Hypertension, often termed the silent killer, is a systemic condition characterized by persistently elevated arterial blood pressure; it is typically associated with cardiovascular hypertrophy. More than 25% of the adult population in developed countries is hypertensive and therefore at risk of heart disease, peripheral vascular disease, end-stage renal disease, and cerebrovascular stroke. The pathogenesis of most hypertensive disorders is complex, because genetic, immune, and environmental factors all may predispose individuals to hypertension. A difficulty faced by physicians when deciding on a therapeutic strategy is that typically, the cause of the hypertension is unknown. Thus, treatment of hypertension remains rather empirical, with physicians choosing among many antihypertensive medications until a drug or drug combination is identified that effectively lowers the blood pressure in the patient. Of those individuals treated, 65% do not meet treatment goals. Therefore, treatment strategies are needed that (1) are preventative, (2) can stop pathological hypertrophy processes or induce the regression of preexisting cardiac hypertrophy, and (3) are efficacious in hypertensive disorders with multiple or unknown cause(s).

Matrix Metalloproteinase-7 and ADAM-12 (a Disintegrin and Metalloproteinase-12) Define a Signaling Axis in Agonist-Induced Hypertension and Cardiac Hypertrophy

Xiang Wang, MSc*; Fung Lan Chow, BSc*; Tatsujiro Oka, MD; Li Hao, MD; Ana Lopez-Campistrous, MSc; Sandra Kelly, MSc; Stephan Cooper, BSc; Jeffrey Odenbach, BSc; Barry A. Finegan, MD; Richard Schulz, PhD; Zamaneh Kassiri, PhD; Gary D. Lopaschuk, PhD; Carlos Fernandez-Patron, PhD

Background—Excessive stimulation of Gq protein–coupled receptors by cognate vasoconstrictor agonists induces a variety of cardiovascular processes, including hypertension and hypertrophy. Here, we report that matrix metalloproteinase-7 (MMP-7) and a disintegrin and metalloproteinase-12 (ADAM-12) form a novel signaling axis in these processes.

Methods and Results—In functional studies, we targeted MMP-7 in rodent models of acute, long-term, and spontaneous hypertension by 3 complementary approaches: (1) Pharmacological inhibition of activity, (2) expression knockdown (by antisense oligodeoxynucleotides and RNA interference), and (3) gene knockout. We observed that induction of acute hypertension by vasoconstrictors (ie, catecholamines, angiotensin II, and the nitric oxide synthase inhibitor NG1-nitro-L-arginine methyl ester) required the posttranscriptional activation of vascular MMP-7. In spontaneously hypertensive rats, knockdown of MMP-7 (by RNA interference) resulted in attenuation of hypertension and stopped development of cardiac hypertrophy. Quantitative reverse-transcription polymerase chain reaction studies in mouse models of MMP-7 knockdown (by RNA interference) and gene knockout revealed that MMP-7 controlled the transcription of ADAM-12, the major metalloproteinase implicated in cardiac hypertrophy. In mice with angiotensin II–induced hypertension and cardiac hypertrophy, myocardial ADAM-12 and downstream hypertrophy marker genes were overexpressed. Knockdown of MMP-7 attenuated hypertension, inhibited ADAM-12 overexpression, and prevented cardiac hypertrophy.

Conclusions—Agonist signaling of both hypertension and hypertrophy depends on posttranscriptional and transcriptional mechanisms that involve MMP-7, which is transcriptionally connected with ADAM-12. Approaches targeting this novel MMP-7/ADAM-12 signaling axis could have generic therapeutic potential in hypertensive disorders caused by multiple or unknown agonists. (Circulation. 2009;119:2480-2489.)

Key Words: gene therapy ■ remodeling ■ metalloproteinases ■ vasculature ■ hypertension ■ hypertrophy
Recently, we proposed an approach to treat hypertension by blocking mediators that are commonly shared by many vasoconstrictors but significantly activated only in response to excessive agonist stimulation. The major vasoconstrictor systems discovered to date (catecholamines, endothelins, and angiotensin II) all use Gq protein–coupled receptors (GqPCRs) as their cognate receptors. GqPCRs act through the activation of the classic phospholipase C/protein kinase C pathway and downstream matrix metalloproteinases (MMPs, such as MMP-2, MMP-7, and MMP-9) and a disintegrin and metalloproteinases (such as [ADAM]-12 and ADAM-17/TACE [tumor necrosis factor–convertase]).


The MMP-7 generated by disrupting the MMP-7 gene through the insertion of a neomycin resistance cassette into the fragment spanning exon 3 and 4. Also-hypertensive (22-week-old) spontaneously hypertensive rats (SHRs) and age-matched Wistar-Kyoto (WKY) rats as well as Sprague Dawley rats (250–350 grams) were purchased from Charles River Laboratories Inc (Wilmington, Del).

**Generation of MMP-7 Knockdown Models**

The sequences of the MMP-7 antisense (active), MMP-7 scrambled (inactive) oligodeoxynucleotides, and MMP-7 small interfering RNA (siRNA) were derived from previous studies. The oligonucleotides were delivered with ALZET osmotic minipumps (DURECT Corp, Cupertino, Calif) implanted subcutaneously on the backs of the animals.

**Data Analysis**

Results are presented as mean±SEM and were analyzed with 1-way ANOVA or t test as appropriate with Jandel SigmaStat 3.5 statistical software. In the echocardiography studies, between-group comparisons of the means were performed by 1-way ANOVA followed by Scheffe’s F correction for multiple comparisons of the means. Statistical significance was considered when $P≤0.05$. Except where indicated otherwise, between 4 and 5 animals were used for each study.

The authors had full access to and take full responsibility for the integrity of the data. All authors have read and agree to the manuscript as written.

**Results**

**MMP-7 as a Mediator in Pharmacologically Induced Acute Hypertension**

To trigger an acute hypertensive response in otherwise normotensive Sprague Dawley rats and C57BL/6 mice, we injected intraperitoneally either PBS (vehicle) or (1) α-adrenergic agonists (phenylephrine and norepinephrine), (2) angiotensin II, or (3) Nω-nitro-L-arginine methyl ester (L-NAME), which elevates blood pressure by blocking basal nitric oxide–dependent vasodilation, thus unmasking secondary vasoconstrictor mechanisms (Figure 1, top panels, and Figure 2).

The involvement of MMPs in these experiments was suggested by effects of doxycycline, a broad-spectrum pharmacological inhibitor of MMP activity. Doxycycline (60 to 120 mg/kg IP) dose-dependently blocked the acute hypertensive responses to α-adrenergic agonists, angiotensin II, and L-NAME in rats (Figure 1, top panels) and mice (Figure 2A).

High-performance liquid chromatography analyses indicated that doxycycline (90 mg/kg IP) resulted in plasma concentrations between 10⁻⁴ and 10⁻² mol/L at 1 and 4 hours after IP injection, respectively (data not shown). These doxycycline concentrations are enough to relax small rat mesenteric arteries in isolation. When we examined arteries collected at a time point that coincided with the maximum elevation in systolic blood pressure induced by phenylephrine, angiotensin II, or L-NAME (ie, 4, 1, or 0.5 hours, respectively), the activity of vascular MMP-7 but not MMP-2 was elevated. The increase in MMP-7 activity was in all cases blocked by the coadministration of doxycycline (Figure 1, bottom panels).

We verified the link between MMP-7 expression and systemic blood pressure regulation in studies summarized in Figures 2A through 2C. In these studies, we examined both C57BL/6 mice in which the MMP-7 gene was disrupted by a neomycin resistance cassette to render them MMP-7⁻/⁻ and...
wild-type C57BL/6 mice that were given MMP-7–specific antisense oligodeoxynucleotides (0.6 mg · kg$^{-1} · d^{-1}$), scrambled (inactive) oligodeoxynucleotides (0.6 mg · kg$^{-1} · d^{-1}$), or PBS for 14 days (through subcutaneous osmotic minipumps). The antisense sequence chosen for the present study was previously validated in vivo and has anticancer activity through the long-lasting knockdown of MMP-7.17 Antisense treatment resulted in a systemic downregulation of MMP-7 activity in various tissues, including aorta, heart, and small intestine (a tissue in which MMP-7 is normally expressed at very high levels21; supplemental Figure I).

Interestingly, resting systolic blood pressure was not significantly affected by doxycycline injections (data not shown), MMP-7 antisense oligodeoxynucleotides, or MMP-7 gene knockout (Figure 2D); however, mice that received MMP-7 antisense oligodeoxynucleotides displayed blunted acute hypertensive responses to norepinephrine, angiotensin II, and L-NAME (versus PBS and versus scrambled oligodeoxynucleotides; Figures 2B, 2E, 2F, and 2G). Similarly, MMP-7$^{-/-}$ mice showed attenuated acute responses to angiotensin II (data not shown) and norepinephrine (Figure 2C). Moreover, MMP-7$^{-/-}$ mice (but not wild-type mice) were resistant to chronic hypertension induced by repeated norepinephrine administration (Figure 3A). Isolated microperfused small mesenteric arteries from MMP-7$^{-/-}$ mice constricted less (versus wild-type mice) in response to luminally delivered boluses of the α-adrenergic agonist phenylephrine (0, 5, or 50 pmol per bolus; Figure 3B). Together, these in vivo and in vitro functional data strongly suggested that vasoconstrictors induce hypertension, at least in part, through the posttranscriptional activation of MMP-7.

MMP-7 as a Mediator of Hypertension and Cardiac Hypertrophy in SHRs

We next examined whether blocking MMP-7 expression would decrease the systolic blood pressure of SHRs, a genetic model in which hypertension is caused by multiple mechanisms, including endothelial dysfunction and upregulated activities of catecholamines (ie, sympathetic system) and angiotensin II.22–24 Figure 4 illustrates results obtained in already-hypertensive 22-week-old SHRs when we targeted...
the MMP-7 gene by RNA interference using an siRNA against the same mRNA sequence targeted by the MMP-7 antisense oligodeoxynucleotides (for an alignment of the sequences, please see supplemental Figure II). MMP-7 siRNA treatment significantly decreased the systolic blood pressure, producing an attenuation of the hypertension that lasted beyond the window of siRNA delivery (Figure 4A). The antihypertensive effects of MMP-7 siRNA treatment were associated with a significant decrease in MMP-7 activity in resistance arteries (Figure 4B). Interestingly, MMP-7 siRNA treatment stopped the progression of cardiac hypertrophy (Figure 5A and 5B; Table) in association with a downregulation of myocardial MMP-7 (Figure 5C). MMP-7 siRNA also decreased the number of nuclei/area unit in histological sections of hearts from treated SHRs, which further confirmed the prevention of cardiac hypertrophy (data not shown). Comparative gross pathology further revealed that treatment with MMP-7 siRNA resulted in an approxi-

![Figure 2.](image-url)

**Figure 2.** Resistance to acute hypertension in mice lacking active MMP-7. A, Time course of systolic blood pressure in wild-type mice administered norepinephrine (NE; 1.5 mg/kg IP) alone or together with doxycycline (Dox; 90 mg/kg IP). *P<0.05 vs NE+Dox. Baseline refers to blood pressure before injection of norepinephrine (t=0). B, Protection from norepinephrine-induced acute hypertension in mice treated with MMP-7 antisense oligodeoxynucleotides (oligos; 0.6 mg · kg$^{-1}$ · d$^{-1}$ for 14 days) vs mice administered scrambled antisense oligodeoxynucleotides (0.6 mg · kg$^{-1}$ · d$^{-1}$ for 14 days). *P<0.05 vs scrambled oligodeoxynucleotides. C, Protection from norepinephrine-induced acute hypertension in MMP-7$^{-/-}$ mice. *P<0.05 vs wild-type. D, Effects of MMP-7 knockdown and MMP-7 gene knockout on resting blood pressure of mice. Left, Resting blood pressure of mice treated with MMP-7 antisense vs scrambled oligodeoxynucleotides for 14 days. Blood pressure at day 15. Right, Resting blood pressure of MMP-7$^{-/-}$ mice vs age-matched wild-type mice. E through G, Top, Time course of systolic blood pressure effects of norepinephrine (1.5 mg/kg IP), angiotensin II (Ang II; 1.5 mg/kg IP), or N$^G$-nitro-L-arginine methyl ester (L-NAME; 15 mg/kg IP) in mice treated with MMP-7 antisense oligodeoxynucleotides (MMP-7 antisense; 0.6 mg · kg$^{-1}$ · d$^{-1}$ for 14 days) vs mice given PBS. E through G, Bottom, Quantitative analysis of the dose response for each vasoconstrictor. Baseline refers to blood pressure before injection of the vasoconstrictor (t=0). *P<0.05 vs PBS. Results are mean±SEM of 4 to 5 mice in each study group.
mately 50% reduction in cardiac hypertrophy versus SHRs given PBS and versus untreated normotensive age-matched WKY rats: heart weight/body weight (WKY) = 3.40 ± 0.01, heart weight/body weight (SHR + MMP-7 siRNA) = 3.99 ± 0.05, heart weight/body weight (SHR + PBS) × 1000 = 4.43 ± 0.09; n = 3 for WKY, n = 4 for both SHR + PBS and SHR + MMP-7 siRNA. We excluded a major contribution of the inflammatory response in these antihypertensive and antihypertrophy effects of MMP-7 siRNA because we did not observe significantly elevated interferon-γ levels in plasma or in the left ventricle of the rats (supplemental Figure III, SHR).

MMP-7/ADAM-12 Signaling Axis

Administration of MMP-7 siRNA (0.4 mg · kg⁻¹ · d⁻¹) for 14 days resulted in a significant downregulation in myocardial MMP-7 mRNA levels in mice (Figure 6A). Interestingly, MMP-7 siRNA inhibited ADAM-12 transcription (Figure 6B) but had otherwise insignificant effects on other genes, including α-skeletal actin, TACE, TIMP-2 (tissue inhibitor of metalloproteinase-2), and MMP-9 (Figure 6C and 6D) and on interferon-γ levels (supplemental Figure III). Like the MMP-7 siRNA, MMP-7 gene knockout resulted in decreased levels of myocardial ADAM-12 mRNA (but normal levels of TACE; Figure 6E). Mice that received MMP-7 siRNA displayed no morphometric or echocardiographic abnormalities (supplemental Table).

Mice given angiotensin II (1.4 mg · kg⁻¹ · d⁻¹ for 10 days) displayed hypertension and left ventricular hypertrophy (Figure 6F and 6G; supplemental Table). Interestingly, continuous angiotensin II infusion inhibited MMP-7 transcription (Figure 6A) but increased transcription of ADAM-12 and hypertrophy marker genes (β-myosin heavy chain, brain natriuretic peptide, and α-skeletal actin; Figure 6B and 6C). Pretreatment with MMP-7 siRNA attenuated angiotensin II–induced hypertension (as expected from studies in Figures 1 through 4), inhibited the angiotensin II–induced overexpression of both ADAM-12 and hypertrophy marker genes (Figure 6B and 6C), and prevented left ventricular hypertrophy (Figure 6F and 6G; supplemental Table). Supporting these observations, MMP-7−/− mice (but not age-matched wild-type mice) exhibited resistance to hypertension (Figure 3A), cardiac hypertrophy (supplemental Figure IVA), and the transactivation of cardiac growth factor receptors, which are purported mediators of agonist-activated ADAM-12β (supplemental Figure IVB).

Discussion

This investigation has resulted in 3 interrelated discoveries: (1) To the best of our knowledge, the present findings suggest for the first time that agonist signaling of both hypertension and cardiac hypertrophy depends on MMP-7 gene expression and activity. (2) We revealed a novel transcriptional link between MMP-7 and ADAM-12, the major disintegrin metalloproteinase implicated in the de-
development of cardiac hypertrophy. (3) We have shown that disrupting the MMP-7/ADAM-12 axis at the level of MMP-7 protects against development of both cardiac hypertrophy and hypertension in simple models (such as mice infused with angiotensin II) and in a complex model (SHR). Thus, targeting the MMP-7/ADAM-12 axis (eg, at the level of MMP-7) could have general therapeutic potential in multiple hypertensive disorders caused by multiple or unknown agonists.

Prior to the present study, many characterizations of MMP-7 in vivo related to cancer or the innate immune response, with the exception of a few recent studies, including one that showed a novel interaction between MMP-7 and connexin-43 in cardiac failure. Our laboratory had proposed a role for MMP-7 in agonist-induced vasoconstriction of isolated arteries on the basis of broad-spectrum pharmacological inhibitor data; however, none of our previous studies could establish its mediator role in agonist-induced hypertension nor its novel involvement in cardiac hypertrophy, a process that invariably develops subsequently to sustained vasoconstrictive agonist stimulation. Prior to this research, MMP-7 and ADAM-12 had been studied separately; however, these separate studies suggested their involvement in cardiovascular hypertrophy processes through a common pathway. Accordingly, an overabundance of vasoconstrictor agonists (as occurs in hypertensive disorders) would enhance their activity through posttranscriptional pathways. Next, the activated MMP-7 and ADAM-12 would cleave and release substrates, including growth factors and inflammatory mediators (such as HB-EGF, transforming growth factor-α, and tumor necrosis factor-α). These mediators then trigger the mitogen-activated protein kinase cascade to promote cardiovascular hypertrophy through the transcriptional activation of immediate-early genes and fetal genes, often referred to as hypertrophy marker genes (Figure 7, module 1).

The data suggest that MMP-7 and ADAM-12 are connected in agonist-induced posttranscriptional and transcriptional events that may ultimately result in the development of hypertension and cardiovascular hypertrophy. We have further revealed novel hierarchical relationships between these metalloproteinases and observed that these relationships are dynamic, because they differ under basal conditions (Figure 7, module 2) versus agonist stimulation (Figure 7, module 3). Under basal conditions, MMP-7 transcriptionally controls the expression of ADAM-12 and downstream hypertrophy marker genes; however, under sustained agonist stimulation, MMP-7 mRNA levels and thereby the contribution of MMP-7 to signaling may decrease, whereas the expression and thereby the contribution of ADAM-12 to signaling may increase. We thus propose the following: (1) MMP-7 may mediate the early posttranscriptional events by which vasoconstrictor agonists trigger an acute elevation of blood pressure (in the short term) and the development of cardiovascular hypertrophy (which is a long-term process). (2) Under sustained agonist stimulation, the overexpression of ADAM-12 may act to inhibit MMP-7 transcription (in a negative feedback loop) while increasing transcription of hypertrophy marker genes. (3) The inhibition of MMP-7 transcription by sustained agonist stimulation may represent a novel physiological compensatory mechanism to counter hypertension and hypertrophy processes.
The therapeutic potential of disrupting the MMP-7/ADAM-12 axis at the level of MMP-7 was evidenced by studies in both mice with agonist-induced hypertension and SHR, a model in which hypertension has multiple or poorly understood causes.22–24 The present data clearly showed that blocking MMP-7 expression could be valuable for attenuating hypertension and preventing the development of cardiac hypertrophy.

Limitations and Future Studies

Although quantitative reverse-transcription polymerase chain reaction provided a reliable, highly sensitive, and quantitative tool, metalloproteinase quantitation by other complementary means remains challenging for various reasons. First, MMP-7 and ADAM-12 genes have very low tissue expression (particularly in the left ventricle). Second, commercial antibodies to these proteins have poor sensitivity or cross-react with many bands on Western immunoblotting, which hampers their unambiguous quantitation. Finally, activity-based determinations are potentially nonspecific and may favor the detection of the more active forms of these metalloproteinases, thus introducing a quantitation bias.

That vasoconstrictors signal through mutually regulated metalloproteinases (and not just through isolated metalloproteinases) is a novel observation that integrates and substantially expands previous research.5–7 This notion is in complete agreement with a previous investigation that

Table. Involvement of MMP-7 in the Development of Cardiac Hypertrophy in the SHR Model: Morphometric, Hemodynamic, and Echocardiographic Results

<table>
<thead>
<tr>
<th>Day 40</th>
<th>PBS</th>
<th>MMP-7 siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS at diastole, mm</td>
<td>1.90±0.03</td>
<td>1.57±0.05*</td>
</tr>
<tr>
<td>LVID at diastole, mm</td>
<td>8.36±0.08</td>
<td>8.63±0.17</td>
</tr>
<tr>
<td>LVPW at diastole, mm</td>
<td>1.74±0.04</td>
<td>1.60±0.03*</td>
</tr>
<tr>
<td>Ejection fraction, %</td>
<td>65.73±1.10</td>
<td>66.06±1.13</td>
</tr>
<tr>
<td>Heart rate, bpm</td>
<td>353.7±14.8</td>
<td>347.7±11.7</td>
</tr>
<tr>
<td>Body weight, g</td>
<td>360.0±7.4</td>
<td>363.7±9.9</td>
</tr>
</tbody>
</table>

IVS indicates interventricular septum; LVID, left ventricular inner diameter; and LVPW, left ventricular posterior wall dimension. Results are mean±SEM. n=4 rats per group.

*P<0.05 vs PBS. Multiple ANOVA with Scheffé’s test.

Figure 5. MMP-7 as a mediator of cardiac hypertrophy in SHR. A, Cardiac hypertrophy in SHR treated with siRNA or PBS. Although the ratio of corrected left ventricle mass (corr. LV mass) to body weight (BW) increased over time in untreated rats, this ratio did not increase in rats that received siRNA. Time axis indicates when M-mode echocardiography analysis was conducted. B, Gross pathology analysis (conducted on day 41) indicated a significantly decreased heart weight (HW) to body weight (BW) ratio. C, Quantitative analysis and representative traces of zymography and Western immunoblotting indicating knockdown of MMP-7 expression. Zymography on casein gels was relatively selective for MMP-7 active form (25 kDa). Western blots show all immunoreactive bands detected with commercially available antibodies to MMP-7. *P<0.05 vs PBS group. Results are mean±SEM of 3 to 4 rats in each group.

That vasoconstrictors signal through mutually regulated metalloproteinases (and not just through isolated metalloproteinases) is a novel observation that integrates and substantially expands previous research.5–7 This notion is in complete agreement with a previous investigation that
detected a differential involvement of multiple metalloproteinases in various forms of cardiomyopathy, including hypertrophic obstructive cardiomyopathy and dilated cardiomyopathy in humans. Future studies should further dissect the dynamics of the metalloproteinase networks that may operate in various models of hypertension and cardiac hypertrophy and in different stages of the development of the disease. Such studies might enable the design of general treatments for hypertensive disorders with complex or unknown causes, such as preeclampsia, which

![Graphs and images related to the study of MMP-7 and ADAM-12 in hypertension and hypertrophy.](http://circ.ahajournals.org/)

Figure 6. Transcriptional relationships between MMP-7, ADAM-12, and hypertrophy marker genes define novel signaling pathways under basal conditions vs sustained agonist stimulation. A through D, Quantitative analysis of mRNA expression levels of indicated genes. Mice were administered either PBS or MMP-7 siRNA (0.4 mg kg⁻¹ · d⁻¹ for 14 days) through a first osmotic minipump followed by the administration of either PBS (ie, basal conditions) or angiotensin II (1.4 mg kg⁻¹ · d⁻¹, for 10 days, ie, from day 5 to day 15; sustained agonist stimulation) through a second osmotic minipump. The mice were euthanized on day 16, and mRNA levels were measured in myocardial tissue (left ventricle) by quantitative reverse-transcription polymerase chain reaction with TaqMan probes. A, B, Analysis of MMP-7 and ADAM-12 expression. C, Analysis of hypertrophy marker genes: β-myosin heavy chain (MHC), brain natriuretic peptide (BNP), and α skeletal actin (alpha-sk-actin). Mice were administered either PBS or MMP-7 siRNA (0.4 mg kg⁻¹ · d⁻¹ for 14 days) through a first osmotic minipump followed by the administration of either PBS (ie, basal conditions) or angiotensin II (1.4 mg kg⁻¹ · d⁻¹, for 10 days, ie, from day 5 to day 15; sustained agonist stimulation) through a second osmotic minipump. The mice were euthanized on day 16, and mRNA levels were measured in myocardial tissue (left ventricle) by quantitative reverse-transcription polymerase chain reaction with TaqMan probes. D, Examples of genes in which mRNA expression levels were unaltered by administration of siRNA to MMP-7. Mice were administered either PBS (ie, basal conditions) or MMP-7 siRNA (0.4 mg kg⁻¹ · d⁻¹ for 14 days). The mice were euthanized on day 16, and mRNA levels were measured in myocardial tissue (left ventricle) by quantitative reverse-transcription polymerase chain reaction with TaqMan probes. E, Quantitative analysis of mRNA expression levels of TACE and ADAM-12 genes in MMP-7⁻/⁻ mice vs age-matched wild-type mice. F, Pretreatment with MMP-7 siRNA before angiotensin II infusion significantly attenuated angiotensin II–induced hypertension in mice. The mice received a first minipump that delivered either vehicle (PBS) or siRNA (0.4 mg kg⁻¹ · d⁻¹ for 14 days). On day 5, the mice were implanted with a second minipump loaded with either PBS or angiotensin II (1.4 mg kg⁻¹ · d⁻¹). G, Pretreatment with MMP-7 siRNA prevented angiotensin II–induced left ventricular hypertrophy. Left, Ratio of corrected left ventricle mass (corr. LV mass; measured by M-mode echocardiography) to body weight (BW). Echocardiographic analysis was conducted 10 days after implantation of a second osmotic minipump (ie, on day 15). Right, Heart weight (HW) to body weight (BW) ratio. Mice were euthanized on day 16. *P<0.05 vs PBS. †P<0.05 vs (PBS + Ang II). Results are mean±SEM of 4 mice in each study group. Ang II indicates angiotensin II.
complicates 5% of all pregnancies worldwide,\(^2^8\) and essential hypertension, which affects 25% of the adult population in developed countries.\(^1\)

**Sources of Funding**

This work was supported by research grants of the Natural Sciences and Engineering Council (NSERC) and the Canadian Institutes of Health Research (CIHR) to Dr Fernandez-Patron, who is also a CIHR and Heart and Stroke Foundation of Canada New Investigator. This work was also supported by CIHR research grants to Drs Kassiiri and Lopaschuk.

**Disclosures**

None.

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**CLINICAL PERSPECTIVE**

Excessive stimulation of Gq protein–coupled receptors by cognate vasoconstrictor agonists induces a variety of cardiovascular processes, including hypertension and hypertrophy. Here, we observed that matrix metalloproteinase-7 and a disintegrin and metalloproteinase-12 (ADAM-12) may form a novel signaling axis in these processes. We suggest further that targeting the matrix metalloproteinase-7/ADAM-12 axis (eg, at the level of matrix metalloproteinase-7) with RNA interference–based approaches could have general therapeutic potential in multiple hypertensive disorders caused by multiple or unknown agonists.
Matrix Metalloproteinase-7 and ADAM-12 (a Disintegrin and Metalloproteinase-12) Define a Signaling Axis in Agonist-Induced Hypertension and Cardiac Hypertrophy

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Circulation. 2009;119:2480-2489; originally published online April 27, 2009; doi: 10.1161/CIRCULATIONAHA.108.835488

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MMP-7 and ADAM-12 define a signalling axis in agonist-induced hypertension and cardiac hypertrophy

Authors: Xiang Wang, MSc; Fung Lan Chow, BSc; Tatsuiro Oka, MD; Li Hao, MD; Ana Lopez-Campistrous, MSc; Sandra Kelly, MSc; Stephan Cooper, BSc; Jeffrey Odenbach, BSc; Barry A. Finegan, MD; Richard Schulz, PhD; Zamaneh Kassiri, PhD; Gary D. Lopaschuk, PhD; and Carlos Fernandez-Patron, PhD

Affiliation: From the Departments of Biochemistry (XW, FLC, LH, AL-C, SC, JO, CF-P), Pediatrics (TO, RS, GL), Pharmacology (RS, GL), Anesthesiology and Pain Medicine (BAF), Physiology (ZK) and the Cardiovascular Research Group (XW, FLC, TO, LH, AL-C, SK, SC, JO, BAF, RS, ZK, GL, CF-P) University of Alberta, Edmonton, AB, Canada.

Xiang Wang and Fung Lan Chow contributed equally to this work.

Corresponding author: Dr. Carlos Fernandez-Patron, Department of Biochemistry, 3-19 Medical Sciences Building, University of Alberta, Edmonton, Alberta T6G 2H7, Canada. Phone: (780) 492 9540, Fax: (780) 492 0095, E-mail: carlos.fernandez-patron@ualberta.ca

Short title: MMP-7 and ADAM-12 in hypertension, hypertrophy
SUPPLEMENTAL METHODS

**Animals** Animal protocols were conducted in accordance with institutional guidelines issued by the Canada Council on Animal Care. All animals were male and housed at the Animal Facility of the University of Alberta until use. MMP-7⁻/⁻ mice and age-matched C57BL/6 (wild type) littermates (12 week old) were purchased from The Jackson Laboratory (Bar Harbor, Maine). The MMP-7⁻/⁻ mice were generated by disrupting the MMP-7 gene through the insertion of a neomycin resistance cassette into the fragment spanning exon 3 and 4. Already-hypertensive (22 week old) spontaneously hypertensive rats (SHR) and age-matched Wistar Kyoto rats (WKY) were purchased from Charles River Laboratories Inc (Wilmington, MA).

**Acute hypertension models** To induce acute hypertension in otherwise normotensive animals, we injected intraperitoneally (i.p.), as previously reported² one of the following pro-hypertensive agents³ (Sigma-Aldrich, Canada): norepinephrine (0.75, 1.5 or 3 mg/kg) or phenylephrine (3 mg/kg), angiotensin II (0.15, 1.5 mg/kg) or L-nitroarginine methyl ester (L-NAME, 10 or 15 mg/kg) in 100 µL PBS. To study the significance of MMPs for the development of acute hypertension, these agents were co-administered with doxycycline (Sigma-Aldrich, 90 mg/kg, i.p.), which pharmacologically inhibits MMPs⁴. The selection of these doses was based on previous reports² and our own experimental observations (see for instance Figure 2E-G).

**Chronic hypertension models** We studied 3 models of chronic hypertension:

1) Angiotensin II-induced hypertension, where mice were infused Ang II (1.4 mg/kg/day) through ALZETosmotic minipumps (DURECT Corporation, Cupertino, CA) implanted subcutaneously on the back of the animals.
2) Norepinephrine-induced hypertension, where mice were injected norepinephrine (1.5 mg/kg, i.p.) once or twice daily for 9 days, as previously reported\(^2\).

3) Spontaneously hypertensive rats.

**Generation of a MMP-7 knock-down model using antisense oligodeoxynucleotides**

Previously validated, commercially available, MMP-7 antisense and scrambled (inactive) oligodeoxynucleotides were synthesized in sufficient scale for *in vivo* studies by Integrated DNA Technologies (Toronto, Ontario)\(^7\)-\(^9\). MMP-7 antisense sequence: 5’-GTATATGATACGATC-3’.

Scrambled sequence: 5’-GTATTAGTATCGAAC-3’. These antisense oligos and their dose were previously reported and produce a knock-down of MMP-7 expression that lasts for at least 40 days in mouse\(^9\). To produce a sustained inhibition of MMP-7 expression in mice, we infused these antisense or scrambled oligodeoxynucleotides (1.31 µmol/kg/d, i.e., 0.6 mg/kg/d) or PBS for 14 days through osmotic minipumps subcutaneously implanted on the back right side of the mice.

**Generation of MMP-7 knock-down models using siRNA** A mouse model of MMP-7 expression knock-down was generated using a siRNA designed as to overlap with the mRNA sequence targeted by the MMP-7 specific antisense oligodeoxynucleotides (for siRNA designs, please see Data supplement Figure II). MMP-7 siRNA, in quantities sufficient for *in vivo* studies, were synthesized by Sigma-Aldrich (Paris). The first two nucleotides of each oligo were 2’-O methylated to increase siRNA stability. The siRNAs were dissolved in PBS prior to use, a dose previously validated\(^10\).

**siRNA studies in mice** As with oligodeoxynucleotides, the siRNAs (30 nmol/kg/d, i.e., 0.4 mg/kg/d) or PBS were infused for 14 days into mice through osmotic minipumps.
siRNA studies in rats The siRNAs (9 nmol/kg/d, i.e., 0.12 mg/kg/d) or PBS were infused for 14 days into SHR through osmotic minipumps.

**Systolic blood pressure measurement** Systolic blood pressure of conscious animals was measured indirectly using a commercially available computerized tail cuff plethysmography system (Kent Scientific Corporation, Torrington, CT).

**Vascular tone studies in microperfusion bioassay system** Mouse mesenteric arteries were dissected and microperfused as described earlier\textsuperscript{11,12} using a Danish MyoTechnology arteriograph system (Aarhus, Denmark). This microperfusion system facilitates the study of vascular reactivity to luminal infusions of drugs as well as to drugs added to the bath (adventitia side). The bioassay closely mimics the \textit{in vivo} situation where adrenergic agonists are administered i.p. and enter the systemic circulation to act on the luminal side of arteries. The arteries were perfused at constant temperature (37°C) and flow rate (2 \( \mu \)L/min) with standard HEPES-PSS (142 mM sodium chloride, 4.7 mM potassium chloride, 1.17 mM magnesium sulfate, 1.56 mM calcium chloride, 1.18 mM potassium phosphate, 10 mM HEPES, 5.5 mM glucose, pH 7.4). In experiments involving the luminal administration of drugs, small volumes (5 \( \mu \)L) of specified drugs were injected into the perfusion line towards the artery. Changes in arterial outer diameter in response to drugs were monitored using a video camera and processed using VediView software (Danish MyoTechnology). The injection of drugs in the line towards the artery, without introducing flow rate change-related artifacts, was facilitated by an HPLC injection valve (Rheodyne Model 9725I, Mandel Scientific Co., ON, Canada).

**Echocardiography** \textit{In vivo} assessment of anatomical structures and hemodynamic function in mice was conducted by echocardiography. The animals were first anaesthetized with 2.0 % Isoflurane, and their cardiac function was subsequently analyzed using a Vevo 770 high-
resolution imaging system (ON, Canada). Three consecutive heartbeats of each frame were
analyzed to measure the wall thickness and end-diastolic (EDD) and end-systolic (ESD) internal
dimensions of the left ventricle (LV). Echocardiographic corrected LV mass (in mg) was
calculated as: \(1.05 \times 0.8 \times [(LVID;d + LVPW;d + IVS;d)^3 - (LVID;d)^3]\) on diastole (d). In the
formula, ID is internal diameter (in mm), PWD is the posterior wall dimension (in mm) and IVS
is the interventricular septum dimension (in mm).

**Sample Preparation** Tissues were washed in isotonic saline buffer, rinsed and weighed (Denver
Instruments model APX-60, Colorado, USA). Protein extraction was done in 25 mM Tris, 62.5
mM NaCl, 1.25 mM PMSF, 62.5 mM Glycerol-2-phosphate, 12.5 mM sodium pyrophosphate,
125 \(\mu\)M NaF, 6.25 \(\mu\)g/ml leupeptine, 312.5 \(\mu\)M sodium orthovanadate, 12.5% glycerol, pH 7.4,
supplemented with 5% SDS and 1% Triton X-100. To determine the protein content, the extracts
were separated by 12% SDS-PAGE followed by densitometric analysis of Coomassie blue
stained bands. Equal protein loads (approximately 50 \(\mu\)g/well) were subsequently subjected to
zymography or western analysis.

**Substrate zymography** To determine MMP-7 and MMP-2 activity levels, tissue lysates were
subjected to electrophoresis on SDS-PAGE gels co-polymerized with casein (2.5 mg/mL) or
gelatin (2 mg/mL), two well characterized substrates of MMP-7 and MMP-2, respectively.
Following electrophoresis, the gel was washed with 2.5% Triton X-100 for 3 x 20 min. Activity
was developed by incubating the gel for 16 hrs at 37°C in enzyme assay buffer (25 mM Tris, 5
mM CaCl\(_2\), 142 mM NaCl, 0.5 mM NaN\(_3\), pH 7.6) supplemented with 1 mM benzamidine and
then the gel was stained with Coomassie blue.

**Western immunoblotting** The extracted proteins were separated using SDS-PAGE gels. The
proteins were transferred to nitrocellulose membrane (BioRad) and blocked in 5% milk or 5%
BSA. Blots were incubated with antibody against MMP-7 (1:250) (Calbiochem, Santa Cruz), ADAM12 (1:1000) (Chemicon) or phopho-ERK1/2 (1:1000) (Sigma-Aldrich), followed by incubation in corresponding secondary antibodies.

**Cryosectioning** Hearts and aortas from SHR treated with PBS, or MMP-7 siRNA (n=3) were embedded in Tissue-Tek (Sakura), frozen in dry ice and stored at -70 ºC. Sections were cut by a Leica microtome and left at room temperature overnight to dehydrate prior to fixation.

**H+E Stain Protocol and Nuclei Count** Cryosections of SHR hearts were fixed in acetone for 5 minutes and allowed to dry. Slides were then re-hydrated in progressively decreasing concentrations of ethanol (100%, 90%, 80%, 50% ethanol then H₂O). The slides were then stained with Gill’s hematoxylin for 5 minutes and washed with warm tap water. The slides were washed twice in 95% ethanol and stained in 0.5% eosin Y for 5 minutes. After staining, the slides were washed three times in 95% ethanol, two times in 100% ethanol, once in propanol followed by two 5 minute immersions in xylene. Slides were then mounted with mounting media and covered by a coverslip. The number of nuclei per unit area was determined from pictures of H+E stained longitudinal cardiac myocytes. Pictures were taken by a DCM500 digital camera on a Kyowa Medlux-12 light microscope at 400x magnification and viewed in ScopePhoto. The total nuclei number of each picture was calculated by adding the nuclei count of each grid section.

**Immunofluorescence** Cryosections of SHR aortae were fixed with acetone and then blocked with 2.5% BSA in TBS-T (20 mM Tris-HCl, 137 mM NaCl, 0.1% Tween-20, pH 7.6) for 30 min. Slides were then incubated with goat anti-MMP-7 antibodies (Santa Cruz Biotechnology) (1:500 dilution each in TBS-T with 2.5% BSA) for 1 hr and washed with TBS-T and 2.5% BSA in TBS-T. Finally the aortas were incubated in Cy3 conjugated rabbit anti-goat (Sigma
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Chemical) and fluorescein conjugated donkey anti-rabbit antibodies (Amersham Life Sciences) (1:500 dilution in TBS-T with 2.5% BSA) for 1 hr. The slides were washed with TBS-T and 2.5% BSA in TBS-T before mounted with DAPI-containing VectaShield mounting medium and viewed at 200x magnification by a Zeiss Axiovert 200M fluorescence.

**Interferon γ Measurement** The level of interferon γ (IFN-γ) in siRNA treated mice or SHR was measured using VeriKine™ Mouse IFN Gamma ELISA kit (PBL biomedical laboratories).

**Genotype analysis** MMP-7 gene knock-out was confirmed by genotype analysis of MMP-7−/− vs. wild type C57BL/6 mice. For the amplification of the MMP-7 gene, specific primers were designed based on *Mus musculus* genomic sequence and synthesized by Sigma-Proligo. The sequence of the upstream primer was (5′-AGACAGCTTCCCCTTGTG-3′). The sequences of the down-stream primers were: (5′-CTGTCGTCCTCACCATCAGT-3′) for wild-type genotyping and (5′-GCTATCAGGACATAGCGTTGG-3′) for MMP-7−/− genotyping following the recommended protocol (Jackson Lab). A 12 µL PCR reaction was performed which included 2 µL genomic DNA, 1 µL 10x High Fidelity PCR Buffer (600 mM Tris-SO₄, pH 8.9, 180 mM Ammonium Sulfate), 1 µL each gene-specific primers 0.2 mM dNTPs, 2 mM MgSO4, and 0.3 unit of Platinum Taq High Fidelity (Invitrogen). The PCR was performed for 25 cycles with temperature at 94 °C for 30 s, 68 °C for 1 min, and 72 °C for 1 min on Mastercycler ep (Eppendorf AG). PCR products were assessed on a 1% agarose/Tris-Acetate-EDTA gel, stained with ethidium bromide and visualised with UV light.

**RNA expression analysis by TaqMan RT PCR** Total RNA was extracted from flash-frozen tissue using Trizol, and cDNA was generated from 1 µg RNA by using a random hexamer. Expression analysis of the reported genes was performed by TaqMan RT-PCR using ABI 7900
sequence detection system. 18S rRNA was used as an endogenous control as described previously\textsuperscript{13}.

**Data analysis** Results are presented as mean ± sem and were analyzed using one-way ANOVA or t-test as appropriate using Jandel SigmaStat 3.5 statistical software. In the echocardiography studies, between-group comparisons of the means were performed by one-way ANOVA followed by Scheffe’s F correction for multiple comparisons of the means. Statistical significance was considered when $p \leq 0.05$. Except where indicated otherwise, between 4 and 5 animals were used for each study.
**SUPPLEMENTAL TABLES**

Supplemental Table. Involvement of MMP-7 in the development of the cardiac hypertrophy induced by angiotensin II (1.4 mg/kg/d for 10 days) in the mouse. Morphometric, haemodynamic and echocardiographic results.

<table>
<thead>
<tr>
<th>Group</th>
<th>IVS;d (mm)</th>
<th>LVID;d (mm)</th>
<th>LVPW;d (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0.68 ± 0.02</td>
<td>3.95 ± 0.04</td>
<td>0.73 ± 0.02</td>
</tr>
<tr>
<td>MMP-7 siRNA</td>
<td>0.67 ± 0.03</td>
<td>3.99 ± 0.09</td>
<td>0.68 ± 0.02</td>
</tr>
<tr>
<td>PBS + AngII</td>
<td>0.83 ± 0.01*</td>
<td>4.62 ± 0.09*</td>
<td>0.85 ± 0.03*</td>
</tr>
<tr>
<td>MMP-7 siRNA + Ang II</td>
<td>0.68 ± 0.04</td>
<td>4.07 ± 0.09</td>
<td>0.69 ± 0.02</td>
</tr>
<tr>
<td>EF (%)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>HR (bpm)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BW (g)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PBS</td>
<td>65.52 ± 2.11</td>
<td>524.7 ± 22.7</td>
<td>25.7 ± 0.2</td>
</tr>
<tr>
<td>MMP-7 siRNA</td>
<td>68.00 ± 2.00</td>
<td>521.1 ± 12.2</td>
<td>27.2 ± 0.5</td>
</tr>
<tr>
<td>PBS + AngII</td>
<td>61.99 ± 1.95</td>
<td>554.1 ± 23.1</td>
<td>24.6 ± 0.7</td>
</tr>
<tr>
<td>MMP-7 siRNA + Ang II</td>
<td>66.28 ± 1.76</td>
<td>546.0 ± 7.3</td>
<td>25.5 ± 0.6</td>
</tr>
</tbody>
</table>

PBS, phosphate buffered saline; d, diastoles; IVS, interventricular septum; LV, left ventricle; ID, inner diameter; PW, posterior wall dimension; EF, ejection fraction; HR, heart rate; BW, body weight. Results are mean ± sem. n = 4 mice for each group.

(*) p < 0.05 vs all groups (i.e., vs. PBS, vs. MMP-7 siRNA and vs. MMP-7 siRNA + Ang II). MultiANOVA with Scheffe's test.

Note: The angiotensin II group consisted of six mice, two of which developed acute systolic dysfunction at the time of echocardiography and were excluded from the analysis. The data indicate the induction of left ventricular hypertrophy and left ventricular dilation by angiotensin II, without significantly affecting contractile function. MMP-7 siRNA treatment protected against these angiotensin II-induced alterations.
SUPPLEMENTAL FIGURES AND FIGURE LEGENDS

Supplemental Figure I  Representative casein zymograms of MMP-7 in indicated tissues showing systemic MMP-7 knock-down in mice administered commercially available antisense oligodeoxynucleotides vs. mice administered vehicle (PBS) or scrambled oligodeoxynucleotides for 14 days. Analysis on day 20. Statistical analysis by t-test. (*): p < 0.05. Results are means ± sem of 4 mice in each group.
Supplemental Figure II Targeted sequences on MMP-7 using siRNAs and antisense oligodeoxynucleotides.

Depicted are the domain structure of MMP-7 protein and the cDNA sequences targeted by the siRNA and antisense oligodeoxynucleotides.
**Supplement Figure III** Assessment of off-target effects. Interferon γ (IFN-γ) levels in spontaneously hypertensive rats (SHR) and mice treated with MMP-7 siRNA or PBS. The rats were subjected to the treatment protocol illustrated in Figure 4; data at day 41. The mice were treated for 14 days and euthanized on day 16. IFN γ levels were measured in indicated tissues by ELISA. (*) \( p < 0.05 \).

Results are means ± sem of 4 animals in each group.
Supplement Figure IV

A, Left panel: Quantitative analysis of the corrected left ventricular mass of MMP-7−/− mice and age-matched wild type mice challenged with norepinephrine (NE, 1.5 mg/kg/d) once or twice daily for 9 days. Right panel: Representative echocardiographic M-mode tracings of the left ventricle. IVS: intraventricular septum. LVID: left ventricular internal dimension. LVPW: Left ventricular posterior wall. (*): p < 0.05 vs. baseline. Results are means ± sem of 4-5 mice per group.

B, Western blot analysis showing the phosphorylation of the cardiac platelet derived growth factor receptor (PDGFR) β in the heart of wild type mice vs. MMP-7−/− mice injected twice daily with norepinephrine (NE) for 9 days and euthanized on day 12. Results are expressed as mean ± sem of 4 mice in each study group.
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