ORIGINAL ARTICLE

Effects of long-term administration of methionine on vascular endothelium in rabbits

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Abstract

Background and Aim: *We studied the effects of long-term methionine administration on the vascular endothelium of Japanese white rabbits.*

Methods and Results: *Eleven rabbits were divided into a control group (n=6) and a methionine-fed group (n=5), and reared for 22 weeks. Blood samples were collected at baseline and after 22 weeks for the measurement of serum homocysteine and cysteine, serum lipids and serum superoxide dismutase activity. At the end of experiments, the animals were sacrificed, and the thoracic aorta was removed for the measurement of isometric tension and histopathological examination.*

The blood samples taken from the methionine group in the 22nd week showed slight but significant increases in serum homocysteine and cysteine levels (Hcy: 13.7±1.4 vs *21.0±4.9,* $p<0.01$*; Cys: 241.6±37.8* vs *342.6±35.0,* $p<0.01$*). In the isometric tension experiments, the methionine group had a significantly decreased* ($p<0.01$) *vasodilatation reaction induced by acetylcholine, an endothelium-dependent vasodilator. The histopathological examination (immunostaining in response to eNOS and tissue factor) showed significant increases in endothelium expression in the methionine group before atherosclerotic changes appeared.*

Conclusions: *The above results suggest that vascular endothelial dysfunction played an important role in the atherosclerosis occurring after excess methionine feeding.*

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Introduction

A number of studies have found that feeding with excess amounts of methionine, an essential amino acid, facilitates atherosclerosis and thrombogenesis (1-3), but the underlying mechanism has not yet been fully elucidated.

After being taken into the body, methionine is activated by ATP and becomes S-adenosylmethionine, which donates a methyl group in the biosynthesis of DNA, lipids and amino acids. S-adenosylhomocysteine is partially produced as a by-product, and becomes homocysteine in hydrolysis. Homocysteine usually converts to methionine as a result of re-methylation but, when excess methionine is present or cysteine synthesis is required, it enters the sulphur transferase pathway, and cystathionine and cysteine are synthesised. Under normal conditions, these reactions occur harmoniously in the body, but when excess methionine is taken in, or when there are abnormalities in the enzymes necessary for re-methylation or in the sulfur transfer pathway, or if vitamin levels are decreased, blood homocysteine and cysteine concentrations increase.

It has recently been discovered that particularly hyperhomocysteinemia is an independent risk factor for atherosclerosis and thrombogenesis. There are many epidemiological studies of the relationships between blood homocysteine concentrations and cerebral infarction,

Key words Methionine, homocysteine, endothelial dysfunction, eNOS, tissue factor

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peripheral vascular diseases and myocardial infarction (4-9), but the mechanism has not yet been fully elucidated. *In vitro*, homocysteine produces active oxygen (10), thus causing injury to the vascular endothelium (11-13) or affecting platelet function (14) and coagulation factors (15), and a recent study has reported that lipid metabolism is also affected (16). However, there are not many published *in vivo* studies, and there are fewer reports (17) concerning the relationship between cysteine and atherosclerosis.

The importance of vascular endothelial function in atherosclerosis has been investigated by many researchers, and it seems certain that vascular endothelial dysfunction facilitates atherosclerosis.

In the present study, Japanese white rabbits were fed excess amounts of methionine over a long period of time in order to investigate whether vascular endothelial function was affected in the same manner as in *in vitro* studies. Histopathological atherosclerotic changes and the effects on lipid metabolism were also examined.

Methods

Animals

Eleven 15-week-old male Japanese white rabbits weighing 2-3 kg were divided into a control group (n=6) and a group fed with methionine (n=5). The average body weights of the two groups were nearly equal.

Treatments

The animals in both groups were reared for 22 weeks, during which the control group was fed RC-4 pellets (Oriental Yeast Co. Ltd.) and the methionine group the same type of RC-4 pellets plus 3% methionine (Wako Pure Chemical Industries Ltd.). Feeding was restricted to 150g per day, but the animals were allowed to drink freely. Blood samples were collected after a 12-hour fast from the auricular veins at baseline and after 22 weeks, when the animals were sacrificed by means of bloodletting under pentobarbital anesthesia and their thoracic aortas were removed. Some of the aortas were used for isometric tension experiments and the others for the preparation of histopathological specimens.

Serum homocysteine and serum cysteine

Serum total homocysteine and acysteine were measured basically according to the method described by Araki *et al* (18). Briefly, total homocysteine and cysteine were reduced or liberated from plasma proteins using tri-n-butylphosphine and were derivated using a thiol-specific fluorogenic agent ABD-F (4-aminosulfonyl-7-fluoro-2,1,3-benzoxdiazole). The derivatives were separated by means of reversed-phase HPLC and detected by fluorescence at excitation and emission wavelengths of respectively 375 and 505 nm.

Serum total cholesterol and triglycerides

Serum total cholesterol and triglyceride levels were determined enzymatically using a Hitachi 7170 automated analyser and the L-type CHO H kit (cholesterol oxidase-HDAOS method, Wako Pure Chemicals) or L-type TG H kit (glycerophosphate oxidase-DAOS, glycerol-blanked method, Wako Pure Chemicals), according to manufacturer's instructions.

Serum LDL- and HDL-cholesterol

Serum LDL- and HDL-cholesterol were determined enzymatically using a Hitachi 7170 automated analyser with the detergent-based homogeneous Cholestest LDL and Cholestest HDL assay kits (Daiich Pure Chemicals), according to the manufacturer's instructions.

Serum superoxide dismutase (SOD) activity

Serum SOD activity was measured by means of an improved nitrite method (19).

Isometric tension measurements

Drugs and reagents

Phenylephrine, dimethyl sulfoxide (Wako Pure Chemical Co. Ltd.), acetylcholine (Ovisot, Daiichi Pharmaceuticals Co. Ltd.), nitroglycerine (nitroglycerine injection, Yoshitomi Pharmaceuticals Co. Ltd.) and NG-monomethyl-L-arginine (L-NMMA) (Research Biochemicals International, RBI).

Preparations of drugs and reagents

Phenylephrine and L-NMMA were dissolved in distilled water. Acetylcholine and nitroglycerine for injection were diluted with distilled water.

Test methods

The thoracic aorta was placed in Krebs-Heseleit solution and cut into approximately 2-mm wide rings, with care being taken not to injure the vascular endothelial cells. The rings were cut open to make rectangular specimens with a length of approximately 1 cm, the ends of which were fixed by pins and silk threads. One end was fixed at the bottom of a Magnus cis-

tern filled with 20 ml of Krebs-Henseleit solution (37°C, aerated with mixed gas of 95% O_2 + 5% CO_2); the other was suspended from an isotonic transducer (TD-112S, Nihon Koden Co. Ltd.) with a 1.0 g load was given. The changes in tension were measured using an automatic Magnus apparatus (IT-400, JT Toshi Co. Ltd.). The specimens were first stabilized by means of three phenylephrine-induced contractions (final concentration: $3x10^{-7}$g/mL), after which acetylcholine, nitroglycerine and L-NMMA were cumulatively added at 5-minute intervals (10-minute intervals for L-NMMA) 15 min after the addition of phenylephrine. Relaxation and contraction were measured, and the differences from the contraction induced by phenylephrine were calculated. Maximal relaxation and contraction measurements were obtained by adding nitroglycerine (final concentration: $2.2x10^{-5}$M) or phenylephrine (final concentration: $3x10^{-5}$ g/mL) as control drugs. The differences between the two values were taken as 100, and the relative values were calculated.

Histopathological specimens

The histopathological specimens were prepared by fixing the thoracic aortas in 10% neutral buffered formalin, embedding them in paraffin and slicing them into 5-μm thick sections. The thin sections were treated with hematoxylin-eosin stain for light microscopy examination.

Immunohistochemistry

The antibodies used were: endothelial nitric oxide synthase (eNOS) (Transduction Laboratories, Lexington, USA, mouse IgG1, 1:1000), angiotensin converting enzyme (ACE)(Chemicon, Temecula, USA, mouse IgG1, 1:100), tissue factor (TF)(American Diagnostica Inc, Greenwich, USA, mouse IgG1, 1:500), intercelluar adhesion molecule 1 (ICAM-1) (DAKO, Denmark, mouse IgG1, 1:300), E-selection (Chemicon, International, Temecula, CA, mouse IgG, 1:300), thrombomodulin (DAKO, Carpinteria, CA, mouse IgG, 1:500). The thin sections were deparaffined with xylene, dehydrated in sequential alcohol baths, and then washed in Tris buffer solution. A catalysed signal amplification system (DAKO, Carpinteria, USA) was used for the immunohistochemical analysis. In addition, primary antibodies were incubated for 60 min and peroxidase activity was visualised by means of diaminobenzidine (Dojin, Kumamoto, Japan), which yields a brown reaction product. The thin sections were washed in tap water, and counterstained with Mayer's hematoxylin. The negative control [non-immune mouse serum (DAKO) instead of the primary antibody] showed negative eNOS, ACE, TF, ICAM-1, E-selection and thrombomodulin immunoreactivity in the sections.

Statistical analysis

Statistical comparisons were performed using the unpaired Student's *t* test for sequential measurements in the control and methionine group. A value of $p<0.05$ was considered statistically significant.

Results

Serum homocysteine and serum cysteine

There was no difference in serum homocysteine levels in the baseline blood samples collected from the two groups (13.4±1.9 nmol/L *vs* 13.0±2.1 nmol/L, N.S.) (Table

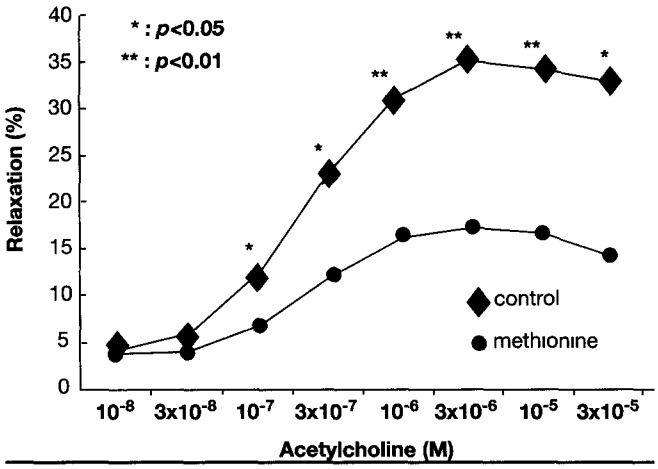
TABLE 1

Serum homocysteine, cysteine, cholesterol and triglyceride levels, and serum SOD activity, in the control group and methionine group at baseline and after 22 weeks. Mean values ±SD. Values of $p<0.05$ are considered significant.

		Week 0 Control	Methionine diet	
t-Hcy	nmol/mL	13.4±1.9	13.0±2.1	N.S
t-Cys	nmol/mL	213.0±30.5	220.7±30.0	N.S
t-Cho	mg/dL	68.0±19.8	42.0±6.5	$p<0.05$
LDL-Cho	mg/dL	36.7±20.8	17.4±5.3	N.S
HDL-Cho	mg/dL	28.3±3.2	21.6±2.7	$p<0.01$
TG	mg/dL	40.3±19.2	44.8±12.5	N.S.
SOD activity	U/mL	46.3±7.1	44.0±6.6	N.S.
			(means±SD)	
		22nd week Control	**Methionine diet**	
t-Hcy	nmol/mL	13.7±1.4	21.0±4.9	$p<0.01$
t-Cys	nmol/mL	241.6±37.8	342.6±35.0	$p<0.01$
t-Cho	mg/dL	18.5±5.3	40.7±12.4	$p<0.01$
LDL-Cho	mg/dL	3.0±1.1	11.2±7.6	$p<0.05$
HDL-Cho	mg/dL	13.3±6.5	29.0±5.4	$p<0.01$
TG	mg/dL	27.8±18.6	24.8±10.3	N.S.
SOD activity	U/mL	40.4±10.0	56.4±20.6	$p=0.13$
			(means±SD)	

SOD superoxide dismutase, Hcy homocysteine, Cys cysteine, t-Cho: total cholesterol, LDL-Cho low-density lipoprotein cholesterol, HDL-Cho high-density lipoprotein cholesterol; TG: triglycerides; N.S.: not significant.

FIGURE 1
Endothelium-dependent vasodilatation in response to acetylcholine. The responses to each concentration are expressed as percentage relaxation of phenylephrine-induced pre-contraction. At a significance level of $p<0.05$, the relaxation rate decreased significantly more in the methionine group than in the control group.



1), but homocysteine in the methionine group had slightly but significantly increased by the 22nd week (13.7±1.4 nmol/L *vs* 21.0±4.9 nmol/L, $p<0.01$) (Table 1).

Serum cysteine levels showed the same trend. There was no significant difference between the baseline blood samples of the two groups (231.0±30.5 nmol/L *vs* 220.7±30.0 nmol/L, N.S.) (Table 1), whereas cysteine levels in the methionine group had increased significantly by the 22nd week (241.6±37.8 nmol/L *vs* 342.6±35.0 nmol/L, $p<0.01$) (Table 1).

Serum total, LDL and HDL cholesterol

Total cholesterol at baseline was significantly higher in the control group (68.0±19.8 mg/dL *vs* 42.0±6.5 mg/dL, $p<0.05$) (Table 1) but, by the 22nd week, it was significantly higher in the methionine group (18.5±5.3 mg/dL *vs* 40.7±12.4 mg/dL, $p<0.01$) (Table 1).

There was no significant difference in LDL cholesterol between the two groups at baseline (36.7±20.8 mg/dL *vs* 17.4±5.3 mg/dL, N.S.), but the methionine group has significantly higher levels by the 22nd week (3.0±1.1 mg/dL *vs* 11.2±7.6 mg/dL, $p<0.05$) (Table 1).

Baseline HDL cholesterol was significantly higher in the control group (28.3±3.2 mg/dL *vs* 21.6±2.7 mg/dL, $p<0.01$) (Table 1) but, by the 22nd week, it was significantly higher in the methionine group (13.3±6.5 mg/dL *vs* 29.0±5.4 mg/dL, $p<0.01$) (Table 1).

Serum triglycerides

There was no significant difference between the two groups at baseline (40.3±19.2 mg/dL *vs* 44.8±12.5 mg/dL, NS) (Table 1) or at the end of the 22nd week (27.8±18.6 mg/dL *vs* 24.8±10.3 mg/dL, NS) (Table 1).

Serum SOD activity

There was no significant baseline difference between the two groups (46.3±7.1 U/mL *vs* 44.0±6.6 U/mL, NS) (Table 1). By the 22nd week, although the difference was not significant, serum SOD activity showed an increasing trend in the methionine group (40.4±10.0 U/mL *vs* 56.4±20.6 U/mL, $p=0.13$) (Table 1). It is said that homocysteine produces active oxygen and, as homocysteine levels in the methionine group increased significantly by the 22nd week, it is possible that the greater serum SOD activity was a reaction intended to compensate for active oxygen production.

Vascular endothelial function

In the isometric tension experiments, the administration of acetylcholine (an endothelium-dependent vasodilator)

FIGURE 2
Endothelium-independent vasodilatation in response to nitroglycerine The responses to each concentration are expressed as percentage relaxation of phenylephrine-induced pre-contraction. At a significance level of $p<0.05$, there was no significant difference between the two groups.

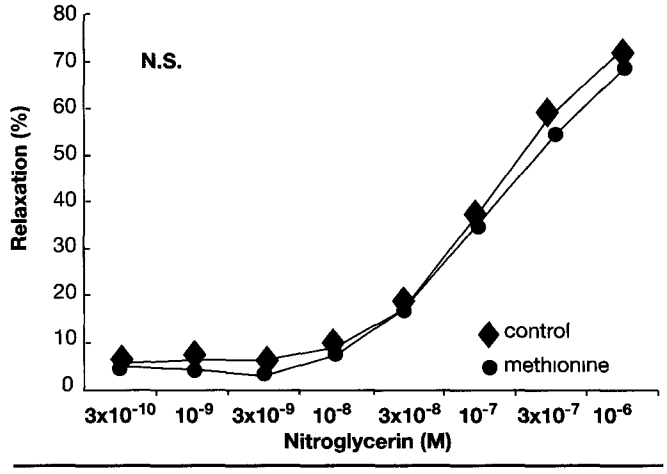
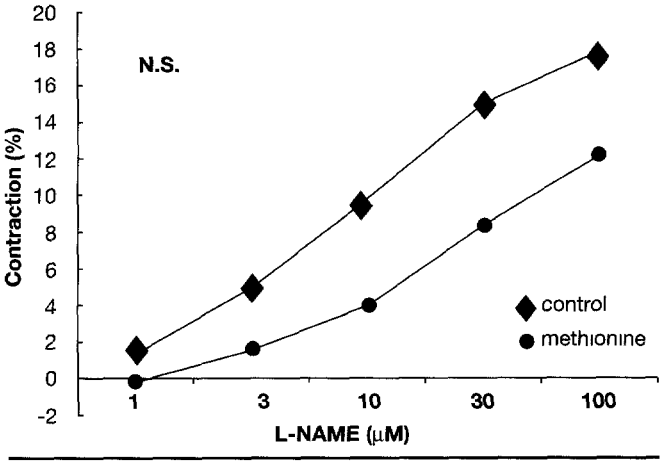


FIGURE 3
Basic secretion of NO following L-NMMA administration. The responses to each concentration are expressed as percentage contraction of phenylephrine-induced pre-contraction. At a significance level of $p<0.05$, there was no significant difference in relative contraction rates in the two groups, but there was a decreasing trend in the methionine group.



led to a significant decrease in the vascular relaxation rate in the methionine group ($p<0.01$) (Fig 1). Following the administration of nitroglycerine (an endothelium-independent vasodilator), there was no significant difference in the vascular relaxation rate between the two groups (Fig 2). These results suggest that vascular endothelial function decreased in the methionine group.

L-arginine is converted to L-citrulline in endothelial cells as a result of enzyme NO synthase, which leads to the formation of NO as a by-product; NO synthesis can therefore be inhibited using L-NAME. Differences in the basic secretion of NO should therefore be reflected by the relative contraction of the intact endothelium of the aortic rings in the presence of L-NAME (20). However, there was a decreasing trend in the methionine group (Fig 3), thus suggesting that basic NO secretion was decreased. The L-NMMA experiments showed no significant difference in the relative contraction rate between the two groups.

Histopathological examination

Light microscopy

As all of the aorta specimens stained with hematoxylin-eosin, light microscopy did not reveal any apparent difference between the two groups. There was also no apparent atherosclerotic change in the methionine group (Fig 4).

FIGURE 4
Light microscopy examination of rabbit thoracic aorta stained with hematoxylin-eosin No atherosclerotic change was observed in the control or methionine group. (x320)

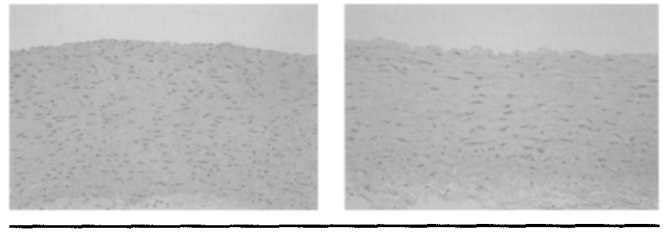


FIGURE 5
Immunostaining of rabbit thoracic aorta in response to tissue factor (TF). There was a significant increase in vascular endothelium expression in the methionine group. (x320)

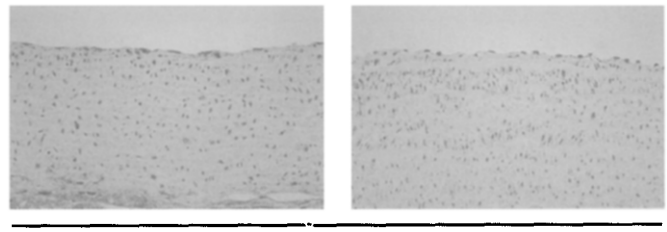
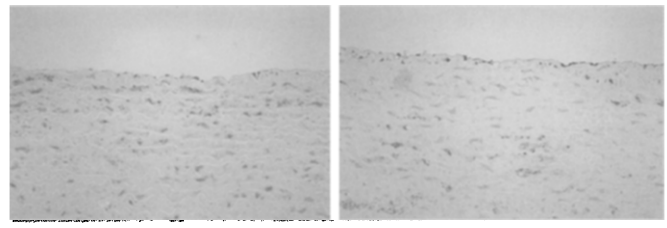


FIGURE 6
Immunostaining of rabbit thoracic aorta in response to eNOS. There was a significant increase in vascular endothelium expression in the methionine group. (x320)



Immunohistochemistry

The expression of TF and eNOS was greater in the vascular endothelium of the methionine group (Figs 5, 6), there

was no between-group difference in the expression of ACE, ICAM-1, E-selectin or thrombomodulin.

Discussion

The results of this study show that long-term excess methionine feeding decreased vascular endothelial function in Japanese white rabbits.

The administration of excess methionine increases the blood concentrations of homocysteine and cysteine, amino acids containing an SH-group whose oxidation leads to the production of active oxygen (10). It is thought that atherosclerosis progresses because the active oxygen directly injures endothelial cells and facilitates the oxidation of lipids (11-13), and our results may support this theory. It is also possible that, although not significant, the increasing trend of serum SOD activity in the methionine group was a compensatory reaction against the production of active oxygen, because all of the studies of homocysteine and SOD activity have reported increases in activity (21-24). However, other studies have reported that the main active oxygen produced by homocysteine is hydrogen peroxide (10), or that the activity of glutathione peroxidase did not increase (21, 25), and so further studies seem necessary.

As it has not been confirmed that methionine has these actions itself, it is interesting that even relatively slight increases in homocysteine and cysteine led to the above results, thus supporting the finding of recent large-scale epidemiological investigations that even mild hyperhomocysteinemia is an independent risk factor for atherosclerosis and thrombogenesis.

Our light microscopy investigations did not reveal any significant atherosclerotic changes in the methionine group, but the results of the isometric tension tests indicated decreased endothelial function and the immunostaining tests showed increased expression of tissue factor and eNOS. Our tissue factor results support previous *in vitro* findings (26, 27). What is particularly interesting is that the increased expression in response to tissue factor is specific to homocysteine and does not occur in the case of SH-group containing analogues such as cysteine; expression has also been induced with methionine (27). This may be one reason for the fact that, although homocysteine is a widely recognised risk factor for atherosclerosis, there are not many reports concerning cysteine. It is possible that methionine plays a role in the onset of atherosclerosis, and that the increase in eNOS antibody is a defensive reaction. The reaction between NO and homocysteine leads to the creation of S-nitrosohomocysteine in the body, which inhibits homocysteine from producing active oxygen (28). S-nitrosohomocysteine itself has vasodilating activity and inhibits platelet agglutination (like NO); it is also said to be more stable in the body than NO (29-33). NO may work against the homocysteine-induced facilitation of atherosclerosis, and *in vitro* studies have reported that homocysteine increases the mRNA levels of NOs (3) and NO production (28). However, when hyperhomocysteinemia persists and the balance is broken down, the active oxygen produced by homocysteine injures the vascular endothelium, and the basic secretion of NO decreases; furthermore, as active oxygen is also believed to inactivate NO, the vascular endothelial injury progresses. The above description seems to explain the results of the present study.

With respect to lipid metabolism, no increase thought to affect the progress of atherosclerosis was observed in either group. In particular, there was no difference in triglyceride levels between the two groups, but cholesterol levels (which were higher in the control group at baseline) became even higher in the methionine group by the 22nd week. As the only difference in the feed given to the two groups was methionine, it is possible that excess methionine intake affected cholesterol metabolism. Although further studies are needed, this finding is in line with the results of another study (16) and, if true, the importance of controlling methionine should be emphasised as a means of preventing arteriosclerosis.

Limitations

Our study has a number of limitations. First of all, the excess administration of dietary methionine to Japanese white rabbits caused vascular endothelium dysfunction, and it is possible that this event was not only due to homocysteine, but also to cysteine and methionine. Both cysteine and homocysteine levels were significantly increased in the methionine group, but the results of our experiments are not sufficient to clarify this question. Many published reports have indicated homocysteine as a risk factor for atherosclerosis, but only a few have investigated the effects of cysteine or methionine. Morita *et al* recently reported that when a carotid arterial balloon was used to cause an injury in hyperhomocysteinemic rats, neointima formation was markedly enhanced in comparison with controls, and concluded that this effect was attributable to homocysteine, but not to cysteine or methionine (34). Furthermore, it has been reported that the methylation of p21ras and inhibition of the activity of extracellular signal-regulated kinase (ERK) 1/2 are involved in impairing endothelial cells, and that homocysteine (but not cysteine) has an

inhibitory effect on kinase activity (35). On the contrary, as mentioned in the Discussion, there has been a report that methionine can also induce the expression of tissue factor. Further investigations are needed.

Secondly, the effects of active oxygen and SOD activity described here are limited to the intracellular level, and serum SOD does not always reflect intracellular SOD levels.

Thirdly, we estimated basal NO secretion on the basis of the relative contraction rate and did not determine absolute NO levels; furthermore, we found no significant difference between the two groups. Nevertheless, on the basis of previous *in vitro* results and as described in the Discussion, it is very likely that NO is closely involved in the mechanism leading to the decreased vascular endothelial function observed by us. Our results seem to support this possibility, although further studies are necessary.

Finally, it should be noted that there was a significant difference in baseline cholesterol levels between the two groups, which gives rise to considerable problems in interpreting the results. However, it is difficult to imagine that the cholesterol levels in either group affect atherosclerosis. Furthermore, as mentioned in the Discussion, it has been previously reported that homocysteine, cholesterol and triglyceride levels are correlated in animal experiments. As the only dietary difference between the two groups was the methionine given to the experimental animals, we think that it is possible to discuss the relationship between methionine and lipid metabolism to some extent.

Despite the above limitations, we believe that our results are of interest as a contribution towards the elucidation of the mechanism of homocysteine-induced atherosclerosis because methionine intake is the only means of producing homocysteine *in vivo*.

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