Lipophilic Compound-Mediated Gene Expression and Implications for Reactive Oxygen Species (ROS)-Related Diseases

A dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Environmental Sciences

by

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Abstract

Cardiovascular disease (CVD) is a major cause of death in developed countries, and most cardiovascular events are secondary to atherosclerosis. Atherosclerosis is viewed as a chronic inflammatory disease with underlying abnormality in redox-mediated signals in the vasculature. Excessive reactive oxygen species (ROS) generation is implicated in CVD. ROS play a role in oxidizing biomolecules, i.e., low-density lipoprotein (LDL), and in altering gene expression, i.e., proinflammatory and atherogenic gene expression. The antioxidant defense system consists of endogenous and exogenous antioxidants. It prevents ROS generation, subsequent oxidative chain reactions, and ROS-induced signaling/gene expression. Conjugated linoleic acid (CLA) isomers and vitamin E have shown antioxidant and anti-atherogenic effects in a limited number of studies. Recent studies have suggested that these two compounds are possible activators of peroxisome proliferator activated receptor gamma (PPARγ), a nuclear transcription factor. Synthetic PPARγ activators have exhibited anti-inflammatory and anti-atherogenic effects by inducing the antioxidant enzymes, Cu/Zn superoxide dismutase (SOD) and catalase. These results indicate that the link exists between these lipophilic compounds, expression of antioxidant enzymes, and PPARγ activation.

This research consists of three review papers and two experimental studies. The objectives of the review papers were to review the roles of ROS and the antioxidant defense system and the potentials of nutritional interventions, CLA isomers and vitamin E, for modulating ROS generation and ROS-related diseases, such as atherosclerosis. The objectives of the two studies were to examine the potentials of nutritional interventions:
the effects of the lipid soluble compounds, CLA isomers and α-tocopherol, on 1) gene regulation of the antioxidant enzymes, Cu/Zn SOD and catalase, and on 2) ROS generation in human umbilical vein endothelial cells.

In the CLA review papers, it was concluded that there are multiple factors to be considered for accurate extrapolation and interpretation of the human and animal studies of CLA isomers, such as inter-/intra-species-genetic differences, tissue specificities, age and health status, dose/concentration, isomer types and their purity, and duration of supplementation. With regard to vitamin E, the effects of vitamin E may be multifunctional: prooxidant and/or antioxidant, inhibitor of ROS-generating enzyme activities, and inducer and/or inhibitor of gene and protein expression, depending on microenvironments. We also conclude that the modulation of ROS-related diseases by CLA isomers and vitamin E may involve the control of redox status by regulating genes whose products influence ROS generation through redox-sensitive transcription factors, such as PPARγ and NF-κB.

The results of our experimental studies support the hypothesis that expression of the antioxidant enzymes is mediated by CLA isomers and α-tocopherol through PPARγ and/or NF-κB. The expression mediated by these compounds appears to be concentration-dependent. The expression of antioxidant enzymes was positively correlated with lipid peroxidation (indicator of ROS generation) exhibiting either stimulatory (prooxidant) or inhibitory (antioxidant) effects on atherogenesis. These results suggest that the amounts of supplemental CLA isomers and α-tocopherol should
be tailored and prescribed to elicit beneficial effects, *i.e.*, minimum ROS generation, at individual levels for preventive and therapeutic strategies.
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Chapter 1

Introduction
Introduction

Oxidative stress refers to the situation in which there is a significant imbalance between oxidants and antioxidants. Oxidants, in particular reactive oxygen species (ROS), lead to oxidative damage to biomolecules, including DNA, proteins, and lipids. The cellular damage has been associated with many diseases, such as cancer and cardiovascular disease. Cardiovascular disease (CVD) is a major cause of death in developed countries, and most cardiovascular events are secondary to atherosclerosis [1]. Atherosclerosis is viewed as a chronic inflammatory disease with an underlying abnormality in redox-mediated signals in the vasculature [2]. Previous animal studies have suggested that ROS have a role in atherosclerosis and other cardiovascular diseases. Major sources of oxidative stress in the vascular wall include: NADPH oxidases, nitric oxide synthases, myeloperoxidase, xanthine oxidase, lipoxygenase/cyclooxygenase, and the mitochondrial respiratory chain/ oxidative phosphorylation [3]. Many cell types, including macrophages/monocytes and vascular endothelial cells, are involved in atherogenesis and inflammation. Macrophages play a role in inflammation and early atherogenesis, in particular, the uptake of oxidized LDL via scavenger receptors leading to foam cell formation and further atherogenic processes. Endothelial cells also play a role in initiating the inflammatory response such as expression of adhesion molecules leading to recruitment of leukocytes. Atherosclerosis and inflammation are associated with ROS generation and NADPH oxidase induction in both macrophages and vascular endothelial cells. Phagocytic NADPH oxidase and endothelial NADPH oxidase are the major sources of ROS in the vasculature [3]. In the oxidation-modification hypothesis, atherogenesis is
thought to be initiated and promoted by oxidation of the LDL lipids, and oxidized LDL is then recognized by the macrophage scavenger receptor, SR-A, leading to foam cell, fatty streak, and plaque formation. Therefore, protection from oxidative damage is crucial to prevention of atherogenic processes.

Epidemiological studies have indicated an inverse relationship between dietary intakes of antioxidant vitamins, such as vitamins A, C, and E, and CVD. The antioxidant defense system consists of endogenous and exogenous antioxidants, and prevents oxidative damage in vivo. Endogenous antioxidants and antioxidant enzymes (i.e. glutathione, catalase, glutathione peroxidase, and superoxide dismutases) prevent ROS generation. Exogenous antioxidants (i.e. vitamin C, E, and beta-carotene) inhibit oxidative chain reactions, serving as radical scavengers [1].

Many studies have shown the role of nutrients, in particular lipid soluble compounds, in regulating gene expression. For example, vitamin A (retinoic acid) serves as a ligand for two different nuclear receptors, RARs and RXRs. The complex of vitamin D [1,25-(OH)2-D3 ] and the vitamin D receptor (VDR) also mediate gene expression by binding to vitamin D-responsive elements (VDREs). Recent studies have suggested that both vitamin E and conjugated linoleic acid (CLA) isomers might modulate gene expressions through peroxisome proliferator-activated receptor gamma (PPARγ) [4–7]. Peroxisome proliferator-activated receptors (PPARs) are a group of nuclear receptors and belong to the steroid hormone receptor superfamily. Three isoforms have been identified: PPARα, PPARδ (or β), and PPARγ. PPARs heterodimerize with the 9-cis retinoic acid receptor (RXR: retinoid X receptor) and bind to peroxisome proliferator response elements
(PPREs: 5’- AGGTCA\textsubscript{n}AGGTCA-3’) located in enhancer sites of regulated genes. PPARs are activated to induce target gene products by their ligands, such as fatty acids and their derivatives (e.g. eicosanoids, prostaglandins) and numerous structurally dissimilar xenobiotics. PPAR\textsubscript{γ} plays a crucial role in controlling insulin sensitization, adipogenesis, and inflammation. Synthetic PPAR\textsubscript{γ} ligands, such as thiazolidinediones (TZDs), have shown various effects: 1) adipocyte differentiation/ lipid metabolism; 2) anti-inflammation; 3) anti-diabetes; and 4) anti-atherosclerosis [8]. In addition, the genes for antioxidant enzymes, superoxide dismutase (SOD) and catalase, may be regulated through PPAR\textsubscript{γ} activation, because PPREs are located in the promoter regions of human Cu/Zn-superoxide dismutase (SOD1) and rat catalase [9-11]. Thus, a link is suggested between the expression of antioxidant enzymes through PPAR\textsubscript{γ} and these lipophilic nutrients, vitamin E and CLA isomers, which have exhibited antioxidant effects in previous atherosclerotic studies.

1) **Conjugated linoleic acids (CLAs)**

CLAs are positional- and stereo-isomers of linoleic acid (ω-6, 18:2). CLAs are found in ruminant food products. Although there are 28 different CLA isomers, the \textit{cis}-9, \textit{trans}-11 CLA isomer is predominantly found in foods and accounts for >90% of CLA intake in human diets. CLA isomers are naturally produced by bacterial hydrogenation and isomerization in the ruminant gut, or they are chemically synthesized by alkali isomerization of linoleic acid. Beneficial effects have been reported for cancer, atherosclerosis, and diabetes in animal models fed CLA isomer supplements. With regard to the effects of supplemental CLA isomers on atherosclerosis, CLA isomers suppress
lesion formation in atherosclerosis-prone apo-E knockout mice and hyperlipidemic rabbits, possibly modulating oxidative damage [7]. CLA isomers have been reported to be possible PPAR\(\gamma\) activators [7].

2) Vitamin E

Vitamin E is a lipid-soluble vitamin, and has eight naturally occurring isoforms, four tocopherols and four tocotrienols. Vitamin E is a well-known antioxidant (hydroxy group on C6) that scavenges free radicals and superoxide and protects polyunsaturated fatty acids from ROS in cell membranes. It has been thought that beneficial effects of vitamin E are attributed to its antioxidant capacity. Although epidemiological studies have indicated the protective effects of vitamin E against atherosclerosis, the results of clinical trials have been controversial. Recent studies have proposed that vitamin E may serve as a gene regulator, in non-antioxidant mechanisms [4, 5], possibly PPAR\(\gamma\)-dependant mechanisms.

This research consisted of three review papers and two experimental studies. The objectives of the review papers were to review the roles of ROS and the antioxidant defense system and the potentials of nutritional interventions, CLA isomers and vitamin E, for modulating ROS-related diseases, such as atherosclerosis. The objectives of the two studies were to examine the potentials of nutritional interventions: the effects of lipid soluble compounds, CLA isomers and \(\alpha\)-tocopherol, on 1) gene regulation of the
antioxidant enzymes, Cu/Zn SOD and catalase, and on 2) ROS generation in human umbilical vein endothelial cells.
References

Chapter 2  Part A

Review of Conjugated Linoleic Acid

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Conjugated Linoleic Acid Modulation of Risk Factors Associated with Atherosclerosis

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Abstract

Conjugated linoleic acid (CLA) has been the subject of extensive investigation regarding its possible benefits on a variety of human diseases. In some animal studies, CLA has been shown to have a beneficial effect on sclerotic lesions associated with atherosclerosis, be a possible anti-carcinogen, increase feed efficiency, and act as a lean body mass supplement. However, the results have been inconsistent, and the effects of CLA on atherogenesis appear to be dose-, isomer-, tissue-, and species-specific.

Similarly, CLA trials in humans have resulted in conflicting findings. Both the human and animal study results may be attributed to contrasting doses of CLA, isomers, the coexistence of other dietary fatty acids, length of study, and inter-and/or intra-species diversities. Recent research advances have suggested the importance of CLA isomers in modulating gene expression involved in oxidative damage, fatty acid metabolism, immune/inflammatory responses, and ultimately atherosclerosis. Although the possible mechanisms of action of CLA have been suggested, they have yet to be determined.
Conjugated linoleic acid

A group of trans-fatty acids, conjugated linoleic acid (CLA) has been purported to have diverse physiological functions and potential health benefits [1-6]. These unique geometric and positional isomers of octadecadienoic acid derived from linoleic acid (18:2n-6) have been found in only a limited number of foods or food products mostly derived from the fat of range animals. The highest levels of CLA are found in ruminant animals (beef, lamb and dairy cows) with beef, milk-fat, and cheese, the most common animal products containing CLA. During the biohydrogenation of linoleic acid to stearic acid, CLA is synthesized in the rumen as an intermediate by gram-negative bacteria, *Butyrivibrio fibrisolvens* [7]. CLA is also found in fish, monogastric animal products, and plant products, however, in much lower concentrations [3]. CLA isomers have been identified during the hydrogenation of fat, e.g., margarine production, and are found primarily in foods considered high in fat. Also, CLA is found in low concentrations in the lipids of human blood, tissue, and milk [8], presumably from dietary intakes. Although there are 28 different CLA isomers, the cis-9, trans-11 CLA isomer is predominantly found in the ruminant foods discussed earlier and accounts for >90% of CLA intake in the human diet [9]. The structures, shown in Figure 1, consist of 18 carbon atoms with two conjugated double bonds separated by a single bond, unlike linoleic acid, which is a non-conjugated diene [1]. The conjugated double bonds of CLA isomers contribute to their higher susceptibility to autooxidation than the non-conjugated bonds of linoleic acid [10]. Differences in chain length, degree of unsaturation, and position and stereoisomeric configuration of the double bonds affect fatty acid oxidation or lipid peroxidation.
Usually, long-chain fatty acids are oxidized more slowly and unsaturated fatty acids are oxidized more rapidly than are saturated fatty acids. Lauric acid is highly oxidized, but PUFAs and monounsaturated fatty acids are fairly well oxidized [11]. Oxidation of the long-chain, saturated fatty acids decreases with increasing carbon number.

Many research groups have looked at the possibility of CLA isomers as anti-carcinogens. Most anti-carcinogens are plant products (phytochemical), therefore, CLA isomers are unusual find because it occurs in the highest concentration in animal products (zoochemical) with only trace amounts found in plant lipids. The possibility of CLA isomers working as a feed efficiency supplement and a lean body mass supplement has also been examined, along with its role in cancer prevention and stimulation of the immune system.

With regard to potential health benefits, considerable attention has been given to anti-carcinogenic effects of CLA isomers; however, less attentions has been devoted toward its usefulness in preventing and reversing atherosclerosis and related diseases. The majority of research studies have been done using experimental animals and in vitro, with only recent investigations showing the effects of CLA isomers on humans. The purpose of this review is to assess and summarize current literature and knowledge on the possible health benefits of CLA isomers, particularly with respect to atherosclerosis as a chronic inflammatory disease.
Cardiovascular disease (CVD) is a major cause of death in developed countries, and most cardiovascular events are secondary to atherosclerosis [12]. CVD causes high medical costs and losses of productivity. The high incidence of CVD mortality and morbidity and the economic toll of CVD emphasize the need for prevention and management of CVD associated risk factors. Although the risks for CVD are multifactorial, the three most important modifiable risk factors for atherosclerosis are: 1) smoking, 2) inactive lifestyle, and 3) elevated blood cholesterol levels from dietary sources. Of particular concern for the elevated blood cholesterol is increased low-density lipoprotein (LDL). Results of extensive epidemiological and clinical research support the direct association between elevated blood cholesterol and CVD risk.

Atherosclerosis is a condition characterized by degeneration and hardening of the walls of the arteries and sometimes the valves of the heart. There is accumulation of lipids and other materials in the arteries which contributes to hypertension and vice versa. Figure 2 schematically illustrates the major points of oxidized LDL in the process of plaque formation. The process of atherosclerosis begins with buildup of soft fatty streaks along the inner arterial walls often at branch points. With age, fatty streaks steadily grow and become hardened with minerals, leading to plaque. Consequently, plaque stiffens and narrows the artery lumen. By middle age, most people have well-recognized plaque formation [13]. Blood platelets respond to plaque as if it was a blood vessel injury produce clots which unlike the normal blood clotting events, do not readily dissolve and instead stick to the plaque, grow and restrict blood flow, i.e., thrombosis. Platelet activity
is under the control of eicosanoids synthesized from 20-carbon omega-6 and omega-3 fatty acids, such as prostaglandins and thromboxanes. Complication may occur when blood clots break free from the walls of arteries and make their way to a smaller artery, and shut off the blood supply to tissue; this produces an embolism.

LDL oxidation is thought to be the first step of atherogenesis, followed by foam cell, fatty streak, and plaque formation. It has been hypothesized that LDL can be transported through endothelial tight junctions and/or endothelial transcytosis from the lumen into the intima [14], in which blood antioxidants are unlikely to be available, and undergo atherogenic oxidative changes. Modified LDL is then taken up by macrophages through multiple pathways. Minimally oxidized LDL (MM-LDL) is recognized by CD14 and toll-like receptor-4 (TLR4) [15], while oxidized LDL binds to scavenger receptors (e.g., CD36, CD68, SR-A1, SR-B1). Aggregated forms of either MM-LDL or native LDL are endocytosed by activated macrophages [16].

Both oxidized LDL and activated macrophages by oxidized LDL uptake affect gene expression in neighboring endothelial cells (ECs), contributing to further atherogenic/inflammatory processes. Studies have documented that the oxidized LDL affect the pattern of gene expression in ECs, leading to up-regulated expression of target molecules. The oxidized LDL-induced molecules in ECs include monocyte chemoattractant proteins (MCPs), macrophage colony stimulating factors (M-CSFs), and cell adhesion molecules (CAMs) [17,18]. MCPs and M-CSFs are induced by MM-LDL, and are released from ECs. MCPs recruit monocytes to the ECs. M-CSFs promote the differentiation and proliferation of monocytes to macrophages (Figure 2). CAMs, cell
surface proteins, are involved in binding with other cells or the extracellular matrix. These molecules contribute to foam cell formation by the recruitment of circulatory monocytes into vascular walls and by the stimulation of monocyte differentiation to macrophages.

The differences in genetic susceptibility to atherosclerosis were investigated using animal and human models [19-22]. ECs from the atherosclerotic prone strain of C57BL/6J mice exhibited dramatic induction of MCP-1 and M-CSF in response to MM-LDL, while ECs from the atherosclerotic resistant strain of C3H/HeJ mice showed little or no induction of MCP-1 or M-CSF. Shi et al. [19] provide the evidence that genetic factors influencing the endothelial response to oxidized LDL contribute to the genetic component in atherosclerosis. Levula et al.’s [20] microarray study reveals the groups of target genes whose expressions are altered by oxidized LDL in human macrophages. The target genes are involved in 1) lipid metabolism, 2) inflammation, growth, and hemostasis, 3) metalloproteinases and tissue inhibitors of matrix metalloproteinases, 4) enzymes, 5) structural and binding proteins, and 6) annexins. The genes involved in inflammation include M-CSF1, MCP1, and ICAM1, all of which are induced in the macrophages by oxidized LDL, and correspond to the results of previous Shi et al.’s EC studies.

Induced expression of CD68 and SR in human macrophages by oxidized LDL was also observed in Levula et al.'s microarray study. In addition, activated macrophages secrete inflammatory cytokines, such as TNF-α, that contribute to the induction of the expression of MCP-1, M-CSF, ICAM1, and VCAM1 in human aortic endothelial cells (HAECs) [21] and the expression of ICAM1 and VCAM1 in human neonatal dermal lymphatic
endothelial cells (HNDLECs) [22] and the development of atherosclerosis. Thus, the pro-
inflammatory gene expression in ECs is mediated by either oxidized LDL or pro-
inflammatory cytokines released from activated macrophages, resulting in augmented 
atherogenic/inflammatory events by recruiting circulatory monocytes.

Moreover, oxidized LDL may modulate the apoptosis of vascular cells. Reeve et al's [23] 
microarray study demonstrated that 221 genes were differentially regulated by oxidized 
LDL in coronary artery smooth muscle cells (CASMC). Of particular interest are 
apoptotic genes, FasL, Bax, and p53, induced by oxidized LDL in CASMC. Oxidized 
LDL induces apoptosis of ECs via the mitogen-activated protein kinase (MAPK) 
pathway [24]. Studies using EC and smooth muscle cell cultures demonstrated that 
multiple apoptotic signaling pathways were affected by ROS [25].

**Roles of ROS in atherogenesis**

Reactive oxygen species (ROS) are implicated in atherogenesis. Risk factors for 
atherosclerosis are associated with an increased arterial wall flux of ROS that not only 
may oxidize biomolecules, but also directly produce phenotypic changes in vascular 
cells, including the induction of adhesion molecules and smooth muscle proliferation 
[26].

**Sources of ROS**

Sources of ROS involved in atherogenesis include NADPH oxidases, nitric oxide 
synthases (NOS), lipoxigenases (LO), cyclooxygenases (COX), and the mitochondrial
respiratory chain [27]. Native LDL is modified by ROS generated by these enzymes in vascular tissues. NADPH oxidase is composed of a number of different subunits. There are seven homologues of the gp91phox (Nox-2) subunit. Nox-4 is predominantly expressed in ECs, though the expressions of Nox-1 and Nox-2 are also detected [28]. During the respiratory burst in phagocytes, NADPH oxidase converts oxygen molecules to superoxide, which is a microbicidal oxygen metabolite. Then, superoxide is converted by superoxide dismutase (SOD) to hydrogen peroxide, which also kills microorganisms. ROS are also produced in ECs by endothelial NADPH oxidase [26,27]. Phagocytic NADPH oxidase and endothelial NADPH oxidase is one of the major ROS sources in the vasculature [27]. Monocyte differentiation to macrophage is associated with the production and the release of ROS possibly through the induction of NADPH oxidase, resulting in further LDL oxidation [29,30]. NADPH oxidase generates superoxide on the extracellular side of the plasma membrane, and the enzyme can trigger intracellular signaling by superoxide transport via chloride channel-3 [31]. Stepp et al. [32] reported that native LDL and MM-LDL differentially increase vascular endothelial superoxide generation in canine carotid arteries, leading to vascular dysfunction and atherogenesis. Native LDL increases superoxide by an endothelial nitric oxide synthase (eNOS)-dependent mechanism whereas MM-LDL induces greater superoxide by the mechanisms dependent on eNOS, xanthine oxidase, and NADPH oxidase. Superoxide production by vascular tissues and its interaction with nitric oxide (NO) play important roles in vascular pathophysiology. Superoxide reacts rapidly with NO, reducing NO bioavailability and producing the oxidative peroxynitrite radical [33]. Endothelial activation via LOX-1 produces additional ROS, generating a positive feedback loop for further LDL oxidation.
ROS generated in a NADPH oxidase-dependent pathway mediate TNF-α-induced MCP-1 expression in ECs, and the induction of MCP-1 expression is suppressed by the antioxidant enzymes, SOD and catalase [35]. Since phagocytic NADPH oxidase is the first line of the host defense system, selective suppression of vascular NADPH oxidase in local inflammatory lesions might be one of the therapeutic strategies [36].

Cyclooxygenases (COX-1 or COX-2) and lipoxygenases (5-, 12-, or 15-LO) also contribute to ROS generation during arachidonic acid (AA) metabolism shown in Figure 3[27,37]. The initial products in AA metabolism are highly reactive peroxides. Overexpression or induction of COX-2 increases ROS in certain cell types [38,39] and the effects of overexpression of COX-2 are cell-/tissue-specific [40]. Constitutive COX-1 and inducible COX-2 catalyze the conversion of free PUFA to prostanoids (prostaglandins and thromboxanes), while LO generates the leukotrienes. Prostanoids and leukotrienes comprise a large and complex family of biologically active lipids derived from PUFA by insertion of molecular oxygen. Collectively, these compounds are termed eicosanoids. Both prostanoids and leukotrienes play important roles in inflammation.

Non-esterified PUFA released from the sn-2 position (middle carbon of glycerol) of the membrane phospholipids by the action of specific phospholipases are substrates for COX, LO, or cytochrome P450 monooxygenases (CYP) [41,42]. The metabolism of AA has two main pathways: the cyclic pathway leading to prostanoid formation and the linear pathway resulting in leukotriene formation. Two molecules of oxygen are added in the first step for the generation of prostaglandin G (PGG). The second step, via the peroxidase activity of COX, converts PGG2 into PGH2, the precursor for either
thromboxanes or other prostaglandins. The peroxidase step is inhibited by aspirin or ibuprofen. LO is also a major source of extracellular superoxide release in a certain cell type during AA metabolism [37]. The LO pathway is responsible for the formation of leukotrienes and hydroxyeicosatetraenoic acids (HETEs). LO isoforms act upon arachidonic acid to form 5-, 8-,12-, or 15-hydroperoxy eicosatetraenoic acids (HPETEs), which are unstable and can be reduced to the hydroxyl derivatives (HETE) \textit{in vivo}. The range of HPETEs with biological activity is known as the leukotrienes. Both COX and LO products diffuse from cells and act locally at nanomolar levels on cell surface receptors linked to G-proteins. Activation of G-protein-associated receptors leads to changes in intracellular cAMP or calcium, which serve as second messengers that activate signaling mechanisms influencing various cellular functions. The COX products are modulators of thromboregulatory and chemotaxic responses, and inflammation. The LO products are involved in vascular permeability, vasoconstriction, and bronchoconstriction. The third route for eicosanoid production is via the CYP, in particular CYP4 family [43], including epoxy derivatives of 20:4 ω-6 that can modulate calcium signaling, channel activity, transporter function, and mitosis. This mechanism seems to be more consequential in cells when COX and LO activities are minimal.

This body of research has verified that multiple enzymes are involved in ROS generation that leads to atherogenesis. Hence, it is speculated that suppressing ROS generation may be a therapeutic target for preventing and alleviating atherosclerosis.
**Oxidation of biomolecules**

ROS play a role in LDL oxidation. LDLs are rich in polyunsaturated fatty acids (PUFAs), which are susceptible to oxidation [12]. Lipid peroxidation can commence by ROS and other mechanisms that result in abstraction of an electron from a PUFA [44]. The sequential carbon-centered radical undergoes rearrangement, and in the presence of oxygen, will add oxygen to form a peroxyl radical (ROO•). Propagation of the free radical reaction can occur by reaction of the peroxyl radical with another PUFA, generating the corresponding fatty acid hydroperoxide (ROOH) and another carbon-centered radical. Other factors, such as Fe^{2+}, and other oxidants can result in an amplification of the free radical process. Vitamin E is a nonenzymatic chain-breaking scavenger of lipid radicals generated in cell membranes; it protects against further lipid peroxidation. Vitamin C is an important antioxidant against lipid peroxidation because it has a high reactivity with the oxygen-centered radical. Oxygen-centered radicals have sufficient polarity to be accessible to the aqueous soluble vitamin C. Also, when vitamin E reacts with a radical, vitamin E is converted to its radical form which can be recycled to reduced vitamin E by reacting with vitamin C. Other reducing compounds such as glutathione and NADPH act in concert with vitamins E and C in an antioxidant cascade.

LDL oxidation also results in changes in apolipoprotein B epitope. The oxidized apolipoprotein portion of LDL is subsequently recognized and internalized by SR-A, whereas the oxidized lipid moiety of LDL is bound to CD36 on macrophages [45].
Inflammation and fatty acids/other protective compounds

**Fatty acids**

The relationship between fatty acids and atherosclerosis and other inflammatory diseases has been suggested by epidemiological, clinical, and in-/ex-vivo studies. Increased intake of saturated fatty acids is positively associated with development of atherosclerosis and inflammation. In contrast, omega-3 (ω-3) fatty acids, such as eicosapentaenoic acid (EPA, C20:5) and docosahexaenoic acid (DHA, C22:6), have shown protective effects against CVD. EPA and DHA are major components of dietary fish and fish oils. Like EPA and DHA, CLA isomers, exhibit protective effects against atherogenesis [46,47] and inflammatory bowel disease (IBD) [5,48,49] and antioxidant effects [50] in in-/ex-vivo studies; however, clinical studies have been inconclusive.

Most of the fatty acids synthesized or ingested have one of two fates: incorporation into triglycerides for the storage of metabolic energy or incorporation into the phospholipid components of membranes. The selection between the alternative fates depends on the need (i.e. growth and starvation) [42]. Fatty acids may differentially affect inflammatory processes and ultimately the etiology of atherosclerosis in three ways as:

1) the components of membrane; fatty acids may serve as precursors of pro- or anti-inflammatory eicosanoids;

2) the components of LDL; fatty acids may differentially modulate recognition of macrophage receptors and subsequent inflammatory processes and atherogenesis; and
3) the regulators of gene expression; fatty acids may differentially regulate inflammatory gene expression, serving as ligands for transcription factors (e.g. PPARs).

Almost all mammalian cells, except red blood cells, produce eicosanoids, which play a role in inflammation. Arachidonic acid (AA, C22:4) is the most important precursor of eicosanoid, and AA is synthesized from linoleic acid (LA, C18:2, \( \omega-6 \)) by elongation and desaturation. In response to hormonal or other stimuli, phospholipase A2, present in most types of mammalian cells, attacks membrane phospholipids, releasing AA from the middle carbon of glycerol. Enzymes of the smooth endoplasmic reticulum then convert AA into eicosanoids, potent biological signaling molecules [42].

LA (18:2, \( \omega-6 \)) and \( \alpha \)-linolenic acid (ALA, 18:3, \( \omega-3 \)) serve as the precursors for longer-chain \( \omega-6 \) (e.g., arachidonic acid: AA) and \( \omega-3 \) fatty acids (e.g., EPA, DHA), respectively. Neither \( \omega-3 \) nor \( \omega-6 \) fatty acids can be synthesized in mammals due to the lack of certain types of desaturases. \( \omega-3 \) Fatty acids cannot be generated from \( \omega-6 \) fatty acids in mammals. Hence, the source of these PUFAs is limited to dietary intake [51]. Dietary EPA, DHA, and CLA can partially replace AA derived from LA in the cell membrane [52,53]. Usually the plentiful LA may exclude these fatty acids from incorporation into membrane phospholipids [54] and/or LDL. However, EPA, DHA and CLA may influence eicosanoid production from AA and subsequent immune and inflammatory processes. For example EPA and DHA decrease the synthesis of pro-inflammatory eicosanoids, such as leukotriene-4 and prostaglandin-2 by replacing AA in phospholipid bilayers and by inhibiting cyclooxygenase activity [55-57]. As components of LDL and/or membrane, these fatty acids may affect the inflammatory gene expression.
by altering signaling pathways. The unusual conformation structures (kinks) in unsaturated fatty acids interfere with the membrane motion [42] and possibly signal transduction. In addition, EPA and DHA reduce the expression of adhesion molecules induced by oxidized LDL in endothelial cells [58-60]. ω-3 PUFAs suppress inflammatory gene expression by inhibiting TLR4 signaling pathway, whereas saturated fatty acids exhibit the opposite effect [61], Thus, subsequently decreasing risk for CVDs.

**Phytochemicals/dietary antioxidants**

Several epidemiological studies have reported an inverse relationship between intake of vegetables and fruits (in particular those rich in antioxidant vitamins including vitamins C and E and β-carotene), and risk for CVD [62-65]. The protective effects of these antioxidant vitamins on atherosclerosis have been intensively investigated in animal and human studies. According to the oxidative modification hypothesis, oxidized LDL is immunogenic and atherogenic and LDL oxidation triggers atherosclerotic processes. Therefore, the protection of LDL from oxidation may be crucial to the prevention of atherosclerosis; the antioxidant components of LDL may prevent LDL oxidation.

Vitamin E is the generic term for all tocopherol and tocotrienol derivatives that exhibit the biological activity of α-tocopherol. There are eight naturally occurring isoforms synthesized in plants. α-Tocopherol is the most biologically and chemically active form of vitamin E. The hydroxyl groups at the C-6 position of tocopherols enable them to scavenge free radicals and superoxide. Although γ-tocopherol is predominant in the American diet, its plasma levels are only 10% of plasma α-tocopherol levels (about 25 μmol/L α-tocopherol) [66].
α-Tocopherol is the major antioxidant in LDL and one LDL particle contains approximately six molecules of α-tocopherol. α-Tocopherol in LDL plays a role in preventing LDL oxidation. Vitamin E depletion in LDL may trigger LDL oxidation; and the addition of micromolar concentrations of vitamin E inhibits LDL oxidation. All other antioxidants, such as γ-tocopherol, carotinoids, and ubiquinol-10, are present in much smaller amounts than α-tocopherol. In contrast to α-tocopherol, carotenoids play only a minor or no role in LDL protection [67]. However, many clinical studies have failed to demonstrate the protective effects of vitamin E. One explanation may be that vitamin E exhibits prooxidant activity in the absence of co-antioxidant compounds capable of reducing the tocopherol radical [68,69]. A similar situation may occur with other antioxidants, such as β-carotene [70-72]. Depending on the concentrations, environmental conditions and presence of oxygen or other oxidants, compounds with antioxidant properties may exhibit prooxidant or other non-antioxidant properties.

Polyphenolic compounds, such as resveratrol and catechins, are derived from plants, and the compounds have shown anti-atherogenic and anti-inflammatory effects. The beneficial effects of the compounds are attributed to their abilities to function as antioxidants by: 1) inhibition of prooxidant enzymes, such as lipoxygenases, cyclooxygenases, and xanthine oxidase, possibly through suppressing the activation of redox-sensitive transcription factors, NF-κB and activator protein-1 (AP-1), and 2) induction of antioxidant enzymes such as glutathione S-transferase, glutathione peroxidase (Gpx), superoxide dismutase (SOD), and catalase [73,74].
**Endogenous/enzymatic antioxidants**

Antioxidant enzymes are involved in the maintenance of intracellular and extracellular reducing reactions [75] and suppress the generation of free radicals as the first line of antioxidant defense [12]. Antioxidant enzymes include superoxide dismutase (SOD) and catalase. SOD is expressed in most cell types, and converts harmful superoxide to less harmful hydrogen peroxide and oxygen. Catalase catalyzes the dismutation of hydrogen peroxide to oxygen and water. Catalase has an iron redox center. Catalase is located predominantly within peroxisomes to protect from hydrogen peroxide generated during fatty acid β-oxidation within the cellular organelles [76].

The antioxidant enzymes play a role in preventing atherogenesis. Increased expression of GR in macrophages reduces atherosclerotic lesion formation in LDL receptor-deficient mice [77]. Over-expression and/or induction of CuZn-SOD and catalase can be beneficial because of: 1) decreases in superoxide levels in ECs; 2) suppression of oxidative stress, e.g., age related; 3) protection against inflammatory events by inhibiting NF-κB activation; and 4) suppression of low-density lipoprotein (LDL) oxidation by ECs [50,76,78-81].

**Target genes/signaling pathway**

Atherosclerosis is a chronic inflammatory disease with an underlying abnormality in redox-mediated signals in the vasculature [82]. ROS play a role in the signaling involved in atherogenic/inflammatory processes. There are two major redox-sensitive signaling
pathways related to the atherogenic/inflammatory processes: NF-κB-, and peroxisome proliferator-activated receptor (PPAR)-mediated pathways. Fatty acids may act as gene regulators. CLA isomers are ligands with high to moderate affinity and activators of PPAR α and γ. CLA isomers may induce responsive genes of both PPAR α and γ in vivo [6]. The trans-10, cis-12 CLA isomer inhibits the NF-κB p50 and p65 subunits binding to DNA [83]. Also, CLA isomers may involve the control of redox status by regulating genes, whose products influence ROS generation, through transcription factors (PPARγ and NF-κB), which are concentration-dependent [84].

**NF-κB**

NF-κB is a redox-sensitive transcription factor expressed in all cell types; it recognizes and binds to specific DNA sequences (5'-GGGRNNYYCC-3'). NF-κB activation is triggered by the IkB kinase (IKK)-mediated degradation of inhibitor κB (IkB), which regulates NF-κB. NF-κB is activated by intra-/extra-cellular ROS and/or ROS-modified target biomolecules, and is involved in regulating immune and inflammatory responses. NF-κB-mediated target genes include: inflammatory cytokines (*e.g.*, TNF-a, IL-1, IL-2, M-CSF), chemokines (*e.g.*, MCP-1), adhesion molecules (*e.g.*, ICAM-1, VCAM-1), inflammatory enzymes (*e.g.*, iNOS, COX-2), and apoptotic regulators (*e.g.*, Fas ligand, Fas, p53) [85].

Oxidized LDL may affect atherogenesis in part via the NF-κB activation pathway.

Oxidized LDL activates NF-κB as well as *C. pneumoniae* [86]. Resveratrol, an antioxidant polyphenol derived from plants, attenuates TNF-α-induced inflammatory gene expression and monocyte adhesion to human coronary arterial endothelial cells
(HCAECs) by inhibiting NF-κB activation, suggesting that the anti-inflammatory actions of resveratrol are responsible for anti-atherogenic effects [78]. Oxidized LDL exerts biphasic effects on NF-κB: 1) inflammatory effects by up-regulating inflammatory gene expression via NF-κB activation at lower concentrations of oxidized LDL; and 2) immunosuppressive effects by inhibiting NF-κB activation triggered by inflammatory agents such as lipopolysaccharide (LPS) at higher concentrations of oxidized LDL [85].

HUVECs incubated with LPS which causes inflammatory gene expression via TLR4 activation, induce the expressions of TLR4, LOX-1, ICAM-1, and E-selectin, and increase monocyte adhesion to endothelium and NF-κB activation levels, suggesting the atherogenic process is mediated through TLR4/NF-κB pathways [87]. There are two types of TLR4/NF-κB pathways identified: MyD88-dependent and independent pathways. MyD88 is a common downstream adaptor molecule for most TLRs, and recruits other molecules required to activate NF-κB. Saturated fatty acids trigger TLR4 and downstream NF-κB activations, resulting in inflammatory gene expression (i.e. COX2 or iNOS). In contrast, unsaturated fatty acids inhibit TLR4/NF-κB activation. This inhibition may be due to the alteration of fatty acid components in membrane lipid rafts, which may lead to the disruption of the recruitment of the downstream signaling components [61].

**PPARs**

PPARs (PPARα, PPARβ, and PPARγ), a group of nuclear receptors, belong to the steroid hormone receptor superfamily [88]. PPARs heterodimerize with the 9-cis retinoic acid receptor (RXR) and bind to peroxisome proliferator response elements (PPREs: 5'-
AGGTCAnAGGTCA-3’) which are located in enhancer sites of target genes (Figure 4). PPARα and PPARγ are expressed in vascular endothelial cells and smooth muscle cells as well as adipose tissues [79,89]. PPARα and PPARγ play a role in inflammation, adipogenesis, and insulin sensitization. Thiazolidinediones (TZDs), a group of synthetic PPARγ ligands, have shown beneficial effects as atheroprotective drugs. 15-Deoxy-
Δ^{12,14}-prostaglandin J2 (15d-PGJ2), an prostanoid, is a natural ligand and an activator for PPARγ. 15-LOX products, 9- and 13-hydroxy-octadecadienoic acids (HODEs), are also PPARγ activators [89]. Therefore, these eicosanoids, 15d-PGJ2 and HODEs, support the implication of PPARγ in inflammation.

PPARγ activation may be involved in oxidized LDL-induced inflammatory gene expression and macrophage lipid metabolism [45,90,91]. Although CD36 is up-regulated by oxidized LDL via PPARγ activation, PPARγ activation suppresses oxidized LDL-induced inflammatory effects by inhibiting inflammatory gene expression. Kunsch and Medforld [82] suggest that PPARγ participates in a positive feedback loop and that alternative or downstream pathways may trigger PPARγ activation resulting in anti-inflammatory effects. For example, 15d-PGJ2 is known to be an endogenous PPARγ activator. 15d-PGJ2 may be a possible anti-inflammatory mediator, though the physiological levels of 15d-PGJ2 may be insufficient to modulate PPARγ activation [92]. PPAR activators are negative regulators of macrophage activation and antagonize the activities of the transcription factors, AP-1, STAT, and NF-κB, involved in inflammatory gene expression [93,94].
Lipoprotein lipase (LPL), a lipolytic enzyme, may play an important role in regulating early atherogenesis. LPL neither acts on nor binds to oxidized LDL. LDL(-) is a form of native LDL containing intermediately modified subfractions with higher electronegative charge and is taken up by LDL receptors. Lipid peroxidation is greater in LDL(-) than in native LDL; LDL(-) exhibits inflammatory effects. Ziouzenkova et al. [95] demonstrated that LPL-treated LDL(-) reduced inflammatory gene expression in human ECs by suppressing NF-κB and AP-1 activations and by increasing the expression of IκB, a target gene for PPARα, via PPARα activation. In contrast, LDL(-) alone increased the inflammatory responses. 9- and 13-HODEs, both known as PPARγ activators, are released during the hydrolysis of both native LDL and LDL(-), resulting in PPARα activation and anti-inflammatory effects.

Expression of antioxidant enzymes, Cu/Zn SOD and catalase, may be modulated through both PPARγ and NF-κB activations. Possible multiple binding sites for PPARγ and NF-κB have been identified: 1) within the promoter region of SOD1, and 2) one binding site for NF-κB within the promoter region of catalase. NF-κB activation is associated with the induction of proinflammatory gene expression. Although Cu/Zn SOD is an antioxidant enzyme, it is induced to convert superoxide to hydrogen peroxide that is still microbicidal; it also serves as a host defense with NADPH oxidase in phagocytes. Furthermore, the treatments of possible PPARγ activators increase both PPARγ and NF-κB DNA binding activities, indicating that these two redox-sensitive transcription factors coordinate and propagate feedback loops between each other.
Interactions between genes and diet: Risk factors

Single-nucleotide polymorphisms (SNPs) are a genetic variation of differences in a single nucleotide between individuals. The gene-diet interaction between common SNPs located in candidate genes and dietary factors related to lipid metabolism has been recently reported [96]. These candidate genes include: APOA1 (75G→A) encoding apolipoprotein A-I, an apolipoprotein of HDL, and PPARA (Leu162Val) encoding PPARα. Ordovas et al. and Tai et al. suggest specific interactions between these polymorphisms and lipid profiles. HDL-cholesterol concentrations increased significantly with increasing PUFA intake in women with the A allele (G/A and A/A) in APOA1, while HDL-cholesterol concentrations decreased as PUFA intake increased in women with the homologous G allele (G/G) in APOA1 [97]. The Leu162Val polymorphism in PPARA is associated with increased plasma concentrations of total cholesterol, LDL cholesterol, and apolipoprotein B [98]. Thus, these interactions influence CVD risk in different directions through effects on two different CVD risk factors: HDL cholesterol through the polymorphism in APOA1 and triacylglycerol through the polymorphism in PPARA. The effects of Pro12Ala polymorphism in PPARG2 on type 2 diabetes and obesity are also reported. The 12Ala allele (Ala/Ala) in PPARG2 confers a reduced risk for type 2 diabetes and decreased obesity-associated insulin resistance in the French Caucasian population [99].

Furthermore, the associations of polymorphisms in genes involved in antioxidant defense systems, with CVD and other diseases, have been proposed. A human sodium-dependent vitamin C transporter, SVCT1, is encoded by SLC23A1, and mediates intestinal absorption and renal absorption of L-ascorbic acid [100]. SLC23A1 appears to have
population-specific variants, and populations with discrete genetic variants might require
different recommended values of vitamin C intake to maintain health and/or to prevent
disease [101]. An antioxidant enzyme, SOD2 (Mn-SOD), is constitutively expressed in
most cells. The SOD2 polymorphism, 16Val homozygous, may be a predisposing factor
for lung cancer, cardiomyopathy, diabetic complications, hypertension, and CVD [102-
105] and may influence longevity [106]. GPX1 encodes Gpx1, and may be a target gene
for exploring roles of its variants in the etiology of various human diseases [107]. Genetic
variations may also affect inflammatory responses. TLR4 is a pattern recognition innate
immunity receptor that binds LPS found in gram-negative bacterial walls and possibly
oxidized LDL [15]. The Asp299Gly TLR4 polymorphism may decrease the risk of
atherosclerosis by reducing TLR4 receptor signaling and subsequent inflammatory
response [61,108].

Thus, genetic variations are widely distributed in various components involved in
atherogenesis. The total genetic variations between individuals may differently influence
the risk for and the etiology of atherosclerosis and CVD. It may be possible to provide
individuals with dietary/therapeutic guidance tailored to their genotypes, given adequate
information on the interaction between specific genetic polymorphisms and diet [109]. In
other words, genetic variations might predict the significant differences in disease
etiology between different species, thus suggesting the limitation of animal studies.
Hence, nutrigenetics and nutrigenomics would be new powerful tools for investigating
the relations between diseases and genes at individual/intra-species levels.
Experimental Studies of CLA isomers

Animal studies

Rabbits

The possibility that the anti-atherogenic properties of CLA isomers may influence atherosclerotic lesions and blood lipid levels has been tested in animal models.

Rabbit studies suggest protective and/or therapeutic effects of CLA isomer treatments. Rabbits fed an atherogenic diet and supplemented with CLA isomer mixture (\textit{cis}-9, \textit{trans}-11 CLA isomer: \textit{trans}-10, \textit{cis}-12 CLA isomer = 1:1; 0.5 g CLA diet/day/rabbit) had significantly less aortic fatty lesions and lower levels of plasma triglycerides and LDL-cholesterol, compared to control animals [110]. A rabbit study by Kritchevsky \textit{et al.} [46] also reported reduced atheromatous lesions to the same extent in all CLA-fed groups (90 days): 1% (final dietary concentration) each of the \textit{cis}-9, \textit{trans}-11 CLA isomer, the \textit{trans}-10, \textit{cis}-12 CLA isomer, and the two isomer mixture, compared to control group.

A dose-dependent effect of CLA isomer mixture (\textit{cis}-9, \textit{trans}-11 CLA isomer: \textit{trans}-10, \textit{cis}-12 CLA isomer \textit{r} = 1:1) on atherosclerotic regression was demonstrated in two rabbit studies [47,111]. New Zealand white rabbits fed a 0.1% (final dietary concentration) CLA isomer mixture diet after receiving an atherogenic diet, showed an inhibition of atherogenesis, while rabbits fed a 1% CLA mixture diet exhibited a 30% regression of established atherosclerosis [47]. Dose-dependent regression of established atherosclerosis was seen in rabbits fed CLA isomer mixtures ranging between 1 and 10 g/kg body weight. However, both serum cholesterol and triglyceride levels were higher in CLA fed...
groups than in control group, despite a dose-dependent reduction of lipid levels within the range of CLA isomer mixtures [111].

**Mice**

Mouse model studies suggest the anti-atherogenic effects of CLA isomers, and some of those studies indicate that the effects of CLA are tissue- (i.e., hepatoxicity described later in this section), isomer-, and dose-specific.

Atherosclerotic prone strain C57BL/6 mice fed an atherogenic diet containing 2.5 or 5 g/kg body weight CLA isomer mixture (cis-9, trans-11 CLA isomer: trans-10, cis-12 CLA isomer = 1:1) for 15 weeks developed higher serum HDL-cholesterol (total cholesterol ratio and lower serum triacylglycerol concentration) than controls. However, despite causing a serum lipoprotein profile considered to be less atherogenic, addition of CLA isomer mixture to the atherogenic diet increased the development of aortic fatty streaks. Mice consuming a diet of 2.5 g CLA isomer mixture/kg body weight, but not 5.0 g CLA isomer mixture/kg body weight, developed a significantly greater area of fatty streaks than the controls [112], suggesting dose-specificity. A study by Arbones-Mainar et al. [113] showed isomer-specific effects on the development of atherosclerosis. The trans-10, cis-12 CLA isomer diet (1% final dietary concentration for 12 weeks fed to apolipoprotein E knockout mice) increased the values of blood lipid, an inflammatory marker (8-iso prostaglandin E), atherosclerotic plaque, and macrophage content and activation. However, the cis-9, trans-11 CLA isomer diet (1%) inhibited atherogenic development. Moreover, de Roos et al. [114] documented that CLA isomers differentially affect plasma lipid levels as well as the markers of insulin resistance and inflammation in
apolipoprotein E knockout mice. The cis-9, trans-11 CLA isomer lowered these values suggesting beneficial properties, whereas the trans-10, cis-12 CLA isomer increased the values indicating detrimental properties. In Nestel et al.'s study [115] using insulin deficient apoE deficient mouse models, 0.9% (final dietary concentration) cis-9, trans-11 CLA isomer diet failed to reduce the severity of aortic atherosclerosis, though plasma triglyceride levels decreased, and HDL cholesterol levels increased.

Hamsters

Like mouse models, hamster models have shown protective effects, some of which are isomer-specific. Hamsters fed a CLA isomer mixture diet (cis-9, trans-11 CLA isomer: trans-10, cis-12 CLA isomer = 1:1, final dietary concentrations 0.06, 0.11, and 1.1%) showed significantly reduced plasma levels in total cholesterol, non-high-density lipoprotein cholesterol, and triglycerides [116]. In Wilson et al.'s study [117] using hamster models, animals on the hypercholesterolemic diet (HCD) supplemented 1% (final dietary concentration) CLA isomer mixture diet (cis-9, trans-11 CLA isomer: trans-10, cis-12 CLA isomer = 1:1) showed 47% fewer aortic fatty streaks and lower plasma cholesterol levels than control. In addition, the CLA isomer mixture diet reduced the development of early aortic atherosclerosis to a greater degree than linoleic acid, possibly through changes in LDL oxidation susceptibility in hypercholesterolemic hamsters. Both the cis-9, trans-11 and trans-10, cis-12 CLA isomer (1% diet of each isomer) fed groups of hamsters had non-significantly decreased fatty streak lesions. However, neither diet affected plasma cholesterol levels [118]. Wilson et al. [119] later reported the adverse effects of the trans-10, cis-12 CLA isomer, but not the cis-9, trans-
11 CLA isomer, suggesting an isomer-dependent effect of CLA on atherogenesis in hypercholesterolemic hamster models. In the Wilson et al's study, hamsters were divided into four groups and were fed for up to 12 weeks: 1) an HCD, 2) an HCD with 0.5% (of diet) cis-9, trans-11 CLA isomer, 3) an HCD with 0.5% (of diet) trans-10, cis-12 CLA isomer, or 4) an HCD with linoleic acid (LA). Both CLA fed groups had lower blood cholesterol levels. However, the trans-10, cis-12 CLA isomer fed group had higher plasma triglyceride and glucose levels compared with the control at 12-weeks of treatment, while the plasma triglyceride and glucose levels of the cis-9, trans-11 CLA isomer fed group were reduced. Wilson et al. concluded that the trans-10, cis-12 CLA isomer may be detrimental if fed separately from the cis-9, trans-11 CLA isomer. In contrast, Navarro et al. demonstrated favorable effects of the trans-10, cis-12 CLA isomer on lipid metabolism in the blood and the liver of hamsters fed an atherogenic diet for 6 weeks and no effects of the cis-9, trans-11 CLA isomer on the same lipid metabolisms [120]. Studies by Valeille et al. [121,122] exhibited the anti-atherogenic and anti-inflammatory effects of the cis-9, trans-11 CLA isomer in hyperlipidemic hamsters.

Inter-/intra-species, tissue-, isomer-specificities

At dietary levels of 0.1–1%, the CLA isomer mixture caused substantial regression of established atherosclerosis in earlier rabbit models [46,47,111]. This was a unique and important finding, because once established, aortic lesions in rabbits will regress only under unusual circumstances. Regression of pre-established lesions has never been achieved by dietary means or by simple pharmacologic intervention in vivo. However, the use of rabbit models for atherosclerotic studies may not be suitable. Unlike humans, the
majority of rabbit blood cholesterol is β-VLDL. Therefore, the aortic lesions caused by feeding atherogenic diets to rabbits may not be comparable to those seen in humans. In contrast, LDLR- or apo-E-deficient mouse models mimic human atherosclerosis [123]. Thus, the effects of dietary CLA isomer supplementation have not been consistent between these different animal models. Even between rats and mice, a different species response to CLA isomers has been indicated. Any response of peroxisome proliferattion to CLA isomers may be greater in mice than in rats [124]. CLA isomers are known to be PPAR activators [6]. Differences in CLA-mediated hepatic gene induction between mice and rats have also been found [125]. Hepatic fat accumulation caused by the trans-10, cis-12 CLA isomer has been reported mainly in mice, and hepatic fat accumulation is associated with the loss of adipose tissue induced by the trans-10, cis-12 CLA isomer [126]. Adipose tissues are important endocrine organs that produce inflammatory mediators such as TNFα and IL-6 and -8, and adipocytokines (adiponectin and leptin). Adipocytokines are key regulators of insulin resistance. Adiponectin and leptin affect immune and inflammatory functions [127]. The dramatic decrease in adiponectin concentrations is important to the development of hepatic steatosis and insulin resistance induced by CLA. Removing CLA from the diet rescued leptin and adiponectin levels and attenuated insulin resistance induced by dietary CLA in mice. However, if a PPARγ activator, rosiglitazone, was added to CLA-TG diet (38.5% trans-10, cis-12 CLA isomer), the reduction in adipose mass and serum leptin and adiponectin levels was reversed [128].
An opposite response to that found in mice, CLA mixture diet (39.2% the cis-9, trans-11 and 38.5% trans-10, cis-12 CLA isomers) reduces hepatic steatosis and plasma lipids in rats [129,130]. Beyond rodent models, the genetic differences between mice and humans should be considered. For example, mice contain more copies of cytochrome P450 than do humans [131]. Cytochrome P450 is involved in microsomal ω-oxidation of fatty acids, eicosanoid synthesis, and detoxification of xenobiotics. The trans-10, cis-12 CLA isomer significantly reduces cytochrome P450 gene expression in mouse livers, and the reduction may contribute to CLA-induced fatty livers as well as the induction of enzymes associated with fatty acid synthesis [126]. Thus, not only are there differences in CLA-mediated cytochrome P450 gene expression, there are additional differences in fatty acid metabolism and eicosanoid formation, between humans and mice. As described in Section 6, single nucleotide polymorphisms (SNPs) among human individuals, such as SNPs in APOA1, PPARA, PPARG, SOD2, Gpx1, and TLR4, may also cause differences in lipid metabolism and the risk for atherosclerosis. In addition, the age of animals fed CLA and examined may be another consideration. Many atherosclerotic studies used adolescent individuals for their animal models. Adolescent animals are still growing; their body composition is still changing. The gene expression profile and sensitivity to and metabolism of chemicals in a developmental stage differ from those in adults. Such differences may make the extrapolation to humans from animals and explanations of study results more difficult. Thus, there are several factors to be considered when investigating the effects of CLA isomers as therapeutic or chemopreventive agents for atherosclerosis: dose-dependency and isomer-specificity, as well as inter- and intra-
species differences. Therefore, genetic and genomic research using human subjects and/or human cells are urgently needed to determine the effects of each isomer.

**Human Studies**

CLA is being sold as a panacea with several alleged benefits including altering body composition, *i.e.*, to reducing obesity and building lean body mass [132]. Safety of long-term (≥ 12 months) CLA supplementation was examined in several clinical trials. A randomized, double-blind study was conducted, in which obese individuals were given 6 g/day of either CLA isomer mixture (*cis*-9, *trans*-11: *trans*-10, *cis*-12 = 50:50) or placebo (high oleic sunflower oil) for 12 months. Although body composition did not differ between the CLA-supplemented group (n = 27) or the placebo group (n = 23), lower levels of adverse effects (alterations in the liver function, glucose and insulin levels, insulin resistance, and white blood cell counts) were observed in the CLA-supplemented group than in the control group. The investigators concluded that CLA isomer mixture as Clarinol™ is safe for use in obese humans for up to one year at the recommended dosage [133]. Another long-term (one year) CLA isomer mixture supplementation study was performed in a double-blind fashion [134]. Healthy overweight humans (n = 180) were randomly divided into three groups: 1) CLA free fatty acid (*cis*-9,*trans*-11: *trans*-10, *cis*-12 = 50:50; 3.6 g CLA isomers/day as FFA forms), 2) CLA-triacylglycerol group (*cis*-9, *trans*-11: *trans*-10, *cis*-12 = 50:50; 3.4 g CLA isomers/day as TAG forms), and 3) placebo (olive oil). The CLA isomer mixture supplementation decreased body fat mass in healthy overweight adult humans. However, there were significant increases in: LDL levels in the CLA-FFA group, HDL levels in the CLA-TAG group, and lipoprotein levels
in both CLA groups. Adverse effects, mostly gastrointestinal, were reported by 11.4% of the subjects, and likely resulted from the daily ingestion of oil or of the gelatin capsule alone. Overall, the adverse effects did not differ significantly between the CLA groups and the placebo group, indicating that CLA isomer mixture was tolerated as well as olive oil as the control. One hundred twenty five of 180 subjects who finished this study, continuously participated in the CLA isomer mixture supplementation study for an additional year, thus, total 2 years [135]. Two-year-CLA isomer mixture supplementation groups significantly reduced body weight, BMI, body fat mass, energy intake and serum leptin levels, compared with the baselines at month 0. However, serum lipoprotein and aspartate amino transferase levels, and whole blood leukocyte and thrombocyte counts were significantly increased in the CLA groups. Gaullier et al. concluded that CLA isomer mixture supplementation for 24 months in healthy, overweight adults was well-tolerated, and that CLA isomer mixture may be beneficial as a weight loss supplement. Another one-year CLA isomer mixture supplementation study [136] was conducted in a randomized, double-blind, placebo-controlled fashion. No significant differences in body weight or body fat regain were observed between the CLA group (cis-9, trans-11: trans-10, cis-12 = 50:50; 3.4 g/day as TAG forms; n = 40) and placebo (4.5 g olive oil; n = 43). No significant differences in adverse effects or indexes of insulin resistance were observed between the groups. However, a significant increase in the number of leukocytes was observed in the CLA group. Although the investigators did not obtain a perfect group match for body weight at randomization, they concluded that the CLA isomer mixture supplementation for one year has no preventative effect on body weight
and body fat regain after the weight loss induced by a low calorie diet for eight weeks in obese subjects.

Many studies have investigated the effects of short-term (mostly 12 weeks or 8 weeks) CLA supplementation. In a six-month double-blind CLA isomer mixture supplementation study [137], 118 healthy overweight and obese adult humans were randomized into two groups supplemented with either 3.4 g/day CLA isomer mixture (cis-9, trans-11: trans-10, cis-12 = 50:50) or placebo. CLA significantly decreased body fat mass, in particular in legs of both males and females and in females with BMI >30 kg/m², at either month 3 or 6, compared with placebo. Lean body mass increased in the CLA supplemented group. The safety parameters including blood lipids, inflammatory and diabetogenic markers remained within the normal range, and adverse events did not differ between the groups in the study. It was concluded that the CLA isomer mixture supplementation in healthy, overweight, and obese subjects decreases body fat mass in specific regions and was well tolerated. The dose-dependent effects of CLA were reported in a CLA isomer mixture supplementation study of 12 weeks [138]. Forty eight obese subjects were divided into three groups: 1) 3.2 g/day CLA (cis-9, trans-11: trans-10, cis-12 = 50:50), 2) 6.4 g/day CLA, and 3) placebo (8 g safflower oil). CLA isomer mixture supplementation at the higher dose increased inflammatory markers, IL-6 and C-reactive protein (CRP), however, remained within normal ranges. A significant increase in lean body mass was also found in the same treatment group. No severe adverse effects were reported. The authors concluded that the CLA isomer mixture intervention was well-tolerated. Beneficial effects on immune functions have been reported in a double-blind, randomized
CLA isomer mixture supplementation study [139]. Twenty-eight healthy adults received either high oleic sunflower oil (placebo) or 3.0 g/day CLA (cis-9, trans-11: trans-10, cis-12 = 50:50; triglyceride form) for 12 weeks. The CLA group showed significantly reduced levels of the proinflammatory cytokines, TNF-α and IL-β, and increased levels of the anti-inflammatory cytokine, IL-10. Immunoglobulin levels were also altered: CLA isomer mixture decreased Ig E levels, and increased both Ig M and Ig A levels. Another CLA isomer mixture supplementation study (2.2 g/day; cis-9, trans-11: trans-10, cis-12 = 50:50; 8 weeks) investigated the effects of CLA isomer mixture on inflammation in a double-blind, randomized, placebo-controlled model using healthy middle-aged males [140]. The CLA isomer mixture supplementation significantly reduced concanavalin A-stimulated peripheral blood mononuclear cell IL-2 secretion, suggesting anti-inflammatory and anti-atherogenic effects of CLA isomer mixture. Other inflammatory markers, IL-6, CRP, and fibrinogen, were not affected in this study. Moloney et al. [141] demonstrated that the CLA isomer mixture supplementation (3.0 g/day; cis-9, trans-11: trans-10, cis-12 = 50:50; 8 weeks) increased total HDL cholesterol concentrations and decreased the ratio of LDL cholesterol to HDL cholesterol without changes in inflammatory markers of CVD in subjects with type 2 diabetes. However, this CLA isomer mixture intervention did not show positive effects on insulin and glucose concentrations among the diabetic patients. In a Swedish study, 53 healthy humans were randomly assigned to CLA isomer mixture supplementation (4.2 g/day; cis-9, trans-11: trans-10, cis-12 = 50:50; 12 weeks) in a double-blind fashion. Supplementation with a CLA isomer mixture reduced the proportion of body fat and affected fatty acid metabolism. However, no effects were found for CLA isomer mixture on body weight,
serum lipids, glucose metabolism or plasminogen activator inhibitor 1 [142]. In Noone et al.’s double-blind placebo-controlled study [143], the CLA isomer mixture treatment (3 g/day; cis-9, trans-11:trans-10, cis-12-CLA = 50:50; 8 weeks) showed reduced plasma triacylglycerol levels in normolipaemic human subjects.

A study using 49 healthy male subjects showed the isomer-/dose-dependent (0.59, 1.19, 2.38 g/day of the cis-9, trans-11 CLA isomer; 0.63, 1.26, 2.52 g/day of the trans-10, cis-12 CLA isomer; 8 weeks) opposite effects of CLA on plasma total cholesterol and LDL-cholesterol levels: hypolipidemic properties of the cis-9, trans-11 CLA isomer and hyperlipidemic properties of the trans-10, cis-12 CLA isomer. However, neither CLA isomer supplementation affected insulin resistance [144]. Using the same healthy male subjects and the same supplementation design, Tricon et al. investigated the effects of two CLA isomers on immune cell functions [145]. The results showed a dose-dependent reduction in the mitogen-induced activation of T lymphocytes and a negative relationship between the mitogen-induced T lymphocyte activation and the contents of each CLA isomer in mononuclear cells, suggesting beneficial effects in inflammatory diseases such as atherosclerosis.

Additional adverse effects of CLA supplementation, in particular trans-10, cis-12 CLA, were reported. Riserus et al. demonstrated that the purified trans-10, cis-12 CLA isomer supplementation (3.4 g/day, 3 months), but not CLA mixture supplementation (3.4 g/day; cis-9, trans-11: trans-10, cis-12 = 50:50; FFA form, 3 months), increased oxidative stress, CRP, and proinsulin levels, and decreased insulin sensitivity in non-diabetic abdominally obese males [146] and in males with metabolic syndrome [147] in two
double-blind, randomized, placebo- (3.4 g/day, olive oil) controlled studies. Like the study by Tricon et al. [144], Riserus et al.'s two studies also suggest the isomer-dependent detrimental effects of CLA. Another unfavorable effect of the trans-10, cis-12 CLA isomer was also reported in a human study examining non-enzymatic and enzymatic lipid peroxidation (8-iso-PGF$_{2α}$ and 15-keto-dihydro-PGF$_{2α}$, respectively) in human plasma and urine. Sixty healthy subjects were divided into six groups: three CLA isomer mixture groups, (3.5 g/day, cis-9, trans-11: trans-10, cis-12 = 50:50, 4 weeks) and three trans-10, cis-12 CLA isomer groups (4.0 g of the trans-10, cis-12 CLA isomer/day, 4 weeks): 1) the CLA supplement alone, 2) with vitamin E (D-α-tocopherol acetate), and 3) with COX-2 inhibitor (refecoxib). Although both CLA isomer mixture and the trans-10, cis-12 CLA isomer supplemetations increased the eicosanoid levels in the urine, the trans-10, cis-12 CLA isomer supplementation with the COX-2 inhibitor suppressed the increase in urinary 15-keto-dihydro-PGF$_{2α}$ levels. This result suggests that increased lipid peroxidation in eicosanoid synthesis may be due to induced COX-2 expression by the CLA supplemetations, in particular the trans-10, cis-12 CLA isomer [148]. Taylor et al. [149] documented that CLA isomer mixture supplemetation (4.5 g/day; cis-9, trans-11: trans-10, cis-12 = 50:50; 12 weeks) impaired endothelial function and increased markers of oxidative stress in 40 healthy white males, suggesting caution in the use of CLA isomers as an aid for weight loss.

Overall, the effects of CLA isomers (or mixture) on atherogenic and/or inflammatory parameters in humans have not been definitive. Although a meta-analysis of 18 CLA human studies (including three single isomer studies) suggests the beneficial use of CLA
isomers only as a body fat reducing supplement [150], the therapeutic potentials of CLA isomers in inflammatory diseases including atherosclerosis remain to be determined. Differences in purity and content of CLA isomers may cause these conflicting results. Impurities might induce undesirable side effects [151]. The variety of CLA isomer content in supplements and/or the differences in CLA dose might cause inconsistent results due to the dose- and/or isomer-dependent effects of CLA suggested by some other studies [138,144,146,147]. Human subjects were not limited in diet (therefore, dietary fat intake, excluding supplemental fat intake, is of concern) and/or physical activities in some study designs. In addition, CLA isomer (or mixture) supplementations contained other fatty acids, including PUFAs and saturated fatty acids, at up to 20% in some study designs. The details of supplemental contents, other than CLA isomers, are not even provided in some other studies. Since CLA isomers are incorporated into membrane phospholipids, they may compete in elongation and desaturation steps with other PUFAs that are precursors of arachidonic acid (AA, 20:3, ω-6). The competition in the incorporation may alter eicosanoid biosynthesis [152], therefore, subsequent immune and inflammatory processes. An experiment by Brown et al. [153] found that the presence of linoleic acid (LA) may affect the possible benefits of CLA isomers. LA, one of the PUFAs present in Western diets and the human body is a possible antagonist to CLA isomers. The plentiful LA may exclude CLA isomers from incorporation into phospholipids and drive it into storage as a component of neutral lipid [52]. LA also inhibits EPA incorporation in membrane phospholipids from fish-oil supplements [54]. Like CLA isomers, dietary EPA and DHA partially replace AA derived from LA in the cell membrane. These ω-3 fatty acids are associated with decreased risk for CVD, and
reduce the formation of pro-inflammatory eicosanoids [53]. The replacement of AA by ω-3 fatty acids may cause the alteration of fatty acid composition in membrane lipid bilayers influencing signaling pathways and subsequent immune and inflammatory processes. The anti-inflammatory effects of CLA isomers observed in a limited number of studies may be attributed to the replacement of AA generated by LA. In addition, saturated fatty acids have been reported to provoke inflammation by inducing pro-inflammatory gene expression through innate immune receptor (TLRs) activation [61]. Thus, the coexistence of other fatty acids might potentially affect the results of any human studies with CLA isomers. Moreover, there may be divergent effects of CLA isomers in obese or diabetic subjects compared to the normal-weight or healthy subjects as well as differences determined by gender and/or genetics, i.e., SNPs in related genes. Further studies are needed to investigate the effectiveness and safety of CLA supplementation and to elucidate these confounding factors.

**CLA mediated gene expression**

*Adiposity plays an important role in fatty acid mobilization, fat storage, and formation of pro-and anti-inflammatory cytokines and chemokines* [128]. As mentioned above, atherosclerosis is viewed as a chronic inflammatory disease affecting lipid profiles. CLA isomers have been shown to influence lipid metabolism associated with inflammation and atherogenesis in *in vitro* studies. One possible mechanism by which CLA isomers could modulate atherogenesis is regulating the production of lipoproteins in the liver. Sterol element binding proteins (SREBP) are a group of membrane-bound transcription factors that bind to their specific DNA binding sites (SRE-1) to activate the expression of target
genes that encode enzymes necessary for lipid synthesis, including the LDL receptor (LDLR) gene in sterol-depleted cells [154]. A study by Ringseis et al. [155] reported that the \textit{trans-10, cis-12} CLA isomer, not the \textit{cis-9, trans-11} CLA isomer, induced LDLR gene expression via SREBP-2 in human hepatoma cells (HepG2). They concluded that the enhanced uptake of VLDL and LDL cholesterols by hepatic LDLR may account for the decreased plasma cholesterol levels in response to CLA isomer (or mixture) supplementations in a limited number of human and animal studies. An alternative pathway for CLA-mediated LDLR expression is also suggested. Yu-Poth et al. [156] demonstrated that a CLA isomer mixture (50:50, 400 \textmu mol/L final concentration) up-regulated LDL receptor (LDLR) mRNA and protein expression at three- to five-fold in HepG2 cells. The results of the study suggest the upregulation of the LDLR gene by CLA through a mechanism that is independent of SREBP-1 and acyl CoA: cholesterol acyltransferase (ACAT).

Monocyte-endothelial interaction is a key step of atherogenesis. However, CLA isomers showed no effects on TNF\textalpha-induced adhesion molecule expression, monocyte adhesion, and chemokine release or on the molecular mechanisms regulating these processes in human aortic endothelial cells [21]. This suggests that the anti-atherogenic effects of CLA isomers may not be associated with the reduction of monocyte-endothelial interactions.

Several studies have investigated the implication of CLA isomers in eicosanoid synthesis and the role of CLA isomers in inflammation and atherogenesis. Dietary CLA may suppress the biosynthetic pathway of AA. CLA may suppress eicosanoid formation via
direct action on COX and LOX, *i.e.*, by inhibiting the expression or the activities of these enzymes [5,6]. Constitutive COX-1 and inducible COX-2 catalyze the conversion of free PUFAs to prostanoids, while LOX generates the leukotrienes. The *trans*-10, *cis*-12 CLA isomer suppresses COX-2 expression and PGE$_2$ release in rat macrophages either by inhibiting NF-κB activation *in vivo* and *in vitro* or by inhibiting the MAPK/ERK/JNK pathway. The trans-10, cis-12 CLA isomer inhibits the NF-κB p50 and p65 subunits binding to DNA [83]. The 50:50 mixture of CLA isomers inhibits the expression of both COX-2 and inducible nitric oxide synthase (iNOS) in LPS activated murine macrophages, resulting in decreases in prostaglandin E$_2$ and NO synthesis [157]. The effects of CLA on prostanoid formation can be either inhibitory or stimulatory, depending on isomer-specificity, chemical forms of CLA isomers (*i.e.*, free fatty acid or esterified forms), or cellular states (*i.e.*, resting or stimulated states) in human endothelial cells and platelets [158]. CLA isomers have also been reported to reduce prostaglandin E2 synthesis in certain cell types in both humans and mice [159].

The beneficial effects of CLA isomers may be attributed to their properties as PPAR$\alpha/\gamma$ activators [5,48,155,160]. Structurally, CLA resembles 13-HODE, as well as 15-HETE and 15d-PGJ2, which were all identified as natural activators of PPAR$\gamma$ [49]. Ringseis et al [160] demonstrated that either the *cis*-9, *trans*-11 or *trans*-10, *cis*-12 CLA isomer (50 μmol/L) reduced AA proportions in human vascular smooth muscle cells (SMCs), TNFα-induced NF-κB DNA binding activity, mRNA levels of enzymes involved in eicosanoid synthesis (*e.g.*, COX-2), and production of PGE2 and PGI2. These CLA isomer treatments increased PPAR$\gamma$ DNA binding activity. Furthermore, a PPAR$\gamma$ repressor
suppressed the inhibitory actions on the eicosanoid formation and NF-κB DNA binding activity in the SMCs. Synthetic PPAR activators exert their anti-inflammatory actions, at least in part, by negatively regulating NF-κB activation [157]. PPARγ and NF-κB may be involved in regulating genes, whose products influence ROS generation that contributes to inflammation and atherogenesis, and these transcription factors coordinate and propagate feedback loops between each other. Thus, anti-inflammatory and anti-atherogenic effects of CLA isomers may be associated with these redox-sensitive transcription factors.

Although CLA isomers and ω-3 PUFAs have shown anti-inflammatory effects, they differ in the nature of their immunomodulatory properties. CLA isomers appear to enhance immune function, while ω-3 PUFAs are immunosuppressive [5]. Tian et al.'s study [51] using rats treated with 300 mg/kg/day of clofibrate, a PPARα activator, for up to 14 days, reported an increase in myocardial DHA proportion and a decrease in the portion of AA that is a precursor of pro-inflammatory eicosanoids (e.g., leukotriene B4 and prostaglandin E2). Tian et al. implicate enhanced uptake of ω-3 PUFAs from blood circulation and/or increased biosynthesis of ω-3 PUFAs in rats treated with the PPARα activator, in those results. Similarly, Attar-Bashi et al.'s human study [161] investigated the effects of CLA isomers mixture [3.2 g/day, cis-9, trans-11: trans-10, cis-12 = 50:50, plus 11 g of alpha-linoleic acid (ALA), 8 weeks] as PPARα activators on DHA (22:6, ω-3) and EPA (20:5, ω-3) biosynthesis from ALA (18:3, ω-3) through Δ5- and Δ6-desaturases, both of which are possible PPARα target genes. The study demonstrated that ALA (18:3, ω-3) plus CLA isomer mixture increased EPA and decreased AA. However,
the CLA isomer mixture supplementation did not affect DHA biosynthesis in humans. DHA synthesis from ALA needs additional peroxisomal oxidation. Thus, CLA isomers may play a role in PPARα-mediated gene expression and ω-3 PUFA-mediated anti-inflammatory effects.

CLA isomers are readily metabolized in vivo via multiple pathways, and elongated and desaturated metabolites of CLA have been detected in the liver and mammary tissue of rats and adipose tissue and sera of humans [6]. Some studies have suggested the involvement of CLA metabolites in anti-atherogenic and ant-inflammatory processes [5,160], though the Δ6-desaturase metabolites of CLA may not be important for the alterations in gene expression induced by CLA [6].

Thus, multiple signaling pathways, such as PPARα, PPARγ, NF-κB, and MAPK/ERK/JNK, may be involved in the anti-inflammatory and anti-atherosclerotic effects of CLA isomers.

**Conclusion**

Conjugated linoleic acid isomers are a group of zoochemicals that have a variety of physiological actions and potential health benefits, e.g., modulation of inflammation, lean body mass, atherosclerosis, and cancer. Most of the health effects of CLA isomers are based on reports with limited power of extrapolation of experiments with animal models and in vitro/ex vivo systems. Multiple factors in earlier animal studies might cause inaccurate extrapolation of CLA isomer effects to humans. First, interspecies-genetic
differences may be a major consideration. Some of the health effects of CLA isomers seen in animal models might be species-specific, and might not be observable in humans. For example, the suppressive effect on hypertension was seen in CLA fed rats [162-165], but not in humans [166]. Second, animal studies used primarily adolescent animals, while human studies used mostly healthy, obese, or diabetic adults. Body composition, sensitivity to and metabolism of chemicals, and gene expression profiles in developing adolescent animals may be different from those seen in adult humans. This could influence the outcome of examining the effects of CLA isomers, in particular, on age-associated events, such as adiposity and atherogenesis. Although atherosclerosis-prone mice (i.e., C57BL/6) mimic humans, animal results have been inconclusive and the efficacy of CLA isomers maybe due to interspecies-gene differences. Third, animal studies have usually been of short durations with high dosages. This is of particular concern because extrapolated CLA isomer dosages for possible human consumption would require dose and duration adjustments for human lifespan and levels at or less than threshold toxicity.

The effects of CLA isomers on body composition have been studied extensively in animals and have recently been repeated in human studies with conflicting findings. In part, the results may be inconclusive because a majority of the studies utilized CLA isomer mixture supplementation. Both dose- and isomer-dependent effects of CLA have been suggested in both the animal and human studies. Further research is needed to identify isomer-and/or dose-related efficacy and toxicity. In addition, recent studies have identified SNPs in genes related to lipid metabolism and antioxidant defense systems. It
may be necessary to investigate the effects of CLA isomers on CVD risk at intra-species levels. Some recent animal studies have reported a positive correlation with other disease prevention and treatment, *e.g.*, diabetes and inflammatory bowel disease [48,49,128,129] leading researchers to further evaluate the possible benefits of CLA isomer supplementation for humans.

Earlier research showed that the ability of CLA isomers to act as anti-carcinogens and protectants against atherosclerosis may be due to its role as an antioxidant. However, in light of current research, the modulation of chronic diseases by CLA isomers may involve the control of redox status by regulating genes, whose products influence ROS generation, through redox-sensitive transcription factors including PPARγ and NF-κB. It is essential that investigation to develop an understanding about the molecular action of CLA isomers be encouraged so that we may learn how to use these compounds as adjuvants in chronic disease therapy.

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Figure 1: Chemical structures of linoleic acid and isomers of conjugated acid (CLA).
Figure 2: Vascular events leading to the development of atherosclerotic lesions. Low density lipoprotein (LDL), scavenger receptor (SR).
Figure 3: Metabolic pathways for the formation of prostaglandins, thromboxanes, and leukotrienes. Prostaglandin H₂ (COX), lipoxygenase (LOX), 12-hydroperoxyeicosatetraenoate (12-HPETE), 5-hydroperoxyeicosatetraenoate (5-HPETE).
**Figure 4: Role of PPARγ.** Peroxisome proliferator response element (PPRE), retinoic X receptor (RXR), retinoic acid (RA), ligands (L).
Chapter 2  Part B

Review of Conjugated Linoleic Acid

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Conjugated Linoleic Acid (CLA)-Mediated Gene Expression and Implications for Atherosclerosis

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Conjugated Linoleic Acid (CLA)-Mediated Gene Expression and Implications for Atherosclerosis

Kritchevsky et al. [1] was first to report the inhibition of atherosclerosis (34%) as well as the regression (30%) of established atherosclerosis in rabbits fed mixtures of conjugated linoleic acid (CLA) isomers (0.1-1.0% of diet) for 90 days. CLAs are geometric and positional isomers of linoleic acid (18:2 ω-6) found in food products derived from ruminants, such as beef, milk, and cheese. CLAs are synthesized in ruminant rumen by gram-negative bacteria, *Butyrivibrio fibrisolvens* [2]. Susceptibility to oxidation of CLAs is higher than that of linoleic acid due to its conjugated double bonds [3,4]. Twenty-eight isomers have been identified with the cis-9, trans-11-CLA isomer being the predominant form in human diets [5]. Both the cis-9, trans-11, and trans-10, cis-12-CLA isomers have been under intensive *in vitro* and *in vivo* studies. Such research indicates many diverse physiological functions and potential health benefits, i.e. anti-obesity, anti-carcinogenesis, anti-diabetes, anti-inflammation, and anti-atherosclerosis [6-13]. Anti-carcinogenic effects in cell culture and animal studies [12,14-16] have been attributed to modulation of initiation, promotion, progression, and metastasis, with alteration of lipid peroxidation, tissue composition of fatty acids, eicosanoid metabolism, gene expression, cell cycle regulation, cell proliferation, and apoptosis [11].

CLA isomers play a role in inflammation [7,10,17-19]. Dietary supplementation of CLA isomer mixtures, but not ω-3 polyunsaturated fatty acid (PUFA), delays the onset of inflammatory bowel disease in a pig model by down-regulating tumor necrosis factor-α (TNF-α) through a peroxisome proliferator-activated receptor γ (PPARγ -dependent
mechanism [19,20]. Supplements of mixtures of CLA isomers appear to improve glucose
tolerance and hepatic lipid composition in rat models [21,22], suggesting anti-diabetic
and anti-obesity effects of CLA isomers. Although the trans-10, cis-12-CLA isomer
decreases adipose tissue lipids, the isomer exhibits adverse effects, induction of hepatic
steatosis and insulin resistance, in mouse models [23,24]. Based on our findings using
human umbilical vein endothelial cells, effects of CLA isomers on lipid peroxidation are
concentration-dependent. Increased lipid peroxidation occurred only at low
concentrations of CLA isomers (5 μmol/L), for which we speculate are due to the
susceptibility of CLA isomers to oxidation [25]. Our findings may partially explain
isomer-, species-, and tissue-specific effects of CLA, i.e. are dose/concentration-related.

CLA isomers are possible ligands/activators for PPARs: PPARγ, PPARα, and
PPARδ [6,19,20] and regulator for NF-κB, a redox-sensitive transcription factor. PPARs,
ligand-dependent transcription factors, belong to a subfamily of the nuclear receptor
superfamily. PPARs heteromerize with the 9-cis retinoic acid X receptor (RXR) and bind
to peroxisome proliferator response elements (PPREs: 5'-AGGTCA nAGGTCA-3')
which are located in enhancer sites of target genes. PPARγ and PPARα play a role in
inflammation, adipogenesis, atherogenesis, and insulin sensitization [26,27]. NF-κB is
expressed in all cell types. NF-κB recognizes and binds to specific DNA sequences (5'-
GGGRNNYYCC-3'). NF-κB is activated by intracellular ROS and/or ROS-modified
target molecules. NF-κB plays a role in regulating immune and inflammatory responses.
Work from our group [25] indicates that CLA isomers may regulate the expression of
antioxidant enzymes, Cu/Zn SOD and catalase, through PPARγ and NF-κB activation in
a concentration-dependent manner. CLA isomers may inadvertently exert adverse effects
under certain conditions, which may be related to increased lipid peroxidation observed at low concentrations of CLA isomers, i.e. due to their structural susceptibility to oxidation. Search results also suggest that multiple transcription factors (e.g. VDR, AP-1, STAT), other than PPARγ and NF-κB are involved in the expression of the antioxidant enzymes.

PPARγ and NF-κB could be involved in regulating genes, whose products influence ROS generation. Hence, activation of these transcription factors may be associated with ROS-related diseases, such as cancer and atherosclerosis [28]. Thus, PPARγ and NF-κB would coordinate and propagate feedback loops, as well as other transcription factors.

In a recent meta-analysis of human clinical studies involving CLA isomer mixture supplementation (n = 15, mostly short-term studies; for less than six months) demonstrated that a dose of 3.2 g CLA mixture/day produced a modest loss in body fat [29]. Other clinical studies support the safety of long-term CLA isomer supplementation (more than one year) [8,30,31]. Subsequently, the U.S. Federal Drug Administration-approved CLA is to be sold as a weight loss supplement over the counter.

The efficacy of CLA isomers on inflammation and atherosclerosis has been inconclusive in other human clinical studies [8,32-39]. Several animal and cell culture studies have exhibited anti-inflammatory and anti-atherosclerotic effects of CLA isomers, in particular the cis-9, trans-11 CLA isomer [1,40-42]. Although speculative, inter-species differences, tissue-specificity, use of different doses/durations of CLA supplementation and using subjects with different conditions (e.g. healthy/normal, obese/overweight, type 2 diabetes) could hinder direct extrapolation to humans. Also,
superimposed are single nucleotide polymorphisms (SNPs) which have been identified within genes related to lipid metabolism and antioxidant defense systems.

These genes include:

- **APOA1** (75G→A) encoding apolipoprotein A-I (an apolipoprotein of HDL),
- **PPARA** (Leu162Val) encoding PPARα,
- **PPARG2** (12Pro/Ala) encoding PPARγ,
- **SLC23A1** (22 SNPs found) encoding sodium-dependent vitamin C transporters 1, and
- **SOD2** (16Val/Val) encoding Mn-SOD.

These SNPs influence risks of CVD, type 2-diabetes, and cancer [43-50]. Further investigations of intra-species differences may provide reasons why the results of clinical studies have not been definitive and perhaps opportunities on how we would overcome limitations of animal studies (inter-species differences) and inaccurate extrapolation from animals to humans.

In conclusion, although protective effects of CLA isomers on inflammation and atherosclerosis have been inconclusive in human studies, efficacy and safety has been shown for weight loss. At the molecular level, CLA isomers appear to be ligands/activators for PPARs and regulators for NF-κB. CLA isomer treatments induce the expression of antioxidant enzymes, Cu/Zn SOD and catalase, in HUVECs, possibly through PPARγ and NF-κB in a concentration-dependent manner; CLA isomer treatments at higher concentration result in decreased autooxidation, and CLA isomer treatments at lower concentrations result in increased lipid oxidation. We have speculated that CLA isomers may be involved in the control of redox status partially by regulating genes, whose products influence ROS generation, through redox-sensitive transcription...
factors, PPARγ and NF-κB. Further research will be necessary to explore dose-, isomer-, tissue-specific effect of CLA supplementation in the prevention of inflammation and atherogenesis. Investigations of intraspecies differences may also account for discrepancies caused by CLA isomer supplementation in humans.
References

Chapter 3

Review of Vitamin E

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Vitamin E-Modulated Gene Expression Associated with ROS Generation

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Abstract

Although the beneficial effects of vitamin E on reactive oxygen species (ROS)-related diseases, such as cardiovascular disease (CVD), have been suggested by epidemiological and animal studies, clinical trials have failed to confirm the efficacy of vitamin E. Vitamin E has a defined role as an antioxidant. Recent studies indicate non-antioxidant roles of vitamin E serving as a regulator of gene/protein expression and enzyme inhibitor. Vitamin E may control ROS generation possibly in both antioxidant-dependent and independent manners, since vitamin E has shown inhibitory effects on activities and expression of ROS generating enzymes. However, the effects of vitamin E appear to depend on microenvironment, such as its concentration and the presence of oxidants/antioxidants. The objective of this article is to review the roles of vitamin E, in particular vitamin E-modulated gene expression, for better understanding the molecular mechanisms of vitamin E and for preventive and therapeutic strategies of ROS-related diseases.

Keywords: Vitamin E; Tocopherol; Antioxidants; Reactive oxygen species
1. Introduction

1.1. Background

Epidemiological studies have indicated an inverse relationship between intakes of antioxidant vitamins, such as vitamins A, C, and E, and cardiovascular disease (CVD) ([Klipstein-Grobusch et al., 2001], [Hung et al., 2005] and [Hung et al., 2004]). CVD is a major cause of mortality in developed countries (Willcox et al., 2004). The anti-atherogenic and anti-inflammatory effects of vitamin E, in particular \( \alpha \)-tocopherol, have been documented both \textit{in vitro} and in animal studies and reviewed ([Cyrus et al., 2003], [Meydani, 2004] and [Tucker and Townsend, 2005]). However, several clinical studies have failed to confirm the efficacy of \( \alpha \)-tocopherol on CVD ([Hodis et al., 2002], [Tucker and Townsend, 2005] and [Munteanu and Zingg, 2007]). Explanations for such inconsistency between \textit{in vitro}, animal studies and clinical studies include: (1) genetic and behavioral factors that contribute to the risk of CVD (Tucker & Townsend, 2005), including polymorphisms in genes associated with lipid metabolism ([Doring et al., 2004] and [Borel et al., 2007]), (2) the interaction with other nutrients (antioxidants) that coexist in diet (Omaye & Zhang, 1998), (3) the needs to optimize physiological concentrations and duration of \( \alpha \)-tocopherol intake ([Robinson et al., 2006] and [Nakamura and Omaye, 2008a]), (4) no animal models that mimic perfectly human atherosclerosis (Cyrus et al., 2003), and (5) intervention at a relatively late stage of CVD (Meydani, 2004). The objective of this article is to review the roles of vitamin E, in particular vitamin E-mediated gene expression associated with ROS generation, for better understanding the molecular mechanisms of vitamin E and for preventive or therapeutic strategies of CVD.
Also, we will briefly examine the association of non-antioxidant roles of vitamin E with reactive oxygen species-(ROS) diseases.

1.2. Structure

Vitamin E is a lipophilic vitamin, which is synthesized by plants, in particular found in seeds and oils. There are eight naturally occurring forms: four tocopherols (α-, β-, γ-, and δ-) and four tocotrienols (α-, β-, γ-, and δ-). Tocopherols consist of a substituted aromatic ring and a saturated isoprenoid side chain, while tocotrienols consist of the ring and an unsaturated isoprenoid side chain with three double bonds. There are eight possible stereoisomers of α-tocopherol (RRR-α-tocopherol; its natural form) due to three stereocenters in its structure (Jensen & Lauridsen, 2007) (Fig. 1). Since esterified forms, including tocopheryl- or tocotirienyl-acetate and succinate, are relatively stable, these forms are commonly used in supplements. Tocotrienols are less stable to high temperature than tocopherols (Combs, 2008). Due to its structural similarity to troglitazone, one of thiazolidines (TZDs), which are used as anti-diabetic drugs, vitamin E may act as ligands for peroxisome proliferator-activated receptors gamma (PPARγ) ([Campbell et al., 2003] and [De Pascale et al., 2006]).

1.3. Absorption

The absorption of vitamin E depends on lipid intake and the secretion of bile and esterases. Synthetic esterified forms of both tocopherols and tocotrienols are hydrolyzed probably by a duodenal mucosal esterase ([O’Byrne et al., 2000] and [Combs, 2008]). Free alcohols are the predominant forms of absorption. All of the vitamers are absorbed
similarly (World Health Organization, 2004). The percentages of vitamin E absorption on a dose administration range from 68% to 33%. Vitamin E deficiency results from fat malabsorption seen in children with cholestatic liver diseases, pancreatitis, or cystic fibrosis due to impaired secretion of bile and pancreatic digestive enzymes (Traber, 2007).

1.4. Transport

Vitamin E is transported by circulating lipoproteins. For example, each low density lipoprotein (LDL) particle contains 6–10 molecules of \( \alpha \)-tocopherol (Stocker, 1999). During the hydrolyzation of lipoproteins by endothelial lipoprotein lipase, a part of vitamin E is released in the plasma and taken up by cells (Munteanu et al., 2004).

Although \( \gamma \)-tocopherol is the predominant form of tocopherol in the American diet and all of the dietary vitamers are absorbed similarly, plasma \( \gamma \)-tocopherol levels are only 10% of plasma \( \alpha \)-tocopherol levels (approximately 25 \( \mu \)mol/L \( \alpha \)-tocopherol). This may be due to hepatic preferential selection of \( \alpha \)-tocopherol by \( \alpha \)-tocopherol binding proteins, such as \( \alpha \)-tocopherol transfer protein (\( \alpha \)-TTP) and tocopherol associated protein (TAPs). \( \alpha \)-TTP and TAPs are found mainly in the liver. These proteins preferentially bind to and transport \( \alpha \)-tocopherol intracellularrly. The proteins also incorporate \( \alpha \)-tocopherol into the nascent very low density lipoprotein (VLDL) in the liver ([Munteanu et al., 2004], [World Health Organization, 2004], [Zingg, 2007] and [Combs, 2008]). \( \alpha \)-TTP plays a role in regulating plasma \( \alpha \)-tocopherol concentrations. \( \alpha \)-TTP is not up-regulated to compensate for the higher turnover or oxidative loss of vitamin E under specific conditions, maintaining optimal levels of \( \alpha \)-TTP in the liver (Bella et al., 2006). The
relative affinities of $\alpha$-TTP for the vitamers are the following: 100% for $RRR$-$\alpha$-tocopherol, 38% for $\beta$-tocopherol, 9% for $\gamma$-tocopherol, 2% for $\delta$-tocopherol, 2% for $\alpha$-tocopherol acetate, 2% for $\alpha$-tocopherol quinine, 11% for $SRR$-$\alpha$-tocopherol, 12% for $\alpha$-tocotrienol, and 9% for trolox (Hosomi et al., 1997). Co-supplementation of $\alpha$-tocopherol and $\alpha$-tocotrienol at equal amounts (2.5 mg of each vitamer/kg body weight) resulted in much higher distribution of $\alpha$-tocopherol than that of $\alpha$-tocotrienol in various organs of rats due to the differences in their binding to $\alpha$-TTP (Khanna et al., 2005). $\alpha$-Tocotrienol produced higher plasma concentrations than $\delta$- and $\gamma$-tocotrienols in a human supplementation study, in which each vitamer was administered at equal doses (250 mg/day), supporting higher affinity of $\alpha$-tocotrienol to $\alpha$-TTP than those of other tocotrienols (O’Byrne et al., 2000). Thus, the physiological levels of vitamers may depend on the differences in their affinities to $\alpha$-TTP and TAPs.

$\alpha$-Tocopherol is the most biologically and chemically active form of vitamin E. Based on rat gestation-resorption bioassay data, the biopotency of $RRR$-$\alpha$-tocopherol (1.49 IU/mg) is approximately higher than those of $RRR$-$\gamma$-tocopherol (0.05 IU/mg), $R$-$\alpha$-tocotrienol (0.32 IU/mg), and $R$-$\beta$-tocotrienol (0.05 IU/mg) (Combs, 2008).

The supplemental intake of $\alpha$-tocopherol at doses of 400–800 IU/day (or 180–360 mg/day) provided the concentrations at 40–60 μM in plasma and at 60–90 nM/10^6 human endothelial cells over a 24-hour supplementation period (Meydani, 2004). Administration of tocotrienols ($\alpha$-, $\delta$-, or $\gamma$-tocotrienol) at doses, 250 mg/day, to hypercholesterolemic subjects resulted in plasma concentrations ranging 0.09–0.98 μmol/L (O’Byrne et al., 2000). Supplementation of mixtures of tocotrienols and
tocopherols (693 mg: 319 mg) to healthy humans provided plasma concentrations at 4.79 mg/mL and 13.46 mg/mL, respectively (Fairus et al., 2006).

1.5. Excretion

Tocopherols and tocotrienols are metabolized and excreted via urine and feces when the vitamers are not recognized by α-TTP and/or α-tocopherol exceeds the binding capacity of α-TTP (e.g., high doses, >50 mg) ([Munteanu et al., 2004], [Sontag and Parker, 2007] and [Combs, 2008]). Less than 1% of the absorbed vitamin E is excreted via urine under conditions of intakes of nutritional levels (Combs, 2008). Certain vitamers, such as γ-tocopherol, all rac-α-tocopherol, and tocotrienols, are metabolized to a higher degree than α-tocopherol ([Doring et al., 2004] and [Khanna et al., 2005]). The vitamers are metabolized similarly to xenobiotics by enzymatic activity of hepatic cytochrome P450 4F2 (CYP4F2) and cytochrome P450 3A (CYP3A) through ω-oxidation, β-oxidation, and conjugation steps ([Sontag and Parker, 2007] and [Traber, 2007]).

1.6. Distribution

Vitamin E is incorporated into the liver, adipose tissues, and non-adipose cells, such as immune cells, smooth muscle cells, and platelets ([Wardlaw and Kessel, 2002] and [Meydani, 2004]). In non-adipose cells, vitamin E is located exclusively in cell and organelle membranes (probably nuclear membranes as well) with intimate contact of polyunsaturated fatty acids (PUFAs) at one molecule for every 2000 phospholipid molecules or a molar ratio of vitamin E to PUFAs, 1:850 ([World Health Organization, 2004] and [Combs, 2008]). Vitamin E is mobilized from adipose tissues by lipolysis
induced under certain conditions, such as intensive exercise, significantly increasing circulating vitamin E levels (Combs, 2008).

1.7. Dietary recommendation

The recommended dietary allowance (RDA) of vitamin E for adults is 15 mg (or 22.4 IU of the natural forms; 33.3 IU of the synthetic forms). The tolerable upper intake levels (UL) of vitamin E, 1000 mg (1500 IU of the natural forms; 2200 IU of the synthetic forms), is more than 65 times greater than the RDA. Extremely high doses of vitamin E interfere the actions of other fat-soluble vitamins, including blood-clotting action of vitamin K in patients on anticoagulant therapy ([Whitney and Rolfes, 2002] and [Combs, 2008]). Based on observational data, vitamin E exerts protective effects on coronary disease at doses of 100 to 400 IU/day (Moats & Rimm, 2007).

1.8. Genetic factors

Fat soluble vitamins, such as vitamins A and E, are transported by lipoproteins. Vitamin E homeostasis is influenced by genetic variation involved in lipid metabolism ([Doring et al., 2004] and [Borel et al., 2007]). For example, Doring et al.’s (2004) in silico study showed that the genes, encoding proteins involved in lipid and drug metabolism, such as lipoprotein lipase (LPL), multidrug resistance protein (MRP2), pregnane X receptor (PXR), cytochrome P450 3A4 (CYP3A4), and CYP4F2, were highly polymorphic. This suggests that the inter-individual genetic variation indirectly influences vitamin E homeostasis and requirements. However, the investigators concluded that genes for α-TTP and TAP, both of which directly regulate vitamin E homeostasis, were not highly
polymorphic. Borel et al. (Borel et al., 2007) demonstrated that genetic variation within the genes, which encode proteins associated with lipoprotein metabolism, such as apoA-IV and SR-BI, influenced plasma levels of vitamin E and carotenoids. Therefore, these studies may offer a partial explanation why clinical studies using heterogenous population failed to exhibit conclusive results of vitamin E supplementation, supporting the assumption that genetic variation alters responses to the supplementation and requirements (Doring et al., 2004).

2. Antioxidant function

The important facets of vitamin E include a methylated 6-chromanol nucleus with a free hydroxyl group (–OH). The hydroxyl group on C-6 is crucial in accommodating an unpaired electron (Fig. 2). Vitamin E scavenges not only free radicals but also superoxide ([Lass and Sohal, 2000] and [Visioli, 2001]). Vitamin E plays a role in protecting PUFA in cell membranes and LDL from oxidation by donating the hydrogen of the hydroxyl group, therefore, blocking the initiation and propagation of lipid peroxidation. The ability of α-tocopherol to scavenge free radicals is the greatest among tocopherols, followed by γ-, β-, and δ-tocopherols (Zingg, 2007). Upon donating the hydrogen, a radical form (e.g., α-tocopheroxyl radical) is generated. α-Tocopheroxyl radical reacts with oxidants 100 times faster than α-tocopherol (Cobbold et al., 2002). α-Tocopheroxyl radical, rather than lipid peroxyl radical (LOO•), is likely the major radical oxidant that carries the chain reaction of lipid peroxidation in human arterial wall (Stocker & Keaney, 2004). The
radical forms are regenerated to their reduced forms by another antioxidant, ascorbic acid (vitamin C), present in the aqueous environment of the intima (Cobbold et al., 2002).

Compounds with antioxidant properties, including $\alpha$-tocopherol and ascorbic acid, serve as prooxidants under certain conditions: e.g., concentrations of each compound, and the presence of oxygen and/or other oxidants and transition metals, and the absence of other antioxidants ([Bowen and Omaye, 1997], [Bowen and Omaye, 1998], [Zhang and Omaye, 2001] and [Nakamura and Omaye, 2004]). In addition, the effects of vitamin E are dose-/concentration-dependent ([Hsieh et al., 2006], [Ahn et al., 2007] and [Nakamura and Omaye, 2008a]). Therefore, a balance of the antioxidant compounds may be needed to meet optimal conditions for protecting from oxidative damage and related implications, including atherosclerosis (Nakamura, 2002).

$\alpha$-Tocotrienol is more potent antioxidant than $\alpha$-tocopherol, due to its higher recycling efficiency from chromanoxyl radicals, its more uniformed distribution in membrane bilayer, and its stronger disordering of membrane lipids (Serbinova et al., 1991). The antioxidant properties of vitamin E depend on unsaturation of isoprenoid tails which may affect mobility through the membrane and location within the membrane. $\alpha$-Tocotrienol is located closer to the membrane surface, serving as a more efficient antioxidant than $\alpha$-tocopherol (Suzuki et al., 1993). The unsaturated isoprenoid tails of tocotrienols also inhibit their own metabolism ($\omega$-oxidation) at high concentrations by disrupting the electron-transfer system of CYP activity, exhibiting vitamer-dependent effects (Sontag & Parker, 2004). In addition, tocotrienols possess powerful neuro-protective, anti-cancer,
and cholesterol lowering properties that often differ from the properties of tocopherols (Sen et al., 2006).

3. Non-antioxidant function

3.1. Redox-sensitive transcription factors: PPARγ and NF-κBp50/p65

There are two major redox-sensitive signaling pathways related to the atherogenic, carcinogenic, and inflammatory processes: PPARγ- and NF-κBp50/p65-mediated pathways.

PPARs are a group of nuclear receptors and belong to the steroid hormone receptor superfamily. There are three isoforms: PPARα, PPARβ, and PPARγ. PPARs heterodimerize with the 9-cis retinoic acid receptor (RXR: retinoid X receptor) and bind to peroxisome proliferator response elements (PPREs: 5′-AGGTCAnAGGTCA-3′) located in enhancer sites of target genes. PPARs induce target gene products by their activators, such as fatty acids, fatty acid derivatives (e.g., eicosanoids, prostaglandins) and numerous structurally dissimilar xenobiotics (peroxisome proliferators, PPs). PPARα and PPARγ are expressed in vascular endothelial cells and smooth muscle cells as well as adipose tissues ([Berger and Moller, 2002] and [Hwang et al., 2005]). PPARα and PPARγ play a role in inflammation, adipogenesis, and insulin sensitization. Synthetic PPARγ activators, such as TZDs, have shown various effects: (1) adipocyte differentiation/lipid metabolism; (2) anti-inflammation; (3) anti-diabetes; and (4) anti-atherosclerosis (Blaschke et al., 2006). In addition, the effects of a group of PPAR
activators and PPs, may be species- and dose-dependent due to their diverse metabolic profiles (O’Brien et al., 2005).

Besides roles of vitamin E as antioxidants ([Tafazoli et al., 2005] and [Tucker and Townsend, 2005]), recent studies suggest the potential of tocopherols as a gene regulator, possibly through PPARγ activation because of the structural similarity of tocopherols to a synthetic PPARγ activator, troglitazone ([Azzi and Stocker, 2000], [Campbell et al., 2003], [Munteanu et al., 2004], [Zingg and Azzi, 2004] and [De Pascale et al., 2006]). Troglitazone is used as an anti-diabetic and anti-inflammatory drug. Campbell et al.’s study (Campbell et al., 2003) showed that both α- and γ-tocopherols increased PPARγ mRNA and protein expression in SW 480 human colon cancer cells, suggesting that the tocopherols up-regulate PPARγ expression and serve as PPARγ activators to regulate target gene expression. The investigators also speculate that the tocopherol-induced PPARγ activation plays a role in preventing cancer and inflammatory diseases, both of which are associated with ROS generation. The link between tocotrienols and PPARγ has been reported in very few studies so far. γ-Tocotrienol treatment at 2.4 μmol/L, but not α-tocopherol, suppressed differentiation of 3T3-L1 preadipocytes to adipocytes by partially lowering PPARγ protein levels, suggesting anti-diabetic effects in an vitamer-specific manner (Uto-Kondo et al., 2009).

NF-κB is another redox-sensitive transcription factor expressed in all cell types. NF-κB recognizes and binds to specific DNA sequences (5′-GGGRNNYYCC-3′). NF-κB activation is regulated by intra-/extra-cellular ROS and/or ROS-modified target biomolecules. NF-κB is involved in regulating immune and inflammatory responses. NF-
κB-mediated target genes include: inflammatory cytokines \([e.g.,\) tumor necrosis factor-\(α\) (TNF-\(α\) interleukin-1 (IL-1), IL-2, macrophage colony stimulating factor (M-CSF)], chemokines \([e.g.,\) monocyte chemotactic protein-1 (MCP-1)], adhesion molecules \([e.g.,\) intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1)], inflammatory enzymes \([e.g.,\) inducible nitric oxide synthase (iNOS), cyclooxygenase-2 (COX-2)], and apoptotic regulators \([e.g.,\) Fas ligand, Fas, p53] (Robbesyn et al., 2004).

Two forms of NF-κB have been investigated: NF-κB p50/p65 heterodimer and NF-κBp50 homodimer, though the former type has been intensively studied. Both NF-κB p50/p65 heterodimer and NF-κBp50 homodimer play a role in regulating inflammatory gene expression probably in an opposite manner ([Kuwata et al., 2003], [Driessler et al., 2004], [Wessells et al., 2004] and [Cao et al., 2006]). The NF-κBp50 subunit DNA binding activity is stimulated by reducing agents (Matthews et al., 1993), including probably \(α\)-tocopherol. NF-κBp50 homodimer competes for the binding sites with transactivating NF-κBp50/p65 heterodimer (Muller et al., 1995); therefore, NF-κB p50 homodimer may inhibit pro-inflammatory gene expression induced by NF-κB p50/p65 activation. Interleukin-10 induced-anti-inflammatory effects include selective induction of NF-κBp50 homodimer DNA binding, resulting in inhibition of transactivation of NF-κB p50/p65 heterodimer transactivation (Driessler et al., 2004).

A GeneChip analysis study, using \(α\)-tocopherol transfer protein knockout mice \((α\)-tocopherol deficient mice\) exposed to cigarette smoke, identified more than 2000 genes modulated by \(α\)-tocopherol in lung tissue. The genes identified in the study include
immune-inflammatory genes, which are regulated through the NF-κB pathway (Gohil et al., 2007).

The inhibitory effects of vitamin E on NF-κBp50/p65 activation have been observed in previous studies. According to Gluert’s review, vitamin E, in particular at high levels, inhibits NF-κBp50/p65 activation in various models. However, it remains to be determined whether the inhibition of NF-κBp50/p65 activation of vitamin E is due to decreased oxidative stress and/or one of vitamin E’s non-antioxidant functions (Glauert, 2007). Traber and Atkinson conclude that vitamin E-, rather than oxidative stress-, mediated signaling pathways are questionable in their review (Traber & Atkinson, 2007). α-Tocopherol dose-dependently regulates signal transduction pathways by mechanisms that are independent of its antioxidant properties at concentrations of 10–50 μmol/L in rat smooth muscle cells (Azzi et al., 1995). Calfee-Mason et al. (Calfee-Mason et al., 2008) reported that dietary α-tocopherol increased apoptosis and decreased oxidative stress and cell proliferation in the absence of the p50 NF-κB subunit, suggesting α-tocopherol-induced pathways independent on NF-κB activation. Another study using cancer cell lines proposes that the involvement of the AP-1 pathway as well as the NF-κB pathway in α-tocopheryl succinate-induced anti-tumor activity (Crispen et al., 2007).

Tocotrienols regulate signal transduction and gene expression at nano-molar or very low micro-molar levels ([Sen et al., 2000] and [Shibata et al., 2008]). Anti-cancer effects of tocotrienols, in particular γ-tocotrienol, in several cell lines have been reported ([Shah and Sylvester, 2005], [Shibata et al., 2008], [Yap et al., 2008] and [Xu et al., 2009.]). γ-Tocotrienol inhibits TNF-α-induced NF-κB activation through inhibition of IκB kinase
activation in a dose-/time-dependent manner, indicating both anti-cancer and anti-inflammatory effects. The inhibition of NF-κB is not cell type-specific (Ahn et al., 2007). Another study (Wu et al., 2008b) also showed anti-inflammatory effects of tocotrienol-rich fraction (TRF; 0.5–5.0 μg/mL) in a dose-dependent manner by inhibiting NF-κB expression in human monocytes.

3.2. Vitamin E-mediated gene expression

3.2.1. Antioxidant enzymes through PPARγ and NF-κB activation

The genes for antioxidant enzymes, Cu/Zn superoxide dismutase (SOD) and catalase, may be regulated through PPARγ activation, because PPREs are located in the promoter regions of human Cu/Zn-superoxide dismutase (SOD1) and rat catalase ([Yoo et al., 1999], [Girnun et al., 2002], [Hwang et al., 2005] and [Nakamura and Omaye, 2008a]). Several studies have demonstrated the beneficial effects of over-expression and/or induction of Cu/Zn SOD and catalase in endothelial cells: (1) decrease in superoxide levels in endothelial cells, (2) suppression of age-related oxidative stress, (3) protection against inflammatory events by inhibiting NF-κB p50/p65 heterodimer activation, and (4) suppression of LDL oxidation ([Yoo et al., 1999], [Inoue et al., 2001], [Girnun et al., 2002], [Kim et al., 2004] and [Hwang et al., 2005]). As described above, vitamin E possesses a structural similarity to a PPARγ activator, troglitazone. The evidence supports the links between α-tocopherol, induction of Cu/Zn SOD and catalase, and PPARγ activation.
Using human umbilical vein endothelial cells (HUVECs) treated with graded concentrations (0–100 μmol/L) of α-tocopherol, we observed five binding sites for PPARγ and six for NF-κB in the promoter region (up to 5000 base upstream) of Cu/Zn SOD gene and one for NF-κB in the promoter region (up to 10,000 base upstream) of catalase gene by a computational analysis. α-Tocopherol treatment at the lowest concentration (10 μmol/L) increased gene/protein expression of Cu/Zn SOD and catalase, the DNA binding activities of PPARγ and NF-κB p50, and lipid peroxidation. The results of our study are consistent with that: (1) α-tocopherol may modulate Cu/Zn SOD and catalase through PPARγ and NF-κB in vascular cells in a concentration-dependent manner, (2) α-tocopherol as a PPARγ activator may up-regulate Cu/Zn SOD as one of the anti-inflammatory gene products via PPARγ activation, and (3) α-tocopherol as a prooxidant (due to autooxidation at the lowest concentration of α-tocopherol) may up-regulate both Cu/Zn SOD and catalase as pro-inflammatory gene products via NF-κB p50/p65 activation. NADPH oxidase and Cu/Zn SOD serve as microcidal ROS sources in innate immunity, generating superoxide and hydrogen peroxide, respectively (Janeway et al., 2005). Therefore, it would not be surprising if the expression of antioxidant enzyme Cu/Zn SOD is also regulated through NF-κBp50/p65 activation involved in regulating pro-inflammatory gene expression. Subsequently, the up-regulation by combinational and/or feedback effects of PPARγ and NF-κB may reduce ROS generation. Our speculation that α-tocopherol-mediated regulation of Cu/Zn SOD through PPARγ is partly supported by the Gohil’s study (Gohil et al., 2007) in which α-tocopherol deficiency models did show the induction of PPARα, mitochondrial manganese SOD (SOD2), and extracellular Cu/Zn SOD (SOD3), but not the induction of PPARγ and
Cu/Zn SOD (SOD1). Thus, α-tocopherol appears to be involved in regulating expression of the antioxidant enzymes, such as Cu/Zn SOD and catalase, via the redox sensitive transcription factors, PPARγ and NF-κB.

A few earlier studies ([Newaz and Nawal, 1999], [van Haaften et al., 2002] and [Lqbal et al., 2004]) have demonstrated effects of tocotrienols on the activities of antioxidant enzymes, though the association of tocotrienols with the expression of antioxidant enzymes has not been documented so far. Both tocopherols and tocotrienols inhibit glutathione S-transferase activity by binding to the enzyme at a hydrophobic pit which the isoprenoidal tails of both tocopherols and tocotrienols fit in van Haaften et al. (2002). Dietary administration of γ-tocotrienol (15–150 mg/kg diet) increases total antioxidant status, including SOD activity, and decreases lipid peroxidation in hypertensive rats (Newaz & Nawal, 1999). TRF treatment reduces glutathione S-transferase activity in rat livers. This reduction may be due to a decrease in toxic substance generation during hepatocarcinogenic processes, suggesting its protective effects on hepatocarcinogenesis (Lqbal et al., 2004).

3.2.2. ROS generating enzymes

Enzymatic sources of ROS include cyclooxygenase (COX), lipoxygenase (LO), NADPH oxidase, and cytochrome P450s (CYPs) ([Li and Shah, 2004] and [Singh and Jialal, 2006]). All of these enzymes are involved in inflammatory processes.

3.2.2.1. Cyclooxygenases (COXs)
COXs catalyze the rate-limiting conversion of free PUFAs to prostanoids, leading to ROS generation. There are three COX isoforms: COX-1, COX-2, and COX-3. COX-1 is constitutively expressed in most cell types. COX-1 maintains prostaglandin levels for cellular homeostasis and platelet aggregation. COX-3 is a variant of COX-1. COX-3 mRNA is expressed mainly in the cerebral cortex and heart (Chandrasekharan et al., 2002).

COX-2 is an immediate early gene whose expression is induced by a variety of pro-inflammatory stimuli, such as pathogens (Combs et al., 2001). Inductive COX-2 plays a role in inflammation by producing larger amounts of prostaglandin E2 (PGE2) than constitutive COX-1 (Iwakiri et al., 2002). COX-2 is preferentially located on nuclear membranes allowing cyclopentenone prostaglandins to directly enter the nucleus and bind to ligand-activated transcription factors (Eibl, 2008). Previous studies have proposed a link between vitamin E, activation of PPARγ and NF-κB, and anti-inflammatory effects, in particular by the inhibition of COX-2 discussed below.

PPARα and PPARγ activators inhibit COX-2 gene expression under certain conditions in various models, exhibiting anti-inflammatory, anti-carcinogenic, and anti-atherogenic effects.

Troglitazone inhibits cell proliferation and induce apoptosis in HepG2 cells partially by down-regulating COX-2 expression (Li et al., 2003). Similarly, 15-deoxy-delta (12,14)-prostaglandin J2 (15d-PGJ2), a natural PPARγ activator, and troglitazone decrease COX-2 expression in human monocyte leukemia cells in vitro, resulting in apoptosis of the cancer cells (Liu et al., 2007). Kulkarni et al.’s study suggests an inverse relationship
between expression of COX-2 and PPARγ and the potential uses of COX-2 and PPARγ expression as biological markers in cancer studies (Kulkarni et al., 2008).

15d-PGJ2 is also a product of COX-2 activity. 15d-PGJ2 has anti-inflammatory properties. Troglitazone and 15d-PGJ2 down-regulate COX-2 expression of cardiac myocytes in a PPARγ dependent manner, exerting anti-inflammatory effects (Mendez & LaPointe, 2003). A PPARα activator, WY14643, inhibits β-amyloid-stimulated gene expression of COX-2, TNF-α, and IL-6, and macrophage differentiation in a dose-dependent manner (Combs et al., 2001).

Conjugated linoleic acids (CLAs) are a group of positional and geometric isomers of linoleic acid (LA). CLA isomers are possible PPARα and PPARγ activators (Belury, 2002). In addition, certain PUFAs, including AA, eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA), and CLA derivatives (conjugated arachidonate), can serve as substrates for COX enzymes, since the PUFAs can be incorporated into cell membranes ([Belury, 2002], [Eibl, 2008] and [Nakamura and Omaye, 2008b]). CLA appears to be a potent free-radical-scavenging agent, which suggests that some CLA-mediated effects may be operated through antioxidant mechanisms (Iwakiri et al., 2002). For example, CLA isomer treatments induce the expression of antioxidant enzymes, Cu/Zn SOD and catalase, in HUVECs, possibly through PPARγ and NF-κB, in a concentration-dependent manner: CLA isomer treatments at high concentrations result in decreased lipid peroxidation, and CLA isomer treatments at low concentrations result in increased lipid peroxidation or autooxidation due to susceptibility of CLA to oxidation. The CLA isomer treatments at high concentrations may induce expression of Cu/Zn SOD
through PPARγ activation, while the CLA isomer treatments at low concentrations may induce expression of Cu/Zn SOD and catalase through NF-κB (Nakamura & Omaye, in press). We have speculated that CLA isomers, as well as α-tocopherol (Nakamura & Omaye, 2008a), may involve the control of redox status partially by regulating genes, whose products influence ROS generation, through redox-sensitive transcription factors, PPARγ and NF-κB.

COX-2 activity inhibition mediated by α-tocopherol contributes to anti-atherogenesis. Wu et al. demonstrated that α-tocopherol dose-dependently inhibited COX activity, not the expression of either COX-1 or COX-2 in human aortic endothelial cells, indicating that the α-tocopherol affected COX activity post-translationally. They also showed that α-tocopherol increased production of vasodilators, prostaglandins E2 and I2, both of which are products of COX activity. The investigators conclude that the net increase in prostaglandin synthesis contributes to preservation of endothelial functions (Wu et al., 2005).

In Egger et al.’s study using mouse neural cells, BV-microglia, α-tocopherol up-regulated microglial protein phosphatase 2A (PP2A) and down-regulated both protein kinase C (PKC) activity and NF-κBp50/p65 activation, resulting in decreases in COX-2 protein levels and prostaglandin E2 production. This suggests that α-tocopherol exerts specific neuro-protective properties and that α-tocopherol could be implicated in prevention of inflammatory and neurodegenerative diseases, such as Alzheimer’s disease (Egger et al., 2003).
COX-2 expression and/or activity are also inhibited by other vitamers. γ-Tocopherol, but not α-tocopherol, inhibits COX-2 activity in lipopolysaccharide (LPS)-activated macrophages IL-1β treated epithelial cells in a non-antioxidant-dependent manner (Jiang et al., 2000). In addition, γ-tocopherol decreases production of pro-inflammatory eicosanoids (prostaglandin E2 and leukotriene B4) and inflammatory damage in rats (Jiang & Ames, 2003). Ahn et al. (2007) demonstrated that γ-tocotrienol, not γ-tocopherol at the same concentration, suppressed NF-κB p50/p65 activation and expression of NF-κB p50/p65-regulated gene products, including COX-2 and ICAM-1, by inhibiting TNF-α-induced phosphorylation and degradation of IκBα, activation of IκBα kinase, and phosphorylation of NF-κB p65. The investigators also showed that glutathione, another antioxidant, had no effects on the γ-tocotrienol-induced down-regulation of NF-κB p50/p65, suggesting that the down-regulation of NF-κB p50/p65 by γ-tocotrienol is not due to alteration of redox status, i.e. non-antioxidant function of the vitamin E analogue (Ahn et al., 2007). δ-Tocotrienol suppresses COX-2 protein expression induced by hypoxia in adenocarcinoma cells, suggesting anti-carcinogenic effects of δ-tocotrienol through the inhibition of angiogenesis (Shibata et al., 2008). TRF results in anti-inflammatory effects by inhibiting expression of both NF-κB and COX-2 in human monocytes (Wu et al., 2008b).

3.2.2.2. Lipoxygenases (LOs)

LOs, iron-containing enzymes, play a catalytic role in the conversion of PUFAs to leukotrienes. LOs also contribute to ROS generation. Devaraj and Jialal demonstrated that α-tocopherol, as well as a 5-LO inhibitor, decreased TNF-α mRNA and protein from
lipopolysaccharide (LPS)-activated human monocytes by inhibiting 5-LO and NF-κB p50/p65 DNA binding activity. The expression of TNF-α appears not to be due to decreased ROS by antioxidant activity of α-tocopherol because other antioxidants, including β-tocopherol, ascorbate, and SOD, failed to exhibit the inhibitory effects (Devaraj & Jialal, 2005). This supports the specific non-antioxidant role of α-tocopherol.

The link between vitamin E and 12/15-lipoxygenase (12/15-LO) has been also reported. In an atherosclerosis-prone mouse study conducted by Zhao et al. (2005), 12/15-LO gene disruption and vitamin E administration (2000 IU/kg diet) reduced atherosclerosis and oxidative stress. However, vitamin E did not show any additional protective effects in the models. This suggests the combinational protection mechanism of vitamin E and 12/15-LO gene disruption. The investigators conclude that 12/15-LO-initiated lipid peroxidation in vivo results in specific oxidized products that participate in chain reactions and decompose to secondary oxidative reaction products susceptible to vitamin E action.

LO sensitivity to vitamin E has been reported. α-Tocopherol binds to a single peptide of 5-LO and irreversibly inhibits its activity in an antioxidant-independent manner (Reddanna et al., 1985). Khanna et al.’s in silico docking studies (Khanna et al., 2003) indicate that α-tocotrienol inhibits the access of arachidonic acid (AA) to the catalytic site of 12-LO by binding to the opening of a solvent cavity close to the active sites.

**3.2.2.3. Resolution lipid mediators**

COX-2 and LOs play a role not only in pro-inflammatory processes, but also an anti-inflammatory (and ultimately anti-atherogenic) process, defined as resolution. Signaling
pathways leading to pro-inflammatory PGE2 to PGD2 actively switch on the transcription of enzymes required for the generation of other classes of AA-derived eicosanoids, such as lipoxins, resolvins, and protectins. Resolvin E and D series and protectins are newly discovered families of lipid mediators which promote the resolution of inflammation by stopping the further recruitment of leukocytes. In the presence of aspirin, acetylated COX-2 converts from EPA and DHA to 18R-hydroperoxyeicosapentaenoic acid [H(p)EPE: a precursor of resolving E1] or 17R-H(p)DHA (a precursor of resolvin D series), respectively, in vascular endothelial cells. The 17R-hydroperoxy precursors, rather than 17S-hydroperoxy precursors, are considered aspirin-triggered lipid mediators (Serhan & Savill, 2005).

The link between the anti-inflammatory process and PPARγ has been documented. Chene et al. reported that γ-linolenic acid (GLA, omega-6) and EPA (omega-3) increased PPARγ activity and induced COX-2 expression via PPARγ activation in human keratinocyte HaCaT cells. The investigators concluded that the induction of COX-2 might be important in the anti-inflammatory and protective mechanism of action of the PUFAs (Chene et al., 2007).

The failure of local endogenous resolution may be implicated in the progress of atherosclerosis. 12/15-LO plays a role in not only the pathogenesis of degenerative diseases (Zhao et al., 2005) but also in the synthesis of anti-inflammatory and pro-resolving lipid mediators, protectins and resolvin D series from DHA. Merched et al. documented that 12/15-LO expression might protect mice against atherosclerosis by producing protectins and resolving D series (Merched et al., 2008).
Genetic variance of 12/15-LO has been reported. Assimes et al. identified a SNP in \textit{ALOX15} which impairs the enzymatic activity of 12/15-LO. The null variant (T560 M) was the most common in Hispanics followed by white/Europeans among the tested population. The variant does not protect against coronary artery disease (CAD) and increases the risk of CAD (Assimes et al., 2008). Hence, the regulation of 12/15-LO expression and/or the use of stable forms of the lipid mediators may be preventive and/or therapeutic strategies for atherosclerosis (Merched et al., 2008).

\textbf{3.2.2.4. Cytochrome P450 monooxygenases (CYPs)}

CYPs are heme-containing enzymes involved in the metabolisms of endogenous and exogenous lipophilic compounds, such as drugs, environmental chemicals, hormones, and fatty acids (Tompkins & Wallace, 2007). CYPs serve as ROS sources, forming reactive intermediate metabolites ([Gottlieb, 2003], [Yasui et al., 2005] and [Nebert and Dalton, 2006]). The CYP 4 family participates in eicosanoid synthesis when COX and LO activities are minimal. In addition, CYP4F2 and CYP3A4 are possible enzymes for vitamin E metabolism ([Kluth et al., 2005] and [Sontag and Parker, 2007]). The CYP1 family plays a role in xenobiotic metabolism.

\(\alpha\)-Tocopherol-mediated expression of CYP families has been reported. \(\alpha\)-Tocopherol increased both protein and catalytic activity levels of CYP1 and CYP2 in rat livers in a dose-dependent manner (Sidorova et al., 2003). \(\alpha\)-Tocopherol inhibited benzo(a)pyrene (BP)-induced CYP1A1 in rat livers (Sidorova & Grishanova, 2005). BP is a polycyclic aromatic hydrocarbon (PAH). PAHs are common environmental pollutants and are metabolized by CYP1A1. This inhibitory effect appears to occur post-translationally,
suggesting that α-tocopherol serves as an inhibitor of CYP1A1 activity and that the inhibitory effect is not associated with the progression of free radical processes. α-Tocopherol prevents the adverse effect of BP by inhibiting CYP1A1 activity in the liver of BP-treated animals though α-tocopherol-induced CYP1A1 activity in the liver of intact animals (Sidorova & Grishanova, 2005). These studies indicate that α-tocopherol acts as a regulator of genes (transcriptionally) and/or their products (post-translationally) and that, at least, the post-translational inhibition is mediated through a free radical and/or ROS-independent mechanism.

Human CYP3A4 is responsible for the metabolism of numerous xenobiotics in the human liver (Zhang et al., 2003). All forms of vitamin E activate gene expression involved in drug metabolism, including CYP3, via the pregnane X receptor (PXR) (Landes et al., 2003). α-Tocopherol, not γ-tocotrienol, increased mRNA levels of Cyp3a11, the analogue of human CYP3A4, at 2.5-fold in mice fed different amounts of RRR-α-tocopherol-containing diet, through PXR activation (Kluth et al., 2005). All four tocotrienols (α-, β-, δ-, and γ-tocotrienols), but not tocopherols, specifically bind to and activate steroid and xenobiotic receptor (SXR) and up-regulate its target gene, CYP3A4, at low micromolar concentrations. The up-regulation is in a tissue-dependent manner (Zhou et al., 2004).

3.2.2.5. NADPH oxidase

Membrane-bound NADPH oxidases are major sources of superoxide in the vasculature. Cachia et al. demonstrated that α-tocopherol inhibited p47 (phox) translocation to the membrane and p47 (phox) phosphorylation in human monocytes by the reduction of protein kinase c (PKC) activity, resulting in decreased superoxide production. The
investigators speculate that the inhibition of PKC activity is due to an interaction between the α-tocopherol molecular structure and PKC, but not directly due to the antioxidant capacity of α-tocopherol (Cachia et al., 1998). Phagocyte NADPH oxidase genes, p47 (phox), p67 (phox), and gp91 (phox), are up-regulated by TNF-α via the NF-κB pathway in human monocyctic cells and monocytes (Gauss et al., 2007).

Transactivation of AP-1, another redox-sensitive transcription factor, is linked to ROS generation and oxidative stress. The transactivation of AP-1 and the production of IL-8 (or CXCL8) induced by UVA in human keratinocytes are inhibited by α-tocopherol, probably via modulating NADPH oxidase activity (Wu et al., 2008a). It has been speculated that the protective effects of α-tocopherol are attributed to its physical and chemical properties, which are involved in antioxidant and non-antioxidant pathways, and that α-tocopherol interacts with endogenous antioxidant enzymes, which are involved in recycling other antioxidants. We proposed that the balance between α-tocopherol and other antioxidants may be a determinant for the functions of α-tocopherol: as an antioxidant to reduce ROS and as a direct gene regulator via the pathways of redox-sensitive transcription factors, PPARγ and NF-κB (Nakamura & Omaye, 2008a). No association of tocotrienols with NADPH oxidase has been documented in PubMed so far.

4. Conclusion

The modes of action of vitamin E may be various regarding the pathology of ROS-related diseases. The effects of vitamin E may be multi-functional: prooxidant and/or
antioxidant, inhibitor of ROS-generating enzyme activities, and inducer and/or inhibitor of gene and protein expression. The effects may depend on microenvironments, such as the presence of oxidants or other antioxidants, the concentrations of vitamin E, and the balance between concentrations of vitamin E and other antioxidants. Therefore, optimal conditions may need to be explored carefully, accounting for such differences in microenvironments.

Vitamin E-mediated gene expression appears to be regulated, at least, via activation of redox-sensitive transcription factors, including PPARγ and NF-κB, whose target genes are associated with ROS generation. Therefore, vitamin E may directly and indirectly control redox status by scavenging free radicals and superoxide and by regulating genes, whose products influence ROS generation, through PPARγ and NF-κB in a combinational and/or feedback-loop manner. Vitamin E may be involved in modulating entire antioxidant defense system in which vitamin E induces endogenous antioxidant enzymes when exogenous antioxidants are imbalanced or limited to reducing ROS. Thus, the vitamin E-mediated gene expression may be either stimulatory or inhibitory, depending on its oxidative status or its concentrations. Further investigation is warranted to determine non-antioxidant roles of vitamin E and how such roles may be favorable by new therapeutically approaches.
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Fig. 1. Vitamin E structures.
Fig. 2. Antioxidant roles of α-tocopherol. α-Tocopherol scavenges radicals in lipid peroxidation.
Chapter 4

Conjugated Linoleic Acid Study

Published as

Conjugated Linoleic Acid isomers’ Roles in the Regulation of PPAR-γ and NF-κB DNA Binding and Subsequent Expression of Antioxidant Enzymes in Human Umbilical Vein Endothelial Cells

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in

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Abstract

Objective

Conjugated linoleic acid (CLA) isomers have shown health benefits. Because CLA isomers may act as activators for peroxisome proliferator-activated receptors and may induce antioxidant enzymes, this study was conducted to examine the effects of CLA isomers on the gene expression of antioxidant enzymes, copper/zinc superoxide dismutase, and catalase in human umbilical vein endothelial cells.

Methods

Human umbilical vein endothelial cells were treated with graded concentrations of the 9-cis, 11-trans or the 10-trans, 12-cis-CLA isomer for 24 h.

Results

The 9-cis, 11-trans-CLA treatments resulted in increases in transcription factor DNA binding activities and expression of antioxidant enzymes at 0–25 μmol/L and an increase in lipid peroxidation only at the lowest concentrations (5 μmol/L). The 10-trans, 12-cis-CLA treatments resulted in increases in transcription factor DNA binding activities at 0–25 μmol/L and highest levels of mRNA of both antioxidant enzymes, superoxide dismutase protein, and lipid peroxidation only at the lowest concentrations (5 μmol/L). The 9-cis, 11-trans-CLA treatments produced expression of antioxidant enzymes, except catalase protein, that were positively correlated with lipid peroxidation. Positive correlations were found between expression of antioxidant enzymes, except catalase protein, and lipid peroxidation for 10-trans, 12-cis-CLA treatments. Although CLA
isomers exhibit mostly stimulatory effects in expression of antioxidant enzymes, interestingly, the lowest concentrations of both CLA isomers resulted in increases in thiobarbituric acid-reactive substance levels.

**Conclusion**

An understanding of the optimal concentrations of CLA isomers, which stimulate the benefits of antioxidant enzyme induction, may require careful CLA titration to determine predictable and dependable therapeutic strategies against adverse effects, such as pro-oxidants.

**Keywords:** Conjugated linoleic acid; Peroxisome proliferator-activated receptor-γ; Endothelial cells; Nuclear factor-κB; Superoxide dismutase; Catalase
Introduction

Conjugated linoleic acid (CLA) isomers are a group of polyunsaturated fatty acids, which are a mixture of positional and stereoisomers of conjugated dienoic octadecadienoate (18:2; linoleic acid). CLA isomers are found in ruminant food products, such as beef and dairy products; they are naturally produced by bacterial hydrogenation and isomerization in the ruminant gut [1]. Although there are 28 different CLA isomers, the cis-9, trans-11 CLA isomer is the predominant compound found in ruminant food products and accounts for >90% of the CLA intake of the human diet [2]. CLA isomers have exhibited many potential health benefits in animal and in vitro studies, such as lean body mass deposition, antidiabetes, anti-inflammation, anticarcinogenesis, and antiatherogenesis [1], [2], [3], [4], [5] and [6].

The CLA isomers may modulate free-radical–induced oxidation by their antioxidant properties, thus serving as an anti-initiator in carcinogenesis [7]. Also, the ability of CLA isomers to decrease aortic plaque formation could be due to changes in low-density lipoprotein (LDL) oxidative susceptibility [4]. CLA isomers induce glutathione (a reducing compound) synthesis without lipid peroxidation in human fibroblasts [8]. In mouse macrophage cells, CLA decreases mRNA expressions of cyclo-oxygenase-2 and inducible nitric oxide synthase, which are sources of oxidants, i.e., oxidative stress [9]. Likewise, the activities of antioxidant enzymes, superoxide dismutase (SOD), catalase, and glutathione peroxidase are induced in cancer cells by CLA isomers [10]. In contrast, CLA treatment increases reduced nicotinamide adenine dinucleotide phosphate (NADPH) oxidase activity and depletes the intracellular pool of reduced glutathione [11].
Of interest are the findings that high concentrations of CLA isomers or \( \alpha \)-tocopherol alleviate CLA-induced cytotoxicity and caspase-related apoptosis in rat hepatoma cells [12] and the antioxidants, \( \alpha \)-tocopherol and butylated hydroxytoluene, attenuate the antiproliferative and proapoptotic effects of CLA isomers in bovine aortic endothelial cells [3]. Furthermore, CLA modulates intracellular reactive oxygen species (ROS) synthesis and cytoplasmic phospholipase A\(_2\) activity, which are associated with oxygenation of arachidonic acid, contributing to the apoptotic process in human macrophages and thus the anti-inflammatory effects of CLA [13]. Overall, these studies suggest a role for CLA in modulating redox balance and support the need for further investigations.

Recently research has focused on the role of CLA isomers as activators for peroxisome proliferator-activated receptors (PPARs) and subsequent modulation of gene expression through PPAR-\( \gamma \) activation. PPAR-\( \gamma \) is one of three PPAR isomers (PPAR-\( \alpha \), PPAR-\( \beta \), and PPAR-\( \gamma \)), a group of nuclear receptors that belong to the steroid hormone receptor superfamily [6]. PPARs heterodimerize with the 9-\( \text{cis} \) retinoic acid receptor (RXR) and bind to peroxisome proliferator response elements (PPREs; 5\( \text{\prime} \)-AGGTCAnAGGTCA-3\( \text{\prime} \)), which are located in enhancer sites of target genes. PPAR-\( \gamma \) plays a role in inflammation, adipogenesis, and insulin sensitization. Thiazolidinediones, a group of synthetic PPAR-\( \gamma \) ligands, have beneficial effects as atheroprotective drugs. In vascular smooth muscle cells, CLA isomers have been shown to increase PPAR-\( \gamma \) DNA binding activity and decrease the DNA binding activity of nuclear factor-\( \kappa \)B (NF-\( \kappa \)B), another transcription factor [14]. Coincidentally, NF-\( \kappa \)B is thought to be a redox-regulated transcriptional
factor expressed in many types of cells and plays a role in regulating the immune response to infection [6] by controlling cell signaling and tumor necrosis factor-α [15].

The antioxidant enzymes SOD and catalase may be regulated by PPAR-γ activation, because PPREs are located in the promoter regions of human copper/zinc SOD (Cu/Zn-SOD; SOD1) and rat catalase [16], [17] and [18]. PPAR-γ is expressed in vascular endothelial cells, smooth muscle cells, adipocytes, and myeloid cells [16]. Overexpression and/or induction of Cu/Zn-SOD and catalase can be beneficial because of 1) decreases in superoxide levels in endothelial cells; 2) suppression of oxidative stress, e.g., age related; 3) protection against inflammatory events by inhibiting NF-κB activation; and 4) suppression of LDL oxidation by endothelial cells [16], [17], [18], [19] and [20]. Overall, this procession of evidence suggests more than a causal link among CLA isomers, expression of antioxidant enzymes, and PPAR-γ activation. The link may be similar to a variety of ligands/activators of PPAR-inducing expression of SOD and catalase [16], [20], [21], [22] and [23]. As part of our investigations of CLA isomers, we undertook the present study to evaluate the concentration-dependent role of 9-cis, 11-trans- and 10-trans, 12-cis-CLA isomers in the regulation of PPAR-γ and NF-κB DNA binding, subsequent expression of SOD and catalase mRNA and protein, and changes in lipid peroxidation (thiobarbituric acid-reactive substance [TBARS]) as an indicator of ROS generation in human umbilical vein endothelial cells (HUVECs).
Materials and methods

Chemicals and reagents

The CLA isomers (9-cis, 11-trans-CLA and 10-trans, 12-cis-CLA) were purchased from Cayman Chemical (Ann Arbor, MI, USA). MCDB131 and 0.25% trypsin/ethylene-diaminetetra-acetic acid were purchased from Invitrogen (Carlsbad, CA, USA). EGM-1 SingleQuiot (fetal bovine serum, human epidermal growth factor, and hydrocortisone), gentamicin, amphotericin-B, vascular endothelial growth factor, human fibroblast growth factor-basic, insulin-like growth factor recombinant 3, ascorbic acid and heparin were purchased from Cambrex Bio Science (Walkersville, Inc., Walkerville, MD, USA). Diethanolamine, trichloroacetic acid, 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid, Tween 20, phosphate buffered saline (PBS), ethyl alcohol, bovine serum albumin, and glutamine were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Carbon dioxide was purchased from local distributors. NaH2PO4, KH2PO4, NaCl, and KCl were purchased from Fisher Chemicals (Springfield, NJ, USA). The nuclear extract and PPAR-γ and NF-κB transcription factor assay kits were purchased from Active Motif (Carlsbad, CA, USA). The SuperScript III CellsDirect cDNA Synthesis System and primers for SOD1 and catalase were purchased from Invitrogen. The SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, CA, USA). The enzyme-linked immunosorbent assay kit (ExtrAvidin Alkaline Phosphatase Staining Kit–Rabbit) was purchased from Sigma-Aldrich Chemical Company. Primary antibodies for Cu/Zn-SOD and catalase were purchased from Fitzgerald Industries.
International, Inc. (Concord, MA, USA) and EMD, Inc. (San Diego, CA, USA), respectively. The SOD and catalase assay kits were purchased from Cayman Chemical.

Cell culture

The HUVECs (Clonetics No. CC-2517), preserved cryogenically, were purchased from Cambrex Bio Science Walkersville, Inc. After thawing, cells were grown in MCDB131 (Invitrogen) supplemented with 2 mmol/L of l-glutamine and EGM-2 SingleQuot (Clonetics CC-4176) containing 2% fetal bovine serum (final concentration), human epidermal growth factor, hydrocortisone, gentamicin, amphotericin-B, vascular endothelial growth factor, human fibroblast growth factor-basic, insulin-like growth factor recombinant 3, ascorbic acid, and heparin. The cells were plated on 75-cm² gelatin-coated (0.5%) flasks and maintained until at 37°C in a humidified atmosphere of 5% CO₂ until confluent. The cells were then subcultured by trypsin, and an aliquot of the cells was cultured in 24-well gelatin-coated plates for RNA isolation and subsequent real-time polymerase chain reaction (PCR) analysis. The tightly confluent monolayers of the fourth to seventh passages were used for the experiments.

Cell treatments

The HUVECs were plated on 75-cm² gelatin-coated flasks or 24-well plates, and confluent cells were treated with vehicle control (0.1%, w/v, ethanol) or with 9-cis, 11-trans- or 10-trans, 12-cis-CLA isomers at 5, 10, 25, and 100 μmol/L for each CLA isomer at 37°C in a humidified atmosphere of 5% CO₂ for 24 h. The concentrations of
CLA treatments were within the range of physiologic blood concentrations used in previous CLA studies [24] and [25].

**Cytoplasmic fraction preparation**

The cytoplasmic fraction of HUVECs was prepared for immunodetection and enzyme activity assays. After treatment with the vehicle control or CLA isomers for 24 h, HUVECs were rinsed, scraped, and suspended in ice-cold PBS (pH 7.4, 10 mmol/L of NaH$_2$PO$_4$, 1.5 mmol/L of KH$_2$PO$_4$, 138 mmol/L of NaCl, 2.7 mmol/L of KCl). The cells were homogenized at maximum speed (Tissue Tearor, model 985-370, Biospec Products, Inc., Bartlesville, OK, USA) for 15 s while keeping the cells cold in an ice bath. Aliquots of the cell homogenate were stored at −70°C until they could be used for testing by the TBARS assay. The remaining cell homogenate was centrifuged at 10 000 × $g$ at 4°C for 15 min. The supernatant was stored at −70°C until it could be used for immunodetection and enzyme activity assays.

**Nuclear extract preparation**

The nuclear fraction of HUVECs was isolated by using a nuclear extract kit. After treatments with the vehicle control or CLA isomers for 24 h, HUVECs previously plated on T75 flasks were rinsed and scraped into ice-cold PBS containing phosphatase inhibitors. The cells were centrifuged at 14 000 × $g$ at 4°C for 30 s, suspended in hypotonic buffer, and lysed with 0.5% (w/v) NP-40. The nuclear pellet was collected after centrifugation of the cell lysate at 14 000 × $g$ at 4°C for 10 min. The suspended nuclear pellet was lysed and centrifuged at 14 000 × $g$ at 4°C for 10 min. The supernatant
(nuclear fraction) was collected, divided into aliquots, and stored at −70°C until they were used for the PPAR-γ transcription factor assay.

**PPAR-γ DNA binding activity assay**

The PPAR-γ DNA binding activity in the nuclear extract of HUVECs was determined by PPAR-γ transcription factor assay (Active Motif). The specific double-stranded DNA sequence containing PPREs (5′- AGGTCAAAGGTCA-3′) was immobilized within the bottom of the wells of a 96-well plate in the enzyme-linked immunosorbent assay. PPARs heterodimerize with 9-cis RXRs (retinoid X receptors), and the dimeric form binds to PPREs. PPARs interact with the upstream extended core hexamer and RXRs occupy the downstream motif. PPAR-mediated transactivation was produced by the combination of PPAR:RXR binding to a PPRE and ligand activation of this complex. The conformational change of PPAR triggered by ligand binding is believed to generate a transcriptionally active complex by forming specific contacts with coactivator proteins, such as the acetyltransferase complex [26]. The nuclear fraction was incubated overnight at 4°C to bind to PPRE immobilized within the bottom of each well. Human PPAR-γ bound to PPRE was detected by spectrophotometric reading of duplicated samples (0–100 µmol/L) at 450 nm after adding the specific primary antibody directed against human PPAR-γ and the secondary antibody conjugated to horseradish peroxidase. According to Active Motif, there should be no cross-reactions with PPAR-α and PPAR-β or -δ.

**NF-κB p50 DNA binding activity assay**
The NF-κB DNA binding activity in the nuclear extract of HUVECs was assessed by the NF-κB transcription factor assay (Active Motif). NF-κB is associated with regulating genes involved in immune and inflammatory responses. The dimeric form of NF-κB recognizes a specific nucleotide sequence (5′-GGGACTTTCC-3′) in the enhancer sites of target genes. The nuclear extract was incubated for 1 h at room temperature with mild agitation to bind to the nucleotide sequence immobilized within the bottom of each well. Primary antibodies specifically bind to NF-κB p50. Subsequently, human NF-κB p50 bound to DNA was measured spectrophotometrically (duplicate samples, 0–25 μmol/L) at 450 nm.

**Quantitative PCR**

The Cu/Zn-SOD and catalase mRNA levels of HUVECs were examined by performing real-time PCR. Total RNA was extracted from HUVECs plated on a 24-well plate using a commercial kit (SuperScript III CellsDirect cDNA Synthesis System, Invitrogen) and was used as a template for cDNA synthesis using oligo(dT) primer. The reverse transcription reaction was performed at 50°C for 20 min, and the reaction was inactivated at 85°C for 5 min. The cDNA was stored at −20°C until quantitative PCR analysis. The primer sets used to amplify Cu/Zn-SOD and catalase cDNAs were SOD-F 5′ CGT GGC CTA GCG AGT TAT GG 3′, SOD-R 5′ TCG AAA TTG ATG ATG CCC TG 3′, catalase-F 5′ AAG ACT GAC CAG GGC ATC AAA 3′, and catalase-R 5′ CCG GAT GCC ATA GTC AGG AT 3′. Quantitative PCR reactions were performed for 40 cycles at 95°C for 15 s and 60°C for 1 min using the SYBR Green PCR Master Mix (Applied Biosystems). Relative expression (duplicated samples) was calculated from cycle
threshold values ($2^{-\Delta\Delta Ct}$ method) using 18S rRNA expression as an internal control for each sample.

**Immunodetection**

The SOD and catalase protein levels in the cytoplasmic fraction of HUVECs were immunologically detected by using the ExtrAvidin Alkaline Phosphatase Staining Kit. Primary antibodies for SOD and catalase were purchased from suppliers (Fitzgerald Industries International, Inc., and EMD Biosciences, Inc., respectively). The cytoplasmic fraction in PBS was incubated in 96-well plates at 4°C overnight. After washing with 0.05% Tween 20 in PBS, block solution (1% bovine serum albumin in PBS) was added into each well to block non-specific binding. Primary antibodies were incubated for 2 h, followed by a 2-h incubation of a biotinylated antibody as a secondary antibody. After adding avidin-bound alkaline phosphatase in 10 mmol/L of Tris (pH 8.2), 150 mmol/L of NaCl, and 0.05% Tween 20, a substrate solution (10 mmol/L of diethanolamine, pH 9.6, 0.5 mmol/L of MgCl$_2$, 0.2% NaN$_3$, 1 mg/mL of $p$-nitrophenyl phosphate) was added and the plates were incubated in the dark at room temperature for 30 min. Absorbance (duplicated samples) was measured at 405 nm spectrophotometrically.

**Enzyme activity assay**

The Cu/Zn-SOD activity in the cytosolic fraction of HUVECs was determined spectrophotometrically by measuring sample-mediated inhibition of xanthine oxidase–dependant superoxide production. Xanthine oxidase was added to the mixture of the cytoplasmic fraction and a chromogenic tetrazolium salt (a radical detector). The
endpoint measurements were performed at 450 nm spectrophotometrically after incubation for 20 min. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical. Absorbance (duplicated samples) was recorded at 450 nm.

Catalase activity in the cytosolic fraction of HUVECs was assessed spectrophotometrically by measuring the quantity of formaldehyde produced. Catalase converts two molecules of hydrogen peroxide to molecular oxygen and two molecules of water (catalytic activity $2 \text{H}_2\text{O}_2 \rightarrow 2 \text{H}_2\text{O} + \text{O}_2$). Catalase also shows peroxidatic activity, in which low-molecular-weight alcohols can serve as electron donors ($\text{H}_2\text{O}_2 + \text{AH}_2 \rightarrow \text{A} + 2 \text{H}_2\text{O}$). The assay method is based on the peroxidatic reaction of catalase with methanol in the presence of an optical concentration of hydrogen peroxide. Purpald, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, used as a chromogen, specifically forms a bicyclic heterocycle with aldehydes. The formaldehyde produced (duplicated samples) was measured at 540 nm. One unit of catalase is defined as the amount of the enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

**TBARS assay**

Oxidative damage in the homogenate of HUVECs was assessed by the TBARS assay. Malondialdehyde is a byproduct of lipid peroxidation and reacts with thiobarbituric acid. The thiobarbituric acid/malondialdehyde complex was measured at 535 nm spectrophotometrically. A solution of 0.375% (w/v) thiobarbituric acid, 15% trichloroacetic acid, and 0.25 N hydrochloric acid was added to the cell homogenate, and the mixture was heated at 100°C for 15 min [27]. The supernatant was collected after
centrifugation of the sample. Absorbance (duplicated samples) was read at 535 nm, and the malondialdehyde (1,1,3,3-tetraethoxypropane, Sigma-Aldrich Chemical Company) equivalent was calculated using a standard curve [28].

**Statistical analysis**

Statistical analyses were performed with SPSS 14.1 for Windows (SPSS, Inc., Chicago, IL, USA). Each treatment group was compared with the control group using one-sided Student's *t* test for analyzing within-group significances. Pearson's correlations were used to analyze between-group differences. Differences with *P* < 0.05 were considered to be statistically significant. All results were expressed as mean ± standard deviation.

**Transcription factor binding site searches**

Transcription factor binding sites were identified using P-Match (BIOBASE Corporation, Beverly, MA, USA). The promoter regions (up to 5000-base upstream of the translational starting site of human *SOD1* and up to 10 000-base upstream of the translational starting site of human catalase) were analyzed to search transcription factor binding sites.

**Results**

**PPAR-γ DNA binding activity**

There were significant increases in PPAR-γ DNA binding activity levels of HUVECs for all the CLA isomer treatments, except the treatment with the 10-trans, 12-cis-CLA isomer at 100 μmol/L compared with the vehicle control (1.4- to 1.8-fold, *P* < 0.005; Fig.
1). Therefore, the 9-cis, 11-trans- and 10-trans, 12-cis-CLA isomers are possible PPAR-γ activators and are associated with PPAR-γ–mediated transcription activation.

**NF-κB p50 DNA binding activity**

All CLA isomer treatments resulted in significant increases in NF-κB DNA binding activity levels of HUVECs ($P < 0.005$) over the control (Fig. 2).

**Quantitative PCR**

Significant increases in Cu/Zn-SOD mRNA levels of HUVECs were shown at all concentrations of the 9-cis, 11-trans-CLA isomer compared with the control (6.7- to 12.4-fold, $P < 0.05$). In contrast, there was a significant increase in Cu/Zn-SOD mRNA levels of HUVECs only at the lowest concentration of the 10-trans, 12-cis-CLA isomer (5 μmol/L, 5.3-fold, $P < 0.005$; Fig. 3). Cu/Zn-SOD mRNA levels were significantly and positively correlated with SOD protein levels and catalase mRNA levels ($P < 0.05$, $r = 0.904$ and 0.944, respectively) in cells with 9-cis, 11-trans-CLA isomer treatments. In cells with 10-trans, 12-cis-CLA isomer treatments, Cu/Zn-SOD mRNA levels were significantly and positively correlated with TBARS levels and catalase mRNA level ($P < 0.05$, $r = 0.890$ and 0.991). However, for Cu/Zn-SOD mRNA there was a non-significant positive correlation with SOD protein levels ($P = 0.053$, $r = 0.873$).

Similarly, significant increases in catalase mRNA levels of HUVECs were found at all concentrations of the 9-cis, 1-trans-CLA isomer (5, 10, 25, 100 μmol/L, 5.5- to 14.7-fold, $P < 0.005$) and at lower concentrations of the 10-trans, 12-cis-CLA isomer (5, 10 μmol/L, 1.7- to 6.2-fold, $P < 0.005$; Fig. 4) compared with the control. Significant positive
correlations were found among catalase mRNA, Cu/Zn-SOD mRNA, and SOD protein levels attributed to the 9-cis, 11-trans and 10-trans, 12-cis-CLA treatments ($P < 0.05$ and $P < 0.005$, respectively; $r = 0.944$ and 0.967, respectively). For 10-trans, 12-cis-CLA isomer treatments, Cu/Zn-SOD and catalase mRNA levels were significantly and positively correlated with TBARS levels ($P < 0.005$, $r = 0.991$).

**Immunodetection**

There were significant increases in SOD protein levels of HUVECs for all CLA isomer treatments except the treatment with 100 μmol/L of the 9-cis, 11-trans-CLA isomer compared with the control (3.0- to 8.9-fold, $P < 0.05$). The highest SOD protein levels were found at the lowest concentrations of 9-cis, 11-trans- and 10-trans, 12-cis-CLA isomers (Fig. 5). In addition, SOD protein levels were significantly and positively correlated with TBARS levels and catalase mRNA levels for the 9-cis, 11-trans- and 10-trans, 12-cis-CLA isomer treatments ($P < 0.05$, $r = 0.939$, 0.967, 0.954, and 0.913, respectively), whereas SOD protein levels exhibited a non-significant positive correlation with Cu/Zn-SOD mRNA levels for 10-trans, 12-cis-CLA isomer treatments ($P = 0.053$, $r = 0.873$).

Significant increases in catalase protein levels of HUVECs were shown for treatments with 5 and 25 μmol/L of the 9-cis, 11-trans-CLA isomer (1.6- to 1.8-fold, $P < 0.05$) compared with the control. All 10-trans, 12-cis-CLA isomer treatments exhibited significant increases in catalase protein levels compared with the control (1.7- to 3.2-fold, $P < 0.05$; Fig. 6).
**Enzyme activity**

A significant increase of Cu/Zn-SOD activity levels of HUVECs was found at 10 μmol/L of the 10-trans, 12-cis-CLA isomer treatment ($P < 0.05$) compared with the control (Fig. 7). No significant increases of Cu/Zn-SOD activity levels of HUVECs were found for any of the 9-cis, 11-trans-CLA isomer treatments. There was no significant increase in catalase activity levels of HUVECs for either CLA isomer treatment compared with the control.

**Thiobarbituric acid-reactive substance**

For the lowest concentration of 9-cis, 11-trans- and 10-trans, 12-cis-CLA isomers, TBARS levels of HUVECs were significantly higher than those of the control (3.0- and 2.0-fold, $P < 0.05$; Fig. 8). For 9-cis, 11-trans-CLA isomer treatments, there was a significant positive correlation between TBARS levels and SOD protein levels ($P < 0.05$, $r = 0.939$), whereas there was a non-significant positive correlation between TBARS levels and catalase mRNA ($P = 0.067$, $r = 0.852$). TBARS levels were significantly and positively correlated with Cu/ZnSOD and catalase mRNA levels ($P < 0.005$, $r = 0.890$ and 0.921, respectively) and SOD protein levels ($P < 0.005$, $r = 0.954$) for 10-trans, 12-cis-CLA isomer treatments.

**Transcriptional factor binding searches**

Five binding sites (-5064, -4317, -3206, -2489, -888) for PPAR-γ were identified in the promoter region of human SOD1 (human Cu/Zn-SOD). Fourteen binding sites for PPAR-α were also found in the same region. We could not find PPREs in the human catalase
promoter region (up to 10 000-base upstream from the translational starting site); however, a PPRE has been identified in the rat catalase promoter region [17]. Six NF-κB binding sites (5′-GGGRNNYYCC-3′: -6437) were found in the SOD1 promoter region (-3866, -3570, -3406, -2406, -1716, -540) and one binding site in the catalase promoter region (-6437). These transcription factors, PPAR-γ and NF-κB, play a key role in inflammation. NF-κB is also known to be a redox-regulated transcription factor nuclear factor [29] and is expressed in most cell types. The number of binding sites of other transcription factors with longer than hexamer recognition (e.g., vitamin D receptor, activator protein 1) in the SOD1 promoter region was 2256, whereas we found only 17 binding sites of other transcription factors (e.g., signal transducers and activators of transcription protein) in the catalase promoter region. This indicates that more transcription factors are involved in SOD1 transcription than in catalase transcription.

Discussion

Several studies examining the effects of the interactions between CLA isomers and PPAR-γ have been reported in the literature, with conflicting results. CLA isomers have been shown to decrease PPAR-γ expression [30], [31] and [32]. Others have found that CLA isomers increase PPAR-γ expression [33], [34], [35], [36], [37], [38], [39], [40], [41] and [42]. Furthermore, studies have indicated that CLA isomers affect PPAR-γ through NF-κB, however with inconsistent findings [43], [44], [45] and [46].
In this study, the results of transcription factor binding site searches and the results of transcription factor DNA binding assays indicate the involvement of PPAR-γ and NF-κB in the expression of antioxidant enzymes. We have also shown the following for 9-cis, 11-trans-CLA treatments: 1) increases in transcription factor DNA binding activities and expression of antioxidant enzymes at 0–25 μmol/L and 2) an increase in lipid peroxidation (TBARS) only at the lowest concentrations (5 μmol/L). For 10-trans, 12-cis-CLA treatments, we found 1) increases in transcription factor DNA binding activities (0–25 μmol/L) and 2) the highest levels of mRNA of both antioxidant enzymes, SOD protein, and TBARS (lipid peroxidation) only at the lowest concentrations.

At least two explanations are possible for the expression of the antioxidant enzymes and lipid peroxidation and ROS generation: 1) PPAR-γ– and 2) NF-κB–mediated expression of antioxidant enzymes. First, CLA isomer-mediated PPAR-γ activation upregulates expression of Cu/Zn-SOD. This upregulation may be a CLA isomer concentration-dependent behavior. As previous studies [16], [20] and [47] have suggested, induction of Cu/Zn-SOD would result in reduction of ROS generation and ultimately in reduction of LDL oxidation and further atherogenesis. Thus, CLA isomer-induced antiatherogenic and anti-inflammation effects may be due to the induction of Cu/Zn-SOD through PPAR-γ activation, leading to decreases in ROS generation.

Another explanation could be the circumstance by which ROS-mediated NF-κB activation regulates the expression of Cu/Zn-SOD and catalase. This mechanism may predominate at low concentrations of CLA isomers. Low CLA concentrations may be inadequate to prevent oxidative events and pro-oxidants. CLA isomers are more...
susceptible to oxidation than linoleic acid due to their conjugated bonds [48]. Others have demonstrated that susceptibility to oxidation of CLA is higher than that of linoleic acid [49]. They also found that CLA-mediated inhibition of lipid oxidation is observed only at high concentrations, but not at low concentrations (1–50 μM). CLA isomers may cause lipid peroxidation by ROS generation [50]. We noted significant increases in lipid peroxidation at the lowest concentrations (5 μmol/L) for both CLA isomers (Fig. 8), which is consistent with the susceptibility of oxidation of CLA isomers and which we speculate occurs at low CLA concentrations. ROS, modified lipids, and/or other biomolecules may serve as second messengers [29] promoting NF-κB activation, leading to the upregulation of the antioxidant enzymes. Oxidized fatty acids induce catalase activity in HUVECs and the induction of cellular antioxidant response prevents further oxidative stress [51]. It is important to note that, although SOD prevents oxidative damage by converting harmful superoxide to less harmful hydrogen peroxide, SOD is microcidal and serves as ROS sources in infection and inflammatory processes. NADPH oxidase and SODs are induced in macrophages and neutrophils during phagocytosis and subsequently produce microcidal cytotoxicity by generating superoxide and hydrogen peroxide, respectively [52]. Therefore, Cu/Zn-SOD may be upregulated by NF-κB activation triggered by ROS (superoxide generated by NADPH oxidase). Subsequently, catalase may be induced by hydrogen peroxide generated by Cu/Zn-SOD, which would occur because of NF-κB activation, i.e., rapid NF-κB activation results from hydrogen peroxide and peroxide-containing molecules in some cell lines [29]. Thus, CLA isomers may act as pro-oxidants and second messengers to activate NF-κB at the low concentration, resulting in NF-κB–mediated gene expression, i.e., CLA may exhibit
unfavorable effects under certain conditions, such as concentrations and the presence of other antioxidants.

However, 9-\textit{cis}, 11-\textit{trans}-CLA isomer, at high concentrations, may play a role in downregulating the expression of antioxidant enzymes by NF-κB, possibly by blocking the NF-κB pathway. High concentrations of the 10-\textit{trans}, 12-\textit{cis}-CLA isomer (100 μmol/L) or α-tocopherol (100 μmol/L) alleviates the CLA isomer-induced cytotoxicity (ROS generation) in rat hepatoma [12]. The 10-\textit{trans}, 12-\textit{cis}-CLA isomer inhibits NF-κB p50/p65 DNA binding [53]. CLA isomers may be less susceptible to oxidation at high concentrations and CLA isomers act as PPAR-γ activators, rather than as pro-oxidants to activate NF-κB, to induce expression of antioxidant enzymes. In addition to induction of antioxidant enzymes, CLA isomers may be involved in other gene expressions whose products influence ROS generation through PPAR-γ and NF-κB. CLA isomer-induced suppression of cyclo-oxygenase-2, a pro-inflammatory enzyme and ROS source, by blocking NF-κB activation, contributes to decreased ROS generation in inflammatory processes. The 9-\textit{cis}, 11-\textit{trans}- and 10-\textit{trans}, 12-\textit{cis}-CLA isomers inhibit tumor necrosis factor-α–induced NF-κB DNA binding activity in a concentration-dependent manner (5–100 μmol/L for each CLA isomer) and inhibit the production of the prostaglandins E$_2$ and I$_2$ in vascular smooth muscle cells [14]. PPAR-γ may be involved in CLA isomer-mediated inhibition of cyclo-oxygenase-2 expression in activated macrophages, suggesting a PPAR-γ–dependent inhibition of NF-κB activation [7]. Also, suppression of endothelial NADPH oxidase expression by CLA-mediated PPAR-γ activation may contribute to reduction of ROS generation, because PPAR-γ activation by its activators is
associated with downregulation of NADPH oxidase in HUVECs [20]. As noted before, endothelial NADPH oxidase is one major ROS source in the vasculature resulting in LDL oxidation.

Peroxisome proliferator-activated receptor-γ and NF-κB may be involved in regulating genes whose products influence ROS generation; therefore, these transcription factor activations may be associated with ROS generation and inflammation and ultimately atherogenic processes. For example, synthetic PPAR activators exert their anti-inflammatory actions, at least in part, by negatively regulating NF-κB activation [7]. Anti-inflammatory effects of CLA isomers are partly involved in PPAR-γ signaling and responsible for the antiatherogenic effects of CLA isomers observed in vivo [14]. Peroxisome proliferator-activated receptor-γ and NF-κB may coordinate and propagate feedback loops. Because the results of transcription factor binding site searches indicate the existence of multiple binding sites for various types of transcription factors, these numerous transcription factors may build a complex network. In addition, gene regulation mediated by transcription factors is tissue- and species-specific. Therefore, adverse and beneficial effects mediated by a single compound might occur simultaneously at different tissues. For example, the CLA isomer-induced loss of adipose tissue in mice is associated with an increase in the amount of fat stored in the liver. The CLA-induced fatty liver results from decreased fatty acid oxidation and increased fatty acid synthesis [36], [54], [55] and [56]. Thus, further investigations and long-term clinical trials are needed to determine the safety and efficacy of CLA isomers.
Conclusion

Further research is necessary to elucidate and separate the CLA isomer-mediated mechanisms associated with the prevention and pathology of arthrosclerosis. We found that the effects of CLA isomers are mostly concentration-dependent in favor of activation of PPAR-γ and NF-κB with subsequent induction of Cu/Zn-SOD and catalase. However, some concentrations of CLA isomers resulted in increased lipid peroxidation. Therefore, CLA isomers may act as beneficial agents or adverse agents, depending on their concentrations and microenvironments, i.e., the concentration determines whether CLA isomers possess an inhibitory or a stimulatory effect in inflammatory and atherogenic processes. Thus, an understanding of the optimal concentrations of CLA isomers that stimulate the benefits of antioxidant enzyme induction may require careful CLA titration to determine predictable and dependable therapeutic strategies.
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Fig. 1. PPAR-γ DNA binding activity levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. With the exception of the 10-trans, 12-cis-CLA isomer treatment at 100 μmol/L, all treatments resulted in significant increases of PPAR-γ DNA binding activities compared with the vehicle control containing neither CLA isomer ($P < 0.005$). Values are means ± SDs. ***$P < 0.005$. CLA, conjugated linoleic acid; PPAR-γ, peroxisome proliferator-activated receptor-γ.
Fig. 2. NF-κB DNA binding activity levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, and 25 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. CLA isomer treatments resulted in significant increases of NF-κB DNA binding activities compared with the vehicle control containing neither CLA isomer ($P < 0.005$). Values are means ± SDs. ***$P < 0.005$. CLA, conjugated linoleic acid; NF-κB, nuclear factor-κB.
Cu/Zn SOD mRNA (A)

Fig. 3. Cu/Zn-SOD mRNA levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. Treatments with the 9-cis, 11-trans-CLA isomer at 5 to 100 μmol/L and the 10-trans, 12-cis-CLA isomer at 5 μmol/L resulted in significant increases of Cu/Zn-SOD mRNA levels compared with the vehicle control containing neither CLA isomer ($P < 0.05$). Values are means ± SDs. *$P < 0.05$ and ***$P < 0.005$. CLA, conjugated linoleic acid; Cu/Zn-SOD, copper/zinc superoxide dismutase.
Fig. 4. Catalase mRNA levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. Treatments with the 9-cis, 11-trans-CLA isomer at 5 to 100 μmol/L and the 10-trans, 12-cis-CLA isomer at 5 and 10 μmol/L resulted in significant increases of catalase mRNA levels compared with the vehicle control containing neither CLA isomer ($P < 0.05$). Values are means ± SDs. ***$P < 0.005$. CLA, conjugated linoleic acid.
Fig. 5. SOD protein levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. With the exception of the 9-cis, 11-trans-CLA isomer at 100 μmol/L, all treatments resulted in significant increases of SOD protein levels compared with the vehicle control containing neither CLA isomer ($P < 0.05$). Values are means ± SDs. *$P < 0.05$, **$P < 0.01$, and ***$P < 0.005$. CLA, conjugated linoleic acid; SOD, superoxide dismutase.
Fig. 6. Catalase protein levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. Treatments with the 9-cis, 11-trans-CLA isomer at 5 and 25 μmol/L and the 10-trans, 12-cis-CLA isomer at 5 to 100 μmol/L resulted in significant increases of catalase protein levels compared with the vehicle control containing neither CLA isomer \((P < 0.05)\). Values are means ± SDs. ** \(P < 0.01\) and *** \(P < 0.005\). CLA, conjugated linoleic acid.
Cu/Zn SOD activity

Fig. 7. Copper/zinc superoxide dismutase enzyme activity levels in human umbilical vein endothelial cells after treatments with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of the 10-trans, 12-cis-CLA isomer. Treatments with the 10-trans, 12-cis-CLA isomer at 10 μmol/L resulted in significant increases of copper/zinc superoxide dismutase enzyme activity levels compared with vehicle control containing no 10-trans, 12-cis-CLA isomer (P < 0.05). Values are means ± SDs. *P < 0.05. CLA, conjugated linoleic acid.
Fig. 8. TBARS levels in human umbilical vein endothelial cells after treatment with graded concentrations of 0, 5, 10, 25, and 100 μmol/L of (A) the 9-cis, 11-trans-CLA isomer or (B) the 10-trans, 12-cis-CLA isomer. Treatments with the 9-cis, 11-trans- and the 10-trans, 12-cis-CLA isomers at 5 μmol/L resulted in significant increases of TBARS levels compared with the vehicle control containing neither CLA isomer (P < 0.05). Values are means ± SDs. *P < 0.05 and **P < 0.01. CLA, conjugated linoleic acid; TBARS, thiobarbituric acid-reactive substance.
Chapter 5

Vitamin E (α-Tocopherol) Study

Published as

α-Tocopherol Modulates Human Umbilical Vein Endothelial Cell Expression of Cu/Zn Superoxide Dismutase and Catalase and Lipid Peroxidation

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in

Abstract

Recent studies suggest the potential of α-tocopherol as a gene regulator, possibly through peroxisome proliferator-activated receptor γ (PPARγ) activation due to the structural similarity of α-tocopherol to a PPARγ ligand, troglitazone. Other investigators have suggested that a link exists between induction of the antioxidant enzymes Cu/Zn superoxide dismutase (SOD) and catalase and PPARγ activation. This study was designed to examine whether α-tocopherol modulates expression of Cu/Zn SOD and catalase in human umbilical vein endothelial cells through redox-sensitive transcription factors, PPARγ, and nuclear factor-κB (NF-κB). α-Tocopherol treatments showed significant increases in both PPARγ (1.4- to 2.2-fold, P < .01) and NF-κB p50 (1.3- to 1.5-fold, P < .005) DNA binding activities compared with vehicle control. Significant increases in Cu/Zn SOD mRNA levels (6.0-fold, P < .005) and catalase mRNA (8.0-fold, P < .005) and its protein levels (2.3-fold, P < .005) and lipid peroxidation levels (5.3-fold, P < .005) were observed at the lowest concentration (10 μmol/L) of α-tocopherol treatments. Both mRNA and protein levels of these 2 antioxidant enzymes were positively associated with lipid peroxidation (P < .05). α-Tocopherol may play a role not only in preventing against oxidative damage as an exogenous antioxidant by scavenging free radicals and superoxide but also in modulating the expression of the endogenous antioxidant enzymes as a gene regulator through PPARγ and NF-κB in the vascular cells. The α-tocopherol-mediated gene expression is either stimulatory or inhibitory, depending on its oxidative status or its concentrations.
**Keywords:** α-Tocopherol; Human; Endothelial cells; PPARγ; NF-κB; Lipid peroxidation; SOD; Catalase

**Abbreviations:** CVD, cardiovascular disease; GPx, glutathione peroxidase; HUVEC, human umbilical vein endothelial cells; LDL, low-density lipoprotein; NF-κB, nuclear factor-κB; PPAR, peroxisome proliferator-activated receptor; PPRE, Peroxisome proliferator response elements; ROS, reactive oxygen species; SOD, superoxide dismutase; TBARS, thiobarbituric acid reactive substance; TZD, thiazolidinedione.
1. Introduction

Epidemiologic studies have shown an inverse relationship between intakes of antioxidant vitamins, such as vitamins A, C, and E, and cardiovascular disease (CVD), which is a major cause of mortality in developed countries [1]. The protective effects of α-tocopherol (vitamin E) on CVD have been demonstrated both in vitro and in animal studies [2] and [3]. However, several clinical studies have failed to confirm the efficacy of α-tocopherol on CVD [3], [4] and [5]. Explanations for the inconsistency between in vitro, animal studies, and clinical studies include (1) inability to accurately measure and/or adjust the cellular environment, such as the effects on tocopherol transport proteins [5]; (2) genetic and behavioral factors that contribute to the risk of CVD [3]; (3) the interaction with other nutrients (antioxidants) that coexist in the diet [6]; (4) the possible need to optimize the concentration and duration of α-tocopherol intake [7]; and (5) interventions at a relatively late stage of CVD [2].

Although α-tocopherol has a defined role as an antioxidant [3] and [8], recent studies suggest the potential of α-tocopherol as a gene regulator, possibly through peroxisome proliferator-activated receptor γ (PPARγ) activation, because of the structural similarity of α-tocopherol to troglitazone, which is both a thiazolidinedione (TZD) and a PPARγ ligand [5], [9] and [10]. Peroxisome proliferator-activated receptors are a group of nuclear receptors and belong to the steroid hormone receptor superfamily. There are 3 isoforms: PPARα, PPARβ, and PPARγ. Peroxisome proliferator-activated receptors heterodimerize with the 9-cis-retinoic acid receptor (retinoid X receptor [RXR]) and bind to peroxisome proliferator response elements (PPREs: 5'-AGGTCAnAGGTC-3')
located in enhancer sites of the target genes. Peroxisome proliferator-activated receptors induce target gene products by their activators, such as fatty acids, fatty acid derivatives (eg, eicosanoids, prostaglandins), and numerous structurally dissimilar xenobiotics (peroxisome proliferators). Peroxisome proliferator-activated receptor α and PPARγ are expressed in vascular endothelial cells and smooth muscle cells as well as adipose tissues [11] and [12]. Peroxisome proliferator-activated receptor α and PPARγ play a role in inflammation, adipogenesis, and insulin sensitization. Synthetic PPARγ activators, such as TZDs, have shown various effects: (1) adipocyte differentiation/lipid metabolism, (2) anti-inflammation, (3) antidiabetes, and (4) antiatherosclerosis [13]. In addition, the effects of a group of PPAR activators and peroxisome proliferators may be species and dose dependent because of their diverse metabolic profiles [14].

Reactive oxygen species (ROS), such as superoxide, are implicated in a variety of diseases, including CVD and cancer. Atherosclerosis results from a series of oxidative processes [4] and is known to be a chronic inflammatory disease with an underlying abnormality in redox-mediated signals in the vasculature [15]. Oxidation of low-density lipoprotein (LDL) is thought of be the first step of atherosclerosis. Oxidized LDL is taken up by macrophage receptors (ie, SRs, CD36), leading to foam cell formation and fatty streaks. Sources of oxidants in the vascular wall include nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, nitric oxide synthases, lipoxygenase, and cyclooxygenase. Phagocytic NADPH oxidase and endothelial NADPH oxidase are the major sources of ROS in the vasculature [16] and [17]. Monocyte differentiation to macrophage is associated with production and release of ROS, possibly through the
induction of NADPH oxidation, resulting in LDL oxidation [17] and [18]. Superoxide production contributes to endothelial cell-induced LDL oxidation in vitro [19]. NADPH oxidases generate superoxide on the extracellular side of the plasma membrane, and the enzyme can trigger intracellular signaling by superoxide transport via chloride channel-3 [20]. Reactive oxygen species are key mediators of signaling pathways, such as the nuclear factor-κB (NF-κB) p50/p65 pathway, which underlie vascular inflammation in atherogenesis, and enhanced ROS plays a causal role in atherosclerosis [21]. Hence, protection from ROS-induced oxidative stress and gene expression is crucial in the prevention of atherogenic processes.

The genes for antioxidant enzymes, superoxide dismutase (SOD) and catalase, may be regulated by PPAR ligands (eg, TZDs) through PPARγ activation because PPREs are located in the promoter regions of human Cu/Zn SOD (SOD1) and rat catalase [11], [22], [23] and [24]. In addition, several studies have shown the beneficial effects of overexpression and/or induction of Cu/Zn SOD and catalase by endothelial cells: (1) decrease in superoxide levels in endothelial cell; (2) suppression of age-related oxidative stress; (3) protection against inflammatory events by inhibiting NF-κB p50/p65 heterodimer activation; and (4) suppression of LDL oxidation [11], [22], [23], [24] and [25]. These effects suggest the links between α-tocopherol and antioxidant enzyme induction, as well as PPARγ activation. The therapeutic benefits of antioxidants in atherosclerosis might be caused by alterations in the molecular regulation of gene expression in endothelial, smooth muscle, and inflammatory cells [15], including the suppression of ROS-induced gene expression in vascular cells. Hence, this study was
designed to examine whether α-tocopherol modulates expression of Cu/Zn SOD and catalase through activation of redox-sensitive transcription factors, PPARγ and NF-κB, in human umbilical vein endothelial cells (HUVECs).

2. Methods and materials

2.1. Chemicals and reagents

α-Tocopherol, diethanolamine, trichloroacetic acid, 1,1,3,3-tetraethoxypropane, 2-thiobarbituric acid (TBA), Tween 20, phosphate-buffered saline (PBS), ethyl alcohol, bovine serum albumin, and glutamine were purchased from Sigma-Aldrich Chemical Company (St Louis, Mo). Carbon dioxide was purchased from local distributors. NaH₂PO₄, KH₂PO₄, NaCl, and KCl were purchased from Fisher Chemicals (Springfield, NJ). The nuclear extract and PPARγ and NF-κB transcription factor assay kits were purchased from Active Motif (Carlsbad, Calif). The SuperScript III CellsDirect cDNA Synthesis System and primers for SOD1 and catalase were purchased from Invitrogen (Carlsbad, Calif). The SYBR Green PCR Master Mix was purchased from Applied Biosystems (Foster City, Calif). The enzyme-linked immunosorbent assay (ELISA) kit (ExtrAvidin Alkaline Phosphatase Staining Kit—Rabbit) was purchased from Sigma-Aldrich. Primary antibodies for Cu/Zn SOD and catalase were purchased from Fitzgerald Industries International, Inc (Concord, Mass), and EMD, Inc (San Diego, Calif), respectively. The Superoxide Dismutase and Catalase Assay Kits were purchased from Cayman Chemical (Ann Arbor, Mich). MCDB131 and 0.25% trypsin-EDTA were
purchased from Invitrogen. EGM-1 SingleQuot (fetal bovine serum [FBS], human epidermal growth factor [hEGF], hydrocortisone), gentamicin, amphotericin B, vascular endothelial growth factor [VEGF], human fibroblast growth factor-basic [hFGF-B], insulin-like growth factor-basic [R³-IGF-1], ascorbic acid, and heparin were purchased from Cambrex Bio Science Walkersville, Inc (Walkersville, Md).

2.2. Cell culture

Primary HUVECs (Clonetics no. CC-2517; preserved cryogenically) were purchased from Cambrex Bio Science Walkersville, Inc. After thawing, cells were grown in MCDB131 (Invitrogen) supplemented with 2 mmol/L l-glutamine and EGM-2 SingleQuot (Clonetics CC-4176) containing 2% FBS (final concentration), hEGF, hydrocortisone, gentamicin, amphotericin B, VEGF, hFGF-B, R³-IGF-1, ascorbic acid, and heparin. The cells were plated on 75-cm² gelatin-coated (0.5%) flasks and maintained at 37°C in a humidified atmosphere of 5% CO₂ until confluent. The cells were then subcultured by trypsin, and an aliquot of the cells were cultured in 24-well gelatin-coated plates for RNA isolation and subsequent real-time polymerase chain reaction (PCR) analysis. The tightly confluent monolayers of the fourth to seventh passage were used for the experiments.

2.3. Cell treatments

Human umbilical vein endothelial cells were plated on 75-cm² gelatin-coated flasks or 24-well plates. The confluent cells (passages 4 to 7) were treated with vehicle control (0.1% wt/vol ethanol) or (±)-α-tocopherol (10, 25, 50, and 100 μmol/L) at 37°C in a
humidified atmosphere of 5% CO₂ for 24 hours. The concentrations of α-tocopherol treatments were within physiologic range [5] and [26]. Different passages of cells treated were immediately isolated each of cytoplasmic fraction, nuclear fraction, total RNA, and cell homogenate.

2.4. Cytoplasmic fraction preparation

The cytoplasmic fraction of HUVECs was prepared for immunodetection and enzyme activity assay. After treatment with either vehicle control or α-tocopherol for 24 hours, HUVECs were rinsed, scraped, and suspended into ice-cold PBS, pH 7.4 (10 mmol/L NaH₂PO₄, 1.5 mmol/L KH₂PO₄, 138 mmol/L NaCl, 2.7 mmol/L KCl). The cells were homogenized at maximum speed (Tissue Tearor, model 985-370; Biospec Products, Inc, Bartlesville, Okla) for 15 seconds, while keeping the cells cold in an ice bath. Aliquots of the cell homogenate were stored at −70°C until they could be used for testing by the TBA reactive substance (TBARS) assay. The remaining cell homogenate was centrifuged at 10,000 g, 4°C for 15 minutes. The supernatant was stored at −70°C until it could be used for immunodetection and enzyme activity assays.

2.5. Nuclear extract preparation

The nuclear fraction of HUVECs was isolated by using a nuclear extract kit. After treatments with either vehicle control or α-tocopherol for 24 hours, HUVECs previously plated on T75 flask were rinsed and scraped into ice-cold PBS containing phosphatase inhibitors. The cells were centrifuged at 14 000g, 4°C for 30 seconds, suspended in hypotonic buffer, and lysed with 0.5% wt/vol NP-40. The nuclear pellet was collected
after centrifugation of cell lysate at 14 000 g, 4°C for 10 minutes. The suspended nuclear pellet was lysed and centrifuged at 14 000 g, 4°C for 10 minutes. Finally, the supernatant (the nuclear fraction) was collected, divided into aliquots, and stored at −70°C until they were used for PPARγ and NF-κB DNA binding activity assays.

2.6. Peroxisome proliferator-activated receptor γ DNA binding activity assay

Peroxisome proliferator-activated receptor γ DNA binding activity in the nuclear extract of HUVECs was determined by PPARγ transcription factor assay (Active Motif). The specific double-stranded DNA sequence containing PPREs (5′-AGGTCAAGGTCA-3′) was immobilized within the bottom of the wells of a 96-well plate in the ELISA. Peroxisome proliferator-activated receptors heterodimerize with 9-cis-retinoic acid receptors (RXRs), and the dimeric form binds to PPRE. Peroxisome proliferator-activated receptors interact with the upstream core hexamer, and RXRs occupy the downstream motif. Peroxisome proliferator-activated receptor–mediated transactivation was produced by the combination of PPAR-RXR binding to a PPRE and ligand activation of this complex. The conformational change of PPAR triggered by ligand binding is believed to generate a transcriptionally active complex by forming specific contacts with coactivator proteins, such as the acetyltransferase complex [27].

The nuclear fraction was incubated overnight at 4°C to bind to PPRE immobilized within the bottom of each well. Human PPARγ bound to PPRE was detected spectrophotometrically at 450 nm after adding the specific primary antibody directed against human PPARγ and the secondary antibody conjugated to horseradish peroxidase.
According to the Active Motif, there should be no cross-reactions with PPARβ (or δ) or PPARα.

2.7. Nuclear factor-κB p50DNA binding activity assay

The NF-κB DNA binding activity in the nuclear extract of HUVECs was assessed by the NF-κB transcription factor assay (Active Motif). Nuclear factor-κB is associated with regulating genes involved in immune and inflammatory responses. The dimeric form of NF-κB recognizes a specific nucleotide sequence (5′- GGGACTTTCC-3′) in the enhancer sites of target genes. The nuclear extract was incubated for 1 hour at room temperature with mild agitation to bind to the nucleotide sequence immobilized within the bottom of each well. Primary antibodies specifically bind to NF-κB p50. Subsequently, human NF-κB p50 bound to DNA was measured spectrophotometrically at 450 nm.

2.8. Quantitative PCR

Cu/Zn SOD and catalase mRNA levels were examined by performing real-time PCR. Total RNA was extracted from HUVECs plated on a 24-well plate using a commercial kit (SuperScript III CellsDirect cDNA Synthesis System; Invitrogen) and was used as a template for complementary DNA (cDNA) synthesis using oligo dT primer. The reverse transcription reaction was performed at 50°C for 20 minutes, and the reaction was inactivated at 85°C for 5 minutes. The cDNA was stored at −20°C until quantitative PCR analysis. The primer sets used to amplify Cu/Zn SOD and catalase cDNAs were the following: SOD-F 5′ CGT GGC CTA GCG AGT TAT GG 3′, SOD-R 5′ TCG AAA
ATG ATG CCC TG 3', catalase-F 5' AAG ACT GAC CAG GGC ATC AAA 3', and catalase-R 5' CCG GAT GCC ATA GTC AGG AT 3'. Quantitative PCR reactions were performed for 40 cycles at 95°C for 15 seconds and 60°C for 1 minute using the SYBR Green PCR Master Mix (Applied Biosystems). Relative expression was calculated from cycle threshold values (2\(^{-\Delta\Delta CT}\) method) using 18S ribosomal RNA expression as an internal control for each sample.

2.9. Immunodetection

Cu/Zn SOD and catalase protein levels in the cytoplasmic fraction of HUVECs were immunologically detected by using a commercial ELISA kit (Sigma-Aldrich). Primary antibodies for Cu/Zn SOD and catalase were purchased from the suppliers (Fitzgerald Industries International, Inc, and EMD Biosciences, Inc, respectively). The cytoplasmic fraction in PBS was incubated on 96-well plates at 4°C overnight. After washing with PBS-T (0.05% Tween 20 in PBS), block solution (1% bovine serum albumin in PBS) was added into each well to block nonspecific binding. Primary antibodies were incubated for 2 hours, followed by 2-hour incubation of a biotinylated antibody as a secondary antibody. After adding avidin bound alkaline phosphatase in tris buffered saline with Tween 20 (TBS-T; 10 mmol/L Tris, pH 8.2, 150 mmol/L NaCl, 0.05% Tween 20), substrate solution (10 mmol/L diethanolamine pH 9.6, 0.5 mmol/L MgCl\(_2\), 0.2% NaN\(_3\), 1 mg/mL p-nitrophenyl phosphate) was added, and the plates were incubated in the dark at room temperature for 30 minutes. Absorbance was measured at 405 nm spectrophotometrically.

2.10. Enzyme activity assay
Cu/Zn SOD activity in the cytosolic fraction of HUVECs was determined spectrophotometrically by measuring sample-mediated inhibition of xanthine oxidase-dependent superoxide production. Xanthine oxidase was added to the mixture of the cytoplasmic fraction and a chromogenic tetrazolium salt (a radical detector). The endpoint measurements were performed at 450 nm spectrophotometrically after incubation for 20 minutes. One unit of SOD is defined as the amount of the enzyme needed to exhibit 50% dismutation of the superoxide radical.

Catalase activity in the cytosolic fraction of HUVECs was assessed spectrophotometrically by measuring the quantity of formaldehyde produced. Catalase converts 2 molecules of hydrogen peroxide to molecular oxygen and 2 molecules of water (catalytic activity). Catalase also shows peroxidative activity, in which low molecular weight alcohols can serve as electron donors. The assay method is based on the peroxidative reaction of catalase with methanol in the presence of an optical concentration of hydrogen peroxide. Purpald, 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole, used as a chromogen, specifically forms a bicyclic heterocycle with aldehydes. The formaldehyde produced was measured at 540 nm. One unit of catalase is defined as the amount of the enzyme that will cause the formation of 1.0 nmol of formaldehyde per minute at 25°C.

2.11. Thiobarbituric acid reactive substance assay

Oxidative damage in the homogenate of HUVECs was assessed by the TBARS assay. Malondialdehyde (MDA) is a byproduct of lipid peroxidation and reacts with TBA. The TBA-MDA complex was measured at 535 nm spectrophotometrically. Thiobarbituric acid-trichloroacetic acid-HCl solution (0.375% wt/vol TBA, 15% trichloroacetic acid,
0.25N HCl) was added to the cell homogenate, and the mixture was heated at 100°C for 15 minutes [28]. The supernatant was collected after centrifugation of the sample. Absorbance was read at 535 nm, and MDA (1,1,3,3-tetraethoxypropane, Sigma) equivalent was calculated using a standard curve [29].

2.12. Transcription factor binding site searches

Transcription factor binding sites were identified using the software program P-Match, (BIOBASE Corporation, Beverly, Mass). The promoter regions (up to 5000-base upstream of the translational starting site of human SOD1 and up to 10 000-base upstream of the translational starting site of human catalase) were analyzed to search transcription factor binding sites.

2.13. Statistical analysis

Statistical analyses were performed with SPSS for Windows version 14.1 (SPSS Inc, Chicago, Ill). Each treatment group was compared with the control group using a 1-sided Student t test for analyzing within-group significances. Pearson correlations were used to analyze between-group differences. Differences with a P value less than .05 were considered to be significant. All results were expressed as means ± SD.
3. Results

3.1. Peroxisome proliferator-activated receptor γ DNA binding activity assay

The mean values of PPARγ DNA binding activity observed at graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol were the following (Δ units): 0.318, 0.457, 0.443, 0.687, and 0.464, respectively. There were significant increases in PPARγ DNA binding activity levels of HUVECs for all treatments of α-tocopherol (1.4- to 2.2-fold, $P < .01$; Fig. 1) compared with the vehicle control. The highest binding activation level was found with the medium concentration of α-tocopherol (50 μmol/L, Fig. 1).

3.2. Nuclear factor-κB p50DNA binding activity assay

The mean values of NF-κB p50 DNA binding activity observed at graded concentrations of 0, 10, and 50 μmol/L of α-tocopherol were the following (Δ units): 0.577, 0.744, and 0.869, respectively. Significant increases in NF-κB DNA binding activity levels of HUVECs were observed at both 10 and 50 μmol/L of α-tocopherol treatment (1.3- to 1.5-fold, $P < .005$; Fig. 2) compared with the vehicle control.

3.3. Quantitative PCR

Mean fold changes of Cu/Zn SOD mRNA vs control observed at graded concentrations of 10, 25, 50, and 100 μmol/L of α-tocopherol were the following (fold increase): 6.43, 0.50, 0.26, and 1.58, respectively. Significant increases in Cu/Zn SOD mRNA levels of HUVECs were exhibited both at the highest and the lowest concentrations of α-tocopherol (10 and 100 μmol/L and 1.6- and 6.0-fold, respectively, $P < .005$; Fig. 3) compared with vehicle control. In contrast, significant decreases in Cu/Zn SOD mRNA
levels were found at the intermediate tocopherol concentrations (25 and 50 μmol/L and 0.5- and 0.3-fold, respectively, \(P < .005\); Fig. 3) compared with vehicle control. Cu/Zn SOD mRNA levels were significantly and positively correlated with Cu/Zn SOD protein, catalase mRNA, catalase protein, and TBARS levels \((P < .05)\) at all concentrations of \(\alpha\)-tocopherol treatment.

Mean fold changes of catalase mRNA vs control observed at graded concentrations of 10, 25, 50, and 100 μmol/L of \(\alpha\)-tocopherol were the following (Δ units): 8.07, 1.46, 1.08, and 0.93, respectively. There was a significant increase in catalase mRNA levels of HUVECs at the lowest concentration of \(\alpha\)-tocopherol (10 μmol/L, 8-fold, \(P < .005\); Fig. 4) compared with vehicle control. Catalase mRNA levels were significantly and positively correlated with catalase protein, Cu/Zn SOD mRNA, Cu/Zn SOD protein, and TBARS levels \((P < .01)\) at all concentrations of \(\alpha\)-tocopherol. There was a nonsignificant positive trend between catalase mRNA and catalase activity \((P = .064)\) at all concentrations of \(\alpha\)-tocopherol treatment.

3.4. Immunodetection

The mean values of SOD protein observed at graded concentrations of 0, 10, 25, 50, and 100 μmol/L of \(\alpha\)-tocopherol were the following (Δ units): \(2.50 \times 10^{-3}\), \(8.75 \times 10^{-3}\), \(2.75 \times 10^{-3}\), \(3.00 \times 10^{-3}\), and \(1.50 \times 10^{-3}\), respectively. In HUVECs, no significant change in Cu/Zn SOD protein levels was found for all concentrations of \(\alpha\)-tocopherol (Fig. 5) compared with vehicle control. Cu/Zn SOD protein levels were significantly and positively correlated with Cu/Zn SOD mRNA levels, catalase protein, catalase mRNA,
catalase activity levels, and TBARS levels \((P < .05)\) at all concentrations of \(\alpha\)-tocopherol treatment.

The mean values of catalase protein observed at graded concentrations of 0, 10, 25, 50, and 100 \(\mu\)mol/L of \(\alpha\)-tocopherol were the following (\(\Delta\) units): \(8.50 \times 10^{-3}\), \(19.75 \times 10^{-3}\), \(10.25 \times 10^{-3}\), \(8.50 \times 10^{-3}\), and \(10.00 \times 10^{-3}\), respectively. A significant increase in catalase protein levels was found at the lowest concentration of \(\alpha\)-tocopherol treatment (10 \(\mu\)mol/L, 2.3-fold, \(P < .005\); Fig. 6) compared with vehicle control. Catalase protein levels were positively correlated with catalase mRNA levels, Cu/Zn SOD protein, mRNA levels, and TBARS levels \((P < .05)\) at all concentrations of \(\alpha\)-tocopherol treatment.

### 3.5. Enzyme activity

The mean values of Cu/Zn SOD enzyme activity observed at graded concentrations of 0, 10, 25, 50, and 100 \(\mu\)mol/L of \(\alpha\)-tocopherol were the following (U/ml): 0.147, 0.124, 0.188, 0.107, and 0.111 \(\mu\)mol/L, respectively (not shown). In HUVECs, no significant change in Cu/Zn SOD activity was found for all of the \(\alpha\)-tocopherol treatments compared with vehicle control. The mean values of catalase enzyme activity observed at graded concentrations of 0, 10, 25, 50, and 100 \(\mu\)mol/L of \(\alpha\)-tocopherol were the following (nmol/min/ml): 1.053, 2.897, 0.813, 1.908, and 0.813, respectively (not shown). There was no significant change in catalase activity at all concentrations of \(\alpha\)-tocopherol compared with vehicle control. There were positive correlation of catalase activity with Cu/Zn SOD protein levels and TBARS levels at all concentrations of \(\alpha\)-tocopherol treatment \((P < .05)\).
3.6. Thiobarbituric acid reactive substance

The mean values of TBARS observed at graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol were the following: 0.180, 0.954, 0.120, 0.180, and 0.173 pmol/L, respectively. Significant increases in TBARS level of HUVECs were exhibited at the lowest concentrations of α-tocopherols (10 μmol/L, 5.3-fold, \( P < .005 \); Fig. 7).

Thiobarbituric acid reactive substance levels were positively correlated with both Cu/Zn SOD and catalase protein levels, both Cu/Zn SOD and catalase mRNA levels, and catalase activity levels \( (P < .05) \) at all concentration of α-tocopherol treatment.

3.7. Transcription factor binding searches

Five possible binding sites (-5064, -4317, -3206, -2489, and -888) for PPARγ were found in the promoter region of human SOD1. Fourteen binding sites for PPARα were also identified in the region. Although a PPRE was identified in the rat catalase promoter region in a previous study [22], we could not identify any PPREs in the human catalase promoter region (approximately 10,000-base upstream from the translational starting site). Interestingly, 6 NF-κB binding sites (5′-GGGRNNYYCC-3′) were found in the SOD1 (-3866, -3570, -3406, -2406, -1716, and -540) and one binding site in the catalase promoter region (-6437). Both of these transcription factors, PPARγ and NF-κB, play a key role in inflammation. Nuclear factor-κB is also known to be a redox-regulated transcription factor [15] and is detected in most cell types. The number of binding sites with longer than hexamer recognition in the SOD1 promoter region was 2256, although there were 17 binding sites in the catalase promoter region. These findings indicate that more transcription factors are involved in SOD1 transcription than catalase transcription.
4. Discussion

Transcription factor search indicated that Cu/Zn SOD is a target gene of PPARγ, and Cu/Zn SOD and catalase are target genes for NF-κB. Furthermore, we found significant increases in PPARγ and NF-κB p50 DNA binding activities, lipid peroxidation, and changes in antioxidant enzyme expression with specific concentrations of α-tocopherol. Our findings support the following suppositions: α-tocopherol is a possible PPARγ activator and α-tocopherol treatments activate PPARγ and NF-κB to induce their target genes in a concentration-dependent manner. Although the α-tocopherol treatments increased PPARγ DNA binding activity, suggesting the potential of α-tocopherol as a PPARγ activator to induce at least Cu/Zn SOD expression, PPARγ DNA binding activity did not show a positive correlation with the expression of either Cu/Zn SOD or catalase. Thus, we speculate that the expression of the antioxidant enzymes may be regulated by combinational and/or feedback effects of PPARγ, NF-κB, and, possibly, other transcription factors as well.

Nuclear factor-κB p50 homodimer and p50/p65 heterodimer are thought to regulate inflammatory gene expression. The NF-κB p50/p65 heterodimer is activated by a variety of cellular responses to stimuli, such as stress, cytokines, free radicals, ultraviolet irradiation, pathogens, and LDL oxidation [5], [30] and [31], and plays a key role in regulating the immune and inflammatory responses. Nuclear factor-κB p50/p65 heterodimer activation requires IκB cleavage [32], which needs an oxidizing milieu [33]. The NF-κB p50/p65 heterodimer appears to be transcriptionally active, resulting in proinflammatory gene expression. Because NF-κB p50 subunit DNA binding activity
depends on the sulphydryl group of a basic amino acid (Cys62) of the subunit, the DNA binding activity is stimulated by reducing agents [34]. Our study showed the significant increases in NF-κB DNA p50 binding activity within a limited range of 10 to 50 μmol/L of α-tocopherol, indicating that the antioxidant activity of α-tocopherol treatment boosts its DNA binding by reducing the sulphydryl group of p50 Cys62. The NF-κB p50 homodimer binds to DNA at the same NF-κB recognition sites. The NF-κB p50 homodimer may function to compete for the binding sites with transactivating the NF-κB p50/p65 heterodimer [35]. The NF-κB p50 homodimer acts as a repressor in the absence of Bcl3, which acts as a coactivator of the NF-κB p50 homodimer. In addition, the NF-κB p50 homodimer-Bcl3 complex serves as an activator to induce anti-inflammatory gene expression. The complex with Bcl3 and/or the NF-κB homodimer induces anti-inflammatory cytokine interleukin 10 and inhibits proinflammatory cytokine tumor necrosis factor (TNF)-α in mouse macrophages [36], [37] and [38], exhibiting an opposite effect of the NF-κB p50/p65 heterodimer.

In this study, the lowest levels of mRNA and protein expressions of the antioxidant enzymes and lipid peroxidation were observed at medium concentrations of α-tocopherol. Compared with the control, significantly high levels of lipid peroxidation were observed only at the lowest concentration of α-tocopherol treatment (10 μmol/L), and lipid peroxidation was positively associated with the expression of Cu/Zn SOD and catalase at all concentrations of α-tocopherol. These findings support the suggestions that (1) the role of α-tocopherol is as an antioxidant to prevent oxidative stress by scavenging superoxide and free radicals at the medium concentrations, and (2) α-tocopherol is
involved in inducing the expression of these antioxidant enzymes observed at the lowest and the highest concentrations of treatment, which may not be optimal for \( \alpha \)-tocopherol to serve as an antioxidant. Thus, the concentration-dependent increase in NF-\( \kappa \)B DNA p50 binding activity may be associated with the formation of the NF-\( \kappa \)B p50 homodimer rather than the NF-\( \kappa \)B p50/p65 heterodimer, which leads to proinflammatory processes, although we were unable to distinguish clearly between these 2 different dimeric forms in this study design. Induction of these antioxidant enzyme expressions may be suppressed by increased NF-\( \kappa \)B p50 homodimer formation or NF-\( \kappa \)B p50-Bcl3 activation, which results in anti-inflammatory gene expression [36], [37] and [38] at medium concentrations of \( \alpha \)-tocopherol treatment. Therefore, induction of antioxidant enzymes may depend on oxidative stress. Induction of antioxidant enzymes may be repressed by total antioxidant capacity via NF-\( \kappa \)B p50 homodimer formation/activation or may be promoted by oxidative stress via NF-\( \kappa \)B p50/p65 heterodimer activation. Subsequently, expression of antioxidant enzymes may be influenced by microenvironment, such as the presence of adequate balance of exogenous antioxidants, and may be regulated through multiple signaling pathways.

Several studies support a link between the transcriptional factors PPAR\( \gamma \) and the NF-\( \kappa \)B p50/p65 heterodimer and antioxidants and/or antioxidant enzymes. Synthetic PPAR\( \gamma \) activators exert their anti-inflammatory actions through inhibiting NF-\( \kappa \)B p50/p65 heterodimer activation [39], [40], [41] and [42], thus, reducing ROS generation. For example, the PPAR\( \gamma \) ligand rosiglitazone has been shown to block inflammatory cytokine synthesis in colonic cell lines by inhibiting NF-\( \kappa \)B p50/p65 heterodimer activation [43].
Similarly, PPARγ activation results in decreases in NF-κB p50/p65 heterodimer activation and expression of NADPH oxidase and adhesion molecule [42] and [44]. Also, PPARγ activators increase both activity and protein expression of Cu/Zn SOD in HUVECs [11] and [24] and mRNA expression of catalase in rat brain cells/microvascular endothelial cells [22] and [45]. The NF-κB p50/p65 heterodimer is activated by superoxide, hydrogen peroxide, and TNF-α, all of which can be modulated by catalase and SOD expression [14] and [46]. It is also known that antioxidants such as polyphenols, α-tocopherol, and ascorbic acid exhibit anti-inflammatory activities by suppressing NF-κB p50/p65 heterodimer activation [30], [47] and [48]. Reactive oxygen species can induce LDL oxidation and superoxide can promote atherogenesis by inducing inflammation via NF-κB p50/p65 heterodimer activation [18]. An inflammatory cytokine, TNF-α, is produced from macrophages and induces endothelial activation, which can be prevented by an inhibitor of NADPH oxidase or SOD plus catalase [49].

Although the expression of rat catalase may be directly regulated by PPARγ, because of the existence of PPRE in its promoter region [22], expression of human catalase may be mediated directly by ROS through signal or as a second messenger via the NF-κB p50/p65 heterodimer pathway. Interestingly, NF-κB p50/p65 heterodimer activation is selectively mediated by hydrogen peroxide [15]. Another antioxidant enzyme, glutathione peroxidase (Gpx), also catalyzes the conversion of hydrogen peroxide to water; catalase does as well. Gpx induction is also ROS dependent, and its up-regulation is prevented by overexpression of extracellular SOD in mouse cardiomyocytes [50]. However, the induction of Gpx expression is associated with the inhibition of NF-κB
p50/p65 heterodimer activation [14]. Although Cu/Zn SOD is an antioxidant enzyme, the enzyme is induced in innate immunity (phagocytosis) to convert superoxide to microcidal hydrogen peroxide, and Cu/Zn SOD serves as a microcidal agent in host defense along with NADPH oxidase in phagocytes [51]. Hydrogen peroxide produced by Cu/Zn SOD may trigger induction of catalase through NF-κB p50/p65 activation. Within the context of our results, PPARγ activation may up-regulate Cu/Zn SOD as one of the anti-inflammatory gene products, and NF-κB p50/p65 activation may up-regulate both Cu/Zn SOD and catalase as proinflammatory gene products. Subsequently, the up-regulation by combinational and/or feedback effects of PPARγ and NF-κB may reduce ROS generation. Therefore, it is reasonable to suggest α-tocopherol may involve the control of redox status by regulating genes, whose products influence ROS generation, through redox-sensitive transcription factors PPARγ and NF-κB.

As our previous studies suggest [6], [26] and [52], α-tocopherol may act as a prooxidant under certain conditions, and therefore, the microenvironmental concentrations of α-tocopherol may be a key determinant for the induction of antioxidant enzymes. In this study, we found both mRNA and protein levels of Cu/Zn SOD and catalase were positively associated with lipid peroxidation within the α-tocopherol treatments. The levels of lipid peroxidation and both the mRNA and protein levels of these antioxidant enzymes were the lowest at medium concentrations of α-tocopherol treatment (25 and/or 50 μmol). Enzyme expression may depend on α-tocopherol levels and their status as an antioxidant or a prooxidant. α-Tocopherol may induce Cu/Zn SOD expression through the NF-κB p50/p65 heterodimer pathway by sending a signal, possibly as ROS or as
itself, in radical form. Then, hydrogen peroxide generated by induced Cu/Zn SOD may stimulate catalase expression through the same pathway.

Coexistence of other exogenous antioxidants is also a key determinant for the oxidative status of $\alpha$-tocopherol. Vitamin C serves as an exogenous antioxidant and may regenerate tocopherols from their radical forms. Vitamin C can also act as a prooxidant in the presence of transition metals in vitro. In addition, vitamins C and E modulate activities of both NADPH oxidase and SOD, which could contribute, at least in part, to decreasing vascular superoxide and improving antioxidant status [53]. Also, age-associated alteration of antioxidant enzyme expression has also been documented [25]. Induction of Gpx expression increases with age and is viewed as a compensatory response that guards against cellular damage in the absence of increased SOD and catalase [25]. Thus, the balance between $\alpha$-tocopherol and other antioxidants may be another determinant for the functions of $\alpha$-tocopherol: as an antioxidant to reduce ROS, which acts as signals to induce Cu/Zn SOD and catalase expression via the NF-\(\kappa\)B p50/p65 pathway, and as a direct gene regulator to induce the expression of the antioxidant enzyme Cu/Zn SOD via the PPAR\(\gamma\) pathway.

For future studies, it may be useful to examine (1) whether significant changes in antioxidant enzyme expression and lipid peroxidation are specific to $\alpha$-tocopherol or other PPAR\(\gamma\) ligands, (2) whether these changes also occur in the presence of other oxidants to determine the prooxidant effect of $\alpha$-tocopherol seen at the lowest concentration (eg, 10 $\mu$mol/L), (3) whether combinational treatments of $\alpha$-tocopherol with another antioxidant (eg, ascorbic acid) alter the pattern by preventing the prooxidant
effects of α-tocopherol, and (4) whether NF-κB p50/p65 DNA binding activity decreases when NF-κB p50 homodimer DNA binding activity increases in use of antibodies specific to NF-κB p65 and p50 (or Bcl3). Such information would be useful in further confirming the current findings.

In conclusion, α-tocopherol may play a role not only in preventing against oxidative stress as an exogenous antioxidant, scavenging free radicals and superoxide, it may also modulate endogenous antioxidant enzymes through PPARγ and NF-κB in vascular cells, serving as a gene regulator. Thus, α-tocopherol may be involved in modulating entire antioxidant defense systems, in which α-tocopherol induces endogenous antioxidant enzymes when exogenous antioxidants are imbalanced or limited to reducing ROS. The α-tocopherol-mediated gene expression may be either stimulatory or inhibitory, depending on its oxidative status or its concentrations. For the development of effective, preventive, and/or therapeutic strategies, optimum levels of antioxidant intakes should be explored to maintain minimum levels of oxidative stress for preventing and attenuating atherosclerosis for each individual.

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Fig. 1. Peroxisome proliferator-activated receptor γ DNA binding activity levels in HUVECs after treatment with graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol. All treatments resulted in significant increases of PPARγ DNA binding activities compared with vehicle control containing no α-tocopherol (10 μmol/L, $P < .01$, 25, 50, and 100 μmol/L, $P < .005$). Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 
Fig. 2. Nuclear factor-κB DNA binding activity levels in HUVECs after treatment with graded concentrations of 0, 10, and 50 μmol/L of α-tocopherol. α-Tocopherol treatments resulted in significant increases of NF-κB DNA binding activities compared with vehicle control containing no α-tocopherol ($P < .005$). Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 
Fig. 3. Cu/Zn SOD mRNA levels in HUVECs after treatment with graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol. α-Tocopherol treatments at 10 and 100 μmol/L resulted in significant increases of Cu/Zn SOD mRNA levels ($P < .005$) compared with vehicle control containing no α-tocopherol. α-Tocopherol treatments at 25 and 50 μmol/L resulted in significant decreases of Cu/Zn SOD mRNA levels ($P < .005$) compared with vehicle control containing no α-tocopherol. Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 
Fig 4. Catalase mRNA levels in HUVECs after treatment with graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol. α-Tocopherol treatment at 10 μmol/L resulted in a significant increase of catalase mRNA levels compared with vehicle control containing no α-tocopherol ($P < .005$). Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 
Fig. 5. Cu/Zn SOD protein levels in HUVECs after treatment with graded concentrations of 0, 10, 25, and 100 μmol/L of α-tocopherol. No significant changes were found in all α-tocopherol treatments (10 μmol/L, $P = .062$) compared with vehicle control containing no α-tocopherol. Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 


Fig. 6. Catalase protein levels in HUVECs after treatment with graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol. α-Tocopherol treatment at 10 μmol/L resulted in a significant increase of Cu/Zn SOD protein levels compared with vehicle control containing no α-tocopherol ($P < .005$). Values are means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 
Fig. 7. Thiobarbituric acid reactive substance levels in HUVECs after treatment with graded concentrations of 0, 10, 25, 50, and 100 μmol/L of α-tocopherol. α-Tocopherol treatment at 10 μmol/L resulted in a significant increase of TBARS levels compared with vehicle control containing no α-tocopherol ($P < .005$). Values are the means ± SD. *$P < .05$, **$P < .01$, and ***$P < .005$. 

![Graph showing MDA pmol/L vs. α-Tocopherol Treatment (μmol/L)](image-url)
Chapter 6

Summary, Conclusions, and Recommendations
Summary

CLA reviews

Previous CLA studies within and/or between humans and animals have shown conflicting findings. There are multiple factors to be considered for accurate extrapolation and interpretation in CLA human and animal studies. The factors include: interspecies/intraspecies-genetic differences, age and health status, dose, isomer types and their purity, and duration of supplementation.

Overproduction of ROS is involved in the etiologies of atherosclerosis and other chronic diseases. Earlier studies have shown that the ability of CLA isomers to act as anticarcinogens and as antiatherosclerotic agents might be due to their roles as antioxidants. However, in light of current research, modulation of ROS-related diseases by CLA isomers may involve the control of ROS generation by regulating genes, whose products influence ROS generation, through redox-sensitive transcription factors such as PPARγ and NF-κB. Further research is warranted to elucidate the CLA isomer-mediated mechanisms associated with ROS-generation and the etiologies of ROS-related diseases, such as atherosclerosis and cancer.

Vitamin E review

Besides the antioxidant function of vitamin E, vitamin E’s multiple functions have been suggested: prooxidant, inhibitor of ROS-generating enzyme activities, and inducer and/or inhibitor of gene and protein expression. The effects may rely on micro-environments including: the presence of oxidants or other antioxidants, the concentrations of vitamin E,
and the balance between concentrations of vitamin E and other antioxidants. Vitamin E may regulate gene expression associated with ROS generation through PPARγ and NF-κB in a combinational and/or feedback-loop manner. The vitamin E-modulated gene regulation may be stimulatory or inhibitory, depending on the oxidative status (i.e., prooxidant activity) and concentrations of vitamin E.

Thus, vitamin E may directly and indirectly control ROS generation by scavenging free radicals and superoxide and by regulating gene expression associated with ROS-generation.

CLA study

This study was conducted to examine the effects of CLA isomers on gene expression of antioxidant enzymes (Cu/Zn SOD and catalase) and on ROS generation in HUVECs. Five possible binding sites for PPARγ were found in the human SOD1 promoter region. Six possible binding sites for NF-κB were found in the promoter region of the human SOD1 and one in that of the human catalase. Both 9-cis, 11-trans and 10-trans, 12-cis CLA isomer treatments significantly increased (p<0.005) PPARγ and NF-κB DNA binding activities at lower concentrations (5-25μmol/L), compared to vehicle control. There were significant increases (p<0.05) in expression of Cu/Zn SOD mRNA and catalase mRNA in all 9-cis, 11-trans CLA treatments. The protein expression of both SOD and catalase significantly increased (p<0.05) in all 10-trans, 12-cis CLA isomer treatments. There were significant increases (p<0.05) in mRNA expression of both Cu/Zn SOD and catalase at lower concentrations (5-10μmol/L) of the 10-trans, 12-cis CLA
isomer. There were significant increases (p<0.05) in lipid peroxidation at only the lowest concentration (5μmol/L) of both the 9-cis, 11-trans and 10-trans, 12-cis CLA isomers. The expression of antioxidant enzymes was positively correlated (p<0.05) with lipid peroxidation. These results indicate that oxidative susceptibilities of CLA isomers cause CLA isomers to possess prooxidant activities at low concentrations of CLA isomers, leading to subsequent ROS generation and expression of the antioxidant enzymes through NF-κB activation. In contrast, CLA isomers might induce the enzyme expression (in particular Cu/Zn SOD) mainly through PPARγ activation at high concentrations of CLA isomers, serving as PPARγ activators.

Thus, the effects of CLA isomers are mostly concentration-dependent in favor of activation of PPARγ and NF-κB with subsequent changes in expression of Cu/Zn SOD and catalase. CLA isomers may act as either beneficial agents or adverse agents, depending on their concentration and microenvironments. Hence, CLA isomers may possess inhibitory and stimulatory effects in atherogenic and inflammatory processes.

Vitamin E study

This study was designed to examine the effects of α-tocopherol on gene expression of Cu/Zn SOD and catalase and on ROS generation in HUVECs. There were significant increases (p<0.01) in PPARγ DNA binding activities in α-tocopherol treatments at 10-100μmol/L and significant increases (p<0.005) in NF-κBp50 DNA binding activities in α-tocopherol treatments at 10-50μmol/L, compared to vehicle control. Significant increases (p<0.005) in Cu/Zn SOD mRNA levels were observed in α-tocopherol
treatments at 10 and 100μmol/L, while there were significant increases (p<0.005) in catalase mRNA levels in α-tocopherol treatment only at 10μmol/L. Interestingly, significant decreases (p<0.005) in Cu/Zn SOD mRNA levels were seen in α-tocopherol treatments at 25 and 50μmol/L. α-Tocopherol treatment at 10μmol/L significantly increased (p<0.005) both catalase protein levels and lipid peroxidation. Positive correlations were found between the expression of the antioxidant enzymes and lipid peroxidation (p<0.05). The results of this study suggest α-tocopherol’s multi-functions: 1) prooxidant that provokes subsequent ROS generation and the expression of the antioxidant enzymes through NF-κBp50/p65 heterodimer activation, 2) antioxidant that prevents ROS generation and suppresses the gene expression of Cu/Zn SOD via NF-κB p50/p65 activation by the formation of NF-κBp50/p50 homodimer, and 3) PPARγ activator that induces the expression of, in particular, Cu/Zn SOD.

The effects of α-tocopherol on the expression of the antioxidant enzymes and lipid peroxidation appear to be concentration-dependent. The α-tocopherol-mediated expression may be either stimulatory or inhibitory, depending on its oxidative status or its concentrations. α-Tocopherol may be involved in modulating the entire antioxidant defense system, in which α-tocopherol induces endogenous antioxidant enzymes when exogenous antioxidants are imbalanced and/or limited in reducing ROS. Thus, α-tocopherol may play a role not only in preventing against oxidative stress as an exogenous antioxidant by scavenging free-radicals and superoxide, but also in modulating endogenous antioxidant enzymes through transcription factors and serving as a gene regulator.
Conclusions and Recommendations

Overall in this research, these lipophilic compounds, CLA isomers and vitamin E, may modulate redox status by regulating genes, whose products influence ROS generation, through transcription factors such as PPARγ and NF-κB. Therefore, the lipophilic compound-mediated gene expression may affect the etiologies of ROS-related diseases, such as atherosclerosis and cancer. According to the results of our experiments, the effects of these lipophilic compounds are concentration-dependent. Therefore, the compounds may act as either beneficial or adverse agents in the processes of ROS-related diseases. The uniform supplementations of these compounds in previous clinical studies failed to show beneficial outcomes, probably at least in part, due to the concentration-dependent effects of these compounds, as well as the differences in microenvironments of the clinical study subjects (i.e., co-existence of other antioxidants) and the genetic variations of the study subjects (i.e., SNPs in genes related to ROS-generation).

We suggest that adequate amounts of supplemental CLA isomers and α-tocopherol should be tailored and prescribed to elicit beneficial effects, i.e., minimum ROS generation and oxidative stress, at individual levels, for preventive and therapeutic strategies.