Attenuation of Oxidative Stress and Remodeling by Cardiac Inhibitor of Metalloproteinase Protein Transfer

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**Background**—Matrix metalloproteinase (MMP) and cardionic inhibitor of metalloproteinase (CIMP) are coexpressed in the heart. Although it is known that oxidative stress activates MMP and CIMP inhibits MMP, it is unclear whether CIMP administration attenuates oxidative stress and MMP-mediated cardiac dilatation.

**Methods and Results**—Arteriovenous fistula (AVF) was created in C57BL/J6 mice, and CIMP was administered to AVF and sham mice by protein transfer into peritoneal cavity by minipump for 4 weeks. Mice were grouped as follows: sham; sham+CIMP; AVF; and AVF+CIMP (n=6). In vivo left ventricular (LV) pressure was measured. Plasma and LV tissue levels of CIMP were measured by Western analysis. LV levels of NADPH oxidase activity, marker of oxidative stress, were increased in AVF mice and decreased in AVF mice treated with CIMP. Compared with sham, CIMP was decreased in AVF mice, and CIMP protein transfer increased plasma and LV tissue levels of CIMP in AVF mice; there was no increase in sham animals. In situ zymography demonstrated a robust increase in MMP activity in the hearts from AVF mice compared with sham, and treatment with CIMP decreased MMP activity. In AVF mice, the cardiac pressure-length relationship was similar to that observed in sham mice after administration of CIMP. Contractile responses of normal LV rings were measured in the presence and absence of CIMP. CIMP shifted the pressure-length relationship to the left, attenuated LV dilatation, and had no effect on CaCl2-mediated contraction.

**Conclusions**—Treatment of AVF mice with CIMP significantly abrogated the contractile dysfunction and decreased the oxidative stress in volume overload–induced heart failure. (Circulation. 2004;109:2123-2128.)

| Key Words: | proteomics ■ nitric oxide ■ NADPH oxidase ■ relaxation ■ heart failure |

Extracellular matrix (ECM) surrounds and tethers individual myocytes and myofilaments, enabling optimal transduction of coordinated force generated by cardiac contraction. Indeed, the structure and function of cardiomyocytes are altered by neutral matrix metalloproteinase (MMP)–mediated degradation of ECM. Although MMPs were activated and the levels of cardiac inhibitor of metalloproteinase (CIMP) were decreased in congestive heart failure (CHF),1–4 the mechanism(s) responsible for these changes is unsubstantiated. Studies have shown that chronic volume overload increases oxidative stress.5–6 Superoxide (O2-) (generated, 2O2-+2H+O2=2H2O + O2•-), dependent and independent of inflammatory or mitochondrial NADPH oxidase7) masked the activity of superoxide dismutase and catalase8–10 and decreased endothelial nitric oxide (NO) availability.11 Both in vivo and in vitro, oxidative stress activates latent resident myocardial MMPs1–4 and increases the levels of cytokines, growth factors, and neurohormones.12–14 A vicious cycle of oxidative stress ensues, in which neurohormones such as angiotensin II15 further increase oxidative stress by lowering the levels of the antioxidants NO-producing bradykinin and prostaglandin.16 In parallel, angiotensin II induces NADPH oxidase.17 In addition, reactive oxygen species (ROS) inactivate tissue inhibitors of metalloproteinase (TIMPs).18 Although ROS are increased in CHF, it is unclear whether increases in ROS caused the increases in MMP activity by attenuating the levels of CIMP.

**Methods**

**Animals**

Wild-type, C57BL/6J, male mice aged 8 to 12 weeks were obtained from Jackson Laboratories (Bar Harbor, Maine). An arteriovenous fistula (AVF) was created in tribromoethanol-anesthetized19 mice (100 mg/kg IP; n=24) between the aorta and the caudal vena cava approximately 1 cm below the kidney with the use of a 30-gauge needle.20 Mice were divided into 4 groups: (1) sham; (2) sham plus CIMP; (3) AVF; and (4) AVF plus CIMP (n=6 in each group). CIMP was purified from mouse hearts in milligram quantity21 and infused by an ALZET micro-osmotic pump model 1002 at 0.25 μL/h, 10 μg/d in the intraperitoneal cavity. Because the binding constant between MMP and TIMP is in the nanomolar range,22 this amount saturates all the binding sites in MMPs. Oxidative stress produced by the fistula and proteolytic activation track together; therefore, to avoid early activation of MMP, CIMP was administered 1 hour before creation of fistula. Because the heart was well compensated after 4 weeks of chronic volume overload due to AVF, mice were anesthetized at 4 weeks. A PE-10 catheter was inserted into common carotid artery and advanced to the left ventricle (LV), and pressure was measured with a Micro-Med pressure transducer.
After 10 minutes of stabilization, maximum LV pressure (LVP\text{max}), its peak time derivatives \( \pm dP/dt\text{max} \), and LV end-diastolic pressure (EDP) were recorded. The heart was excised under deep anesthesia. Lung and body weights were measured, LV and right ventricle (RV) were separated and weighed, and LV rings were prepared. To measure NO, ROS, and NADPH oxidase, fresh LV tissue was homogenized.

**Ex Vivo Study**

Cardiac muscle function determined in isolated papillary muscle preparation does not represent function in the entire transmural myocardial wall, and Langendorff preparations do not differentiate the specific contributions of regional ischemia, hypertrophy, stunning, and/or hibernation and RV function. To address these limitations, we examined function in cardiac rings. Preparations from hypertensive rats\(^2\) produced pressure-volume curves similar to those obtained in the Langendorff preparation.\(^2\) Rings can be prepared to include or exclude the homogeneous or inhomogeneous regions of the transmural myocardial wall\(^2\) and to distinguish regional differences in contractile function. The “donut”-shaped LV rings were mounted by 2 wires in a tissue myobath\(^2\),\(^2\) containing different doses of CIMP. One of the 2 wires was connected to a force transducer. The ring was stretched and brought to resting tension (measured in grams), and 20 mmol/L CaCl\(_2\) was added to the bath. To keep the spherical shape of the ring, a pediatric esophagus balloon was placed inside the ring. The tension in grams was converted to dynes per square centimeter of ring tissue, and the tension generated in grams was converted to atmospheric pressure (mm Hg). For cardiac dilatation, after contraction to 20 mmol/L CaCl\(_2\), active MMP-2 was added. The percent dilatation was based on 100% CaCl\(_2\) contraction. To minimize differences due to geometric alignment of the different muscle layers in the endomyocardium, midmyocardium, and epimyocardium, the rings were rotated \(90^\circ\); the contractile data were repeated, and the average of the 2 was recorded. The bubbling of oxygen at 20 psi was sufficient to avoid ischemia up to 40 minutes as measured by release of creatine phosphokinase–MB, a cardiac muscle–specific enzyme released during injury.\(^2\) The drug was also diffusible at this pressure.\(^2\) To minimize differences due to the weight of the ring, the tension in grams was normalized to the weight of the ring in grams.

**LV Levels of NO, ROS, and NADPH/NAD Oxidase**

Concentration of total LV NO was estimated by measurements of total nitrate/nitrite. Oxidative stress was assessed by measuring LV ROS by incubating LV tissue homogenates with 2,7’-dichlorofluorescein (DCFH); although generation of O\(_2^-\) is transient, O\(_3^-\) and H\(_2\)O\(_2\) (2OH\(^-\)) are stable. DCFH acquires fluorescence properties on reaction with ROS and yields fluorescent product dichlorofluorescein. This product was detected by a 530-nm emission when excited at 485 nm. Levels of NADPH oxidase were measured.\(^2\)

**Western Blot Analysis of CIMP**

Levels of CIMP in plasma and LV tissue homogenates were measured by Western blot analysis under reducing conditions.

**In Situ MMP Activity**

To determine cardiac hypertrophy, fibrosis, and dilation, LV tissue sections were stained with trichrome blue. Myocyte size was measured by a micrometer. Fibrosis was measured by estimating blue stain (arbitrary unit per centimeter). Because MMP/CIMP complex is dissociated in SDS-PAGE zymography, in situ zymography was performed to measure total MMP activity.\(^2\) Collagen degradation was measured by incubation with 5 \(\mu\)mol/L MMP-2 for 0.5 and 5 minutes.\(^2\)

**Statistical Analysis**

Values are given as mean±SEM (n=6 in each group). Differences between groups were evaluated by ANOVA, followed by the Bonferroni post hoc test, focusing on the effects of volume overload (sham mice compared with AVF mice [asterisk]) and treatment (AVF+CIMP-treated mice compared with AVF mice [double asterisk]). \(P<0.05\) was considered significant.

**Results**

**Oxidative Stress in CHF**

Levels of NO were decreased and levels of ROS were increased in AVF hearts compared with sham controls. Treatment with CIMP increased NO and decreased ROS. Levels of NADPH oxidase were substantially increased in AVF mice compared with sham and were decreased with CIMP treatment (Table).

**In Vivo CIMP Administration: Plasma and LV Levels of CIMP**

CIMP was purified to homogeneity from normal mouse hearts. Histological analysis revealed perivascular and interstitial fibrosis and myocyte hypertrophy in LV of AVF mice (Figure 1, Table). LV weight was significantly increased in AVF hearts compared with sham and was decreased in AVF mice treated with CIMP (Table). Administration of CIMP by protein transfer into the peritoneal cavity increased plasma levels of CIMP in AVF mice. The addition of CIMP to sham mice did not further increase plasma levels of CIMP (Figure 2). Basal levels of CIMP were much greater in the LV than the plasma, and the levels of CIMP in the LV were increased by CIMP administration (Figures 2 and 3). There were no further increases in CIMP levels in the LV of sham animals (Figure 3). These results suggest a threshold level of CIMP in plasma and LV of normal mice.

**Total MMP Activity**

LV gelatinolytic activity was significantly increased in AVF compared with sham controls (Figure 4). Treatment with CIMP in AVF mice ameliorated the activation of MMP.

**In Vivo Protective Role of CIMP in LV Stress**

The slope of the pressure-length relationship was decreased in AVF mice compared with sham controls. Treatment with CIMP increased the slope in AVF mice (Figure 5). The force generated (in dynes per square centimeter of tissue) was reduced in AVF hearts and ameliorated by CIMP.

**Effect of CIMP on In Vivo Systolic and Diastolic Function**

LV systolic force, ie, LVP\text{max}/LV weight, was decreased in AVF hearts compared with sham, and the levels of EDP were increased. Treatment with CIMP ameliorated the abnormal pressure–derived indices of cardiac systolic and diastolic dysfunction in AVF mice (Table). LV chamber size was enlarged in AVF mice. Treatment with CIMP decreased this enlargement (Table).

**Ex Vivo CIMP Effect on Cardiac Stress and Contraction in Normal Hearts**

In normal cardiac rings, CIMP shifted the stretch-strain relationship to the left (Figure 6). The dose-response curves of CaCl\(_2\)-mediated contraction demonstrated no difference in contraction in the presence versus absence of CIMP.
Ex Vivo MMP-2–Induced Cardiac Dilatation Was Inhibited by CIMP

To determine whether MMP-2 induces cardiac dilatation, active myocardial MMP-2 (5 μmol/L) was added to normal rings in a myobath. Within 0.5 minute, there was significant collagen disruption (Figure 7) and cardiac dilation. Moreover, the increases in cardiac dilatation by MMP-2 were dose dependent, and the addition of CIMP ameliorated cardiac dilation (Figure 7).

Discussion

Results of the study show that administration of CIMP by protein transfer ameliorates cardiac dysfunction secondary to a decrease in oxidative and proteolytic stresses after chronic volume overload–induced heart failure. Several mechanisms
may be responsible for the reduction of oxidative stress by CIMP. First, CIMP inhibited MMP and consequently preserved ECM connections and increased cardiac muscle synchronized strength. Second, CIMP induced NO in endocardial endothelial cells and increased cardiac muscle relaxation. In addition, increased NO decreased MMP activity. Third, CIMP decreased NADPH oxidase in endocardial endothelial cells, and, finally, CIMP behaved like an oxidative sink by generating nitrotyrosine.

Differential cellular functional roles of the TIMPs have been suggested. For example, TIMP-1 has been shown to demonstrate antimitogenic activity. We previously have shown that TIMP-1 has proliferative activity in endothelial cells. In addition, TIMP-2 has been shown to be a growth-stimulatory protein for transformed fibroblasts. Baker et al demonstrated that TIMP-3 induced apoptosis in vascular smooth muscle cells and regression of neointimal growth. CIMP (TIMP-4) induced apoptosis in transformed cardiac fibroblast cells and did not affect normal cells. Inhibitors of neutral proteinases are sensitive to oxidative inactivation. In normal physiological conditions, a critical balance between neutral serine proteinase inhibitor and metalloproteinase inhibitor is maintained. During inflammatory pathogenesis, oxidants disrupt this balance. Serine proteinases degrade the inhibitors of metalloproteinase, and metalloproteinases degrade the inhibitors of serine proteinase. Therefore, a

Figure 3. LV tissue levels of CIMP. AVF was created in normal wild-type mice. Sham surgery was performed in control group. AVF (AV) and sham mice were administered CIMP by intraperitoneal minipump for 4 weeks. LV tissue levels of CIMP were measured by quantitative 10% SDS-PAGE Western blot analysis. A, Lane 1, AVF mice; lane 2, AVF mice plus CIMP; lane 3, sham; lane 4, sham plus CIMP. Levels of actin were used as control. B, Histogramic presentation of scanned data for LV CIMP. *P = 0.005; **P = 0.03 (n = 6).

Figure 4. In situ zymography of LV rings. A, Ring from AVF group at 4 weeks; B, ring from AVF+CIMP group at 4 weeks; C, ring from sham mice. Arrows indicate inner side (endocardium) of LV. Results suggest that there is more MMP activity in the endocardium of AVF hearts than sham and that CIMP inhibits this MMP activation. Cardiac rings after function measurements were laid on top of 1% gelatin containing acrylamide gels. Gels with LV ring were incubated for 18 hours at 37°C. Gels were stained for lytic activity. Magnification X40. D, Scanned data of MMP activity in arbitrary units [AU] of in situ zymography. Each bar represents mean ± SEM from n = 6 in each group. The treatment of rings, taken from AVF mice, used for in situ zymography in the presence of EDTA abolished the lytic activity, but not by phenylmethylsulfonyl fluoride, suggesting MMP activation in LV of AVF mice. *Sham to AVF mice; **AVF+CIMP treatment mice compared with AVF mice.

Figure 5. A, LV pressure-length relationship. The ring in a myobath was stretched with sequential increments (mm). The corresponding tension, converted to atmospheric pressure (mm Hg), was recorded. Rings were prepared from AVF mice, AVF mice treated with CIMP for 4 weeks, sham mice, and sham mice treated with CIMP. B, Histogramic presentation of tension of in AVF, AVF + CIMP; sham, and sham + CIMP mice. Each bar represents mean ± SEM (n = 6). *Sham to AVF mice; **AVF+CIMP treatment mice compared with AVF mice.

Figure 6. Effect of CIMP on cardiac stretching, relaxation, and contraction. LV rings from normal mice were prepared. Rings were stretched to 60% of length, then relaxed to 25% of resting tension in a myobath. Contraction was induced by adding 20 mmol/L CaCl2 in the bath. Three separate baths were used for 3 separate rings. Each bath contained PSS (25 mL) and 0, 10, and 20 µmol/L CIMP, respectively. All experiments were performed at 37°C in circulating water-jacketed bath. The tension in grams was recorded continuously during the experiment.
vicious proteolytic cascade is initiated during oxidative stress. CIMP has residues (tyrosine and cysteine) that can perturb oxyradicals and oxidative stress and that impair its ability to inhibit metalloproteinase. Our results suggest that CIMP reduced the oxidative stress in AVF mice by decreasing ROS and NADPH oxidase and increasing NO. The in vivo CIMP protein transfer increased CIMP in the myocardium and attenuated volume overload-mediated cardiac remodeling.

O’Brien and Moore suggested that collagen degradation leads to a shift in cardiac pressure-volume curves in normal hearts. In human CHF, baseline LV hypertrophy was associated with increases in the serum levels of collagen-derived peptides. Decreased levels of TIMPs were also associated with hypertrophic and stunned myocardium. Previously, we demonstrated that metalloproteinases dilated and decreased cardiac tensile stress. To determine whether the activation of MMP played a significant role in the development of LV hypertrophy and wall stress, and especially to determine whether LV hypertrophy will normalize wall stress and cardiac dilatation will increase wall stress, it was important to inhibit oxidative stress and myocardial MMP. In this regard, CIMP inhibited ECM disruption and preserved myocardial contractility. CIMP may also increase NO and improve LV relaxation, resulting in a decrease in EDP. To determine whether in vivo CIMP treatment ameliorates MMP-mediated cardiac dilatation, CIMP was administered to AVF mice. The levels of CIMP in AVF mice were increased to normal levels after CIMP protein transfer in plasma as well as in LV tissue. Previously, we have shown increased MMP-2 and -9 activity in heart failure, including AVF, and more recently increased MMP-2 and -9 in atrial failure. Total MMP activity was reduced in the LV of AVF mice after CIMP administration, and LV diastolic function was improved. Although we did not measure anatomic collagen breaks, our results suggest that diastolic dysfunction and cardiac remodeling were reversed by CIMP in chronic volume-overloaded AVF mice. Reversal of AVF-induced decrease in cardiac force is consistent with the notion that CIMP prevented ECM disruption around myocytes and preserved the synchronization of cardiac muscle strength as well as relaxation. In vitro, we demonstrated that the addition of active MMP-2 caused cardiac dilatation, and CIMP inhibited MMP-mediated cardiac dilatation, in part by decreasing oxidative stress.

Potential Limitations
In AVF, increased or nearly normal collagen content has been demonstrated. The seeming paradox that fibrosis, increased MMP activity, and decreased TIMP-4 track together can be explained by the following scenario. During increases in load, particularly with reductions of endothelial NO synthase, latent MMPs are activated and dilate the heart. The LV compensates by developing hypertrophy and rearranging the ECM. The media of the vessel and basement membrane of capillary endothelium containing substantial amounts of elastin and ultrastructural collagen are responsible for interstitial connections. Constitutively expressed MMP-2 and inducible MMP-9 degrade elastin as well as ultrastructural (ie, newly synthesized) collagen efficiently. Because turnover of ultrastructural collagen and elastin is remarkably lower than that of oxidized collagen, degraded ultrastructure collagen and elastin are replaced by oxidatively modified stiffer collagen.

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References
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