Matrix Metalloproteinase-2 Contributes to Ischemia-Reperfusion Injury in the Heart

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Background—Matrix metalloproteinases (MMPs) contribute to collagen degradation and remodeling of the extracellular matrix after myocardial infarction; however, their role in myocardial dysfunction immediately after ischemia and reperfusion is unknown.

Methods and Results—We measured the release of MMPs into the coronary effluent of isolated, perfused rat hearts during aerobic perfusion and reperfusion after ischemia. Aerobically perfused control hearts expressed pro-MMP-2 and MMP-2, as well as an unidentified 75-kDa gelatinase. These enzymes were also detected in the coronary effluent. After 20 minutes of global no-flow ischemia, there was a marked increase in pro-MMP-2 in the coronary effluent that peaked within the first minute of reperfusion. The release of pro-MMP-2 into the coronary effluent during reperfusion was enhanced with increasing duration of ischemia and correlated negatively with the recovery of mechanical function during reperfusion ($r^2 = 0.99$). MMP-2 antibody (1.5 to 15 μg/mL) and the inhibitors of MMPs doxycycline (10 to 100 μmol/L) and $\alpha$-phenanthroline (3 to 100 μmol/L) improved whereas MMP-2 worsened the recovery of mechanical function during reperfusion.

Conclusions—These results show that acute release of MMP-2 during reperfusion after ischemia contributes to cardiac mechanical dysfunction. The inhibition of MMPs may be a novel pharmacological strategy for the treatment of ischemia-reperfusion injury. (Circulation. 2000;101:1833-1839.)

Key Words: ischemia ■ reperfusion ■ metalloproteinases ■ myocardium

Matrix metalloproteinases (MMPs) belong to the zinc-containing endopeptidases.1 The MMPs are synthesized in a latent form (zymogen or pro-MMP) and are activated by proteolytic cleavage of an amino-terminal domain2 or conformational changes such as those induced by oxidative stress.3 They are involved in the remodeling of the extracellular matrix in tissue during various physiological and pathological conditions, such as embryonic development, inflammation, and cancer.4 Extracellular matrix is important in the structure as well as the function of the heart and vascular tissues.5 After myocardial infarction, increased activity of MMPs may be responsible for the degradation of extracellular matrix.6,7 Uprogulation of myocardial MMPs has also been associated with fetal development, ventricular remodeling in heart failure, and in neointima formation after arterial injury.8–10

Among the metalloproteinases, MMP-2 (pro-MMP-2, 72 kDa; active enzyme: MMP-2, 62 kDa) and MMP-9 (pro-MMP-9, 92 kDa; active enzyme: MMP-9, 84 kDa) have been found in endocardial and subendocardial layers and interstitial tissue.11 Both are involved in the degradation of collagen type IV, a major component of the basement membrane. Changes in MMP activity or expression that affect matrix remodeling occur on the time scale of hours to days. However, these enzymes may also stimulate cellular transduction processes in a rapid way before changes in the collagen matrix occur.12 Indeed, we have previously identified MMP-2 in human platelets13 and found that MMP-mediated platelet aggregation results in a rapid translocation (within seconds) and release of this enzyme from platelets.13,14 Thus, MMP-2 release in platelets mediates an acute signaling response in platelets that results in their aggregation. Therefore, we wished to investigate whether there could be a role for MMPs in the heart in the setting of acute myocardial ischemia-reperfusion injury.

Methods

This investigation conforms to the Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care (revised 1993).

Materials

Male Sprague-Dawley rats (250 to 300 g) were used for the experiments. Polyclonal MMP-2 antibody and purified rabbit IgG (negative control for the MMP-2 antibody) were prepared as de-
scribed.14 The supernatant from phorbol ester–activated human fibroblast HT1080 cells (American Type Culture Collection), which contains large amounts of pro-MMP-2, MMP-2, pro-MMP-9, and MMP-9, was used as a standard. All other reagents were purchased from Sigma.

A semipurified preparation of MMP-2 from HT1080 cell–condi-
tioned medium was prepared in bulk by affinity chromatography with gelatin-sepharose.15 The protein content was estimated by ultraviolet spectrometry at 280 nm.

Heart Perusions

Hearts were rapidly excised from pentobarbital-anesthetized rats and briefly rinsed by immersion into ice-cold Krebs-Henseleit buffer. Spontaneously beating hearts were perfused via the aorta at a constant pressure of 60 mm Hg with Krebs-Henseleit buffer at 37°C as previously described.16

A water-filled latex balloon connected to a pressure transducer was inserted into the left ventricle through an incision in the left atrium and through the mitral valve, and the volume was adjusted to achieve an end-diastolic pressure of 8 to 12 mm Hg. Heart rate and left ventricular pressure were monitored on a polygraph. The RPP was calculated as the product of heart rate and left ventricular developed (systolic minus end-diastolic) pressure. Coronary flow was measured with an in-line ultrasonic flow probe (Transonic Systems Inc) positioned proximal to the perfusion cannula. Hearts maintained a steady state of coronary flow, heart rate, and left ventricular developed pressure for at least 80 minutes after stabilization as previously reported.16

Ischemia and Reperfusion Protocol

After 25 minutes of aerobic perfusion, hearts were subjected to 15, 20, or 25 minutes of global no-flow ischemia induced by clamping of the aortic inflow line. This was followed by 30 minutes of aerobic reperfusion when the clamp was reopened. Coronary effluent (6 mL) was collected for determining MMP activities at times ending at 25 minutes of aerobic perfusion and at 1, 2, 5, 10, 20, and 30 minutes of reperfusion. Because equal volume samples were collected, the period of time required to collect samples varied during early reperfusion. The average time required to collect the coronary effluent samples was 26 seconds for the aerobic perfusion sample and between 26 and 60 seconds for the reperfusion samples. The samples were stored at 4°C and processed on the same day.

Preparation of Heart Extracts

Hearts were freeze-clamped and crushed at liquid N2 temperature and then homogenized by sonication in 50 mmol/L Tris-HCl (pH 7.4) containing 3.1 mmol/L sucrose, 1 mmol/L DTT, 10 μg/mL leupeptin, 10 μg/mL soya bean trypsin inhibitor, 2 μg/mL aprotinin, and 0.1% Triton X-100. The homogenate was centrifuged at 10 000 g for 10 minutes, and the supernatant was collected and stored at −80°C until use.

Modulation of Ischemia-Reperfusion by MMP-2 or Its Inhibitors

In some experiments after 15 minutes of aerobic perfusion, either semipurified MMP-2 (100 ng/mL) or MMP-2 antibody (1.5 to 15 μg/mL), doxycycline (10 to 100 μmol/L)17 or o-phenanthroline (3 to 100 μmol/L),17 or o-phenanthroline (100 μmol/L), or phosphoramidon (20 μmol/L)13,14 was infused for the last 10 minutes of aerobic perfusion. The solutions were infused at a constant rate of 0.1 mL/min, and the concentrations of the stock solutions were prepared on the basis of an average coronary flow of 14 mL/min. The vehicles for the test agents were as follows: semipurified MMP-2, 17 mmol/L NaCl; MMP-2 antibody, Krebs-Henseleit buffer solution of rabbit IgG (15 μg/mL); doxycycline and phosphoramidon, water; and o-phenanthroline, aqueous DMSO such that the concentration of DMSO reaching the heart was <0.2% (vol/vol).

Measurement of MMPs by Zymography

Samples of coronary effluent (6 mL) were concentrated ~30-fold in volume in Centricon 10 concentrating vessels (5000g, 4°C, Amicon Inc) and analyzed for gelatinolytic activity by zymography.13,14 To investigate the inhibitory profile of some reagents on the gelatinolytic activities of MMPs, o-phenanthroline (100 μmol/L), doxycycline (10 to 100 μmol/L), or phosphoramidon (20 μmol/L) was added to the incubation buffer during the overnight incubation in some experiments. To quantify the activities of the detected enzymes, zymograms were digitally scanned, and the band intensities were analyzed14 and expressed as a specific activity per milligram protein in the coronary effluent.

Western Blot Analysis

Protein (40 μg) from heart extracts prepared as above was applied to 7% polyacrylamide gels. Electrophoresis was carried out under reducing conditions.20 After electrophoresis, samples were electroblotted onto polyvinylidene difluoride membranes (Schleicher and Schuell) and probed with MMP-2 antibody (1 μg/mL).14

Determination of Neutralizing Activity of MMP-2 Antibody

Human recombinant MMP-2 (10 ng; Oncogene) was preincubated for 15 minutes at 37°C with 2 μg of MMP-2 antibody in 60 μL of 50 mmol/L Tris-HCl buffer (pH 7.6) containing 5 mmol/L CaCl2 and 150 mmol/L NaCl. Gelatin (150 ng: prepared from fetal bovine skin collagen type I, a gift from Dr Paul Scott, University of Alberta) was added, and the mixture was incubated for 60 minutes at 37°C. Gelatin degradation products were analyzed by SDS-PAGE under nonreducing conditions as mentioned above. The protein bands were visualized by silver staining.21

Protein Assay

Protein concentrations were measured either by the Bradford protein assay (BioRad) or by the bicinchoninic acid assay (Sigma) with BSA as a standard.

Statistical Analysis

Data are expressed as mean±SEM. One-way or 2-way ANOVA (simple or repeated measures) or Student’s t test was used as appropriate. Pearson’s correlation test was used to analyze the relationship between 2 continuous variables. A value of P<0.05 was considered statistically significant.

Results

Expression and Release of MMPs into Coronary Effluent During Aerobic Perfusion

Gelatinolytic activities were detected by zymography at 75, 72, and 62 kDa in the myocardium from aerobically perfused hearts (Figure 1A). The 72- and 62-kDa forms were identified as pro-MMP-2 and MMP-2, respectively, by comparison to the HT1080 cell–derived standard. In addition, MMP-2 was detected in this tissue by Western blot analysis (Figure 1A). As in the profile observed in heart tissue, at 25 minutes of aerobic perfusion, 3 bands of gelatinolytic activity (75, 72, and 62 kDa) were also observed in the coronary effluent (Figure 1A). Quantitative analysis of the zymograms from the coronary effluents showed the following rank order of activities: 72>>62>unidentified 75 kDa (Figure 1B). Gelatinolytic activities in heart tissue were inhibited during zymography in the presence of o-phenanthroline or doxycycline but not by phosphoramidon, suggesting that the bands represent MMP activity (Figure 1C). Pro-MMP-9 and MMP-9 activities were not detected by zymography of heart tissue or coronary effluents obtained during aerobic perfusion.
Release of MMP-2 into Coronary Effluent During Reperfusion After Ischemia

The specific activities of 72- and 62-kDa MMPs in coronary effluents collected during aerobic reperfusion and during reperfusion after 20 minutes of global no-flow ischemia showed that within the first minute of reperfusion, there was an acute enhancement in the release of pro-MMP-2 into the coronary effluent from the heart, as shown by the marked increase in the 72-kDa activity, compared with the baseline coronary effluent sampled during aerobic perfusion (Figure 2A). The 72-kDa activity peaked in the first minute of reperfusion (3.5 times baseline values) and gradually decreased to baseline aerobic values after 10 minutes of reperfusion (Figure 2B). An increase in the 62-kDa activity was also found in the coronary effluent after reperfusion, which peaked at 5 minutes of reperfusion (20 times baseline values) (Figure 2B). After 20 minutes of reperfusion, both specific activities in the coronary effluents returned to baseline aerobic values. An increase in 75-kDa activity was also observed during reperfusion (Figure 2A). Concomitantly with the enhanced release of pro-MMP-2 and MMP-2 into the coronary effluent during reperfusion, a significant decrease in their heart tissue content (by ~69% and ~71%, respectively) was observed at the end of 30 minutes of reperfusion (Figure 2C).

Ischemia Duration, MMP-2 Release, and Recovery of Myocardial Function During Reperfusion

The release of pro-MMP-2 into the coronary effluent during reperfusion in hearts subjected to 15, 20, or 25 minutes of ischemia was compared (Figure 3). During the first minute of reperfusion, the 72-kDa specific activity progressively enhanced with increasing duration of ischemia (Figure 3A and 3B). This increased pro-MMP-2 release was accompanied by a concomitant decrease in the recovery of rate-pressure product (RPP) at 30 minutes of reperfusion (Figure 3C). There was an inverse correlation between the pro-MMP-2 activity in the coronary effluent and the recovery of RPP ($r^2 = 0.99, P < 0.05$).

Effect of MMP-2 on Functional Recovery of Ischemic-Reperfused Hearts

Control hearts subjected to a shorter (15 minutes) duration of ischemia showed a complete recovery of mechanical function after 30 minutes of aerobic reperfusion. In contrast, infusion of 100 ng/mL of semipurified MMP-2 significantly impaired the recovery of RPP (Figure 4).

Effects of MMP Inhibitors on Functional Recovery of Ischemic-Reperfused Hearts

In control hearts, the recovery of RPP during reperfusion after 20 minutes of global no-flow ischemia was significantly depressed compared with preischemia values (Figure 5A). There was a concentration-dependent increase in the recovery of RPP in hearts treated with MMP-2 antibody but not in those treated with rabbit IgG (Figure 5A). A gelatin degradation assay showed that this antibody inhibited the activity of MMP-2 (data not shown).

Doxycycline (10 to 100 µmol/L) and o-phenanthroline (3 to 100 µmol/L), but not phosphoramidon (20 µmol/L),...
concentration-dependently improved the recovery of RPP during reperfusion (Figure 5B). MMP-2 antibody, doxycycline, and phosphoramidon did not affect coronary flow or RPP during aerobic perfusion (data not shown). However, o-phenanthroline at 10 μmol/L (data not shown) and 100 μmol/L significantly depressed RPP (Figure 5B) and at 100 μmol/L also significantly increased coronary flow during aerobic perfusion (from 12.8±0.3 to 14.3±0.6 mL/min, n=6, P<0.05).

We also examined the relationship between the ability of doxycycline to inhibit MMP activity and its ability to im-

Figure 2. Gelatinolytic activities in coronary effluent or ventricles of aerobically perfused rat hearts and during reperfusion after ischemia. A, Representative zymogram showing gelatinolytic activities in coronary effluent samples from perfused heart before 20 minutes of global no-flow ischemia (Aerobic indicates 25 minutes perfusion) and during subsequent reperfusion. B, Densitometric analysis of specific gelatinolytic activities of pro-MMP-2 (72 kDa) and MMP-2 (62 kDa) in coronary effluent samples (n=5). C, Densitometric analysis of gelatinolytic activities in heart tissue from aerobically perfused hearts and after 30 minutes of reperfusion (n=6). *P<0.05 vs respective aerobic value (1-way repeated-measures ANOVA). +P<0.05 vs respective aerobic value (Student’s t test).

Figure 3. Relationship between duration of ischemia, release of pro-MMP-2, and recovery of RPP during reperfusion. A, Representative zymogram of coronary effluent from a control heart (at 25 minutes of aerobic perfusion) and during first minute of reperfusion in hearts subjected to either 15, 20, or 25 minutes of ischemia. B, Densitometric analysis of gelatinolytic activities of pro-MMP-2 in coronary effluents in experiments as in A. Control indicates gelatinolytic activity in effluent at 25 minutes of aerobic perfusion. C, Recovery of RPP at 30 minutes of reperfusion. Control indicates RPP at 25 minutes of aerobic perfusion. *P<0.05 vs control (n=3 for each group, ANOVA).

Figure 4. Effect of semipurified MMP-2 (100 ng/mL) on recovery of RPP during reperfusion after 15 minutes of ischemia. MMP-2 or vehicle alone was infused for 10 minutes before ischemia and for the first 10 minutes of reperfusion period. *P<0.05 vs control (n=5 for each group, ANOVA).
prove the recovery of RPP in hearts subjected to ischemia-reperfusion injury. Doxycycline (10 to 100 \( \mu \text{mol/L} \)), when added during zymography, caused a concentration-dependent inhibition of pro-MMP-2 activity in control hearts (Figure 6A and 6B). Doxycycline, when infused into hearts at the same concentrations, concentration-dependently improved the recovery of RPP as measured at 30 minutes of reperfusion (Figure 6C). There was a significant correlation between the inhibition of pro-MMP-2 activity and the recovery of RPP caused by doxycycline \( (r^2=0.98, P<0.01) \).

### Discussion

We studied the expression and liberation of MMPs in isolated rat hearts during aerobic perfusion and reperfusion after global no-flow ischemia.

#### Expression and Release of MMPs in Aerobically Perfused Hearts

Gelatinolytic activities corresponding to molecular weights of \( \approx75 \), 72, and 62 kDa were expressed in heart tissue. The 72- and 62-kDa forms corresponded to pro-MMP-2 and MMP-2, respectively, and have already been described in human, rat, and porcine hearts.\(^8\)–\(^11\) This is the first report to show that pro-MMP-2, MMP-2, and 75-kDa gelatinases are released into the coronary effluent of aerobically perfused rat hearts and that pro-MMP-2 constitutes the major gelatinase activity. In the heart, MMP-2 is ubiquitously distributed and has been localized to endothelial, endocardial, and subendocardial layers as well as to the mesenchymal cells.\(^8\)–\(^11\) We did not identify any pro-MMP-9 or MMP-9 activity in the heart tissue or coronary effluent. The 75-kDa gelatinase may be a modified form of MMP-2 or an activation product of MMP-9 by the action of neutrophil elastase.\(^22\)

#### Expression and Release of MMPs in Hearts Subjected to Ischemia-Reperfusion Injury

The release of MMPs from the heart into the coronary effluent was immediately increased as a consequence of ischemia and reperfusion. The release of pro-MMP-2 and MMP-2 peaked during the first and fifth minutes of reperfusion, respectively, and their release was enhanced with increasing duration of ischemia. Moreover, the release of MMPs was associated with impaired mechanical function of...
the heart, because both chemically unrelated inhibitors of MMP activity (doxycycline and o-phenanthroline) and MMP-2 antibody were able to improve the functional recovery of ischemic-perfused hearts. Ischemia-reperfusion resulted in a clear-cut depletion of pro-MMP-2 and MMP-2 activity in the myocardium, showing that the activation and release of MMP-2 into the perfusate is a consequence of this injury.

Ischemia-reperfusion injury of the porcine heart in vivo increased MMP-1 and MMP-9 activities in the myocardium, an effect ascribed to infiltrating leukocytes. If blood leukocytes were the main source of MMP-9 during reperfusion, this would explain the lack of detectable MMP-9 activity in our experiments using crystalloid buffer-perfused, isolated hearts.

Role of MMP-2 in the Development of Myocardial Ischemia-Reperfusion Injury

These findings suggest a novel role for MMP-2 in the development of myocardial stunning in the reperfusion period after ischemia. The evidence for the involvement of this enzyme in the injury is compelling: (1) there was a marked release of pro-MMP-2 during reperfusion, (2) there was a negative correlation between pro-MMP-2 release during reperfusion and functional recovery of hearts subjected to increasing duration of ischemia, (3) infusion of semipurified MMP-2 worsened the recovery of function after ischemia and reperfusion, (4) there was a positive correlation between the ability of the MMP inhibitor doxycycline to improve the recovery of mechanical function and its ability to inhibit myocardial MMP-2 activity, and (5) there was a protective effect of a neutralizing antibody to MMP-2 in ischemic-reperfused hearts.

Mechanisms of Activation and Action of MMP-2 in Ischemia-Reperfusion Injury

Pro-MMPs may be activated through the breakage of the zinc-cysteine bond, which exposes its catalytic site, followed by proteolytic activation, or through oxidant-induced conformational changes without a change in molecular weight. Indeed, the powerful oxidant peroxynitrite activated pro-MMP-2 in human smooth muscle cells and purified procollagenase in activated neutrophils. The biosynthesis of peroxynitrite in the heart is greatly enhanced during the first minute of reperfusion after ischemia. Because the time courses of peroxynitrite and pro-MMP-2 release are similar, we suggest that this oxidant may be involved in pro-MMP-2 activation during reperfusion. Endogenous tissue inhibitors of metalloproteinases (TIMPs) also control the expression of MMPs, followed by doxycycline and then by phosphoramidon, which failed to inhibit MMPs. Rohde et al recently showed that an MMP inhibitor attenuates left ventricular dilation in a mouse infarct model.

Tetracycline antibiotics, including doxycycline, have been shown to possess additional activities as inhibitors of MMPs, independent of their antibacterial activity. Recent epidemiological studies have indicated a reduced risk of first-time acute myocardial infarction in patients receiving only tetracycline- or quinolone-type antibiotics. Some forms of heart diseases may be associated with bacterial infections, such as chlamydia, and this may at least partially explain the beneficial effect of these antibiotics in preventing heart attacks. Our data, however, suggest that protective actions of tetracyclines on the myocardium may indeed be due to inhibition of MMP-2 activity. Phosphoramides and tetracyclines might also have additional effects on free radical generation, cell growth, and vascular reactivity.

MMPs and Ischemia-Reperfusion Injury In Vivo

Our findings in the isolated perfused heart indicate that myocardium subjected to ischemia-reperfusion injury releases MMP-2 and that its liberation is of pathological significance for the development of mechanical dysfunction. In the setting of ischemia-reperfusion in vivo, activation of blood cells, such as platelets and leukocytes, is likely to result in the release of MMP-2, MMP-9, and possibly other proteases that may also contribute to the development of myocardial stunning. Clearly, more work is necessary to explore the biological and pharmacological significance of the liberation of MMPs during ischemia-reperfusion injury.

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