Published in final edited form as:
Arterioscler Thromb Vasc Biol. 2013 June ; 33(6): 1339–1349. doi:10.1161/ATVBAHA.113.301533.

Impaired coronary collateral growth in the metabolic syndrome is in part mediated by MMP12-dependent production of endostatin and angiostatin

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Abstract

Objective—We have previously shown that transient coronary artery occlusion stimulated coronary collateral growth (CCG) in healthy (SD) but not in metabolic syndrome (JCR) rats. Here, we sought to determine whether matrix metalloproteinases (MMPs) negatively regulate CCG in the metabolic syndrome via release of endostatin and angiostatin.

Approach/Results—Rats underwent transient, repetitive LAD occlusion (RI) for 0-10 days. CCG was measured in the collateral-dependent (CZ) and normal (NZ) zones using microspheres, MMP activation by Western blot, and endostatin and angiostatin by ELISA on days 0, 3, 6, 9 or 10 of RI. Endostatin and angiostatin were increased in JCR but not in SD rats on days 6 and 9 of RI. Increased endostatin and angiostatin correlated with increased MMP12 (~4 fold) activation in JCR but not in SD rats on days 6 and 9 of RI. Inhibition of MMP12 in JCR rats nearly completely blocked endostatin (~85%) and angiostatin (~90%) generation and significantly improved CCG (CZ flow was ~66% of NZ flow vs. ~12% for JCR RI).

Conclusions—Compromised CCG in the metabolic syndrome is in large part due to increased MMP12 activation and consequent increased generation of endostatin and angiostatin, which inhibits late-stage collateral remodeling.

Keywords

coronary collateral growth; MMPs; angiostatin; endostatin

Introduction

Transient repetitive coronary artery occlusion and resultant myocardial ischemia (RI) stimulate adaptive enlargement of pre-existing high resistance arterioles with minimal to no blood flow to conduit arteries in a process termed coronary collateral growth (CCG).[1] Well-

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Disclosures: None

developed coronary collaterals provide an alternative source of blood supply and can help preserve myocardial function in the setting of total coronary occlusion.[1] Clinically, patients with stable angina have decreased incidence of fatal myocardial infarction, which is associated with better developed collateral networks.[2] However, development of collateral networks is severely impaired in patients with metabolic syndrome.[3-7] Similarly, CCG is impaired in our metabolic syndrome rat model (JCR:LA-cp or JCR).[8] JCR rats mimic the complex pathology of human metabolic syndrome. By 8 weeks of age, the rats develop obesity with fatty liver, insulin resistance with glucose intolerance, complex dyslipidemia (low HDL, high LDL and vLDL), and vasculopathy characterized by decreased endothelium-dependent and -independent vasorelaxation and intimal lesions morphologically identical to early atherosclerotic lesions in humans. By 12 weeks of age, JCR rats exhibit wide-spread atherosclerosis and evidence of myocardial and cerebral (micro)infarctions. In addition, like the development of metabolic syndrome and cardiovascular disease in humans, the apparent complexity of the cardio-metabolic phenotype exhibited by JCR rats is suspected to be multifactorial and polygenetic in etiology, arising from a combination of environmental and genetic factors.[9] Thus, the JCR rat acts as an unparalleled rodent model for the study of CCG in the metabolic syndrome.

Temporally regulated reorganization of the extracellular matrix (ECM) is an integral part of collateral remodeling. First, the ECM acts as a mechanical barrier to the migration of endothelial cells (ECs) and vascular smooth muscle cells (VSMCs), which must be removed to allow for effective cell proliferation and migration in the early stages of CCG then reformed to allow for collateral vessel maturation in the late stages of CCG. In addition, the ECM also acts as a repository for pro-angiogenic growth factors such as the vascular endothelial growth factor (VEGF) and the fibroblast growth factor (FGF) as well as for anti-angiogenic peptides such as angiostatin and endostatin. The extent of CCG depends on the balance between the pro-angiogenic growth factors and the anti-angiogenic peptides. Therefore, ECM degradation, through the release of the above mentioned angiogenic factors can either promote or impede collateral development.

Angiostatin and endostatin have been negatively associated with coronary collateral development in patients with coronary artery disease[10] and type II diabetes[11]. Chronic hyperglycemia was also shown to inhibit CCG via an increase in angiostatin in a dog model.[12] Angiostatin is a 38 kDa cleavage product of plasminogen[13], which has been shown to exert its anti-angiogenic effects via induction of endothelial cell (EC) apoptosis[14-17] and impairment of EC proliferation[18], migration, and tube formation[19]. Endostatin is a 20 kDa cleavage product of collagen XVIII.[20] It also functions to induce EC apoptosis thorough multiple signaling pathways[21, 22] and diminish endothelial cell migration, adhesion, and proliferation[23, 24]. Targets of angiostatin and endostatin in collateral growth have not been investigated but are presumed to also be ECs.

Several proteases, including matrix metalloproteinases (MMPs), have been shown to be able to generate angiostatin and endostatin by cleavage of plasminogen and collagen XVIII, respectively, in vitro and in cell culture studies. MMP2[25] and MMP12[26] have been shown to release angiostatin in an in vitro model of lung metastasis. MMP12 has also been shown to inhibit microvascular EC proliferation in vitro by generating angiostatin.[26, 27] MMP3[28], MMP7[29], and MMP9[29] have been shown to be able to release angiostatin in in vitro studies in which human plasminogen was incubated with various MMPs. In an in vitro assay, MMPs 1, 2, 3, 9, 12, 13, 14 and 20 were able to release endostatin from a fragment of human collagen XVIII.[30] One study showed that MMP12 releases endostatin to inhibit VEGF-induced chemotaxis of osteoclasts.[31] Very little is known about MMP8 and its ECM substrates; however, the other two collagenase, MMP1 and MMP13, have been shown to generate endostatin in in vitro assays.[30] The ability of these MMPs to generate these anti-

angiogenic peptides in vivo, especially during collateral development, has not been evaluated. MMP2 and MMP9 are the only MMPs that have been studied in collateral growth. One study correlated their increased expression with actively remodeling collaterals.[32] However their decreased activation has been correlated with decreased CCG in humans with type II diabetes.[11] Furthermore, we have recently definitively shown that they are required for CCG and that they are not activated in metabolic syndrome[33] and several studies demonstrated lack of correlation between increased angiostatin and endostatin levels and increased MMP2 or MMP9 activation in humans with impaired CCG[10, 11], suggesting that MMP2 and MMP9 are not the MMPs responsible for the generation of angiostatin and endostatin associated with decreased coronary collateral development. The role of the other members of the MMP family has not been studied in association with collateral development. Moreover, it is not known whether their activation is altered in metabolic syndrome.

Our results in this study demonstrated that elevated angiostatin and endostatin levels in the metabolic syndrome rat model (JCR) correlated with enhanced MMP12 and MMP8 activation. Therefore, the goal of this study was to determine if MMP12 and/or MMP8 were involved in the corruption of CCG in the metabolic syndrome via upregulation of angiostatin and endostatin in the later stages of CCG.

Materials and Methods

See online Supplement.

Results

MMP12 and MMP8 activation is increased in the later stages of CCG in the metabolic syndrome but not in the normal rat model

Of the MMPs which have been shown to be able to generate angiostatin and endostatin in in vitro or cell culture studies, MMPs 1, 2, 3, 9, 12, 13, and 14, we have found MMPs 2, 9 and 14 (MT1-MMP) to be activated in response to RI and required for CCG in the normal rat phenotype (SD rat)[33] (Figure I, Supplement). Importantly, of these MMPs, we have found only MMP12 to be activated in the metabolic syndrome (JCR) rats in response to RI. Moreover, in our screen of MMPs, which are known to be expressed in the cardiovascular system[34], by expression of the pro and active forms of the MMP proteins, we have found only one additional MMP to be activated in response to RI, MMP8 (Figure 1 and Figure I, Supplement).

Basal MMP12 expression was ~2 fold higher in the JCR phenotype; however, there was no difference in its basal activation between the two phenotypes. RI resulted in a significant increase in pro-MMP12 on day 3 of RI in the SD rats (~5 fold), but this did not translate into an increase in its activation on any day of the RI protocol in the SD animals. Pro-MMP12 was also increased in the JCR rats on days 3 (~3 fold), 6 (~5 fold), and 9 (~5 fold) of RI. However, in contrast to the SD rats, MMP12 was also highly activated beginning at day 6 of RI (~4 fold vs. baseline) in the JCR animals. This activation remained sustained for the duration of the RI protocol (Figure 1A). Increased RI-induced MMP12 expression and activation in the heart did not translate into increased RI-induced MMP12 expression or activation in the plasma of JCR rats (Figure III, Supplement). Basal expression of MMP8 was ~2 fold higher in the JCR animals; however, RI did not significantly alter MMP8 expression in either phenotype. MMP8 activity was increased ~2 fold on day 3 of the RI protocol in the SD rat. In the JCR rat, MMP8 activation was greater in magnitude and the timing of activation was shifted from the early to the later staged of CCG. MMP8 was maximally activated on days 6 (~4 fold) and 9 (~3 fold) of RI (Figure 1B).

All changes were observed in the CZ only. RI did not induce any changes in MMP12 or MMP8 expression or activation in the NZ (Figure 3 and data not shown)

Elevated myocardial angiostatin and endostatin levels in the later stages of CCG in the metabolic syndrome correlate with increased MMP12 and MMP8 activation

Basal angiostatin and endostatin levels were similar between the two phenotypes. RI did not alter either angiostatin or endostatin in the CZ of the SD rats. In contrast, both angiostatin and endostatin levels were markedly upregulated on days 6 (~3 fold and 2 fold, respectively) and 9 (~2 fold and 2 fold, respectively) of RI in the CZ of the JCR rats. Because the angiostatin and endostatin fragments are inaccessible to the respective antibodies within the tertiary structures of intact plasminogen and collagen XVIII in the ELIZA assays, the observed increases specifically represent cleaved angiostatin and endostatin. Neither endostatin nor angiostatin were altered by RI in the NZ of either rat phenotype (Figure 2A and 2B). Thus, increased angiostatin and endostatin levels correlate with the increased activation of MMP8 and MMP12 in the later stages of CCG in the metabolic syndrome animals.

To determine whether the concentrations of angiostatin and endostatin generated by RI in JCR rats induced the classical endothelium-specific anti-angiogenic effects of these peptides, we examined whether they decreased VEGF-induced HCAEC proliferation and survival. While neither angiostatin (750 pg/ml) nor endostatin (1000 pg/ml) alone were able to significantly decrease the VEGF-induced increase in HCAEC number or survival (data not shown), results in Figure 2C demonstrate that their combined effect on both parameters was significant.

Inhibition of MMP12 activation decreased myocardial endostatin and angiostatin levels and partially restored coronary collateral growth in the metabolic syndrome

To determine whether MMP12 activation alone was responsible for the increased level of angiostatin and/or endostatin in response to RI in the metabolic syndrome, JCR rats were treated with the specific MMP12 inhibitor (3.5 mg/kg/day, 1/day, i.p.) on days 6-9 of the RI protocol. Complete inhibition of MMP12 activation was confirmed by Western blot analysis of the active (MW=22 kDa) MMP12 band (Figure 3A). MMP12 expression was not affected. MMP8 activation was not affected (Figure 3A) confirming the specificity of the inhibitor. MMP12 inhibition resulted in a significant reduction in both endostatin and angiostatin levels (85% and 90%, respectively) at day 10 of RI (Figure 3B). Moreover, inhibition of MMP12 partially restored CCG in JCR rat as evident by increased artery/arteriolar density (~2 50μM arteries/mm^2 vs. ~1 20 μM arteriole in JCR RI vs. ~3 60 μM arteries in SD RI) and increased collateral-dependent flow (CZ/NZ flow ratio was 0.66±0.05 vs. 0.12±0.02 in JCR RI vs. 0.87±0.04 in SD RI[33]) and completely restored RI-dependent angiogenesis as indicated by an increase in capillary density equivalent to that observed in normal animals (Figures 3C and Figure 5). Nearly identical results were obtained when MMP12 expression was decreased (~80%) using shRNA, delivered to JCR rats via a lentiviral vector (Figure 3). Control-Lnv, carrying a non-targeting shRNA, had no effect (data not shown).

To determine whether additional inhibition of MMP8 would have an additional beneficial effect on CCG in the metabolic syndrome, in addition to the specific MMP12 inhibitor to inhibit MMP12 activity, we used the MMP8 inhibitor (0.25 mg/kg/day, 1/day, i.v.) to inhibit MMP8 activity at days 6-9 of RI in JCR rats. The MMP8 inhibitor may inhibit the other collagenases, MMP1 and MMP13 at similar doses; however, since neither MMP1 nor MMP13 are activated at these time points in the JCR rat (Figure I, Supplement), the non-specific effects on the other collagenases were not a concern. The collagenase inhibitor

successfully and completely inhibited MMP8 activation without effecting MMP8 expression, and did not alter the effectiveness of the MMP12 inhibitor on MMP12 activity (Figure 4A). Concomitant inhibition of MMP12 and MMP8 resulted in a slightly greater but statistically insignificant reduction in endostatin levels (90% vs. 85% with MMP12 inhibition alone), while the extent of reduction in angiostatin levels remained unchanged (Figure 4B). The additional inhibition of MMP8 likewise did not have any greater of an effect on CCG recovery or angiogenesis then MMP12 inhibition alone (CZ/NZ flow ratio was 0.62±0.08) (Figure 4C).

To confirm that MMP12 but not MMP8 plays the major role in the regulation of angiostatin and endostatin production and CCG in metabolic syndrome, we also inhibited MMP8 alone. Complete inhibition of MMP8 activation had no effect on RI-induced angiostatin or endostatin production (Figure 4B), CCG or angiogenesis in JCR rats (Figure 4C).

Angiostatin and endostatin infusion decreased coronary collateral growth in normal animals

Finally we tested the effect of the amounts of angiostatin and endostatin produced in the JCR rats on CCG in the normal animals to provide proof-of-concept as well as examine the physiological relevance of these amounts. Infusion of 750 pg/ml/day angiostatin and 1000 pg/ml/day endostatin (days 0-10 or 6-9 of RI) moderately but significantly decreased RI-induced angiogenesis and CCG in SD rats while having predictably no effect on pre-existing collaterals and capillary networks (Figure 5). These results suggest that angiostatin and endostatin amounts produced in JCR rats are physiologically significant. The caveat to these conclusions is that ECs of SD rats are normal while JCR rats display marked endothelial dysfunction[9]; thus, these anti-angiogenic peptides would be predicted to more severely impact the already apoptosis-prone JCR ECs.

Discussion

The most important novel findings of this study are that: 1) MMP12 and MMP8 are differentially regulated in the normal vs. the metabolic syndrome phenotype during the process of CCG, 2) the temporal pattern of MMP12 and MMP8 activation in the later stages of CCG correlates with elevated levels of angiostatin and endostatin in the metabolic syndrome, and 3) inhibition of MMP12 but not MMP8 activation blocks RI-induced generation of angiostatin and endostatin and significantly improves CCG in the metabolic syndrome.

Proteolysis of ECM and plasma proteins is a common theme in inhibition of angiogenesis. Many inhibitors of tumor angiogenesis, such as angiostatin, endostatin, serpin antithrombin, tumstatin, canstatin, vasostatin, restin, and arrestin are proteolytic fragments.[20, 35-43] Of these, angiostatin and endostatin have been investigated in collateral growth. Results in our study demonstrating elevated levels of angiostatin and endostatin during the later stages of repetitive ischemia in the metabolic syndrome are in agreement with previous studies which showed that these anti-angiogenic peptides were increased in hyperglycemic dogs with impaired CCG during the later stages of repetitive ischemia[12] and in humans with type II diabetes and impaired coronary collateral development[10, 11].

The mechanism by which angiostatin and endostatin inhibit collateral growth has not been investigated, but is inferred from a related process of tumor angiogenesis. It has been shown that angiostatin directly targets only EC proliferation and survival and does not affect either tumor cells or other cells of non-endothelial origin *in vitro*.[44] In agreement with these findings, results in this study show that the concentrations of angiostatin and endostatin generated in the metabolic syndrome animals can suppress EC proliferation and survival. It

was shown that angiostatin directly induces apoptosis of ECs without a compensatory increase in proliferation, resulting in a net reduction of viable ECs via altering focal adhesion kinase signaling and disrupting normal focal adhesion turnover.[45, 46] Secondly, angiostatin directly reduces EC migration, proliferation and tube formation by inhibiting FGF and VEGF-dependent ERK1/2 activation.[47] Lastly, angiostatin may mediate its anti-angiogenic effects by binding to the cell surface ATP synthase and depriving ECs of ATP.[15] Like angiostatin, endostatin does not affect the *in vitro* proliferation of tumor cells nor other non-endothelial cell lines; thus, its actions appear to be EC-specific.[20, 21] Endostatin increases EC apoptosis by reducing the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL[21] and inducing Caspase 3 activation[48]. Furthermore, endostatin inhibited ERK1/2 phosphorylation in VEGF- and FGF-stimulated primary arterial EC cultures without interfering with growth factor receptor binding.[49] Our unpublished observations suggest that ERK1/2 activation is also impaired in the metabolic syndrome during CCG (Rocic, unpublished data, [2008]).

MMPs, non-MMP elastases and plasmin are capable of generating angiostatin from plasminogen, while a wide variety of MMPs are able to generate endostatin from collagen XVIII. Of the possible candidates relevant to the cardiovascular system, our results show that only MMP12 and MMP8 activation correlate with increased levels of angiostatin and endostatin in the later stages of CCG in metabolic syndrome. Our findings further indicate that MMP12 is the primary regulator of angiostatin and endostatin in the metabolic syndrome since its inhibition alone was sufficient to completely block their generation, additional inhibition of MMP8 had no further effect and MMP8 inhibition alone likewise had no effect. MMP12 has extensive activity against elastin but is also capable of degrading proteoglycans, fibronectin, laminin, non-fibrilar collagen including collagen XVIII, heparin sulfate, plasminogen and vitronectin. Importantly, it has been shown to be anti-angiogenic in several studies in cancer angiogenesis. MMP12 was decreased in breast carcinoma compared to normal mammary cells and these breast carcinoma lines were more efficient in capillary tube formation than the normal mammary cells.[50] Decreased MMP12 expression correlated with decreased expression of angiostatin.[51] MMP12 was also able to generate angiostatin from purified plasminogen in vitro[27] and in an in vitro model of lung metastasis[26]. MMP12 has also been shown to inhibit microvascular endothelial cell proliferation in vitro by generating angiostatin.[26, 27] One study showed that MMP12 can release endostatin, which inhibited VEGF-induced chemotaxis of osteoclasts.[31]

Not much is known about MMP8. Its role in collateral growth or angiogenesis is completely unknown, as are its substrates. However, MMP8 belongs to the family of interstitial collagenases, which in the cardiovascular system, includes MMP1 and MMP13, and its active site is structurally identical to that of MMP1 and MMP13. Neither MMP1 nor MMP13 are activated during coronary collateral remodeling in either the normal or the metabolic syndrome rat phenotype. Thus, it is reasonable to propose that MMP8 may be the representative active interstitial collagenase under these circumstances. Interstitial collagenases cleave collagen types I and III, which are components of the myocardial interstitium and vascular adventitia. MMP1 has been shown to suppress angiogenesis in sarcoma; its silencing significantly increased mean vascular volume per unit volume of tumor.[52] MMP 13 has been found to have a direct role in the formation of endostatin. Because both MMP1 and MMP13 share the collagen XVIII cleavage site, MMP1 was also thought to mediate its anti-angiogenic effects via endostatin production.[52]

Paradoxically, many of the same proteases that release anti-angiogenic peptides are also capable of releasing pro-angiogenic growth factors and stimulate angiogenesis. For example, plasmin mediates cell invasion into neighboring tissues, which requires angiogenesis.[53, 54] MMP1 effectively releases pro-angiogenic growth factors, VEGF and FGF, from the

ECM.[55, 56] This is interesting since in our study, in addition to being activated late in CCG in the metabolic syndrome animals, which fail to grow collaterals, MMP8 was also activated early during CCG in the normal animals. Thus, our data suggest that MMP8 may play a differential role in CCG in normal vs. the metabolic syndrome phenotype. Depending on the timing of its activation, it may release either pro-angiogenic growth factors from their binding proteins in the ECM (early in CCG) or anti-angiogenic peptides (late in CCG). The extent of collateral growth is determined by the balance of the pro-angiogenic growth factors and anti-angiogenic peptides. Therefore, our results in this study further support the concept that ECM proteolysis within the context of collateral growth is a temporally tightly regulated process, which can be profoundly affected by the temporal regulation of MMPs.

Regarding the possible cause of the later and sustained increase in MMP8 activation in the metabolic syndrome, MMP8 is a neutrophil collagenase (collagenase-2). Although transient accumulation of a low number of neutrophils and other inflammatory cells early in collateral growth has been associated with beneficial effects on collateral remodeling[57], prolonged or excessive neutrophil accumulation and inflammation which characterizes the vasculature of type II diabetes and metabolic syndrome is strongly negatively correlated with CCG.[58, 59] Also, increases in the expression, activation, and collagenase activity of MMP8 and MMP13 have been shown in response to Angiotensin II (Ang II) administration.[60] We have previously shown that infusion of a hypertensive dose of Ang II negatively affected collateral growth. Moreover and explicitly relevant to the scenario in the current study, Ang II type I receptor (AT_1R) levels are increased in JCR rats and AT_1R blockade partially but significantly restored CCG in the JCR rat.[61] Another possibility is that MMP8 upregulation in the late stages of CCG in metabolic syndrome may serve some other as of yet unknown function or be coincidental.

Our results suggest that increased angiostatin and endostatin production provide a very substantial impediment to coronary collateral development in the metabolic syndrome since their downregulation results in ~65% recovery of coronary blood flow to the collateral-dependent zone. Upstream regulators of MMP12 activation in collateral growth are not known. The mechanisms responsible for impaired collateral remodeling in the metabolic syndrome have not been completely elucidated, but involve increased oxidative stress and altered redox-dependent signaling[8, 62], which might provide an upstream activation mechanism. All MMPs have a disulfide bond in their hinge region[63], which makes them directly susceptible to oxidation by reactive oxygen species (ROS), which results in their activation[63, 64]. We have previously shown that oxidative stress is highly elevated in response to RI in the metabolic syndrome and that reduction in oxidative stress significantly improved CCG in the metabolic syndrome.[8, 62] Results in the present study are compatible with the idea that observed recovery of CCG in response to oxidative stress reduction was in part due to the downstream inhibition of MMP12 activation and the consequent decrease in angiostatin and endostatin generation.

A limitation of our study inherent to the rat model of coronary occlusion, where it is not possible to isolate collateral vessels from the heart tissue, is that we did not determine the cellular source of the active MMPs in this study. The most likely source of both MMP12 and MMP8 are inflammatory cells, specifically macrophages and/or neutrophils. Metabolic syndrome is a chronic pro-inflammatory state, where the vasculature is characterized by increased infiltration of neutrophils and monocytes, and increased adhesion and extravasation of inflammatory cells into the vascular wall.[65, 66] The infiltration of inflamatory cells, especially neutrophils and monocytes, is a normal and required component of collateral remodeling[57] which further increases the total number of these cells in and around the forming collaterals in the metabolic syndrome. Our findings are consistent with the idea that these highly elevated numbers of neutrophils, adherent monocytes and/or

extravasated tissue macrophages in the metabolic syndrome result in the release of high levels of MMP12 and MMP8, which lead to generation of high levels of angiostatin and endostatin, which are sufficient to significantly contribute to collateral growth impairement in the metabolic syndrome. However, endothelial cells[50, 67] and VSMC[68] have also been shown to be able to secrete MMP12; therefore, other cell types cannot be excluded as possible contributors to the production of these MMPs. Our results showing ~2 fold higher basal MMP12 activation in the plasma of JCR rats is also intriguing. This may be largely a consequence of adipose tissue macrophage-mediated MMP12 production.[69] While clearly not a consequence of cardiac repetitive ischemia and probably of little predictive value within the context of coronary collateral growth, the contributing value of this factor to impaired CCG in metabolic syndrome is difficult to predict.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

None

Sources of Funding: NIH R01 HL093052

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Significance

This study identifies MMP12 as a novel positive regulator of angiostatin and endostatin production, which inhibit coronary collateral development in a rat model of metabolic syndrome. Inhibition of MMP12 activation significantly improved coronary collateral development in the metabolic syndrome animals thus characterizing MMP12 as a potentially important target for revascularization therapy through collateral growth. Furthermore, this study catalogues MMPs which are activated during coronary collateral remodeling in metabolic syndrome vs. the normal phenotype thus identifying targets for future investigation.

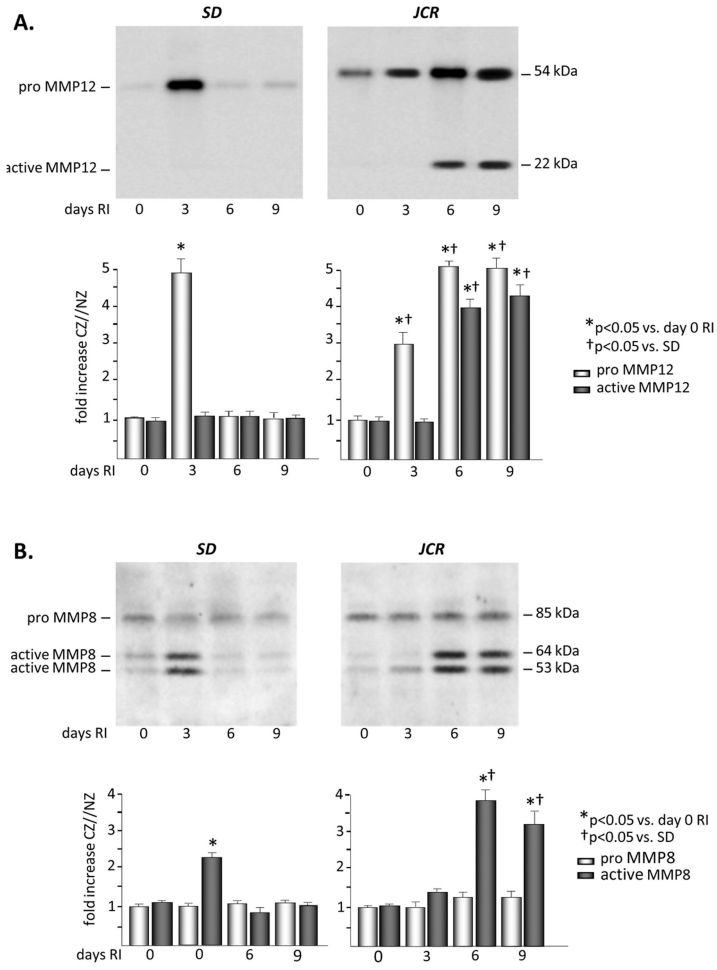


Figure 1.

SD or JCR rats were sacrificed on day 0, 3, 6 or 9 of RI. Tissue samples were collected from the NZ or the CZ. Pro and active MMP12 (A) and MMP8 (B) expression was determined by Western Blot in SD (left) and JCR (right) rats. Top: Representative Western blot using the anti-MMP12 and anti-MMP8 antibodies. Bottom: Cumulative data, *p<0.05 vs. day 0 RI, †p<0.05 vs. SD, n=5.

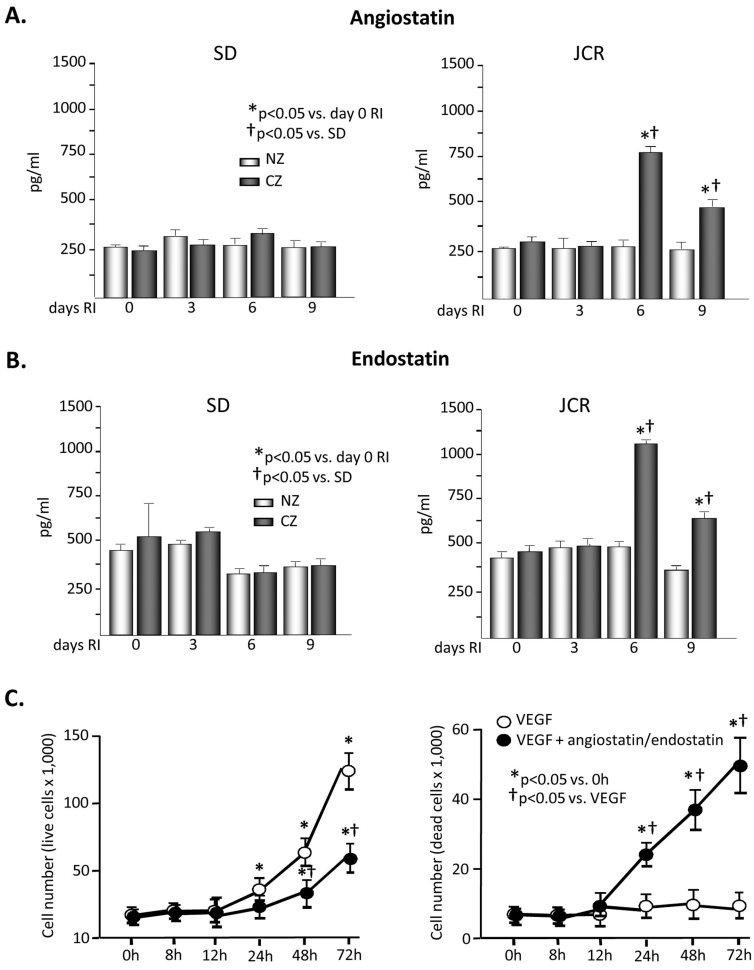
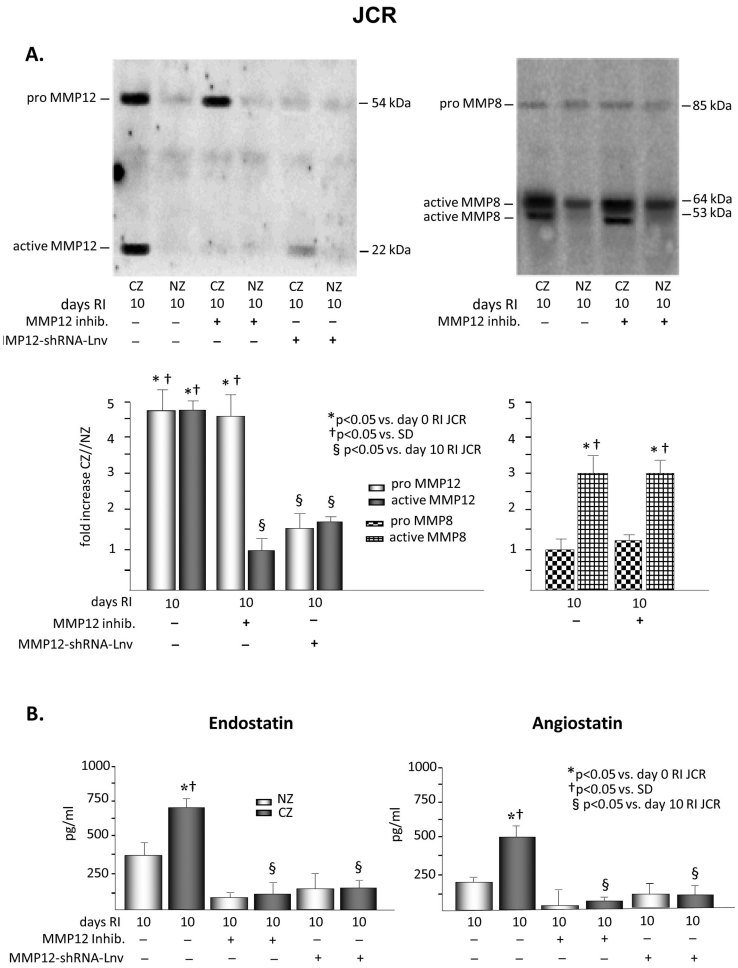


Figure 2.

SD or JCR rats were sacrificed on day 0, 3, 6 or 9 of RI. Tissue samples were collected from the NZ or the CZ. Myocardial angiostatin (**A**) and endostatin (**B**) levels were determined by ELISA in SD (left) and JCR (right) rats. *p<0.05 vs. day 0 RI, †p<0.05 vs. SD, n=5. **C**. HCAEC were treated with VEGF (50 ng/ml) or VEGF (50 ng/ml) + angiostatin (750 pg/ml) + endostatin (1000 pg/ml) for the times indicated. Live and dead cells were counted. *p<0.05 vs. 0 hours, †p<0.05 vs. VEGF, n=3.



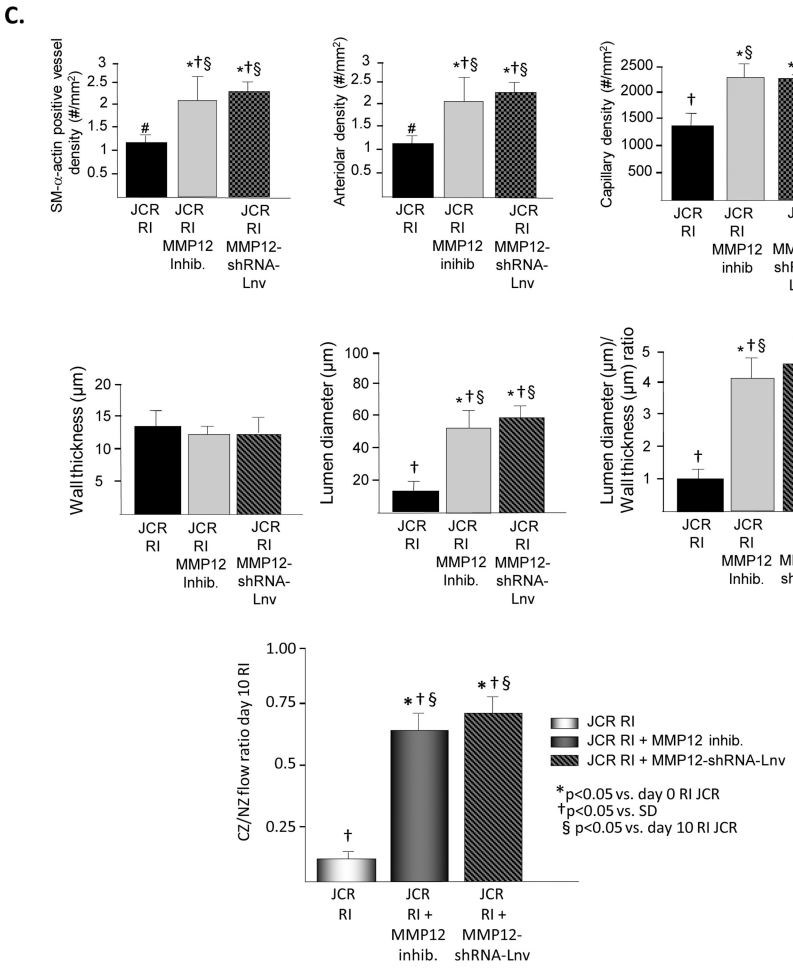
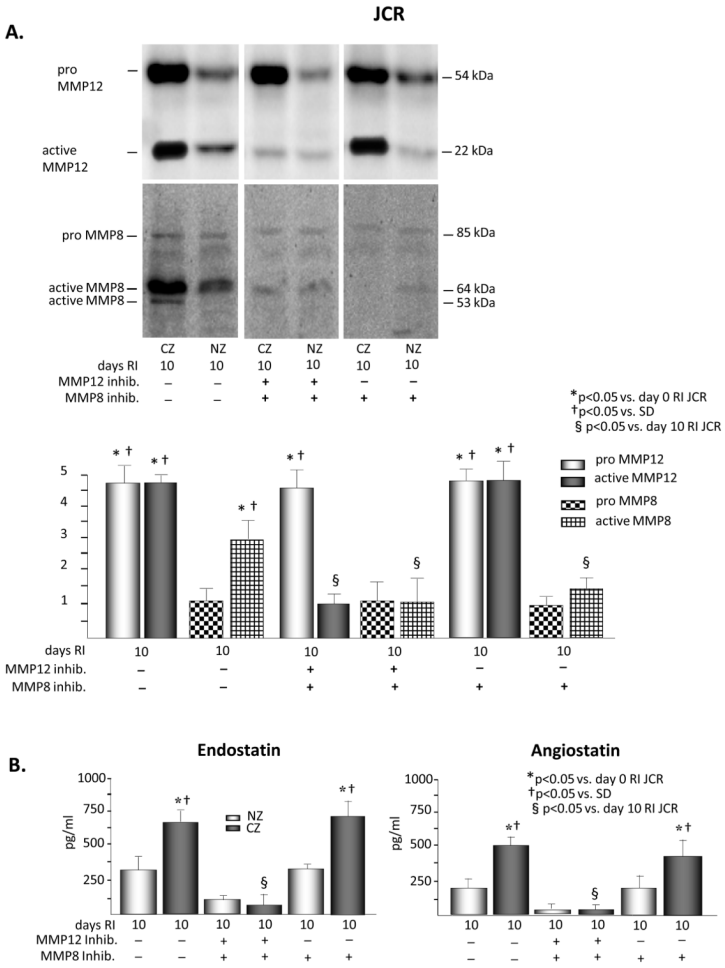


Figure 3.

JCR rats were treated the MMP12 inhibitor or MMP12-shRNA-Lnv as indicated and underwent 10 days of RI. **A**. Tissue samples were collected from the NZ or the CZ. Pro and active MMP12 and MMP8 expression was determined by Western Blot. Top: Representative Western blot detecting pro and active forms MMP12 (left) and MMP8 (right). Bottom: Cumulative data. **B**. Tissue samples were collected from the NZ or the CZ. Myocardial endostatin (left) and angiostatin (right) levels were determined by ELISA. **C**. Top: Arteriolar (SM-a-actin positive vessels >20μM) and capillary (vessels <20μM) densities, wall thickness, lumen diameter and lumen diameter/wall thickness ratio of collateral arteries (anti-SM-a-actin and PCNA positive arteries) were determined in cardiac (CZ) cross-sections on day 10 RI. Bottom: Coronary blood flow was measured in the CZ and the NZ using microspheres during LAD occlusion and is expressed as the ratio between CZ and NZ flows at day 10 of RI. *p<0.05 vs. SD sham, †p<0.05 vs. SD RI, §p<0.05 vs. JCR RI, n=3 for MMP12 inhibitor, n=5 for MMP12-shRNA-Lnv.



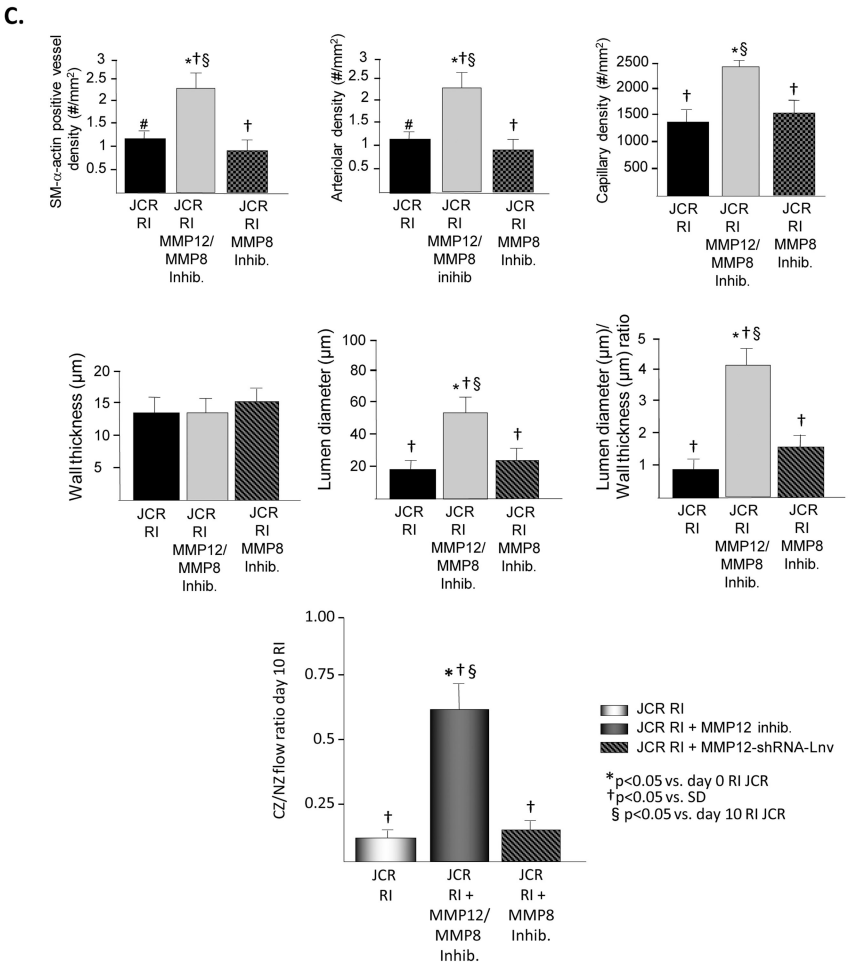


Figure 4.

JCR rats were treated the MMP12 and the MMP8 inhibitor as indicated and underwent 10 days of RI. **A**. Tissue samples were collected from the NZ or the CZ. Pro and active MMP12 and MMP8 expression was determined by Western Blot. Top: Representative Western blot detecting pro and active forms MMP12 and MMP8. Bottom: Cumulative data, **p<0.05 vs. day 0, †p<0.05 vs. SD, §p<0.05 vs. day 10 RI, n=3. **B**. Tissue samples were collected from the NZ or the CZ. Myocardial endostatin (left) and angiostatin (right) levels were determined by ELISA. **p<0.05 vs. day 0, †p<0.05 vs. SD, §p<0.05 vs. day 10 RI, n=3. **C**. Top: Arteriolar (SM-a-actin positive vessels >20μM) and capillary (vessels <20μM) densities, wall thickness, lumen diameter and lumen diameter/wall thickness ratio of collateral arteries (anti-SM-a-actin and PCNA positive arteries) were determined in cardiac (CZ) cross-sections on day 10 RI. Bottom: Coronary blood flow was measured in the CZ and the NZ using microspheres during LAD occlusion and is expressed as the ratio between CZ and NZ flows at day 10 of RI. **p<0.05 vs. SD sham, †p<0.05 vs. SD RI, §p<0.05 vs. JCR RI, n=3.

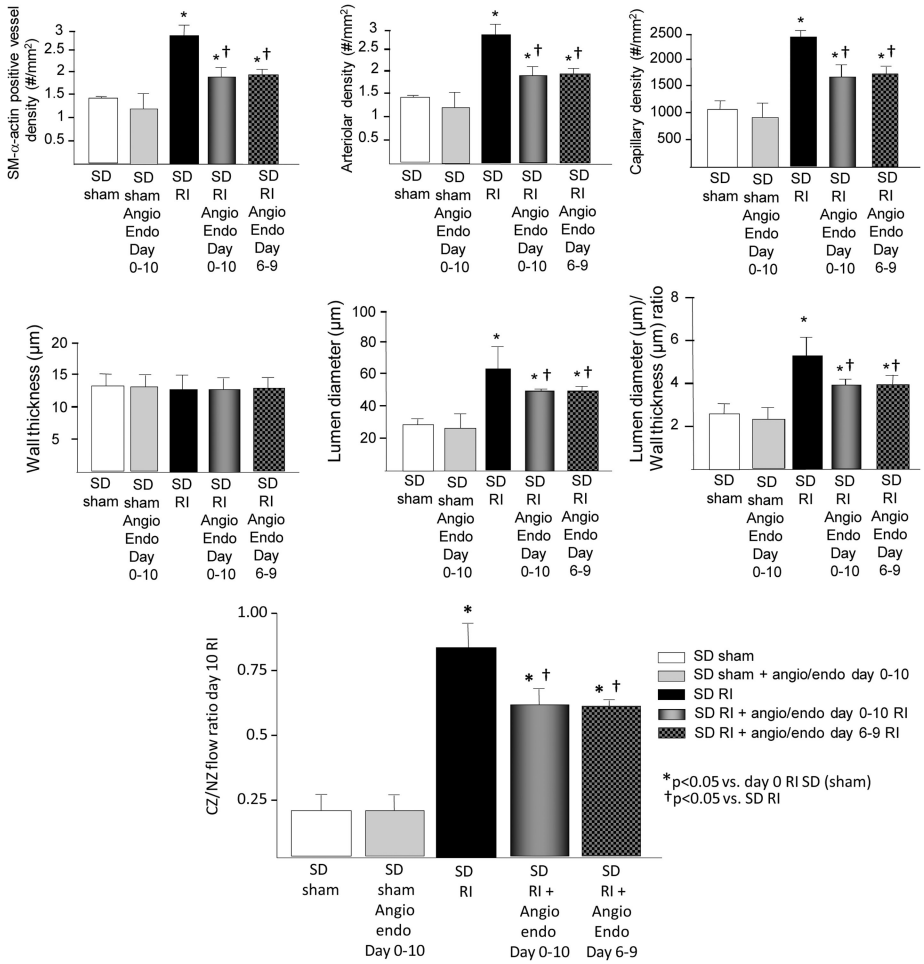


Figure 5.

SD rats were treated angiostatin (750 pg/kg/day) and endostatin (1000 mg/kg/day) on days 0-10 RI or 6-9 RI as indicated and underwent 10 days of RI. Top: Arteriolar (SM-a-actin positive vessels >20μM) and capillary (vessels <20μM) densities, wall thickness, lumen diameter and lumen diameter/wall thickness ratio of collateral arteries (anti-SM-a-actin and PCNA positive arteries) were determined in cardiac (CZ) cross-sections on day 10 RI. Bottom: Coronary blood flow was measured in the CZ and the NZ using microspheres during LAD occlusion and is expressed as the ratio between CZ and NZ flows at day 10 of RI. **p<0.05 vs. SD sham, †p<0.05 vs. SD RI, §p<0.05 vs. JCR RI, n=5.