Basic Res Cardiol 103:431–443 (2008)
DOI 10.1007/s00395-008-0727-y

ORIGINAL CONTRIBUTION

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Inhibiting matrix metalloproteinase-2 reduces protein release into coronary effluent from isolated rat hearts during ischemia-reperfusion

Received: 26 November 2007
Returned for 1. Revision: 13 December 2007
1. Revision received: 17 March 2008
Returned for 2. Revision: 3 April 2008
2. Revision received: 8 April 2008
Accepted: 28 April 2008
Published online: 28 May 2008

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Abstract *Background* Previous studies have shown that the disruption of the coronary endothelium and the increase in its permeability during ischemia-reperfusion (I/R), are linked to matrix metalloproteinase-2 (MMP-2) activity. Studies from our group have shown that during I/R, activity of MMP-2 in the coronary effluent increases and this increase is associated with cardiac dysfunction, which in turn, can be prevented by MMP inhibitors. Therefore, we hypothesize that inhibiting MMPs reduces the MMP-2 dependent disruption of the coronary endothelium and subsequent protein release during I/R. *Methods* Isolated rat hearts were perfused in the Langendorff mode at a constant pressure and subjected to 15, 20 or 30 min no-flow ischemia followed by 30 min of reperfusion. The MMP inhibitors, o-phenanthroline (Phen, 100 μM) or doxycycline (Doxy, 30 μM) an inhibitors of MMPs, were added to the perfusion solution 10 min before ischemia and for the first 10 min of reperfusion. The coronary effluents were collected during perfusion for protein analysis. Creatine kinase was measured as an index of cellular damage. Endothelial integrity was assessed by measuring coronary flow and by measuring the levels of serotransferrin and interstitial albumin in the coronary effluent. Additionally, damage to the endothelium was assessed histologically by light microscopy analysis of the cellular structure of the myocardium. MMP-2 activity was measured by zymography in hearts subjected to 15, 20 and 30 min of ischemia without reperfusion. *Results* MMP-2 activity was increased in heart tissue at the end of ischemia and was correlated with duration of ischemia. The post-ischemia decrease in coronary flow, and the increase in the release of serotransferrin and albumin were attenuated by Phen. Edema (another indirect marker of endothelial damage) was observed in I/R heart and the edema was abolished in I/R heart treated with MMP inhibitors. *Conclusion* MMP inhibition not only reduces cardiac mechanical dysfunction but also reduces endothelial damage resulting from cardiac I/R injury.

Key words ischemia-reperfusion – endothelium – matrix metalloproteinases – proteomics

Abbreviations I/R: Ischemia-reperfusion, MMPs: Matrix metalloproteinases, Phen: *o*-Phenanthroline, Doxy: Doxycycline

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Introduction

The damage that occurs after the restoration of the blood flow to ischemic tissues or organs has deleterious consequences in various clinical settings such as thrombolytic therapy, coronary angioplasty, cardiopulmonary bypass, and organ transplantation [3, 25]. Despite the variations in anatomy and physiology of different tissues or organs, the basic molecular and cellular mechanisms of I/R injury are essentially similar throughout the body. Moreover, in I/R, where microvascular dysfunction involves endothelial injury, there is increased production of reactive oxygen species (ROS) [12, 28, 39, 41].

Over the past few decades, studies focused on understanding the mechanisms of the increased endothelial permeability in I/R [36, 38], have revealed that a basic phenomenon in I/R injury is the disruption of the interaction of the endothelial cytoskeleton and cell–cell adhesive junction which controls the transendothelial flux of fluid and macromolecules [15, 42]. The disassembly of endothelial cell–cell junctions increases the permeability of the vascular endothelium, which allows proteins [7] and various cytotoxic and inflammatory mediators to infiltrate into the affected tissues [3, 8]. However, the precise molecular mechanisms responsible for the disassembly of the cell–cell junctions are not known.

The first evidence suggesting that matrix metalloproteinases (MMPs) are important in I/R induced increased endothelial permeability, was revealed by Soccal and colleagues [37] using a lung I/R model. Next Gasche and colleagues showed that inhibition of MMP-2 and MMP-9 prevented blood-brain barrier dysfunction after transient focal cerebral ischemia [10]. It is worth remembering that the activity of these MMPs is regulated by ROS [13, 21, 27], a very important mediator of I/R injury, and that the increase of ROS has a negative impact on the mechanical function of the heart [40]. Indeed, our results from a separate ongoing study on the role of MMP-2 in a rat model of endotoxemia, where ROS play a very important role [20, 33], show that increased plasma MMP-2 activity can be associated with increased endothelial permeability [22]. However, there is still no solid evidence whether increased activity of MMP-2 during cardiac I/R contributes to the disruption of the endothelial layer and increase of protein secretion in to the coronary circulation.

MMP-2 is a proteolytic enzyme which plays important roles in a variety of physiological processes, including morphogenesis, angiogenesis, cell migration, wound healing, and cartilage and bone repair. It is best known for its degradation of extracellular proteins and remodeling of the extracellular matrix over days to weeks. However, a decade ago we discovered a new intercellular function of MMP-2 (as an agonist of platelet aggregation) with a time frame of action of seconds [30]. This led to a series of studies of the role of cardiac MMP-2 during I/R injury. These investigations revealed that MMP-2 contributes to acute I/R induced cardiac dysfunction and that inhibition of MMP-2 protects the heart from I/R injury [4]. We have also shown that an intracellular contractile machinery regulatory protein, troponin I (TnI), is degraded by MMP-2 [41]. By using a pharmaco-proteomics approach to analyze protein changes in I/R hearts, we discovered that myosin light chain 1 (MLC1) is another target for MMP-2 [32]. These observations provide the impetus for the development of a novel pharmacological strategy in the treatment of I/R injury and a new paradigm pertaining to the site of MMP-2 activity. The novel aspects of the action of MMP-2 in heart injury has been reviewed by Schulz [34] and the role of MMPs in cardiac diseases has been reviewed by Spinale [38].

Understanding both the extracellular and the intracellular actions of MMP-2 is fundamental for understanding the molecular mechanisms of I/R injury. In the present study, using the proteomics approach [32], we investigated the hypothesis that in addition to MMP-2's well known role in remodeling the extracellular matrix, and its novel intracellular action of degrading contractile proteins, that increased MMP-2 activity is involved in the disruption of endothelial integrity which results in increased protein level in coronary circulation.

Materials and methods

Heart preparations

Male Sprague-Dawley rats (300–350 g) were anesthetized with an injection of sodium pentobarbital (40 mg/kg, i.p.). The hearts were rapidly excised and briefly rinsed by immersion in ice-cold Krebs–Henseleit buffer. Spontaneously beating hearts were placed in a water-jacketed chamber (EMKA Technologies) to maintain their temperature at 37°C. Hearts were perfused in the Langendorff mode at a constant pressure of 60 mm Hg with Krebs-Henseleit buffer at 37°C containing (in mM): NaCl (118), KCl (4.7), KH_2PO_4 (1.2), $MgSO_4$ (1.2), $CaCl_2$ (3.0), $NaHCO_3$ (25), glucose (11), and EDTA (0.5), and gassed continuously with 95% O_2/5% CO_2 (pH 7.4).

A water-filled latex balloon connected to a pressure transducer was inserted through an incision in the left atrium into the left ventricle through the mitral valve.

The volume was adjusted to achieve an end diastolic pressure of 10 mm Hg. Stock solutions of various reagents were infused into the heart via a side-port proximal to the aortic cannula at a constant rate of 0.1 mL min^{-1} by a Gilson mini pump (Minipuls 3).

Coronary flow (an indirect index of endothelial integrity/function), coronary perfusion pressure, heart rate, and left ventricular pressure were monitored using an EMKA recording system with IOX2 software (EMKA Technologies). Left ventricular developed pressure (LVDP) was calculated as the difference between systolic and diastolic pressures of the left ventricular pressure trace. The rate pressure product (RPP) was calculated as the product of heart rate and LVDP.

Ischemia/reperfusion protocol

To determine if the duration of ischemia has an effect on MMP-2 activity, a set of hearts (n = 4 per group) were subjected to 15, 20 or 30 min of no-flow ischemia induced by clamping the aortic inflow line after a 25 min aerobic stabilization period. At the end of ischemia, the hearts were frozen in liquid nitrogen and processed for zymography. MMP-2 activities from those hearts were compared to hearts perfused aerobically for 25 min. The perfusion protocol of hearts subjected to ischemia only is illustrated in Fig. 1a.

In a separate set of experiments, following 25 min of perfusion (stabilization period), hearts were subjected to 15, 20 or 30 min global, no-flow ischemia (n = 4). This was followed by 30 min of aerobic reperfusion by reopening the clamp (Fig. 2a). The hearts were frozen and processed for zymography as described above.

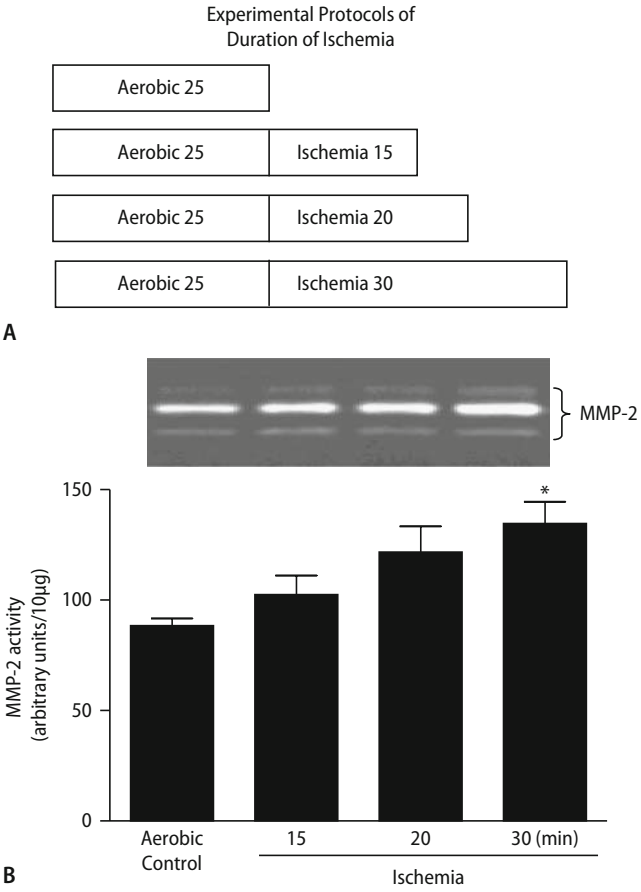

Fig. 1 The effect of duration of ischemia on MMP-2 activity in isolated rat hearts. **a** Experimental protocol of ischemia. **b** Densitometric analysis of activities of MMP-2 in hearts subjected to different durations of ischemia. A representative zymogram is shown in **b**. * $P < 0.05$ versus aerobic controls

Modulation of ischemia/reperfusion injury by phenanthroline, an inhibitor of MMP-2

There are no commercially available selective/specific inhibitors of MMPs. The use of a neutralizing antibody to inhibit MMP-2 [4] would be the best choice, however the neutralizing antibody is not selective due the high homology of primary structure within the MMP family, particularly between MMP-2 and MMP-9 (46.5% identity in 692 amino acid overlap). In some experiments, after 15 min of aerobic perfusion, synthetic inhibitors of MMPs activity, *o*-phenanthroline (Phen, 100 µmol/L, n = 4) or doxycycline (Doxy, 30 µmol/L, n = 4) [4] were infused into hearts for the last 10 min of aerobic perfusion and for the first 10 min of reperfusion. As a control of possible non-specific actions of Phen, some hearts were perfused in presence or absence of Phen in aerobic conditions for the last 45 min of the perfusion protocol (n = 3).

The coronary effluents were collected at 25 and 45 min of the perfusion protocol (Fig. 4a). The average time required to collect 24 mL of the coronary effluent samples was 87 s for the aerobic perfusion sample and between 87 and 200 s for the reperfusion samples.

Measurement of MMP-2 by gelatin zymography

Gelatin zymography was performed as described before [4]. Briefly, preparations of samples were applied to 8% polyacrylamide gel copolymerized with 2 mg/mL gelatin. After electrophoresis, gels were rinsed three times for 20 min each in 2.5% Triton X-100 to remove SDS. Then the gels were washed twice in incubation buffer (50 mmol/L Tris–HCl, 5 mmol/L $CaCl_2$, 150 mmol/L NaCl and 0.05% NaN_3) for 20 min each at room temperature, and placed in incubation buffer at 37°C. The gels were stained in 2% Coomassie

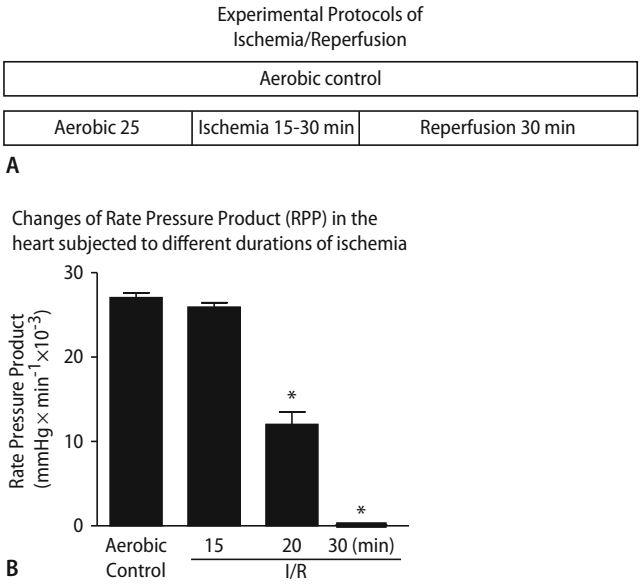

Fig. 2 Correlation of MMP-2 activity with rate pressure product (*RPP*) in isolated rat hearts. **a** Experimental protocol of ischemia-reperfusion (I/R) injury. **b** RPP of aerobic control hearts and those subjected to 15–30 min of ischemia and 30 min reperfusion. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

Brilliant blue R250, 25% methanol, 10% acetic acid for 2 h and then destained for 1 h in 2% methanol/4% acetic acid. Gelatinolytic activities were detected as transparent bands against the background of Coomassie Brilliant blue-stained gelatin. Enzyme activity was quantified by densitometry analysis of gelatinolytic bands and expressed as arbitrary units per μg protein.

Protein concentration of the coronary effluent

Coronary effluent samples of 24 mL were concentrated in Centricon-10 concentrating vessels (Amicon). The final volume of concentrate was measured by gravimetry, and adjusted to the same final volume for each sample (80 μL). Protein concentration in the concentrate as well as from heart homogenates, was determined by the BioRad protein assay using bovine serum albumin as the standard. The protein concentration in coronary effluent before concentration was calculated and expressed as μg/mL.

2D electrophoresis

Concentrated effluent samples of 20 μL were applied to each of 11 cm immobilized linear pH gradient (3–10) strips (IPG, BioRad) and equilibrated for 16–18 h at 20°C in rehydration buffer. For isoelectrofocusing, a BioRad Protean isoelectrofocusing cell was used with the following conditions at 20°C with fast voltage ramping: Step 1, 15 min with end voltage at 250 V; Step 2, 150 min with end voltage at 8,000 V; Step 3, 35,000 V-hours (approximately 260 min). After IEF, the strips were equilibrated according to the manufacturer's instructions. Second dimension of 2D electrophoresis was then carried out with Criterion precast gels (8%–16%) (BioRad). After separation, proteins were detected with the Silver Staining Plus kit (BioRad). To minimize variation in staining, all gels were stained in the same bath. The reproducibility of 2D electrophoresis and quality of protein loading has been previously verified by us [31, 32].

Image analysis

Developed gels were scanned using a GS-800 calibrated densitometer (BioRad). Quantitative analysis of MMP-2 bands from zymography and spot intensity from 2D gels were measured using QuantityOne 4.6 and PDQuest 7.1 software respectively (BioRad).

Mass spectrometry

Selected protein spots, were manually excised from the 2D gel, and processed using a MassPrep Station from Micromass, following the methods supplied by the manufacturer. Briefly, the excised gel fragment containing the protein spot was first distained, reduced, alkylated, digested with trypsin and extracted. Mass analysis of the trypsin digest was performed on a MALDI-TOF Voyager DE-Pro from Applied Biosystems. A mass deviation of 0.2 was tolerated and one missed cleavage site was allowed. Resulting values from mass spectrometry analysis for monoisotopic peaks were used for searches against NCBInr and Swiss-Prot databases with Rattus Norvegicus specified. We used the Mascot (http://www.matrixscience.com) search engine for protein identification. The Mowse scoring algorithm [29] was used for justification of the accuracy of protein identification and is incorporated in the Mascot search engine.

Fixation of the hearts for histochemistry

Following the experimental procedure (see Ischemia/reperfusion protocol), the hearts were gently disconnected from Langendorff apparatus and again rapidly (less than 10 s) canulated to fixation pump (SAGE Instruments). Primary fixative was then perfused through the heart at 15 mL/min for 5 min. The pri-

mary fixative consisted of 0.9% formaldehyde; 2.5% glutaraldehyde in 0.09 M sodium cacodylate (320 mOsM) buffer. Hearts were removed from the perfusion pump and were fixed in the same fixative for 15 min. The hearts were then sectioned open and 1 mm sections cut horizontally through the heart and were immerse fixed in the same fixative for an additional 15 min. The heart sections were then washed in 0.08 M cacodylate buffer for 10 min and subsequently post-fixed using an osmium blacks rich solution (1% osmium tetroxide; 1.8% potassium ferrocyanide in 0.08 M sodium cacodylate) at room temperature for 2 h. Tissue was then washed 2 times for 10 min with 0.08 M cacodylate buffer. The heart tissue was then dehydrated in ascending ethanol solutions and subsequently embedded in epon araldite.

Once polymerized, the blocks of heart tissue were trimmed and sectioned on a Reichert-Jung Ultracut ultramicrotome. Plastic sections (2 μm) were cut, mounted on glass slides and stained with toluidine blue. Slides were examined on a Nikon Eclipse E600 microscope.

Statistical analysis

One-way analysis of variance (ANOVA) was used to compare the differences between the groups. Analysis of spot density of the 2D gels was carried out by *t* tests and Mann–Whitney *U* tests. Post-hoc analysis was performed using Tukey–Kramer multiple comparisons test. A P value less than 0.05 was considered statistically significant. Data are expressed as the mean ± standard error in all figures.

Results

Duration of ischemia and MMP-2 activity in the myocardium

The experimental protocol and corresponding activities of MMP-2 in the hearts subjected to 15, 20 or 30 min of ischemia are shown in Fig. 1a. An activity of MMP-2 is higher in ischemic hearts than in aerobic controls (Fig. 1b). The increase in MMP-2 activity is positively correlated with the duration of ischemia ($r^2 = 0.9539$, $P < 0.05$).

Mechanical function of the I/R heart and myocardial MMP-2 activity

The activity of MMP-2 in the myocardium and the mechanical function of the aerobic control hearts and those subjected to 15, 20 or 30 min of ischemia are compared in Fig. 2. The recovery of RPP during reperfusion following 20 or 30 min of global, no-flow ischemia was depressed when compared to the aerobic controls (Fig. 2b). A negative correlation between RPP and duration of ischemia was observed ($r^2 = 0.7802$, $P < 0.05$). There was also a significant inverse correlation between the MMP-2 activity in the myocardium (Fig. 1b) and the recovery of RPP (Fig. 2b) following 30 min of reperfusion ($r^2 = 0.9256$, $P < 0.05$).

Coronary flow in the I/R heart and MMP-2 activity

Coronary flow was measured as an indirect index of endothelial function. The coronary flow and MMP-2 activity from the hearts subjected to 15, 20 or 30 min of ischemia were compared. The coronary flow during reperfusion following 20 and 30 min of global, no-flow ischemia was significantly depressed when compared to the aerobic controls (Fig. 3a). A negative correlation between coronary flow and duration of ischemia was observed ($r^2 = 0.7664$, $P < 0.05$, not shown). There was also a significant inverse correlation between the MMP-2 activity in the myocardium and the coronary flow following 30 min of reperfusion ($r^2 = 0.9049$, $P < 0.05$) (Fig. 3b).

Inhibiting MMP-2 improves coronary flow and the mechanical function of the heart following I/R injury

The endothelial function was indirectly assessed by measurement of coronary flow and the contractile mechanical function was evaluated by RPP. These parameters were measured in aerobically perfused hearts (control) and in those, which were subjected to I/R injury. Two additional groups of hearts, aerobic control and I/R, were perfused with 100 μ/mL Phen or 30 μM Doxy (Fig. 4a). The coronary flow and RPP of the hearts after 30 min of reperfusion following 20 min of global no-flow ischemia were significantly reduced in I/R group compared with the control hearts (Fig. 4b, c). The administration of Phen or Doxy significantly improved coronary flow and mechanical function versus hearts subjected to I/R alone (69.1% ± 1.7 and 75.4 ± 2.6 Vs. 57.9% ± 4.9 and 72.9% ± 8.4 and 66.9 ± 3.6 Vs. 40.0% ± 4.6, respectively, $P < 0.05$, Fig. 4b,c). Because Phen is also ROS scavenger a separate group of hearts was subjected to aerobic perfusion with this MMP inhibitor. Phen showed no changes in either coronary flow or RPP in comparison to control hearts (data not shown).

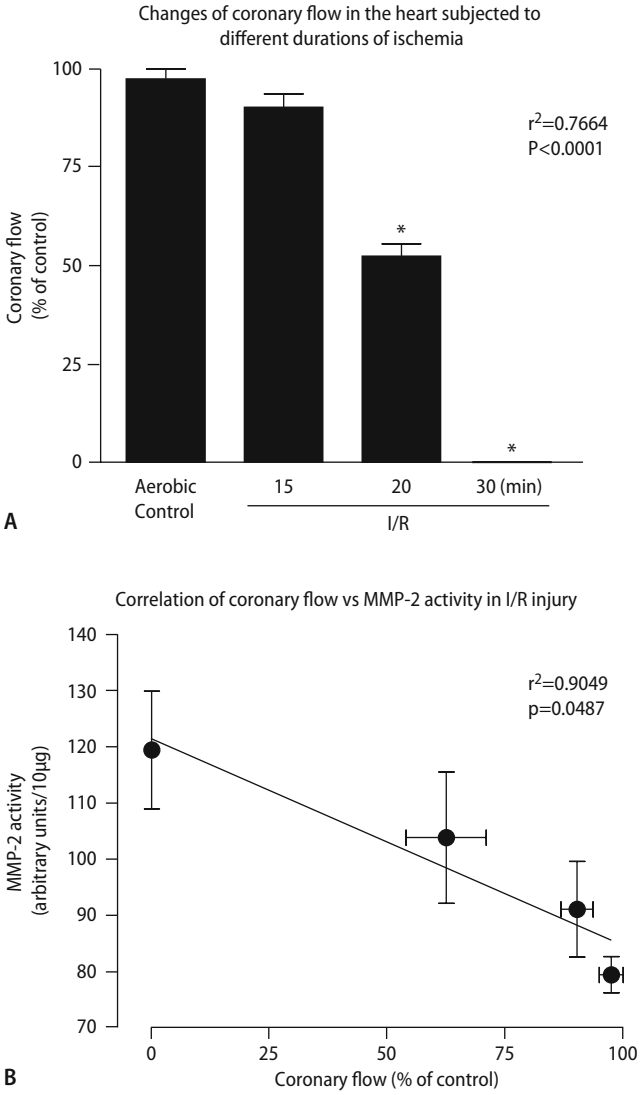

Fig. 3 Coronary flow of aerobic control hearts and those subjected to ischemia-reperfusion. **a** Changes of coronary flow (expressed as percent of aerobic control) in relation to duration of ischemia. **b** Correlation of MMP-2 activities with coronary flow. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

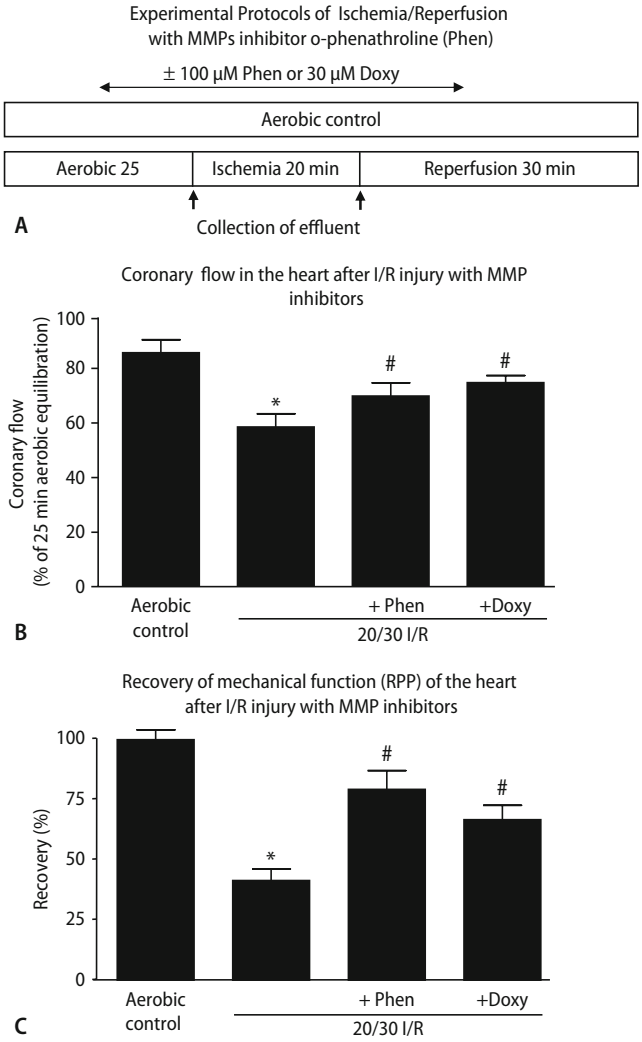

Fig. 4 The effect of MMP-2 inhibition on coronary flow and cardiac mechanical function in isolated rat hearts subjected to I/R. **a** Experimental protocol of heart protection from ischemia-reperfusion (I/R) injury with phenathroline (Phen, 100 µM) or doxycyclin (Doxy, 30 µM) as a MMP inhibitors. The arrows denote the time-point when coronary effluent was collected. **b** Coronary flow in the heart after I/R injury in the presence or absence of Phen or Doxy. **c** Recovery of cardiac mechanical function in hearts subjected to I/R injury in the presence or absence of Phen or Doxy. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

Protection against edema formation with MMP inhibitors

The loss of microvascular integrity is linked to a reduction of negatively charged molecules at the endothelial cell surface, and, this is associated with myocardial tissue edema which contributes to increased microvascular fluid loss and to cardiac dysfunction after I/R injury [11]. Histological analysis of endothelial integrity in I/R triggered edema in myocardium is shown in Fig. 5.

In the control heart (Fig. 5a), the endothelial linings of the capillaries and larger blood vessels are intact, with minimal extracellular space and normal-appearing myocytes. In contrast, the I/R heart (Fig. 5b) shows marked edema, with an increase in the extracellular space (*), vacuolated myocytes in several areas (cells in the circle), and disrupted integrity of the endothelium of several of the larger capillaries (the arrows), and of the connective tissue.

The I/R hearts treated with Phen or Doxy (MMP inhibitors) demonstrate a preservation of the extracelluar space compared to the I/R heart (Fig. 5c, d). The longitudinal sections of the same hearts show that Phen better protects the connective tissue. However,

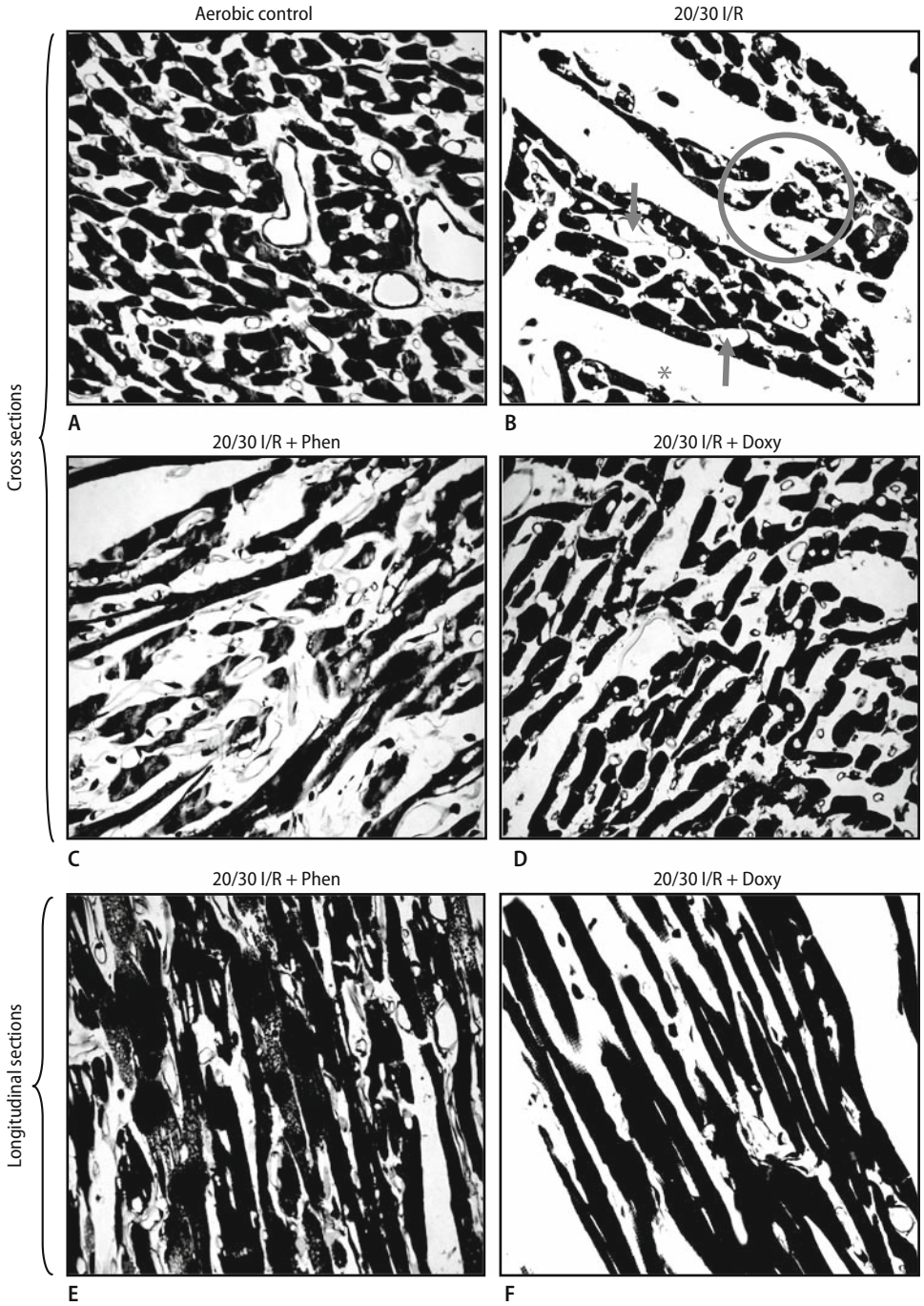

Fig. 5 Effects of MMP on edema formation. Cross sections of the control heart (**a**), and **b** the heart subjected to I/R. **c**, **d** I/R hearts treated with Phen or Doxy, respectively. **e**, **f** Longitudinal sections of the same hearts treated with Phen or Doxy, respectively. *Asterisk* shows edema, with an increase of the extracellular space, circle shows vacuolated cells, and *arrows* point to disrupted endothelium of larger capillaries

there are areas of the Phen heart that show marked cell degeneration compared to the heart treated with Doxy (Fig. 5e, f).

Protein release into the coronary effluent during I/R

The amount of total protein released into the effluent was assessed at two time points, after 25 min of aerobic perfusion and after 45 min of length of experiment when reperfusion was started (see Fig. 4a). We did not observe differences in protein levels of effluents collected at 25 min of perfusion. In contrast during the first minute of reperfusion (at 45 min) the protein content in the effluent of I/R hearts was significantly increased in comparison to aerobic control (3.28 ± 0.31 μg/mL Vs. 0.1 ± 0.1 μg/mL) (Fig. 6a). Phen and Doxy significantly decreased overall protein

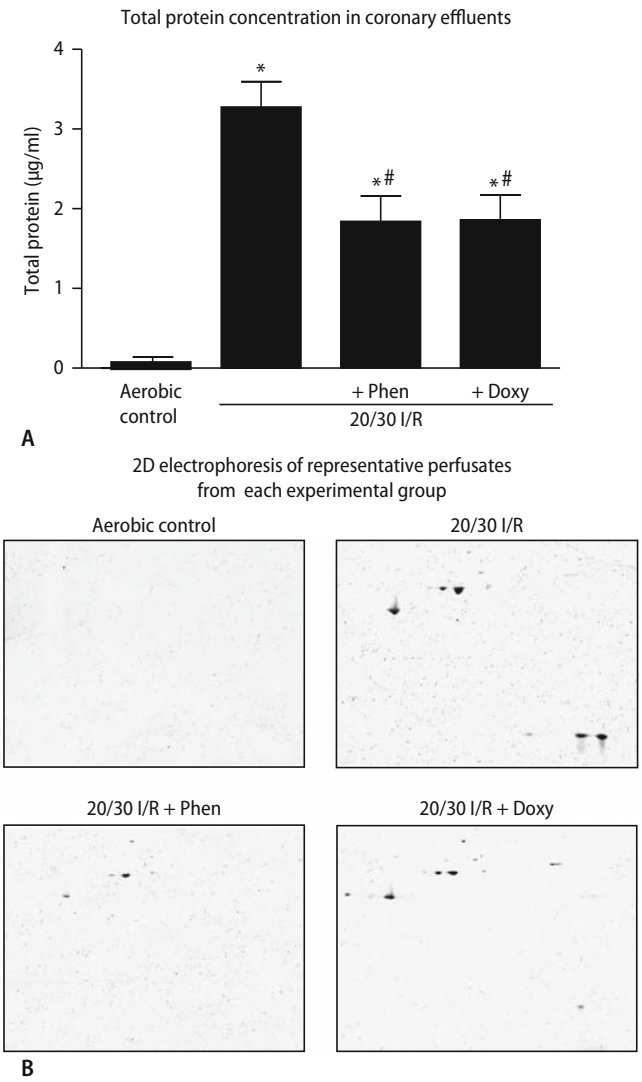

Fig. 6 Quantitative analysis of protein in the coronary effluent from control hearts and from hearts subjected to I/R injury and perfused with or without of Phen or Doxy. **a** Total protein, **b** 2D electrophoresis of representative samples from each experimental group. Note the increased amount of protein spots in the reperfusate collected from I/R hearts and decreased amount of the protein spots in the reperfusates collected from I/R hearts protected with MMP inhibitors. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

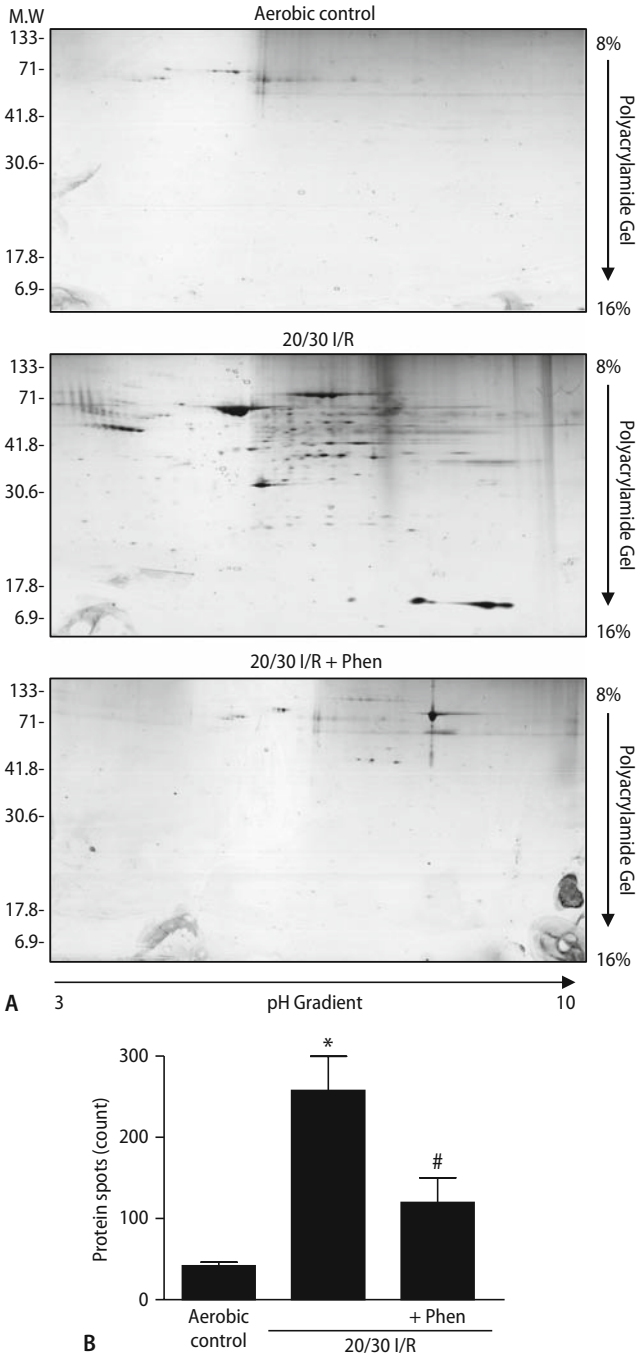

Fig. 7 Analysis of proteoms of perfusates/reperfusates by 2D Electrophoresis. **a** Representative 2D gels of coronary effluent proteins collected from aerobic control hearts and from those subjected to I/R injury and perfused with or without of Phen. **b** Quantitative analysis of amount of protein spots in the coronary effluent from control hearts and from hearts subjected to I/R injury and perfused with or without of Phen

release after I/R compared to I/R alone (1.84 ± 0.32 µg/mL and 1.86 ± 0.31 µg/mL, respectively) (Fig. 6a).

Next, 2D electrophoresis was used for analysis of reperfusate proteomes (sample collected at the first min of reperfusion) and compared to perfusate proteomes of sample collected at 45 min aerobic perfusion. Because, simultaneous analysis by 2D electrophoresis of all 16 effluent samples is technically difficult (limit to analysis of 12 sample, for explanation see references [31, 32]), first we analyzed only one representative sample from each experimental group (Fig. 6b). Phen showed better inhibitory effect of protein spots release than Doxy (Fig. 6b). Because

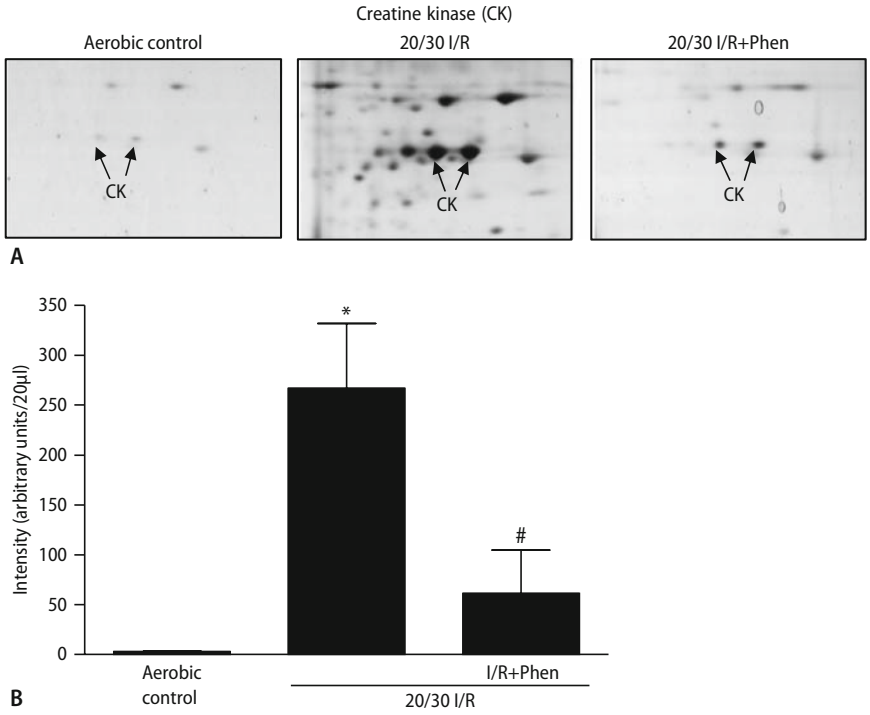

Fig. 8 Effect of I/R on the release of creatine kinase (*CK*) into the effluent. **a** Representative magnification of the region where two different isoforms of the protein were identified in the aerobic control, I/R and I/R + Phen. **b** Quantitative analysis of the CK. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

Phen also has better protective effect of mechanical function of the heart (Fig. 4c), shows better preservation of extracellular matrix in I/R hearts (see Fig. 5 and references 4, 32), and is more potent MMP-2 inhibitor then Doxy [4], we decided to use Phen in this study of reperfusate proteomes.

2D gel electrophoresis of effluents collected during the first minute of reperfusion showed that effluents from I/R hearts have several times more measurable protein spots (257 ± 42) in comparison to control (42 ± 5) and hearts perfused with Phen (120 ± 30) (Fig. 7b). Representative 2D gels from each group are shown in Fig. 7a.

Effects of *o*-phenantroline on heart injury

Changes in creatine kinase (CK) levels are associated with the degree of heart injury. Thus, we evaluated the effects of inhibiting MMP-2 on cardiac CK release into the perfusate during I/R. We detected at least two different molecular forms of CK in each group, more acidic A and more basic B (Fig. 8a). We quantified each spot and the total release of CK is shown in Fig. 7b. Quantitative analysis showed that very little CK was released from control hearts. A significant increase in the release of CK was observed when hearts were subjected to I/R (Fig. 8b). The release of CK was significantly reduced by Phen (61.4 ± 43.2 Vs. 267.1 ± 64.8 AU/20 μL).

Evaluation of the endothelial barrier integrity during I/R

Our goal was to evaluate changes to endothelial integrity by I/R and whether the endothelim can be protected with the MMP inhibitor Phen. To do this, we measured the level of the interstitial proteins (markers of endothelial integrity), such as serotransferrin and albumin, released into the effluent during I/R. Measurement of transferrin is sensitive and specific for determination of vascular permeability [6] and was used for assessment of microvascular permeability to protein in lung I/R injury [37]. We found four and five protein spots with different isoelectric points (pI) that were identified as being different molecular forms of serotransferrin and albumin respectively (Fig. 9a). The existence of these variations in pI of serotransferrin and albumin can be generally explained by post-translational modifications (for example phosphorylation) of protein molecules by which some of the molecules become more acidic or more basic. We quantified each spot and grouped them for densitometric analysis of the overall release of each protein. The amount of serotransferrin observed in the effluent was significantly higher in I/R hearts (Fig. 9b) and Phen attenuated its release (773 ± 49 Vs. 257 ± 74 AU). A similar pattern was observed for albumin (1132 ± 101 Vs. 547 ± 172 AU) (Fig. 9c). The results of identification are shown in Table 1.

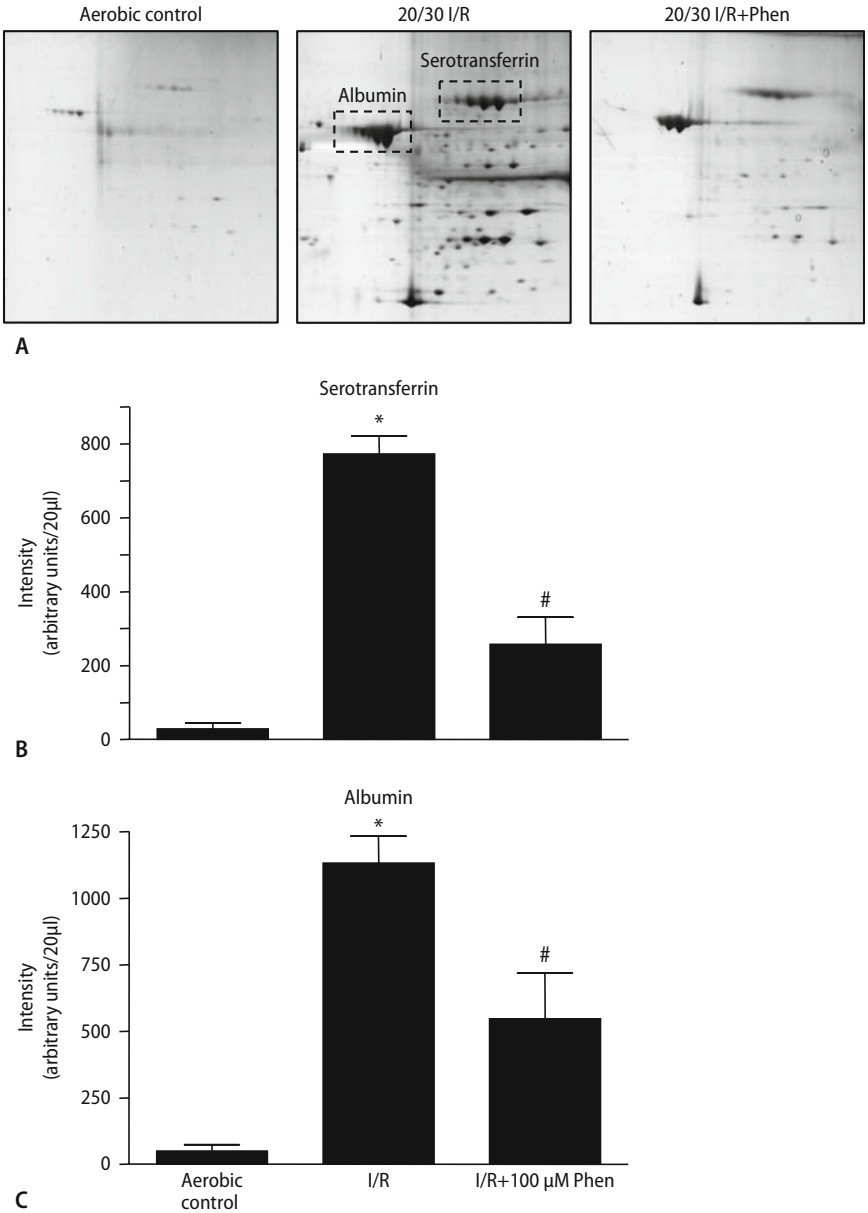

Fig. 9 Release of serotransferrin and albumin into the coronary effluent as markers of endothelial integrity during I/R. **a** Representative magnifications of the 2D gels from Aerobic control, I/R and I/R + Phen, highlighting the location and the different forms of both proteins. **b** Quantitative analysis of the release of serotransferrin into the coronary effluent during the first minute of reperfusion. **c** Quantitative analysis of the release of interstitial albumin into the coronary effluent from perfusates collected at the first min of reperfusion. * $P < 0.05$ versus aerobic controls; # $P < 0.05$ versus I/R only

Discussion

The concept of the endothelium as a highly specialized organ with variety of very specific tasks and functions, has become widely accepted [14, 26]. The dysfunction of this organ has been described in many pathological states, such as sepsis, thrombotic thrombocytopenic purpura, diabetes, hypertension and I/R. The disruption of the endothelium has a

Table 1 Results of identification of protein spots using Mascot search engine

Identified protein	Mass searched (*n*)	Mass matched (*n*)	Sequence coverage (%)	Probability based Mowse score[a]	
				Threshold ($P < 0.05$)	Observed score
Albumin	29	16	25	57	199
Serotransferrin	97	21	30	57	150
Creatine kinase	87	15	34	57	102

[a] −10log(*P*) where *P* is the probability that the observed match is a random event. Individual ion scores >26 or >41 indicate identity or extensive homology ($P < 0.05$)

negative impact on vascular permeability and ultimately leads to coronary artery diseases and heart failure. It has been shown that, while myocardial function has already recovered, endothelial cells are more severely impaired than smooth muscle cells, and that this injury persists beyond myocardial stunning. Thus, endothelial-dependent dysfunction can still impair vasoreactivity while ventricular dysfunction has already resolved [9].

Several studies have been focused on the endothelial permeability, and possible mechanisms for controlling the protein release to the circulation. Yet, studying changes in the heart vasculature during I/R injury has been difficult, however, in the last decade a pharmaco-proteomics approach for the study of cardiovascular diseases has been introduced [2, 23, 33]. To date, studies using this approach, have identified changes in more than forty proteins in chronic cardiovascular diseases [16], such as dilated cardiomyopathy [1, 5, 18], varying degrees of I/R injury [35], and heart failure [17, 19].

In the present study on the possible mechanism responsible for damage of the endothelium in acute heart injury, we used the proteomics approach to study proteins released from the myocardium as a result of endothelium injury, and to measure if the change in their levels is associated with I/R injury. We also determined if the contribution of MMP-2 to the damage of endothelial integrity/function can be minimized with MMP inhibitors.

In this study, we have shown for the first time that the increase of MMP-2 activity in ischemic hearts correlates with the duration of ischemia (Fig. 1). Furthermore, we have shown (Fig. 7) that approximately 250 proteins are released into the effluent in the first minute of reperfusion whereas non-ischemic hearts release fewer than 30 proteins in the corresponding time frame. Administration of Phen or Doxy reduced the release of protein to the effluent from I/R hearts (Fig. 6a). This can be explained by the fact that MMP inhibitors acted also by preventing the disruption of the endothelial barrier as indicated by the inhibition of edema formation and decreased release levels of interstitial albumin and serotransferrin, markers of endothelial integrity (Figs. 5, 9). An interesting observation from this part of our study is the difference in the protein map (proteome) of I/R hearts comparing control to I/R plus Phen. Those differences can be explained by protein posttranslational modifications, such as phosphorylation, hydroxylation, nitration and nitrosylation [24] triggered by myocardial infarction or I/R.

There are three limitations to this study: first both Phen and Doxy are broad-spectrum, non-selective and non-specific MMP inhibitors; second is the lack of verification of these results with using MMP-2 knockout mice model; and third is lack of detection of MMP-9 in myocardium perfused ex vivo with Krebs–Henseleit buffer. In the setting of I/R in vivo studies, activation of blood cells is likely to result in the release of MMP-2, MMP-9, and possibly other proteases, that may also contribute to the development of endothelium injury. Obviously, more work is required to study the biological and pharmacological significance of the inhibition of MMP-2 during I/R injury.

In conclusion, this study shows for the first time that MMP inhibition not only reduces cardiac mechanical dysfunction but also reduces the enhanced endothelial permeability that is expressed by reduction of protein release to coronary effluent. Therefore, we have demonstrated that inhibiting MMP-2 protects the heart from I/R injury, not only by preventing degradation of intracellular proteins [32, 41], but also by protecting the integrity of the endothelium. The results from this project as well as from our previous work show that using MMP inhibitors may provide benefits in the treatment of cardiac diseases not only through protecting cardiac myocytes but also by protecting the integrity of endothelium and possibly intracellular membranes.

Acknowledgments We thank Dr. J. S Richardson for help with manuscript preparation. This work was supported by grants from Canadian Institutes of Health Research and the Heart and Stroke Foundation of Canada.

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