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Reduction of myocardial infarct size by doxycycline: A role for plasmin inhibition

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Abstract

Myocardial ischemia-reperfusion (I/R) is associated with the activation of matrix metalloproteinases (MMPs) and serine proteases. We hypothesized that activation of MMPs and the serine protease plasmin contribute to early cardiac myocyte death following I/R and that broad-spectrum protease inhibition with doxycycline (DOX) preserves myocyte viability. Rats treated daily with or without DOX beginning 48 h prior to experimentation were subjected to 30 min of coronary occlusion and 2 days of reperfusion. DOX pre-treatment reduced infarct size by 37%. DOX attenuated increases in MMP-9 and plasmin levels as determined by gelatin zymography and immunoblot, respectively. Neutrophil extravasation was unaltered by DOX as assessed by myeloperoxidase (MPO) activity. To examine the contribution of MMP-9 and plasmin to myocyte injury, cultures of neonatal rat ventricular myocytes (NRVMs) were treated for 48 h with 83 kDa MMP-9 or plasminogen in the presence or absence of DOX. MMP-9 treatment did not affect myocyte viability. Plasminogen treatment led to increased plasmin activity, resulting in loss of β_1-integrin, NRVM detachment and apoptosis. DOX co-treatment inhibited plasmin activity and preserved NRVM attachment, whereas co-treatment with the broad-spectrum MMP inhibitor GM6001 had no effect. These results indicate that plasmin causes disruption of myocyte attachment and viability independently of MMP activation *in vitro* and that inhibition of plasmin by DOX may reduce I/R-induced myocyte death *in vivo* through the inhibition of plasmin. (Mol Cell Biochem **270:** 1–11, 2005)

Key words: plasmin/plasminogen system, proteases, myocardial ischemia-reperfusion, matrix metalloproteinase

Introduction

Reperfusion of the myocardium following acute coronary occlusion has been shown to salvage myocytes and reduce infarct size [1]; however, its benefit is limited due to reperfusion injury [2]. Upon reperfusion, the extravasation of neutrophils and monocytes, release of inflammatory cytokines, generation of reactive oxygen species, and release of proteolytic enzymes contribute to additional myocyte dysfunction and death beyond that generated by ischemia alone [2]. Reperfusion injury consists of both necrotic and apoptotic forms of cell death [3], and strategies aimed at reducing the extent of apoptosis or necrosis have been demonstrated to reduce infarct size [4–6]. In adherent cell types, apoptotic cell death can be induced by disrupting interactions with the underlying extracellular matrix (ECM). Disruption of normal cell-ECM interactions has been shown to contribute to the induction of myocyte apoptosis both *in vitro* [7] and *in vivo* [8].

We and others have demonstrated that ECM degradation occurs within minutes of myocardial ischemia and may be mediated in part by the early activation of matrix metalloproteinases (MMPs) [9, 10]. MMPs are a family of zinc-dependent endopeptidases that are expressed as inactive

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zymogens and activated through proteolytic processing by other proteases [11]. In studies using isolated perfused rat hearts, MMP inhibition reduces ischemia-reperfusion (I/R)-induced troponin I degradation and improves recovery of mechanical function [12]. However, other ECM-degrading proteases are activated within the ischemic myocardium, including serine proteases of the plasminogen/plasmin system [13]. In addition to its well-known role in clot lysis, plasmin also mediates pericellular proteolysis of ECM proteins both directly and through the activation of MMPs [13]. Increased plasmin levels have been observed following coronary occlusion [14]. The inactive plasminogen zymogen is proteolytically activated to plasmin by plasminogen activators. Urokinase-type plasminogen activator is upregulated following coronary occlusion in the pig [15] and in the mouse predisposes the heart to rupture following coronary occlusion [14]. Broad-specrum serine protease inhibition with aprotinin attenuates creatine kinase release following I/R and improves recovery of mechanical function in the rat [16] and dog [17]. While these results suggest that MMPs and/or plasmin may have a role in mediating early myocardial ischemic injury, their effects on myocardial infarct size following I/R have not been investigated *in vivo*.

Doxycycline (DOX), a member of the tetracycline family of antibiotics, has been shown to inhibit MMP expression and activity [18] and to preserve cardiac function following I/R injury [12]. However, recent reports indicate DOX also directly inhibits cysteine protease activity [19] and indirectly inhibits serine protease activity via inhibition of MMP-mediated degradation of endogenous serine protease inhibitors (SERPINs) [20, 21]. In this study we report that DOX treatment attenuated increases in plasmin and MMP-9 activities following myocardial I/R injury and reduced infarct size *in vivo*. Using an *in vitro* culture system, plasmin but not MMP-9 induced myocyte death by detachment, and this was inhibited by DOX. These results suggest that DOX may reduce myocyte death *in vivo*, at least in part, through the inhibition of plasmin.

Materials and methods

Doxycycline treatment

Adult male Sprague-Dawley rats (Harlan, Indianapolis, IN) weighing 250–300 g were used. DOX (30 mg/kg per day; Sigma, St. Louis, MO) or water was administered orally beginning 48 h before surgery and continuing 48 h post-surgery. This DOX dose effectively blocks MMP activity in models of tissue injury and healing in the rat [22, 23]. For infarct size determination, groups included untreated ($n = 8$) and DOX-treated ($n = 8$) I/R rats. For biochemical measurements, groups included untreated ($n = 11$) and DOX-treated ($n = 12$) I/R rats and untreated ($n = 5$) and DOX-treated ($n = 5$) sham rats. All procedures were approved by the Institutional Animal Care and Use Committee and conform to published NIH guidelines for animal research.

Surgery

Animals were anesthetized by intraperitoneal injection of ketamine (100 mg/kg) and xylazine (10 mg/kg), intubated, and positive-pressure ventilated. Following a left thoracotomy, the left anterior descending coronary artery was ligated for 30 min, released and the suture left in place as a point of reference. The chest was closed and the animal allowed to recover. Successful occlusion and reperfusion were verified by visual inspection of left ventricle (LV) color. Sham animals were treated identically, except the ligature was not tightened.

Determination of infarct size

At 48 h post-I/R, infarct size was determined as previously described [24]. Trypan blue (0.4%) was used to delineate the area at risk. Triphenyltetrazolium chloride staining was used to distinguish between viable and infarcted myocardium.

Tissue collection

At 48 h post-I/R, the hearts were rapidly excised and perfused with cold cardioplegia to remove blood. LV freewall (ischemic region) and septum were separated and homogenized on ice in lysis buffer (50 mmol/L Tris pH 7.4, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$, 0.2 mmol/L NaN_3, 0.1% Triton X-100). Lysates were cleared by centrifugation at 12,000 g for 10 min at 4 °C.

Gelatin zymography

Gelatin zymography was performed as previously described [25]. An internal control (human MMP-2/MMP-9, Chemicon, Temecula, CA) was loaded to normalize between gels. Bands of gelatinolytic activity were digitally quantified (Kodak 1D, Eastman Kodak, Rochester, NY).

Immunoblots

Immunoblotting was performed as previously described [26]. Blots were probed with a rabbit polyclonal anti-plasminogen antibody (1:250, Biogenesis Ltd., Kingston, NH), a mouse monoclonal anti-plasminogen antibody [27] (1:200), a mouse monoclonal anti-α-actinin antibody (1:400, Upstate U.S.A., Charlottesville, VA), or a rabbit polyclonal anti-β_{1D}-integrin

antibody [28] (1:10,000) followed by appropriate horseradish peroxidase-conjugated secondary antibodies.

Myeloperoxidase (MPO) Assay

The MPO assay was performed as previously described [26] with modifications. Tissue samples were homogenized in MPO lysis buffer (50 mmol/L KH_2PO_4 pH 6.0, 0.5% hexadecyltrimethylammonium bromide) and incubated on ice for 30 min. Following centrifugation, the supernatants were reacted with 0.4 mmol/L tetramethylbenzidine (Sigma) and 0.006% H_2O_2 in 50 mmol/L phosphate at pH 6.0. Absorbance was monitored and MPO activity expressed as mOD/min per gram of protein.

Plasminogen and MMP-9 stimulation on neonatal rat ventricular myocytes (NRVMs)

NRVMs were prepared as previously described [29] and maintained in serum-free media (80% DMEM, 20% M199). NRVMs were stimulated with bovine plasminogen (Sigma), porcine or human plasmin (Sigma), or 83 kDa human recombinant active MMP-9 (Calbiochem, La Jolla CA) for 16–48 h. In studies involving DOX or the broad-spectrum MMP inhibitor GM6001 (Sigma), the inhibitor was added 1 h prior to stimulation. At the end of the experiment, plasmin activity was assessed by reacting culture media with 0.5 mmol/L N-p-tosyl-gly-pro-lys-pNA (Sigma) in Tris buffer (50 mmol/L, pH 7.5) and continually monitoring the increase in absorbance at 405 nm.

Global MMP activity was assessed by reacting culture media with 10 μmol/L OmniMMP substrate (Mca-pro-leu-gly-leu-Dpa-ala-arg, BIOMOL Research Laboratories, Plymouth Meeting, PA) in assay buffer (50 mmol/L Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$). To assess gelatinase activities, culture media was subjected to gelatin zymography as described above.

Cell detachment assay

Cell detachment was assessed as previously described [30]. Following treatment, the wells were rinsed with PBS, and the remaining viable adherent cells were assessed using a methylthiazoletetrazolium (MTT)-based in vitro toxicology assay kit (Sigma) per manufacturer instructions.

Annexin V labeling of apoptotic NRVMs

NRVMs were grown on gelatin-coated coverslips. Annexin V and propidium iodide labeling were performed per manufacturer instructions (BD ApoAlert Annexin V, BD Biosciences, San Diego, CA). The cells were then fixed with 3.4% formaldehyde and counterstained with 4′,6-diamidino-2-phenylindole dihydrochloride (DAPI, Sigma).

Inhibition of plasmin by DOX

Plasmin (100 nmol/l) was incubated with various concentrations of DOX in assay buffer (50 mM Tris pH 7.5, 150 mmol/L NaCl, 5 mmol/L $CaCl_2$) for 1 h at room temperature. N-p-tosyl-gly-pro-lys-pNA was then added to a final concentration of 0.5 mmol/L and the increase in absorbance at 405 nm was continuously monitored. The data were fit to the sigmoidal Hill equation: $Y = [\mathrm{DOX}]^n/([\mathrm{DOX}]^n + k^n)$. Y is the rate of substrate hydrolysis as a fraction of maximal substrate hydrolysis, n is the Hill coefficient, and k is the DOX concentration at which activity is half maximal (IC_{50}).

Statistical analysis

Results are expressed as mean ± S.E.M. Comparisons between means were analyzed, as appropriate, by student's *t*-test or one-way ANOVA followed by Bonferroni *t*-test. A value of $p < 0.05$ was considered statistically significant.

Results

Doxycycline reduced infarct size

To assess the effects of DOX on infarct size following myocardial I/R injury, rats were subjected to 30 min of coronary occlusion followed by 2 days of reperfusion. In the vehicle group, the mean area at risk was 46.0 ± 1.6% of the left ventricle (LV) and the infarct area was 39.9 ± 5.0% of the area at risk. In the DOX-treated group, the mean area at risk was comparable (50.0 ± 3.1% of LV), but the infarct area was significantly reduced to 25.2 ± 4.9% of the area at risk (Fig. 1).

Doxycycline attenuated MMP-9 upregulation in the ischemic region

Gelatin zymography of tissue homogenates from the ischemic region and septum revealed bands corresponding to 92 kDa MMP-9 and 72 and 75 kDa MMP-2 (Fig. 2A). Densitometric analysis revealed MMP-9 levels were increased 3-fold in the ischemic region of the I/R group compared with Sham ($p < 0.05$), and this increase was attenuated in the I/R+DOX group ($p < 0.05$) (Fig. 2B). MMP-9 levels in the septum remained unchanged between groups; no differences in MMP-2 levels were observed in any group.

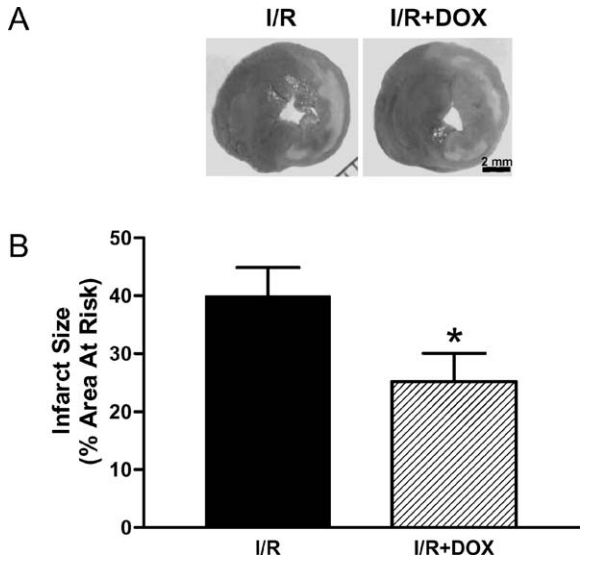

Fig. 1. Reduction of myocardial infarct size with DOX pre-treatment. A, Representative control (I/R) and DOX-treated (I/R + DOX) infarcted hearts stained with trypan blue and triphenyltetrazolium chloride for infarct size determination. B, Morphometric analysis of infarct size in I/R ($n = 8$) and I/R + DOX ($n = 8$). Values are mean ± S.E.M. $^{*}p < 0.05$.

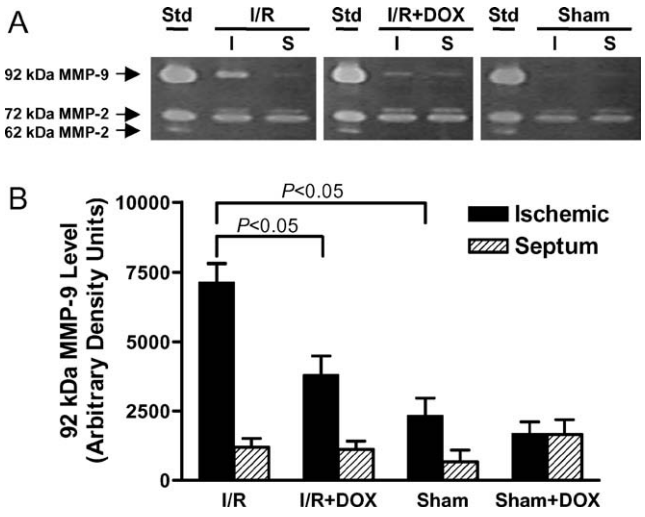

Fig. 2. Reduction of 92 kDa MMP-9 levels in the ischemic region with DOX pre-treatment. A, Representative gelatin zymograms of myocardial MMP-2 and MMP-9 levels in control (I/R) and DOX-treated (I/R + DOX) infarcted hearts and sham hearts. 'I' and 'S' indicate ischemic region and septum, respectively. 'Std' indicates human MMP-2/MMP-9 standard. B, Densitometric analysis of 92 kDa MMP-9 zymographic activity in I/R ($n = 11$), I/R + DOX ($n = 12$), Sham ($n = 5$), and Sham + DOX ($n = 5$). Values are mean ± S.E.M..

Doxycycline did not inhibit the inflammatory response

Since MMP-9 is associated with neutrophil infiltration in other models of MI [26, 31], we assessed the capacity of DOX to inhibit neutrophil accumulation in the myocardium by measuring MPO activity. MPO is an enzyme located within the primary granules of neutrophils and has commonly been used as a marker of tissue neutrophil content [32]. In both the I/R and I/R + DOX groups, MPO activity in the ischemic region was significantly higher compared to septum, indicating infiltration of neutrophils into the ischemic region. No significant difference in MPO activity in the ischemic region was observed between the I/R and I/R + DOX groups, indicating DOX treatment did not inhibit neutrophil infiltration into the ischemic region (Fig. 3).

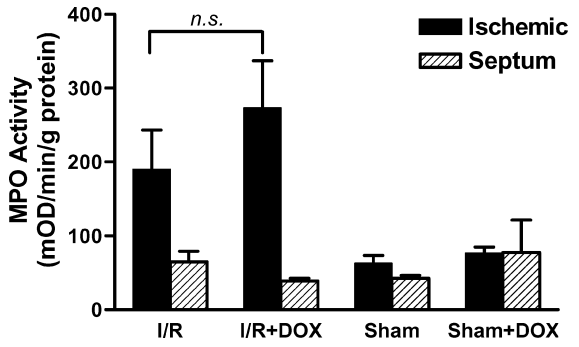

Fig. 3. Myocardial myeloperoxidase (MPO) activity in control (I/R, $n = 11$) and DOX-treated (I/R + DOX, $n = 12$) ischemic hearts and control (Sham, $n = 5$) and DOX-treated (Sham + DOX, $n = 5$) sham hearts. No significant difference was observed between I/R and I/R + DOX ischemic regions. Values are mean ± S.E.M.. n.s. = not significant.

Doxycycline reduced plasmin levels in the ischemic region

Since DOX has inhibitory effects on proteases other than MMPs, we assessed the effects of DOX on plasminogen and plasmin levels in the I/R hearts. Immunoblots revealed an increase in plasminogen in the ischemic region compared to septum in both the I/R group and the I/R + DOX group ($p < 0.05$) (Figs. 4A and B). DOX treatment tended toward reduced plasminogen levels in the ischemic region compared with the untreated I/R group, but this did not reach statistical significance (Fig. 4B).

An immunoreactive band of 68 kDa also appeared in the ischemic region of the I/R and I/R + DOX groups, consistent with the active plasmin form of the protease (Fig. 4A). Plasmin levels were significantly increased in the ischemic region compared to septum in I/R and I/R + DOX. DOX treatment significantly reduced plasmin levels in the ischemic region of I/R + DOX compared with I/R (Fig. 4C).

MMP-9 did not cause NRVM detachment or death

Given the enhanced levels of MMP-9 observed with I/R injury *in vivo*, we investigated the effects of recombinant, 83 kDa active MMP-9 on NRVMs *in vitro*. MMP-9 was added

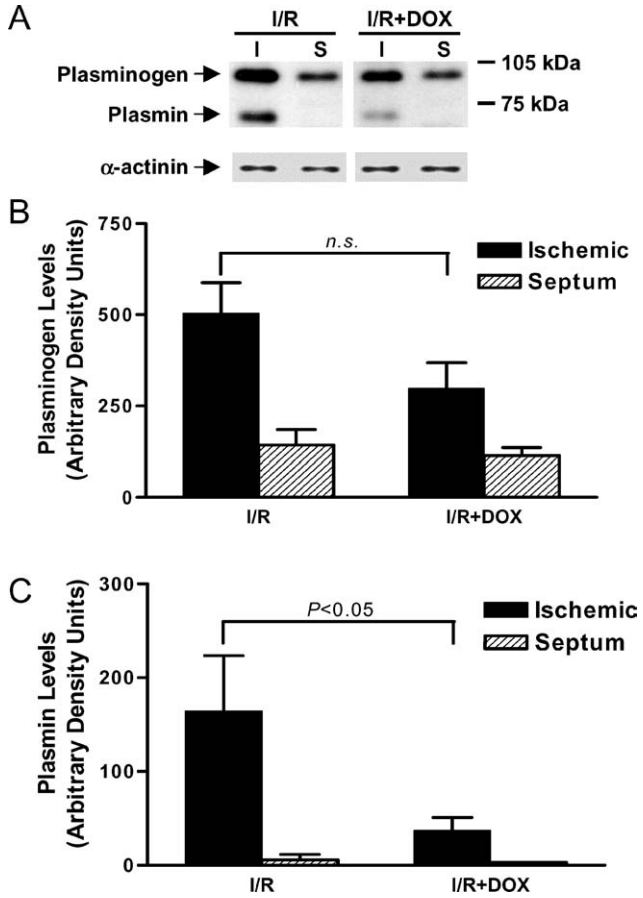

Fig. 4. Reduction of plasmin levels in the ischemic region with DOX pretreatment. A, Representative immunoblots of myocardial plasminogen and plasmin levels in control (I/R) and DOX-treated (I/R + DOX) infarcted hearts. 'I' and 'S' indicate ischemic region and septum, respectively. B and C, Densitometric analysis of plasminogen and plasmin levels in I/R ($n = 11$) and I/R + DOX ($n = 12$). Values are mean ± S.E.M.. n.s. = not significant.

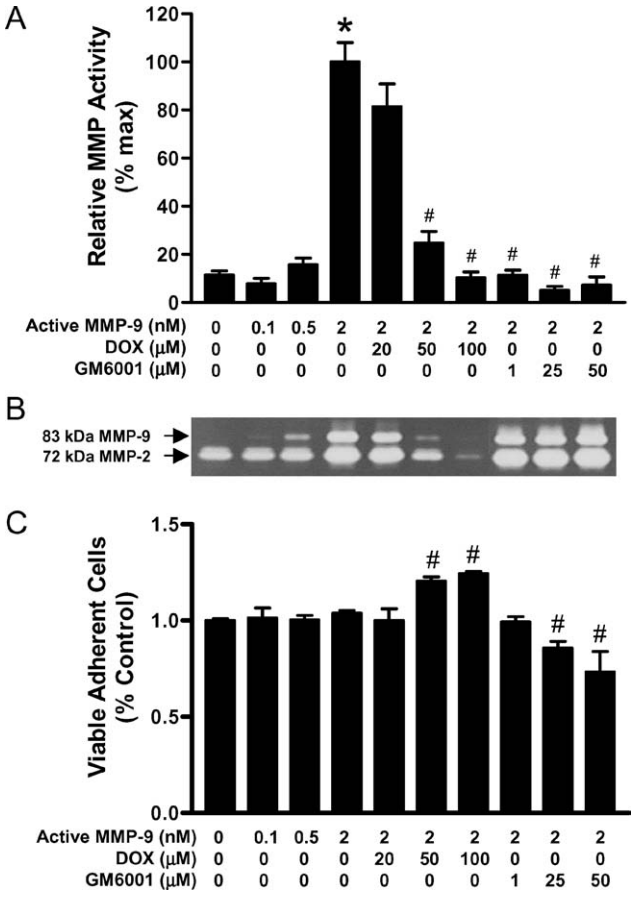

Fig. 5. Effects of DOX co-treatment on MMP activity and cell detachment in cultures of NRVMs stimulated with 83 kDa active MMP-9 for 48 h. A, Dose-dependent increase in MMP activity and inhibition by DOX and GM6001 as assessed by hydrolysis of a peptide substrate. Activities are expressed as the percentage of maximal activity. B, Representative gelatin zymogram following stimulation of NRVMs with 83 kDa MMP-9 in the presence or absence of inhibitors. Gel lanes correspond to columns in A. C, Remaining viable adherent cells as assessed by reduction of MTT. Values are normalized to unstimulated cells. Data are expressed as mean ± S.E.M. from experiments performed at least in triplicate from two different cell preparations. $^{*}p < 0.05$ vs. unstimulated control. $^{\#}p < 0.05$ vs. 2 nM active MMP-9-stimulated only.

incrementally to NRVMs up to a concentration of 2 nM, which corresponds to serum concentrations of MMP-9 reported in humans post-MI [33]. DOX co-treatment resulted in a dose-dependent inhibition of MMP activity, and the broad-spectrum MMP inhibitor GM6001 completely inhibited MMP activity by 1 μmol/L (Figs. 5A and B). Interestingly, media from DOX-treated cells showed a dose-dependent loss of MMP zymographic activity, whereas GM6001-treated cells retained MMP zymographic activity (Fig. 5B).

MMP-9 did not cause cell detachment nor induce cell death at any concentration tested (Fig. 5C). A slight but significant decrease in the number of viable adherent cells was observed with 25 and 50 μmol/L GM6001, likely due to cytotoxicity at high concentrations. The small increase in the number of viable adherent cells with 50 and 100 μmol/L DOX compared to controls (Fig. 5C) suggests that DOX may reduce NRVM detachment that occurs under normal, untreated culture conditions.

NRVMs convert plasminogen to plasmin

Addition of plasminogen to NRVMs for 48 h led to a dose-dependent increase in plasmin activity as assessed by hydrolysis of a plasmin-specific peptide substrate (Fig. 6A). Since plasmin is a known activator of MMPs, we also measured MMP activity following 48 h of plasminogen stimulation using the OmniMMP substrate. However, no increase in MMP activity was observed (Fig. 6B). Interestingly, the treatment of NRVM and cardiac fibroblast co-cultures with plasminogen yielded dose-dependent increases in MMP activation (data not shown), implicating fibroblasts as the primary source of MMPs in the heart.

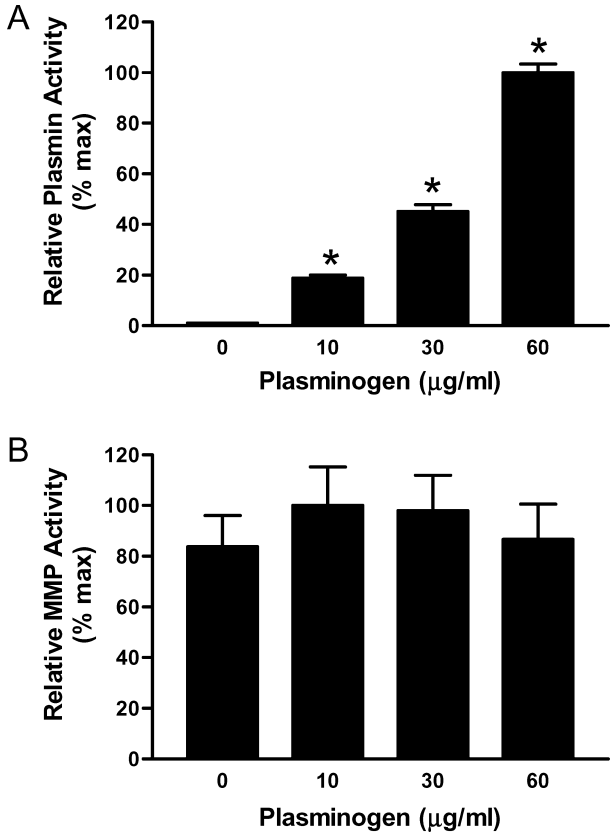

Fig. 6. Effects of plasminogen treatment on plasmin and MMP activities in cultures of NRVMs. Plasmin activity (A) and global MMP activity (B) induced by 48 h of plasminogen treatment as assessed by hydrolysis of peptide substrates. Data are mean ± S.E.M. from experiments performed at least in triplicate from two different cell preparations. $^{*}p < 0.05$ vs. unstimulated.

Plasmin induced NRVM detachment and death

The addition of 60 μg/mL plasminogen to NRVMs for 48 h resulted in cell rounding and a loss of cell-cell and cell-matrix interactions (Fig. 7A). MTT assay results showed that ~50% of the cells were detached by 48 h of stimulation with 60 μg/mL plasminogen (Fig. 7B). To verify that cell detachment was due to plasmin activity, NRVMs were stimulated with 60 μg/mL purified plasmin. Cell detachment occurred more rapidly with plasmin than plasminogen, as 92% of the NRVMs were detached by 16 h of stimulation (data not shown). Immunoblots of cell lysate against β_{1D}-integrin revealed an immunoreactive doublet of ~125 kDa, corresponding to the mature and precursor forms of the protein as previously described [28]. Plasmin stimulation resulted in the loss of mature β_{1D}-integrin (Fig. 7C), the primary β_1-integrin isoform expressed in the postnatal heart [34].

Plasminogen-treated NRVMs showed increased membrane staining with annexin V and increased nuclear fragmentation (Fig. 7D). The cells did not stain with propidium iodide, indicating maintenance of membrane integrity. Surprisingly, many of the detached cells continued to spontaneously beat, also indicating membrane integrity. Thus, these data are consistent with early apoptotic cell death.

Doxycycline inhibited plasmin activity in culture and preserved NRVM attachment

We assessed the effects of DOX on plasminogen-treated NRVMs. DOX co-treatment resulted in a dose-dependent decrease in plasmin activity, with an apparent IC_{50} of ~36 μmol/L (Fig. 8A). DOX also preserved NRVM attachment in the presence of 60 μg/mL plasminogen in a dose-dependent manner, with 50 μmol/L completely preserving attachment (Fig. 8B). However, the broad-spectrum MMP inhibitor GM6001 did not inhibit plasmin activity (Fig. 8A) nor did it preserve myocyte attachment (Fig. 8B). DOX also completely preserved levels of mature β_{1D}-integrin while GM6001 did so to a much lesser extent (Fig. 7C).

To verify that the reduction in plasmin activity by DOX was due to the direct inhibition of plasmin rather than inhibition of the conversion of plasminogen to plasmin, NRVMs were stimulated with purified plasmin (60 μg/mL) for 16 h in the presence or absence of DOX. 100 μmol/L DOX inhibited plasmin activity by 72% ($p < 0.05$) (Fig. 8A), demonstrating plasmin to be a direct target of DOX.

Media from NRVMs treated for 16 h with 60 μg/mL plasmin or plasminogen revealed a diffuse immunoreactive doublet at the approximate molecular weight of pure plasmin (Fig. 8C). Interestingly, DOX co-treatment reduced plasmin levels in the media of both the plasmin-treated (lane 3) and plasminogen-treated cells (lane 5).

Doxycycline directly inhibited plasmin

The addition of DOX to plasmin (100 nmol/L) in buffer resulted in a dose-dependent inhibition of plasmin activity with an IC_{50} of 18.4 μmol/L as determined by hydrolysis of a peptide substrate (Fig. 9).

Discussion

The unique observations from this study were that DOX pretreatment significantly reduced infarct size 2 days post-I/R in the rat heart *in vivo*. The smaller infarct size correlated with reduced MMP-9 and plasmin levels in the ischemic region, suggesting a possible role for these proteases in mediating I/R injury. The assessment of MMP-9 and plasmin effects on cultured NRVMs demonstrated that physiological concentrations of plasmin, but not MMP-9, were capable of

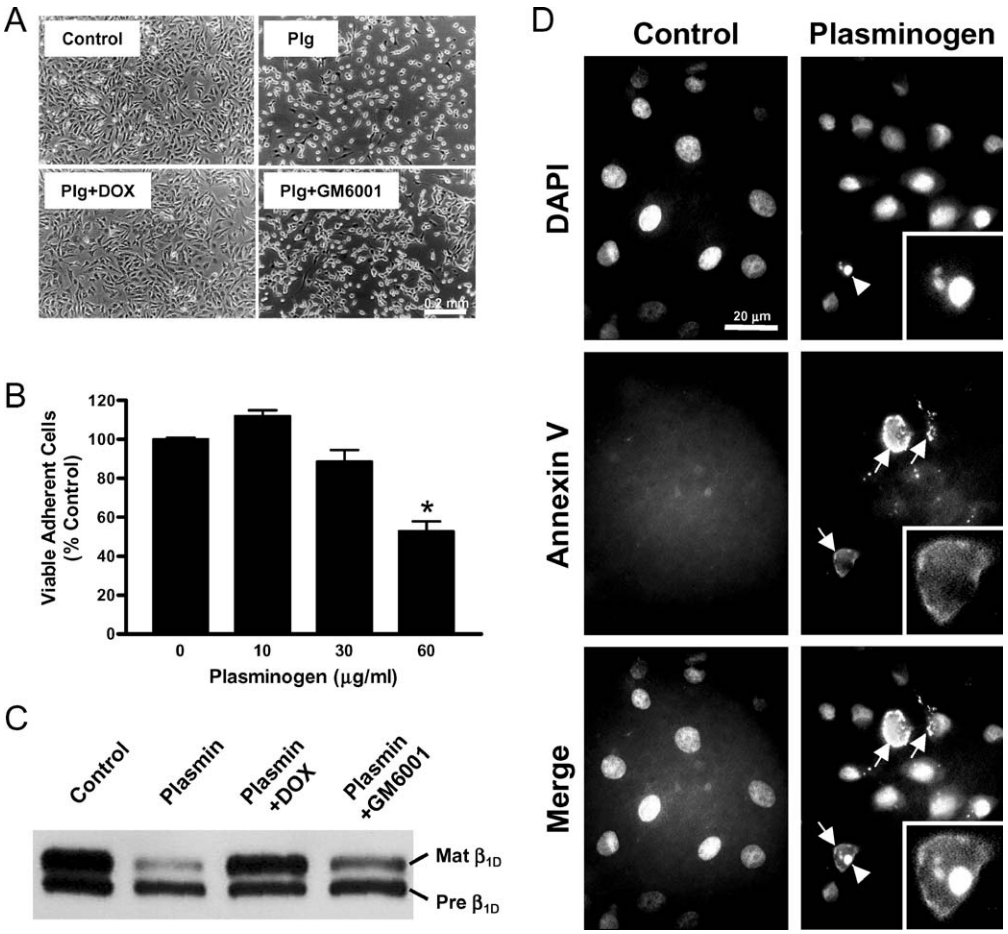

Fig. 7. Neonatal rat ventricular myocyte (NRVM) detachment and death following 48 h of plasminogen stimulation. A, Representative phase contrast microscopy images of untreated NRVMs and NRVMs stimulated with plasminogen (60 μg/mL) with or without DOX (100 μmol/L) or GM6001 (50 μmol/L). B, Remaining viable adherent NRVMs following plasminogen (60 μg/mL) stimulation as assessed by reduction of MTT. Values are normalized to the unstimulated control. C, Immunoblot of β_{1D}-integrin in cell lysate from NRVMs stimulated for 16 h with plasmin (60 μg/mL) with or without DOX (100 μmol/L) or GM6001 (50 μmol/L). 'Mat β_{1D}' and 'Pre β_{1D}' indicate mature and precursor β_{1D}-integrin, respectively. D, Annexin V and DAPI staining of NRVMs stimulated with plasminogen (60 μg/mL). Annexin V staining localized to the membrane of apoptotic cells (arrows). Many of the annexin V positive cells showed nuclear fragmentation (arrowhead, magnified view in *inset*). Cells were negative for propidium iodide staining. Data are mean $\pm$ S.E.M. from experiments performed at least in triplicate from two different cell preparations. $^*p < 0.05$ vs. unstimulated.

inducing cell death by detachment. DOX inhibited plasmin activity in culture and preserved NRVM attachment, suggesting that DOX may reduce myocyte death *in vivo*, at least in part, through the inhibition of plasmin.

Previous studies demonstrated latent (92 kDa) and active (83 kDa) MMP-9 are upregulated within hours of I/R and are primarily derived from neutrophils [26, 31]. We observed increases in 92 kDa MMP-9 in the ischemic region 48 h post-I/R correlating with increased neutrophil content. However, increases in 83 kDa active MMP-9 were not observed. A plausible explanation for lack of increased 83 kDa MMP-9 is that MMP-9 activation and subsequent clearance from the myocardium may occur within the first 48 h following I/R, since neutrophil accumulation peaks within the first 24 h of reperfusion in the rat [35].

We found that pretreatment of rats with DOX reduced tissue levels of 92 kDa MMP-9 within the ischemic region but did not alter neutrophil content, suggesting that MMP-9 levels may not necessarily follow neutrophil infiltration. Reductions in MMP-9 levels without a concomitant reduction in inflammatory cell infiltrate have also been reported with DOX treatment in a mouse model of abdominal aortic aneurysm [36]. Furthermore, MMP-9-deficient mice subjected to I/R showed only slight reductions in neutrophil infiltration into the I/R region [14]. Thus, our results indicate that although DOX reduces 92 kDa MMP-9 levels, it does not appear to affect the acute inflammatory response following I/R.

The integrity of cell-matrix interactions is necessary for myocyte survival both *in vitro* [7, 37] and *in vivo* [8]. To investigate whether reduced infarct size was due to reduced

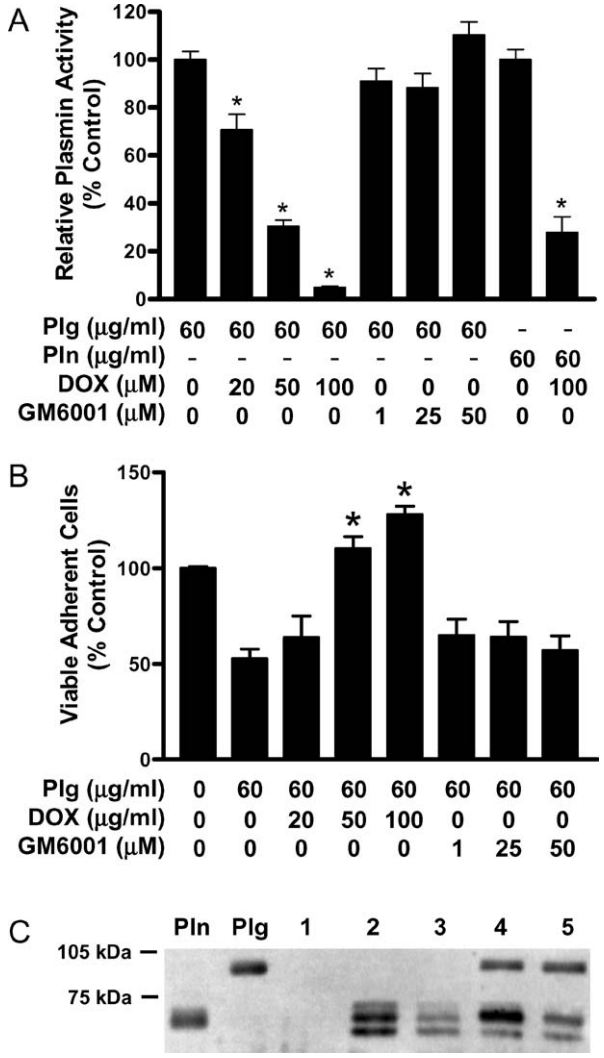

Fig. 8. Effects of DOX co-treatment on plasmin activity and cell detachment in cultures of NRVMs. A, Inhibition of plasmin activity by DOX and GM6001 following 48 h of plasminogen (Plg) stimulation or 16 h of plasmin (Pln) stimulation as assessed by hydrolysis of a peptide substrate. Activity is expressed as the percentage of activity observed without inhibitors. B, Remaining viable adherent cells following 48 h of plasminogen (60 μg/mL) stimulation as assessed by reduction of MTT. Values are normalized to unstimulated cells. C, Representative immunoblot of media from NRVMs treated for 16 h with 60 μg/mL Pln (lane 2), 60 μg/mL Pln + 100 μmol/L DOX (lane 3), 60 μg/mL Plg (lane 4), or 60 μg/mL Plg+100 μmol/L DOX (lane 5). Lane 1 is untreated control. 'Pln' and 'Plg" indicate plasmin and plasminogen standard, respectively. Data are mean ± S.E.M. from experiments performed at least in triplicate (Plg) or duplicate (Pln) from two different cell preparations. $^*p < 0.05$ vs. no inhibitor.

MMP-9 activity with DOX, we investigated the effects of MMP-9 on cell survival in cultures of NRVMs. Addition of physiological concentrations of MMP-9 did not alter NRVM attachment or viability, suggesting MMP-9 alone is not sufficient to directly disrupt myocyte-matrix interactions. DOX and GM6001 both effectively inhibited global MMP activity

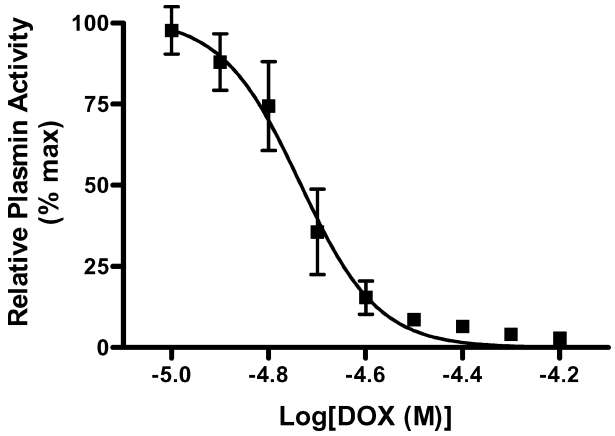

Fig. 9. Inhibition of purified human plasmin by DOX. Plasmin was preincubated with DOX for 1 h. Data was fit to the sigmoidal Hill equation and the IC50 determined to be 18.4 μmol/L. Data points are shown as mean ± S.E.M. from 3 independent experiments.

as assessed by OmniMMP substrate hydrolysis. Interestingly, whereas GM6001 did not inhibit MMP-2 or MMP-9 zymographic activity due to dissociation of the inhibitor during electrophoresis, DOX reduced MMP-2 and MMP-9 zymographic activity. This suggests DOX treatment causes a loss of functional MMP-2 and MMP-9 protein or that DOX does not dissociate from the MMP during zymography. Therefore, MMP inhibition by DOX appears to occur by different mechanisms than GM6001.

Increases in the serine protease plasmin and its zymogen, plasminogen, were also noted within the ischemic region 2 days following I/R. A study by Heymans *et al.* [14] showed similar increases in plasminogen and plasmin levels within the infarct region of mice. Plasminogen is produced primarily by the liver and is maintained in plasma at a high concentration of ~220 μg/mL in rats [38]. The increased plasminogen levels observed following I/R suggest leakage of plasminogen into the myocardium due to increased microvascular permeability. uPA expression has been shown to be increased in the ischemic heart [15], likely promoting activation of extravasated and endogenous plasminogen and increasing plasmin levels within the myocardium.

Studies using knockout mice have suggested a central role for plasmin in mediating inflammatory, wound healing, and remodeling processes post-MI either directly or by activation of MMPs [14, 39]. uPA knockout mice subjected to MI show reduced plasmin levels and impaired leukocyte infiltration, reduced incidence of cardiac rupture, reduced MMP-2 and MMP-9 activities, and impaired healing and scar formation [14]. These observations suggest that plasmin activation may mediate ECM degradation, allowing infiltration of inflammatory and wound-healing cells, but also potentially reducing the tensile strength of the infarcted myocardium. Myocyte death occurs *in vivo* as a result of disruption of

normal myocyte anchorage [8], suggesting ECM proteolysis by plasmin or subsequent activation of MMPs could disrupt myocyte-matrix interactions and lead to cell death.

To determine whether plasmin could degrade myocyte-matrix attachments and whether DOX had any effect on this process, we assessed effects of plasmin with or without DOX co-treatment on cultured NRVMs. Plasmin is a serine protease with trypsin-like specificity capable of directly degrading various adhesive glycoproteins, such as fibronectin, laminin, and vitronectin [30–41], and indirectly degrading collagen via activation of MMPs [13]. Previous studies have demonstrated plasmin-mediated disruption of cell-matrix interactions in several adherent cell types: cultured vascular smooth muscle cells convert plasminogen to plasmin, resulting in fibronectin proteolysis and cell detachment and death [30], and plasminogen stimulation of CHO cells causes fibronectin and laminin degradation, inducing detachment and death [40]. In addition, stimulation of human keratinocytes with plasminogen resulted in proteolysis of vitronectin and cell detachment [41].

In agreement with previous studies [30, 40, 41], we observed that stimulation of NRVMs with plasminogen led to dose- and time-dependent increases in plasmin activity and that the conversion of plasminogen to plasmin occurred via endogenous plasminogen activators. Increased plasmin activity yielded morphological changes, such as cell rounding and loss of intercellular contacts, and loss of β_{1D}-integrin leading to cell detachment. Evidence of nuclear fragmentation and annexin V labeling confirmed an apoptotic mode of cell death. We did not observe MMP activation in NRVMs stimulated with plasminogen, suggesting that MMPs did not contribute to NRVM detachment. This was further supported by the inability of the broad-spectrum MMP inhibitor GM6001 to preserve β_{1D}-integrin and cell attachment. These data suggest that the direct proteolytic degradation of cell-matrix attachments by plasmin induces NRVM detachment and apoptosis.

Pre-treatment of the NRVM cultures with DOX prior to plasminogen stimulation preserved NRVM attachment and resulted in a dose-dependent inhibition of plasmin activity, with an IC_{50} of $\sim$36 μmol/L. The inhibition of plasmin activity upon stimulation with purified plasmin (as opposed to plasminogen) demonstrated the inhibitory effect of DOX was subsequent to plasminogen activation, however inhibition of plasminogen activators by DOX was not tested. Sorsa *et al*. [20] suggest an indirect inhibition of serine proteases by DOX via inhibition of MMP-mediated degradation of the serine protease inhibitor α_1-antitrypsin. Grenier *et al*. [21] showed DOX preserves α_1-antitrypsin levels and inhibits the trypsin-like activity of periodontal pathogen *T. denticola*. Contrary to these previous studies [20, 21], we showed that MMP inhibition by DOX played no role in inhibiting plasmin activity in culture since GM6001 treatment had no effect on plasmin activity despite complete inhibition of MMP activity. Therefore, the ability of DOX to inhibit plasmin and preserve NRVM attachment was specific and independent of MMP inhibition.

Similar to our zymography data showing that DOX co-treatment reduced MMP protein levels in NRVMs stimulated with MMP-9, immunoblot analysis showed DOX co-treatment also reduced the amount of plasmin in NRVMs stimulated with plasminogen or plasmin. The mechanism by which DOX exerts this effect is uncertain. Autoproteolysis of plasmin is known to occur [42], and it is possible that DOX accelerates this process. Also, cellular uptake and degradation of plasmin is known to be mediated by LDL receptor-related proteins [43], and it is possible that DOX may promote this process. The reduction in plasmin levels in culture by DOX correlates well with our observations of a 76% reduction in plasmin levels with DOX treatment in the rat post-I/R. Whether the mechanism by which plasmin is eliminated in culture contributes to the loss of plasmin *in vivo* post-I/R remains to be determined. These unique, and as of yet undescribed, capabilities of DOX offer tempting possibilities for treatment of pathologies where protease activation underlies the disease process.

Although we demonstrated that plasmin induced NRVM detachment and apoptosis *in vitro*, one limitation of this study is that we have not directly demonstrated that this occurs *in vivo* following I/R injury. Our determination of infarct size using TTC only discriminates between viable and nonviable tissue irrespective of whether cell death occurred by apoptosis or necrosis. However, given the high levels of plasminogen present in the bloodstream and tissue, it is reasonable to expect that plasmin activation plays an important role in inducing cell death during I/R. Secondly, the reduction of plasmin levels by DOX and reduced infarct size *in vivo* coupled with the potent inhibition of plasmin by DOX *in vitro* suggest that the inhibition of plasmin by DOX contributed to the reduction in infarct size. However, it is also possible that the reduced plasmin levels occurred secondarily to reduced infarct size via an alternate cardioprotective mechanism of DOX that was not explored in this study. Thirdly, the response of neonatal myocytes to plasmin may not completely parallel the response of adult myocytes *in vivo*. NRVMs were selected as the model of choice rather than adult myocytes due to the relative stability of NRVMs in culture over several days. Whereas adult myocyte cultures are fraught with loss of cylindrical morphology and detachment of calcium-intolerant cells early in primary culture, NRVMs are routinely cultured for days to weeks for investigation of myocyte morphology, function, and viability and the effects of experimental agents on these parameters. Therefore, NRVMs are a robust and suitable model to study the effects of protease-induced myocyte injury.

In conclusion, DOX treatment reduced infarct size *in vivo*, possibly by inhibiting plasmin, a serine protease capable of degrading myocyte-matrix attachments. Further exploration

of the pathophysiological role of plasmin in mediating myocardial I/R injury is thus warranted.

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References

1. Braunwald E: Myocardial reperfusion, limitation of infarct size, reduction of left ventricular dysfunction, and improved survival. Should the paradigm be expanded? Circulation 79: 441–444, 1989
2. Park JL, Lucchesi BR: Mechanisms of myocardial reperfusion injury. Ann Thorac Surg 68: 1905–1912, 1999
3. Kajstura J, Cheng W, Reiss K, Clark WA, Sonnenblick EH, Krajewski S, Reed JC, Olivetti G, Anversa P: Apoptotic and necrotic myocyte cell deaths are independent contributing variables of infarct size in rats. Lab Invest 74: 86–107, 1996
4. Parsa CJ, Kim J, Riel RU, Pascal LS, Thompson RB, Petrofski JA, Matsumoto A, Stamler JS, Koch WJ: Cardioprotective effects of erythropoietin in the reperfused ischemic heart: a potential role for cardiac fibroblasts. J Biol Chem 279: 20655–20662, 2004
5. Stadler B, Phillips J, Toyoda Y, Federman M, Levitsky S, McCully JD: Adenosine-enhanced ischemic preconditioning modulates necrosis and apoptosis: Effects of stunning and ischemia-reperfusion. Ann Thorac Surg 72: 555–563; discussion 563–554, 2001
6. Wakiyama H, Cowan DB, Toyoda Y, Federman M, Levitsky S, McCully JD: Selective opening of mitochondrial ATP-sensitive potassium channels during surgically induced myocardial ischemia decreases necrosis and apoptosis. Eur J Cardiothorac Surg 21: 424–433, 2002
7. Sabri A, Alcott SG, Elouardighi H, Pak E, Derian C, Andrade-Gordon P, Kinnally K, Steinberg SF: Neutrophil cathepsin G promotes detachment-induced cardiomyocyte apoptosis via a protease-activated receptor-independent mechanism. J Biol Chem 278: 23944–23954, 2003
8. Ding B, Price RL, Goldsmith EC, Borg TK, Yan X, Douglas PS, Weinberg EO, Bartunek J, Thielen T, Didenko VV, Lorell BH: Left ventricular hypertrophy in ascending aortic stenosis mice: anoikis and the progression to early failure. Circulation 101: 2854–2862, 2000
9. Villarreal F, Omens J, Dillmann W, Risteli J, Nguyen J, Covell J: Early degradation and serum appearance of type I collagen fragments after myocardial infarction. J Mol Cell Cardiol 36: 597–601, 2004
10. Etoh T, Joffs C, Deschamps AM, Davis J, Dowdy K, Hendrick J, Baicu S, Mukherjee R, Manhaini M, Spinale FG: Myocardial and interstitial matrix metalloproteinase activity after acute myocardial infarction in pigs. Am J Physiol Heart Circ Physiol 281: H987–994, 2001
11. Nagase H, Woessner JF, Jr.: Matrix metalloproteinases. J Biol Chem 274: 21491–21494, 1999
12. Cheung PY, Sawicki G, Wozniak M, Wang W, Radomski MW, Schulz R: Matrix metalloproteinase-2 contributes to ischemia-reperfusion injury in the heart. Circulation 101: 1833–1839, 2000
13. Collen D: Ham-Wasserman lecture: Role of the plasminogen system in fibrin-homeostasis and tissue remodeling. Hematology (Am Soc Hematol Educ Program) 1–9, 2001
14. Heymans S, Luttun A, Nuyens D, Theilmeier G, Creemers E, Moons L, Dyspersin GD, Cleutjens JP, Shipley M, Angellilo A, Levi M, Nube O, Baker A, Keshet E, Lupu F, Herbert JM, Smits JF, Shapiro SD, Baes M, Borgers M, Collen D, Daemen MJ, Carmeliet P: Inhibition of plasminogen activators or matrix metalloproteinases prevents cardiac rupture but impairs therapeutic angiogenesis and causes cardiac failure. Nat Med 5: 1135–1142, 1999
15. Knoepfler PS, Bloor CM, Carroll SM: Urokinase plasminogen activator activity is increased in the myocardium during coronary artery occlusion. J Mol Cell Cardiol 27: 1317–1324, 1995
16. Gurevitch J, Barak J, Hochhauser E, Paz Y, Yakirevich V: Aprotinin improves myocardial recovery after ischemia and reperfusion. Effects of the drug on isolated rat hearts. J Thorac Cardiovasc Surg 108: 109–118, 1994
17. McCarthy RJ, Tuman KJ, O'Connor C, Ivankovich AD: Aprotinin pretreatment diminishes postischemic myocardial contractile dysfunction in dogs. Anesth Analg 89: 1096–1100, 1999
18. Golub LM, Lee HM, Ryan ME, Giannobile WV, Payne J, Sorsa T: Tetracyclines inhibit connective tissue breakdown by multiple non-antimicrobial mechanisms. Adv Dent Res 12: 12–26, 1998
19. Imamura T, Matsushita K, Travis J, Potempa J: Inhibition of trypsin-like cysteine proteinases (gingipains) from Porphyromonas gingivalis by tetracycline and its analogues. Antimicrob Agents Chemother 45: 2871–2876, 2001
20. Sorsa T, Lindy O, Konttinen YT, Suomalainen K, Ingman T, Saari H, Halinen S, Lee HM, Golub LM, Hall J, *et al.*: Doxycycline in the protection of serum alpha-1-antitrypsin from human neutrophil collagenase and gelatinase. Antimicrob Agents Chemother 37: 592–594, 1993
21. Grenier D, Plamondon P, Sorsa T, Lee HM, McNamara T, Ramamurthy NS, Golub LM, Teronen O, Mayrand D: Inhibition of proteolytic, serpinolytic, and progelatinase-b activation activities of periodontopathogens by doxycycline and the non-antimicrobial chemically modified tetracycline derivatives. J Periodontol 73: 79–85, 2002
22. Curci JA, Petrinec D, Liao S, Golub LM, Thompson RW: Pharmacologic suppression of experimental abdominal aortic aneurysms: Acomparison of doxycycline and four chemically modified tetracyclines. J Vasc Surg 28: 1082–1093, 1998
23. Lamparter S, Slight SH, Weber KT: Doxycycline and tissue repair in rats. J Lab Clin Med 139: 295–302, 2002
24. Tiefenbacher CP, Kapitza J, Dietz V, Lee CH, Niroomand F: Reduction of myocardial infarct size by fluvastatin. Am J Physiol Heart Circ Physiol 285: H59–64, 2003
25. Hawkes SP, Li H, Taniguchi GT: Zymography and reverse zymography for detecting MMPs and TIMPs. In: I. M. Clark, (ed.). Matrix Metalloproteinase Protocols, Humana Press, Inc., Totowa, NJ, 2001, pp. 399–410
26. Romanic AM, Harrison SM, Bao W, Burns-Kurtis CL, Pickering S, Gu J, Grau E, Mao J, Sathe GM, Ohlstein EH, Yue TL: Myocardial protection from ischemia/reperfusion injury by targeted deletion of matrix metalloproteinase-9. Cardiovasc Res 54: 549–558, 2002
27. Pozzi A, Moberg PE, Miles LA, Wagner S, Soloway P, Gardner HA: Elevated matrix metalloprotease and angiostatin levels in integrin alpha 1 knockout mice cause reduced tumor vascularization. Proc Natl Acad Sci U.S.A. 97: 2202–2207, 2000
28. Pham CG, Harpf AE, Keller RS, Vu HT, Shai SY, Loftus JC, Ross RS: Striated muscle-specific beta(1D)-integrin and FAK are involved in cardiac myocyte hypertrophic response pathway. Am J Physiol Heart Circ Physiol 279: H2916–2926, 2000
29. Iwaki K, Sukhatme VP, Shubeita HE, Chien KR: Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. J Biol Chem 265: 13809–13817, 1990

30. Meilhac O, Ho-Tin-Noe B, Houard X, Philippe M, Michel JB, Angles-Cano E: Pericellular plasmin induces smooth muscle cell anoikis. Faseb J 17: 1301–1303, 2003
31. Lindsey M, Wedin K, Brown MD, Keller C, Evans AJ, Smolen J, Burns AR, Rossen RD, Michael L, Entman M: Matrix-dependent mechanism of neutrophil-mediated release and activation of matrix metalloproteinase 9 in myocardial ischemia/reperfusion. Circulation 103: 2181–2187, 2001
32. Griswold DE, Hillegass LM, Hill DE, Egan JW, Smith EF, 3rd: Method for quantification of myocardial infarction and inflammatory cell infiltration in rat cardiac tissue. J Pharmacol Methods 20: 225–235, 1988
33. Kaden JJ, Dempfle CE, Sueselbeck T, Brueckmann M, Poerner TC, Haghi D, Haase KK, Borggrefe M: Time-dependent changes in the plasma concentration of matrix metalloproteinase 9 after acute myocardial infarction. Cardiology 99: 140–144, 2003
34. Ross RS, Borg TK: Integrins and the myocardium. Circ Res 88: 1112–1119, 2001
35. Smith EF, 3rd, Egan JW, Bugelski PJ, Hillegass LM, Hill DE, Griswold DE: Temporal relation between neutrophil accumulation and myocardial reperfusion injury. Am J Physiol 255: H1060–1068, 1988
36. Kaito K, Urayama H, Watanabe G: Doxycycline treatment in a model of early abdominal aortic aneurysm. Surg Today 33: 426–433, 2003
37. Heidkamp MC, Bayer AL, Kalina JA, Eble DM, Samarel AM: GFP-FRNK disrupts focal adhesions and induces anoikis in neonatal rat ventricular myocytes. Circ Res 90: 1282–1289, 2002
38. Owens MR, Cimino CD: Biosynthesis of plasminogen by the perfused rat liver. J Lab Clin Med 105: 368–373, 1985
39. Creemers E, Cleutjens J, Smits J, Heymans S, Moons L, Collen D, Daemen M, Carmeliet P: Disruption of the plasminogen gene in mice abolishes wound healing after myocardial infarction. Am J Pathol 156: 1865–1873, 2000
40. Rossignol P, Ho-Tin-Noe B, Vranckx R, Bouton MC, Meilhac O, Lijnen HR, Guillin MC, Michel JB, Angles-Cano E: Protease-nexin-1 inhibits plasminogen activation-induced apoptosis of adherent cells. J Biol Chem 279: 10346–10356, 2003
41. Reinartz J, Schafer B, Batrla R, Klein CE, Kramer MD: Plasmin abrogates alpha v beta 5-mediated adhesion of a human keratinocyte cell line (HaCaT) to vitronectin. Exp Cell Res 220: 274–282, 1995
42. Gately S, Twardowski P, Stack MS, Cundiff DL, Grella D, Castellino FJ, Enghild J, Kwaan HC, Lee F, Kramer RA, Volpert O, Bouck N, Soff GA: The mechanism of cancer-mediated conversion of plasminogen to the angiogenesis inhibitor angiostatin. Proc Natl Acad Sci USA 94: 10868–10872, 1997
43. Strickland DK, Kounnas MZ, Argraves WS: LDL receptor-related protein: a multiligand receptor for lipoprotein and proteinase catabolism. Faseb J 9: 890–898, 1995