Contribution of Dietary Intakes of Antioxidants to Homocysteine-Induced Low Density Lipoprotein (LDL) Oxidation in Atherosclerotic Patients

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Purpose: Elevated circulating oxidized low density lipoprotein (Ox-LDL) levels are associated with increased risk of atherosclerosis, which may be due to high plasma homocysteine (Hey) and low intakes of antioxidants. We investigated the contribution of dietary intakes of antioxidants to Hey-induced LDL oxidation in atherosclerotic patients (AP) and controls. Materials and Methods: Male AP (n = 101) who were confirmed by coronary angiography and 91 controls were evaluated by blood biochemistry and dietary intakes. To determine whether homocysteine is an independent risk factor for atherosclerosis, subjects were divided into three groups; low- (< 6.9 uM/L), normal- (7 uM-12 uM/L) and high- (≥ 12.1 uM/L) Hcy. Results: Plasm levels of homocysteine and LDL were higher, but plasma apo A-I in HDL and folate were lower in the AP group. The odds ratio (OR) for the risk of atherosclerosis was 3.002 [95% confidence interval (CI), 1.27-7.09] for patients in the highest tertile with homocysteine (≥ 12.1 uM/L). AP having high homocysteine levels had low intakes of vitamin A, β-carotene and vitamin C. By logistic regression analysis, age, body mass index (BMI), plasma LDL, plasma folate, and low intakes of vitamin A and β-carotene were found to be risk factors for atherosclerosis in patients with high-Hcy, but dietary B vitamins including folate were not. Conclusion: A high-Hcy level was a risk factor for atherosclerosis in patients with high Ox-LDL levels. High intakes of antioxidants appeared to be a protective factor for atherosclerosis, perhaps exerting a pro-oxidative effect on LDL when combined with high levels of Hcy and LDL. However, more evidence for the benefits of B vitamins as a homocysteine-lowering therapy is needed.

Key Words: Atherosclerosis, homocysteine, oxidized low density lipoprotein, folate, Vit A, β-carotene

INTRODUCTION

Cardiovascular disease (CVD)-related chronic diseases are the leading cause of death in societies with a large aged population such as Korea and Western
countries. Elevated levels of homocysteine (Hcy) above 12.1 μmol/L have been shown to double the risk of pathophysiological conditions such as atherosclerosis, myocardial infarction, cerebral or peripheral vascular diseases.1 Meta-analysis of 20 prospective studies indicated that the odds ratios (OR) for the risk of ischemic heart disease and stroke were 1.32 [95% confidence interval (CI), 1.19-1.45] and 1.59 (95% CI, 1.29-1.96) for every 5 μmol/L increase in Hcy.2 Elevated Hcy may contribute to progressive atherosclerosis by several mechanisms, including arterial endothelial function impairment, oxidative stress induction, and sclerosis by several mechanisms, including arterial endothelial function impairment, oxidative stress induction, and thrombosis.1,3

A recent randomized clinical trial, however, did not support Hcy-lowering vitamin supplements to prevent heart diseases.4 Since Hcy is a branch-point intermediate of cystathionine and methionine metabolism, folate, B6, and B12 are essential to the conversion of Hcy. It has been shown that not only dietary B vitamin deficiency but also genetic disorders, certain drugs, and renal impairment are related to hyperhomocysteinemia.5,6 Folate and B vitamin supplements decrease blood Hcy levels and carotid intima-media thickening, but they do not inhibit LDL oxidation, a marker of atherosclerosis.7-8 Attempts to delineate causative factors of atherosclerosis have often investigated production of reactive oxygen species (ROS) and reduction of availabilities of endothelial nitric oxide (NO).9 Hcy is known to induce oxidative stress and ROS or lipid peroxides (LPO) production, and decrease NO availability.11,12 Hcy-induced ROS has been shown to be related to impaired mitochondrial NF-κB activation and NO bioavailability in endothelial cells, cardiac myocytes, fibroblasts and leukocytes, however, they return to basal levels by antioxidants.3 Therefore, Hcy affects the molecular structure or the activation of control agents which participate in the oxidative mechanism of atherosclerosis.

The aim of the current study was to investigate whether elevated Hcy levels are associated with oxidation of LDL, and further explore the contribution of dietary intakes of B vitamins or antioxidants to Hcy-induced LDL oxidation in atherosclerosis.

**MATERIALS AND METHODS**

**Subjects and samples**
Healthy or atherosclerotic male subjects were recruited from among in- and out-patients of Korea University College of Medicine in Seoul, Korea, after approval from Biomedical Human Subjects Review Committee. After first screening (n = 340) with a health questionnaire (family history, exercise), subjects were further evaluated with a dietary survey and anthropometry [body mass index (BMI), blood pressure (BP)]; biochemical tests were performed for 192 final subjects. The 192 subjects were divided into atherosclerosis patients (AP, n = 101) or control (n = 91) groups after AP were diagnosed by coronary angiography by a cardiologist. The coronary artery lesion was defined where stricture levels of Lt. main, Lt. anterior descending artery, left circumflex, or right coronary artery were over 50%. To determine whether Hcy is an independent risk factor for CVD, subjects were assigned to one of three Hcy levels, using the European Concerted Action Projects (ECAP) cut-off value.13 Dietary surveys were performed using the CANpro 3.0 software (developed by Korean Nutrition Society). Survey results on dietary intakes and biochemistry were statistically analyzed. None of the participants were using vitamin and/or mineral supplements.

**Biochemical measurements**

Cholesterol and triglycerides (TG) were analyzed by enzymatic Kits (Sigma Co, St Louis, MO, USA).14,15 After HDL and LDL were separated by density gradient ultracentrifugation, apo A-I in HDL and apo B-100 in LDL were quantified by Western blotting analysis (antibodies; Santa Cruz, CA, USA). In order to measure Ox-LDL, pure LDL was high-speed centrifugated using KBr, at a density of 1.019-1.063 g/mL. During centrifugation, 0.1% EDTA and 0.02% sodium azoid were added. Phosphate-buffered saline (PBS) was used for dialysis of centrifugated LDL. SDS-polyacrylamine gel electrophoresis (PAGE) was used to verify LDL purification.16 The purified LDL was dialyzed for a day, and then LPO [malondialdehyde (MDA)] production was analyzed. The thiobarbituric acid (TBA) method (modified Packer and Smith method) was used to obtain correct amount of lipid peroxide with the MDA produced. By using 1,1,3,3-tetraethoxypropane as the standard reagent, comparisons were made between different treatments according to thiobarbituric acid reactive substances (TBARS) values from different amounts of 1 mg protein. Butylhydroxytoluen (BHT), sulfuric acid, and Na-WO₄ were mixed with LDL, and the mixture was centrifugated. The liquid that rose was taken; NaOH and 1% TBA were added and heated for 60 minutes at 100°C. After centrifugation at 3,000 rpm for 10 minutes, absorbance was read.17-19 After the protein content of LDL was measured, units of LDL per mg protein were used to express the amount. Total plasma Hcy (sum of free-, protein-bound and mixed disulfide forms) in all samples was determined by the use of “Abbott IMX system Homocysteine E/B3D390, 77-0650/R2” kit (ABBOTT Plasma Total Hcy Diagnostic kit; Abbott, Abbott Park, IL, USA) in one run.19 By processing Hcy molecules (protein, residual thiol, or sulfide combined) with DTT, a crystalline form of Hcy can be obtained. By applying an S-adenosyl-L-homocysteine
(SAH) hydrolase to the crystalline with cholesterol, the amount of Hcy that combines with adenosine to form SAH was measured. For determination of vitamin B12 and folate in serum and plasma, vitamin B12/folate kit (Diagnostic Products Corporation, Los Angeles, CA, USA) was used according to the method of Solid Phase No Boil Dual-count. 20: 200 µL of serum was mixed with sample buffer, and the mixture was left for 30 minutes at room temperature. The supernatant after centrifugation at 2,000 g for 15 minutes was discarded. The radioactivity from 125I-folate and 57Co-vit B12 in the pellet was measured by Gamma counter Cobra II. For the amount of vitamin B12, pg/mL was multiplied by 0.7378 to convert to pmol/L. For folate, ng/mL was multiplied by 2.266 to convert to pmol/L. The clinical reference value for blood vitamin B12 is 200-950 pg/mL, and 3.0-17 ng/mL for blood folate.

Statistical analysis
Using SAS statistical software (Carey, NC, USA), ANOVA and independent t-test were performed. Statistical significance was accepted at p-value < 0.05. Relationships among variables were assessed by correlation analysis. Univariate and multivariate logistic regression analyses were used to obtain the crude and adjusted OR (AOR) and 95% CI.

RESULTS

Demographics of subjects
Average age of the AP group was 56.2 years, while the average age of the control group was 42.4 years. The two groups exhibited no differences in blood pressure and BMI (Table 1). Over 60% of the AP subjects exhibited other co-morbidities also, whereas 73% of controls were disease-free. The incidences of hypertension and diabetes were 35% and 16%, respectively, in the AP (data not shown). Smoking and drinking rates were similar in both groups, but the amount and length of the use were much higher in the AP; 47.7% of total AP smoked more than 1 pack of cigarettes/day (control: 32.2%) and 35.5% of the AP drank 2-4 times/week (control: 28.8%). The AP also had higher coffee consumption rate (5 cups/day) than the control (data not shown).

Table 1. Mean Values (± SD) of General Characteristics, Blood Parameters and Daily Nutrient Intakes of Folate and Antioxidants Related to Atherogenic Risk Factors in AP and Control Groups

|                         | AP group (n = 101) | Control (n = 91) | p value |
|-------------------------|-------------------|-----------------|---------|
| Age (yrs)               | 56.2 ± 12.4       | 42.4 ± 8.8      | 0.002   |
| BMI (kg/m²)             | 24.7 ± 3.1        | 23.9 ± 2.3      | 0.087   |
| Blood Parameters        |                   |                 |         |
| Systolic BP (mmHg)      | 131.5 ± 21.7      | 129.9 ± 11.4    | 0.560   |
| Diastolic BP (mmHg)     | 81.2 ± 13.7       | 82.3 ± 9.23     | 0.554   |
| HCY (µM/L)              | 12.98 ± 6.82      | 11.19 ± 4.54    | 0.032   |
| T-C (mg/dL)             | 186.5 ± 39.0      | 179.6 ± 35.4    | 0.267   |
| HDL-C (mg/dL)           | 40.3 ± 12.0       | 39.9 ± 22.3     | 0.877   |
| LDL-C (mg/dL)           | 119.2 ± 36.6      | 108.3 ± 33.6    | 0.049   |
| Ox-LDL* (µM/mg)         | 249.0 ± 112.0     | 225.7 ± 90.6    | 0.115   |
| TG (mg/dL)              | 135.0 ± 80.9      | 156.9 ± 75.7    | 0.097   |
| Apo A-1 (mg/dL)         | 118.6 ± 18.7      | 134.8 ± 19.1    | 0.000   |
| Apo B-100 (mg/dL)       | 87.2 ± 19.8       | 92.3 ± 22.4     | 0.091   |
| Vit B12 (pg/mL)         | 425.0 ± 144.0     | 448.0 ± 161.0   | 0.312   |
| Folate (ng/mL)          | 6.6 ± 2.7         | 7.7 ± 4.0       | 0.025   |
| Dietary intakes of Folate and Antioxidants† |                   |                 |         |
| Folate (µg)             | 277.4 ± 108.5     | 302.6 ± 108.8   | 0.130   |
| Vit A (µg RE)           | 964.6 ± 571.2     | 815.9 ± 471.8   | 0.063   |
| β-carotene (µg)         | 4790 ± 3174       | 4261 ± 2804     | 0.246   |
| Vit C (mg)              | 93.9 ± 50.2       | 124.1 ± 63.9    | 0.001   |
| Vit E (mg)              | 12.7 ± 7.6        | 13.8 ± 5.8      | 0.326   |

BMI, body mass index; Hcy, homocysteine; T-C, total cholesterol; LDL, low density lipoprotein; HDL, high density lipoprotein; TG, triacylglycerol; Vit, vitamin; BP, blood pressure.
*Oxidized LDL; malondialdehyde (µM) concentration per mg LDL protein.
†Adjusted by the calorie intake.
Blood lipid profiles, folate & B12 in AP or hyperhomocysteinemia

In the AP, blood Hcy and LDL cholesterol levels were higher and apo A-I in HDL and folate were lower, exhibiting characteristic lipid spectrum of atherosclerosis (Table 1). Plasma Hcy and LDL can be viewed as pro-arteriosclerosis factors, and HDL’s apo A-I as an anti-arteriosclerosis factor. However, no apparent differences in total cholesterol (T-C), HDL, TG, and apo B-100 were observed between AP and control. Blood Hcy and Ox-LDL were higher in AP, suggesting that Hcy plays a role in LDL oxidation. Blood folate levels were lower in AP, whereas vitamin B12 levels were similar. In order to verify the changes in lipid profiles, resulting from the effects of vitamins and Ox-LDL on Hcy concentrations, subjects were divided into 3 groups depending on Hcy levels: low-Hcy (≤ 6.9 µM/L), normal (7 µM-12 µM/L) and high-Hcy (≥ 12.1 µM/L) (Table 2). Ox-LDL levels were not different between AP and control, but significantly higher in the normal and high-Hcy groups than in the low-Hcy group (Table 2). The positive association of LDL with Ox-LDL was stronger in high-Hcy subjects than that in total subjects, normal or low Hcy subjects (data not shown). The increased Ox-LDL in the high-Hcy group can be explained as LDL oxidation caused by the increase in blood Hcy. Blood folate levels were significantly different (p < 0.05) among the 3 Hcy groups, and it was negatively correlated with Hcy (Fig. 1). There was no correlation between vitamin B12 and Hcy even though blood folate and vitamin B12 were positively correlated (data not shown). The blood lipid levels did not change according to Hcy levels and were not correlated with Hcy.

Dietary intakes in AP or hyperhomocysteinemia

The relative percentages of carbohydrate, protein and fat intakes per total calories were similar in AP and controls,
even though AP had lower energy intakes (data not shown), suggesting that AP might have attempted to lose weight, since they had slightly, but not significantly, higher BMIs. Dietary intakes of other B vitamins were not different between the two groups, except vitamin B1 and B2 which were lower in AP. Vitamin B12 intake could not be evaluated because of insufficient data. There were no differences in any dietary intakes except vitamin A, β-carotene and vitamin C among the three Hcy groups (Table 2). Hcy was not affected by dietary folate intakes in this study, and the pattern of correlation between dietary folate and Hcy or plasma folate were not obvious (Fig. 1). Therefore, low dietary folate in AP might have not induced hyperhomocysteinemia in this study. However, dietary intakes of vitamin A, and its precursor, and vitamin C were significantly lower with increasing Hcy. The negative correlations between dietary vitamin A ($r^2 = -0.226$) or β-carotene ($r^2 = -0.233$) antioxidants and Ox-LDL were also significant in AP ($p < 0.001$) compared to no correlations in controls (data not shown).

**Risk factors for atherosclerosis according to Hcy levels**

Using the ECAP cut-off value ($\geq 12.1$ uM/L), 26.6% of the total Korean male subjects had hyperhomocysteinemia, and 69.3% and 4.2% had normal and low-Hcy levels, respectively. The frequency of atherosclerosis was substantially increased if the blood Hcy was higher than $\geq 12.1$ uM/L (OR, 2.887; 95% CI, 1.51-5.53) and this status was stronger after adjusting for age and BMI (AOR, 3.002; 95% CI, 1.27-7.09)(data not shown). To identify links between risk factors for atherosclerosis and high blood Hcy levels, all anthropometric and biochemistry variables and daily nutrient intakes were subjected to logistic regression analysis (Table 3). The AOR for atherosclerosis was 4.420 (95% CI, 1.542-12.663) with high blood Hcy and LDL levels compared to when LDL was not included as a factor.

![Fig. 1. Correlation between plasma folate or dietary folate and Hcy concentration in adult Korean men. Plasma folate levels were significantly and negatively correlated with Hcy concentration, but not with dietary folate.](image)

| Variables                  | Adjusted OR (95% CI)¹  |
|----------------------------|------------------------|
| Blood HCY ²                | 3.002 (1.269 - 7.088) ³ |
| Dietary Folate             | 1.001 (0.997 - 1.005)   |
| Plasma Folate              | 0.797 (0.670 - 0.949) ³ |
| LDL                        | 1.014 (1.000 - 1.028) * ³ |
| Dietary β-carotene (or vit A)³ | 1.000 (1.000 - 1.000)  |

Hcy, homocysteine; LDL, low-density lipoprotein; BMI, body mass index.

* $p<0.05$.

† $p<0.01$.

² Risk factors for atherosclerosis were analyzed by logistic regression analysis. All variables of odds ratios for atherosclerosis were adjusted by age and BMI.

³ High blood Hcy (serum HCY levels $\geq 12.1$ uM/dL).

⁴ Same when vit A variable was included.
When dietary folate and plasma were added, the AOR of high-Hcy for atherosclerosis was not changed. After dietary vitamin A and $\beta$-carotene were included as antioxidants, the AOR of high-Hcy for atherosclerosis was reduced to 4.206 (95% CI, 1.457-12.142). We demonstrated that the most important risk factor for atherosclerosis was LDL and the least important risk factor was plasma folate in hyperhomocysteinemia. When participants were divided into tertiles of mean Hcy and LDL concentrations, 20.7% of subjects in the 3rd tertile of Ox-LDL (276.13-745 uM/mg LDL) came under two combined categories with the highest Hcy (11.89-50 uM/L) and LDL levels (124.81-244 mg/dL) (Fig. 2). The probability of subjects having the highest Ox-LDL while also having low Hcy and low LDL levels was only 3.4%. We found that the greatest progression of atherosclerosis was observed with the highest Ox-LDL simultaneously with both the highest Hcy and LDL concentrations. This means that a high circulating Hcy level was a risk factor for atherosclerosis only when the subject had also elevated LDL levels.

DISCUSSION

In this study, AP exhibited multiple complications causing co-morbidities of metabolic syndrome such as hypertension and NIDDM, which were similar to the distribution of complications seen in Korean hyperlipoproteinemia. The ECAP study found that elevated Hcy was an independent risk factor for Hcy and CVD, causing multiple effects on the risks of smoking, hypertension, and hypercholesterolemia. It has been reported that 85.2% of Korean AP smoke 20 cigarettes/day or more, and 85.4% of AP smoked more than one pack of cigarettes/day for the last month. Since elevated Hcy strongly correlates with smoking and low blood folate levels were found in the PRIME case-control study, Hcy levels might be a secondary risk factor to low blood folate and smoking. Elevated Hcy may also be involved in hypertension because it is associated with impaired endothelial function due to the generation of oxidative stress, VCAM-1 and reduction of NO bioavailability. High dietary intake of sodium is also a cause of hypertension. Since Koreans customarily consume large amounts of traditional salty fermented foods such as kimchi or soybean paste, sodium intake is almost 3 times higher than the DRI established by WHO (2 g/day). In American adults, blood Hcy levels are 5-15 µmol/L, and Hcy concentration of the control (healthy) group is around 7-12 µM/L. In Jacques’ study, male coronary artery disease patients (age, 43-57 years) had Hcy concentrations of 13.66 uM/L while the control had 10.93 uM/L concentration. If the total blood Hcy is low, the death rate of CVD is low. Every 5 µM/L increase in the Hcy concentration increases the risk of CVD by 50%, and TC levels by 20 mg/dL. While doubts were raised concerning the cause-effect relationship between the Hcy and CVD, we found that Hcy is definitely higher in AP than in controls. The mechanisms possibly responsible for causing endothelial dysfunction include changes in LDL, and Ox-LDL, since higher LDL, which is associated with oxidation of LDL was observed in the AP. We also found that elevated Hcy makes the correlation between LDL and Ox-LDL much stronger. In Mansoor’s research, Hcy levels and Ox-LDL were higher in subjects with CAD than in control. The oxidation of LDL is increased by the combination of thiolactone and apo B’s free lysyl epsilon amino residue. When LDL is reacted with Hcy-thiolactone in methionine, which is an explicit initiator of arteriosclerosis, LDL-binding thiol is increased by 250 nM per mg of LDL protein. The free amino- or thiol-adducted LDL causes aggregation, and increases LDL uptake in macrophages and atheroma production by lipids. In Marek’s research, LDL oxidation in the aortic cell walls was 40% higher in hyper-homocysteinemia (≥ 14.0 µM/L) compared to control. Another mechanism by which Hcy may cause LDL oxidation is a possible deformation of LDL through Hcy autoxidation, which causes the oxidation of side chains of LDL such as fatty acids or apo B-100. Both Hcy and Ox-LDL could participate in thrombosis by increasing VCAM-1 and ICAM-1, caused by endothelial cell activation due to fibrinogen-platelet GPIIIb-
llla formation. Ox-LDL affects both initial and progressive stages of atherosclerosis.\textsuperscript{11,12,23,32} On the other hand, circulating Hcy reduced NO-induced detoxification, vasodilation, and endothelial function.\textsuperscript{30} NO participates in a metabolic pathway (S-nitroso-HCY) that is able to protect against Hcy-induced endothelial oxidative damage.

Since Hcy is a branch-point intermediate of cystathionine and methionine metabolism, the deficiency of B vitamins (folate, vitamin B\textsubscript{6} and B\textsubscript{12}) might cause hyperhomocysteinemia.\textsuperscript{43} In animal and clinical tests, vitamin B\textsubscript{6} and folate deficiency is related to increased blood Hcy levels, but the correlation between Hcy and B vitamins is not clear.\textsuperscript{34,35} In the Hcy-lowering Trial’s Collaboration Research, an additional 7\% supplement of vitamin B\textsubscript{12} reduced fasting Hcy levels and decreased hypercysteinemia by 25\%, however, vitamin B\textsubscript{6} had no effect on Hcy levels.\textsuperscript{5,25} In Vernaelen’s research, where AP was given 5 mg of folate and 250 mg of vitamin B\textsubscript{12}, the Hcy level decreased from 14.7 \text{µM/L} to 7.4 \text{µM/L}, showing a higher reduction rate than in the placebo group (12.0 \text{µM/L}).\textsuperscript{36} Supplementation of folate (2.5 mg), vitamin B\textsubscript{6} (25 mg), and B\textsubscript{12} (500 \text{µg}) for one year reduced Hcy from 10.5 to 6.56 \text{µM/L} and carotid intima-media thickness by 5.3\%.\textsuperscript{6,37} However, Young and Woodside reported that supplementary folate and vitamin B\textsubscript{12} lower blood Hcy levels, but they cannot stop LDL oxidation.\textsuperscript{7} Vitamins B\textsubscript{6} or B\textsubscript{12} and the C677T NTHFR polymorphism have not been associated with carotid intima-media thickness.\textsuperscript{9} Food-based feeding trials have shown a reduction in blood Hcy in subjects who consume fortified cereals or whole grains in combination with fruits, vegetables and low fat dairy products.\textsuperscript{9,38} However, more evidence is needed for the association of vitamin B\textsubscript{12} and Hcy levels with the pro-oxidative mechanism of LDL and the dietary benefits of Hcy-lowering therapy for atherosclerosis. The effects of antioxidants on decreasing the oxidation of LDL may be an important observation when blood LDL and Hcy levels are increased. Because low intakes of vitamin A, \textit{β}-carotene and vitamin C are associated with Hcy levels, even though the biochemical data did not support that the Hcy-induced oxidative damage is protected by antioxidants. Both hydro- and lipid-soluble antioxidants have been shown to be depleted in oxidative endothelial damage.\textsuperscript{10,39} Our findings confirmed a role of antioxidant compounds, particularly \textit{β}-carotene, against the oxidative damage promoted by Hcy and elevated LDL levels. Nevertheless, it needs to be further evaluated in conjunction with markers of oxidative damage.

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