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Generation of Nitrotyrosine Precedes Activation of Metalloproteinase in Myocardium of Hyperhomocysteinemic Rats

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ABSTRACT

The hypothesis is that homocysteine decreases endothelial nitric oxide (NO) availability by generating nitrotyrosine. In the absence of NO, and in an attempt to reduce endocardial load by dilatation, the matrix metalloproteinase (MMP) is activated. To address this hypothesis, homocysteine (0.67 mg/ml) was administered in drinking water of Sprague–Dawley rats for 8 weeks. To elicit the reversible effects of homocysteine, homocysteine was removed from the water after 8 weeks. The plasma levels of homocysteine were 2.79 ± 0.5 μ*M* in control (*n* = 6), measured by spectrofluorometry. The levels of homocysteine increased to 22 ± 1.3 and 17 ± 2.8 μ*M* following 4 (*n* = 6) and 8 (*n* = 6) weeks of homocysteine treatment, respectively. The level of homocysteine decreased to 5.8 ± 1.0 μ*M* (*n* = 6) when homocysteine was removed from the drinking water. The mean arterial pressure (MAP) of control rats was 108 ± 10 mm Hg and increased to 128 ± 2 and 130 ± 3 mm Hg following 4 and 8 weeks of homocysteine treatment, respectively. When homocysteine was removed from the drinking water, the MAP was decreased to 118 ± 3 mm Hg. Left ventricle (LV) parameters were measured by a catheter in the LV through right common carotid artery in anesthetized rats. The LV tissue was analyzed for MMP activity by zymography. Levels of nitrotyrosine and cardiospecific tissue inhibitor of metalloproteinase-4 (TIMP-4/CIMP) were measured by western blot analysis using the respective antibodies. The specific bands in zymographic gel and western blot were scanned and normalized with β-actin. The results suggest a continuous increase in nitrotyrosine levels at 4 and 8 weeks after homocysteine administration. The removal of homocysteine did not decrease the levels of nitrotyrosine. The zymographic analysis revealed a temporal increase in MMP-2 activity from 4 to 8 weeks post homocysteine administration. However, removal of homocysteine did not decrease the MMP-2 activity. The cardiac active diastolic function, −dP/dt, was decreased at 4 weeks and stayed depressed up to 12 weeks. The end-diastolic pressure started increasing at 8 weeks; at this point the MMP-2 activity was also increased. The results suggest that in the absence of endothelial NO, and in an attempt to reduce LV load, MMP-2 is activated and CIMP is inactivated, by increasing nitrotyrosine. *Antioxid. Redox Signal.* 4: 799–804.

INTRODUCTION

HOMOCYSTEINE has emerged as a causative factor for cardiovascular disease (10). In an acute study, we have demonstrated that homocysteine impairs endocardial endothelium by decreasing the bioavailability of nitric oxide (NO) (24). In a chronic study of 4 weeks of hyperhomocysteinemia, Ungvari *et al.* (25) have demonstrated that the reduced activity of NO in arterioles may contribute to the microvascular impairment by homocysteine. The *in vivo* treatment of homocysteine for 12 weeks induces apoptosis in capillary endothelium and may reduce oxygen supply to the myocardium

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causing global ischemia (14). However, the molecular mechanism of endocardial endothelium dysfunction by homocysteine is unclear. Impairment of NO generation is the footprint of endothelial dysfunction. The ability of homocysteine to play a role in nitration of proteins is fundamentally important as a potential mechanism for regulation of protein function (29). NO, in conjunction with superoxide, forms peroxynitrite. In the presence of thiol, the peroxynitrite generates nitrotyrosine in the proteins (2, 13, 22). Because accumulation of plasma homocysteine reduces the levels of cysteine (27) and glutathione peroxidase activity (26), therefore, in hyperhomocysteinemia, homocysteine is the primary thiol in regulating redox reaction; also, the levels of Cu^{2+} ions are increased in the conditions of high homocysteine (7, 28). The Cu^{2+} ion catalyzes the formation of peroxynitrite. In culture conditions, inhibition of cytokine-induced NO synthase reduced both expression and activity of matrix metalloproteinase (MMP) (20). In contrast, cytokine inducible MMPs in immortalized cells were not modified by NO synthase inhibition (12). Oxygen species stimulate MMPs (18), and *in vivo* inhibition of NO production increases MMP activity in other tissue (17). The reasons for such diverse effects of NO on MMPs are not clear. However, a differential regulation of MMPs, release, and activation *in vivo* versus *in vitro* may account for this discrepancy. Although homocysteine activates MMP and decreases NO production, the exact mechanism of this association is unclear. We hypothesize that homocysteine activates metalloproteinase by decreasing NO availability and by generating nitrotyrosine.

MATERIALS AND METHODS

Creation of hyperhomocyst(e)inemia

The condition of chronic hyperhomocyst(e)inemia was created by adding homocyst(e)ine (0.67 mg/ml) to the drinking water of male Harlan's Sprague–Dawley rats, 275–325 g (14). Twenty rats received homocysteine. After 8 weeks of homocysteine administration, homocysteine was withdrawn from the drinking water. The protocol was further continued for 4 weeks without homocysteine. Rats at 4, 8, and 12 weeks (n = 6) were anesthetized with Inactin (100 mg/kg). This anesthesia has minimal effect on cardiovascular function (5). The hemodynamic parameters were measured as described (15). One milliliter of blood was collected by a catheter in the femoral artery. The plasma was separated and homocysteine was measured. The values of data from untreated rats at 0 and 12 weeks were used as control. The 24-h urine was collected, prior to anesthetizing, from each rat in metabolic cages. All rats were given standard rat chow and water *ad libitum*. All studies conformed with the principles of 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.

Plasma homocyst(e)ine and urinary protein

Homocysteine was measured by modification of the procedure by Frantzen *et al.* (8). In brief, the plasma was reduced by trace amounts of reduced glutathione. The homocysteine was converted to *S*-adenosyl-l-homocysteine by incubating the plasma with *S*-adenosyl-L-homocysteine hydrolase (Sigma Chemical Co.) and adenosine. The fluorescence of the incubate, *S*-adenosyl-L-homocysteine was measured at 422 nm when excited at 320 nm. The standard of *S*-adenosyl-L-homocysteine (Sigma Chemical Co.) was used as reference. Total urinary protein was measured by the Bio-Rad dye binding assay (4).

Preparation of left ventricle (LV) tissue homogenate and MMP-2 zymography

LV tissue homogenates were prepared as described (23). Because MMP-2 is present across the species, we measured MMP-2 activity in the LV of rats from the above study groups by gelatin-zymography. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis containing 1% gelatin was used as impregnated substrate for MMP (23). The gels were stained by Coomassie Blue, and lytic activity in the band was scanned by a Bio-Rad GS-700 densitometer. The lytic band intensity was normalized with β-actin band.

Nitrotyrosine, TIMP-4, and actin western blots

The levels of nitrotyrosine and cardiospecific tissue inhibitor of metalloproteinase-4 (TIMP-4/CIMP) (9) were measured by Western blot analysis using mouse monoclonal anti-nitrotyrosine antibody (Upstate Biotechnology) and rabbit anti-TIMP-4 antibody (Chemicon). The specificity of antibodies was established by immunoprecipitating the antigen, prior to loading onto the gel, by antibody-conjugated agarose beads (Upstate Biotechnology). To determine whether total proteins loaded onto the gel were identical, β-actin western blots were performed, using anti-actin antibody (Sigma Chemical Co.). The alkaline phosphatase conjugated secondary antibody was used as the detection system. Bands on blots were scanned by a Bio-Rad GS-700 densitometer.

In vivo *assessment of hemodynamic and LV parameters*

For mean arterial pressure (MAP), a fluid-filled arterial catheter (PE-50 tubing) was inserted into the right femoral artery in anesthetized rats. The arterial catheter was 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. After 10-min stabilization, the MAP and heart rate were recorded. To measure LV pressure (LVP), a catheter connected to a pressure transducer was advanced to the LV via the right carotid artery to record LVP. The pressure transducer was calibrated and electronically interfaced to a PC for analog-to-digital conversion, storage, and analysis of data. Ten minutes after insertion of the ventricular catheter, resting LV parameters were recorded. Only the data where systolic blood pressure in aortic root and LV was identical in the same animal were used. The maximum derivative of the falling LVP ($-dP/dt$) and rise ($+dP/dt$) were calculated. Because $-dP/dt$ is afterload-dependent, to correct for the afterload, $-dP/dt$ was divided by MAP (15). To determine LV

muscle strength, LVP maximum was normalized with heart weight (6). After the functional measurements, the heart was arrested in diastole by injecting intravenously 0.2 ml/100 g body weight of a 20% solution of KCl, rapidly excised, and placed in cold freshly prepared physiological salt solution. The ventricles were separated and weighed.

Statistical analysis

Data are presented as means ± SEM. Because results at 4, 8 and 12 weeks were compared with control at 0 weeks, the significance of data was tested by Student's unpaired *t* test. Control data were collected from untreated rats at 0 and 12 weeks.

RESULTS

Homocysteine, LV hypertrophy, and tissue injury

The levels of plasma homocysteine increased at 4 and 8 weeks of homocysteine administration, and stayed elevated, compared with control, after the withdrawal of homocysteine. The heart weight/body weight ratio was 3.3 ± 0.4, 5.6 ± 0.5, 4.0 ± 0.4, and 4.1 ± 0.3 × 10^{-3} for control and at 4, 8, and 12 weeks post homocysteine administration, respectively (Table 1). These data suggest LV hypertrophy in homocysteinemic rats. The levels of urinary protein excretion were increased at 4 and 8 weeks post homocysteine administration and stayed elevated after removal of homocysteine (Table 1).

Levels of nitrotyrosine

The average levels of nitrotyrosine concentration were increased approximately twofold at 4 weeks when compared with control and stayed elevated up to 8 weeks. There was no decrease in the levels of nitrotyrosine contents after removal of homocysteine at 12 weeks (Fig. 1).

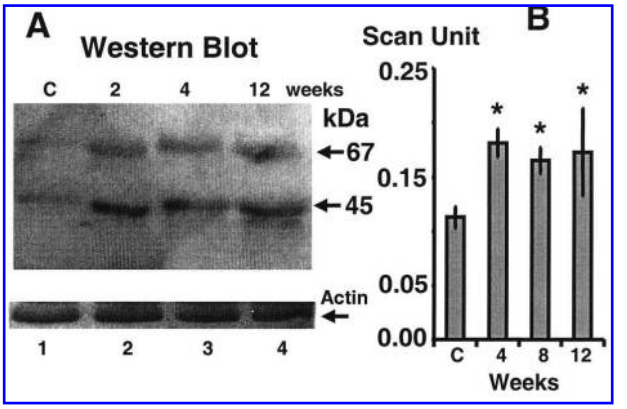

FIG. 1. (**A**) Representative western blot analysis of nitrotyrosine contents in the LV. LV tissue homogenates were prepared. Lane 1, 0-week control (C); lane 2, 4 weeks; lane 3, 8 weeks; lane 4, 12 weeks post homocysteine administration. Actin was used as reference in these blots. (**B**) The scanned means ± SEM of data from western blots are reported. Each column represents mean ± SEM of six rats. *$p < 0.005$. compared with control.

MMP-2 activity

The average value of MMP-2 activity in the LV was increased robustly at 8 weeks and stayed elevated after homocysteine withdrawal at 12 weeks. At 4 weeks, the MMP-2 activity was at its basal level (Fig. 2). The results suggested

TABLE 1

Group	*Control$_{0wk}$*	*4 wk*	*8 wk*	*12 wk*	*Control$_{12wk}$*
n	6	6	6	6	6
BW	311 ± 19	279 ± 18	302 ± 9	334 ± 11	338 ± 21
H	2.79 ± 0.5	22.0 ± 1.3*	17.0 ± 2.8*	5.81 ± 1.1*	2.93 ± 0.7
UP	0.43 ± 0.12	0.70 ± 0.21*	1.05 ± 0.085*	1.41 ± 0.58*	0.42 ± 0.11
HW	1.02 ± 0.03	1.56 ± 0.24*	1.21 ± 0.12*	1.38 ± 0.08*	1.12 ± 0.08
HW/BW (10^3)	3.3 ± 0.4	5.6 ± 0.5*	4.0 ± 0.4	4.1 ± 0.3	3.3 ± 0.03
MAP	108 ± 10	128 ± 2*	130 ± 3*	118 ± 3*	111 ± 8
SP	125 ± 4	146 ± 8*	146 ± 7*	134 ± 11*	126 ± 3
DP	90 ± 6	110 ± 5	112 ± 8	98 ± 7	91 ± 4
HR	337 ± 12	351 ± 27	353 ± 23	341 ± 16	335 ± 19
LVPmax	122 ± 8	128 ± 6	128 ± 9	121 ± 12	123 ± 8
EDP	3.8 ± 0.8	3.7 ± 0.5	5.1 ± 0.4*	7.5 ± 0.8*	3.5 ± 0.6
−dP/dt	7965 ± 121	5358 ± 90*	6815 ± 175*	6113 ± 225*	8163 ± 90
(−dP/dt)/MAP (s)	83 ± 12	49 ± 4*	52 ± 5*	48 ± 6*	74 ± 3
LVPmax/HW mm Hg/g)	120 ± 17	82 ± 9*	105 ± 28	87 ± 23*	110 ± 8

Plasma homocysteine (H; μmol/L), urinary protein (UP; mg/day/kg), body weight (BW; g), and heart weight (HW; g) were measured. The mean arterial pressure (MAP; mm Hg), systolic blood pressure (SP; mm Hg), diastolic blood pressure (DP; mm Hg), and heart rate (HR; beats/min) were measured by PE-50 catheter in the left femoral artery. Left ventricle pressure maximum (LVPmax; mm Hg), end-diastolic pressure (EDP; mm Hg), and first derivative of fall in systolic pressure (−dP/dt, mm Hg/s) were measured by a catheter in the LV via right carotid artery. Means ± SEM are reported.

*$p < 0.05$ when compared with control at 0 wk of homocysteine treatment.

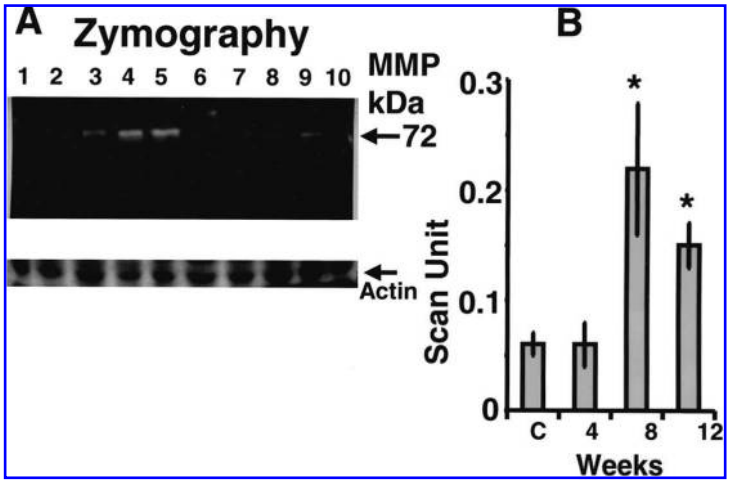

FIG. 2. (**A**) Representative zymographic analysis of MMP-2 activity in the LV. Lanes 1 and 2, 4-week; lanes 3 and 4, 8-week; lanes 5 and 6, 12-week period post homocysteine administration; lanes 7 and 8, 0-week control (C); and lanes 9 and 10, 12 week control (C). Actin from these blots was used as reference. (**B**) The scanned means ± SEM of data from zymographic gels are reported. Each column represents mean ± SEM of six rats. *$p <$ 0.01, compared with control.

increase MMP-2 activity between 4 and 8 weeks during homocysteine administration.

TIMP-4

The average levels of cardiospecific TIMP-4 were increased at 4 weeks and decreased at 8 weeks when compared with control. There was no significant change in the levels of TIMP-4 at 12 weeks of homocysteine withdrawal (Fig. 3). These results suggested temporal changes in the levels of TIMP-4 by homocysteine administration.

MAP

The MAP was increased 20 mm Hg at 4 weeks post homocysteine administration and stayed elevated up to 8 weeks. The removal of homocysteine did not decrease the MAP to control levels (Table 1).

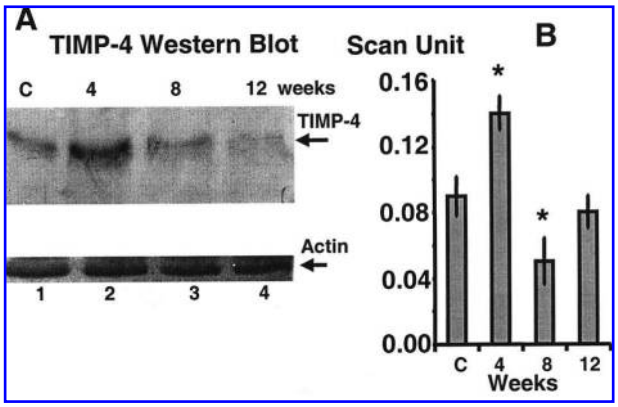

FIG. 3. (**A**) Representative western blot analysis of cardiospecific TIMP-4 in the LV. Lane 1, 0-week control (C); lane 2, 4 weeks; lane 3, 8 weeks; lane 4, 12 weeks post homocysteine administration. Actin from these blots was used as reference. (**B**) The scanned means ± SEM are reported from western blots. Each column represents mean ± SEM of six rats. *$p <$ 0.05, compared with control.

LV function

The cardiac muscle strength (LVP maximum/heart weight) was decreased at 4 weeks as compared with control, and the active diastolic LV function was decreased at 4 weeks post homocysteine administration and stayed depressed up to 12 weeks after removal of homocysteine (Table 1). The LV end-diastolic pressure (EDP) was increased at 8 weeks post homocysteine administration and stayed elevated up to 12 weeks (Fig. 4, and Table 1). These results suggest differential regulation of LV systolic and diastolic function by homocysteine.

DISCUSSION

The results of this study support the notion that the induction of oxidative stress and increased nitrotyrosine formation lead to activation of MMP in the LV of homocysteinemic rats. The removal of homocysteine from drinking water does not reverse the injury caused by chronic homocysteine administration. Because previous studies have demonstrated renal tubular hyperplasia by homocysteine (14), therefore, we may suggest that the increase in the excretion of urinary protein is associated, in part, with renal injury. The loss of cardiac muscle strength is correlated with increased nitrotyrosine genera-

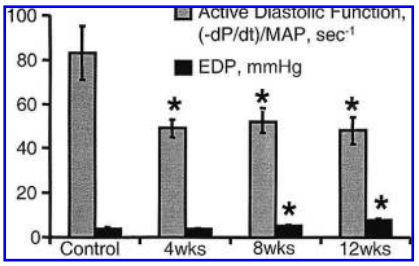

FIG. 4. Active diastolic function, (−dP/dt)/MAP, and EDP. A fluid-filled PE-50 catheter was inserted into the LV, and LV parameters were measured. Each column represents mean ± SEM of six rats. *$p <$ 0.05, compared with control.

tion. The increased EDP was associated with increased MMP-2 activity and decreased levels of cardiospecific TIMP-4.

The plasma levels of homocysteine increase within days after homocysteine administration in the drinking water. The levels of homocysteine at 8 weeks decrease slightly as compared with 4 weeks. This may suggest uptake of homocysteine by tissue. In fact, we previously demonstrated increased levels of homocysteine in the cardiac, aortic, and renal tissue post homocysteine administration (14). The removal of homocysteine from drinking water did not reverse completely the levels of plasma homocysteine and also the levels of proteinurea caused by homocysteine did not reverse after withdrawal of homocysteine (Table 1).

The levels of nitrotyrosine increase at 4 weeks, whereas MMP-2 activity increases at 8 weeks post homocysteine administration. Therefore, it appears that nitrotyrosine generation (Fig. 1) occurs prior to the activation of MMP-2 (Fig. 2). This is consistent with the notion that a decrease in NO availability leads to increased MMP-2 activity. Nitrotyrosine remains elevated despite reduction in plasma homocysteine. The study further elucidates the mechanism by which homocysteine generates nitrotyrosine in the sense that decreased NO availability by homocysteine is associated with generation of nitrotyrosine and MMP activation. Under these conditions, there was no induction of MMP-9. It is true that MMP-13 is interstitial collagenase in rodents. However, MMP-2 is present across the species; it is a good interstitial collagenase (1) as well as degrades elastin (21). Therefore, we measured MMP-2. Because under this condition there was no change in the levels of β-actin, to normalize the MMP-2 activity, we used β-actin as reference. The levels of TIMP-4 were increased at 4 weeks and decreased at 8 weeks (Fig. 3). This may suggest that initially the levels of TIMP-4 are increased, presumably, due to compensatory response to homocysteine overloading and oxidative stress caused by homocysteine. The levels of TIMP-4, however, decreased at 8 weeks, suggesting a role of MMP activation and TIMP-4 inactivation, causing collagenolysis post homocysteine administration. The levels of TIMP-4 returned to the control value post homocysteine withdrawal. These results may suggest the differential role of nitrotyrosine, MMP-2, and TIMP-4 post homocysteine administration in cardiac remodeling, structure, and function.

The arterial pressure was increased within 4 weeks post homocysteine administration (Table 1). The removal of homocysteine did not decrease the MAP to basal levels, suggesting a role of homocysteine in the alteration of vascular structure and function. Previously, others and we have demonstrated that homocysteine causes alterations in the structure and function of the vessel wall and reduces lumen diameter (14, 19). This may suggest that arterial damage caused by homocysteine is irreversible, and this causes persistent vascular resistance and blood pressure. The combination of results on nitrotyrosine levels and MAP suggests an association between formation of nitrotyrosine and increased MAP.

The LV muscle strength was decreased within 4 weeks post homocysteine administration, and the active diastolic function was decreased (Table 1). This may suggest a link between decreased cardiac muscle strength and generation of nitrotyrosine with increased levels of TIMP-4. However, EDP was not affected until 8 weeks post homocysteine administration (Fig. 4 and Table 1). These results may suggest that in the absence of NO, and in an attempt to reduce LV load, activation of MMP causes LV dilatation. The increased levels of MMP/TIMP have been demonstrated in human heart systolic and diastolic dysfunction (11). Previous studies from our laboratory in a load-free aortic culture condition demonstrated that homocysteine induces the generation of nitrotyrosine and activation of metalloproteinase (16). Therefore, it may suggest that temporal changes in the levels of nitrotyrosine and activation of MMP in hyperhomocysteinemia are probable causes of temporal alteration in hemodynamic parameters. Previously, we (23) and Bescond *et al.* (3) have demonstrated direct activation of MMP by pathophysiological concentration of homocysteine. Here we suggest that due to the antioxidant nature of NO, MMP is in the latent form. However, during high homocysteine and oxidative conditions, the labile residues, tyrosine, and arginine in MMP or in other protein/ligand are nitrated via the formation of peroxynitrite leading to the activation of MMP (Fig. 5).

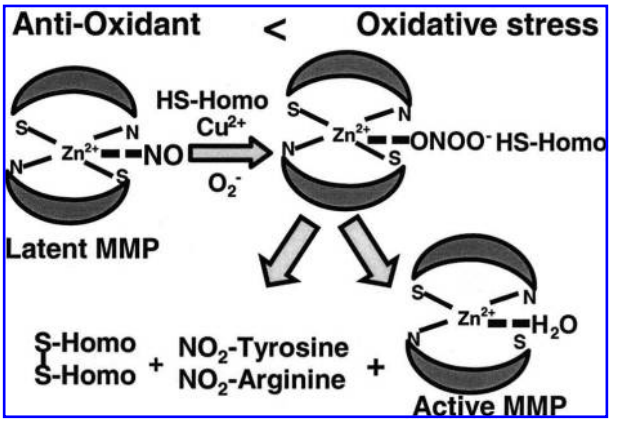

FIG. 5. A plausible mechanism of formation of nitrotyrosine, nitroarginine and activation of latent resident MMP by oxidative stress caused by homocysteine. HS-Homo, homocysteine; Homo-S-S-Homo, homocystine; $ONOO^-$, peroxynitrite.

ACKNOWLEDGMENTS

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ABBREVIATIONS

CIMP, cardiac inhibitor of metalloproteinase; EDP, end-diastolic pressure; LV, left ventricle; LVP, left ventricle pressure; MAP, mean arterial pressure; MMP, matrix metalloproteinase; NO, nitric oxide; TIMP, tissue inhibitor of metalloproteinase.

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