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Methionine, a Limiting Amino Acid in Soy Protein, Accelerates the Development and Progression of Atherosclerosis?

Young-Sun Song

School of Food Science and Nutrition, Biohealth Product Research Center,
Inje University, Gimhae 621-749, Korea. E-mail: fdsnsong@inje.ac.kr

INTRODUCTION

Several lines of evidences suggest that animal proteins may exert atherogenic effects as compared to plant proteins. A possible explanation how animal proteins become atherogenic is their high methionine content and methionine/protein ratio (Toborek and Hennig, 1996). The mechanisms by which methionine may stimulate the development of atherosclerosis are not fully understood. One of the possibilities is injury of endothelial cells by homocysteine, an intermediate formed during methionine metabolism. It has been suggested that homocysteine induced endothelium injury involves oxidative damage (Frauscher et al., 1995; Symons et al., 2002; Toborek et al., 1995; Young et al., 1997). Toxic and inflammatory effects of homocysteine on endothelial cells were studied comprehensively *in vitro* (Hofmann et al., 2001; Huang et al., 2001b; Welch et al., 1998). Several animal studies have accumulated that increased lipid peroxidation by hyperhomocysteinemia (HHcy) is a mechanism of methionine-induced atherosclerosis (Durand et al., 1997; Toborek et al., 1995; Young et al., 1997). Indeed, Hofmann et al. (2001) found that HHcy enhanced NFκB activation and expression of VCAM-1, MMP-9, and tissue factor in vasculature of apoE-null mice. Symons et al. (2002) reported that HHcy impairs endothelium-dependent relaxation of coronary resistance vessels, increased carotid arterial permeability, and initiates arterial stiffening in rats by increased oxidative stress. HHcy following oral methionine load (100 mg/kg B.W) in humans confirmed that endothelial dysfunction is closely related with lipid peroxidation (Constans et al., 1999; Domagala et al., 1997; Hanratty et al., 2001). From all these findings, these elevated plasma homocysteine and oxidative stress induced by methionine supplementation seem to contribute to the development of atherosclerosis.

Previous studies have emphasized that dietary methionine induced HHcy accelerates vascular oxidative injury. If oxidized lipids or oxidative stress were responsible for the development of vascular inflammation and atherosclerosis, these phenomena might be induced in any tissue that accumulated oxidized lipids. Macrophages play essential roles in inflammation and premature atherosclerosis by producing pro-inflammatory mediators, trapping oxidized LDL via scavenger receptors, and transforming into foam cells. Liver is the organ where methionine metabolism occurs and production and degradation of apoB-100 containing lipoproteins. Davis and Hui (2001) proposed that atherosclerosis is a liver disease of the heart. Recently, Werstuck et al. (2001) also reported that homocysteine induced endoplasmic reticulum (ER) stress causes dysregulation of the cholesterol and triglyceride biosynthetic pathways, which might be a possible mechanism of atherosclerosis observed in HHcy. This paper will cover the potential mechanisms of atherosclerosis by methionine-induced HHcy in C57BL/6 and retired apoE -/- mice.

The Effect of Methionine-induced HHcy on the Development of Atherosclerosis

Multiple lines of evidences have been supported that mehionine induced HHcy is critical for the vascular endothelial damage. Increases of oxidized lipids, protein and DNA fragmentation and inflammatory mediators upon HHcy have been described *in vitro* and *in vivo* studies (Hofmann et al., 2001; Huang et al., 2001a; Welch et al.,

1998; Young et al., 1997). In our study, HHcy was induced by feeding high methionine (2%), and low folate (1 mg/kg) diet for 12 wk to C57BL/6 mice. Plasma homocysteine level of mice fed methionine enriched diet was 5 times higher than mice fed control diet, even though HHcy resulted in growth retardation, consistent with previous reports (Huang et al., 2001a; Yagasaki et al., 1986; Zhou et al., 2001). We also found several indicators of oxidative stress, including increased superoxide anion level in macrophages (Fig. 1), elevated TBARS and lowered GSH concentrations in livers of C57BL/6 mice. Several animal and human studies have shown that HHcy is positively correlated with increased lipid peroxidation, represented by TBARS (Toborek et al., 1995, 1996; Young et al., 1997), or total antioxidant capacity (Abdelfatah et al., 2002), which supports our result. Acute oral methionine loading test in humans also demonstrated significant elevation of TBARS at 4 and 6 h after methionine loading, which correlated with raised plasma homocysteine concentrations (Domagala et al., 1997). The increased concentration of TBARS suggests an increase in reactive oxygen species (ROS). Excess amounts ROS increase the oxidative stress in the body. ROS actively participate in the oxidative deterioration of lipids, proteins and DNA, altering protein function, cellular homeostasis, and gene expression (Altavila et al., 2001; Bagchi et al., 1998; Liao et al., 1993; Werstuck et al., 2001; Wheeler et al., 2001). To combat against these disturbances by oxidative stress induced by homocysteine, GSH might be the first line of antioxidant defense toward oxidative stress (Dumaswala et al., 1999). In our study, GSH concentration was significantly lowered by 12 wk of methionine feeding, which was comparable to the dada published by several researchers (Henning et al., 1989; Toborek et al., 1995). Demonstrated decreases in hepatic GSH concentration contribute to increased hepatic TBARS level in methionine-fed mice, since GSH serves as a non-enzymatic free radical scavenger. However, concentration and duration period of the elevated plasma homocysteine may affects GSH concentrations in liver. Indeed, Huang et al. (2001a) observed GSH was unaffected, while TBARS was elevated, by 4 wk of folate depletion in rats with modest HHcy (26 μmol/L), and suggested that homeostasis of GSH in modest HHcy was not disrupted by the 4-wk experimental period.

Intracellular antioxidative enzymes such as Cu,Zn-SOD, catalase, glutathionine peroxidase (GSH-Px) and glutathione reductase prevent cells from free radical-mediated disturbances by scavenging ROS and products of lipid peroxidation. The higher expression of hepatic antioxidative enzymes, Cu,Zn-SOD, catalse, glutathionine reductase and

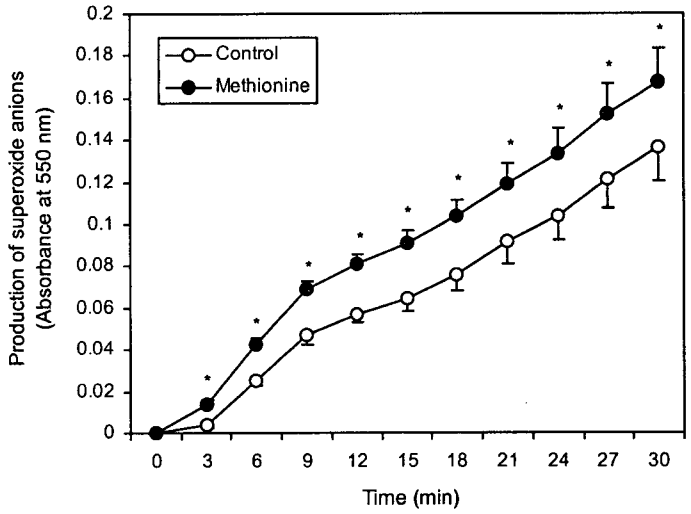

Fig. 1. Superoxide generation by peritoneal macrophages. Thioglycollate-elicited peritoneal macrophages were harvested from mice fed methionine and control diets, n=8. Absorbance at 500 nm reflects production of superoxide anion due to activity of ferricytochrome C. Asterisks denote a significant difference from control at $p<0.05$.

GLO, was observed in mice fed methionine enriched diet than mice fed control diet, which may represent a protective response of the tissue to an increased intracellular concentration of ROS (Oteiza et al. 2001). This is consistent with the results of Toborek et al. (1995, 1996), who found the increased antioxidative enzyme activities were associated with increased TBARS levels in livers and aorta of rabbits fed methionine for 9 mo, respectively. However, Huang et al. (2001a) reported that modest HHcy (26.4 μmol/L) in Wistar rats lowered GPx and Cu,Zn-SOD activities, whereas catalase was unaffected. One interesting data found in our study is homocysteine-induced oxidative stress also increased expression of GLO, the rate-limiting enzyme of ascorbate synthesis. This founding supports that oxidative stress by HHcy induces genetic expression of antioxidative enzymes which help not only reactive oxygen scavenging but also universal antioxidation.

The earliest sign of atherosclerosis is a purely inflammatory lesion that forms in response to arterial injury (Collins and Cybulsky, 2001; Takacs et al., 2001). HHcy induced by methionine-enriched, folate, B_{12} deficient diet in apoE knockout mice promoted nuclear translocation of NFκB, a redox-activated inflammatory transcription factor, concomitant increase in expression of VCAM-1 and tissues factor (Hofmann et al., 2001). Homocysteine now appears to initiate a cascade of inflammatory pathways, possibly initiated by NFκB, that promote atheroscleotic lesions (Brand et al., 1996).

NFκB, inducible transcription factor, regulates the expression of many target genes involved in immune and inflammatory responses. NFκB exists in a latent form in the cytoplasm of cells comprising a transcriptionally active dimer bound to inhibitor protein, IκB. NFκB is activated in response to various extracellular stimuli, including cytokines, lipopolysaccharide and oxidative stress (Bowie et al., 1997; Bowie and O'Neill, 2000; O'Connell et al., 1998; Schreck et al., 1992). Oxidative stress is defined as an increase in intracellular reactive oxygen species (ROS) such as H_2O_2, superoxide (O_2-) or hydroxy radical (· OH). It has been proposed that reactive oxygen species may be involved in the activation of NFκB and the role of ROS in NFκB activation is cell specific. ROS generated by NADPH oxidase are required for NFκB activation by pro-inflammatory cytokines in macrophages/ monocytes (Bonizzi et al., 1999). This was proved in our study. Indeed, superoxide anion production and NFκB activation were both elevated in peritoneal macrophages of mice fed methionine-enriched diet (Fig. 2).

Challenges for this study is to determine whether HHcy also promotes oxidative stress and modulates NFκB ctivation in other tissues except vascular tissue, such as peritoneal macrophages and the livers. Our study demonstrated that HHcy induced by methionine in C57BL/6 mice promoted NFκB activation in both peritoneal macrophages and the livers significantly. Although there is no consensus as to pathogenetic significance of NFκB in macrophages and liver, there is few evidences that inducible nitric oxide synthase (iNOS) expression is increased in macrophages and smooth muscle cells in NFκB activation (Hattori et al., 2001; Lo et al., 2002; Welch et al., 1998). Over-expression of iNOS results in increased production of NO and its derivatives, such as peroxynitrite and nitrogen dioxide. Excessive amounts of reactive nitrogen species (RNS) increase local oxidative stress, promoting the oxidative deterioration of lipids and atherosclerosis (Welch et al., 1998; Yen and Lai, 2002). Arnalich et al. (2001) also reported that NFκB activation is dependent on the intracellular GSH deficiency, associated with enhanced serum TBARS in non-insulin dependent diabetic patients. These observations clearly support our findings that oxidative stress led to enhanced response of NFκB activation in peritoneal macrophages of methionine-fed mice.

There has been a new suggestion that ER stress caused by HHcy, leading to increased hepatic biosynthesis and uptake of cholesterol and triglycerides, likely explains the development of hepatic steatosis and possibly atherosclerotic lesions observed in HHcy (Werstuck et al., 2001). However, we found hepatic cholesterol and triglyceride

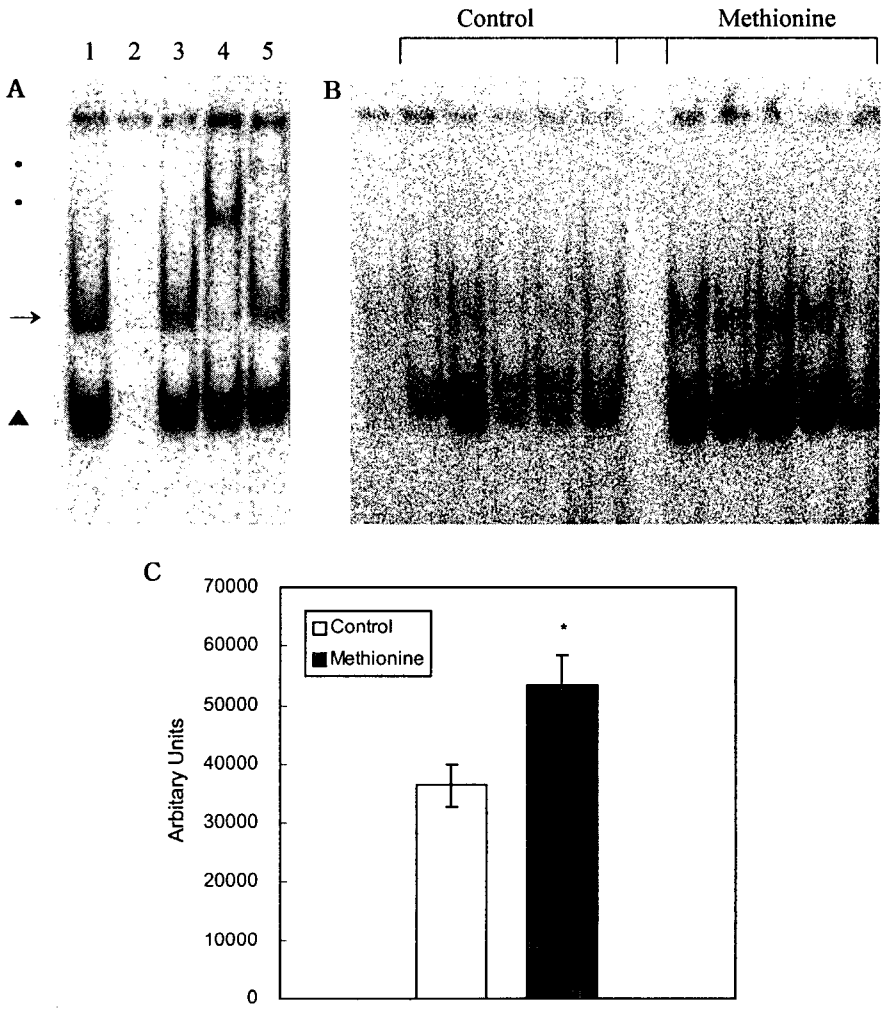

Fig. 2. NFκB binding activity of nuclear extracts from peritoneal macropahges. NFκB binding activity was evaluated by electrophoretic mobility shift assay. (A) Supershift assay of NFκB. Lane 1=nuclear extract from macrophages; lane 2=nuclear extract from macrophages incubated with 100-fold unlabeled NFκB nonspecific oligonucleotide; lane 3～5= nuclear extracts from macrophages with 2 μg of normal rabbit serum, anti-p65, and anti-p50, respectively. Circles indicate the bands shifted by the antisera. The arrow indicates p50/p65 heterodimeric NFκB. The triangle indicates unidentified NFB. (B) NFκB binding activity of nuclear extracts of peritoneal macrophages harvested from mice fed methionine and control diets for 12 wk. Lanes 1～6: control, lanes 8～12: methionine. (C) Each value is expressed as the relative intensity of radioactivity. Asterisk denotes a significant difference from control at $p<0.05$.

was not changed and decreased respectively, in mice fed methionine compared to those fed control diet. Similar result was obtained by Yagasaki et al. (1986), who observed no change in hepatic cholesterol and triglyceride in rats fed methionine, possibly due to lowered feed intake and weight gain of mice fed methionine diet (Fau et al., 1988; Yagasaki et al., 1986). Furthermore, hepatic toxicity of methionine has been proposed by Toborek et al. (1996), who observed that methionine treatment for 9 mo in rabbits caused the inflammatory infilteration of the portal triads, but preserved the lobular architecture of the livers. They suggested increased lipid peroxidation may be responsible, at least in part, for methionine toxicity of liver. Our biochemical analysis on ALT and AST activity did not support that liver function might be damaged, possibly due to HHcy or oxidative stress. Patho-biological significance of NFκB in relation to athrosclerosis in peritoneal macrophages and livers has to be investigated further in the near future.

The Effect of Methionine-induced HHcy on the Progression of Atherosclerosis

Our study designed to determine the chronic effect of methionine-induced HHcy on the progression of atherosclerosis and its possible mechanisms in old apoE mice (52 wk-old) showed that HHcy modified lipoprotein cholesterol distribution and enhanced oxidative stress, without influencing atherosclerotic lesion size. Despite the elevated hepatic oxidative stress by HHcy, hepatic GSH level, lipid profiles, and activities of AST and ALT of mice fed methionine diet was not significantly different for those fed control diet. Similar observation was made by Huang et al. (2001a), who observed GSH was unaffected, while TBARS was elevated, by 4 wk of folate depletion in rats with modest HHcy (26 μmol/L), and suggested that homeostasis of GSH in modest HHcy was not disrupted by the 4-wk experimental period. However, several lines of evidences suggested that elevated oxidative stress depleted GSH pool, a front line of antioxidative processes. Contradictory result of our constant GSH from these observations might be due to slightly elevated glutathione reductase expression.

Intracellular antioxidative enzymes prevent cells from free radical-mediated disturbances by scavenging ROS and products of lipid peroxidation. The significantly higher expression of Cu,Zn-SOD and slightly elevated expression of catalse, glutathionine reductase and GLO was observed in mice fed methionine enriched diet than mice fed control diet (Fig. 3), which may represent a protective response of the tissue to an increased intracellular concentration of ROS (Oteiza et al. 2001). This is consistent with the results of Toborek et al. (1995, 1996), who found the increased antioxidative enzyme activities were associated with increased TBARS levels in livers of rabbits fed methionine for 9 month.

It is postulated that enhanced oxidative stress in livers might potentiate NFκB activation, which is supported by few researchers, who found NFκB activation and concomitant rise of oxidative stress in the cholestatic liver of BDL mice (Miyoshi et al., 2001; Singh et al., 1992) and Kupffer cells (Wheeler et al., 2001). Our study demonstrated that HHcy induced by methionine in retired apoE -/- mice did not promote NFκB activation of livers. However, Hofmann et al. (2001) observed that HHcy promoted nuclear translocation of NFκB, a redox-activated inflammatory transcription factor, concomitant increase in expression of VCAM-1 and tissues factor in young apoE -/- mice (12 wk-old). These observations support our findings that oxidative stress led to enhanced response of NFκB activation, suggesting that hypercholesterolemia itself induces systemic oxidative stress, while HHcy does not further contribute to the activation of NFκB induced by the hypercholesterolemia in 52 wk-old apoE mice.

No elevation of plasma cholesterol level by HHcy in methionine fed apoE -/- mice is consistent with Hofmann et al. (2001) and Zhou et al. (2001). However, opposing data have been reported by several researchers, who found that plasma cholesterol level was significantly enhanced by dietary methionine supplementation (0.8%), while triglyceride remained unaffected in Wistar rats (Sugiyama et al. 1986). Yagasaki et al. (1986) reported that methionine elevated serum cholesterol level at a supplemented amount of 0.3%, then returned it to the control level above 0.6% of methionine supplementation in Wistar rats. Methionine supplementation to the soy protein at the level of methionine in casein did not affect plasma cholesterol, while elevated plasma triglycerides in Sprague Dawley rats (Kern et al., 2002), possibly by the inhibition of fatty acid oxidation (Frauscher et al., 1995). It seems to be that plasma cholesterol level is affected by animal strains, duration period and level of methionine fed.

ApoE -/- mice, used in this study, have markedly increased cholesterol concentrations, which are five times normal plasma cholesterol (Zhang et al., 1992), since apoE is involved in the clearance of chylomicrons and VLDL. Lack of apoE, therefore, accumulates plasma cholesterol-rich remnants whose prolonged circulation should be atherogenic. In the mutants lacking apoE fed methionine or control diets for 20 wk, 86% of the total cholesterol was carried in VLDL and LDL. Interestingly, differences in lipoprotein profiles of mice fed methionine from those of control mice

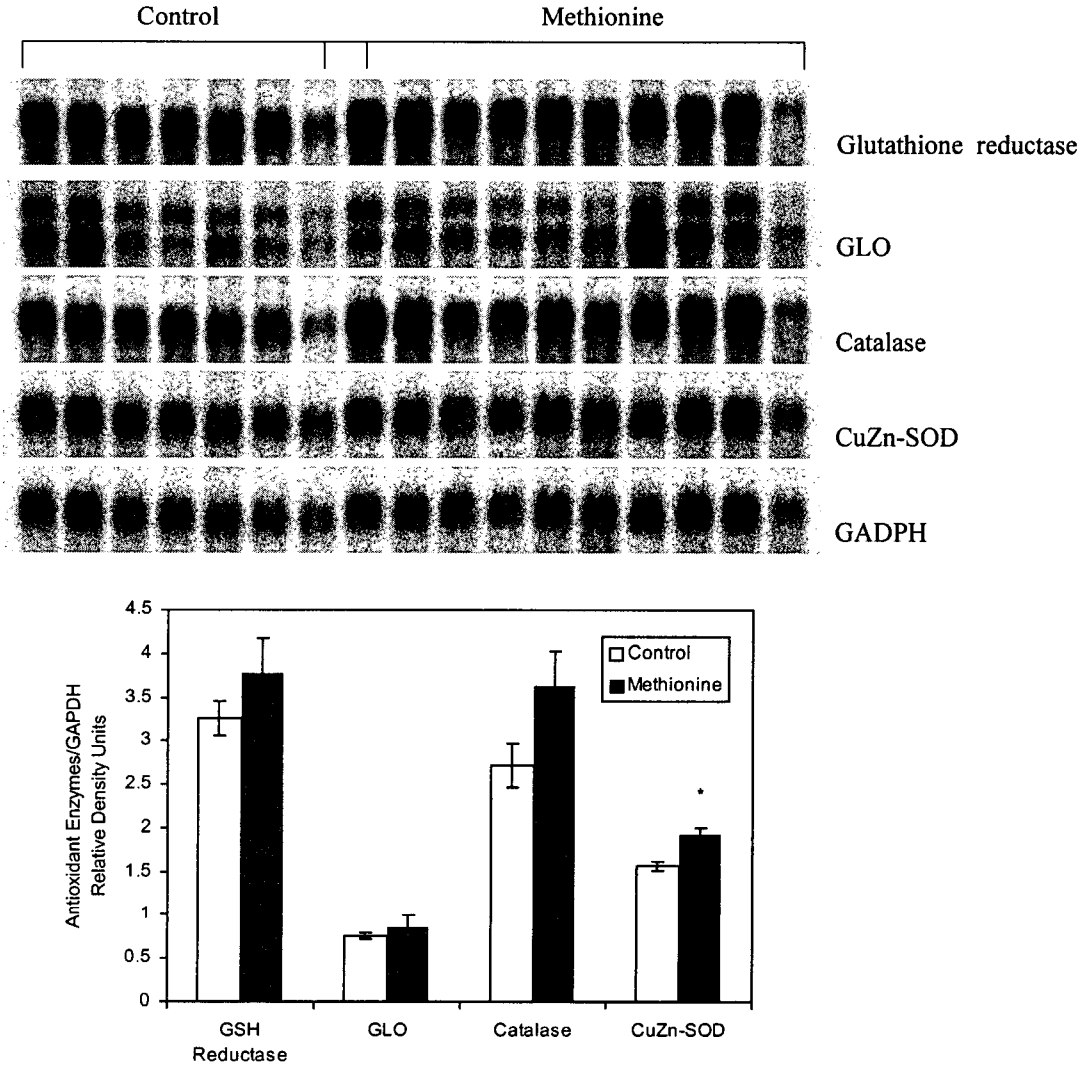

Fig. 3. Hepatic mRNA expression of antioxidative enzymes in ApoE -/- mice fed methionine (n=9) and control (n=8) diets for 20 wk. Data were normalized to GAPDH expression. Asterisks denote a significant difference from control. $p<0.05$. GLO=gulonolactone oxidase; Cu,Zn-SOD=CuZn superoxide dismutase; GAPDH=glyceraldehydes 3-phosphate dehydrogenase.

were revealed by FPLC, where VLDL cholesterol was increased by 35% , while LDL cholesterol was decreased by 36% in mice fed methionine to those fed control (Fig. 4). This tendency was more evident in mice fed methionine-enriched diet for 20 wk than mice fed 10 wk. Even though the mechanisms to explain the shift of cholesterol between VLDL and LDL is not known, it is noteworthy that the reactive species has been implicated in signal transduction (Marumo et al., 1997), raising the possibility that HHcy modulates lipoprotein metabolism.

A moderate linear association was found between homocysteine level and plaque score, which suggests that higher plasma homocysteine levels appears to have associations with increased severity of carotid atherosclerotic plaques (Sasaki et al., 2002). It has been suggested that homocysteine is a late stage predictor of adverse cardiovascular events. However, a human case-control and cross-sectional study has been failed to support that HHcy is directly involved in the development and progression of atherosclerosis (Pasterkamp et al., 2002). Indeed, studies on the relationship between homocysteine levels and the stage of atherosclerotic disease did not show consistent results. Few animal studies have been supported that HHcy was associated with significant increase in

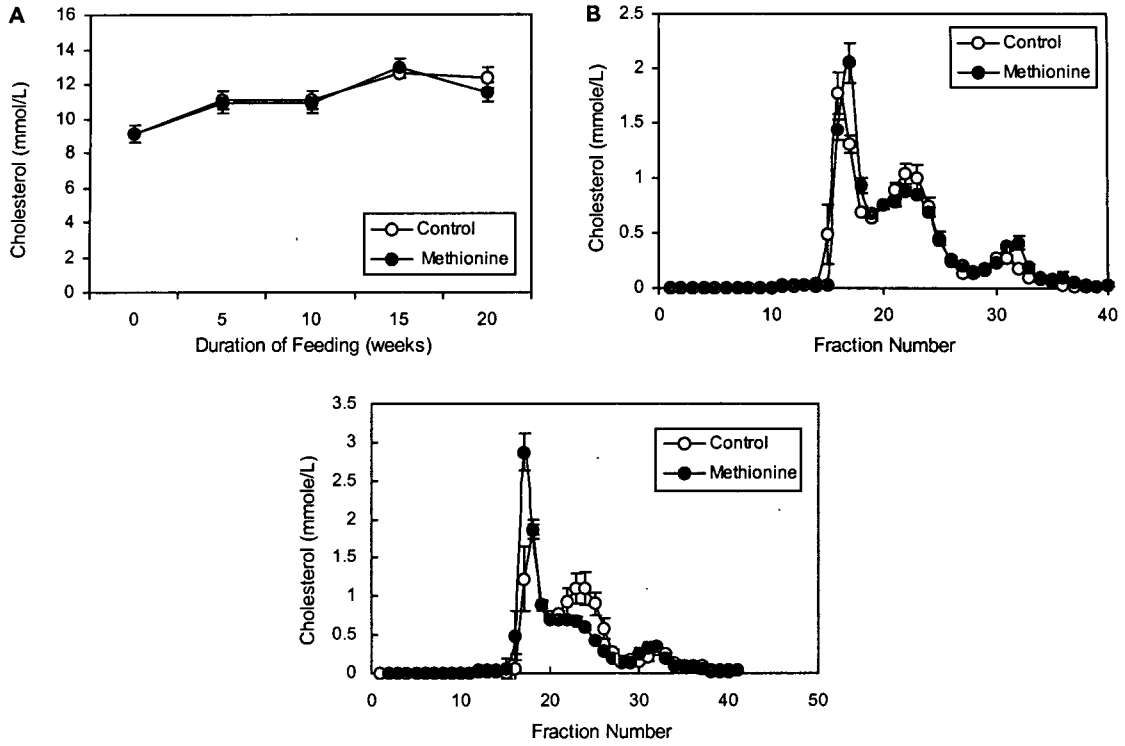

Fig. 4. Cholesterol distribution in lipoproteins of ApoE -/- mice fed methionine for 10 and 20 wk. (A) Changes of cholesterol level during methionine feeding for 20. (B) Cholesterol distribution in lipoproteins at 10 wk. (C) Cholesterol distribution in lipoproteins at 20 wk.

arterial plague size in young apoE -/- mice (Hofmann et al., 2001; Zhou et al., 2001). Indeed, Zhang et al. (1992) found evidence of fatty streaks in the proximal aorta in 3 month-old apoE knockout mice fed mouse chow. The lesions progressed with age, and near total occlusion at the entrance of a coronary artery was seen in 8-monthold apoE knockout mice. However, long term feeding (12 month) of methionine in apoE -/- mice developed same amount of atherosclerosis as compared to control, even though larger plaque size was observed in methionine-fed mice after 3 months. These findings indicates that HHcy affects the development of early lesions more than it does the progression of established plaques (Zhou et al., 2001) and supports our results as we have observed that HHcy induced methionine feeding for 20 wk did not accelerate the progression of atherosclerosis in retired apoE-deficient mice (52 wk-old).

CONCLUSION

It is well documented that hyperhomocysteinemia is an independent risk factor for atherosclerosis, but whether elevated plasma homocysteine contributes to the development and progression of atherosclerotic lesion in young and old animals with hypercholesterolemia, a risk factor of atherosclerosis, is still unknown. However, our data clearly shows that HHcy induced by methionine enriched, low folate diet accelerates oxidative stress and NFκB activation in peritoneal macrophage and livers of young C57BL/6 mice. These results confirm that HHcy induced by methionine may promote disturbances in lipid peroxidation, antioxidant processes, and inflammatory mediator activation as possible mechanisms of its atherogenic influence. However, atherosclerotic lesion area was not changed by methionine feeding in retired ApoE knockout mice. These results suggest that HHcy induced by methionine may not contribute to the progression of atherosclerotic lesion in old animals with other risk factors

for atherosclerosis. Taken together, these findings indicate that HHcy affects the development of early lesions more than it does the progression of established plaques.

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