Original Article

Dysfunction of blood pressure regulation in hyperhomocysteinemia model in rats

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ABSTRACT — Hyperhomocysteinemia (HHC) has been reported to be one of risk factors for cardiovascular disease. We investigated the effects of HHC on blood pressure regulation and its association with damage to the thoracic aorta and imbalance of redox in plasma in rats. Rats were fed a methionine enriched diet (Met diet) or a methionine and cholesterol (Met+Chol diet) enriched diet for 16 weeks to create a subchronic HHC model, in which the plasma concentration of homocysteine was about 7 times higher than that of control rats. The increase in systolic blood pressure (Δ -SBP) from sympathetic stimulation by L-epinephrine was 2- to 3-fold larger in HHC model in rats than that in control rats after several weeks of the treatment. These findings suggest that HHC deteriorates vaso-regulatory function, which could bring on an increased risk of cardiovascular events in humans. In addition, some of the elastic lamellae in the aorta were disrupted in the HHC group. However, the content of cross-linkages which give elasticity and mechanical strength in the lamellae, was not significantly different between HHC and control rats. Also plasma concentrations of thiobarbituric acid reactive substance and glutathione as indicators for redox balance in plasma were not different. In conclusion, the deterioration of vaso-regulation in HHC model in rats might be caused by the damage to elastic lamellae in the aorta, and not by oxidative stresses.

Key words: Hyperhomocysteinemia, Homocysteine, Cardiovascular event, Vaso-regulation, Elastic lamellae

INTRODUCTION

Homocysteine (Hcy), an amino acid having a thiol group, is synthesized *in vivo* by the demethylation of methionine. It is metabolized further with sulfur transition to cysteine through cystathionine, or returned again to methionine by methylation in the presence of 5-methyl-tetrahydrofolic acid and trimethylglycine as a methyl donor. In healthy individuals, plasma concentration of Hcy is maintained constant by metabolic regulation as just described, even though that of methionine is raised by ingestion of foods.

Hyperhomocysteinemia (HHC) is well known as a genetic disease with cystathionine-synthetase deficiency, but also is a life style-related disease associated with deficiency of vitamins such as vitamin B₆, B₁₂ and folic acid, and other factors such as age, smoking and alcohol abuse (Zhou *et al.*, 2014). It was reported that patients with homocystinuria, whose plasma concentration of Hcy was

markedly higher than in healthy individuals, commonly develop arteriosclerosis and thrombotic lesions early in life (McCully *et al.*, 1969). Many researchers reported that HHC is an independent risk factor for cardiovascular events (Boushey *et al.*, 1995; Refsum *et al.*, 1998; Nygård *et al.*, 1997). Moreover, some reports have shown that the plasma concentration of Hcy is correlated with systolic blood pressure in humans (Nygård *et al.*, 1995; Lim and Cassano, 2002). Recently, HHC was reported to be associated with the blood pressure response to mental stress and masked hypertension (Tayama *et al.*, 2006; Yücel *et al.*, 2014).

Some reports have suggested the mechanism by which HHC increases the risk of cardiovascular events. For example, Charpiot *et al.* (1998) demonstrated that disruption of elastic lamellae in the aorta occurred in a HHC model in minipigs. Zulli *et al.* (1998a) reported that, in a subchronic HHC model created by feeding rats a methionine rich diet for 15 weeks, disruption of aortic elastic

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lamellae and thickening of the aortic wall were observed, and that such changes were further potentiated by feeding cholesterol in addition to methionine. Moreover, Raposo *et al.* (2004) reported that a high concentration of Hcy inhibited the activity of lysyl oxidase, which is required for the formation of cross-linkage structures in elastic lamellae, and downregulated *lysyl oxidase* gene expression in vascular endothelial cells. Based on these facts, it was expected that a high concentration of Hcy would disturb the elastic lamellae and reduce the cross-linkages in the aorta, and that such damage of the aorta might reduce the vaso-regulation and cause hypertension. Previously, it was reported that repeated methionine administration did not alter the blood pressure in normotensive rats (Matthias *et al.*, 1996; Ungvari *et al.*, 1999). However the effect of methionine-enriched diet on the ability to regulate blood pressure in response to some stimulation, such as sympathetic drugs or some stresses, *in vivo* still remains unclear.

On the other hand, there are some reports about endothelial dysfunction due to the oxidative stress generated from Hcy in the HHC condition. Tawakol *et al.* (1997) indicated that HHC is associated with impaired endothelium-dependent vasodilation in a comparative study between HHC and control human subjects. Lang *et al.* (2000) demonstrated that Hcy inhibited endothelium-dependent relaxation of rabbit aortic rings with an increase in intracellular levels of $O_2(-)$ in the endothelial cells in an *in vitro* study. Tsai *et al.* (1994) also reported that Hcy led to an increase of smooth muscle cells in the rat aorta and a decrease of DNA synthesis in endothelial cells from human umbilical veins.

The mechanism by which HHC affects blood pressure remains unclear. Thus, in the present study, the effect of the HHC on vaso-regulation, structure of the blood vessels and redox imbalance, and the relationships among these factors were investigated using a HHC model in rats fed a methionine enriched diet.

MATERIALS AND METHODS

Chemicals, reagents and diets

Methionine, Hcy, pyridylethyl-cysteine and *N*-acetyl-L-cysteine (NAC) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). *O*-phthalaldehyde and tris (2-carboxyethyl) phosphine was purchased from Tokyo Chemical Industry Co., Ltd. (Tokyo, Japan). L-Epinephrine, human angiotensin II and ethylenediaminetetraacetic acid (anhydrous) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 4-Fluoro-7-sulfobenzofurazan, ammonium salt (SBD-F) was purchased

from Dojindo Laboratories (Kumamoto, Japan). All other reagents and solvents in this study were commercially available and were either extra pure, guaranteed, high-performance liquid chromatography (HPLC) or liquid chromatography-mass spectrometry (LC/MS) grade.

All animal diets were purchased from Nosan Corporation (Kanagawa, Japan). Labo MR Stock® was used as a control diet and contained 0.26% (w/w) methionine and 0.025% (w/w) cholesterol. The composition of methionine and cholesterol in special diets was the same as those in the reports by Zulli *et al.* (1998a, 1998b), as follows. A methionine enriched diet (Met diet) contained 2% (w/w) methionine; a cholesterol enriched diet (Chol diet) contained 2% (w/w) cholesterol; and a methionine and cholesterol enriched diet (Met+Chol diet) contained 2% each of methionine and cholesterol. The content of the other ingredients in these special diets was the same as in the control diet.

Animals

Twenty male Sprague-Dawley rats (5 weeks old) were obtained from Japan SLC Inc. (Shizuoka, Japan) and used after 1 week of acclimatization. All rats were housed in a temperature ($23 \pm 1^\circ\text{C}$) and humidity ($55 \pm 5\%$)-controlled room with 12-hr light/dark cycle. Water and diet were available *ad libitum* throughout the study. All experimental animals were handled in accordance with the institutional and national guidelines for the care and use of laboratory animals.

Treatment of animals

The rats were divided into 4 groups ($n = 5$) at random, and used for the study consisting of consecutive 3 periods. In the 1st phase, the acclimatization period, all rats were fed with control diet for 1 week. Then in the 2nd phase, the testing period, rats in each respective group were fed the Met diet, Chol diet, Met+Chol diet or control diet for 16 weeks. In the last phase, recovery period, the diet for all groups was returned to the control diet for 3 weeks.

About 0.4 mL of blood were collected serially from each rat with heparinized syringes under isoflurane anesthesia between 9:00 and 10:00 AM at weeks 0 (the end of the acclimatization period), 2, 4, 6, 8, 10, 12, 14 and 16 of the testing period and week 3 of the recovery period. In addition, on the first day of the recovery period, about 0.15 mL of blood was collected at 3, 6, 9, 12 and 24 hr after the return to control diet (9:00, 12:00, 15:00, 18:00, 21:00 and the following day at 9:00). Plasma samples were subsequently obtained by centrifugation at 10,000 g and 4°C for 1 min, and stored at -80°C until the measurement of total Hcy, methionine, chole-

terol, triglyceride concentration, thiobarbituric acid reactive substances (TBARS) and aspartate aminotransferases (AST) activity.

Blood pressure was serially measured at week 0 (the end of the acclimatization period), at weeks 4, 6, 8, 10, 12, 14 and 16 of the testing period, and at week 2 of the recovery period; blood collection and blood pressure measurement were done on different days. The day after the last blood pressure measurement, the thoracic aorta was isolated after the rats were euthanized by exsanguinations under isoflurane anesthesia, and prepared for histological evaluation and the determination of cross-linkages in thoracic aortas.

Measurement of blood pressure

Blood pressure with or without sympathetic stimulation was measured as described in our previous report (Miyajima *et al.*, 2013). Briefly, systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured by a non-invasive tail-cuff/pulse transducer and a Lab Chart 7 recorder (AD Instruments Pty Ltd., Bella Vista, Australia) under pentobarbital anesthesia. Ten min before measurement of blood pressure, 10 µg/kg L-epinephrine was administered subcutaneously to stimulate the sympathetic nervous system. In addition, at week 2 of the recovery period the effects of angiotensin II (0.7 µg/kg) as another vasopressor were measured. The difference between SBP before and after administration of the vasopressor (Δ -SBP) was calculated.

Plasma concentrations

Total Hcy, cysteine and glutathione (GSH):

Total Hcy, which consists of oxidized forms and disulfide forms of Hcy; cysteine and glutathione were determined by HPLC after complete reduction followed by fluorescent-labeling as follows. Fifty microliters of plasma was mixed with 25 µL of 1 mM NAC (internal standard) solution and 10 µL of 100 g/L tris(2-carboxyethyl)phosphine solution. The mixture was incubated at room temperature for 30 min to reduce disulfide bonds. After the reduction, the mixture was mixed with 90 µL of 100 g/L trichloroacetic acid and centrifuged at 13,000 g and 4°C for 10 min to precipitate the proteins. Then, 50 µL of supernatant was mixed with 10 µL of 1.55 M NaOH and 125 µL of 125 mM borate buffer (pH9.5) containing 4 mM EDTA. In addition, the sample was mixed with 50 µL of 1 mg/mL SBD-F solution and the mixture was incubated at 60°C for 60 min for the derivatization of Hcy, NAC and other thiol groups. After cooling to room temperature, the samples were used for HPLC analysis.

The derivatized Hcy, NAC, cysteine, GSH and other endogenous thiol groups were separated by a dual pump HPLC system (Shimadzu, Kyoto, Japan) using a Wakosil-5C18RS column (4.6 × 150 mm, 5 µm; Wako Pure Chemical Industries, Ltd.) attached to a TSKgel guardgel ODS-80Ts (3.2 × 15 mm, 5 µm; Tosoh Corporation, Tokyo, Japan). The mobile phase consisted of 100 mM acetate buffer (pH 4.5) and methanol, and was delivered at a flow rate of 1.0 mL/min. During the measurement, the proportion of methanol in the mobile phase was increased linearly from 3% at the start to 23% at 2.0 min, decreased to 3% at 6.0 min, and held at this proportion until the next analysis. The fluorescence intensity was measured at 515 nm with excitation at 385 nm.

Methionine:

Methionine was determined by HPLC analysis after derivatization using *O*-phthalaldehyde according to the method reported by Sun *et al.* (2005) with a slight modification. Briefly, 30 µL of acetonitrile containing 50 µM of butylamine as an internal standard was added to 10 µL of plasma and mixed well, then the sample was centrifuged at 9,000 g and 4°C for 2 min. Ten microliters of the supernatant was mixed with 5 µL of 80% methanol buffered with sodium borate (pH 10.5) containing 0.8 mg/mL of *O*-phthalaldehyde and 1.6 mg/mL of NAC. The mixture was incubated at room temperature for 90 sec, and then 150 µL of sodium acetate buffer (100 mM, pH 7.0) was added and mixed well to stop the derivatization. The derivatized samples were used for HPLC analysis.

The derivatized methionine was detected by the same HPLC system and columns as described above. The mobile phase consisted of solvent A (tetrahydrofuran/methanol/100 mM acetate buffer, pH 7.0, v/v/v = 5/95/900) and solvent B (methanol), and was delivered at a flow rate of 1.8 mL/min. During the measurement, the proportion of solvent B in the mobile phase was held at 20% from the start to 2.0 min, increased linearly from 20% to 60% from 2.0 to 14.0 min, decreased to 20% at 16.0 min, and held at this proportion until the next analysis. The fluorescence intensity was measured at 450 nm with excitation at 350 nm.

Total cholesterol, triglyceride and AST:

Total cholesterol (TC) concentration in plasma was measured by Cholesterol E-test Wako kit (Wako Pure Chemical Industries, Ltd.). Triglyceride concentration in plasma was measured by Triglyceride E-test Wako kit (Wako Pure Chemical Industries, Ltd.). AST activity in plasma was measured at weeks 0 and 16 of the testing period by Transaminase CII-test Wako kit (Wako Pure

Chemical Industries, Ltd.). All assays were conducted using 20 μ L of plasma according to their instructions.

TBARS:

TBARS was measured using TBARS assay kit (Cayman Chemical Company, Ann Arbor, MI, USA) according to the instructions with slight modification. Twenty microliters of plasma was mixed with 20 μ L of 10% SDS solution and 800 μ L of 37 mM TBA solution (containing 19.9% acetic acid and 0.2 M NaOH). The mixture was heated at 100°C for 1 hr, and then cooled on ice for 10 min. The mixture was centrifuged at 1,600 g and 4°C for 10 min, and then 600 μ L of the supernatant was taken and mixed with the same volume of 1-butanol. The mixture was centrifuged again at 1,600 g and 4°C for 15 min. The fluorescence intensity of the supernatant was measured at 590 nm with excitation wave length of 540 nm.

Histological analyses of elastic lamellae

The histological sections of aorta were prepared as described in our previous report (Miyajima *et al.*, 2013). Briefly, fragments of thoracic aortas were fixed with the 1/2-Karnovsky' solution, and the samples were embedded in paraffin and sliced at 5 μ m thickness. The sections were stained with the Elastica-van Gieson methods and were observed with a light microscope ($\times 200$).

Contents of cross-linkages in thoracic aorta

Desmosine, isodesmosine, pyridinoline and deoxypyridinoline were measured to evaluate the damage of elastin and collagen in the thoracic aorta according to our previous report (Muto *et al.*, 2013) with slight modification. Briefly, approximately 5 mm long fragments of thoracic aorta were lyophilized and their dry weight was measured. HCl was added to the lyophilized samples, which were then heated at 110°C for 24 hr and cooled at room temperature. The sample was lyophilized again and the residue was re-dissolved in mobile phase with pyridile-

thyl-cysteine (an internal standard). The samples were analyzed by API-3200 LC-MS/MS system (AB Sciex, Framingham, MA, USA).

Data analyses

One-way analysis of variance with Tukey's multiple comparison tests was used to determine the statistical significance. Differences were considered statistically significant when $p < 0.05$.

RESULTS

Plasma concentrations of Hcy and selected endogenous substances

To determine the Hcy exposure during the testing and recovery periods, the plasma concentrations of total Hcy were measured. In the Met and Met+Chol groups, the average plasma concentrations were 29.5 ± 6.7 and 39.0 ± 7.6 μ M, which were 7.4 and 9.8 times higher than those in the control group, respectively, in the testing period (Table 1). These results confirmed that rats in the Met and Met+Chol groups developed HHC during the treatment. In the recovery period, during the 24 hr period after returning to the control diet the plasma concentration in the Met and Met+Chol groups quickly returned to the control level (Fig. 1).

The plasma concentrations of Hcy related metabolites were also measured. At week 2 of the testing period, the plasma concentrations of methionine, a precursor of Hcy, were about 19 times higher in the Met group and 23 times higher in the Met+Chol group compared to those in the control group (Fig. 2A, Table 1). In both groups, such significantly high concentrations were sustained in the testing period. The concentration in the Chol group was almost the same as that in the control group throughout the study. At week 3 of the recovery period the methionine levels in all groups were almost the same as in the control group. On the other hand, plasma concentrations of cysteine, which is a further metab-

Table 1. Average plasma concentration (μ M) of selected endogenous substances.

	Control	Chol	Met	Met+Chol
Homocysteine	4.0 ± 0.8^c	6.0 ± 1.0^c	29.5 ± 6.7^b	39.0 ± 7.6^a
Methionine	35 ± 4^c	33 ± 3^c	465 ± 47^b	626 ± 53^a
Cysteine	178 ± 9^{ab}	187 ± 7^a	163 ± 8^c	170 ± 9^{bc}
Triglyceride	120 ± 8^a	137 ± 11^a	84 ± 14^b	97 ± 12^b
Total cholesterol	67.6 ± 9.4^a	73.8 ± 10.2^a	67.4 ± 18.7^a	77.1 ± 18.5^a

Average concentration of selected endogenous substances between the week of 2 to 16 of the testing period are shown as mean $\pm$ S.D. ($n = 5$). Superscript letters indicate results of pairwise comparisons. Within each row, the means followed by different letters are significantly different ($p < 0.05$, Tukey's multiple comparison test).

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Fig. 1. Plasma concentration-time profiles of total Hcy in the testing period and the recovery period. Data are shown as mean $\pm$ S.D. ($n = 5$).

Fig. 2. Plasma concentration-time profiles of methionine (A), cysteine (B), triglyceride (C), and total cholesterol (D) in the testing and the recovery period. Data are shown as mean $\pm$ S.D. ($n = 5$).

Fig. 3. SBP (A) and DBP (B) in the testing and recovery period. Data are shown as mean $\pm$ S.D. ($n = 5$).

Fig. 4. Changes of blood pressure in response to L-epinephrine in the testing period. [Change in systolic blood pressure (Δ SBP)] = [SBP after L-epinephrine administration] - [SBP before the administration]. Data are shown as mean $\pm$ S.D. ($n = 5$). * $p < 0.05$ between groups analyzed by Tukey's multiple comparison test.

olite of Hcy in the methionine metabolic pathway, did not vary greatly among all the groups throughout the study (Fig. 2B, Table 1).

The mean plasma concentration of total cholesterol was not significantly different between the control group and the other groups in the testing period (Table 1). The average plasma concentration of triglyceride was significantly lower in the Met group and the Met+Chol group compared with the control group in the testing period (Table 1). However, since the differences between the groups treated with and without methionine were relative-

ly small, it was thought that they were of little physiological significance.

Blood pressure and its regulation

In the absence of vasopressor stimulation, neither SBP nor DBP was significantly different between the control group and the other groups throughout the study except at 10 weeks in the testing period (Fig. 3). The Δ -SBP, the difference between SBP before and after administration of the vasopressor, was calculated as an indicator for the ability to regulate a temporary increase in blood pressure

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Fig. 5. Changes of blood pressure in response to L-epinephrine and angiotensin II in the recovery period. Rats were fed with Met+Chol, Met, Chol or control diet in the testing period, then their food were returned to control diet for more than 2 weeks in the recovery period. Data are shown as mean $\pm$ S.D. ($n = 5$). * $p < 0.05$ between groups analyzed by Tukey's multiple comparison test.

(Fig. 4). In the Met+Chol group at week 4 in the testing period, the Δ -SBP induced by L-epinephrine was 18.1 ± 2.6 mmHg, which was significantly (about 1.6-fold) larger than that in the control group (11.4 ± 1.8 mmHg). Also in the Met+Chol and Met groups at week 6 of the testing period, the Δ -SBP were 21.0 ± 3.2 and 17.3 ± 3.8 mmHg, respectively, both of which were significantly larger than that in control group (10.4 ± 2.3 mmHg). Until the end of the testing period, the significant enhancement of Δ -SBP was observed in both the Met and Met+Chol groups compared to the control group. Furthermore, at week 2 of the recovery period, this significant enhancement of Δ -SBP was still observed in the Met+Chol and Met groups not only when L-epinephrine was used as the vasopressor but also when angiotensin II was used (Fig. 5).

Histopathological observation

The histopathological changes of the elastic lamellae of the thoracic aorta were evaluated by optical microscopy (Fig. 6). The elastic lamellae in the aorta of control rats were clearly observed as thick bands without any branching. In the rats with HHC, some of the lamellae were disrupted and swollen. These histopathological changes in HHC were consistent with those in the previous report (Zulli *et al.*, 1998a), although they were less severe.

Contents of the cross-linkages of elastin and collagen in the aorta

Desmosine and isodesmosine are the specific cross-linkages in elastin, and largely contribute to its elasticity (Thomas *et al.*, 1963; Partridge *et al.*, 1966). The contents of desmosine and isodesmosine were determined as indicators for damage to elastin by Hcy. Pyridinoline and deoxypyridinoline are the specific cross-linkages in collagen, and contribute to its mechanical strength (Eyre *et al.*, 1984). The contents of pyridinoline and deoxypyridinoline were measured as indicators for the damage of collagen by Hcy. The contents of desmosine, isodesmosine and pyridinoline were not significantly different among the groups (Fig. 7). The deoxypyridinoline content in all groups was below the lower limit of quantification (6 ng/mL).

Concentration of TBARS and GSH, and transaminase activity in the plasma

The plasma concentrations of TBARS and GSH were measured as indicators of lipid peroxidation and antioxidant activity in plasma. Neither indicator was significantly different among the groups throughout the study (Fig. 8). The AST activity in plasma was measured as an indicator of liver function. The activity (IU/L) in

Fig. 6. Representative micrographs of elastic lamellae in the thoracic aorta of rats fed with control (A), Chol (B), Met (C), or Met+Chol (D) diet. Optical microscopy with Elastica-van Gieson method. Original magnification, 200 $\times$. Bars, 40 μ m. A) L, lumen; I, intima; M, media; Ad, adventia; El, elastic lamellae. C), D) the arrows indicate the representative parts where lamellae are disrupted and swollen.

the groups at week 16 of the testing period were as follows; Control, 7.3 ± 0.9 ; Chol, 9.6 ± 2.6 ; Met, 14.8 ± 6.1 ; Met+Chol, 8.6 ± 2.3 . The activity of the Met group was statistically significant higher compared to the Met+Chol and control groups ($p < 0.05$). However, since the differences were relatively small, it was thought that they were of little physiological significance.

DISCUSSION

In general, the severity of human HHC is classified into 3 grades based on the plasma level of Hcy, mild (15-30 μ M), moderate (30-100 μ M) and severe (more than 100 μ M). In the present study, since the plasma

concentrations of Hcy in the Met and Met+Chol groups were in the range of 20-50 μ M (Fig. 1 and Table 1), our HHC model in rats could be regarded as mild or moderate HHC. Zulli *et al.* (1998b) reported that using older Wistar-Kyoto rats (28 weeks old at the start of treatment) the plasma concentrations of Hcy were about 85 μ M in Met groups and 140 μ M in Met+Chol groups, values which were higher than in our study using Sprague-Dawley rats that were 6 weeks old at the start of treatment (Fig. 1, Table 1). Addition of cholesterol to the Met diet boosted the Hcy concentration in plasma by 20 to 30% compared with the Met diet in the present study, which effect was also milder than that in the report mentioned above. The milder increase of the Hcy concentration in

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Fig. 7. Contents of desmosine (A), isodesmosine (B) and pyridinoline (C) in thoracic aorta in rats fed with Met+Chol, Met, Chol or control diets. Data are shown as mean $\pm$ S.D. ($n = 5$). * $p < 0.05$ between groups analyzed by Tukey's multiple comparison test.

Fig. 8. Average plasma TBARS levels (A) and GSH concentration (B) during the testing period. TBARS values are expressed as equivalent malondialdehyde values. Data are shown as mean $\pm$ S.D. ($n = 5$). * $p < 0.05$ between groups analyzed by Tukey's multiple comparison test.

this study might be caused by the differences of the experimental conditions such as age and strain of rats. Meanwhile, the mechanism of the enhancement of increasing Hcy concentration in plasma remains unclear.

The results in this study suggested that the damage of elastic lamellae by the treatment was not serious enough to affect blood pressure in the resting condition. No differences in either SBP or DBP were observed among all the groups, regardless of the plasma Hcy concentration (Fig. 3). In general, blood pressure is mainly determined by the blood volume and the vascular caliber, and is regu-

lated by the elasticity of the aorta to keep a moderate pulse pressure (i.e., the difference between SBP and DBP). With such mild impairment in the lamellae, blood pressure was well regulated by other factors, such as blood volume and/or vascular caliber. On the other hand, the Δ -SBP induced by sympathetic stimulation with L-epinephrine was significantly larger in the Met or Met+Chol group compared with the non-HHC group (Fig. 4). Thus this enhancement of Δ -SBP might occur because the increase in blood pressure by L-epinephrine overwhelmed the capacity for regulation. Moreover, similar results were obtained from

the experiments using angiotensin II instead of L-epinephrine in the rats at week 2 of the recovery period (Fig. 5). These results exhibit two very interesting features. One is that the enhancement of the Δ -SBP was sustained for at least 2 weeks in the recovery period when plasma Hcy concentration had returned to normal level. This feature suggested that these harmful changes in vaso-regulation continued for more than 2 weeks after the Hcy concentration level normalized, implying the possibility of an irreversible dysfunction of vaso-regulation. The other feature is that the response occurred with vasopressors acting on different receptors. L-epinephrine and angiotensin II increase blood pressure via the constriction of blood vessels by mainly acting on the α 1 adrenergic receptor and angiotensin II receptor, respectively. These facts suggest the possibility that the response is not dependent on receptors of these vasopressors. Meanwhile, since in any HHC model plasma methionine level is higher than in normal animals, the possibility that the findings in this study were due to high exposure of methionine is not completely denied. We already reported that penicillamine induced disruption of elastic lamellae and dysfunction of vaso-regulation in rats (Muto *et al.*, 2013). As one of the mechanisms for the disruption, it was thought the free SH group of penicillamine binds to extracellular matrices (Deshmukh and Nimni, 1969). Hcy, which has similar structure to penicillamine, might bind to collagen via that SH group. However since methionine has sulfur but not free SH residues in its structure, methionine is not thought to be the main factor for the harmful changes in vaso-regulation in the present study.

The elastic lamellae were partially disrupted in this rat model for HHC (Fig. 6), although the severity of disruption was clearly milder than that in the report by Zulli *et al.* (1998a). This difference could be explained by the differences in plasma concentration of Hcy, age and strain of rats. In addition, the cross-linkages of elastin (desmosine and isodesmosine) and collagen (pyridinoline and deoxypyridinoline) were measured as other indicators for damage to the elastin and collagen. In our previous study using rats, it was demonstrated that repeated administration of D-penicillamine (D-PA), which is a structural homologue to Hcy, disrupted elastic lamellae with a decrease of these cross-linkages and caused dysfunction of vaso-regulation (Muto *et al.*, 2013). All of the cross-linkages in the aorta tended to be slightly decreased in the Met or Met+Chol groups compared to the control group, but were not significantly different between the treatments in this study (Fig. 7). These results indicate that such mild damage of elastic lamellae in the present study was too little to affect the content of the cross-linkages. How-

ever, the possibility was not ruled out that Hcy disrupted both elastin and collagen in the same manner as penicillamine, because the contents of cross-linkages tended to be lower in the HHC model in rats (Met and Met+Chol groups) compared with the non-HHC rats (Chol and control groups). If the exposure to Hcy were higher and/or longer, the contents of cross-linkages could be decreased significantly also in this model.

Although in general Hcy induces oxidative stress, there was no significant difference of redox indicators (TBARS and GSH in plasma) among all groups (Fig. 8). Thus, at least in this study the redox environment in plasma was not affected in rats in the HHC model, indicating that oxidative stress was not a factor in the deterioration of the ability of the thoracic aorta to buffer blood pressure.

The present study demonstrated that subchronic HHC enhanced the Δ -SBP after sympathomimetic stimulation, but did not affect the normal blood pressure, indicating that the capacity for vaso-regulation was reduced probably by the enhancement of the elastic lamellae disruption, and not by oxidative stress. Moreover the severity of HHC was potentiated by cholesterol intake, even though in this study it was very mild. However, in patients, considering that exposure to high concentrations of Hcy spans more than several years, the disruption of elastic lamellae and dysfunction of vaso-regulation could occur with high frequency, and accordingly increase the risk for cardiovascular events. To minimize this dysfunction of vaso-regulation, future studies are needed to develop new therapies or drugs for lowering and shortening Hcy exposure.

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Conflict of interest---- The authors declare that there is no conflict of interest.

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