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Journal of Nutritional Biochemistry 17 (2006) 780–786
Journal of Nutritional Biochemistry

Carotenoids enhance phosphorylation of Akt and suppress tissue factor activity in human endothelial cells

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Received 17 October 2005; received in revised form 14 December 2005; accepted 19 January 2006

Abstract

Enhanced production of tissue factor has been linked to development of cardiovascular disease due to endothelial activation, resulting in thrombosis of blood vessels. Epidemiological studies reported that diet-derived antioxidants might suppress and/or delay progression of cardiovascular disease. Detailed molecular level studies are needed to understand this effect with prevention as a goal. Water-dispersible forms of various carotenoids (β-carotene, lutein and lycopene) from natural sources in microemulsion were used to study effects of carotenoids on tissue factor activity in human endothelial cells. All carotenoids studied suppressed tissue factor activity ($P<.01$) and gene expression in human endothelial cells. Our study also demonstrated that addition of Akt-specific inhibitor reversed the inhibitory effect of carotenoids on tissue factor activity, indicating that carotenoids enhanced phosphorylation of Akt and suppressed tissue factor activity in endothelial cells by this mechanism.

Keywords: Akt; Carotenoids; Endothelial cells; Oxidative stress; Tissue factor

1. Introduction

Tissue factor, a transmembrane glycoprotein with an apparent molecular weight of 47 kDa, is expressed in many cells including smooth muscle cells, fibroblasts, monocytes and activated endothelial cells [1]. Activation of tissue factor plays a key role in vascular thrombosis through the conversion of fibrinogen to fibrin [2]. Vessel injury results in an increase of tissue factor expression and activation of tissue factor: factor VIIa complex, which leads to fibrin deposition and activation of platelets [3].

Increase of oxidative stress in the vascular system has been reported to enhance tissue factor production in response to activated platelets, resulting in development of unstable atherosclerotic plaque with resultant thrombosis [4,5]. Pathophysiological pathways increase production rates of reactive oxygen species and/or decrease capacity to remove reactive oxygen species [6–8]. Studies over the last few decades suggest that diet-derived antioxidants may help to prevent and/or delay progression of the pathogenic pathways, modifying the risk of cardiovascular disease caused by oxidative damage to endothelial cells [9–11].

While β-carotene has been studied for several decades, lycopene, a red color pigment found in tomatoes, has recently attracted interest for its beneficial effects in cardiovascular disease and cancer [11]. Lycopene has been found to suppress progression of coronary heart disease and atherosclerosis by reducing oxidative modification of low-density lipoprotein [9]. Lycopene, an acyclic form of β-carotene, exhibits higher singlet oxygen quenching capacity than β-carotene due to a higher number of double bonds [12]. It is also resistant to heat during cooking, which makes lycopene a good source of diet-derived antioxidant.

Lutein is a xanthophyll carotenoid with antioxidant activity. An inverse relationship between the serum concentration of lutein and ocular diseases, such as age-related macular degeneration and cataract, has been reported [13]. Epidemiological, in vitro and animal studies have found that increased dietary intake of lutein was protective against development of early atherosclerosis [14]. However, other

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doi:10.1016/j.jnutbio.2006.01.006

epidemiological study showed inconsistent association between high serum concentration of lutein and lower risk for cardiovascular disease [15].

A growing number of studies support the beneficial effects of high plasma concentrations of carotenoids in prevention of cardiovascular disease [9–11]. In addition, available data continue to demonstrate beneficial effects of moderate consumption (five to nine servings per day) of fruits and vegetable rich in carotenoids for human health [16]. Several recent clinical trials, however, showed no statistically significant association between β-carotene supplements and cardiovascular disease [17,18]. Thus, detailed molecular level studies are necessary to dissect mechanisms implicated in effects of carotenoids on vascular diseases. We took advantage of water-dispersible forms of various dietary carotenoids in microemulsion to study molecular effects of carotenoids on tissue factor activity in human endothelial cells.

2. Methods

2.1. Human endothelial cells

Human umbilical vein endothelial cells (HUVECs) and human aortic endothelial cells (HAECs) obtained from healthy adults were purchased from Cambrex Bioscience (Walkersville, MD). Human endothelial cells were maintained in endothelial growth medium-2 according to the manufacturer's instructions (Cambrex Biosciences). Endothelial growth medium-2 is composed of basic endothelial medium-2 and supplements [human epidermal growth factor, vascular endothelial growth factor, fibroblast growth factor-b, insulin-like growth factor-1, gentamicin/amphotericin B, hydrocortisone, 2% or 5% fetal bovine serum (FBS) and ascorbic acid]. All human endothelial cells used in this study were within the third to sixth passages.

2.2. Carotenoids

Due to extremely low solubility of carotenoids in culture media [19], various carotenoids dissolved in microemulsion were used in this study. All carotenoids used in this study were extracted from natural sources (algae *Dunaliella salina* for β-carotene, marigold flower *Tagetes erecta* for lutein and tomatoes for lycopene) and emulsified into a glycerol/water aqueous phase. Lycopene, lutein, β-carotene and vehicle (microemulsion without carotenoids) were generous gifts from Cognis Australia (Victoria, Australia). Natural β-carotene exists in two isoforms, cis and trans. The ratios of cis and trans isomer forms in β-carotene are 50:50.

2.3. Tissue factor activity assay

Human endothelial cells were grown in a humidified incubator with 5% CO_2 at 37°C. When cells in six-well plates reached approximately 90% confluency, the medium was replaced with phenol red-free Dulbecco's modified Eagle medium (DMEM) (Invitrogen) containing 0.5% FBS with or without various carotenoids (final concentration, 0.5–10 μmol/L). Cells were maintained in medium containing low serum for 16 h and stimulated with human thrombin (Sigma) at a final concentration of 3 U/ml for 7 h. When necessary, cells were incubated in the medium containing 4 μmol/L Akt-specific inhibitor IV for 2 h (Calbiochem) before addition of thrombin. Cells were rinsed twice with 1× phosphate-buffered saline (PBS), and tissue factor was extracted in 200 μl of extraction buffer (50 mmol/L Tris–HCl, pH 7.4, 100 mmol/L NaCl and 0.1% Triton X-100) by repeated cycles of freezing and thawing.

Tissue factor activity was determined using the Actichrome kit (American Diagnostica) according to the manufacturer's instruction. Briefly, 25 μl of each cell extract in a 96-well plate was mixed with the same volume of human factor VIIa and factor X. Spectrozyme FXa substrate was added to the samples, and the samples were incubated for 1 h at 37°C. The enzymatic reaction was terminated by addition of 50 μl of glacial acetic acid. Optical density at 405 nm was measured using a microplate reader (Bio-Rad). Optical density readings of each cell extract without human factor VIIa and factor X were used as blanks for calibration. Total protein concentration of each extract was determined using Bradford assay (Bio-Rad) and was used to normalize tissue factor activity. Optical density of cell extract without thrombin treatment was taken as 1 in each set of experiments for statistical analysis. At least three independent experiments were performed for analysis of tissue factor activity for each cell type.

2.4. Semiquantitative reverse transcriptase-polymerase chain reaction

Total RNAs from human endothelial cells grown on 75-cm^2 culture flasks under the same conditions used for the

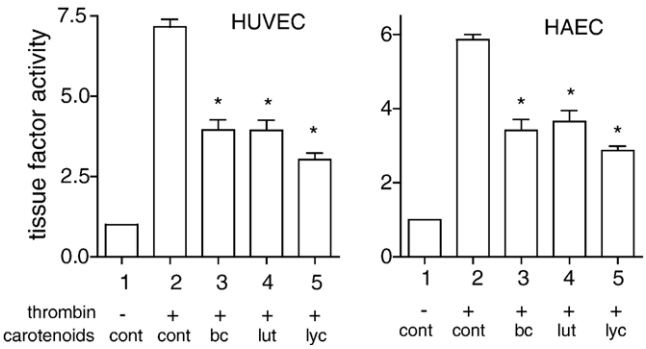

Fig. 1. Effects of various carotenoids on tissue factor activity in endothelial cells. Tissue factor activity was determined using the Actichrome kit. Carotenoids used were 10 μmol/L of β-carotene (bc), lutein (lut) and lycopene (lyc). Control (cont) is a microemulsion without carotenoids. Optical measurement of tissue factor activity without thrombin treatment was calculated as 1 in each set of experiment for statistical analysis. Total protein concentration of each extract was determined using Bradford assay (Bio-Rad) and was used to normalize tissue factor activity. Results are presented as means±S.E.M.; *n* (numbers of experiments performed)=3. **P*<.01 vs. Lane 2. Statistical comparison of data was determined using two-way ANOVA followed by the Bonferroni posttest adjustment.

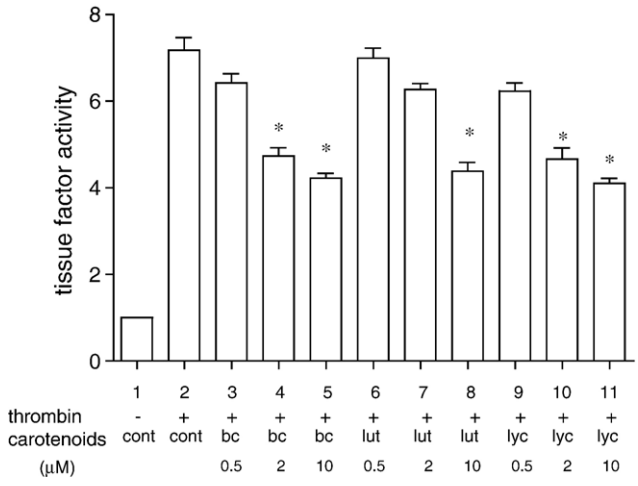

Fig. 2. Effects of various concentrations of carotenoids on tissue factor activity. Tissue factor activity in HUVEC was determined as described in Fig. 1. Carotenoids used were 0.5, 2 and 10 μmol/L of β-carotene (bc), lutein (lut) and lycopene (lyc). Control (cont) is a microemulsion without carotenoids. Results are presented as means±S.E.M.; $n=3$. *$P<.01$ vs. Lane 2. Statistical comparison of data was determined using one-way ANOVA followed by the Bonferroni posttest adjustment.

tissue factor activity assay were isolated using Trizol (Invitrogen) extraction, according to the manufacturer's instructions. Complementary DNAs (cDNAs) from total RNA samples (200 ng each) were synthesized in 25 μl at 60°C using gene-specific primers and ThermoScript reverse transcriptase polymerase chain reaction (RT-PCR) system (Invitrogen), according to the manufacturer's instructions. Primers used for cDNA preparation were ^{531}ATCCCGGAGGCTTAGGAAAGTGTT^{514} for tissue factor and ^{638}TTAATGTCACGCACGATTTCCCGC^{615} for β-actin. One fifth (tissue factor) or one tenth (β-actin) of 35 cycles of PCR products (362 base pair DNA fragment for tissue factor and 192 base pair DNA fragment for β-actin) obtained from 2 μl of each cDNA sample were separated on 2% SeaKem agarose gels (Cambrex) and stained by SYBR Green I. Expression levels of tissue factor and β-actin were determined using densitometry.

2.5. Western blot analysis

When cells reached approximately 80% confluency, cells were treated with vehicle or 10 μmol/L carotenoids for 7 h. The medium was replaced with DMEM containing 0.5% FBS with or without carotenoids, and cells were further incubated for 2 h. Cells were rinsed with 1× PBS, and whole cell lysates were prepared by lysis buffer (0.5% sodium deoxycholate, 1% nonidet P-40, 10% glycerol, 0.5 mmol/L phenylmethylsulfoxide and 0.1% sodium deoxysulfate in 1× PBS). Proteins were separated on Laemmli-polyacrylamide gel, transferred to a membrane, detected by immunoblotting with anti-Akt or antiphosphorylated Akt antibodies (Santa Cruz) and visualized by Immun-Blot amplified alkaline phsophatase kit (Bio-Rad).

2.6. Statistical analysis

Means and S.E.M.'s were calculated. Statistical comparison of data was determined using one- or two-way analysis of variance (ANOVA), followed by the Bonferroni posttest adjustment. A value of $P<.05$ was considered significant. Data are presented as mean±S.E.M. Numbers of experiments performed for each cell type and assay is presented as n.

3. Results

While an inverse relationship between the increased serum concentration of carotenoids and risk factors of cardiovascular disease has been clinically and epidemiologically studied [9–11], molecular analysis of the beneficial effects of carotenoids using a cell culture system has been hampered by insolubility of carotenoids in water. A recent study demonstrated that food-grade microemulsion based on nonionic emulsifiers enhanced lycopene solubility [19]. Carotenoids in microemulsion are similar to carotenoids in mixed lipid micelle form [20], which can be absorbed by duodenal mucosal cells [21]. Absorption of carotenoids in microemulsion by in vitro cultured cells has been reported [20]. We took advantage of water-dispersible forms of

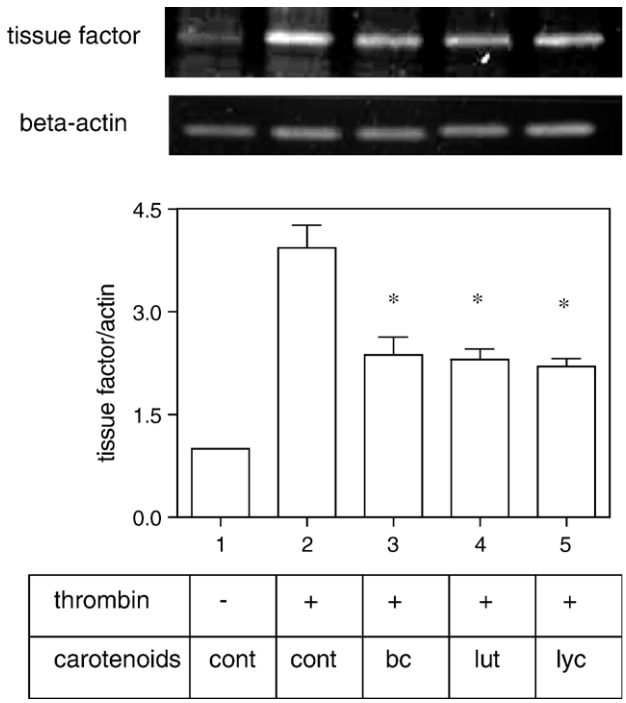

Fig. 3. Effects of various carotenoids on tissue factor gene expression in human endothelial cells. Semiquantitative RT-PCR of total RNA from endothelial cells grown in the medium containing microemulsion alone without carotenoids (cont), 10 μmol/L of β-carotene (bc), lutein (lut) or lycopene (lyc) was performed as described in the Methods section. β-Actin was used as an internal control for RNA concentration from each sample. Quantitative analysis was performed from three different sets of experiment. A ratio of tissue factor to actin from control was calculated as 1 in each set of experiment. *$P<.05$.

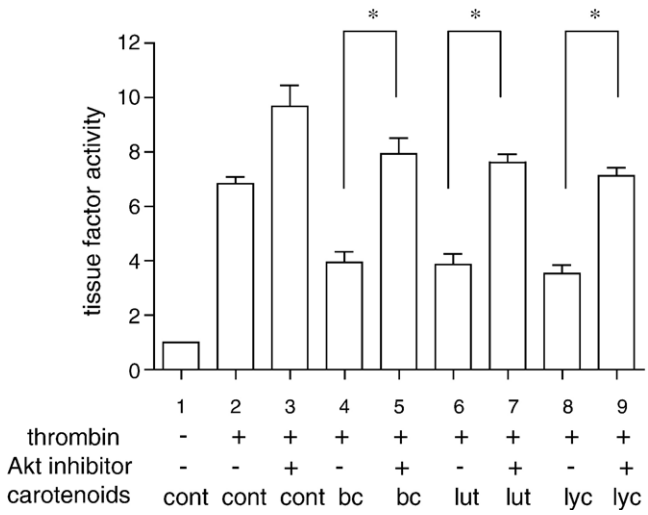

Fig. 4. Akt-specific inhibitor reversed the inhibitory effect of carotenoids on tissue factor activity. Tissue factor activity was determined as described in Fig. 1. Cells were incubated in the medium containing Akt-specific inhibitor (4 μmol/L) for 2 h prior to thrombin treatment, where indicated. Results are presented as means±S.E.M.; *n* (numbers of experiments performed)=3. **P*<.01 vs. lanes without Akt inhibitor. Statistical comparison of data was determined using one-way ANOVA followed by the Bonferroni posttest adjustment.

carotenoids in microemulsion (Cognis) to analyze effects of various carotenoids on tissue factor activity in human endothelial cells. Since this study is intended to analyze effects of dietary carotenoids in endothelial cells, all carotenoids examined in this study are from natural sources.

Treatment of both human umbilical vein and aortic endothelial cells (HUVEC and HAEC) with thrombin (final concentration, 3 U/ml) for 7 h markedly increased tissue factor activity in endothelial cells, consistent with a previous report [20]. As shown in Fig. 1, incubation of human endothelial cells in the medium containing β-carotene (*n*=3, *P*<.01 vs. Lane 2), lutein (*n*=3, *P*<.01 vs. Lane 2) or lycopene (*n*=3, *P*<.01 vs. Lane 2) prior to thrombin treatment suppressed thrombin-induced tissue factor activity. The variation of effects among carotenoids was statistically insignificant at the concentration of 10 μmol/L (*n*=3, *P*>.05). Difference of the inhibitory effects of carotenoids on tissue factor activity between endothelial cells from two different sources (HUVEC and HAEC) was also statistically insignificant (*n*=3, *P*>.05), indicating that the inhibitory effect of carotenoids on tissue factor activity was not dependent on endothelial cell type. Incubation of cells in the medium containing microemulsion alone (without carotenoids) showed no statistically significant effect on suppression of tissue factor activity, indicating that the microemulsion used to solubilize carotenoids did not suppress tissue factor activity.

Since cells absorb carotenoids in microemulsion by a mechanism involving passive diffusion [21], concentration of carotenoids in the culture media may influence the inhibitory effect of carotenoids on tissue factor activity. We, therefore, evaluated whether concentration of carotenoids in the culture media influenced the inhibitory effect of carotenoids on suppression of tissue factor activity in human endothelial cells. Fig. 2 shows that the concentration of lutein in the culture medium needs to be higher than that of β-carotene or lycopene to show a statistically significant effect on suppression of tissue factor activity in human endothelial cells. While the effect of β-carotene (*P*<.05 vs. Lane 2) and lycopene (*P*<.05 vs. Lane 2) on suppression of tissue factor activity was statistically significant, that of lutein (*P*>.05 vs. Lane 2) was statistically insignificant at 2 μmol/L concentration. Lutein in a higher concentration (10 μmol/L) did show a statistically significant effect (*P*<.01 vs. Lane 2) on suppression of tissue factor activity.

In order to determine if the effect of carotenoids on suppression of tissue factor activity is at the transcriptional level, a semiquantitative RT-PCR assay was performed using total RNA prepared from endothelial cells grown under the same conditions as the tissue factor activity assay. The expression level of β-actin was used as an internal control of messenger RNA (mRNA) concentrations in each sample. Consistent with a previous report [5], treatment of endothelial cells with thrombin induced expression of tissue factor mRNA. Incubation of endothelial cells in the culture media containing various carotenoids suppressed expression levels of tissue factor mRNA (Fig. 3). Results in Figs. 1 and 3 indicate that carotenoids

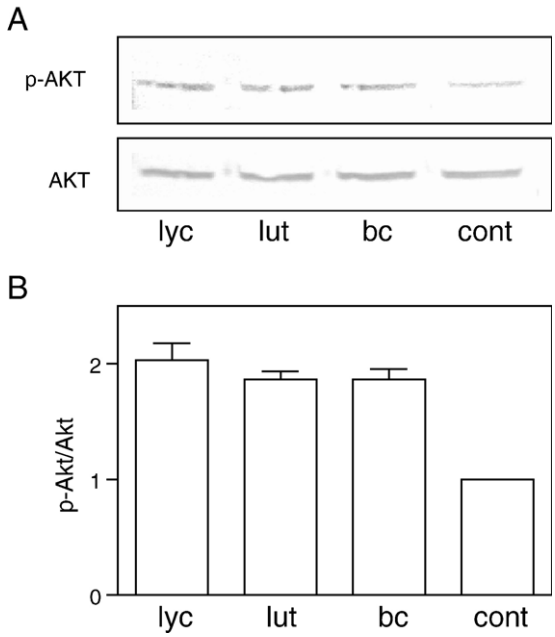

Fig. 5. Carotenoids enhance phosphorylation of Akt. Human endothelial cells were grown in the growth medium containing microemulsion alone without carotenoids (cont), 10 μmol/L β-carotene (bc), lutein (lut) or lycopene (lyc). Proteins from total cell lysates were separated on 10% polyacrylamide-SDS gel, transferred to a membrane and detected by immunoblotting with anti-Akt and antiphosphorylated Akt (p-Akt) antibodies (A). Western blot analysis was performed from three different sets of experiment (B). A ratio of p-Akt to Akt was calculated as 1 in each set of experiment. *P*<.05 (lyc, lut, bc vs. cont).

suppress tissue factor activity in endothelial cells by suppression of gene expression.

We also analyzed if carotenoids suppress tissue factor activity through regulation of signaling pathways. Our study of carotenoids on cell death indicated that carotenoids enhanced Akt activity and suppressed apoptosis in endothelial cells (unpublished observation). Phosphoinositol-3 kinase (PI3K) phosphorylates Akt, resulting in transportation of Akt into the nucleus and activation of the protein. Therefore, a highly specific Akt-specific inhibitor IV (Calbiochem) that does not interfere with PI3K was used in this study. As shown in Fig. 4, incubation of endothelial cells in the medium containing Akt-specific inhibitor IV for 2 h reversed the inhibitory effect of carotenoids on tissue factor activity ($n=3$, $P<.01$; lanes with vs. without Akt inhibitor). Since phosphorylation of Akt at Thr^{308} and Ser^{473} is required for Akt activity [22,23], we further analyzed whether carotenoids enhanced phosphorylation of Akt. Fig. 5 demonstrated that incubation of cells in the medium containing carotenoids indeed increased amounts of the phosphorylated form of Akt, indicating that carotenoids enhanced phosphorylation of Akt and suppressed tissue factor activity in endothelial cells by this mechanism.

4. Discussion

Tissue factor is not detected on the luminal surface of the vascular endothelium in the normal physiological condition [3]. Pathological conditions, especially enhanced oxidative stress by NADP oxidase, in vascular cells have been reported to increase tissue factor expression and activity [4,5]. Pathological up-regulation of tissue factor activity within the vasculature induces thrombosis and results in vascular disease states, such as atherosclerosis, and this effect is also observed in sepsis. Creasey et al. [24] in an animal study demonstrated inhibition of the tissue factor: factor VIIa complex decreased mortality of sepsis. In atherosclerosis, tissue factor expressed in atherosclerotic plaque initiates thrombosis upon plaque rupture [25]. Higher tissue factor levels in atherosclerotic lesions removed by atherectomy from patients with unstable angina compared with stable angina have been reported [26]. Thus, inhibition of pathological up-regulation of tissue factor activity may reduce thrombosis associated with vascular diseases, such as atherosclerosis and sepsis.

A recent study demonstrated that statins, inhibitors of the cholesterol synthesis pathway, inhibited tissue factor activity and gene expression in human endothelial cells by suppression of Rho kinase and Akt-dependent pathways [27]. Another study indicated that statins reduced the production of reactive oxygen species in endothelial cells via activation of thioredoxin, indicating that the pleiotropic protective effects of statins in cardiovascular diseases may result from their function as antioxidant [28].

Our study of carotenoids on tissue factor activity demonstrates the following:

1. All three carotenoids tested (β-carotene, lutein and lycopene) suppressed thrombin-induced tissue factor activity, but lutein was effective only at a higher concentration in various human endothelial cells (Figs. 1 and 2).
2. Carotenoids suppress tissue factor activity by suppression of gene expression at the transcriptional level (Fig. 3).
3. Carotenoids suppress tissue factor activity by enhancing phosphorylation of Akt (Figs. 4 and 5).

To our knowledge, this is the first report demonstrating that carotenoids suppress tissue factor activity through enhanced Akt signaling pathway. Previous studies using endothelial and monocytic cells demonstrated that activation of PI3K pathway suppressed tissue factor expression [29,30].

Carotenoids from food sources are first absorbed by duodenal mucosal cells in mixed lipid micelle form by passive diffusion [21]. Chylomicrons transport carotenoids from the intestinal mucosal cells to the bloodstream via the lymphatics. Carotenoids are transported in the plasma exclusively by lipoproteins [21]. Studies by Reddy et al. and Clevidence and Bieri [31,32] demonstrated that lycopene and β-carotene were mainly found in low-density lipoprotein, whereas lutein was predominantly found in high-density lipoprotein.

The Los Angeles Atherosclerosis Study [14] showed mean plasma concentrations of β-carotene, and lutein from all subjects were 0.74 and 0.28 μmol/L. According to the Kuopio Ischaemic Heart Disease Study [33], a mean concentration of plasma lycopene from all subjects was 0.17 μmol/L. Microemulsion was used to deliver carotenoids to endothelial cells in this study. Carotenoids in microemulsion are similar to carotenoids in the mixed lipid micelle form [20] that can be absorbed by duodenal mucosal cells [21]. However, intake efficiency of carotenoids in microemulsion by endothelial cells is not known at this moment. This makes it difficult to compare the concentration of carotenoids in microemulsion used in this study with the serum concentrations of carotenoids in human.

A majority of clinical studies demonstrated inverse association between higher plasma concentration of lycopene and lower risk of cardiovascular disease [9–11]. However, the beneficial effect of routine supplementation of vitamins and β-carotene for lowering risk factors for cardiovascular disease is still under debate [17,18]. Buijssse et al. [34] found an inverse association between cardiovascular mortality and plasma concentration of carotene in the elderly with body mass indexes <25. Rowley et al. [10] in an epidemiological study reported that abnormally high mortality from coronary heart disease in the aboriginal population in Australia might result from low plasma concentrations of carotenoids. Asplund [18] in a systemic

study of dietary effects of carotenoids demonstrated that intake (five to nine servings per day) of fruits and vegetables rich in carotenoids was associated with lower mortality of cardiovascular disease. However, the same study found no statistically significant association between β-carotene supplementation and cardiovascular disease. Thus, detailed molecular level studies such as those in this report are necessary to dissect mechanisms implicated in effects of carotenoids on vascular diseases in order to further develop recommendations for dietary and therapeutic approaches.

Our study of the effects of carotenoids on tissue factor activity showed a universal inhibitory effect. A study by Martin et al. [35] demonstrated that the efficiency of lycopene to suppress cell adhesion of monocytes to human endothelial cells, an initial step of atherosclerotic plaque instability and vessel thrombosis, was markedly higher than that of β-carotene or lutein. A study by Fuhrman et al. [36] found that the efficiency of lycopene to inhibit oxidation of low-density lipoprotein was higher than that of β-carotene. Therefore, lycopene may in fact provide better protective effects against development of atherosclerosis than β-carotene and lutein by suppression of multiple aspects of atherosclerosis. Further studies of these mechanisms of the effects of carotenoids on vascular endothelium are required to develop preventive and therapeutic strategies for prevention of cardiovascular disease.

Acknowledgments

The authors thank Dr. David Groves for the discussion of the results and Anne Baker for the careful reading of the manuscript. The research of this study was conducted at the Department of Biological Sciences, Cameron University. This study was supported by the Mary E. Groff Foundation of Pennsylvania and by the McMahon Foundation of Oklahoma.

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