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Hyperhomocyst(e)inemia induces multiorgan damage

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Abstract Hyperhomocyst(e)inemia has been associated with the development of hypertension, stroke, and cardiovascular, cerebral/neuronal, renal, and liver diseases. To test the hypothesis that homocyst(e)ine plays an integrated role in multiorgan injury in hypertension, we employed: (1) spontaneously hypertensive rats (SHR) in which endogenous homocyst(e)ine levels are moderately high (18.1 ± 0.5 μM); (2) control age- and sex-matched Wistar Kyoto (WKY) rats in which homocyst(e)ine levels are normal (3.7 ± 0.3 μM). To create the pathophysiological condition of hyperhomocyst(e)inemia, 20 mg/day homocyst(e)ine was administered for 12 weeks in (3) SHR (SHR-H) and in (4) WKY (WKY-H) rats. (5) Endogenous homocyst(e)ine levels were reduced slightly but not significantly from 18.1 ± 0.5 μM to 12.5 ± 0.7 μM in SHR by folic acid administration (SHR-F). Plasma and tissue levels of homocyst(e)ine were determined by HPLC and spectrophotometric methods. Plasma and sympathetic ganglion (neuronal) matrix metalloproteinase (MMP) activity was measured by zymography. Activity of neuronal MMP was increased in hyperhomocyst(e)inemic rats as compared with controls. Mean arterial pressure (mmHg) was 95 ± 5, 126 ± 8, 157 ± 10, 188 ± 5, and 165 ± 12 in WKY, WKY-H, SHR, SHR-H, and SHR-F, respectively. Urinary protein (mg/day) was 0.11 ± 0.03, 0.88 ± 0.22, 0.47 ± 0.10, 0.89 ± 0.21, and 0.81 ± 0.21 in WKY, WKY-H, SHR, SHR-H, and SHR-F, respectively, as measured by the Bio-Rad dye binding assay. The relationships between increased arterial pressure, plasma homocyst(e)ine, and urinary protein were delineated. Plasma and neuronal creatinine phosphokinase (CK) isoenzymes were measured by agarose gel electrophoresis. All three CK isoenzymes, i.e., MM, MB, and BB, specific for skeletal, cardiac, and nerve tissue, respectively, were induced following 12 weeks' hyperhomocyst(e)inemia, suggesting multiorgan injury by homocyst(e)ine. Homocyst(e)ine induces endocardial endothelial cell (capillary) apoptosis and may reduce capillary cell density. Structural damage to aorta, myocardium, kidney, and renal-ureter was analyzed by histology. Results suggested an integrated physiological role of homocyst(e)ine in injury to the endothelial/epithelial cell lining in the respective organs.

Key words Nerve · Capillary endothelium · Renal-ureter · Hypertrophy · Heart failure

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Introduction

Half of dietary methionine is converted to homocyst(e)ine.[1] A diet (fruits and vegetables) which limits methionine and homocyst(e)ine[1] reduces hypertension.[2] The body's inability to clear the metabolic by-product, homocyst(e)ine, creates hyperhomocyst(e)inemia. A concentration of plasma homocyst(e)ine greater than 20 μM is associated with an increased risk of mortality by 35%.[3] Hyperhomocyst(e)inemia is associated with the development of premature arterial fibrosis with peripheral vascular, cerebral vascular, neurogenic, liver, hypertensive heart, and renal diseases, and myocardial infarction as well as venous thromboembolism.[4–7] A relationship between conventional and emerging risk factors, and markers of end-organ damage has been suggested.[8] Hyperhomocyst(e)inemia has been associated with systolic hypertension.[9] Hypertension induces multifactorial target organ injury.[10,11]

The common denominator among all the above organs is the endothelium/epithelium lining. It is known that homocyst(e)ine damages endothelium.[4] Elevated plasma homocyst(e)ine has been suggested as a marker of endothelial dysfunction[12] and adverse extracellular matrix (ECM) remodeling in atherosclerosis.[13,14] Adverse ECM remodeling and end-organ damage are associated with increased levels of creatinine phosphokinases (MM, MB,

BB isoforms) in spontaneously hypertensive rats (SHR).[15] The volume retention by the kidney causes hypertension.[16] A reduced glomerular filtration rate (GFR) leads to increased plasma homocyst(e)ine.[17] To test the hypothesis that elevated homocyst(e)ine levels are associated with increased blood pressure and markers of cell and organ injury, we employed: (1) SHR in which GFR is reduced[18] and endogenous homocyst(e)ine levels are moderately high;[19] (2) control age- and sex-matched Wistar Kyoto (WKY) rats in which homocyst(e)ine levels were normal. To create the pathophysiological conditions of hyperhomocyst(e)inemia, homocyst(e)ine was administered in (3) SHR (SHR-H) and in (4) WKY (WKY-H) rats. (5) Endogenous homocyst(e)ine levels were reduced in SHR by folic acid administration (SHR-F). Results suggested that elevation of homocyst(e)ine induces hypertension, and targets organ injury.

Materials and methods

Creation of hyperhomocyst(e)inemia

Since homocyst(e)ine is associated with hypertension[9] and SHR have higher levels of homocyst(e)ine as compared with age- and sex-matched control WKY rats,[19] we created a condition of chronic hyperhomocyst(e)inemia by adding DL-homocyst(e)ine (Sigma, St. Louis, MO, USA) (0.67 mg/ml) in drinking water of normal and SHR rats. We did not administer methionine since methionine induces moderate homocyst(e)inemia and may affect overall protein synthesis. We created hyperhomocyst(e)inemia by directly administering homocyst(e)ine as follows: (1) spontaneously hypertensive rats (SHR) of 32–36 weeks, 325–350 g (Charles River Laboratories) in which endogenous homocyst(e)ine levels are moderately high;[19] (2) control age-sex matched Wistar Kyoto (WKY) rats in which homocyst(e)ine levels are normal. To create the pathophysiological condition (30 μM) of hyperhomocyst(e)inemia, 20 mg/day homocyst(e)ine was administered for 12–15 weeks in (3) SHR (SHR-H), and in (4) WKY (WKY-H) rats. (5) Endogenous homocyst(e)ine levels were reduced in 32–36-week-old SHR rats by folic acid administration for 12–15 weeks (SHR-F). Folic acid was administered in drinking water at 0.04 mg/ml. All rats were given standard rat chow containing 2.84% folic acid, 0.37% methionine, and 0% homocyst(e)ine, and water ad libitum. The level of water intake and changes in body weight were measured every other day. To determine plasma homocyst(e)ine and creatinine phosphokinase (CK) activity, 0.5 ml blood was collected every second day from the tail veins and transferred to tubes containing ethylenediaminetetraacetic acid (EDTA). The plasma was separated by centrifuging the blood at 1000 rpm for 20 min. All studies conformed with the principles of the National Institutes of Health Guide for the Care and Use of Laboratory Animals, and the protocol was approved by the University of Mississippi Medical Center Institutional Animal Care and Use Committee.

To optimize the dose of homocyst(e)ine, rats were given 0.1, 0.4, and 0.67 mg/ml homocyst(e)ine in the drinking water for 4 days. Rats ingested 3.7 ± 0.5, 12.8 ± 2.5, and 20.1 ± 1.8 mg homocyst(e)ine/day, respectively. Within 2 days, the plasma level of total homocyst(e)ine at a dose of 0.67 mg/ml was elevated to pathophysiological amounts (20–60 μM). This concentration is also found in human plasma.[3] Therefore, we selected a dose of 0.67 mg/ml of homocyst(e)ine to be added to the drinking water for further studies.

Hemodynamic parameters

To determine whether homocyst(e)ine ameliorates hypertension, blood pressure was recorded. At the end of 12 weeks, homocyst(e)ine- and folic acid-treated rats were anesthetized with Inactin (100 g/kg i.p.). This anesthesia has a minimal effect on cardiovascular function.[20] A catheter was inserted through the femoral artery (PE 50 tubing) and connected to a pressure transducer (Micro-Med Corp.) positioned at the level of the heart. Pulsatile arterial pressure signals were sent to an analog-to-digital converter and analyzed by computer using customized software. Following a 10-min stabilization period, mean arterial pressure (MAP), systolic and diastolic blood pressure, and heart rate were measured.

Measurements of plasma creatine phosphokinase (CK) activity

Tissue-specific injury was determined by measuring creatine phosphokinase (CK) isoforms in plasma.[21] Isoform CK BB is primarily nerve- and kidney-specific; MB is cardiac; and MM is cardiac and skeletal muscle-specific. From each rat, 5 μl of plasma was mixed with 5 μl of mercaptoethanol and loaded onto the 1% agarose gels. The gels were preequilibrated with CK substrate and catalysts as recommended by Sigma.[22,23] The gels were run at 175 V for 45 min. The standard (ST) amounts of CK isoforms were loaded parallel to the samples. Based on the densitometric intensity (I) of the samples, the amount of specific CK isoform was estimated as follows: $\text{Amount}_{ST}/I_{ST} \times I_{\text{Sample}}$.

To determine whether homocyst(e)ine injured nerves in the above five different study groups of rats, sympathetic ganglions were isolated and homogenized and CK activity was estimated.

Urinary protein

To determine proteinurea in hyperhomocyst(e)inemic rats, 24-h urine was collected in metabolic cages. Urinary protein was measured by the Bio-Rad dye binding assay, using bovine serum albumin as the standard.

Measurements of plasma redox-thiols and homocyst(e)ine

Plasma total homocyst(e)ine is the sum of all forms, i.e., 70% bound to albumin and other proteins, 1% reduced

(homocysteine), and 29% oxidized disulfide (including homocystine).[1] Plasma total redox-thiols were measured by titrating the -SH group with dithio-bis-nitrobenzoate (DTNB) in the presence of a minimal reducing agent. After incubation at 37°C for 3.5 h all thiol and disulfides were exchanged with DTNB and produced color at 412 nm. The concentration of thiols was calculated using an extinction coefficient of $13\,600\,M^{-1}cm^{-1}$ at 412 nm. Total homocyst(e)ine was isolated from plasma and tissue homogenates by high-performance liquid chromatography (HPLC), and the isolated fraction of the homocyst(e)ine peak were quantitated using spectrophotometry at 412 nm and the extinction coefficient.[13]

Preparation of tissue homogenates

Aorta, kidney, heart, and sympathetic ganglion were cleaned of external tissue and tissue homogenates were prepared as described.[24] A Bio-Rad dye binding assay was applied to estimate total protein.

Zymography

To determine total plasma matrix metalloprotein (MMP) activity in rats from the above five study groups, gelatin zymography was performed as described.[24] Twenty microliters of plasma was loaded onto the gel under nonreducing conditions. The lytic band intensity in the gel was scanned with a Bio-Rad gel scanner (GS-700). The intensity at 92 and 72 kDa for gelatinase A and B were plotted. To determine whether homocyst(e)ine activated neuronal MMP, gelatin zymography on extracts from the sympathetic ganglion was performed.

Histological analysis

At the time of sacrifice, a portion of the aorta, heart, kidney, and renal-ureter was collected in 10% zinc formalin for histology. The tissue sections from control, homocyst(e)ine-treated experimental rats were prepared as described.[24] Tissue sections were stained with hematoxylin and eosin (H&E), and Masson's trichrome stain was used for collagen and proteoglycans. Optical light microscopy was performed at 10× and 40× magnification.

TUNEL

Homocyst(e)ine injures endothelium. To determine whether homocyst(e)ine causes endocardial endothelial (capillary) injury, serial tissue sections, as used for histology, were labeled with transferase deoxyuridine nick end labeling (TUNEL) according to the instructions of the manufacturer (Oncogene Research Products, Fluorescein-FragEL, cat. #QIA39), for identification of nicked DNA.

Assessment of tissue injury

Aortic medial thickness was measured by a digital micrometer. To measure cardiac injury, TUNEL-positive cells were counted per cm^2. Renal tubular cell density was measured as tubular hyperplasia. Renal-ureter wall thickness was measured.

Statistical analysis

Data are presented as mean ± SEM. Results in Tables 1 and 2, metabolic data, blood pressure, heart rate, and CK activity were compared between WKY-H and WKY; SHR-H and SHR; and SHR-F and SHR. The significance of the data was tested using Student's unpaired *t*-test.

Results

Hyperhomocyst(e)inemia

To confirm whether homocyst(e)ine administration leads to an increase in plasma homocyst(e)ine, homocyst(e)ine was measured. Plasma homocyst(e)ine in homocyst(e)ine-treated normotensive rats (WKY-H) averaged 32.4 ± 0.7 μM at day 2 of homocyst(e)ine administration compared with 3.7 ± 0.3 μM in untreated rats. The values were stable over the 12-week period (Table 1).

Blood pressure and redox homocyst(e)ine

To determine whether homocyst(e)ine induces vascular resistance, blood pressure was measured. Increased homocyst(e)ine levels were associated with increases in blood pressure (Table 1). The addition of homocyst(e)ine to drinking water caused roughly similar absolute increases in blood pressure in SHR and WKY rats (~30 mm of MAP in each). Folic acid treatment reduced plasma homocyst(e)ine, slightly but not significantly, from 18.1 ± 0.5 to 12.5 ± 0.7 μM. These results suggested that homocyst(e)ine induces hypertension in both normotensive Wistar rats and SHR at the pathophysiological concentrations found in humans.

Tissue levels of homocyst(e)ine

To determine whether increased plasma homocyst(e)ine leads to an increase in tissue homocyst(e)ine, homocyst(e)ine levels in the cardiovascular system were measured. Cardiac tissue homocyst(e)ine was 422 ± 32 and 49 ± 8 ng/mg in the homocyst(e)ine-treated WKY rats and control groups, respectively, suggesting a significant ($P = 0.005$) increase in cardiac tissue levels of homocyst(e)ine in homocyst(e)ine-treated rats. In WKY-H, most of the stored homocyst(e)ine was in the heart, with a much lower increase in the kidney and aorta. In SHR-H, there was

Table 1. Food, water intake, body, heart and kidney weights, urinary protein, plasma, cardiac, aortic, and kidney tissue homocyst(e)ine (H), and hemodynamic parameters and activity of plasma creatine phosphokinase isoforms in WKY, WKY-H, SHR, SHR-H, and SHR-F

	WKY	WKY-H	SHR	SHR-H	SHR-F
n	10	7	10	9	5
Water (ml/day)	28.6 ± 4.5	28.8 ± 4.2	31.8 ± 5.1	33.5 ± 5.0	32.6 ± 7.3
Food (g/day)	21.7 ± 1.5	22.6 ± 0.5	24.4 ± 1.6	24.8 ± 2.7	25.1 ± 2.5
Body weight (BW) (g)	311 ± 3	337 ± 5	326 ± 2	340 ± 4	335 ± 2
Heart weight (HW) (g)	1.06 ± 0.02	1.39 ± 0.04	1.36 ± 0.03	1.46 ± 0.02	1.37 ± 0.24
Kidney weight (KW) (g)	1.26 ± 0.03	1.46 ± 0.05	1.25 ± 0.02	1.36 ± 0.03	1.21 ± 0.14
HW/BW ($\times 10^3$)	3.41 ± 0.35	4.12 ± 0.56*	4.17 ± 0.28	4.29 ± 0.68	4.09 ± 0.22
KW/BW ($\times 10^3$)	4.05 ± 0.48	4.33 ± 0.63*	3.83 ± 0.39	4.00 ± 0.12	3.62 ± 0.35
Urine protein (mg/day)	0.11 ± 0.03	0.88 ± 0.22*	0.47 ± 0.10	0.89 ± 0.21*	0.81 ± 0.21
Plasma H (μM)	3.7 ± 0.3	32.4 ± 0.7*	18.1 ± 0.5	28.4 ± 1.8*	12.5 ± 0.7
Cardiac H (ng/mg)	49 ± 8	422 ± 32*	225 ± 18	648 ± 38*	205 ± 12
Aorta H (ng/mg)	36 ± 4	113 ± 8*	265 ± 32	486 ± 47*	198 ± 23
Kidney H (ng/mg)	97 ± 20	134 ± 9*	212 ± 45	598 ± 34*	206 ± 11
Hemodynamic parameters					
MAP (mmHg)	95 ± 5	126 ± 8*	157 ± 10	188 ± 5*	165 ± 12
HR (beats/min)	268 ± 33	342 ± 35	316 ± 16	313 ± 24	333 ± 39
SBP (mmHg)	117 ± 22	134 ± 25*	190 ± 8	220 ± 24*	196 ± 14
DBP (mmHg)	81 ± 18	88 ± 9*	132 ± 8	161 ± 9*	141 ± 12
Plasma creatine phosphokinase activity					
CK MM (μg/ml)	0.1 ± 0.02	12.6 ± 1.5*	2.5 ± 0.5	14.7 ± 3.0*	2.2 ± 0.4
CK MB (μg/ml)	0.05 ± 0.01	6.3 ± 1.2*	1.8 ± 0.3	8.5 ± 1.5*	1.4 ± 0.3
CK BB (μg/ml)	0.011 ± 0.002	2.8 ± 0.25*	0.5 ± 0.1	4.5 ± 0.25*	0.35 ± 0.05

Each rat was anesthetized by Inactin (100 mg/kg, i.p.). Heart rate (HR), mean arterial pressure (MAP), systolic blood pressure (SBP), diastolic blood pressure (DBP), and creatine phosphokinase (CK) at 12 weeks are recorded. After hemodynamic measurement, the tissue was isolated. Data are presented as mean ± SEM

* $P < 0.05$ when WKY-H was compared with WKY, and SHR-H was compared with SHR

substantial storage in all three organs, although the baseline was higher to begin with (Table 1).

Plasma MMP activity

To determine whether homocyst(e)ine induces cardiovascular remodeling, MMP activity was measured. Gelatinase A and B activity was increased significantly ($P < 0.05$) in the plasma of homocyst(e)ine-treated WKY and SHR rats as compared with control WKY and SHR. Specifically gelatinase B was induced to a greater extent than gelatinase A by homocyst(e)ine (Fig. 1). These results suggest generalized MMP activation in hyperhomocyst(e)inemia.

Pressure and proteinuresis

To determine whether homocyst(e)ine induces multiorgan injury, urinary protein concentrations were measured. Elevation in plasma and tissue levels of homocyst(e)ine were associated with increases in MAP and urinary protein in WKY and SHR (Table 1). The higher blood pressure in untreated SHR than in untreated WKY is, in part, associated with increased urinary protein. The SHR-F group showed no significant change in blood pressure, but manifested a drastic increase in urinary protein, which would argue that blood pressure is not the (sole) cause of renal insufficiency. It is plausible that blood pressure and/or elevated homocyst(e)ine (or an interaction of both) cause organ injury.

In vivo molecular assay of target organ injury by homocyst(e)ine

As a marker of target organ injury, serum CK isoenzyme in controls was measured, 2 and 12 weeks following homocyst(e)ine treatment (Fig. 2A). The results suggested that at 2 weeks following homocyst(e)ine infusion, target organ injury was minimal. However, 12 weeks after homocyst(e)ine administration, MB activity was significantly ($P = 0.01$, WKY-H compared with WKY and SHR-H compared with SHR) increased. These data may suggest that the elevated homocyst(e)ine is one of the causes of hypertension and the associated increase in CK MB activity (Table 1). Since all three CK isoforms (BB, MB, MM) were increased in hyperhomocyst(e)inemic animals, this suggested multiorgan injury, including the kidney, which may be one of the causes of the increase in blood pressure in hyperhomocyst(e)inemic rats. The neuronal CK BB activity (Fig. 2B) was analyzed in an extract prepared from isolated sympathetic ganglia from homocyst(e)ine-treated and control rats. Results suggested a specific increase in CK BB activity in the ganglia of the homocyst(e)ine-treated rats (Table 1). Also, MMP activity was increased in the sympathetic nerves of the homocyst(e)ine-treated rats (Fig. 2C). These results may suggest that homocyst(e)ine is associated with multiorgan injury and activated neuronal MMP, leading to sympathetic impairment.

Cardiac apoptosis

To determine whether homocyst(e)ine causes capillary endothelial cell apoptosis, TUNEL labeling was performed

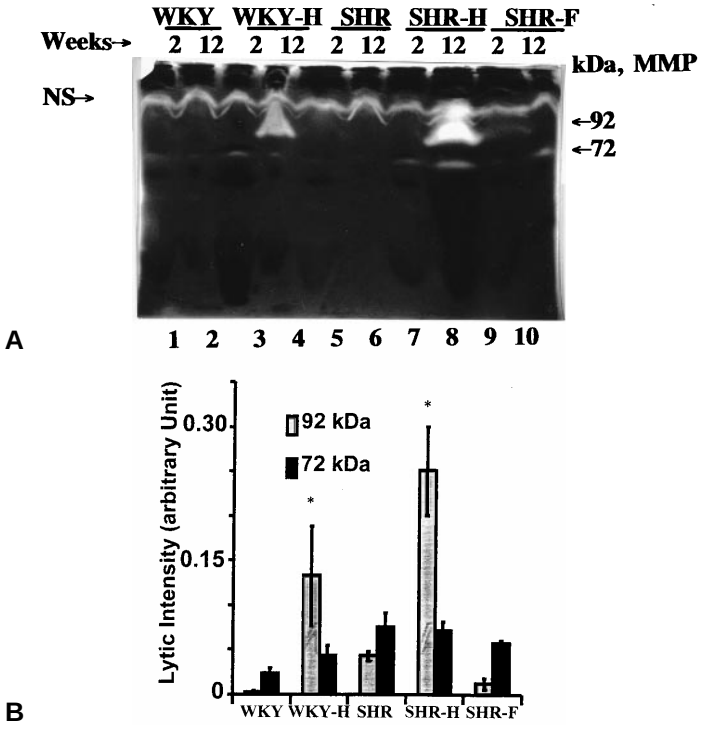

Fig. 1. **A** Plasma matrix metalloproteinase (*MMP*) activity of WKY, SHR, WKY-H, SHR-H, and SHR-F. Rats treated for 2 and 12 weeks with homocyst(e)ine were killed. The plasma was separated and analyzed on gelatin zymography. Identical amounts of total protein were loaded onto the gel. *NS*, nonspecific proteinase (i.e., during electrophoresis some fraction of MMP binds to the matrix and plasma macromolecules with very high affinity so that it cannot be dissociated by electrophoresis). **B** Scanned lytic intensity (arbitrary units) of gelatinase b (92 kDa MMP) and gelatinase a (72 kDa MMP) of 12-week-treated rats. Each bar is an average of data from five rats in each group. $*P < 0.05$ when WKY-H was compared with WKY and SHR-H was compared with SHR

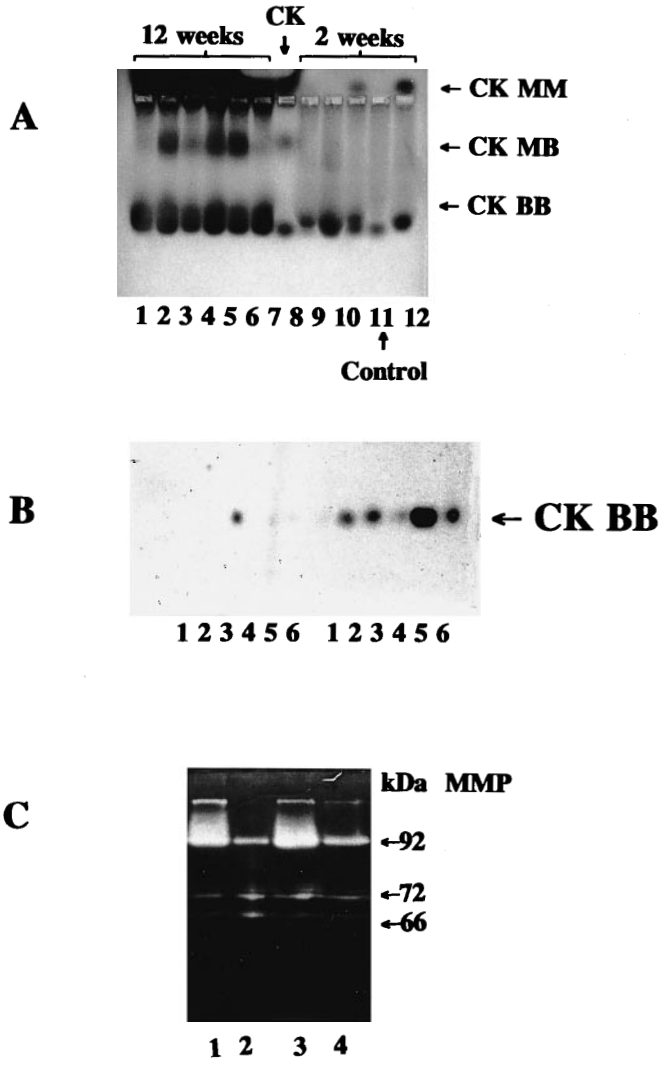

Fig. 2. **A** Molecular analysis of target organ injury by homocyst(e)ine. Plasma creatinine phosphokinase (*CK*) activity of MM, MB, and BB isoenzymes was measured in 2- and 12-week homocyst(e)ine-treated WKY rats. *Lanes 1–6*, 12-week homocyst(e)ine-treated rats; *lane 7*, standard CK isoenzymes; *lanes 8–10 and 12*, 2-week homocyst(e)ine-treated rats; *lane 11*, control rats. **B** CK BB activity in isolated sympathetic ganglion from control (first, $n = 6$) and homocyst(e)ine-treated rats (second, $n = 6$). **C** Gelatinolytic activity in isolated sympathetic ganglion extract of control (*lanes 2 and 4*) and homocyst(e)ine-treated (*lanes 1 and 3*)-rats

in myocardium. There were inhomogeneous TUNEL-positive cells throughout the myocardium. Representative TUNEL-positive cells observed in homocyst(e)ine-treated WKY rats are shown in Fig. 3. The results suggested endothelial cell degeneration by hyperhomocyst(e)inemia.

Effect of homocyst(e)ine on cardiovascular structure

To determine structural alteration by homocyst(e)ine, histological analysis was performed. Homocyst(e)ine induced elastic breakdown in aortas (Fig. 4). The endothelial cell lining was disrupted. The aortas were thickened, dilated, and hypertrophied, suggesting vascular injury associated with high homocyst(e)ine. Endocardial and interstitial tissue of the heart was stained with trichrome (Fig. 4). The findings suggested endocardial and interstitial fibrosis in hyperhomocyst(e)inemia. There was interstitial fibrosis and hyperplasia in the renal tubular epithelial layer in rats treated with homocyst(e)ine (Fig. 4).

The renal ureter wall in homocyst(e)ine-treated rats was significantly thickened compared with that of control rats (Fig. 5). The ureter diameter was reduced. The epithelial cell layer was wavy and inhomogeneous. The basement membrane was disrupted and smooth muscle cells were highly polarized. Basement membrane, collagen, and proteoglycans were increased.

The quantitative analysis of tissue injury demonstrated aortic, myocardial, renal, and ureter injury by homocyst(e)ine. Treatment with folic acid reduces aortic, kidney, and ureter injury but has no effect on myocardial apoptosis by homocyst(e)ine (Table 2).

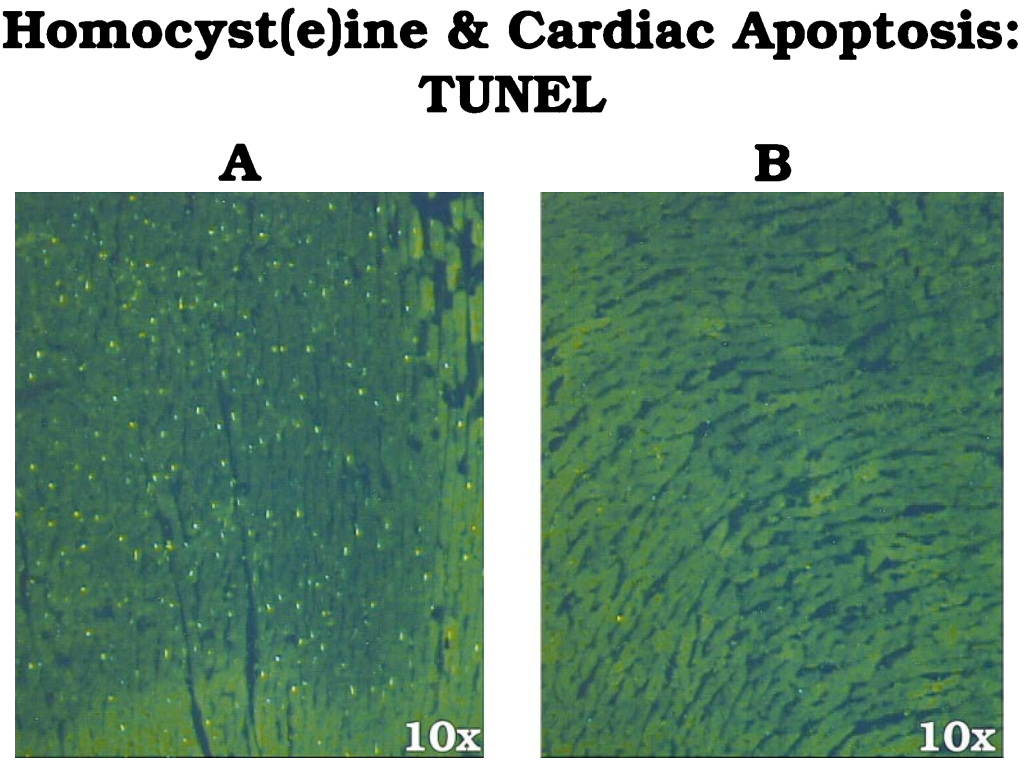

Fig. 3A,B. Representative cardiac tissue sections were labeled for transferase deoxyuridine nick end labeling (*TUNEL*)-positive cells. Cardiac apoptosis in homocyst(e)ine-treated WKY was observed (**A**). The TUNEL assay was performed according to the instructions of the manufacturer (Oncogene Research Products). There were more TUNEL-positive cells in homocyst(e)ine-treated rats than in the control (**B**) (×10)

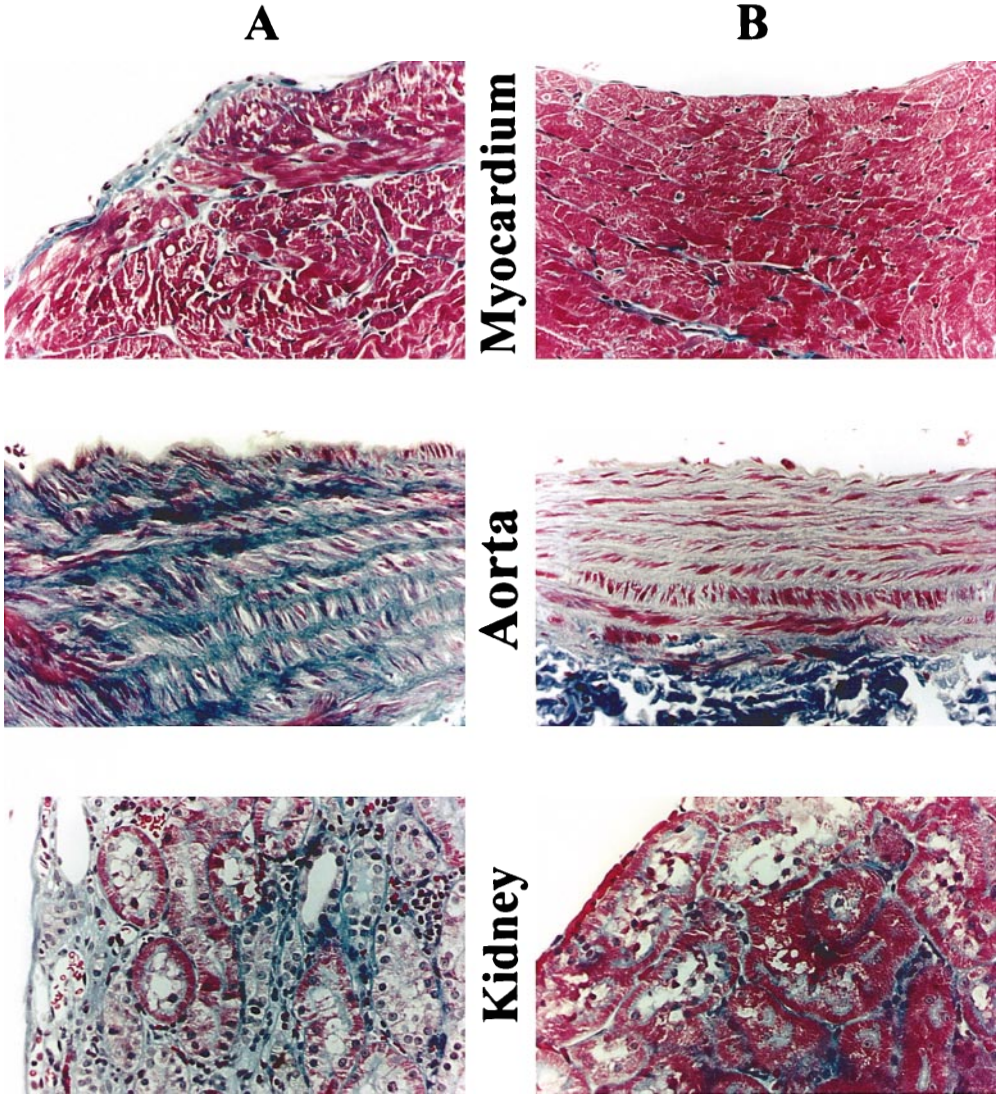

Fig. 4A,B. Trichrome stain of myocardial endocardium, aorta, and kidney. **A** WKY rats treated with homocyst(e)ine for 12 weeks; **B** tissue from control WKY rats. Tissue sections were prepared from similar sites. The blue stain demonstrates collagenous matrix in the endocardium and interstitium in the heart. In the aorta, the blue-green stain in the adventitia demonstrates the connective tissue matrix, particularly collagen and proteoglycans. The medial lining of the elastic laminae was disrupted and smooth muscle cells are hypertrophied in homocyst(e)ine-treated rats. In kidney, tubular epithelial hyperplasia and interstitial fibrosis was observed in homocyst(e)inemic rats (×40)

Table 2. Quantitative analysis of histological assessment of target organ injury: aorta (medial thickness, μm), endocardium (apoptotic nuclei/cm^2), kidney (number of epithelial cells/cm^2), ureter (wall thickness, μm) in WKY, WKY-H, SHR, SHR-H, and SHR-F

	WKY	WKY-H	SHR	SHR-H	SHR-F
n	10	7	10	9	5
Aorta (medial thickness)	87 ± 5	150 ± 20*	145 ± 24	163 ± 18*	135 ± 19*
Endocardium (apoptotic nuclei)	1.2 ± 0.4	8.5 ± 1.2*	3.2 ± 0.5	7.6 ± 1.7*	3.6 ± 0.7
Kidney (tubular hyperplasia)	6 ± 1	11 ± 2*	8 ± 2	15 ± 3*	5 ± 1*
Ureter (wall thickness)	52 ± 6	95 ± 11*	76 ± 8	102 ± 19*	55 ± 11*

The data are presented as mean ± SEM
* $P < 0.05$ when WKY-H was compared with WKY and SHR-H was compared with SHR, and SHR-F was compared with SHR

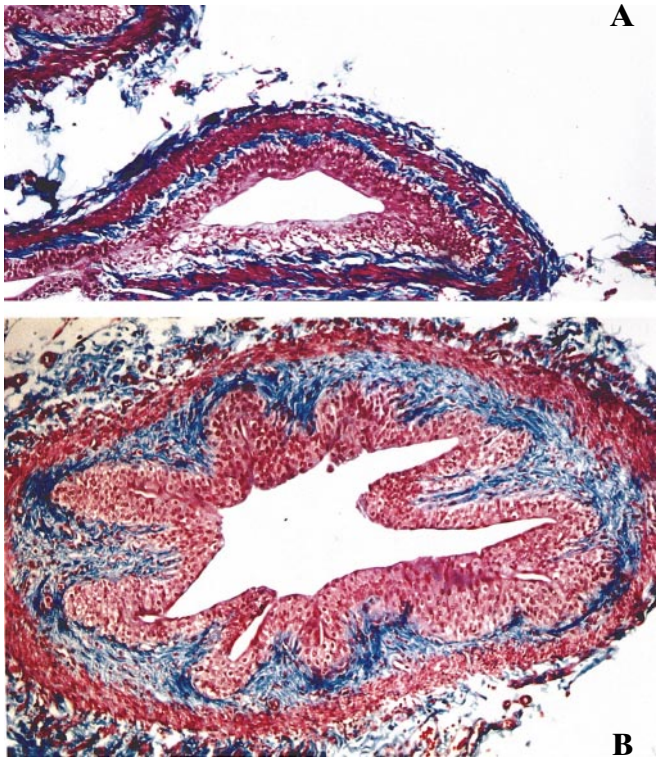

Fig. 5A,B. Trichrome staining of renal ureter: **A** Control; **B** homocyst(e)ine-treated rat. The blue stain demonstrates connective tissue matrix, particularly collagen and proteoglycans (×10)

Discussion

The plasma and tissue levels of homocyst(e)ine achieved in WKY and SHR (Table 1) were in the range of pathophysiological concentrations found in human.[3] Hyperhomocyst(e)inemia is associated with neuronal and cardiovascular-renal injury. Increased plasma homocyst(e)ine is one of the causes of increased neuronal MMP activity and systolic hypertension. A relationship between proteinurea, homocyst(e)ine, and increased arterial pressure was delineated.

Previous studies to induce hyperhomocyst(e)inemia and vascular dysfunction by feeding animal methionine or vitamin B6-deficient diets resulted in only a modest increase in plasma homocyst(e)ine,[25] and tissue homocyst(e)ine was not measured in those studies. We observed increased tissue levels of homocyst(e)ine in the SHR model of homocyst(e)inemia (Table 1). Elevated levels of homocyst(e)ine result in decreased levels of cyst(e)ine[26] and glutathione peroxidase activity.[27] Rolland et al.[25] have demonstrated no significant alteration in creatinine clearance in hyperhomocyst(e)inemic rats. Therefore, the physicochemical redox is facilitated primarily by the elevated homocyst(e)ine during hyperhomocyst(e)inemia. The mechanism by which homocyst(e)ine causes hypertension is probably by neutralizing the redox-bioactivity of nitric oxide.[1]

High urinary protein excretion was still observed after folic acid treatment, in spite the decrease in plasma homocyst(e)ine and high creatine kinase levels in untreated SHR (Table 1). Therefore, it is likely that factors such as blood pressure and other circulating components, independent of homocyst(e)ine, may in part cause proteinurea. Since folic acid reduces homocyst(e)ine only by converting it back to methionine, these data may suggest that older SHR, having developed hypertension (32 weeks), have mechanisms of homocyst(e)ine accumulation other than folic acid deficiency. It is conceivable that the SHR model is hetero- or homozygous in the activity of cystathione β synthase (CBS) or that the reduced glomerular filtration rate is the primary mechanism of retention of plasma homocyst(e)ine in SHR. The possibility exists that early (at 2 weeks of age) treatment with folic acid may reduce both the homocyst(e)ine and blood pressure in SHR. Such studies are in progress.

Decreased nitric oxide production is associated with increased MMP activity.[28] Homocyst(e)ine decreases the bioavailability of endothelial nitric oxide.[27] In an ex vivo experiment, we previously demonstrated that homocyst(e)ine induced cardiovascular MMP.[13] In homocyst(e)inemic patients and animals, basement membranes are broken.[25] The degradation of the basement membrane implies activation of MMP.[29] Elevated plasma MMP activity is associated with increased cardiovascular injury.[30,31] Plasma spillover of MMP from various tissues can be employed as a marker of adverse ECM remodeling, tissue injury, and aortic dilatation.[32] Here we demonstrated that the levels of plasma MMP were increased in homocyst(e)ine-treated rats and that folic acid

supplementation reduces MMP activity (Fig. 1), suggesting an alteration in the ECM homeostasis in hyperhomocyst(e)inemia.

The role of neuronal MMP in ECM remodeling is unclear. However, physical connections between ECM and the nerve terminal that can release activators have been suggested.[33] MMP has been demonstrated in human central nervous system.[34] Histologically, it was found that immediately and during the first 2 days after catecholamine administration the myocardial collagenous network became disrupted and partly disappeared. The collagenase and peptidase activity was elevated.[35] Norepinephrine induces collagen expression in cardiac fibroblasts.[36] Proteinase originating from cervical sympathetic ganglia has been identified.[37] The MMP activity in homocyst(e)inemic rats was increased (Figs. 1 and 2). This may suggest a role of neuronal degeneration and adverse ECM remodeling in hyperhomocyst(e)inemia.

Creatine phosphokinase isoforms (BB, MB, MM) are induced in heart failure.[15,21] Homocyst(e)ine induces all three creatine phosphokinase isoforms (Fig. 2). These isoforms are tissue-specific, i.e., BB is primarily nerve- and kidney-specific, MM is a skeletal muscle enzyme, and MB is cardiac-specific, therefore, their expression indicates injury to the respective tissue. Our data suggest a relationship between hypertension and proteinurea in hyperhomocyst(e)inemic rats, and indicate a role of homocyst(e)ine in increased blood pressure, proteinurea, and organ damage.

Proteinolysis may, in part, mediate the activation of neutral MMP and metalloendopeptidase. A 94-kDa endopeptidase from the proximal tubule brush border, physiologically released into the urine, with apical membrane fragments, has been demonstrated.[38] The increase in urinary protein may indicate severe protein degradation and activation of neutral MMP and endopeptidase.

Both pressure and volume overload reduce myocardial capillary endothelial cell density.[39] Also, myocardial apoptosis is induced by viral infection.[40] It is apparent that homocyst(e)ine instigates endothelial cell injury in hypertension. The labeling of TUNEL-positive cells in the myocardium suggests that homocyst(e)ine induces apoptotic cascade in myocardial endothelial cells (Fig. 3).

Histological analysis of aortas, endocardium, and kidney suggested significant dilatation and fibrosis in aortas, endocardium, and renal proximal tubules. The epithelial and endothelial cells were disrupted (Fig. 4). Similar aortic lesions were observed by Matthias et al.[19] The epithelial cell hyperplasia in the renal tubule may suggest tubular hypertrophy and renal injury in hyperhomocyst(e)inemia. MMP-2 has been shown to induce growth of renal mesangial cells in situ.[41] The enlargement of the renal ureter wall (Fig. 5) was similar to the changes observed in polycystic kidney and prune belly syndromes.[42,43] The thickening of the renal-ureter wall causes urinary tract obstruction and may reduce excretion of the metabolic by-product, homocyst(e)ine. This, in turn, may cause volume retention and hypertension.

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