regions containing novel candidate genes that may be linked to cardiovascular disease. (38) For example, using quantitative trait locus analysis, Welch et al. identified a region on chromosome 6 that was highly linked to atherosclerosis in LDL-receptor knockout mice. (30) This region most likely corresponds to chromosome 12p13-12 in humans, which contains the gene for the newly identified lectin-like oxidized LDL receptor (LOX-1). Association studies have also supported the influence of genetics in atherosclerosis. In this respect, a number of genetic polymorphisms related to endothelial dysfunction, oxidative stress, and lipid metabolism have been shown to correlate with the risk of cardiovascular disease. (38) While genes contribute to variation within populations, population migration studies clearly show that the environment explains much of the variation in disease incidence between populations. (24) Thus, cardiovascular disease results from the combination of genetic susceptibility and an unhealthy environment.

Genetics of Cardiovascular Risk Factors

There have been few studies evaluating the genetic contribution to endothelial function. Data from the Framingham Heart Study suggest that the heritability of

endothelium-dependent vasodilation (as assessed by venous occlusion plethysmography) is modest, with an estimate of 12-14% in a large community-based cohort. (135) Wang et al. found that ~30% of the variation in plasma NO_x levels is due to the additive effect of genes. (136) In terms of oxidative stress, plasma total antioxidant capacity has a reported heritability of ~51%, while ~20-35% of the variation in plasma hydrogen peroxide production is due to heritable factors. (84,137) Studies also indicate that the concentration of conjugated dienes extracted from LDL (a marker of LDL oxidation), as well as the formation of peroxynitrite, and hence nitrotyrosine, may also be under genetic control. (138,139) Much stronger genetic influences have been found for plasma lipoprotein-lipid levels, with heritability estimates ranging from 50-83% for total cholesterol, LDL-C, HDL-C, and TG. (140)

Gene-Environment Interactions

As mentioned previously, high dietary fat intake and physical inactivity are two major risk factors for cardiovascular disease. Although these two factors are largely environmental, genetic factors may partly explain the effect of diet and exercise on the susceptibility to atherogenesis. (38) Significant gene-diet interactions have been found for endothelium-dependent vasodilation, plasma ox-LDL levels, and plasma lipid levels. (46,141,142) Similarly, according to the 2004 human gene map for performance and health-related fitness phenotypes, significant gene-exercise interactions have been observed for phenotypes related to endothelial function (i.e. forearm blood flow), oxidative stress (i.e. antioxidant enzymes), and plasma lipid levels. (45)

Summary

Both genetic and environmental factors influence risk for atherosclerosis. A number of genetic polymorphisms have been associated with cardiovascular disease, as well as the risk factors for cardiovascular disease, including endothelial dysfunction, oxidative stress, and dyslipidemia. At the same time, genetic factors can modulate the response to environmental factors, such as diet and exercise, and these interactions may have a profound effect on disease risk.

Lectin-like Oxidized LDL Receptor

As mentioned above, the oxidative modification of LDL is a key step in the initiation and progression of atherosclerosis. It has long been known that ox-LDL is taken up by macrophages and smooth muscle cells through a variety of scavenger receptors, ultimately leading to foam cell formation. (143) More recently, it has been shown that vascular endothelial cells can also internalize and degrade ox-LDL, but this occurs through a receptor-mediated pathway that does not involve the classic scavenger receptors. In 1997, Sawamura et al. identified the lectin-like ox-LDL receptor-1 (LOX-1) as the major receptor for ox-LDL in endothelial cells. (144) LOX-1 is a Type II membrane protein that is comprised of four domains: a short N-terminal cytoplasmic domain, a transmembrane domain, a neck domain, and a C-terminus extracellular lectin domain. (143) The lectin domain has been identified as the ox-LDL-binding domain, as electrostatic interactions between basic residues in the lectin domain of LOX-1 and negatively charged residues in ox-LDL appear to be critical for LOX-1 activity. Moreover, cysteine residues in the neck domain are thought to be involved in the

formation of LOX-1 homodimers, and the quaternary structure of this receptor may also affect its binding activity. (145)

In addition to ox-LDL, LOX-1 exhibits binding activity for multiple cellular ligands that are implicated in the pathogenesis of atherosclerosis, including apoptotic/aged cells, activated platelets, bacteria, and leukocytes. (143) Given these findings, it has been suggested that under physiological conditions, LOX-1 plays a role in host defense or helps to scavenge or clean up cellular debris and other related material. This is consistent with the fact that LOX-1 shares significant homology to natural killer cell receptors involved in immune responses. However, in pathological states, LOX-1 may be involved in the uptake of ox-LDL and cellular ligands, which induces endothelial cell activation, leukocyte adhesion, cellular apoptosis, smooth muscle cell transformation, and macrophage lipid accumulation. Moreover, LOX-1 activation has been shown to stimulate several intracellular signaling pathways that regulate the expression of genes related to atherosclerosis. (6)

Several lines of evidence support the role of LOX-1 in atherosclerosis. (143) LOX-1 is expressed *in vivo* in aortic, carotid, thoracic, and coronary arteries and veins. While it is predominantly found in endothelial cells, LOX-1 is also expressed in macrophages, smooth muscle cells, fibroblasts, and platelets. *In vitro*, basal expression of LOX-1 in endothelial cells is very low. (6) However, LOX-1 can be upregulated by pro-inflammatory, vasoactive, pro-oxidant, and mechanical stimuli. (6,7) Similarly, *in vivo*, basal LOX-1 expression is very low, but is enhanced in diseases that affect the vasculature, including hypertension, hyperlipidemia, and diabetes. (6) In addition, LOX-1 mRNA and protein expression is undetectable or negligible in normal arteries, but is

prominent in atherosclerotic vessels. (146) Evidence suggests that LOX-1 plays an important role in ox-LDL uptake by endothelial cells and subsequent vascular dysfunction in early atherosclerotic lesions. In advanced lesions, LOX-1 may be involved in ox-LDL uptake by macrophages and smooth muscle cells and the formation of foam cells, as well as the destabilization and rupture of atherosclerotic plaques. (143,146) Thus, it appears that LOX-1 is involved in multiple events in atherosclerosis and its complications.

LOX-1 and Endothelial Dysfunction

Recently, it has been demonstrated that ox-LDL via LOX-1 significantly decreases eNOS mRNA and protein expression in cultured endothelial cells. (10) In addition, Cominacini et al. found that ox-LDL-induced LOX-1 activation reduces the intracellular NO concentration in basal and stimulated endothelial cells, largely due to increased NO inactivation by superoxide. (9) Consistent with a reduction in NO bioactivity, Nagase et al. reported that LOX-1 may be involved in impaired endothelium-dependent vasodilation in hypertension. (11) At the same time, NO deficiency induced by eNOS inhibitors leads to increased LOX-1 mRNA and protein expression and uptake of ox-LDL in cultured endothelial cells. (147) Thus, a positive feedback loop can develop with LOX-1 and NO concentrations that exacerbates endothelial dysfunction.

LOX-1 and Oxidative Stress

Ox-LDL via LOX-1 may induce oxidative stress by increasing the production of superoxide and hydrogen peroxide. (8) This is supported by the findings that LOX-1 activation is associated with increased NADPH oxidase subunit expression and enzyme activity. (12) The binding of ox-LDL to LOX-1 may also promote the formation of

peroxynitrite (and potentially nitrotyrosine), by increasing the reaction between NO and superoxide. (9) Ox-LDL has also been found to decrease superoxide dismutase activity and increase lipid peroxidation in cultured endothelial cells, and these effects are potentially mediated by LOX-1. (64) Given that LOX-1 not only increases ROS, but is also increased by ROS and ox-LDL, this can further upregulate LOX-1 expression, leading to the formation of a positive feedback loop. (7)

LOX-1 and Dyslipidemia

To date, no studies have examined the effects of LOX-1 activation on dyslipidemia. However, Hu et al. showed that ox-LDL via LOX-1 decreases the expression of LDL receptors in cultured endothelial cells via increased superoxide production. (13) While vascular endothelial cells are not major sites for cholesterol uptake, small amounts of LOX-1 have been found in the liver, which removes up to 70% of circulating LDL. (65) Several studies suggest that hepatic endothelial cells and macrophages express LOX-1, and thus may be involved in the uptake of ox-LDL. (148,149) Duryee et al. found that injection of aldehyde-modified proteins into rats significantly increased LOX-1 expression in isolated liver endothelial cells. (148) In addition, co-incubation of primary hepatocytes (the major liver cells involved in LDL metabolism) with liver endothelial cells has been shown to significantly enhance the ability of hepatocytes to take up LDL *in vitro*. (150) Taken together, these findings suggest that LOX-1 may lead to dyslipidemia by reducing receptor-mediated uptake of LDL in the liver.

Soluble LOX-1

Like many cell-surface receptors with a single transmembrane domain, LOX-1 can be cleaved at the juxtamembrane region, most likely by serine proteases, and secreted in a soluble form. (27) Two cleavage sites have been identified in the neck region of LOX-1 (Arg86-Ser87 and Lys89-Ser90), generating similar proteins that are indistinguishable by SDS-PAGE. Studies have shown that soluble membrane receptors can modulate disease activity by binding to its ligand and preventing ligand uptake by the receptor at the cell-surface. In addition, elevated levels of soluble membrane proteins in plasma may reflect increased expression of membrane-bound proteins and disease activities. Hayashida et al. recently examined serum soluble LOX-1 levels in patients with CAD and noncardiac inflammatory diseases. (28) They found that soluble LOX-1 levels did not reflect general inflammation or lesion size, but rather instability of atherosclerotic plaques, as patients with acute coronary syndromes had significantly higher levels than other patients. Thus, the measurement of soluble LOX-1 in plasma may potentially be useful in predicting atherosclerotic disease progression in humans.

LOX-1 Gene

The LOX-1 gene has been localized to region 12.3-13.2 on the short arm of chromosome 12. (143) As mentioned earlier, this region has been linked to atherosclerosis in hypercholesterolemic mice. (30) This region has also be associated with familial hypertension. (29) LOX-1 is encoded by 6 exons that span ~15 kb in the human genome and codes for a protein of 273 amino acids. (143) Yamanaka et al. demonstrated that the 5'-untranslated region (UTR) of the human LOX-1 gene is a functional promoter that contains putative binding sites for multiple transcription factors.

(65) The presence of these regulatory elements suggests that LOX-1 gene expression may be regulated by inflammatory cytokines and/or growth factors. (65) In addition, seven polymorphisms have been identified in the LOX-1 gene that may be associated with cardiovascular disease. (31) These include three polymorphisms located within intron 4, two located within intron 5, one in exon 4, and one in the 3'UTR. The 5 intronic polymorphisms and the one in the 3'UTR are in complete linkage disequilibrium and behave as a single variant, while these polymorphisms are also highly linked to the polymorphism in exon 4, with a reported $D' = 0.87$. (31,37)

LOX-1 3'UTR C/T Polymorphism

The LOX-1 3'UTR polymorphism involves a C-to-T substitution located 188 bp downstream of the stop codon. (32) Using data from the WISE Study, Chen et al. found that in white women, the frequency of the 3'UTR/T allele significantly increased as the stenosis severity increased from normal/minimal (<20%) to significant ($\geq 50\%$). (32) In addition, IgG antibodies to ox-LDL were significantly higher in the 3'UTR/T allele carriers compared to the CC homozygotes, suggesting that this polymorphism may influence CAD risk by affecting ox-LDL metabolism. Mango et al. examined 150 patients with acute myocardial infarction (as determined by electrocardiographic changes, increased serum activities of at least two enzymes, and coronary angiography) and 103 clinically healthy controls with normal arteries (as determined by coronary angiography), but at least one conventional risk factor. (31) The 3'UTR/T allele was associated with a 3.7-fold greater risk for acute myocardial infarction, with allele frequencies of 64% and 45% in patients and controls, respectively. Recently, Trabetti et al examined 350 patients with significant coronary stenosis (>50%) and 327 subjects with normal coronary

arteries, as determined by coronary angiography. (37) The CAD subjects were further divided into those with and without acute myocardial infarction. In contrast to the previous studies, they found no difference in 3'UTR/T allele frequencies between patients with and without acute myocardial infarction (54% vs. 56%, respectively), between CAD patients and controls (55% vs. 51%, respectively), or between patients with acute myocardial infarction and controls (54% vs. 51%). (37) However, there was a slightly positive association between the 3'UTR polymorphism and CAD (odds ratio = 1.42, $p=0.065$).

In the Chen et al. study mentioned previously, DNA binding assays demonstrated that the 3'UTR polymorphism affects the binding of a putative transcription factor in an allele-specific manner, with the T allele having a 3-fold lower binding affinity compared to the C allele. (32) In addition, it was recently shown that intronic SNPs in LOX-1, which are in complete linkage disequilibrium with the 3'UTR C/T polymorphism, regulate the expression of a functional splicing isoform of the LOX-1 gene, known as LOXIN. (34) The splicing introduces a premature stop codon that removes exon 5 and generates a truncated protein (199 amino acids) lacking two-thirds of the lectin domain (i.e. the ligand-binding domain). Examination of the subcellular and membrane distribution of the two isoforms revealed that LOXIN accumulates in the perinuclear region and fails to localize at the cell membrane. The intronic polymorphisms modulate the relative abundance of the LOX-1 and LOXIN transcripts, such that the ratio of LOX-1 to LOXIN is significantly higher (33%) in macrophages from subjects homozygous for the "risk" haplotype (i.e. CTGGTT) compared with subjects homozygous for the "non-risk" haplotype (i.e. GGAAGC). Moreover, increased levels of LOXIN in isolated

macrophages were associated with reduced apoptosis in response to ox-LDL. The mechanism behind the protective effect of LOXIN may involve blocking the transport of LOX-1 receptors to the cell membrane or the formation of inactive heterodimers with LOX-1, both of which would serve to decrease the number of functional receptors able to bind ox-LDL on the cell surface.

LOX-1 G501C Polymorphism

The SNP in exon 4 is a missense mutation that involves a G-to-C transition at nucleotide 501 (G501C), resulting in a nonconservative amino acid change in codon 167 (Lys to Asn, K167N). (35) The amino acid residue 167 is located in the lectin domain of LOX-1, which is the ligand-binding domain. Basic residues in the lectin domain are important for strengthening ligand binding, and substitution of these residues causes reduced binding and internalization of ox-LDL. (36) As such, the LOX-1 G501C polymorphism may affect the metabolism of ox-LDL and consequently, have a protective effect against atherogenesis. However, the literature has been largely inconsistent.

To date, two studies have explored the relationship between the G501C polymorphism and cardiovascular disease in Japanese subjects. The first study by Tatsuguchi et al. examined 102 patients with myocardial infarction (defined as a history of chest pain associated with regional ST segment elevation and increased serum creatine kinase) and 102 normolipidemic age- and sex-matched controls. (35) In this study, the 501C allele was associated with a 3-fold greater risk for myocardial infarction. The frequency of the 501C allele was 21% in patients and 9% in controls. In addition, the percentage of subjects with the GC or CC genotype was significantly higher in the patient group than in the control group (38% vs. 18%, respectively). In a later study, Ohmori et

al. examined this polymorphism in 586 patients undergoing coronary angiography for suspected CAD. (33) Study patients were categorized into three groups: 1) normal/minimal ($\leq 25\%$) stenosis; 2) mild (26-50%) stenosis; and 3) significant ($>50\%$) stenosis. Patients with significant stenosis were further divided into two groups based on the presence of myocardial infarction, confirmed by the elevation of cardiac enzymes or diagnostic changes on electrocardiograms. In contrast to the previous study, they found that the percentage of CAD patients carrying the 501C allele did not differ between those with and without myocardial infarction (34% vs. 37%, respectively). They did, however, find a significant genotype association with CAD, as the 501C allele frequency was lower in patients with significant stenosis compared to those with normal/minimal stenosis (21% vs. 28%, respectively). Moreover, the G501C polymorphism was inversely associated with CAD severity, independent of traditional risk factors (odds ratio = 0.61).

Two separate studies have also investigated the LOX-1 G501C polymorphism in an Italian population. Mango et al. reported a lower 501C allele frequency in patients than controls (4% vs. 9%, respectively). (31) In addition, they found that the G501C polymorphism demonstrated only a weak inverse association with acute myocardial infarction. Trabetti et al. attempted to clarify the association between the LOX-1 G501C polymorphism and cardiovascular disease. (37) They found no difference in the 501C allele frequency between CAD patients and controls (both 8%), between patients with and without acute myocardial infarction (8% vs. 7%), or between patients with myocardial infarction and controls (both 8%). In addition, the distribution of G501C genotypes in CAD patients did not show any significant difference with increasing number of stenosed vessels. However, the GG genotype frequency in patients with three

obstructed vessels was significantly lower than in patients with one or two obstructed vessels (odds ratio = 0.47). Interestingly, this is the opposite of what Ohmori et al. found in the Japanese subjects. (33) Taken together, it appears that the LOX-1 G501C polymorphism may be associated with cardiovascular disease, although it is unclear which allele is the risk allele. Discrepancies in the current studies may be due to differences in the subject populations or in the selection of patients and controls.

Summary

LOX-1 plays a critical role in atherosclerosis, from endothelial activation and plaque formation in the early stages, to plaque instability and rupture in the advanced stages. These effects may be partly due to the fact that LOX-1 activation by ox-LDL induces endothelial dysfunction, oxidative stress, and potentially dyslipidemia. The role of LOX-1 in cardiovascular disease is supported by the findings that the LOX-1 gene has been linked to atherosclerosis and hypertension. The LOX-1 3'UTR polymorphism regulates gene expression and has been positively associated with both CAD and myocardial infarction. The LOX-1 G501C polymorphism has also been associated with cardiovascular disease, although whether this association is positive or negative remains to be elucidated.

Dietary Fat Intake

Fatty acids have a diverse array of functions that affect many structural, metabolic, and regulatory components of cells and tissues. (92) Not only are they of vital importance as an energy nutrient, but they are also required for membrane synthesis, protein and carbohydrate modification, assembly of structural elements, production of signaling compounds, and solubilization of various cellular and extracellular constituents.

(151) Depending on the number of double bonds found in the hydrocarbon chain, fatty acids can be classified as either saturated (SFAs, no double bonds) or unsaturated (multiple double bonds). Unsaturated fatty acids can be further classified into monounsaturated fatty acids (containing 1 double bond) or polyunsaturated fatty acids (PUFAs, containing >1 double bond), with the latter group containing the important n-3 and n-6 PUFA.

It is well known that different types of fatty acids have different health effects.

(41) SFAs increase plasma cholesterol levels and the risk for atherosclerotic and thrombotic events. (67) On the other hand, both n-6 and n-3 fatty acids are associated with lower risk of CAD, although through different biological pathways. (41) Clinical studies have shown that n-6 PUFAs reduce risk primarily by lowering plasma cholesterol levels. (40) Of these, linoleic acid (18:2n-6), the major n-6 fatty acid in the diet, appears to have the most potent effect. n-3 fatty acids protect against atherosclerosis by reducing arrhythmia, inflammation, and thrombosis, and improving cardiac function, fibrinolysis, and vascular tone. (39,40) The most important n-3 PUFAs are α -linolenic acid (18:3n-3), which is found in plant foods, and eicosapentaenoic acid (EPA, 20:5n-3) and docosahexaenoic acid (DHA, 22:6n-3), which are found primarily in fish. (40)

Although fatty acids are essential to life, excessive levels of dietary fat or an imbalance of saturated versus unsaturated fat has been implicated in the onset and progression of several chronic diseases, including atherosclerosis, dyslipidemia, inflammation, diabetes, and obesity. (92) Accordingly, the current American Heart Association (AHA) dietary guidelines advocate a total fat intake of $\leq 30\%$ of energy and a saturated fat intake of $< 10\%$ of energy. (67) It has also been recommended that PUFAs

contribute between 4-10% of energy. (40) It is becoming increasingly recognized that the type of fat is more important than the total amount of fat in determining the risk of CAD. (41) Using 14-year follow-up data from the Nurses' Health Study, it was estimated that replacing 5% of energy from saturated fat with unsaturated fat can reduce risk by 42%. (41) In addition, the ratio of PUFAs to SFAs (P:S ratio) was strongly associated with a lower risk of CAD, and after adjustment for confounding factors, the relative risk of CAD was 0.79 for each 0.2 unit increment in the ratio.

Dietary Fat and Endothelial Dysfunction

Dietary fatty acids can have significant effects on endothelial function. (71) Diets high in SFAs have been shown to promote platelet activation, thrombosis, and vasoconstriction. (70) Such diets also lead to major morphological changes in the vascular wall related to atherogenesis, including endothelial damage, intimal thickening, leukocyte adhesion/infiltration, foam cell formation, and smooth muscle cell proliferation. In contrast, n-3 fatty acids have been shown to reduce blood pressure, increase systemic arterial compliance, and improve FBF and vascular reactivity. (39) These improvements may be due to increased NO generation or bioactivity. Linoleic acid also increases eNOS activity and NO formation, in part, by increasing intracellular BH₄ concentrations. (152) However, this may occur as a compensatory response to linoleic acid-induced oxidative stress. Consistent with this, there is some evidence to suggest that linoleic acid causes endothelial activation and injury, promotes smooth muscle cell proliferation, elicits the production of inflammatory cytokines and cellular adhesion molecules, induces apoptosis, disrupts endothelial cell integrity, impairs endothelial barrier function, and alters cell morphology. (47,71,152,153)

Fuentes et al. looked at the effects of replacing a high-fat diet (38% total fat, 20% SFAs, and 6% PUFAs; P:S ratio ~0.3) with a low-fat diet (28% total fat, <10% SFAs, 6% PUFAs; P:S ratio ~0.6) in hypercholesterolemic men. (69) They found that reducing the total fat and SFA intake had no effect on endothelium-dependent dilation of the brachial artery following ischemia (9.9% and 11.1%, for high-fat diet and low-fat diet, respectively). However, in normocholesterolemic subjects, endothelium-dependent dilation of the brachial artery following ischemia was ~50% lower after a high-SFA diet (37% total fat, 19% SFAs, 4% PUFAs; P:S ratio ~0.2) compared with a high-PUFA diet (36% total fat, 9% SFAs, 15% PUFAs; P:S ratio ~1.67). (154) These findings suggest that the relative amount of PUFAs and SFAs, rather than the total amount of fat, has a greater effect on endothelial function. The presence/absence of cardiovascular risk factors may also influence the effect of dietary fat intake on endothelial function. It has also been shown that in healthy subjects 4 weeks of a high-linoleic acid diet (34.4% total fat, 10.6% SFAs, 12.3% PUFAs; P:S ratio ~1.16) decreases urinary NO_x excretion by 38%. (155) In contrast, Sato et al. found no difference in serum NO_x levels between ApoE-deficient mice fed a diet rich in linoleic acid (9.5% SFAs and 75.4% PUFAs; P:S ratio ~7.9) and mice fed a diet rich in SFAs (45% SFAs and 9.8% PUFAs; P:S ratio ~0.21). (156)

Dietary Fat and Oxidative Stress

Besides affecting endothelial function, dietary fatty acids may also have important implications for the development of oxidative stress. Several studies have shown that a high SFA intake may increase oxidative stress by increasing lipid peroxidation and decreasing antioxidant enzymes. (70,157) This may be due to the hypercholesterolemic effect of SFAs, whereby elevated plasma LDL levels can lead to increased retention and

oxidation of LDL in the arterial wall. (1) Moreover, hypercholesterolemia is associated with increased superoxide production and NO inactivation. (131) Due to their high degree of saturation, PUFAs are highly susceptible to damaging, free radical-initiated lipid peroxidation. Consequently, PUFAs also tend to be positively associated with oxidative stress. (153,157) However, this may depend on the type of PUFA, as n-3 PUFAs have been found to suppress ROS production, upregulate gene expression of antioxidant enzymes, and downregulate genes associated with ROS production. (39) On the other hand, linoleic acid has been shown to induce superoxide production, increase plasma and urinary concentrations of lipid peroxidation products, promote NO inactivation, and lead to nitrotyrosine formation. (70,152,155)

Diniz et al. found that a high-SFA diet (28.8% total fat, 82% SFAs, 8% PUFAs; P:S ratio ~0.10) increased hydroperoxide concentrations and decreased glutathione peroxidase activity in cardiac tissue of male Wistar rats compared to control rats (3.8% total fat, 49% SFAs, 39% PUFAs; P:S ratio ~0.79) (157) However, the effect of a high-PUFA diet (28.8% total fat, 37% SFAs, 48% PUFAs; P:S ratio ~1.3) appeared to be even greater. Cardiac tissue from rats on the high-PUFA diet had higher concentrations of lipoperoxides and hydroperoxides, and lower superoxide dismutase and catalase activities compared to cardiac tissue from both rats on the high-SFA diet and controls. In addition, diets high in linoleic acid have been shown to increase serum TBARS levels and urinary isoprostane levels (markers of lipid peroxidation) in both mice and humans. (155,156) On the other hand, other studies have shown that PUFAs promote less or similar oxidative damage compared to SFA. (158-160) For example, Miller et al. reported that the DASH diet (27% total fat, 6% SFA, 8% PUFA; P:S ratio ~1.3) significantly lowered urinary

isoprostane excretion and increased serum antioxidant capacity compared to an average American diet (37% total fat, 16% SFA, 8% PUFA; P:S ratio ~0.5). (161) The DASH diet also increased the concentration of antibodies to ox-LDL, which may be associated with enhanced immunoprotective mechanisms. Thus, both PUFAs and SFAs can cause oxidative stress, although overall, PUFA may still be more protective against atherosclerosis.

Dietary Fat and Plasma Lipid Levels

There are remarkable differences between SFAs and PUFAs with respect to their effects on plasma lipid levels. (40) In general, SFAs increase plasma levels of total cholesterol, LDL-C, and TG. This occurs most likely via increased hepatic cholesterol synthesis and increased expression of apolipoproteins B-100 and C-III, which are involved in lipid transport and metabolism. (94) On the other hand, n-6 PUFAs, specifically linoleic acid, decrease total cholesterol and LDL-C by enhancing hepatic receptor-mediated clearance of LDL and concomitantly reducing LDL cholesterol production. (68) The n-3 PUFAs have little effect on cholesterol, but may reduce plasma TG levels by enhancing hepatic β -oxidation of fatty acids, thereby inhibiting TG secretion from the liver. Interestingly, the traditional dietary recommendations can negatively impact HDL and its subclasses. (132) For example, a reduction in total fat and SFAs has been shown to decrease total HDL, as well as HDL₂ and HDL₃, when compared with the average American diet.

Substantial research has assessed the impact of dietary fat on plasma lipid levels, and the current research highlights the importance of the relative amounts of individual fatty acids. (153) Consistent with this, controlled clinical trials have shown that replacing

SFAs with PUFAs is more effective in lowering serum cholesterol and reducing the risk of CAD than simply reducing total fat consumption. (41) Studies have predicted that a 1% increase in PUFAs (without any corresponding changes in other fatty acid classes) would be expected to lower total cholesterol by an average of 0.024 mmol/L (~1 mg/dL). (153) Likewise, simply increasing SFAs by 1% would be expected to increase total cholesterol by an average of 0.054 mmol/L (~2 mg/dL). Moreover, diets low in SFAs (8-9% energy) and high in PUFAs (14-21% energy) reduce LDL-C levels by 13-15%, which is associated with a 25-43% reduction in CAD events. (40)

Gene-Diet Interactions

Fatty acids can influence many cellular pathways by altering gene expression. (92) The simplest mechanism for fatty acid regulation of gene expression is for the fatty acid (or a metabolite) to directly bind to and regulate the activity of a transcription factor, ultimately influencing gene transcription. (92) Fatty acids can also indirectly affect the nuclear abundance of the transcription factor by influencing mRNA turnover. Thus, the fatty acid-mediated regulation of diverse biological pathways is of considerable interest. Microarray analysis has revealed that n-3 PUFAs modulate the expression of genes involved in cell adhesion, oxidative stress response and antioxidant enzyme activities, apoptosis, cell growth and proliferation, cell signaling and transduction, lipid metabolism, cellular transport, and membrane localization. (93) Similarly, palmitic acid (16:0) has been shown to alter the hepatic expression of genes involved in lipid transport and metabolism, cell growth and proliferation, cell signaling, protein synthesis, cytoskeletal structure, and oxidative stress response. (94) These effects can ultimately alter cell function, development, or maturation.

While fatty acids are important regulators of gene expression, these effects may be highly variable, as genetic polymorphisms have been shown to influence the response to dietary fat. (46) In particular, studies have demonstrated significant gene-diet interactions for markers of endothelial dysfunction, oxidative stress, and dyslipidemia. (46,47,141,142) It has been shown that a high-fat, high-cholesterol diet increases LOX-1 expression in rabbits, and this may be due to an increase in linoleic acid. (162) Recently, Maingrette et al. found that linoleic acid increases LOX-1 expression and ox-LDL uptake in endothelial cells. (47) These effects were mediated by the binding of nuclear proteins to a regulatory sequence in the LOX-1 gene promoter and subsequent superoxide production via NADPH oxidase. (47) In contrast, palmitic acid and DHA did not affect LOX-1 protein expression. Taken together, these findings suggest that differences in dietary fatty acid composition (i.e. PUFA vs. SFA intake) may affect LOX-1 expression. Moreover, polymorphisms in the LOX-1 gene could influence changes in endothelial function, oxidative stress, and plasma lipid levels in response to dietary fat.

Summary

The relative amount of PUFAs and SFAs in the diet is an important determinant of disease risk, whereby a high P:S ratio may reduce risk for cardiovascular disease. Although PUFAs offer many cardioprotective benefits, certain PUFAs can promote endothelial dysfunction and oxidative stress. Thus, it is necessary to identify the optimal level of dietary PUFAs that maximally and favorably affect the greatest number of cardiovascular risk factors. Given the inter-individual variation in dietary response and disease susceptibility, it may be of interest to consider the interaction between the dietary P:S ratio and LOX-1 gene variation.

Aerobic Exercise Training

A number of studies have shown that moderate-intensity physical activity reduces the incidence of all-cause mortality, particularly deaths due to cardiovascular disease. (163) There is also convincing evidence that exercise training can help to slow, halt, and even reverse the progression of atherosclerotic CAD. Risk factors such as hypertension, obesity, and hyperlipidemia may respond favorably to physical activity, thereby protecting against cardiovascular disease. However, the effect of exercise on traditional risk factors does not solely account for the magnitude of risk reduction. (43) Hence, physical activity reduces the morbidity and mortality associated with atherosclerotic CAD through direct (cardiovascular) and indirect (risk factor modification) mechanisms, independent of other interventions. (163) The baseline physical activity level plays an important role in this respect, as physical activity generally has greater cardiovascular effects among sedentary individuals compared to active individuals. (44,126)

Shear Stress

Vascular endothelial cells are constantly exposed to blood flow, which generates a frictional force per unit area known as hemodynamic shear stress. (88) The magnitude of shear stress can be estimated in most of the vasculature by Poiseuille's law, which states that shear stress is directly proportional to blood flow viscosity and inversely proportional to the third power of the internal radius. Shear stress of physiological and elevated magnitudes ($>15 \text{ dyne/cm}^2$) increases the production of vasodilators, growth inhibitors, fibrinolytics, and antioxidants, and decreases the production of vasoconstrictors, growth promoters, inflammatory mediators, oxidants, and adhesion molecules. Shear stress is an important component of exercise, and sufficient activity-related elevations in shear stress

can promote the development of an atheroprotective endothelium, thus attenuating (and potentially reversing) cardiovascular disease. (26,88)

Characteristics of the Training Program

It is clinically important to select the appropriate intensity of exercise because very intense exercise can be hazardous to the vascular wall. (79) A study by Goto et al. suggests that low intensity exercise (25% VO₂ max) may fall below the threshold for improvements in NO-related vascular function, while moderate intensity exercise (50% VO₂ max) significantly enhances NO bioactivity. (79) Although NO production has been shown to progressively increase as exercise intensity increases, any improvement in vascular function as a result of enhanced NO production may be abolished through increased scavenging by free radicals generated at high exercise intensities (75% VO₂ max). (75,79) In contrast, changes in plasma lipid levels with exercise training appear to be related to the amount and not the intensity of exercise. (164) Specifically, high amounts of exercise (~17-18 miles/week) have been shown to result in significantly greater improvements in the plasma lipoprotein-lipid profile compared to lower amounts of exercise (~11 miles/week). This is consistent with the threshold established from a recent review paper which indicates that 15-20 miles/week of exercise is necessary to produce the desired lipid changes. (42)

The duration of exercise (i.e. length of training program) may also be of significance, as short-term and long-term training adaptations may be quite different. (43) Animal studies involving both the peripheral and coronary vasculature suggest that short-term exercise training (as few as 7-10 days) enhances eNOS activity, NO production, and NO bioactivity, producing a short term buffer to the increased shear stress associated with

exercise. However, after extended training (>16 weeks), at least in the peripheral circulation, shear stress is “structurally” normalized and endothelial NO bioactivity returns towards initial levels. In terms of oxidative stress, there is insufficient data to establish a time course for changes in oxidants and antioxidants with exercise training. Similarly, the relationship between the duration of training and the observed changes in plasma lipid levels remains unclear, although it has been suggested that at least 12 weeks of exercise training is required to increase HDL-C levels. (126)

Exercise Training and Endothelial Function

The effect of exercise training on endothelial function has been studied in patients with cardiovascular disease, in individuals with cardiovascular risk factors, and in healthy subjects. (43) Exercise training attenuates the paradoxical vasoconstriction in CAD patients and increases blood flow in response to pharmacological agents. (26,43,74) Exercise training is also associated with improvements in measures of NO vasodilator function in subjects with cardiovascular risk factors. (43,75,77) A large number of studies show that even in normal control animals and healthy subjects, exercise training can augment endothelial function. However, it appears that a beneficial effect of exercise training in those with *a priori* normal vascular function may require a higher volume or intensity of exercise. (43) The evidence strongly suggests that subjects with impaired endothelial function may be more amenable to training-induced improvements in endothelium-dependent vasodilation and may respond more rapidly to training than healthy subjects. But, as mentioned earlier, these adaptations may only be transient, and may disappear in the longer term. Moreover, the observed effects on endothelial function also depend on the vascular bed examined (coronary, cerebral, skeletal muscle, etc) and

the position in the arterial tree (conduit artery, smaller artery, arteriole, and branch order of the arteriole). (77)

Most studies indicate that plasma and urinary NO_x levels are higher after exercise training in both healthy subjects and CAD patients. (17,165,166) Similarly, in hypercholesterolemic subjects, Lewis et al. found that 4 weeks of cycling training increased basal, but not stimulated, NO production. (72) Before training, there was a positive forearm NO_x arteriovenous difference indicating net consumption of NO_x, while there was a negative forearm NO_x arteriovenous difference after training, indicating net production of NO_x. These results have been confirmed in cross-sectional studies. For example, Rodriguez-Plaza et al. found that urinary NO_x excretion increased with increasing levels of physical activity, such that highly trained runners (90 km/wk) had urinary NO_x levels that were 2-fold higher than well-trained men (64 km/wk), 7-fold higher than sedentary individuals, and 37-fold higher than CAD patients. (165)

One possible mechanism for the beneficial effect of exercise training on endothelial function is an increase in NO bioactivity. (75) Shear stress is thought to be the most important physiological stimulus for endothelial NO release, and this may be mediated by shear stress response elements in the eNOS gene. (74,167) Exercise training, probably via an increase in shear stress, exerts its beneficial effects on endothelial function by activation of several signal transduction pathways, which lead to increased eNOS mRNA and protein expression and increased NO production. (74,75,168) Regular exercise training also enhances eNOS activity by increasing eNOS phosphorylation. (74) Moreover, the ratio of phosphorylated to unphosphorylated eNOS appears to be increased after exercise training, and this has been associated with an improvement in endothelial

function. (74) In addition to increases in NO production, exercise training may also decrease NO inactivation by reducing its degradation by free radicals or by directly decreasing free radical production. (43)

Regular aerobic exercise also leads to functional and histological alterations in the vascular endothelium, resulting in enhanced vascular structure and function. (75) There is an evolving hypothesis that exercise training induces structural enlargement of conduit vessels. This adaptation is dependent upon shear stress-mediated NO release and may serve to mitigate the increases in shear stress brought about by repeated exercise bouts. (43) Over time, an increase in arterial diameter reduces the shear stress signal associated with a given exercise-induced elevation in blood flow and allows NO bioactivity to return towards pre-training levels. (43,77) This is consistent with the restoration of eNOS expression and endothelium-dependent vasodilation to control levels in the fully-trained state. (77)

Exercise Training and Oxidative Stress

It has been reported that the massive increase in oxygen uptake that occurs in skeletal muscle during exercise is associated with an increase in the generation of ROS. (75) During exercise, ROS can potentially be produced from several cellular sources, including the mitochondrial electron transport chain, xanthine oxidase, NADPH oxidase, and myeloperoxidase. (87,114,169) At the same time, antioxidant enzymes may be activated selectively during an acute bout of strenuous exercise depending on the oxidative stress imposed on the specific tissues, as well as the intrinsic antioxidant defense capacity. (114) Superoxide dismutase, catalase, and glutathione peroxidase provide the primary defense against ROS generated during exercise, and the activities of

these enzymes have been shown to increase in response to exercise in both animals and humans. Thus, while a single bout of exercise may initially cause oxidative stress, chronic exercise training contributes to favorable changes in vascular gene expression that may reduce oxidative stress or attenuate exercise-induced tissue damage. (44,80)

Exercise training may improve oxidative stress by decreasing the formation of oxidants. It has been shown that exercise training decreases NADPH oxidase expression and activity and superoxide production in animal models. (81,170) In addition, myeloperoxidase activity decreased by 29% after 12 weeks of exercise training in individuals with elevated cardiovascular risk. (169) A reduction in the activities of ROS-producing enzymes can ultimately lead to a reduction in the oxidative damage of lipids and proteins. Some studies, but not all, have shown significant reductions in ox-LDL and lipid peroxidation with exercise training. (73,78,80,81,170-173) LDL resistance to oxidation may also be increased with exercise training. (78,172,173) On the other hand, nitrotyrosine levels appear to be unchanged after training. (80,81)

Exercise training also improves oxidative stress by increasing antioxidant activity. Exercise training enhances the protein levels and enzymatic activities of cytosolic and mitochondrial superoxide dismutase in the vascular endothelium and smooth muscle cells. (75) While this appears to be a direct effect of shear stress, exercise training indirectly increases the expression and activity of extracellular superoxide dismutase. (85) In fact, NO produced by endothelial cells in response to shear stress stimulates extracellular superoxide dismutase expression in adjacent smooth muscle cells. This NO-mediated regulation very likely represents an important feed-forward mechanism, whereby NO released from the endothelium ultimately enhances its own bioactivity by

reducing superoxide-mediated degradation as it traverses between the two cells. It has also been shown that 3 months of endurance training significantly decreases plasma levels of extracellular superoxide dismutase by 22%. (174) Taken together, it appears that exercise training may enhance the abundance of extracellular superoxide dismutase in the vascular wall, where it is needed to perform its important biological function. Exercise training also stimulates enzymatic activities of catalase, glutathione peroxidase, and glutathione reductase. (73,78,86) Consistent with upregulation of antioxidant enzymes after training, Fatouros et al. found that 16 weeks of endurance exercise training increased total antioxidant capacity in older men. (80)

ROS-mediated signaling appears to be an important mechanism for the improvement in oxidative stress that occurs with exercise training. (86) It is thought that oxidative stress is beneficial for atherosclerosis through the induction of antioxidant responses in the vascular wall. This induction of antioxidant enzymes would not only minimize oxidative damage, but also reduce the generation of oxidants. Lauer et al. found that hydrogen peroxide is critically involved in the up-regulation of eNOS by exercise, as three weeks of exercise training significantly increased eNOS expression in control mice, but not in transgenic mice overexpressing catalase. (87) The effect of hydrogen peroxide on exercise-induced eNOS expression is supported by the concomitant increase in superoxide dismutase activity, which facilitates the generation of hydrogen peroxide from superoxide. These authors concluded that complete suppression of cellular ROS production is most likely undesirable, for it may lead to a loss of ROS-mediated signaling events. Consistent with these findings, vitamin E supplementation has been shown to inhibit the induction of vascular antioxidants during exercise training in mice. (86)

Exercise Training and Plasma Lipoprotein-Lipid Levels

Regular physical activity has beneficial effects on plasma lipoprotein and lipid levels. (42) Both cross-sectional and longitudinal exercise training studies indicate that plasma TG levels are usually, but not always, decreased. (42,126) This decrease is often related to the baseline concentration, as well as the amount and intensity of exercise. (42) Similarly, HDL-C generally increases with exercise training, and this too is related to the amount and intensity of exercise. (42,126) Exercise training-induced increases in HDL-C levels range from 2 to 8 mg/dL, and the increases in HDL-C with training seem to primarily involve the HDL₂ fraction. (42,126) In contrast, cross sectional and longitudinal exercise training studies do not support a training-induced change in plasma cholesterol concentrations. (42) Plasma LDL-C levels are also not usually lower after aerobic exercise training. For a reduction in total cholesterol or LDL-C to occur with exercise training, a reduction in body weight, body fat, or dietary fat must accompany the exercise training program.

The exercise training-induced changes in plasma lipid and lipoprotein levels can be explained in part by changes in the activities of lipolytic enzymes and lipid transfer proteins. (42) The enzyme lipoprotein lipase is bound to the capillary walls of most tissues and is especially active in adipose tissue, heart muscle, and skeletal muscle. Lipoprotein lipase hydrolyzes TG found in large TG-rich lipoproteins and participates in the formation of HDL particles. Increased plasma lipoprotein lipase activity is often reported after exercise training, and this is generally associated with increases in HDL-C and HDL₂-C. Hepatic lipase is bound to the liver capillary endothelium and functions similarly to lipoprotein lipase. In particular, hepatic lipase hydrolyzes TG and

phospholipids in LDL and HDL and helps convert HDL₂ to HDL₃. (172) Cross-sectional studies show that hepatic lipase activity may be lower in trained subjects, although it is unclear if exercise training significantly affects hepatic lipase activity. (42,175,176) Cholesterol ester transfer protein mediates the exchange of TG and cholesterol esters between HDL and LDL, and in doing so, contributes to the conversion of HDL₂ to HDL₃. (42) Cholesterol ester transfer protein also participates in reverse cholesterol transport and helps remove excess cholesterol from the body. The response of cholesterol ester transfer protein to exercise training is unclear, as increases, decreases, and no changes have been reported. (42)

Gene-Exercise Interactions

As mentioned earlier, shear stress is a major component of exercise due to substantial increases in blood flow. (26) The effects of shear stress on the vascular wall are mediated by the regulation of gene expression, and this occurs via activation of shear stress response elements in the promoter region of various genes. (74,95) Compared to no-flow conditions, Wasserman et al. found that 24 hrs of laminar shear stress at 10 dyne/cm² upregulated the endothelial expression of genes associated with antioxidant, anti-inflammatory, anti-proliferative, anti-apoptotic, and differentiative activities, and downregulated the expression of genes required for cell cycle progression and cellular proliferation. (95) This gene expression pattern likely generates endothelial cells that are protected from apoptosis, inflammation, and oxidative stress. As such, exercise training may have a significant impact on gene expression via increases in blood flow, and hence shear stress.

While an exercise-induced increase in shear stress offers a cardioprotective benefit, a great deal of heterogeneity has been observed in the response to exercise training. (45) Genetic polymorphisms in genes relevant to atherosclerosis have been shown to influence changes in FBF, ox-LDL levels, and lipoprotein-lipid levels. Shear stress response elements have been identified in the 5'-UTR of LOX-1, and Murase et al. demonstrated that physiological levels of laminar shear stress (1-15 dyne/cm²) upregulate LOX-1 mRNA and protein expression in endothelial cells. (29,48) Their results also show that the initial changes in fluid shear stress may be sufficient for induced expression of LOX-1 and that sustained application is not necessarily required. (48) Taken together, these findings suggest that exercise-induced increases in shear stress may affect LOX-1 expression. Moreover, polymorphisms in the LOX-1 gene could influence changes in endothelial function, oxidative stress, and plasma lipid levels in response to exercise training.

Summary

Exercise training reduces risk for cardiovascular disease by improving endothelial function, oxidative stress, and plasma lipid levels. The underlying mechanisms for these improvements involve enhanced lipid transport and metabolism and increases in shear stress, which promote anti-inflammatory, antioxidant, and anti-atherogenic events in the vascular wall. These training adaptations may be dependent on a number of variables, including exercise intensity, duration, and volume, and may be transient or persist for longer periods. Moreover, the response to training is highly variable and may be influenced by genetic polymorphisms. Given the inter-individual variation in training

response and disease susceptibility, it may be of interest to consider the interaction between aerobic exercise training and LOX-1 gene variation.

Concluding Remarks

LOX-1 is an important mediator of endothelial dysfunction and oxidative stress, and recent evidence suggests that LOX-1 may also be involved in lipid metabolism and cholesterol uptake. Accordingly, LOX-1 expression has been strongly associated with the initiation and progression of atherosclerosis. Soluble forms of LOX-1 in plasma may also be related to the disease process. The LOX-1 gene has been linked to cardiovascular disease in different animal models, and genetic polymorphisms in LOX-1 may influence the presence and severity of CAD and the risk for acute myocardial infarction. Given that LOX-1 expression is upregulated by linoleic acid and shear stress, it is possible that polymorphisms in the LOX-1 gene may explain the inter-individual variation in the responses to diet and exercise. Thus individually and collectively, the LOX-1 gene, dietary fat intake, and aerobic exercise training may have significant effects on the events relevant to atherogenesis.

REFERENCE LIST

1. Stocker R, Keaney JF, Jr. Role of oxidative modifications in atherosclerosis. *Physiol Rev* 84: 1381-478, 2004.
2. Chisolm GM, Steinberg D. The oxidative modification hypothesis of atherogenesis: an overview. *Free Radic Biol Med* 28: 1815-26, 2000.
3. Mertens A, Holvoet P. Oxidized LDL and HDL: antagonists in atherothrombosis. *FASEB J* 15: 2073-84, 2001.
4. Salvayre R, Auge N, Benoist H, Negre-Salvayre A. Oxidized low-density lipoprotein-induced apoptosis. *Biochim Biophys Acta* 1585: 213-21, 2002.
5. Young IS, McEneny J. Lipoprotein oxidation and atherosclerosis. *Biochem Soc Trans* 29: 358-62, 2001.
6. Mehta JL, Chen J, Hermonat PL, Romeo F, Novelli G. Lectin-like, oxidized low-density lipoprotein receptor-1 (LOX-1): a critical player in the development of atherosclerosis and related disorders. *Cardiovasc Res* 69: 36-45, 2006.
7. Nagase M, Ando K, Nagase T, Kaname S, Sawamura T, Fujita T. Redox-sensitive regulation of lox-1 gene expression in vascular endothelium. *Biochem Biophys Res Commun* 281: 720-5, 2001.
8. Cominacini L, Pasini AF, Garbin U, Davoli A, Tosetti ML, Campagnola M, Rigoni A, Pastorino AM, Lo C, V, Sawamura T. Oxidized low density lipoprotein (ox-LDL) binding to ox-LDL receptor-1 in endothelial cells induces the activation of NF-kappaB through an increased production of intracellular reactive oxygen species. *J Biol Chem* 275: 12633-8, 2000.
9. Cominacini L, Rigoni A, Pasini AF, Garbin U, Davoli A, Campagnola M, Pastorino AM, Lo C, V, Sawamura T. The binding of oxidized low density lipoprotein (ox-LDL) to ox-LDL receptor-1 reduces the intracellular concentration of nitric oxide in endothelial cells through an increased production of superoxide. *J Biol Chem* 276: 13750-5, 2001.
10. Mehta JL, Li DY, Chen HJ, Joseph J, Romeo F. Inhibition of LOX-1 by statins may relate to upregulation of eNOS. *Biochem Biophys Res Commun* 289: 857-61, 2001.
11. Nagase M, Hirose S, Sawamura T, Masaki T, Fujita T. Enhanced expression of endothelial oxidized low-density lipoprotein receptor (LOX-1) in hypertensive rats. *Biochem Biophys Res Commun* 237: 496-8, 1997.

12. Shin HK, Kim YK, Kim KY, Lee JH, Hong KW. Remnant lipoprotein particles induce apoptosis in endothelial cells by NAD(P)H oxidase-mediated production of superoxide and cytokines via lectin-like oxidized low-density lipoprotein receptor-1 activation: prevention by cilostazol. *Circulation* 109: 1022-8, 2004.
13. Hu B, Li D, Sawamura T, Mehta JL. Oxidized LDL through LOX-1 modulates LDL-receptor expression in human coronary artery endothelial cells. *Biochem Biophys Res Commun* 307: 1008-12, 2003.
14. Raitakari OT, Celermajer DS. Testing for endothelial dysfunction. *Ann Med* 32: 293-304, 2000.
15. Davignon J, Ganz P. Role of endothelial dysfunction in atherosclerosis. *Circulation* 109: III27-III32, 2004.
16. Yang Z, Ming XF. Recent advances in understanding endothelial dysfunction in atherosclerosis. *Clin Med Res* 4: 53-65, 2006.
17. Maeda S, Tanabe T, Otsuki T, Sugawara J, Iemitsu M, Miyauchi T, Kuno S, Ajisaka R, Matsuda M. Moderate regular exercise increases basal production of nitric oxide in elderly women. *Hypertens Res* 27: 947-53, 2004.
18. Cai H, Harrison DG. Endothelial dysfunction in cardiovascular diseases: the role of oxidant stress. *Circ Res* 87: 840-4, 2000.
19. Ma XL, Lopez BL, Liu GL, Christopher TA, Gao F, Guo Y, Feuerstein GZ, Ruffolo RR, Jr., Barone FC, Yue TL. Hypercholesterolemia impairs a detoxification mechanism against peroxynitrite and renders the vascular tissue more susceptible to oxidative injury. *Circ Res* 80: 894-901, 1997.
20. Leeuwenburgh C, Hardy MM, Hazen SL, Wagner P, Oh-ishi S, Steinbrecher UP, Heinecke JW. Reactive nitrogen intermediates promote low density lipoprotein oxidation in human atherosclerotic intima. *J Biol Chem* 272: 1433-6, 1997.
21. Itabe H. Oxidized low-density lipoproteins: what is understood and what remains to be clarified. *Biol Pharm Bull* 26: 1-9, 2003.
22. Shishehbor MH, Aviles RJ, Brennan ML, Fu X, Goormastic M, Pearce GL, Gokce N, Keaney JF, Jr., Penn MS, Sprecher DL, Vita JA, Hazen SL. Association of nitrotyrosine levels with cardiovascular disease and modulation by statin therapy. *JAMA* 289: 1675-80, 2003.
23. Havel RJ, Rapaport E. Management of primary hyperlipidemia. *N Engl J Med* 332: 1491-8, 1995.
24. Lusis AJ. Atherosclerosis. *Nature* 407: 233-41, 2000.

25. Dugi KA, Rader DJ. Lipoproteins and the endothelium: insights from clinical research. *Semin Thromb Hemost* 26: 513-9, 2000.
26. Laroia ST, Ganti AK, Laroia AT, Tendulkar KK. Endothelium and the lipid metabolism: the current understanding. *Int J Cardiol* 88: 1-9, 2003.
27. Murase T, Kume N, Kataoka H, Minami M, Sawamura T, Masaki T, Kita T. Identification of soluble forms of lectin-like oxidized LDL receptor-1. *Arterioscler Thromb Vasc Biol* 20: 715-20, 2000.
28. Hayashida K, Kume N, Murase T, Minami M, Nakagawa D, Inada T, Tanaka M, Ueda A, Kominami G, Kambara H, Kimura T, Kita T. Serum soluble lectin-like oxidized low-density lipoprotein receptor-1 levels are elevated in acute coronary syndrome: a novel marker for early diagnosis. *Circulation* 112: 812-8, 2005.
29. Aoyama T, Sawamura T, Furutani Y, Matsuoka R, Yoshida MC, Fujiwara H, Masaki T. Structure and chromosomal assignment of the human lectin-like oxidized low-density-lipoprotein receptor-1 (LOX-1) gene. *Biochem J* 339 (Pt 1): 177-84, 1999.
30. Welch CL, Bretschger S, Latib N, Bezouevski M, Guo Y, Pleskac N, Liang CP, Barlow C, Dansky H, Breslow JL, Tall AR. Localization of atherosclerosis susceptibility loci to chromosomes 4 and 6 using the Ldlr knockout mouse model. *Proc Natl Acad Sci U S A* 98: 7946-51, 2001.
31. Mango R, Clementi F, Borgiani P, Forleo GB, Federici M, Contino G, Giardina E, Garza L, Fahdi IE, Lauro R, Mehta JL, Novelli G, Romeo F. Association of single nucleotide polymorphisms in the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction. *J Med Genet* 40: 933-6, 2003.
32. Chen Q, Reis SE, Kammerer C, Craig WY, LaPierre SE, Zimmer EL, McNamara DM, Pauly DF, Sharaf B, Holubkov R, Bairey Merz CN, Sopko G, Bontempo F, Kamboh MI. Genetic variation in lectin-like oxidized low-density lipoprotein receptor 1 (LOX1) gene and the risk of coronary artery disease. *Circulation* 107: 3146-51, 2003.
33. Ohmori R, Momiyama Y, Nagano M, Taniguchi H, Egashira T, Yonemura A, Nakamura H, Kondo K, Ohsuzu F. An oxidized low-density lipoprotein receptor gene variant is inversely associated with the severity of coronary artery disease. *Clin Cardiol* 27: 641-4, 2004.
34. Mango R, Biocca S, del VF, Clementi F, Sangiuolo F, Amati F, Filareto A, Grelli S, Spitalieri P, Fiesi I, Favalli C, Lauro R, Mehta JL, Romeo F, Novelli G. In vivo and in vitro studies support that a new splicing isoform of OLR1 gene is protective against acute myocardial infarction. *Circ Res* 97: 152-8, 2005.

35. Tatsuguchi M, Furutani M, Hinagata J, Tanaka T, Furutani Y, Imamura S, Kawana M, Masaki T, Kasanuki H, Sawamura T, Matsuoka R. Oxidized LDL receptor gene (OLR1) is associated with the risk of myocardial infarction. *Biochem Biophys Res Commun* 303: 247-50, 2003.
36. Chen M, Inoue K, Narumiya S, Masaki T, Sawamura T. Requirements of basic amino acid residues within the lectin-like domain of LOX-1 for the binding of oxidized low-density lipoprotein. *FEBS Lett* 499: 215-9, 2001.
37. Trabetti E, Biscuola M, Cavallari U, Malerba G, Girelli D, Olivieri O, Martinelli N, Corrocher R, Pignatti PF. On the association of the oxidised LDL receptor 1 (OLR1) gene in patients with acute myocardial infarction or coronary artery disease. *Eur J Hum Genet* 14: 127-30, 2006.
38. Tuomisto TT, Binder BR, Yla-Herttuala S. Genetics, genomics and proteomics in atherosclerosis research. *Ann Med* 37: 323-32, 2005.
39. Mori TA, Beilin LJ. Omega-3 fatty acids and inflammation. *Curr Atheroscler Rep* 6: 461-7, 2004.
40. Upritchard JE, Zeelenberg MJ, Huizinga H, Verschuren PM, Trautwein EA. Modern fat technology: what is the potential for heart health? *Proc Nutr Soc* 64: 379-86, 2005.
41. Hu FB, Manson JE, Willett WC. Types of dietary fat and risk of coronary heart disease: a critical review. *J Am Coll Nutr* 20: 5-19, 2001.
42. Durstine JL, Grandjean PW, Cox CA, Thompson PD. Lipids, lipoproteins, and exercise. *J Cardiopulm Rehabil* 22: 385-98, 2002.
43. Green DJ, Maiorana A, O'Driscoll G, Taylor R. Effect of exercise training on endothelium-derived nitric oxide function in humans. *J Physiol* 561: 1-25, 2004.
44. Kojda G, Hambrecht R. Molecular mechanisms of vascular adaptations to exercise. Physical activity as an effective antioxidant therapy? *Cardiovasc Res* 67: 187-97, 2005.
45. Wolfarth B, Bray MS, Hagberg JM, Perusse L, Rauramaa R, Rivera MA, Roth SM, Rankinen T, Bouchard C. The human gene map for performance and health-related fitness phenotypes: the 2004 update. *Med Sci Sports Exerc* 37: 881-903, 2005.
46. Ye SQ, Kwiterovich PO, Jr. Influence of genetic polymorphisms on responsiveness to dietary fat and cholesterol. *Am J Clin Nutr* 72: 1275S-84S, 2000.

47. Maingrette F, Renier G. Linoleic acid increases lectin-like oxidized LDL receptor-1 (LOX-1) expression in human aortic endothelial cells. *Diabetes* 54: 1506-13, 2005.
48. Murase T, Kume N, Korenaga R, Ando J, Sawamura T, Masaki T, Kita T. Fluid shear stress transcriptionally induces lectin-like oxidized LDL receptor-1 in vascular endothelial cells. *Circ Res* 83: 328-33, 1998.
49. American College of Sports Medicine. ACSM's Guidelines for Exercise Testing and Prescription. 6th ed. Baltimore: Lippincott Williams & Wilkins, 2000.
50. Mazess RB, Barden HS, Bisek JP, Hanson J. Dual-energy x-ray absorptiometry for total-body and regional bone-mineral and soft-tissue composition. *Am J Clin Nutr* 51: 1106-12, 1990.
51. Nicklas BJ, Rogus EM, Colman EG, Goldberg AP. Visceral adiposity, increased adipocyte lipolysis, and metabolic dysfunction in obese postmenopausal women. *Am J Physiol* 270: E72-E78, 1996.
52. Seals DR, Hagberg JM, Hurley BF, Ehsani AA, Holloszy JO. Endurance training in older men and women. I. Cardiovascular responses to exercise. *J Appl Physiol* 57: 1024-9, 1984.
53. Corretti MC, Plotnick GD, Vogel RA. Technical aspects of evaluating brachial artery vasodilatation using high-frequency ultrasound. *Am J Physiol* 268: H1397-H1404, 1995.
54. Data SA, Roltsch MH, Hand B, Ferrell RE, Park JJ, Brown MD. eNOS T-786C genotype, physical activity, and peak forearm blood flow in females. *Med Sci Sports Exerc* 35: 1991-7, 2003.
55. Holvoet P, Harris TB, Tracy RP, Verhamme P, Newman AB, Rubin SM, Simonsick EM, Colbert LH, Kritchevsky SB. Association of high coronary heart disease risk status with circulating oxidized LDL in the well-functioning elderly: findings from the Health, Aging, and Body Composition study. *Arterioscler Thromb Vasc Biol* 23: 1444-8, 2003.
56. Allain CC, Poon LS, Chan CS, Richmond W, Fu PC. Enzymatic determination of total serum cholesterol. *Clin Chem* 20: 470-5, 1974.
57. Friedewald WT, Levy RI, Fredrickson DS. Estimation of the concentration of low-density lipoprotein cholesterol in plasma, without use of the preparative ultracentrifuge. *Clin Chem* 18: 499-502, 1972.
58. Sampson EJ, Demers LM, Krieg AF. Faster enzymatic procedure for serum triglycerides. *Clin Chem* 21: 1983-5, 1975.

59. Gidez LI, Miller GJ, Burstein M, Slagle S, Eder HA. Separation and quantitation of subclasses of human plasma high density lipoproteins by a simple precipitation procedure. *J Lipid Res* 23: 1206-23, 1982.
60. Warnick GR, Benderson J, Albers JJ. Dextran sulfate-Mg²⁺ precipitation procedure for quantitation of high-density-lipoprotein cholesterol. *Clin Chem* 28: 1379-88, 1982.
61. Wilund KR, Colvin PL, Phares D, Goldberg AP, Hagberg JM. The effect of endurance exercise training on plasma lipoprotein AI and lipoprotein AI:AII concentrations in sedentary adults. *Metabolism* 51: 1053-60, 2002.
62. Chen X, Levine L, Kwok PY. Fluorescence polarization in homogeneous nucleic acid analysis. *Genome Res* 9: 492-8, 1999.
63. Puccetti L, Pasqui AL, Pastorelli M, Ciani F, Palazzuoli A, Gioffre W, Auteri A, Bruni F. 3'UTR/T polymorphism of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) is associated with modified anti-platelet activity of atorvastatin in hypercholesterolemic subjects. *Atherosclerosis* 183: 322-8, 2005.
64. Li D, Yang B, Mehta JL. Ox-LDL induces apoptosis in human coronary artery endothelial cells: role of PKC, PTK, bcl-2, and Fas. *Am J Physiol* 275: H568-H576, 1998.
65. Yamanaka S, Zhang XY, Miura K, Kim S, Iwao H. The human gene encoding the lectin-type oxidized LDL receptor (OLR1) is a novel member of the natural killer gene complex with a unique expression profile. *Genomics* 54: 191-9, 1998.
66. Executive Summary of The Third Report of The National Cholesterol Education Program (NCEP) Expert Panel on Detection, Evaluation, And Treatment of High Blood Cholesterol In Adults (Adult Treatment Panel III). *JAMA* 285: 2486-97, 2001.
67. Krauss RM, Eckel RH, Howard B, Appel LJ, Daniels SR, Deckelbaum RJ, Erdman JW, Jr., Kris-Etherton P, Goldberg IJ, Kotchen TA, Lichtenstein AH, Mitch WE, Mullis R, Robinson K, Wylie-Rosett J, St JS, Suttie J, Tribble DL, Bazzarre TL. AHA Dietary Guidelines: revision 2000: A statement for healthcare professionals from the Nutrition Committee of the American Heart Association. *Circulation* 102: 2284-99, 2000.
68. Wijendran V, Hayes KC. Dietary n-6 and n-3 fatty acid balance and cardiovascular health. *Annu Rev Nutr* 24: 597-615, 2004.
69. Fuentes F, Lopez-Miranda J, Sanchez E, Sanchez F, Paez J, Paz-Rojas E, Marin C, Gomez P, Jimenez-Pereperez J, Ordovas JM, Perez-Jimenez F. Mediterranean and low-fat diets improve endothelial function in hypercholesterolemic men. *Ann Intern Med* 134: 1115-9, 2001.

70. Bayindir O, Ozmen D, Mutaf I, Turgan N, Habif S, Gultur C, Parildar Z, Uysal A. Comparison of the effects of dietary saturated, mono-, and n-6 polyunsaturated fatty acids on blood lipid profile, oxidant stress, prostanoid synthesis and aortic histology in rabbits. *Ann Nutr Metab* 46: 222-8, 2002.
71. Toborek M, Lee YW, Garrido R, Kaiser S, Hennig B. Unsaturated fatty acids selectively induce an inflammatory environment in human endothelial cells. *Am J Clin Nutr* 75: 119-25, 2002.
72. Lewis TV, Dart AM, Chin-Dusting JP, Kingwell BA. Exercise training increases basal nitric oxide production from the forearm in hypercholesterolemic patients. *Arterioscler Thromb Vasc Biol* 19: 2782-7, 1999.
73. Napoli C, Williams-Ignarro S, de NF, Lerman LO, Rossi L, Guarino C, Mansueto G, Di TF, Pignalosa O, De RG, Sica V, Ignarro LJ. Long-term combined beneficial effects of physical training and metabolic treatment on atherosclerosis in hypercholesterolemic mice. *Proc Natl Acad Sci U S A* 101: 8797-802, 2004.
74. Hambrecht R, Adams V, Erbs S, Linke A, Krankel N, Shu Y, Baither Y, Gielen S, Thiele H, Gummert JF, Mohr FW, Schuler G. Regular physical activity improves endothelial function in patients with coronary artery disease by increasing phosphorylation of endothelial nitric oxide synthase. *Circulation* 107: 3152-8, 2003.
75. Higashi Y, Yoshizumi M. Exercise and endothelial function: role of endothelium-derived nitric oxide and oxidative stress in healthy subjects and hypertensive patients. *Pharmacol Ther* 102: 87-96, 2004.
76. Hambrecht R, Wolf A, Gielen S, Linke A, Hofer J, Erbs S, Schoene N, Schuler G. Effect of exercise on coronary endothelial function in patients with coronary artery disease. *N Engl J Med* 342: 454-60, 2000.
77. Rush JW, Denniss SG, Graham DA. Vascular nitric oxide and oxidative stress: determinants of endothelial adaptations to cardiovascular disease and to physical activity. *Can J Appl Physiol* 30: 442-74, 2005.
78. Elosua R, Molina L, Fito M, Arquer A, Sanchez-Quesada JL, Covas MI, Ordonez-Llanos J, Marrugat J. Response of oxidative stress biomarkers to a 16-week aerobic physical activity program, and to acute physical activity, in healthy young men and women. *Atherosclerosis* 167: 327-34, 2003.
79. Goto C, Higashi Y, Kimura M, Noma K, Hara K, Nakagawa K, Kawamura M, Chayama K, Yoshizumi M, Nara I. Effect of different intensities of exercise on endothelium-dependent vasodilation in humans: role of endothelium-dependent nitric oxide and oxidative stress. *Circulation* 108: 530-5, 2003.

80. Fatouros IG, Jamurtas AZ, Villiotou V, Pouliopoulou S, Fotinakis P, Taxildaris K, Deliconstantinos G. Oxidative stress responses in older men during endurance training and detraining. *Med Sci Sports Exerc* 36: 2065-72, 2004.
81. Rush JW, Turk JR, Laughlin MH. Exercise training regulates SOD-1 and oxidative stress in porcine aortic endothelium. *Am J Physiol Heart Circ Physiol* 284: H1378-H1387, 2003.
82. Frost MT, Halliwell B, Moore KP. Analysis of free and protein-bound nitrotyrosine in human plasma by a gas chromatography/mass spectrometry method that avoids nitration artifacts. *Biochem J* 345 Pt 3: 453-8, 2000.
83. Kamisaki Y, Wada K, Nakamoto K, Kishimoto Y, Kitano M, Itoh T. Sensitive determination of nitrotyrosine in human plasma by isocratic high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 685: 343-7, 1996.
84. Wang XL, Rainwater DL, VandeBerg JF, Mitchell BD, Mahaney MC. Genetic contributions to plasma total antioxidant activity. *Arterioscler Thromb Vasc Biol* 21: 1190-5, 2001.
85. Fukai T, Siegfried MR, Ushio-Fukai M, Cheng Y, Kojda G, Harrison DG. Regulation of the vascular extracellular superoxide dismutase by nitric oxide and exercise training. *J Clin Invest* 105: 1631-9, 2000.
86. Meilhac O, Ramachandran S, Chiang K, Santanam N, Parthasarathy S. Role of arterial wall antioxidant defense in beneficial effects of exercise on atherosclerosis in mice. *Arterioscler Thromb Vasc Biol* 21: 1681-8, 2001.
87. Lauer N, Suvorava T, Ruther U, Jacob R, Meyer W, Harrison DG, Kojda G. Critical involvement of hydrogen peroxide in exercise-induced up-regulation of endothelial NO synthase. *Cardiovasc Res* 65: 254-62, 2005.
88. Malek AM, Alper SL, Izumo S. Hemodynamic shear stress and its role in atherosclerosis. *JAMA* 282: 2035-42, 1999.
89. Hein TW, Liao JC, Kuo L. oxLDL specifically impairs endothelium-dependent, NO-mediated dilation of coronary arterioles. *Am J Physiol Heart Circ Physiol* 278: H175-H183, 2000.
90. Fleming I, Mohamed A, Galle J, Turchanowa L, Brandes RP, Fisslthaler B, Busse R. Oxidized low-density lipoprotein increases superoxide production by endothelial nitric oxide synthase by inhibiting PKC α . *Cardiovasc Res* 65: 897-906, 2005.
91. Thomas SR, Chen K, Keaney JF, Jr. Oxidative stress and endothelial nitric oxide bioactivity. *Antioxid Redox Signal* 5: 181-94, 2003.

92. Jump DB. Fatty acid regulation of gene transcription. *Crit Rev Clin Lab Sci* 41: 41-78, 2004.
93. Lapillonne A, Clarke SD, Heird WC. Polyunsaturated fatty acids and gene expression. *Curr Opin Clin Nutr Metab Care* 7: 151-6, 2004.
94. Swagell CD, Henly DC, Morris CP. Expression analysis of a human hepatic cell line in response to palmitate. *Biochem Biophys Res Commun* 328: 432-41, 2005.
95. Wasserman SM, Mehraban F, Komuves LG, Yang RB, Tomlinson JE, Zhang Y, Spriggs F, Topper JN. Gene expression profile of human endothelial cells exposed to sustained fluid shear stress. *Physiol Genomics* 12: 13-23, 2002.
96. El-Sayed MS, Sale C, Jones PG, Chester M. Blood hemostasis in exercise and training. *Med Sci Sports Exerc* 32: 918-25, 2000.
97. Ishibashi Y, Takahashi N, Shimada T, Sugamori T, Sakane T, Umeno T, Hirano Y, Oyake N, Murakami Y. Short duration of reactive hyperemia in the forearm of subjects with multiple cardiovascular risk factors. *Circ J* 70: 115-23, 2006.
98. Tagawa T, Imaizumi T, Endo T, Shiramoto M, Harasawa Y, Takeshita A. Role of nitric oxide in reactive hyperemia in human forearm vessels. *Circulation* 90: 2285-90, 1994.
99. Wilkinson IB, Webb DJ. Venous occlusion plethysmography in cardiovascular research: methodology and clinical applications. *Br J Clin Pharmacol* 52: 631-46, 2001.
100. Sakurai K, Sawamura T. Stress and vascular responses: endothelial dysfunction via lectin-like oxidized low-density lipoprotein receptor-1: close relationships with oxidative stress. *J Pharmacol Sci* 91: 182-6, 2003.
101. Tedgui A, Mallat Z. Anti-inflammatory mechanisms in the vascular wall. *Circ Res* 88: 877-87, 2001.
102. Libby P, Aikawa M, Kinlay S, Selwyn A, Ganz P. Lipid lowering improves endothelial functions. *Int J Cardiol* 74 Suppl 1: S3-S10, 2000.
103. Calabresi L, Gomaschi M, Franceschini G. Endothelial protection by high-density lipoproteins: from bench to bedside. *Arterioscler Thromb Vasc Biol* 23: 1724-31, 2003.
104. Li H, Wallerath T, Munzel T, Forstermann U. Regulation of endothelial-type NO synthase expression in pathophysiology and in response to drugs. *Nitric Oxide* 7: 149-64, 2002.
105. Kiechle FL, Malinski T. Nitric oxide. Biochemistry, pathophysiology, and detection. *Am J Clin Pathol* 100: 567-75, 1993.

106. Dusse LM, Silva RM, Vieira LM, das Gracas CM. Does plasma nitrite determination by the Griess reaction reflect nitric oxide synthesis? *Clin Chim Acta* 362: 195-7, 2005.
107. Lauer T, Kleinbongard P, Kelm M. Indexes of NO bioavailability in human blood. *News Physiol Sci* 17: 251-5, 2002.
108. Baylis C, Vallance P. Measurement of nitrite and nitrate levels in plasma and urine--what does this measure tell us about the activity of the endogenous nitric oxide system? *Curr Opin Nephrol Hypertens* 7: 59-62, 1998.
109. Archer S. Measurement of nitric oxide in biological models. *FASEB J* 7: 349-60, 1993.
110. Tanaka S, Yashiro A, Nakashima Y, Nanri H, Ikeda M, Kuroiwa A. Plasma nitrite/nitrate level is inversely correlated with plasma low-density lipoprotein cholesterol level. *Clin Cardiol* 20: 361-5, 1997.
111. Yoon Y, Song J, Hong SH, Kim JQ. Plasma nitric oxide concentrations and nitric oxide synthase gene polymorphisms in coronary artery disease. *Clin Chem* 46: 1626-30, 2000.
112. Nedeljkovic ZS, Gokce N, Loscalzo J. Mechanisms of oxidative stress and vascular dysfunction. *Postgrad Med J* 79: 195-9, 2003.
113. Chen K, Keaney J. Reactive oxygen species-mediated signal transduction in the endothelium. *Endothelium* 11: 109-21, 2004.
114. Ji LL. Antioxidants and oxidative stress in exercise. *Proc Soc Exp Biol Med* 222: 283-92, 1999.
115. Sucu N, Unlu A, Tamer L, Aytacoglu B, Ercan B, Dikmengil M, Atik U. 3-Nitrotyrosine in atherosclerotic blood vessels. *Clin Chem Lab Med* 41: 23-5, 2003.
116. Fukuyama N, Takebayashi Y, Hida M, Ishida H, Ichimori K, Nakazawa H. Clinical evidence of peroxynitrite formation in chronic renal failure patients with septic shock. *Free Radic Biol Med* 22: 771-4, 1997.
117. Kaur H, Halliwell B. Evidence for nitric oxide-mediated oxidative damage in chronic inflammation. Nitrotyrosine in serum and synovial fluid from rheumatoid patients. *FEBS Lett* 350: 9-12, 1994.
118. ter Steege JC, Koster-Kamphuis L, van Straaten EA, Forget PP, Buurman WA. Nitrotyrosine in plasma of celiac disease patients as detected by a new sandwich ELISA. *Free Radic Biol Med* 25: 953-63, 1998.

119. Rader DJ, Dugi KA. The endothelium and lipoproteins: insights from recent cell biology and animal studies. *Semin Thromb Hemost* 26: 521-8, 2000.
120. Holvoet P, Vanhaecke J, Janssens S, Van de WF, Collen D. Oxidized LDL and malondialdehyde-modified LDL in patients with acute coronary syndromes and stable coronary artery disease. *Circulation* 98: 1487-94, 1998.
121. Hulthe J, Fagerberg B. Circulating oxidized LDL is associated with subclinical atherosclerosis development and inflammatory cytokines (AIR Study). *Arterioscler Thromb Vasc Biol* 22: 1162-7, 2002.
122. Nordin FG, Hedblad B, Berglund G, Nilsson J. Plasma oxidized LDL: a predictor for acute myocardial infarction? *J Intern Med* 253: 425-9, 2003.
123. Meisinger C, Baumert J, Khuseyinova N, Loewel H, Koenig W. Plasma oxidized low-density lipoprotein, a strong predictor for acute coronary heart disease events in apparently healthy, middle-aged men from the general population. *Circulation* 112: 651-7, 2005.
124. Dart AM, Chin-Dusting JP. Lipids and the endothelium. *Cardiovasc Res* 43: 308-22, 1999.
125. Ahmed SM, Clasen ME, Donnelly JE. Management of dyslipidemia in adults. *Am Fam Physician* 57: 2192-8, 1998.
126. Leon AS, Sanchez OA. Response of blood lipids to exercise training alone or combined with dietary intervention. *Med Sci Sports Exerc* 33: S502-S515, 2001.
127. Napoli C, Lerman LO. Involvement of oxidation-sensitive mechanisms in the cardiovascular effects of hypercholesterolemia. *Mayo Clin Proc* 76: 619-31, 2001.
128. Meyer JW, Schmitt ME. A central role for the endothelial NADPH oxidase in atherosclerosis. *FEBS Lett* 472: 1-4, 2000.
129. Steinberg D, Parthasarathy S, Carew TE, Khoo JC, Witztum JL. Beyond cholesterol. Modifications of low-density lipoprotein that increase its atherogenicity. *N Engl J Med* 320: 915-24, 1989.
130. Feron O, Dessy C, Moniotte S, Desager JP, Balligand JL. Hypercholesterolemia decreases nitric oxide production by promoting the interaction of caveolin and endothelial nitric oxide synthase. *J Clin Invest* 103: 897-905, 1999.
131. Landmesser U, Hornig B, Drexler H. Endothelial dysfunction in hypercholesterolemia: mechanisms, pathophysiological importance, and therapeutic interventions. *Semin Thromb Hemost* 26: 529-37, 2000.
132. Morgan J, Carey C, Lincoff A, Capuzzi D. High-density lipoprotein subfractions and risk of coronary artery disease. *Curr Atheroscler Rep* 6: 359-65, 2004.

133. Young CE, Karas RH, Kuvin JT. High-density lipoprotein cholesterol and coronary heart disease. *Cardiol Rev* 12: 107-19, 2004.
134. Rapp RJ. Hypertriglyceridemia: a review beyond low-density lipoprotein. *Cardiol Rev* 10: 163-72, 2002.
135. Benjamin EJ, Larson MG, Keyes MJ, Mitchell GF, Vasan RS, Keaney JF, Jr., Lehman BT, Fan S, Osypiuk E, Vita JA. Clinical correlates and heritability of flow-mediated dilation in the community: the Framingham Heart Study. *Circulation* 109: 613-9, 2004.
136. Wang XL, Mahaney MC, Sim AS, Wang J, Wang J, Blangero J, Almasy L, Badenhop RB, Wilcken DE. Genetic contribution of the endothelial constitutive nitric oxide synthase gene to plasma nitric oxide levels. *Arterioscler Thromb Vasc Biol* 17: 3147-53, 1997.
137. Lacy F, Kailasam MT, O'Connor DT, Schmid-Schonbein GW, Parmer RJ. Plasma hydrogen peroxide production in human essential hypertension: role of heredity, gender, and ethnicity. *Hypertension* 36: 878-84, 2000.
138. Kujala UM, Ahotupa M, Vasankari TJ, Kaprio J, Tikkanen MJ. Familial aggregation of LDL oxidation. *Scand J Clin Lab Invest* 57: 141-6, 1997.
139. Rossi GP, Cesari M, Zanchetta M, Colonna S, Maiolino G, Pedon L, Cavallin M, Maiolino P, Pessina AC. The T-786C endothelial nitric oxide synthase genotype is a novel risk factor for coronary artery disease in Caucasian patients of the GENICA study. *J Am Coll Cardiol* 41: 930-7, 2003.
140. Perusse L, Rice T, Despres JP, Bergeron J, Province MA, Gagnon J, Leon AS, Rao DC, Skinner JS, Wilmore JH, Bouchard C. Familial resemblance of plasma lipids, lipoproteins and postheparin lipoprotein and hepatic lipases in the HERITAGE Family Study. *Arterioscler Thromb Vasc Biol* 17: 3263-9, 1997.
141. Leeson CP, Hingorani AD, Mullen MJ, Jeerooburkhan N, Kattenhorn M, Cole TJ, Muller DP, Lucas A, Humphries SE, Deanfield JE. Glu298Asp endothelial nitric oxide synthase gene polymorphism interacts with environmental and dietary factors to influence endothelial function. *Circ Res* 90: 1153-8, 2002.
142. Pitsavos C, Panagiotakos D, Trichopoulou A, Chrysoshoou C, Dedoussis G, Chloptsios Y, Choumerianou D, Stefanadis C. Interaction between Mediterranean diet and methylenetetrahydrofolate reductase C677T mutation on oxidized low density lipoprotein concentrations: the ATTICA study. *Nutr Metab Cardiovasc Dis* 16: 91-9, 2006.
143. Chen M, Masaki T, Sawamura T. LOX-1, the receptor for oxidized low-density lipoprotein identified from endothelial cells: implications in endothelial dysfunction and atherosclerosis. *Pharmacol Ther* 95: 89-100, 2002.

144. Sawamura T, Kume N, Aoyama T, Moriwaki H, Hoshikawa H, Aiba Y, Tanaka T, Miwa S, Katsura Y, Kita T, Masaki T. An endothelial receptor for oxidized low-density lipoprotein. *Nature* 386: 73-7, 1997.
145. Xie Q, Matsunaga S, Niimi S, Ogawa S, Tokuyasu K, Sakakibara Y, Machida S. Human lectin-like oxidized low-density lipoprotein receptor-1 functions as a dimer in living cells. *DNA Cell Biol* 23: 111-7, 2004.
146. Kataoka H, Kume N, Minami M, Moriwaki H, Sawamura T, Masaki T, Kita T. Expression of lectin-like oxidized LDL receptor-1 in human atherosclerotic lesions. *Ann N Y Acad Sci* 902: 328-35, 2000.
147. Smirnova IV, Sawamura T, Goligorsky MS. Upregulation of lectin-like oxidized low-density lipoprotein receptor-1 (LOX-1) in endothelial cells by nitric oxide deficiency. *Am J Physiol Renal Physiol* 287: F25-F32, 2004.
148. Duryee MJ, Freeman TL, Willis MS, Hunter CD, Hamilton BC, III, Suzuki H, Tuma DJ, Klassen LW, Thiele GM. Scavenger receptors on sinusoidal liver endothelial cells are involved in the uptake of aldehyde-modified proteins. *Mol Pharmacol* 68: 1423-30, 2005.
149. Van Berkel TJ, De Rijke YB, Kruijt JK. Different fate in vivo of oxidatively modified low density lipoprotein and acetylated low density lipoprotein in rats. Recognition by various scavenger receptors on Kupffer and endothelial liver cells. *J Biol Chem* 266: 2282-9, 1991.
150. Nahmias Y, Casali M, Barbe L, Berthiaume F, Yarmush ML. Liver endothelial cells promote LDL-R expression and the uptake of HCV-like particles in primary rat and human hepatocytes. *Hepatology* 43: 257-65, 2006.
151. German JB, Dillard CJ. Saturated fats: what dietary intake? *Am J Clin Nutr* 80: 550-9, 2004.
152. Saraswathi V, Wu G, Toborek M, Hennig B. Linoleic acid-induced endothelial activation: role of calcium and peroxynitrite signaling. *J Lipid Res* 45: 794-804, 2004.
153. Kris-Etherton PM, Hecker KD, Binkoski AE. Polyunsaturated fatty acids and cardiovascular health. *Nutr Rev* 62: 414-26, 2004.
154. Keogh JB, Grieger JA, Noakes M, Clifton PM. Flow-mediated dilatation is impaired by a high-saturated fat diet but not by a high-carbohydrate diet. *Arterioscler Thromb Vasc Biol* 25: 1274-9, 2005.
155. Turpeinen AM, Basu S, Mutanen M. A high linoleic acid diet increases oxidative stress in vivo and affects nitric oxide metabolism in humans. *Prostaglandins Leukot Essent Fatty Acids* 59: 229-33, 1998.

156. Sato M, Shibata K, Nomura R, Kawamoto D, Nagamine R, Imaizumi K. Linoleic acid-rich fats reduce atherosclerosis development beyond its oxidative and inflammatory stress-increasing effect in apolipoprotein E-deficient mice in comparison with saturated fatty acid-rich fats. *Br J Nutr* 94: 896-901, 2005.
157. Diniz YS, Cicogna AC, Padovani CR, Santana LS, Faine LA, Novelli EL. Diets rich in saturated and polyunsaturated fatty acids: metabolic shifting and cardiac health. *Nutrition* 20: 230-4, 2004.
158. Oliveros LB, Videla AM, Gimenez MS. Effect of dietary fat saturation on lipid metabolism, arachidonic acid turnover and peritoneal macrophage oxidative stress in mice. *Braz J Med Biol Res* 37: 311-20, 2004.
159. Schwab US, Vogel S, Lammi-Keefe CJ, Ordovas JM, Schaefer EJ, Li Z, Ausman LM, Gualtieri L, Goldin BR, Furr HC, Lichtenstein AH. Varying dietary fat type of reduced-fat diets has little effect on the susceptibility of LDL to oxidative modification in moderately hypercholesterolemic subjects. *J Nutr* 128: 1703-9, 1998.
160. Yu-Poth S, Etherton TD, Reddy CC, Pearson TA, Reed R, Zhao G, Jonnalagadda S, Wan Y, Kris-Etherton PM. Lowering dietary saturated fat and total fat reduces the oxidative susceptibility of LDL in healthy men and women. *J Nutr* 130: 2228-37, 2000.
161. Miller ER, III, Erlinger TP, Sacks FM, Svetkey LP, Charleston J, Lin PH, Appel LJ. A dietary pattern that lowers oxidative stress increases antibodies to oxidized LDL: results from a randomized controlled feeding study. *Atherosclerosis* 183: 175-82, 2005.
162. Chen H, Li D, Sawamura T, Inoue K, Mehta JL. Upregulation of LOX-1 expression in aorta of hypercholesterolemic rabbits: modulation by losartan. *Biochem Biophys Res Commun* 276: 1100-4, 2000.
163. Smith JK. Exercise and atherogenesis. *Exerc Sport Sci Rev* 29: 49-53, 2001.
164. Kraus WE, Houmard JA, Duscha BD, Knetzger KJ, Wharton MB, McCartney JS, Bales CW, Henes S, Samsa GP, Otvos JD, Kulkarni KR, Slentz CA. Effects of the amount and intensity of exercise on plasma lipoproteins. *N Engl J Med* 347: 1483-92, 2002.
165. Rodriguez-Plaza LG, Alfieri AB, Cubeddu LX. Urinary excretion of nitric oxide metabolites in runners, sedentary individuals and patients with coronary artery disease: effects of 42 km marathon, 15 km race and a cardiac rehabilitation program. *J Cardiovasc Risk* 4: 367-72, 1997.
166. Edwards DG, Schofield RS, Lennon SL, Pierce GL, Nichols WW, Braith RW. Effect of exercise training on endothelial function in men with coronary artery disease. *Am J Cardiol* 93: 617-20, 2004.

167. Marin J, Rodriguez-Martinez MA. Role of vascular nitric oxide in physiological and pathological conditions. *Pharmacol Ther* 75: 111-34, 1997.
168. Booth FW, Chakravarthy MV, Spangenburg EE. Exercise and gene expression: physiological regulation of the human genome through physical activity. *J Physiol* 543: 399-411, 2002.
169. Richter B, Niessner A, Penka M, Grdic M, Steiner S, Strasser B, Ziegler S, Zorn G, Maurer G, Simeon-Rudolf V, Wojta J, Huber K. Endurance training reduces circulating asymmetric dimethylarginine and myeloperoxidase levels in persons at risk of coronary events. *Thromb Haemost* 94: 1306-11, 2005.
170. Laufs U, Wassmann S, Czech T, Munzel T, Eisenhauer M, Bohm M, Nickenig G. Physical inactivity increases oxidative stress, endothelial dysfunction, and atherosclerosis. *Arterioscler Thromb Vasc Biol* 25: 809-14, 2005.
171. Vasankari TJ, Kujala UM, Vasankari TM, Ahotupa M. Reduced oxidized LDL levels after a 10-month exercise program. *Med Sci Sports Exerc* 30: 1496-501, 1998.
172. Ziegler S, Schaller G, Mittermayer F, Pleiner J, Mihaly J, Niessner A, Richter B, Steiner-Boeker S, Penak M, Strasser B, Wolzt M. Exercise training improves low-density lipoprotein oxidability in untrained subjects with coronary artery disease. *Arch Phys Med Rehabil* 87: 265-9, 2006.
173. Sanchez-Quesada JL, Ortega H, Payes-Romero A, Serrat-Serrat J, Gonzalez-Sastre F, Lasuncion MA, Ordonez-Llanos J. LDL from aerobically-trained subjects shows higher resistance to oxidative modification than LDL from sedentary subjects. *Atherosclerosis* 132: 207-13, 1997.
174. Ookawara T, Haga S, Ha S, Oh-ishi S, Toshinai K, Kizaki T, Ji LL, Suzuki K, Ohno H. Effects of endurance training on three superoxide dismutase isoenzymes in human plasma. *Free Radic Res* 37: 713-9, 2003.
175. Halle M, Berg A, Konig D, Keul J, Baumstark MW. Differences in the concentration and composition of low-density lipoprotein subfraction particles between sedentary and trained hypercholesterolemic men. *Metabolism* 46: 186-91, 1997.
176. Williams PT, Krauss RM, Wood PD, Lindgren FT, Giotas C, Vranizan KM. Lipoprotein subfractions of runners and sedentary men. *Metabolism* 35: 45-52, 1986.