

## Chronic Mild Hyperhomocysteinemia Induces Aortic Endothelial Dysfunction but Does Not Elevate Arterial Pressure in Rats

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### Key Words

Arterial pressure · Endothelial dysfunction · Homocysteine · Hyperhomocysteinemia

### Abstract

Mild hyperhomocysteinemia is prevalent in the general population and has been linked to endothelial dysfunction and high arterial pressure (AP) in clinical studies. The present study was designed to determine whether a rise in AP was induced by mild hyperhomocysteinemia and whether the potential rise in AP is secondary or prior to endothelial dysfunction. Experiments were performed in a rat model of mild hyperhomocysteinemia induced by oral administration of homocysteine for 1–4 months. Aortic endothelial dysfunction was observed 2 months after homocysteine treatment while endothelium-independent vasodilation was normal. In parallel, homocysteine treatment increased phenylephrine-induced contraction in aortas with endothelium, but did not modify the contraction in aortas without endothelium, suggesting a decrease of basal NO production. In conscious unrestrained rats, AP was not significantly different 1, 2, 3 and 4 months after homocysteine treatment. In correlation, endothelial function of a resistance vessel (mesen-

teric artery), mainly non-NO nonprostanoid factor mediated, was preserved, indicating that homocysteine treatment only affected the NO pathway. In conclusion, mild hyperhomocysteinemia alone is not sufficient to elevate arterial blood pressure, at least in the rat model. Aortic endothelial dysfunction produced by mild hyperhomocysteinemia is independent of hemodynamic factors.

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### Introduction

Homocysteine (Hcy) is a sulfur-containing amino acid that is derived from the metabolism of the essential amino acid methionine. Plasma Hcy levels are determined by genetic and nutritional factors. Mutations in the genes for enzymes involved in Hcy metabolism, methionine-rich diets, and deficiencies in vitamins B<sub>6</sub>, B<sub>12</sub>, and folic acid are associated with the elevation of the plasma Hcy levels [1–3]. Hyperhomocysteinemia is generally defined as a plasma Hcy level >12 μM, and roughly divided into mild (<30 μM), intermediate (30–100 μM), and severe (>100 μM) hyperhomocysteinemia [1–3].

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Mild hyperhomocysteinemia has been reported to be prevalent in the general population and to be an independent risk factor for atherosclerosis in the coronary, cerebral, and peripheral circulations [1, 2, 4]. The mechanisms by which mild hyperhomocysteinemia is atherogenic have not been fully elucidated. Endothelial dysfunction seems to play a major role, since it is considered as an early step in the pathogenesis of atherosclerosis, and commonly observed in acute and chronic mild hyperhomocysteinemia, whether induced experimentally or occurring naturally [5–14]. Several clinical studies have also shown an association between plasma Hcy and arterial pressure (AP) or hypertension [15–23]. Moreover, it has been proposed that mild elevations in plasma Hcy levels may contribute to elevations in AP levels, and that part of the cardiovascular effects of Hcy are caused by the change in AP [3, 21–23]. Thus, it appears that high AP per se may be another mechanism underlying the atherogenic effect of mild hyperhomocysteinemia.

Although several groups have published studies on this topic in the past, a direct relation between mild hyperhomocysteinemia, endothelial dysfunction and the increase in AP remains unclear. The current study was designed to evaluate whether or not a long-term exposure to moderately elevated plasma Hcy induced a rise in AP and also to determine whether the potential rise in AP is secondary or prior to endothelial dysfunction. Experiments were performed in a rat model of mild hyperhomocysteinemia induced by oral administration of Hcy. AP was monitored in conscious unrestrained rats for up to 4 months after treatment with Hcy. Aortic and mesenteric reactivity was examined in order to determine the endothelial function in this model.

## Methods

### *Animals*

Male, 12-week-old Wistar rats were purchased from Charles River Laboratories (L'Arbresle, France) and allowed 1 week to acclimatize. They were housed in individual cages with free access to food and water. All surgical and experimental procedures were performed in accordance with the recommendations of the French Accreditation of Laboratory Animal Care.

### *Experimental Protocol*

At the age of 13 weeks, rats were divided into control and Hcy-treated groups. In Hcy-treated rats, *DL*-Hcy (Sigma, St. Quentin Fallavier, France) was chronically given through the drinking water. During the treatment, drinking water was changed three times a week, water intake was recorded three times a week, and body weight was measured once a week. AP, plasma parameters and aortic reactivity were measured 1 and 2 months after treatment. As no change in

hemodynamic parameters was detected after 1 and 2 months of treatment, AP recording and plasma parameters were followed for up to 3 and 4 months in same rats treated with *DL*-Hcy. In addition, mesenteric microvascular reactivity was measured in the prolonged study, in order to know whether the response of resistance vessels is consistent with the demonstration of systemic blood pressure.

### *Measurement of Blood Pressure*

AP was continuously recorded in conscious unrestrained rats [24]. Briefly, the rats were anesthetized with a mixture of ketamine (100 mg/kg, i.p.) and acepromazine (5 mg/kg, i.p.). A polyethylene catheter (PE-10 connected to PE-50) was chronically placed into the lower abdominal aorta via the left femoral artery for measurement of AP and heart rate. The catheter was tunneled subcutaneously and exteriorized through the interscapular skin. After 2 days of recovery, the aortic catheter was connected to a pressure transducer via a rotating swivel that allowed the rat to move freely in the cage. After approximately 3 h of habituation, the AP signal was digitized by a microcomputer for 2 h (12.00–14.00 h). Systolic AP, diastolic AP, and heart rate values from every heart beat were determined on-line. Using off-line analysis, the mean values of these parameters were calculated for a period of 2 h and served as systolic AP, diastolic AP, and heart rate. The standard deviation of the pressure during a 2-hour interval was calculated and defined as the quantitative parameter of blood pressure variability.

### *Determination of Plasma Parameters*

After AP recording, the rat was fasted overnight, and then anesthetized with sodium pentobarbital (30 mg/kg, i.p.). The thoracic cavity was opened. Blood was removed from the inferior vena cava into pre-chilled tubes containing EDTA, cooled in an ice-cold water bath, and immediately centrifuged at 4°C for plasma isolation. For each rat, two aliquots of plasma were stored at –20°C until assayed. One was used to measure plasma total cholesterol, high-density lipoprotein cholesterol, triglycerides, glucose, creatinine, and urea. Another one was used to measure plasma total *L*-Hcy using an enzyme immunoassay kit (Axis® Homocysteine EIA, Orange Medical, Brussels, Belgium) [25]. Total *L*-Hcy is referred to as *L*-Hcy, mixed disulfide and protein-bound forms of *L*-Hcy. The *L*-form of Hcy is the only form present in the blood. In enzyme immunoassays, the calibrators, samples and controls were all measured in duplicate. A four-parameter logistic curve fit was used to set up the calibration curve and to calculate the values of unknown samples and known controls.

### *Vascular Reactivity*

After drawing blood from the inferior vena cava, the descending thoracic aorta and the mesenteric artery (second branch) were immediately removed into cold Krebs solution of the following composition (mM): NaCl 112.0, KCl 5.0, CaCl<sub>2</sub> 2.5, MgSO<sub>4</sub> 1.2, KH<sub>2</sub>PO<sub>4</sub> 1, NaHCO<sub>3</sub> 29.7, glucose 11.5 and EDTA 0.016, and aerated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. For the mesenteric artery, 10 mM HEPES were used instead of NaHCO<sub>3</sub>, and the solution was bubbled with 25% O<sub>2</sub> and 5% CO<sub>2</sub>. Adhering fat and connective tissue of the vessels was removed.

*Aortic Reactivity.* Aorta was cut into 2- to 3-mm-wide rings. In some rings the endothelium was removed mechanically. Changes in isometric tension were recorded using an IT1-25 transducer and an IOX computerized system (EMKA Technologies, Paris, France) in conventional 20-ml organ bath chambers. Each aortic ring was allowed to equilibrate for 90 min at a resting tension of 2.5 g, and

**Table 1.** Plasma parameters after 1–4 months of treatment with Hcy

	1 month		2 months		3 months		4 months	
	control (n = 4)	Hcy (n = 4)	control (n = 5)	Hcy (n = 7)	control (n = 4)	Hcy (n = 4)	control (n = 5)	Hcy (n = 5)
Total cholesterol, mM	1.39 ± 0.15	1.40 ± 0.10	1.56 ± 0.28	1.69 ± 0.08	1.51 ± 0.17	1.30 ± 0.07	1.79 ± 0.34	1.64 ± 0.13
HDL cholesterol, mM	0.81 ± 0.08	0.80 ± 0.04	0.81 ± 0.16	0.83 ± 0.08	0.81 ± 0.06	0.73 ± 0.04	0.88 ± 0.10	0.93 ± 0.09
Triglycerides, mM	1.06 ± 0.19	1.48 ± 0.14	1.09 ± 0.16	1.08 ± 0.15	1.30 ± 0.13	1.20 ± 0.23	1.06 ± 0.10	1.05 ± 0.15
Glucose, mM	8.65 ± 0.63	11.14 ± 0.65*	8.47 ± 0.78	9.20 ± 0.50	8.61 ± 0.41	9.90 ± 0.20	8.40 ± 0.36	8.85 ± 0.40
Creatinine, μM	53.8 ± 3.5	57.5 ± 2.1	56.0 ± 4.0	59.1 ± 1.5	57.4 ± 2.3	56.8 ± 4.0	50.0 ± 3.4	56.4 ± 2.9
Urea, mM	7.50 ± 0.70	8.72 ± 0.43	7.18 ± 0.77	7.69 ± 0.36	7.40 ± 0.75	7.50 ± 0.50	7.76 ± 0.26	8.00 ± 0.76

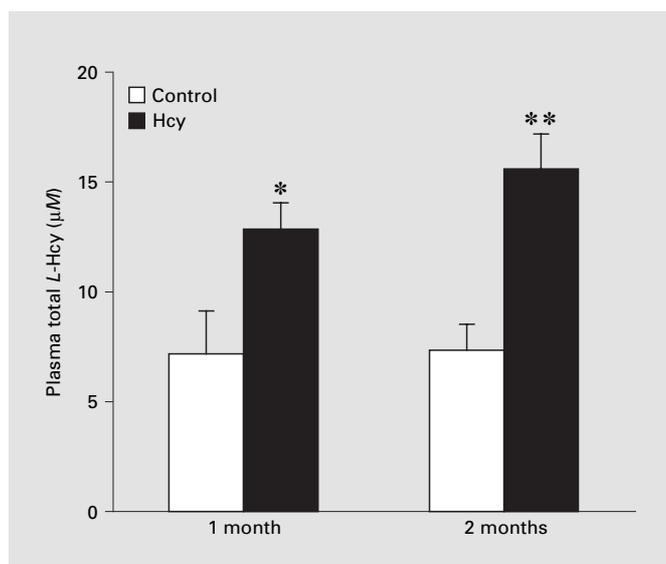
HDL = High-density lipoprotein. \*  $p < 0.05$  vs. control.

then exposed to high potassium (80 mM). When the high-potassium-induced contraction reached a plateau, the rings were rinsed with Krebs solution, and allowed to re-equilibrate for 30 min. Thereafter, some rings with endothelium were contracted with a submaximal concentration of phenylephrine ( $10^{-6}$  M) and relaxed with cumulative concentrations of the endothelium-dependent vasodilator, acetylcholine ( $10^{-8}$  to  $10^{-5}$  M). The other rings with or without endothelium were contracted with cumulative concentrations of phenylephrine ( $10^{-9}$  to  $10^{-5}$  M) to estimate basal production of NO. These rings were then rinsed with Krebs solution, allowed to re-equilibrate for 30 min, contracted with a submaximal concentration of phenylephrine ( $10^{-6}$  M in rings with endothelium and  $10^{-7}$  M in rings without endothelium), and relaxed with cumulative concentrations of the endothelium-independent vasodilator, sodium nitroprusside ( $10^{-10}$  to  $10^{-5}$  M).

**Mesenteric Reactivity.** A mesenteric segment of 1.5–3 mm was cannulated at both ends in a video-monitored perfusion system (LSI). Pressure was controlled by a servo-perfusion system, and flow (10 μl/min) was generated by a peristaltic pump. Changes in diameter studied at 75 mm Hg perfusion pressure were monitored continuously. After stabilization, flow was stopped and the arterial segment was exposed to 40 mM KCl for 10 min before being washed. Thereafter, phenylephrine ( $8 \times 10^{-8}$  M) plus 5-hydroxytryptamine ( $8 \times 10^{-8}$  M) was added and the effects of cumulative concentrations of extraluminally ACh ( $10^{-9}$  to  $10^{-6}$  M) were evaluated. In some experiments performed only in control rats, a pre-incubation with N<sup>ω</sup>-nitro-L-arginine (LNA,  $10^{-4}$  M, NOS inhibitor) alone or LNA ( $10^{-4}$  M) combined with indomethacin ( $10^{-5}$  M, a cyclooxygenase inhibitor), and Ca<sup>2+</sup>-activated K<sup>+</sup> channel inhibitors on endothelial cells [charybdotoxin ( $10^{-7}$  M, intermediate conductance channel inhibitor) and apamin ( $10^{-6}$  M, small conductance channel inhibitor)] was performed. In the present study, contraction was expressed as an increased tension (g) or reduced diameter (μm), and relaxation was expressed as a percentage (%) of the maximal contraction.

#### Data Analysis

All data are reported as means ± SEM. Statistical analysis was performed with two-tailed Student's unpaired t test, except for the vascular reactivity study, for which two-way ANOVA for repeated measures was used to compare two curves. The threshold for statistical significance was  $p < 0.05$ .

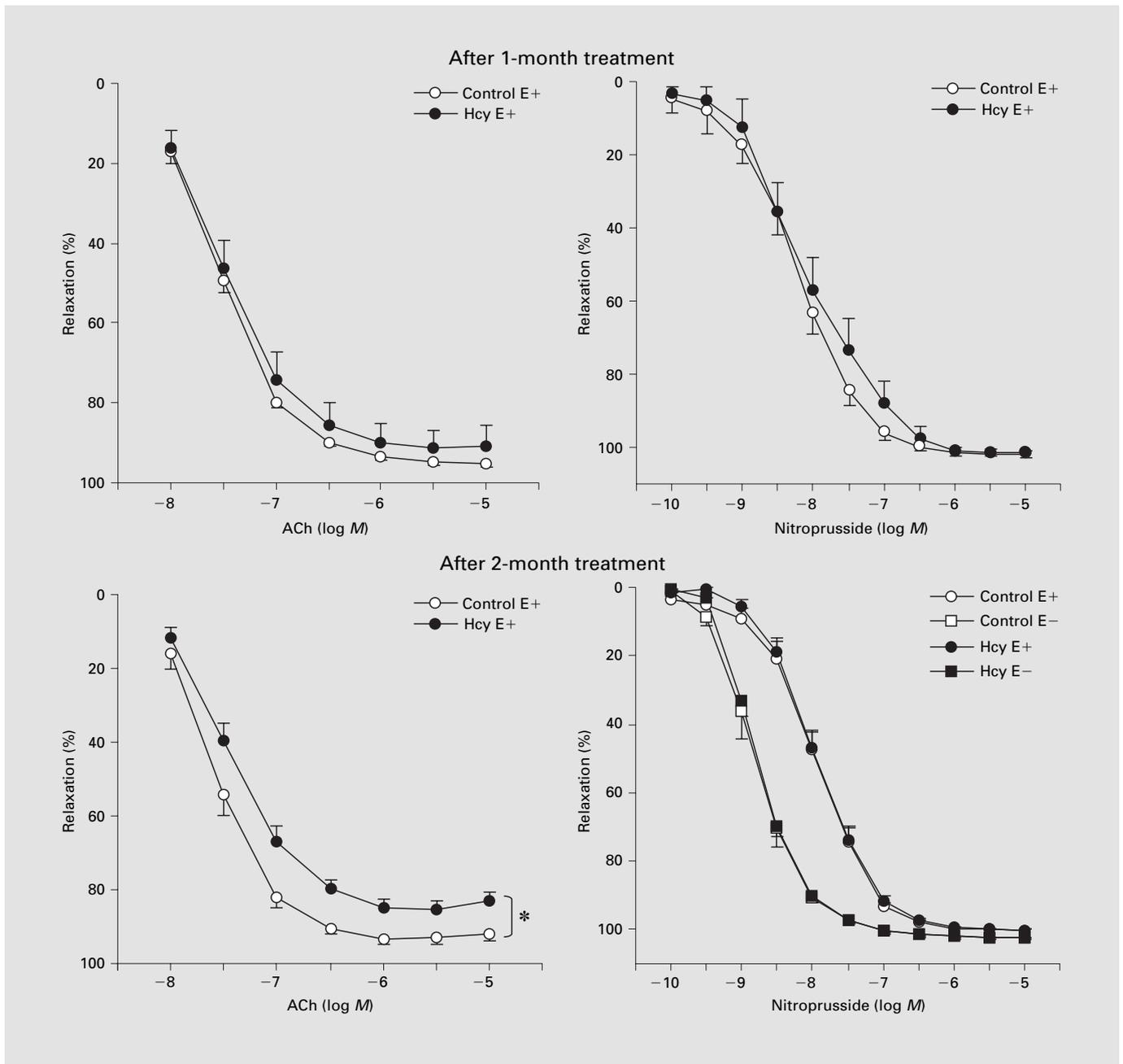


**Fig. 1.** Plasma total L-Hcy levels after 1 and 2 months of treatment with Hcy. n = 4–7. \*  $p < 0.05$ , \*\*  $p < 0.01$ , vs. control.

## Results

### Data from 1- and 2-Month Hcy-Treated Rats

**DL-Hcy Dose and Body Weight.** The average dose of DL-Hcy during the treatment was 94 mg/kg/day, which was calculated according to the DL-Hcy concentration in drinking water, water intake and body weight. There was no significant difference in body weight between control and Hcy-treated rats before and during the treatment (before treatment:  $352 \pm 3$  vs.  $354 \pm 2$  g,  $p > 0.05$ ; 1 month after the treatment:  $443 \pm 4$  vs.  $446 \pm 3$  g,  $p > 0.05$ ; 2 months after treatment:  $486 \pm 9$  vs.  $486 \pm 4$  g,  $p > 0.05$ ).



**Fig. 2.** Aortic relaxation induced by ACh and sodium nitroprusside after 1 and 2 months of Hcy treatment. E+ = With endothelium; E- = without endothelium. n = 4-7. \* p < 0.05.

*Plasma Total L-Hcy.* Plasma total L-Hcy levels were significantly increased after oral administration of DL-Hcy. Compared with the corresponding controls, they were 1.79-fold higher in 1-month Hcy-treated rats and 2.12-fold higher in 2-month Hcy-treated rats (fig. 1). Other plasma parameters such as total cholesterol, high-densi-

ty lipoprotein cholesterol, triglycerides, creatinine, and urea were not significantly different between control and Hcy-treated rats 1 and 2 months after treatment (table 1). Plasma glucose was slightly higher in Hcy-treated rats compared to control rats 1 month after treatment. However, this parameter disclosed no significant difference

between control and Hcy-treated rats 2 months after treatment, suggesting that the elevation of plasma glucose is a transient rather than a persistent change during the chronic treatment with Hcy.

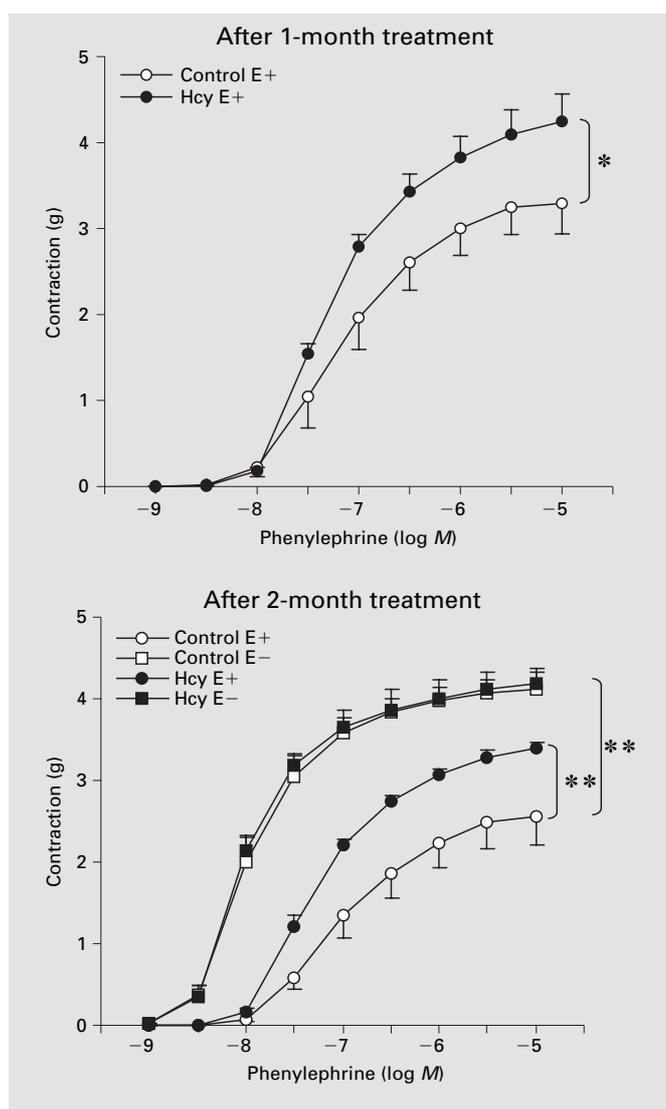
**Blood Pressure and Its Variability.** Systolic AP, diastolic AP and their respective variability remained unchanged after 1 and 2 months of treatment with Hcy (table 2). There was no difference in heart rate between control and Hcy-treated groups. Other hemodynamic parameters such as pulse pressure, mean AP, and their respective variability did also not differ between Hcy-treated rats and control rats (data not shown).

**Aortic Reactivity.** The contractions induced by high potassium (80 mM) were not significantly different between control and Hcy-treated groups 1 and 2 months after treatment in aortas with and without endothelium (after 1 month of treatment/E+:  $2.96 \pm 0.25$  vs.  $3.12 \pm 0.27$  g,  $p > 0.05$ ; after 2 months of treatment/E+:  $2.93 \pm 0.17$  vs.  $3.17 \pm 0.12$  g,  $p > 0.05$ ; after 2 months of treatment/E-:  $3.74 \pm 0.25$  vs.  $3.64 \pm 0.13$  g,  $p > 0.05$ ). There was no significant difference in endothelium-dependent relaxations induced by cumulative concentrations of acetylcholine between the 1-month Hcy-treated group compared with the corresponding controls, whereas they were significantly decreased in the 2-month Hcy-treated group compared with the corresponding controls (fig. 2). Endothelium-independent relaxations induced by cumulative concentrations of sodium nitroprusside were not significantly different between control and Hcy-treated groups in aortas with and without endothelium (fig. 2). Increased phenylephrine-induced contractions were obtained in rings with endothelium treated by Hcy. In contrast, in rings without endothelium, Hcy did not modify the contractility (fig. 3).

#### Data from the Prolonged Observations

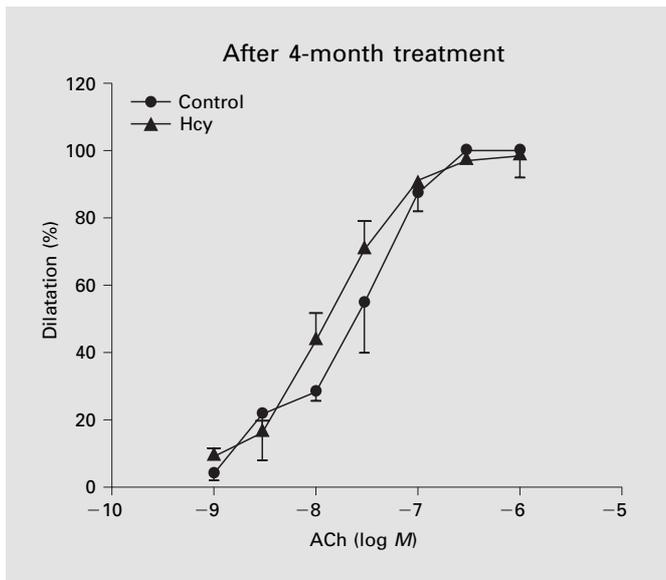
**Plasma Parameters and Hemodynamic Data.** The effects of Hcy on plasma parameters and blood pressure were also studied 3 and 4 months after treatment with Hcy. Total plasma L-Hcy levels were persistently higher (2.08-fold increase) in Hcy-treated rats compared to control rats, although other plasma parameters remained unchanged (table 1). All measured hemodynamic parameters, including AP and its variability, were not significantly different between Hcy-treated and control rats 3 and 4 months after treatment (table 2).

**Mesenteric Reactivity.** After 4 months of treatment, diameters of the mesenteric artery at baseline ( $231.67 \pm 4.10$  vs.  $234.75 \pm 5.78$ ) and at 75 mm Hg perfusion pressure ( $360.0 \pm 8.2$  vs.  $343.8 \pm 8.9$   $\mu$ m) were not signifi-

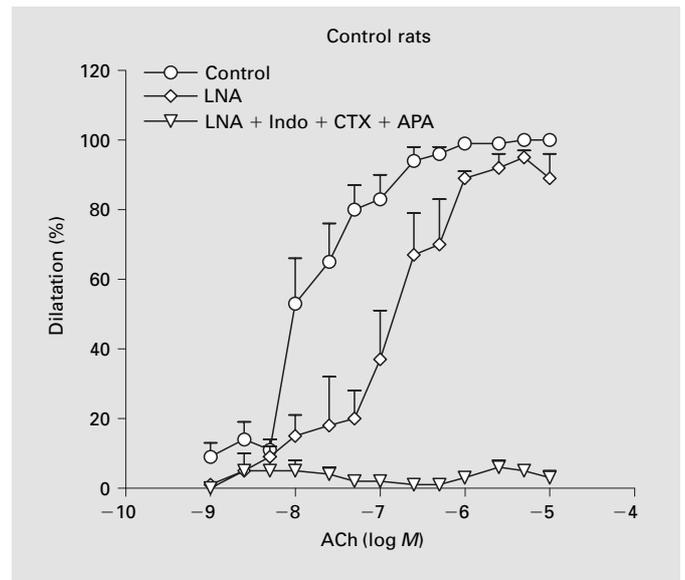


**Fig. 3.** Aortic contraction induced by phenylephrine after 1 and 2 months of Hcy treatment. E+ = With endothelium; E- = without endothelium.  $n = 4-7$ . \*  $p < 0.05$ , \*\*  $p < 0.01$ .

cantly different between both groups (control vs. Hcy). Similarly, the amplitudes of contraction induced by 40 mM potassium ( $176.5 \pm 6.25$  vs.  $189.2 \pm 7.27$   $\mu$ m) or by the agonists ( $48.2 \pm 2.0$  vs.  $52.6 \pm 2.7$  % of KCl-induced contraction) were comparable in control and Hcy-treated groups. Dilatations induced by cumulative concentrations of acetylcholine were not significantly changed after 4 months of Hcy treatment (fig. 4) while they were totally suppressed by the combination including LNA, charybdotoxin and apamin and partially inhibited by LNA alone (fig. 5).



**Fig. 4.** Mesenteric dilatation induced by ACh after 4 months of treatment with Hcy. n = 5.



**Fig. 5.** Mesenteric dilatation induced by ACh in control rats in the presence of  $10^{-4}$  M LNA or a combination of  $10^{-4}$  M LNA,  $10^{-5}$  M indomethacin (Indo),  $10^{-7}$  M charybdotoxin (CTX) and  $10^{-6}$  M apamin (APA). n = 5.

**Table 2.** AP and its variability (APV) after 1, 2 and 4 months of treatment with Hcy

	1 month		2 months		4 months	
	control (n = 4)	Hcy (n = 4)	control (n = 5)	Hcy (n = 5)	control (n = 5)	Hcy (n = 5)
Systolic AP, mm Hg	143 ± 6	142 ± 7	144 ± 5	145 ± 7	146 ± 5	144 ± 7
Systolic APV, mm Hg	6.00 ± 0.36	6.18 ± 0.40	6.19 ± 0.36	6.95 ± 0.30	8.20 ± 0.24	8.16 ± 0.34
Diastolic AP, mm Hg	108 ± 5	107 ± 6	110 ± 4	110 ± 6	111 ± 3	107 ± 3
Diastolic APV, mm Hg	5.58 ± 0.28	6.02 ± 0.35	5.74 ± 0.27	6.20 ± 0.41	6.56 ± 0.35	6.56 ± 0.17
Heart rate, beats/min	364 ± 13	361 ± 12	366 ± 14	363 ± 14	334 ± 7	306 ± 11

## Discussion

Chronic hyperhomocysteinemia can be produced by several pathways affecting Hcy metabolism: (1) chronic treatment with high methionine and/or low folate, vitamins B<sub>12</sub> and B<sub>6</sub> [5, 6, 26], in which methionine is a precursor to form Hcy; folate and vitamin B<sub>12</sub> are involved in the remethylation of Hcy to methionine, and vitamin B<sub>6</sub> is a necessary cofactor in the degradation of Hcy to cysteine; (2) deletion of genes coding for certain enzymes in mice, such as cystathionine β-synthase (for the degradation of Hcy to cystathionine) [7], methylenetetrahydrofolate reductase (for the production of active folate, i.e. 5-methyltetrahydrofolate) [27], and methionine synthase

(for the remethylation of Hcy to methionine) [28], and (3) chronic treatment with Hcy to directly increase plasma Hcy levels [29]. In the present study, this latter method was used to induce mild hyperhomocysteinemia in rats; the plasma Hcy levels were 1.8- to 2.1-fold higher than in controls. We did not use the first methods, because methionine and folate may have direct effects on endothelial function, and methionine may affect overall protein synthesis [2, 29].

Acetylcholine is an endothelium-dependent vasodilator commonly used to evaluate aortic endothelial function. In the rat aorta, inhibitors of NOS abolished the relaxation to acetylcholine indicating that it is mediated by NO [30]. The present results show that 1 month after

treatment with Hcy, aortic relaxation to acetylcholine tended to decrease, although nonsignificantly, compared to normal controls. Our results are consistent with previous observations in a rat model of mild hyperhomocysteinemia produced by methionine [26]. However, after 2 months of treatment with Hcy, the aortic relaxation to acetylcholine was significantly diminished. This impaired response is due to endothelial dysfunction, since the ability to relax of the vascular smooth muscle cells was normal, as evidenced by the normal endothelium-independent relaxation to sodium nitroprusside. In rings with endothelium, the increased contractions to phenylephrine after 1 or 2 months of Hcy treatment were similar, but lower than the augmentation following the removal of the endothelium. These results suggest that Hcy causes an early decrease in basal NO production, in agreement with other models of early atherosclerosis, e.g. apolipoprotein-E-deficient mice [31].

Because the resistance vessels determine systemic blood pressure, the reactivity of the mesenteric artery was also evaluated. Four months of Hcy treatment did not affect the reactivity to agonists or endothelial function. Indeed, in correlation with the absence of hypertensive effect, vasodilatation to acetylcholine, mainly non-NO nonprostanoid factor mediated [32], was preserved, indicating that Hcy first affected the NO pathway.

On the contrary, in a murine model of hyperhomocysteinemia endothelial dysfunction was reported in microvessels measuring 20–30  $\mu\text{m}$  in diameter due to cystathionine  $\beta$ -synthase deficiency [7, 33] or in human small coronary arteries after acute methionine treatment [34, 35], which could affect by itself vascular reactivity. These discrepancies could be explained by differences in species or in vessel diameter which determined the relative contribution of NO in endothelium-dependent vasodilatation to agonists [36]. A recent study [37] also showed that chronic hyperhomocysteinemia decreased vasodilatation mediated by endothelium-derived hyperpolarizing factor (EDHF) in the renal vasculature. However, hyperhomocysteinemia was obtained with a diet enriched in methionine, which, as already said, induced confounding issues. Indeed, it was suggested that rather than homocysteine itself, another component of the methionine-homocysteine cycle may be responsible for the endothelial dysfunction.

The present results also suggest that aortic endothelial dysfunction induced by mild hyperhomocysteinemia depends on its duration. The endothelial dysfunction caused by homocysteine under acute conditions is also time dependent; since low concentrations of Hcy (10–50  $\mu\text{M}$ )

induced vascular endothelial dysfunction only after prolonged incubation (for 24 h) [8]. Clinical observations reported an association between mild hyperhomocysteinemia and endothelial dysfunction only in middle-aged and elderly subjects [38]. However, endothelium-dependent dilatations are impaired in young children with severe hyperhomocysteinemia and homocystinuria [2], indicating that the concentration of Hcy is another major determinant for endothelial dysfunction. In acute studies, rat aortas incubated with high concentrations of Hcy (1,000  $\mu\text{M}$ ) for 3 h exhibited a marked reduction in endothelium-dependent relaxation [39]. Coexistence of hyperhomocysteinemia with other environmental stimuli may have a synergetic effect on endothelial function. Thus, although a mild elevation (1.4-fold) of plasma Hcy for 4 weeks had no significant effect, by itself, on endothelial function, its coexistence with high salt intake significantly diminished it [26]. In vitro, incubation with Hcy 100  $\mu\text{M}$  for 30 min reduced acetylcholine-induced relaxation only in the presence of  $\text{Cu}^{2+}$  [39].

Two major pathways have been proposed to explain hyperhomocysteinemia-induced endothelial dysfunction [2]. One is the formation of reactive oxygen species due to autooxidation, activation of oxidant systems, and inhibition of antioxidant systems producing cytotoxic action and reducing the bioavailability of NO. Another is the generation of asymmetric dimethylarginine, an endogenous inhibitor of eNOS which is formed when *L*-arginine is methylated, thus decreasing NO production. It is unknown whether both mechanisms are involved in the present model of mild hyperhomocysteinemia. The present results only suggest that, in this model, the endothelial dysfunction is not due to a high arterial blood pressure, an unfavorable lipid profile, or an abnormal glucose level, factors which may play a role in endothelial dysfunction in other models of hypertension, atherosclerosis and diabetes [40], since these parameters remained unchanged in the rat model of mild hyperhomocysteinemia.

In the current study, the main objective was to provide direct evidence as to whether or not arterial blood pressure is indeed elevated by mild hyperhomocysteinemia. Intra-arterial pressure was obtained by continuous recording of beat-to-beat AP in conscious unrestrained rats, which is more accurate than previously used methods in hyperhomocysteinemic rats, such as indirect AP measurement in restrained rats [26] and direct intra-arterial pressure measurements in anesthetized rats [6, 29]. Indeed, restraining the animals or anesthetic drugs affect AP and other cardiovascular parameters [41]. AP variability was also measured, since it is a risk factor involved in cardio-

vascular damage [24]. The hemodynamic measurements were obtained monthly up to 4 months after initiating the treatment with Hcy. The results revealed no differences in the hemodynamic parameters between control and Hcy-treated groups. Other investigators reported unchanged systolic AP in mild hyperhomocysteinemic rats obtained with methionine, although the exposure to mild hyperhomocysteinemia was short (4 weeks), and the AP was measured in restrained or anesthetized rats [6, 26]. Acute intravenous injection or infusion of high doses of Hcy to induce higher plasma Hcy levels (65–1,000  $\mu\text{M}$ ) did not alter mean AP in the rat [42, 43]. Therefore, mild hyperhomocysteinemia per se has no effect on AP and its variability, at least in this species. The association between mild hyperhomocysteinemia and higher AP observed in human studies [3, 15–23] remains to be explained, perhaps by the combined effects of Hcy and other environmental stimuli.

The limitation of the present study is the relatively small sample. Nevertheless, the variability between the two groups was low and the conclusion was statistically validated. Moreover, our recent study performed in Sprague-Dawley rats also showed unchanged AP in Hcy-

treated compared to control rats 4 months after treatment (systolic AP:  $141 \pm 5$  vs.  $142 \pm 6$  mm Hg,  $n = 8$  in each group,  $p > 0.05$ ) [Chao-Yu Miao et al., unpubl. data].

The present study did not exclude the possibility that severe hyperhomocysteinemia with prolonged exposure may induce hypertension. In fact, a marked rise in mean AP (31 mm Hg) was found in Wistar rats with higher levels of plasma Hcy (9-fold) for 12–15 weeks, in which AP was measured under anesthesia 10 min after catheterization [29]. In that study, older rats were used, with the Hcy treatment starting at about 35 weeks of age and AP recorded at about 50 weeks. It is unknown whether or not in older rats severe hyperhomocysteinemia is easier to produce and results in higher AP.

In summary, the present chronic study demonstrated the occurrence of aortic endothelial dysfunction without elevation in AP and its variability after up to 4 months of exposure to mild hyperhomocysteinemia. These results indicate that endothelial dysfunction produced by mild hyperhomocysteinemia is independent of hemodynamic factors, and that mild hyperhomocysteinemia alone is not sufficient to elevate AP.

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