ß-CAROTENE AND α-TOCOPHEROL INHIBIT THE DEVELOPMENT OF ATHEROSCLEROTIC LESIONS IN HYPERCHOLESTEROLEMIC RABBITS

by

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A DISSERTATION

Presented to the Faculty of
The Graduate College at the University of Nebraska

In Partial Fulfillment of Requirements

For the Degree of Doctor of Philosophy

Major: Interdepartmental Area of Nutrition

Under the Supervision of Professor Judy A. Driskell

Lincoln, Nebraska

November, 1995

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Ann Arbor, MI 48103

DISSERTATION TITLE

Beta-carotene and alpha-tocopherol inhibit the development of atherosclerotic lesions in hypercholesterolemic rabbits

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University of Nebraska, 1995

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Male New Zealand White rabbits were made hypercholesterolemic by feeding an atherogenic diet (0.5% cholesterol, 3% peanut oil, and 3% coconut oil) with or without antioxidants. The antioxidant treatments were intravenous injection of ß-carotene (25 mg/kg/BW, twice weekly), dietary α-tocopherol (0.5%), and a combination of both. Rabbits treated with ß-carotene had significantly higher plasma and lipoprotein ß-carotene concentrations at 4 and 8 weeks, and significantly lower total and LDL cholesterol concentrations, thoracic atherosclerotic lesion area, and aortic intimal thickness at 8 weeks, but had no effects on TBARS and lag time values compared to control. Rabbits treated with dietary α-tocopherol had significantly higher plasma and lipoprotein α-tocopherol concentrations and lag time values at 4 and 8 weeks, significantly lower TBARS values at 4 and 8 weeks, and total atherosclerotic lesions and aortic intimal thickness at 8 weeks compared to the control. Rabbits treated with a combination of both antioxidants had significantly higher plasma and lipoprotein ß-carotene and α-tocopherol concentrations and lag time values at 4 and 8 weeks, and significantly lower TBARS values at 4 and 8 weeks, as well as significantly lower total atherosclerotic lesion area and aortic intimal thickness at 8 weeks compared to control, but not the ß-carotene or the α-tocopherol

groups. These data suggest that the antihypercholesterolemic effect of ß-carotene and antioxidant effect of α-tocopherol may benefit the rabbits fed an atherogenic diet by inhibiting the development of atherosclerosis.

ACKNOWLEDGEMENTS

The author wishes to express his most sincere appreciation to the many people who assisted and advised in the research. I especially want to thank the following people:

Dr. Judy A. Driskell, the major advisor, who gave help, advice, and encouragement throughout the entire project;

Dr. Mary M. Beck, Dr. Nancy M. Betts, Dr. Patricia C. Crews, Dr. James E. Kinder, Dr. Terry J. Klopfenstein, and Dr. Rodney A. Moxley, for their support, encouragement, and advice;

David Giraud, LaVonne Johns, Robin Olmscheid, Gene Steppat, and Wen Li Young, for their insights and technical assistance which ultimately led to the successful completion of this work;

Last, Bei Cheng, my wife, whose help and support throughout this past years made the completion of this work possible.

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INTRODUCTION

Coronary heart disease (CHD), primarily caused by atherosclerosis, remains by far the leading cause of death in United States (Barber & Harris 1994, Reynolds 1994, Gaziano 1994). It is responsible for one of every three deaths in women as well as men. Thus, any intervention that can reduce CHD risk could have a tremendous public health impact among U.S. adults. Over the past several decades, the atherogenic potential of low-density lipoprotein (LDL) cholesterol has been identified (Witztum 1994, Berliner et al 1995). Recent evidence suggests that oxidation of LDL may enhance its atherogenicity (Steiberg et al 1989, Luc & Fruchart 1993), raising the possibility that antioxidant vitamins, such as ß-carotene and α-tocopherol, may reduce the risk of CHD (Frei 1995, Gaziano 1994, Hennekens 1994, Hoffman et al 1995).

ß-carotene is a very effective quencher of singlet oxygen (Foote & Denny 1968, Stahl & Sies 1993) and can also act as a chain-breaking antioxidant under certain conditions (Burton & Ingold 1984). When added in micromolar concentrations in vitro, ß-carotene appears to inhibit the oxidation of fatty acids and liposomes (Krinsky 1989, Palozza & Krinsky 1992, Liebler 1993). ß-carotene is transported predominantly with LDLs (Clevidence & Bieri 1993, Traber et al 1994a) and dietary supplementation with ß-carotene leads to considerably increased ß-carotene levels in the LDLs (Princen et al 1992, Reaven et al 1993, Jialal & Grundy 1993, Reaven et al 1994, Gaziano et al 1995). Significantly lower LDL ß-carotene levels are observed in coronary disease patients and smokers (Croft et al 1991).

It is observed in vitro that as long as there are antioxidants present in LDL, the rate of lipid peroxidation is low and then starts to increase once the antioxidants have been used up (Esterbauer et al 1992, Lynch et al 1994). Among those endogenous antioxidants, ß-carotene is the last antioxidant to be consumed when LDL is exposed to oxidizing conditions (Lynch et al 1994). Depletion of ß-carotene corresponds with formation of conjugated dienes, increased susceptibility to further oxidation, and aggregation of apolipoprotein B-100 (Chang et al 1994).

Addition of exogenous ß-carotene to LDL in vitro has yielded inconsistent results. Jialal et al (1991), Lavy et al (1993), and Packer (1993) reported strong protective effects of added ß-carotene against cell-free and macrophage-mediated LDL oxidation and subsequent uptake by the macrophage scavenger receptor. ß-carotene is also effective in preventing the oxidation of LDL and lipoprotein(a), an LDL-like particle that has been implicated as an independent risk factor for atherosclerosis (Naruszewicz et al 1992). In contrast, two groups of investigators have found no protection against endothelial cell-mediated or Cu^{2+}-induced LDL oxidation, even though large excesses of ß-carotene were added (Van Hinsbergh et al 1986, Gaziano et al 1995).

In vivo, several studies have found that ß-carotene supplementation did not confer increased protection against LDL to oxidation despite a 2.5- to 35-fold increase in LDL ß-carotene levels (Princen et al 1992, Abbey et al 1993, Jialal & Grundy 1993, Reaven et al 1993, Reaven et al 1994, Gaziano et al 1995). However, Allard et al (1993) found that ß-carotene supplementation significantly reduced the

lipid peroxidation measured by breath-pentane output (BPO) in smokers. Smith et al (1994) reported that the injection of 20 mg/day ß-carotene significantly lowered plasma malondialdehyde (MDA), and increased lag time for LDL conjugated diene formation in Yucatan Miniature Swine.

It remains possible that the ß-carotene contained within the LDL particle may inhibit LDL oxidation in vivo, but the ex vivo assay of LDL oxidation does not readily mimic the in vivo process. On the other hand, ß-carotene may inhibit oxidation of LDL not from within the particle but by reducing oxidation in atherosclerotic plaques where ß-carotene clearly accumulates (Prince et al 1988, Mitchell et al 1993). Preincubation of co-cultures of aortic endothelial cells and smooth muscle cells with ß-carotene prevented LDL oxidative modification and its induction of monocyte transmigration (Navab et al 1991). Reduction of atherosclerotic plaques in the thoracic aorta and lesions/serum cholesterol by ß-carotene supplementation were observed in animal studies (Smith et al 1994, Ziemlanski & Panczenko-Kresowska 1994). Furthermore, epidemiological and clinical data have shown a decreased risk of CHD with increased ß-carotene intake (Gaziano & Hennekens 1993, Hennekens 1994, Riemersma 1994, Gaziano 1994, Hoffman & Garewall 1995, Machlin 1995).

α-tocopherol, biologically and chemically the most active form of vitamin E, is by far the most abundant antioxidant in LDL (Frei 1994). It belongs to the first line of antioxidant defenses (Jessup et al 1990, Frei and Gaziano 1993, Smith et al 1993, Lynch et al 1994). Enrichment of LDL with α-tocopherol in vitro has been shown to

inhibit LDL oxidation by endothelial cells (Van Hinsbergh et al 1986), macrophages (Leak & Rankin 1990), monocytes (Cathcart et al 1985), and Cu^{2+} (Esterbauer et al 1991). In vivo, almost all of the studies that employed supplementation with α-tocopherol found significantly increased resistance of LDL to oxidation by Cu^{2+} (Dieber-Rotheneder et al 1991, Jialal & Grundy 1992a,b, Princen et al 1992, Reaven et al 1993, Reaven & Witztum 1993a, Suzukawa et al 1995), macrophages (Jessup et al 1990), and endothelial cells (Reaven et al 1993, Reaven & Witztum 1993).

Recent data on the potential antiatherogenic effects of vitamin E in animal studies are inconsistent, depending on atherogenicity of diet, the stage of atherosclerotic lesion development at the time of antioxidant intervention, and the vitamin E levels administered. Proatherosclerotic (Dam 1944, Godfried et al 1989, Keaney et al 1994), antiatherosclerotic (Wilson et al 1978, Westrope et al 1982, Wójcicki et al 1991, Bocan et al 1992, Verlangieri & Bush 1992, Williams et al 1992, Prasad & Kalra 1993, Keaney et al 1994, Kleinveld et al 1994, Kazdova et al 1995, Lafont et al 1995), or inconclusive effects of vitamin E feeding were observed (Godfried et al 1989, Bocan et al 1992, Willingham et al 1993, Keaney et al 1994). However, epidemiological and clinical data have shown a decreased risk of CHD with increased vitamin E (Gaziano & Hennekens 1993, Hennekens 1994, Riemersma 1994, Gaziano 1994, Hoffman & Garewall 1995, Machlin 1995).

Because of their similar chemical properties and distributions in LDLs, the relationship between ß-carotene and α-tocopherol has attracted much attention. Leibovitz et al (1990) showed that dietary ß-carotene had an inhibitory effect on the

lipid peroxidation of rat liver tissue in cooperation with α-tocopherol. Palozza & Krinsky (1992) found a synergistic effect of ß-carotene and α-tocopherol on free-radical-mediated lipid peroxidation of liver microsomes. Ojima et al (1993) demonstrated that ß-carotene may protect α-tocopherol from oxidative loss by singlet oxygen. Terao et al (1980) indicated that α-tocopherol prolonged the inhibitory effect of ß-carotene on singlet oxygen-initiated photooxidation by preventing the decomposition of ß-carotene. Kagan et al (1992) reported that vitamin E may protect ß-carotene in LDL against peroxyl radicals.

The domestic rabbit (*Oryctolagus cuniculus dom*) is the first and most frequently used animal in atherosclerosis research (Vesselinovitch 1988). The New Zealand White rabbit is particularly sensitive to dietary cholesterol, easy to feed and handle, large enough for considerable tissue and blood samples, and develops atherosclerotic lesions relatively quickly and easily (Adams et al 1972, Shore & Shore 1976, Pescador 1978, Beynen et al 1984, Clarkson et al 1988). The predominant evidence suggests that older animals are more susceptible to atherosclerosis than younger animals (Spagnoli et al 1991).

The usual atherogenic diet consists of commercial rabbit chow supplemented with 0.2 to 3% cholesterol and/or 4 to 8% fat (Jokinen et al 1985). To maintain the general health of rabbits, dietary cholesterol should not exceed 0.5% when the diet already contains added fat (Jayo et al 1994). Human-like atherosclerotic lesion can be produced in rabbits by producing endogenous hypercholesterolemia by feeding a diet containing hydrogenated coconut oil and peanut oil (St. Clair 1983, Clarkson et al

1988). Bocan et al (1993) suggested that a diet with 0.5% cholesterol, 3% peanut oil, and 3% coconut oil would be good for atherosclerotic lesion development.

The objectives of this study were to investigate:

1). Whether supplementation with ß-carotene or α-tocopherol or a combination of both to rabbits fed with an atherogenic diet would decrease plasma lipid and lipoprotein levels.

2). Whether enrichment of LDL by giving ß-carotene or α-tocopherol or a combination of both to rabbits fed with an atherogenic diet would reduce the formation of conjugated dienes and MDA.

3). Whether supplementation with ß-carotene or α-tocopherol or a combination of both to rabbits fed with an atherogenic diet would reduce the area of aortic atherosclerosis and the intimal thickness of aorta.

The overall goal of this study was to determine whether ß-carotene or α-tocopherol or combination of both would inhibit the development of atherosclerotic lesions in rabbits fed with atherogenic diet.

LITERATURE REVIEW

Atherosclerosis

Atherosclerosis is a degenerative disease of larger- and medium-sized arteries characterized by hardening and losses of elasticity of the arterial walls narrowing the lumen of the arteries due to local thickening of the intima, the innermost part of the arterial wall (Ross 1992, Kumar et al 1992). Atherosclerosis is the principal cause of cardiovascular and cerebrovascular disease leading to ischemic heart disease (IHD), myocardial infarction (MI, the most serious form of IHD), and ischemic stroke (Barber & Harris 1994). No disease in the United States is responsible for more deaths than heart disease. In 1989, nearly 43% of the deaths in the United States were due to some form of heart disease (Barber & Harris 1994). In 1993, an estimated 1.5 million Americans had heart attacks, with approximately 500,000-600,000 resulting deaths. The estimated cost of coronary heart disease in 1993 was 51.6 billion (Reynolds 1994).

The atherosclerosis lesion develops in several stages (Ross 1993). Initially, fatty streaks are formed, which are characterized by the presence of lipid-laden, so called foam cells. Fatty streaks may then develop into fibrous plaques, which consist of a dense connective tissue cap covering an area containing a number of different cell types, including foam cells, macrophages, smooth muscle cells, and T lymphocytes. At this stage foam cell necrosis occurs and cell debris, extracellular lipid, and cholesterol crystals are deposited. The final stages of atherosclerosis are characterized by mature, fibrous plaques formed by continued proliferation of myoinitimal cells,

foam and endothelial cell necrosis, hemorrhage, mural thrombosis, and calcium deposition.

The Oxidation Hypothesis of Atherosclerosis

In the most widely accepted current concept of atherogenesis, oxidation of LDL plays a pivotal role (Steinberg et al 1989, Luc & Fruchart 1993). This hypothesis originates with the seminal observation made by Goldstein et al (1979) that macrophages in culture are converted into lipid-laden cells in the presence of chemically altered LDL, but not native LDL. One chemical modification that very effectively converts LDL into a form rapidly internalized by macrophages is oxidation (Henriksen et al 1981). These findings stimulated a great number of studies that led to formulation of the "oxidation hypothesis of atherosclerosis".

Mechanisms of LDL Oxidation

LDL oxidation in atherosclerosis may be mediated by all cell types found in vascular walls (Chang et al 1994). All major cell types of the arterial wall (including endothelial cells, smooth muscle cells, and macrophages) are capable of oxidizing LDL (Holvoet & Collen 1994, Penn & Chisolm 1994, Berliner et al 1995). LDL oxidation does not occur in the presence of serum and requires trace amounts of redox-active metals (Cu or Fe) (Lynch 1993), which are contained in increased levels in arterial walls, suggesting that LDL is not oxidized in the circulation (Smith et al 1992). The nature and sources of the oxidations that initiate formation of oxidatively modified LDLs in the arterial walls have been the subject of much speculation. Extracellular superoxide anion radicals, nitric oxide radicals, myeloperoxides, and 15-

lipoxygenases are possible mediators (Chang et al 1994).

Superoxide anion radicals ($O_2^{-\cdot}$), a cell-derived oxidant from macrophages or smooth muscle cells, might be responsible for initiation of oxidation (Hiramatsu et al 1987, Jessup et al 1993, White et al 1994). Superoxide anion radicals are involved in autoxidation of extracellular thio compounds (Sparrow & Olszewski 1993) and react with nitric oxide radicals to form peroxynitrite anions, which decompose into the hydroxyl radicals ($OH^{\cdot}$), a potent mediator of lipoprotein oxidation (White et al 1994). Nitric oxide radicals ($NO^{\cdot}$) are potentially relevant to LDL oxidation by endothelial cells and macrophages. Chang et al (1994) demonstrated that $NO^{\cdot}$ under certain circumstances may contribute to oxidative modification of LDL. Recently, the myeloperoxidase-derived oxidants hypochlorite- and tyrosyl- radicals have been suggested to convert LDL to a form taken up at increased rates by macrophages (Hazell & Stocker 1993, Daugherty et al 1994). Finally, 15-lipoxygenase-derived products have been implicated in LDL oxidation by endothelial cells and macrophages (Parthasarathy et al 1989, Rankin et al 1991, Folcik et al 1995).

One of the initial events in cell-mediated and metal ion-induced LDL oxidation presumably is the peroxidation of polyunsaturated fatty acids (PUFA) on LDL-surface phospholipids (Hoffman & Garewal 1995). Once formed, the carbon-centered PUFA radical reacts very quickly with molecular oxygen to yield a lipid peroxyl radical which in turn abstracts a hydrogen atom from an adjacent PUFA, yielding a lipid hydroperoxide and a new PUFA radical. It is the latter reaction that carries on the lipid peroxidation chain. If no chain termination takes place, a single initiating event

could convert all LDL PUFAs into lipid hydroperoxides (Esterbauer et al 1993). The antioxidants in LDL compete with chain propagation by very efficiently scavenging lipid peroxyl radicals. Before substantial amounts of lipid hydroperoxides are formed, LDL becomes depleted of its antioxidants, with α-tocopherol being consumed first and ß-carotene last (Esterbauer et al 1992, Lynch et al 1994).

Following initiation, free radicals rapidly attack the core lipids, resulting in the formation of phospholipid hydroperoxides (Witztum 1993). Phospholipid hydroperoxides then are hydrolyzed to lysophospholipids and fatty acid hydroperoxides. Fatty acid hydroperoxides subsequently break down to a whole array of products, including reactive aldehydes such as malondialdehyde and 4-hydroxynonenal. These aldehyde products react with the positively charged ϵ-amino groups of lysine residues in the apolipoprotein B-100 moiety of LDL, forming Schiff's bases and lead to an increased negative net charge of the LDL particle. As a consequence, LDL is no longer recognized by the normal (apo B/E) LDL receptor. Instead, macrophages take up LDL by the oxidatively modified LDL receptors (i.e., the scavenger receptors), unlike the apolipoprotein B/E receptor, which is not subject to down-regulation by intracellular cholesterol content (Goldstein et al 1979, Steinberg et al 1989). Uptake of oxidatively modified LDL by macrophages via the scavenger receptor is three to ten times more rapid than uptake of native LDL (Steinberg et al 1989, Holvoet & Collen 1994). Therefore, the macrophages accumulate large amounts of lipids from oxidized LDLs and eventually are converted into foam cells, which are the hallmark of atherosclerotic fatty streaks (Frei 1994).

Role of Oxidized LDL in Atherosclerosis

It is generally accepted that the initiation of atherosclerotic lesion formation involves alteration of the structural or functional integrity of the endothelial barrier, which may be the consequence of increased endocytic activity in cells exposed to atherogenic levels of LDLs (Holland et al 1992, Ross 1993), allowing a net influx of lipoproteins from the circulating plasma into the subendothelium (Ross 1993, Consigny 1995). Once beneath the endothelium, LDL becomes trapped. This trapping increases the probability that LDL will be modified (Consigny 1995).

Increased adherence of monocytes to arterial endothelium constitutes one of the early visible changes in experimental atherosclerotic animals (Joris et al 1983, Back et al 1995, Ross 1995). Oxidized LDL stimulates monocyte adhesion by inducing the expression of monocyte-specific binding molecules on the endothelial cell surface (Penn & Chisolm 1994). These molecules are monocyte chemotactic protein-1 (MCP-1), granulocyte colony-stimulating factor(GM-CSF), and macrophage colony-stimulating factor (M-CSF), which can cause increased endothelial adhesiveness and stimulate proliferation and differentiation of macrophages in endothelial and smooth muscle cells (Cushing et al 1990, Lehr et al 1991, Ross 1995). M-CSF induces the expression of scavenger receptors at the surface of macrophage, resulting in enhanced uptake of oxidized LDL and in enhanced foam cell generation (Ishibash et al 1990). Oxidized LDL enhances both smooth muscle cell migration via induction of platelet-derived growth factor in macrophages and smooth muscle cells and smooth muscle cell proliferation via induction of basic fibroblast growth factor in endothelial cells