Effect of a methionine-supplemented diet on the blood pressure of Wistar–Kyoto and spontaneously hypertensive rats

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The objectives of the present work were to evaluate the effect of a methionine-supplemented diet as a model of hyperhomocysteinaemia on the systolic blood pressure (BP) and vasomotor functions of aortic rings in Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR). WKY and SHR rats, randomised into four groups, were fed a normal semisynthetic diet or a methionine (8 g/kg)-supplemented diet for 10 weeks. Systolic BP was measured non-invasively. At the end of the experiment, plasma homocysteine, methionine, cysteine and glutathione levels were determined. Vasoconstriction and vasodilatation of aortic rings were measured. The methionine-supplemented diet induced a significant increase in plasma homocysteine and methionine concentration in both WKY and SHR rats, an increase in plasma cysteine concentrations in WKY rats and an increase in the glutathione concentration in SHR. The systolic BP of WKY rats fed the methionine-supplemented diet increased significantly ($P < 0.01$), whereas systolic BP was reduced in SHR. An enhanced aortic responsiveness to noradrenaline and a decreased relaxation induced by acetylcholine and bradykinin were observed in the WKY rats fed the methionine-enriched diet. In SHR, the bradykinin-induced relaxation was reduced, but the sodium nitroprusside response was increased. In conclusion, a methionine-enriched diet induced a moderate hyperhomocysteinaemia and an elevated systolic BP in WKY rats that was consistent with the observed endothelial dysfunction. In SHR, discrepancies between the decreased systolic BP and the vascular alterations suggest more complex interactions of the methionine-enriched diet on the systolic BP. Further investigations are needed to understand the paradoxical effect of a methionine-rich diet on systolic BP.

Hypertension: Homocysteine: Aortic rings

Increased plasma total homocysteine is an established independent risk factor for the development of atherosclerotic vascular disease. Epidemiological studies have shown that even mild hyperhomocysteinaemia (HHcy) is associated with coronary artery disease (Kawashiri *et al.* 1999; Abdelmouttaleb *et al.* 2000), myocardial infarction (Al-Obaidi *et al.* 2000; Christensen *et al.* 2000; Senaratne *et al.* 2000), peripheral arterial disease (Taylor *et al.* 1999; Mansoor *et al.* 2000), cerebrovascular disease (Zhang *et al.* 1998; Moller *et al.* 2000), stroke (Van Beynum *et al.* 1999; Hankey & Eikelboom, 2001), cardiac allograft vasculopathy (Cooke *et al.* 2000; Parisi *et al.* 2000) and death from coronary artery disease. Plasma homocysteine (Hcy)

concentration is a function of the complex interactions between genetic and environmental factors, such as vitamin status, hormonal factors, lifestyle and diet (Nygard *et al.* 1998). Enzyme abnormalities (cystathionine β-synthase and methyltetrahydrofolate reductase mutations) and vitamin deficiencies (folic acid, vitamin B_{12} and vitamin B_6) involved in the metabolism of Hcy may lead to increased plasma Hcy concentrations.

The mechanisms linking HHcy to the development and progression of vascular disease have not been fully explained (Nygard *et al.* 1997; Van Guldener & Stehouwer, 2000). One mechanism appears to be endothelial injury and dysfunction, which may be mediated by oxidant stress with

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a depletion of biologically active NO (Kanani *et al.* 1999; Zhang *et al.* 2000). An elevated concentration of asymmetrical dimethylarginine, which is an endogenous inhibitor of NO synthase, has also been proposed (Boger *et al.* 2001). Elevated Hcy has been associated with alterations in endothelial morphology (Harker *et al.* 1976; Matthias *et al.* 1996) and endothelium-dependent vasodilatation in animals and human subjects (Ungvari *et al.* 1999; Hanratty *et al.* 2001). Furthermore, HHcy promotes the growth of vascular smooth muscle cells and the production of collagen (Majors *et al.* 1997). Both experimental and clinical studies suggest that the alterations of vascular function and histology depend on the effect of HHcy on endothelial function, smooth muscle cell function, vascular wall structure and properties, and the localisation in the vascular tree (Van Guldener & Stehouwer, 2000). Stimulation of vascular smooth muscle cell proliferation and impairment of vasoregulatory mechanisms would lead to an increase in peripheral resistance and thus to hypertension. However, there is little evidence linking HHcy to hypertension and what there is, is controversial. Several studies have reported that HHcy may be related to hypertension in human subjects (Malinow *et al.* 1995; Sutton-Tyrrell *et al.* 1997; Fiorina *et al.* 1998) or animals (Rolland *et al.* 1995; Matthias *et al.* 1996). On the other hand, it has also been reported that subjects with a common mutation in the methylenetetrahydrofolate reductase gene, which may account for elevated plasma Hcy concentration, had a significantly lower blood pressure (BP) than subjects with other genotypes in the overall population (Nakata *et al.* 1998).

In the present study, the effect of a methionine (Met)-enriched diet (Durand *et al.* 1997; Ungvari *et al.* 1999), which induces mild HHcy, was evaluated during the development of genetic hypertension in spontaneously hypertensive rats (SHR); the contractile function of aortic rings was also studied. In addition, biochemical studies were carried out to determine, not only the plasma level of Hcy, but also that of its metabolic derivatives, Met and cysteine (Cys), and of glutathione, which may be influenced by HHcy and interfere with the oxidative toxic effect of Hcy.

Methods

Chemicals and reagents

L-Noradrenaline hydrochloride, acetylcholine chloride, sodium nitroprusside, bradykinin, calcium chloride, DL-Met, DL-homocysteine, tri-*n*-butylphosphine and 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide were purchased from Sigma (La Verpillère, France). Other reagents were of analytical grade.

Animals

SHR and Wistar–Kyoto (WKY) male rats weighing 80–100 g were purchased from IFFA CREDO, L'Arbesle, France. The rats were housed in polyethylene cages in an environment with a controlled temperature of $22 \pm 2°C$, constant humidity (50–60 %) and a 12 h light–dark cycle. They had free access to food and distilled water.

Experimental procedures

Animals were randomised into four groups (*n* 12 per group). WKY and SHR control groups were fed for 10 weeks on a normal semisynthetic diet, which comprised (/kg diet): casein 200 g, cellulose 60 g, maize starch 400 g, granulated sugar 210 g, maize oil 25 ml, peanut oil 25 ml, vitamin mix 10 g, mineral mix 70 g. The diet contained (/kg): Met 6·50 g, vitamin B_6 10 mg, folic acid 0·05 mg, vitamin B_{12} 0·05 mg. Experimental groups WKY-Met and SHR-Met were fed for the same period with the semisynthetic diet that was supplemented with an additional 8 g DL-Met/kg, giving a total of 14·50 g Met/kg diet (Table 1).

Systolic BP was measured in unanaesthetised restrained and previously warmed rats (5 min in a thermostatic box at 37°C) by the indirect tail-cuff method using a sphygmomanometer (PE-3000; Narco-Biosystems, Houston, TX, USA). The cuff was inflated rapidly at an inflation–deflation rate of 15 mmHg/s and systolic BP was monitored on a physiographical recorder (MK-III; Narco-Biosystems). After discarding the lowest and highest values, the average of six clear readings was calculated for each rat. Animals were acclimated to this method of BP measurement for 2 weeks before the onset of the experiment, and the systolic BP was measured 0, 1, 3, 5, 7 and 10 weeks after the beginning of the experiment.

On the day 42 of the experimental period, each rat was housed alone in a polyethylene cage and for 2 d the quantity of food and drink consumed and urine produced was measured. Urine (5 ml) from each rat was stored at $-20°C$ in polyethylene tubes until analysis.

At the end of the experiment, the rats were anaesthetised with sodium pentobarbital (60 mg/kg body weight,

Table 1. Composition of the vitamin and mineral mix

Vitamin mix composition (mg/kg)	
Retinol	600
Cholecalciferol	6·25
Thiamin	2000
Riboflavin	1500
Niacin	7000
Pyridoxine	1000
Biotin	15000
Cyanocobalamin	5
Ascorbic acid	80000
α-Tocopherol	17000
Menadione	40
Nicotinamide	100
Choline	1360
Folic acid	5
Paraaminobenzoic acid	50
Mineral mix composition	
Calcium (g/kg)	100
Potassium (g/kg)	60
Sodium (g/kg)	40
Magnesium (g/kg)	10
Iron (g/kg)	3
Phosphorus (g/kg)	77·5
Manganese (g/kg)	0·8
Copper (g/kg)	0·125
Cobalt (mg/kg)	0·9
Zinc (g/kg)	0·45
Iodine (mg/kg)	4·9

intraperitoneally). Abdominal aorta blood was collected and placed in EDTA-vacutainer and sodium heparinate-vacutainer tubes, immediately cooled on ice and centrifuged at 4000 rpm for 10 min at 4°C. Portions of plasma were stored at -20°C until analysis. The thoracic aorta was dissected for the study of vascular reactivity.

Ex vivo isolated aorta preparation

The aorta was rapidly placed in a physiological salt solution (37°C) composed of: 118 mM-NaCl, 1·2 mM-KH$_2$PO$_4$, 2·5 mM-CaCl$_2$, 4·7 mM-KCl, 1·2 mM-MgSO$_4$, 25 mM-NaHCO$_3$, 12 mM-glucose (pH 7·4).

The aorta was cleaned of surrounding connective and fat tissue and cut into rings about 1–2 mm wide, which were suspended between two stainless-steel hooks immersed in 10 ml physiological salt solution kept at 37°C and constantly bubbled with O$_2$–CO$_2$ (95:5, v/v). During the equilibration period (90 min), the bathing solution was changed every 15 min and the resting tension was frequently adjusted to 2 g (Laurant et al. 1995).

The presence of undamaged endothelial cells was confirmed by 10^{-6} M-acetylcholine-induced relaxation in preparations precontracted by 10^{-7} M-noradrenaline, and the rings presenting < 50 % relaxation were discarded. The efficacy of endothelium denudation, which was performed by gently rubbing the aortic rings, was ascertained by loss of 10^{-6} M-acetylcholine-induced relaxation in preparations precontracted by 10^{-7} M-noradrenaline. The reference contraction used for calculations was determined with a 100 mM-KCl physiological salt solution (solution described earlier with 100 mM-KCl and 18 mM-NaCl).

In the first series of experiments, which were performed on aortic rings with endothelium, a cumulative concentration–response curve to noradrenaline (10^{-10}–10^{-5} M) was obtained. Then, with rings precontracted with a concentration of noradrenaline inducing 70 % of maximal contraction, a cumulative concentration–response curve to acetylcholine (10^{-9}–10^{-4} M) or bradykinin (10^{-10}–10^{-5} M) was recorded.

In experiments that were performed with rings without endothelium, a cumulative concentration–response curve to sodium nitroprusside (10^{-11}–10^{-6} M) and a cumulative concentration–response curve to CaCl$_2$ (10^{-6}–10^{-2} M) after a re-equilibration in Ca-free K depolarising Krebs solution (100 mM-KCl, 18 mM-NaCl and without added CaCl$_2$) were obtained.

Biochemical analysis

Determination of plasma methionine, homocysteine, cysteine and glutathione. Total Hcy, Cys and glutathione concentrations in plasma were measured by HPLC with fluorometric determination after derivation of thiols with 7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide according to Durand et al. (1996). Total Met concentration was measured electrochemically. Briefly, 200 µl plasma and 20 µl 2·5 mM-N-acetylcysteine (used as internal standard for the determination of Hcy, Cys and glutathione) were treated with 20 µl tri-n-butylphosphine (100 ml/l dimethyl-formamide) for 30 min at 4°C in order to release thiols

from plasma proteins and reduce them. Proteins were precipitated with 200 µl cold 0·6 M-perchloric acid. After 10 min centrifugation at 4000 g, the supernatant fraction was strained with a 0·2 µM polytetrafluoroethylene filter (Interchim, Montluçon, France) and immediately measured.

Fluorescence detection of homocysteine, cysteine and glutathione. 20 µl 1·55 M-NaOH, 250 µl 0·125 M-borate buffer (pH 8) and 30 µl 4·6 mM-7-fluoro-2,1,3-benzoxadiazole-4-sulfonamide solution (in borate buffer) were added to 100 µl filtered supernatant fraction. After derivation at 50°C for 20 min, the samples were rapidly cooled and 40 µl 1 M-HCl was added.

Samples (20 µl) were analysed by HPLC (ESA 580 Kontron instruments 400; Kontron; Strasbourg, France) equipped with a fluorescence detector (Bio-Tek, SFM 25; Kontron) set at λ_{ex} 385 nm and λ_{em} 515 nm. The eluent phase consisted of 0·1 M-KH$_2$PO$_4$ buffer (pH 3·25) containing acetonitrile (100 ml/l), and the rate was fixed at 1·2 ml/min. Separation was carried out on a 250 × 4·6 mm, 5 µm diameter Nucleosil C$_{18}$ analytical column (Interchim Montluçon France) maintained at 35°C. Hcy, Cys and glutathione concentrations were determined by the area quotient of N-acetylcysteine and Hcy, Cys and glutathione peaks respectively, after standard calibration of thiols.

Electrochemical detection of methionine. Samples were analysed by HPLC (ESA 580, Kontron instruments 400; Kontron) associated with a Coulochem II detector (ESA, model 5200) equipped with a dual analytical cell (model 5010) and a guard cell (model 5020). For optimum detection of Met, the electrode potentials for the guard cell, electrode 1 and electrode 2 were set at +1200, +450 and +1000 mV respectively. The mobile phase was 0·02 M-NaH$_2$PO$_4$ buffer (pH 2·5) and the rate was fixed at 1·8 ml/min. Separation was carried out on a 150·0 × 4·6 mm, 5 µm diameter C$_{18}$ column (Sigma). Filtered supernatant fraction (20 µl), diluted in the mobile phase, was injected. A calibration curve of Met was plotted in the mobile phase.

Determination of sodium content in plasma and urine. Na in plasma and urine was analysed by electrolyte analyser AVL-988 (AVL Instruments Médicaux, Cergy Pontoise, France) using a Na-selective electrode after appropriate dilution in distilled water.

Statistical analysis

Data were expressed as mean values with their standard errors. For each variable, statistical analysis was performed with two-way ANOVA, the two factors being rat strain and treatment, followed by inter-group pair-wise comparisons with Tukey's *post-hoc* test when significant differences were detected.

Results

Change in body weight and organ weight

The change of the body weight of rats over the 10 weeks of the experiment was similar in the four experimental groups, though WKY rats were significantly ($P < 0·05$) lighter than SHR rats.

In a similar manner, the livers and hearts of the SHR rats were significantly ($P<0.01$) heavier than those of WKY rats. There was no significant difference between the weights of the kidneys of the two strains. The Met-enriched diet had no significant effect upon either total body or isolated organ weight (Table 2 and Fig. 1).

Table 2. Organ weights in control and methionine-supplemented Wistar–Kyoto and spontaneously hypertensive rats after 10 weeks of treatment‡

(Mean values with their standard errors for twelve rats per group)

| | WKY | | | | SHR | | | |
| | Control | | Met | | Control | | Met | |
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Kidney (g)	1·21	0·02	1·22	0·02	1·28	0·05	1·18	0·03
Liver (g)	10·80	0·30	10·72	0·14	13·08††	0·65	12·27*	0·39
Heart (g)	1·09	0·02	1·11	0·06	1·34††	0·04	1·20	0·03

WKY, Wistar–Kyoto rats; SHR, spontaneously hypertensive rats; Met, methionine.
Mean value was significantly different from that at the WKY—Met group: * $P<0.05$.
Mean values were significantly different from those of the WKY control group; †† $P<0.01$.
‡ For details of diets and treatments, see Table 1 and p. 540.
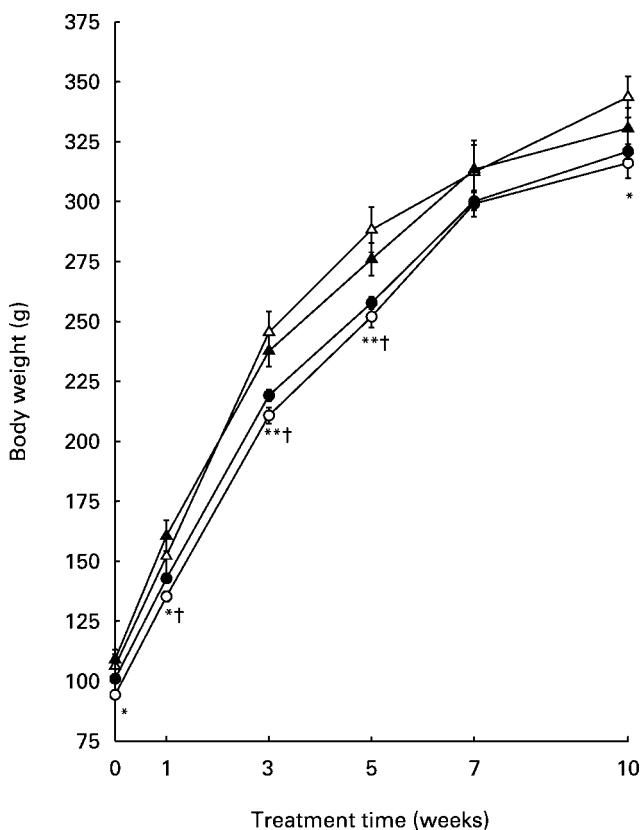
Fig. 1. Effect of a control or methionine (Met)-supplemented diet on change in body weight in Wistar–Kyoto (WKY) and in spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for the SHR control group were significantly different from those of the WKY control group: * $P<0.05$, ** $P<0.01$. Mean values for the SHR-Met group were significantly different from those of the WKY-Met group: † $P<0.05$.

Metabolism and plasma and urine sodium levels

There was a tendency for control SHR rats to have a lower food and water intake and urine volume compared with WKY animals, though this did not achieve statistical significance. These values were normalised in SHR rats on the high-Met diet. Otherwise, none of these variables were different between the different groups (Table 3).

The daily Met intake was similar in WKY and SHR rats and the extra Met intake was about 500 mg/kg body weight per d in the Met-supplemented rats.

Plasma and urine Na levels were also similar in all groups of animals.

Total plasma homocysteine, methionine, cysteine and glutathione levels

After 10 weeks of a Met-supplemented diet, plasma Hcy concentrations were 2·3-fold ($P<0.01$) higher in WKY rats and 3·9-fold ($P<0.01$) higher in SHR rats than base-line values. The plasma Met level was also significantly ($P<0.01$) higher in WKY and SHR rats on the high-Met diet. The Met-supplemented diet also induced an increase in plasma Cys concentration in WKY rats compared with SHR rats, where it had no effect ($P<0.01$). Plasma gluta-thione concentration was significantly higher ($P<0.01$) in SHR than in WKY rats on either diet, and the Met-supplemented diet induced a further increase of the glutathione level in SHR rats ($P<0.05$) (Fig. 2).

Effect of methionine-supplemented diet on systolic blood pressure

At the beginning of the experiment, the systolic BP of WKY and SHR rats were similar at about 100–110 mmHg. By the third week of the experiment, the systolic BP of SHR was considerably elevated ($P<0.01$) compared with those of WKY rats, irrespective of the diet. Starting from the fifth week, the Met-supplemented diet significantly ($P<0.01$) increased the systolic BP of WKY rats and significantly ($P<0.05$) decreased the systolic BP of SHR rats. Thus, the Met-supplemented diet induced a lesser development

of hypertension in SHR, while an increase of the systolic BP was observed in the WKY rats (Fig. 3).

Aortic reactivity to noradrenaline and calcium chloride

Noradrenaline evoked a more effective constriction of aortic rings from WKY-Met rats (half effective concentration (EC_{50}) 0.70×10^{-8} (SEM 0.09×10^{-8}) M) than WKY control rats (EC_{50} 1.22×10^{-8} (SEM 0.13×10^{-8}) M, $P < 0.05$). The maximum contraction was not modified (94.0 (SEM 1.9) v. 86.5 (SEM 1.5) %) (Fig. 4)

There was no significant difference between the responses to noradrenaline of aortic rings from either Met-rich or control diet SHR rats.

High concentrations of Ca^{2+} induced a greater constriction of aortic rings from SHR rats fed with a Met-supplemented diet than those from SHR rats on control diet. Aortic rings from WKY rats fed on either diet showed no difference in their reaction to Ca^{2+} (Fig. 5).

Aortic reactivity to acetylcholine, bradykinin and sodium nitroprusside

Aortic rings from control diet WKY rats were more sensitive to acetylcholine-induced relaxation than those from WKY rats on Met-supplemented diet (EC_{50} 4.97×10^{-8} (SEM 0.83×10^{-8}) and 8.18×10^{-8} (SEM 1.02×10^{-8}) M respectively ($P < 0.05$)). The Met-rich diet did not induce a significant difference in the responses of aortic rings acetylcholine in SHR (Fig. 6).

In both normotensive and hypertensive rats, low concentrations of bradykinin ($10^{-9} - 10^{-7}$ M) elicited greater relaxation of aortic rings from control diet rats than from rats on Met-supplemented diet, whereas the EC_{50} were similar ($\times 10^{-7}$ M): WKY control 4.43 (SEM 1.30), SHR control 4.00 (SEM 0.83), WKY-Met 2.63 (SEM 0.95), SHR-Met 3.31 (SEM 0.62); Fig. 7).

Aortic rings from SHR rats were significantly ($P < 0.01$) more sensitive to sodium nitroprusside than those from
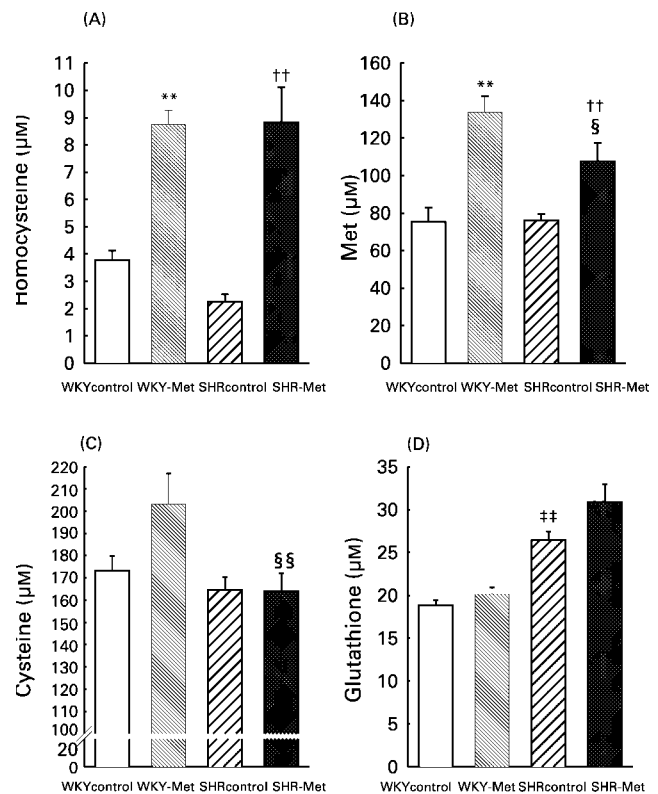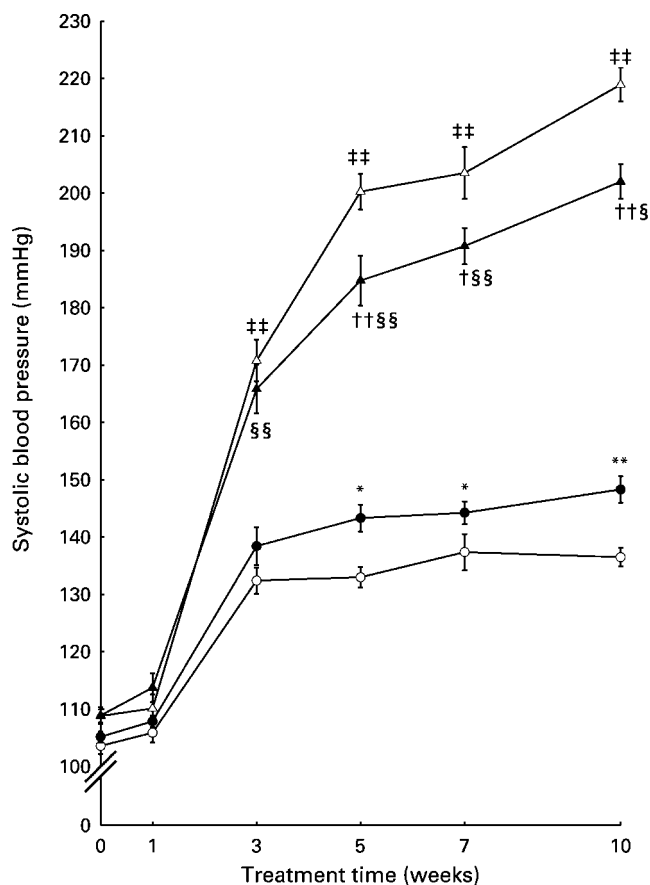

Fig. 2. Effect of a control or methionine (Met)-supplemented diet on plasma homocysteine (A), Met (B), cysteine (C) and glutathione (D) in Wistar−Kyoto (WKY) and in spontaneously hypertensive rats (SHR). For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for WKY-Met group were significantly different from those of WKY control group: **$P < 0.01$. Mean values for SHR-Met group were significantly different from those of SHR control group: ††$P < 0.01$. Mean value for the SHR control group was significantly different from that of WKY control group: ‡‡$P < 0.01$. Mean values for SHR-Met group were significantly different from those of WKY-Met: §$P < 0.05$, §§$P < 0.01$.

Table 3. Daily methionine intake (mg/kg body weight), food and water intake (g and ml/kg per d), urine volume (ml/kg per d), plasma sodium (mM) and urine sodium (mmol/kg per d) in control and methionine-supplemented Wistar−Kyoto and spontaneously hypertensive rats*

(Mean values with their standard errors for twelve rats per group)

	WKY				SHR			
	Control		Met		Control		Met	
	Mean	SEM	Mean	SEM	Mean	SEM	Mean	SEM
Food intake (g/kg per d)	57.4	1.2	60.1	2.1	53.6	2.0	59.8*	1.7
Met intake (mg/kg per d)	373.1	7.8	871.5††	30.4	348.4	13.0	867.1**	24.7
Water intake (ml/kg per d)	115.3	9.8	120.1	9.7	94.3	7.4	117.6	12.2
Urine volume (ml/kg per d)	62.8	8.7	62.5	6.5	42.7	6.1	62.2	8.6
Plasma sodium (mM)	140.1	0.47	139.3	0.39	139.2	0.35	140.2	0.38
Urine sodium (mmol/kg per d)	5.2	0.3	4.8	0.3	4.3	0.5	4.8	0.3

WKY, Wistar−Kyoto rat; SHR, spontaneously hypertensive rats; Met, methionine.
Mean values were significantly different from those of SHR control group: *$P < 0.05$, **$P < 0.01$.
Mean value was significantly different from that of WKY control group: ††$P < 0.01$.
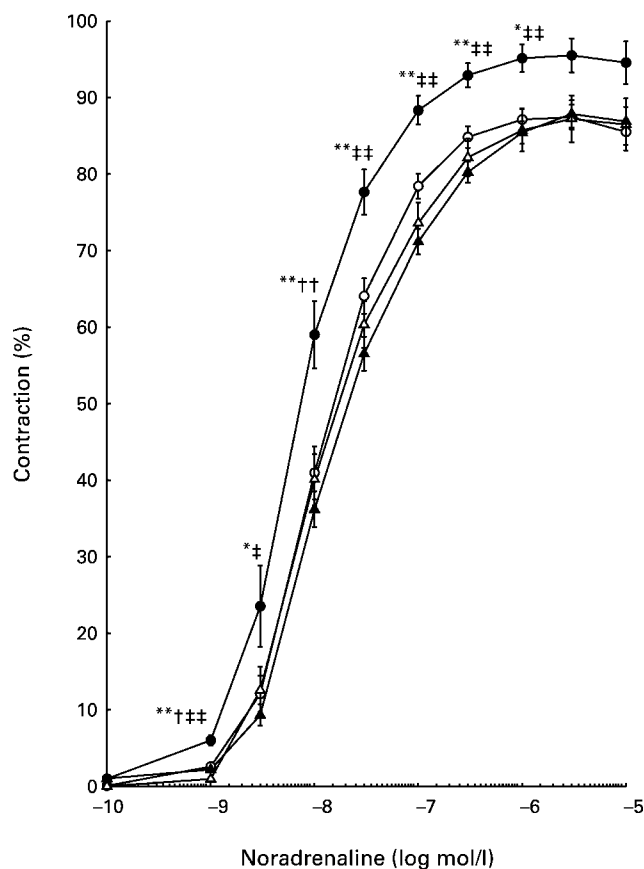‡ For details of diets and procedures, see Table 1 and p. 540.

Fig. 3. Effect of a control or methionine (Met)-supplemented diet on systolic blood pressure in Wistar–Kyoto (WKY) and in spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for WKY-Met group were significantly different from those of WKY control group: *$P<0.05$, **$P<0.01$. Mean values for SHR-Met group were significantly different from those of SHR control group: †$P<0.05$, ††$P<0.01$. Mean values for SHR control group were significantly different from those of WKY control: ‡‡$P<0.01$. Mean values for SHR-Met group were significantly different from those of WKY-Met: §§$P<0.01$.

Fig. 4. Effect of a control or methionine (Met)-supplemented diet on the action of noradrenaline on isolated aortic rings from Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for WKY-Met group were significantly different from those of WKY control group: *$P<0.05$, **$P<0.01$. Mean values for SHR control group were significantly different from those of WKY control group: †$P<0.05$, ††$P<0.01$. Mean values for SHR-Met group were significantly different from those of WKY-Met: ‡$P<0.05$, ‡‡$P<0.01$.

WKY rats. In normotensive WKY rats, sodium nitroprusside induced a similar relaxation of aortic rings from animals fed either control or Met-enriched diet. In SHR rats, the Met-supplemented diet was associated with an increased to sodium nitroprusside at concentrations $>10^{-8}$ M and a maximum of relaxation of 109·9 (SEM 4·6) *v.* 98·8 (SEM 0·4) % respectively ($P<0.01$) (Fig. 8).

Discussion

The present study showed that a Met-enriched diet, while inducing a similar 3-fold increase of plasma Hcy in WKY and SHR rats, was associated with an increase of the systolic BP in WKY rats after the 5th week of the diet and a decrease of the systolic BP of SHR rats after the 3rd week of the diet. The absence of variation in BP that has been reported previously for normotensive rats may be related to different experimental conditions such as a shorter diet regimen
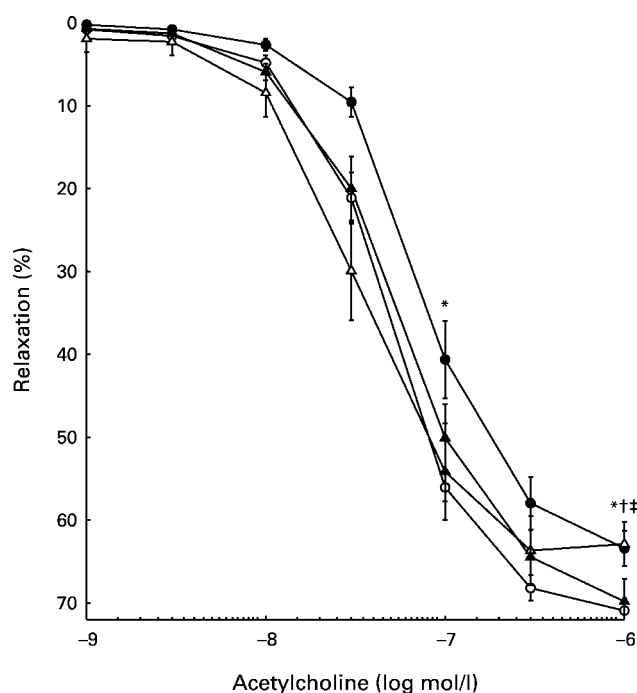
(Ungvari *et al.* 1999) or different conditions of Met administration in smaller samples of older rats (Matthias *et al.* 1996). However, 4 months of a Met-enriched diet was reported to induce both systolic and diastolic hypertension in normotensive Gotingen minipigs (Rolland *et al.* 1995). In human patients, elevated Hcy levels were either strongly and independently associated with isolated systolic hypertension (Sutton-Tyrrell *et al.* 1997) or associated with higher diastolic and mean arterial BP (Fiorina *et al.* 1998). Given the biological mechanisms which have been proposed in support of the relationship between HHcy and cardiovascular diseases and which include endothelial injury and dysfunction, modification of vascular wall structure and properties, one would predict a positive association between Hcy and BP such as we found in WKY rats. Therefore, the slower increase in systolic BP of SHR rats observed from the 3rd week of the Met-enriched diet was surprising. Recently, an attenuation of the development of hypertension was reported
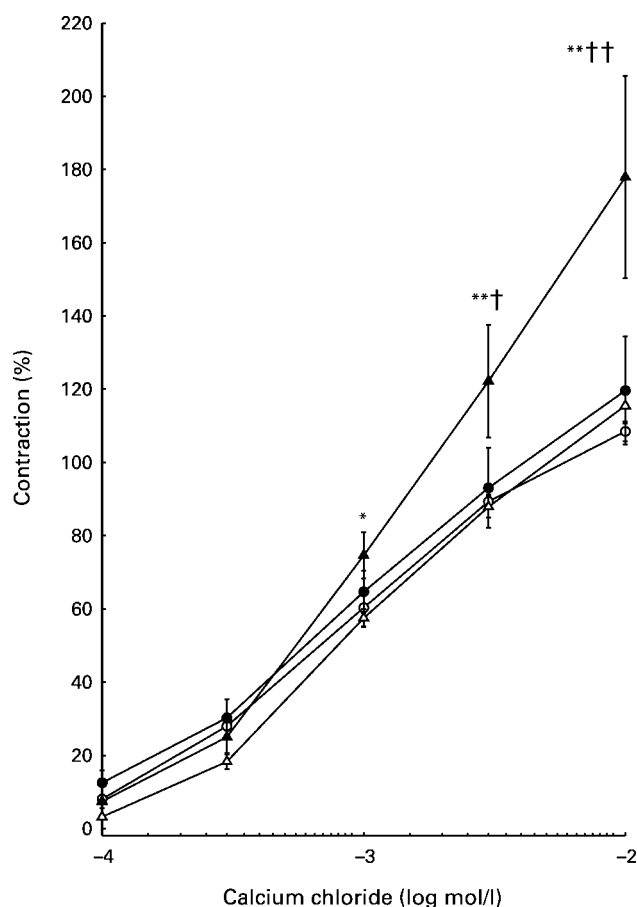
Fig. 5. Effect of a control or methionine (Met)-supplemented diet on the action of calcium chloride on isolated aortic rings from Wistar−Kyoto (WKY) and spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for SHR-Met group were significantly different from those of SHR control group: $*P<0.05$, $**P<0.01$. Mean values for SHR-Met group were significantly different from those of WKY-Met group: $†P<0.05$, $††P<0.01$.

Fig. 6. Effect of a control or methionine (Met)-supplemented diet on the relaxation in response to acetylcholine of isolated, precontracted aortic rings from Wistar−Kyoto (WKY) and spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for WKY-Met group were significantly different from those of WKY control group: $*P<0.05$. Mean value for SHR-Met group was significantly different from that of SHR control group: $†P<0.05$. Mean value for SHR control group was significantly different from that of WYK control group: $‡P<0.05$.

in female SHR, whereas an accelerating effect was observed in males after Met overloading which induced a similar 3-fold increase in serum Hcy in each sex (Yen & Lau, 2002). These discrepancies of effect observed with similar elevation of Hcy in different strains and sexes suggest that the association that has sometimes been observed between plasma Hcy and BP is unlikely to be causal.

Since Hcy leads to endothelial dysfunction and other vascular damage, we investigated the contractile and relaxant properties of isolated aortic rings from the four different experimental groups. In WKY rats fed the Met-enriched diet, an enhanced aortic constriction to noradrenaline was observed. This was in accordance with previous reports on rat skeletal muscle arterioles (Ungvari et al. 1999) showing that this increased responsiveness to noradrenaline was associated with impaired endothelial NO function. The sensitivity of vascular smooth muscle cells to NO of WKY-Met rats to the NO donor sodium nitroprusside was not different from that of the other groups. However, their reactions to acetylcholine and

bradykinin, which release NO from endothelial cells, were reduced. This suggests an impaired agonist-induced synthesis, release or bioavailability of endothelial NO. Our present results are in accordance with results from large vessels from animals (Lentz et al. 1996; Lentz, 1997) and human subjects (Tawakol et al. 1997; Hanratty et al. 2001) and in arterioles from animals (Eberhardt et al. 2000; Ungvari & Koller, 2001). The decreased flow-mediated vasodilatation of the brachial artery observed in healthy men after oral Met loading that induced an acute elevation of Hcy are in agreement with these observations (Chambers et al. 2001). The endothelial cell dysfunction in the WKY-Met rats is consistent with the observed increase in systolic BP in this group.

In SHR, the decreased sensitivity to acetylcholine and bradykinin in aortic rings of rats fed the Met-rich diet was not in agreement with the systolic BP reduction effect. It is possible that the Met-rich diet could have induced endothelial dysfunction in both SHR and WKY rats, but that the hypertensive consequence of this effect was masked in SHR rats by another mechanism. SHR rats express a higher level of NO than the normotensive strain (Yen & Lau, 2002), which was indicated here by a tendency to a higher sensitivity to acetylcholine and bradykinin of the SHR v. WKY rats. In this case, the consequence
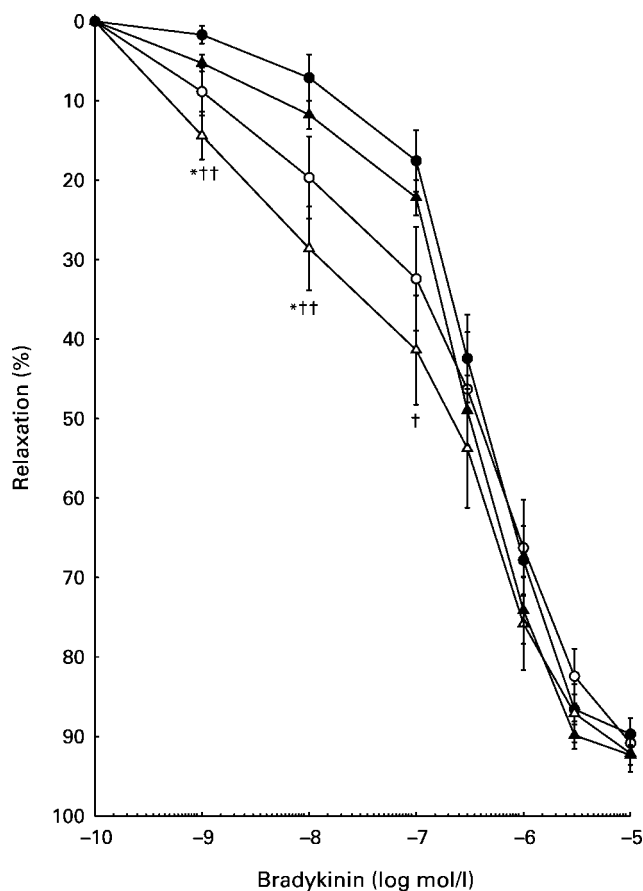
Fig. 7. Effect of a control or methionine (Met)-supplemented diet on the relaxation in response to bradykinin of isolated, precontracted aortic rings from Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for WKY-Met group were significantly different from those of WKY control group: *$P<0.05$. Mean values for SHR-Met group were significantly different from those of SHR control group: †$P<0.05$, ††$P<0.01$.

Fig. 8. Effect of a control or methionine (Met)-supplemented diet on the relaxation in response to sodium nitroprusside of isolated, precontracted aortic rings from Wistar–Kyoto (WKY) and spontaneously hypertensive rats (SHR). (○), WKY control; (●), WKY-Met; (△), SHR control; (▲), SHR-Met. For details of diets and procedures, see Table 1 and p. 540. Values are means with their standard errors shown by vertical bars for twelve rats per group. Mean values for SHR-Met group were significantly different from those of SHR control group: *$P<0.05$, **$P<0.01$. Mean values for SHR control group were significantly different from those of WKY control group: †$P<0.05$, ††$P<0.01$. Mean values for the SHR-Met group were significantly different form those of WKY-Met group: ‡$P<0.05$, ‡‡$P<0.01$.

of the deleterious effect of Met would be less pronounced. In this way, the enhanced aortic constriction to noradrenaline observed in the WKY rats was not present in SHR.

Endothelial NO synthase has been suggested as a potential target for Hcy with controversial results showing up- or down-regulation of NO production (Zhang *et al.* 2000). Since tissue endothelial NO synthase expression is affected in SHR (Bauersachs *et al.* 1998), increased levels of Hcy may induce different effects in WHY and SHR rats. Moreover, a more pronounced responsiveness of vascular smooth muscle cells to direct stimulation by the higher concentrations of the NO donor sodium nitroprusside was observed in the SHR-Met. The higher sensitivity to Ca also found in the SHR-Met group may suggest a differential response to the Met-rich diet of smooth muscle cells in SHR and WKY rats. Therefore, that Hcy, similar to a voltage-operated Ca channel inhibitor, decreased arteriolar smooth muscle intracellular Ca levels (Ungvari & Koller, 2001) may be related to the decreased systolic BP in SHR. However, whether these

vascular alterations were directly connected to change of BP is unknown and the differential vasopressive effect of the Met-rich diet may be due to other mechanisms. In our present experimental conditions, systolic BP was not related to body weight, which was similar in animals fed either control or Met-enriched diet. Renal function also seemed to be unaffected by the diet, since kidney weight, urinary output, urinary and plasma Na concentrations were similar in each group.

Another question is whether the effects observed with Met-enriched diet were specifically related to Hcy or to another metabolite of the Met–Hcy pathway. Hcy is a metabolite of Met by transformation of Met into *S*-adenosylmethionine by methionine *S*-adenosyltransferase and then into *S*-adenosylhomocysteine by demethylation and finally into Hcy by *S*-adenosylhomocysteine hydrolase. Once

formed, Hcy can be metabolised via two pathways. The first is the remethylation of Hcy to Met, and the second is a reaction of transsulfuration in which Hcy is transformed into Cys and cystathionine. In the present study, HHcy was induced by adding Met to the diet (Durand *et al.* 1997). Plasma Hcy increased about 3-fold, but the Met and cysteine levels were also modified. The WKY rats, in which the Met-enriched diet induced an increase of systolic BP, showed a greater increase of the methioninaemia and an increase of plasma Cys levels. Although Met and cysteine have a cyto-toxic action (Anderson & Meister, 1989), there have been no reports to indicate that a high level of Met or cysteine would elicit adverse vascular changes and induce an increase in BP.

Other metabolic pathways can be modified by a high-Met diet. The most abundant thiol-reducing agent, glutathione, can act directly as a major antioxidant and as a cofactor for enzymes such as glutathione peroxidase, glutathione reductase and glutathione transferase. We found that the plasma glutathione level, which is higher in SHR than in WKY rats, was further increased in the SHR-Met group. Increased oxidant stress imparted by HHcy contributes to endothelial dysfunction (Kanani *et al.* 1999; Eberhardt *et al.* 2000) and vascular damage (Rolland *et al.* 1995). The increase in plasma glutathione levels may then reflect an elevation of endothelial glutathione content, which could prevent the deleterious effects of HHcy. This mechanism may be implicated in the lowered systolic BP observed in SHR. Other antioxidant systems such as superoxide dismutase (Zhang *et al.* 1998), catalase (Starkebaum & Harlan, 1986) and ascorbic acid (Chambers *et al.* 1999) are known to decrease Hcy-induced endothelial dysfunction, and Hcy decreases bioavailable NO by a mechanism involving glutathione peroxidase (Upchurch *et al.* 1997).

In conclusion, our present results showed that a Met-enriched diet induced a moderate HHcy in both SHR and WKY rats, but thiol compounds such as Met, Cys and glutathione were elevated differently in the two rat strains. The Met-enriched diet was associated with an increase in systolic BP in WKY rats, which is consistent with the observed endothelial dysfunction. In SHR, the discrepancies between the decreased systolic BP and the modified vascular responses suggest complex interactions of the Met-enriched diet on the systolic BP. The differential vasopressive effect of the Met-rich diet on the two rat strains which were obtained with similar elevation of Hcy suggests that the association between plasma Hcy and BP is unlikely to be causal. Further investigations are needed to understand the paradoxical effect of a Met-rich diet on systolic BP in WKY and SHR rats.

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