Tumor Necrosis Factor-α–Converting Enzyme Is a Key Regulator of Agonist-Induced Cardiac Hypertrophy and Fibrosis

Xiang Wang, Tatsuiro Oka, Fung L. Chow, Stephan B. Cooper, Jeff Odenbach, Gary D. Lopaschuk, Zamanek Kassiri, Carlos Fernandez-Patron

Abstract—Cardiac remodeling is associated with hypertrophy and fibrosis processes, which may depend on the activity of matrix metalloproteinases (MMPs) and “a disintegrin and metalloproteinases” (ADAMs). We investigated whether ADAM-17 (tumor necrosis factor-α–converting enzyme [TACE]) plays a role in agonist-induced cardiac remodeling and the relationships established among TACE, MMP-2, and ADAM-12. We targeted TACE in rodent models of spontaneous and agonist-induced hypertension using RNA interference combined with quantitative RT-PCR, activity determinations, and functional studies. Treatment of spontaneously hypertensive rats with previously validated TACE small-interfering RNA for 28 days resulted in systemic knockdown of TACE expression. TACE knockdown effectively stopped the development of cardiac hypertrophy. Mice receiving angiotensin II (1.4 mg/kg per day for 12 days) exhibited cardiac hypertrophy, as well as fibrosis, which was associated with elevated myocardial expression of molecular markers of hypertrophy (α-skeletal actin, β-myosin heavy chain, and brain natriuretic peptide) and fibrosis (collagen types I and III and fibronectin), as well as MMP-2 and ADAM-12. Treatment with TACE small-interfering RNA (but not with PBS or luciferase small-interfering RNA) inhibited TACE expression, thus preventing angiotensin II–induced cardiac hypertrophy and fibrosis. Moreover, knockdown of TACE inhibited angiotensin II–induced overexpression of markers of myocardial hypertrophy and fibrosis, as well as ADAM-12 and MMP-2. These findings provide the first in vivo evidence that agonist-induced cardiac hypertrophy and fibrosis processes are signaled through TACE, which acts through novel pathways involving transcriptional regulation of ADAM-12 and MMP-2. Targeting TACE has potential therapeutic importance for modulating agonist-induced cardiac remodeling. (Hypertension. 2009;54:400-00.)

Key Words: cardiac hypertrophy ■ hypertension ■ metalloproteinase ■ ADAM-17/TACE ■ Gq protein–coupled receptor agonist ■ RNA interference

Cardiac remodeling is a major hallmark of hypertensive disorders and is associated with the development of cardiac hypertrophy (ie, an increase in cell size of individual cardiomyocytes), which causes thickening of the myocardium. Although initially compensatory, sustained hypertrophic growth is pathological, in part, because of its association with the development of fibrosis (ie, increased synthesis and deposition of extracellular matrix proteins), which disrupts the normal structure and contractile properties of the myocardium.1 Pathological cardiac remodeling is thus, detrimental for cardiac function and may cause cardiac dysfunction, myocardial stiffness, and increased risk of heart failure, sudden death, and stroke.2–5 However, it remains unclear how and why such apparently distinct processes as cardiac hypertrophy and fibrosis develop with hypertension and whether pressure overload in hypertension is causal.

Our laboratory is investigating the general hypothesis that cardiac remodeling may be associated with hypertension simply because high blood pressure, hypertrophy, and fibrosis share common inducers, which signal through largely overlapping pathways. Among the common inducers of high blood pressure, hypertrophy, and fibrosis are vasoconstrictive agonists, eg, catecholamines, endothelins, and angiotensin (Ang) II.6–9 These agonists all act on Gq protein–coupled receptors, which, in turn, activate the classic phospholipase C/protein kinase C pathway and reactive oxygen species, leading to the activation of downstream matrix metalloproteinases (MMPs) and “a disintegrin and metalloproteinases” (ADAMs). Among them, ADAM-12 and ADAM-17 (tumor necrosis factor-α–converting enzyme [TACE]) are present in the cardiovascular and endocrine systems.7–13 ADAM-12 and TACE are synthesized and stored in...
the rough endoplasmic reticulum until they mature in a late Golgi compartment. Their maturation after agonist stimulation involves the removal of the prodomain from the precur-

sor protein. The activated metalloproteinases from both MMP and ADAM families are able to cleave a host of common substrates, including extracellular matrix (ECM) proteins (eg, collagens), proinflammatory mediators (eg, tumor necrosis factor-α), and growth factors (eg, transforming growth factor-α and heparin-binding epidermal growth factor–like growth factor), to signal through their receptors and downstream mitogen-activated protein kinases, which transcriptionally activate the expression of immediate-early genes and fetal genes, including hypertrophy markers. There is an increasing evidence that different metalloproteinases, including MMP-2, MMP-7, ADAM-12, and TACE, may, thus, mediate tissue remodeling and injury in both cardiovascular and renal systems.

The similar tissue localization, agonist-activation profile, substrates, and signaling pathways of some of the growth factor sheddases, eg, ADAM-12 and TACE, suggest a redundancy of their functions in vivo, particularly in signaling of cardiovascular growth processes. ADAM-12 has previously been directly implicated in cardiac hypertrophy. However, the involvement of TACE in cardiac hypertrophy has been suggested but not yet demonstrated in vivo. Although TACE is a key mediator of Ang II–induced renal injury and fibrosis, it is unknown whether TACE mediates the development of agonist-induced cardiac fibrosis.

TACE plays a role in the development of cardiac hypertrophy and fibrosis, it would be important to determine whether and, if so, how TACE and ADAM-12 coordinate each other’s expression and functional redundancy. Would their relationships follow a hierarchical pattern? Would such hierarchical relationships be of significance for the mechanisms and treatment of pathological cardiac remodeling?

Here we start to address these important questions by focusing on the role of TACE in the development of agonist-induced cardiac hypertrophy and fibrosis processes. Our findings establish a novel central role for TACE in signaling of both processes, upstream of MMP-2 and ADAM-12.

**Materials and Methods**

Please see the online Data Supplement (available at http://hyper.ahajournals.org) for the expanded Methods section.

**Generation of TACE Knockdown Models Using Small-Interfering RNA**

The animal (mouse and rat) model of TACE expression knockdown was generated using a previously validated TACE small-interfering RNA (siRNA) (for siRNA design, please see Figure 1) synthesized by Sigma-Aldrich. Luciferase (Luc) siRNA (antisense: 5′-GUAUCUCUCAUAGCCUUAdTdT; sense: 5′-mGmAGAAGCUUGAUUCUUUGCdTdT) was used as the control. The first 2 nucleotides of each strand were 2′-O methylated to increase siRNA stability. The siRNAs were dissolved in PBS before use.

**siRNA Studies in Rats**

siRNA (9 nmol/kg per day, ie, 0.12 mg/kg per day) or PBS was infused for 14 days into spontaneously hypertensive rats (SHRs) through subcutaneously implanted ALZET osmotic minipumps (DURECT Corporation) in the back of the animals.

**siRNA Studies in Mice**

siRNA (30 nmol/kg per day, ie, 0.4 mg/kg per day) or PBS was infused for 14 days into C57bl/6J mice through subcutaneously implanted osmotic minipumps. Another group of mice received siRNA (15 μg per mouse, ie, 0.45 mg/kg) or PBS by injection via the jugular vein following a recently described method. The injection was conducted every 5 days to maintain the knockdown effects.

**Mouse Model of Ang II–Induced Hypertension and Cardiac Hypertrophy**

Male C57BL/6J mice were infused with Ang II (1.4 mg/kg per day) through subcutaneously implanted osmotic minipumps for 12 days.

**RNA Expression Analysis by TaqMan RT-PCR**

Total RNA was extracted from flash-frozen tissue using TRIzol (Invitrogen), and cDNA was generated from 1 μg of RNA using a random hexamer. Expression analysis of the reported genes was performed by TaqMan RT-PCR using the ABI 7900 sequence detection system. 18S rRNA was used as an endogenous control, as described previously.

**Results**

**TACE Mediates Cardiac Hypertrophy in Genetically Hypertensive Rats**

We examined whether blocking TACE expression in already-hypertensive 22-week-old SHRs would affect systolic blood pressure and/or development of cardiac hypertrophy. To inhibit TACE gene expression, we chose an RNA interference–based approach using a previously validated siRNA with the exception that we introduced a chemical modification (2′-O methylation) on the 5′ end of the double-stranded RNA molecule to enhance its resistance to nucleases in vivo (Figure 1). The administration of TACE siRNA for 30 days through a subcutaneous osmotic minipump (protocol depicted in Figure 2A) effectively stopped the progression of cardiac hypertrophy, as evidenced by M-mode echocardiography and gross pathology studies (Figure 2B and 2C and Table 1). In addition, the cross-sectional width of cardiomyocytes was decreased on average by 38% in SHRs receiving TACE
siRNA versus SHRs receiving vehicle (PBS; n = 3 rats in each study group; P < 0.05 by t test), confirming the antihypertrophic effects of TACE siRNA.

Rats receiving TACE siRNA had significantly lower TACE immunoreactivity and TACE proteolytic activity (Figures 2D and S1 and data not shown), MMP-2 activity (Figure 2D), and extracellular signal-regulated kinase 1/2 phosphorylation (Figure S2) compared with rats receiving an unrelated

Table 1. Involvement of TACE in the Development of Cardiac Hypertrophy in the SHR Model: Echocardiographic Results

<table>
<thead>
<tr>
<th>Day 40</th>
<th>PBS</th>
<th>TACE siRNA</th>
</tr>
</thead>
<tbody>
<tr>
<td>IVS, d, mm</td>
<td>1.90±0.03</td>
<td>1.54±0.03*</td>
</tr>
<tr>
<td>LVID, d, mm</td>
<td>8.36±0.08</td>
<td>8.23±0.17</td>
</tr>
<tr>
<td>LVPW, d, mm</td>
<td>1.74±0.04</td>
<td>1.56±0.02*</td>
</tr>
<tr>
<td>EF, %</td>
<td>65.7±1.1</td>
<td>66.6±0.7</td>
</tr>
<tr>
<td>HR, bpm</td>
<td>354±15</td>
<td>349±16</td>
</tr>
<tr>
<td>BW, g</td>
<td>360.0±7.4</td>
<td>350.2±10.1</td>
</tr>
</tbody>
</table>

IVS indicates interventricular septum; LVID, left ventricle; ID, inner diameter; LVPW, posterior wall dimension; EF, ejection fraction; HR, heart rate; BW, body weight. Results are mean±SEM. n=4 rats per group.

*P < 0.01 vs PBS by multi-ANOVA with Scheffe’s test.

RNA or vehicle (PBS). These data indicated that the TACE siRNA effectively decreases TACE expression and activity, as well as TACE downstream signaling. Interestingly, treatment with TACE siRNA did not decrease the blood pressure in the SHR model (Figure 3A) despite TACE siRNA causing a significant inhibition of TACE proteolytic activity in resistance arteries of the rats (Figure 3B). Taken together, these observations provide strong in vivo evidence that TACE expression regulates development of cardiac hypertrophy, but TACE may not play a major role in the regulation of blood pressure in hypertension.

**TACE and ADAM-12 Form a Novel Signaling Axis In Vivo**

To further clarify how TACE knockdown impacts the development of cardiac hypertrophy, we conducted functional studies, quantitative RT-PCR, and activity determinations in mice where TACE was knocked down by in vivo RNA interference. Administration of TACE siRNA (0.4 mg/kg per day) through subcutaneous osmotic minipumps for 14 days in mice resulted in a significant downregulation in myocardial TACE mRNA levels (Figure 4A) and proteolytic activity (Figure 4B).
To substantiate the effects of TACE siRNA, we conducted a small additional study (n=2 mice per group) using a different route of siRNA administration (ie, intravenous). Mice were injected with TACE siRNA (15 μg every 5 days) into their jugular vein, a method that was shown to effectively knock down the expression of cardiac target proteins. TACE expression was decreased by 30±5% in mice that received TACE siRNA by intravenous injection versus mice that received PBS. Mice that received TACE siRNA did not display any echocardiographic abnormalities (Table 2).

Figure 3. Effects of TACE knockdown on blood pressure of SHRs. A, Time course of the systolic blood pressure of already-hypertensive SHRs treated with either TACE siRNA or vehicle (PBS). B, Demonstration of vascular TACE knockdown. Quantitative analysis indicates that, in small (resistance) mesenteric arteries, TACE proteolytic activity was significantly knocked down by siRNAs to TACE, as determined by a commercially available fluorogenic peptide cleavage assay. *P<0.05 vs PBS group. Results are mean±SEM of n=4 rats in each group.

Figure 4. TACE, MMP-2, and ADAM-12 genes define a novel metalloproteinase signaling network. Quantitative analysis of gene expression (ie, mRNA levels) for: (A and B) TACE, (C and D) MMP-2, and (E) ADAM-12. Mice (12 weeks old) were administered either PBS or TACE siRNA (0.4 mg/kg per day for 14 days) through a first osmotic minipump followed by the administration of either PBS or Ang II (1.4 mg/kg per day for 12 days, ie, from day 5 to day 16), through a second osmotic minipump. The mice were euthanized on day 16. Expression: mRNA levels were measured in myocardial tissue (left ventricle) by quantitative TaqMan RT-PCR and normalized by 18S rRNA. Activity: TACE proteolytic activity was determined using a commercially available fluorogenic peptide. MMP-2 activity was measured by gelatin zymography. ADAM-12 antibodies were not sensitive enough to allow a reliable quantification of ADAM-12 in the left ventricle of mice. *P<0.05 vs PBS. +P<0.05 vs PBS+Ang II. Results are mean±SEM of n=4 mice in each study group.
Interestingly, knockdown of TACE resulted in the down-regulation of MMP-2 mRNA levels (Figure 4C) and activity (Figure 4D), as well as in ADAM-12 gene expression (Figure 4E). However, the siRNA had otherwise insignificant effects on other genes, including MMP-9, tissue inhibitor of metalloproteinase 3 (an endogenous inhibitor of TACE), and interferon-γ (data not shown; n = 4 mice; P > 0.05 by t test). In mice receiving siRNA by jugular vein injection, the level of interferon-γ was also unaffected (data not shown). The unchanged level of interferon-γ demonstrated that the small RNA-induced innate immune response was not activated. This further suggested that the observed downregulation of MMP-2 or ADAM-12 was caused by a decrease in TACE expression rather than a nonspecific effect of the TACE siRNA.

As expected, the blood pressure of mice receiving Ang II through osmotic minipumps (1.4 mg/kg per day for 10 days) was significantly elevated versus mice receiving PBS (BP[Ang II] = 188 ± 1 mm Hg; BP[PBS] = 133 ± 2 mm Hg; P < 0.001 by t test; n = 4 mice per study group). Mice receiving either TACE siRNA or Luc siRNA (0.4 mg/kg per day) by osmotic minipumps also developed hypertension on infusion of Ang II for 10 days (BP[TACE siRNA + Ang II] = 167 ± 13 mm Hg; BP[Luc siRNA + Ang II] = 180 ± 22 mm Hg; n = 4 mice per group).

Similarly, after 10 days of Ang II infusion, blood pressure was elevated in mice receiving PBS, TACE siRNA, or Luc siRNA by jugular vein injection (BP[Ang II] = 179 ± 4 mm Hg; BP[TACE siRNA + Ang II] = 173 ± 3 mm Hg; BP[Luc siRNA + Ang II] = 179 ± 3 mm Hg; n = 3 to 4 mice per study group). Interestingly, in mice that received TACE siRNA by intravenous injection (but not in those that received TACE siRNA by osmotic minipumps) there seemed to be a delay in the onset of the hypertension. Indeed, after 5 days of Ang II infusion, blood pressure was higher in mice that received either PBS or Luc siRNA by intravenous injection versus those that received TACE siRNA (BP[PBS + Ang II] = 150 ± 3 mm Hg; BP[Luc siRNA + Ang II] = 164 ± 9 mm Hg; BP[TACE siRNA + Ang II] = 122 ± 11 mm Hg; n = 3 to 4 mice per study group).

Mice that received Ang II (1.4 mg/kg per day) for 12 days displayed elevated expression and proteolytic activity of TACE, MMP-2, and ADAM-12 (Figures 4 and S3), as well as left ventricular hypertrophy (Table 2 and Figures 5, 6, and S4), which was associated with an overexpression of myocardial hypertrophy marker genes (brain natriuretic peptide and α-skeletal actin and β-myosin heavy chain; Figures 5, 6, and S4). Cardiac fibrosis, the increased deposition of ECM proteins in myocardium that is typically associated with pathological cardiac hypertrophy, was also induced by Ang II, as shown by the increased expression of the ECM proteins, collagen types I and III, and fibronectin (Figures 5, 6, and S4).

Pretreatment with TACE siRNA through osmotic minipumps decreased cardiac TACE levels and activity (Figure 4) and fully protected the mice from Ang II–induced left ventricular hypertrophy and cardiac fibrosis (Table 2 and Figure 5). The protective effect of TACE siRNA was associated with the normalization of the expression of MMP-2, ADAM-12 (Figure 4), hypertrophy marker genes, and ECM proteins (Figure 5). Unlike TACE siRNA, the treatment of mice with an siRNA against Luc did not protect from Ang II–induced left ventricular hypertrophy and fibrosis, as evidenced by M-mode echocardiography, gross pathology studies, and RT-PCR analysis of molecular markers of hypertrophy and fibrosis (Figure 6).

Furthermore, excluding a role of the administration route in the protective effects of TACE siRNA, in mice receiving siRNA by jugular vein injection, TACE siRNA also blocked the Ang II–induced expression of TACE (Figure S3A), MMP-2 (Figure S3B), and ADAM-12 (Figure S3C). TACE siRNA, but not Luc siRNA, prevented Ang II–induced cardiac hypertrophy, as indicated by gross pathology (Figure S4A), cardiomyocyte cross-sectional area (Figure S4B), and expression of hypertrophic markers (Figure S4C). In addition, knockdown of TACE by siRNA also protected mice from Ang II–induced cardiac fibrosis, as determined by cardiac interstitial collagen staining with Picrosirius red (Figure S4D) and expression of collagen I (Figure S4E).

These findings are the first evidence that the down-regulation of TACE expression may prevent the transcription of metalloproteinases, eg, MMP-2 and ADAM-12, and downstream hypertrophy markers, which together could act as effectors of TACE in agonist-induced cardiac hypertrophy.

**Discussion**

This investigation has resulted in several novel observations. First, to our knowledge, our findings suggest for the first time in vivo that agonist-induced cardiac hypertrophy and fibrosis are signaled through ADAM-17/TACE, and decreasing TACE activity by RNA interference protects from cardiac hypertrophy and fibrosis in models of hypertension. Furthermore, our data suggest that TACE may act by promoting the transcription of metalloproteinases (eg, MMP-2 and ADAM-12) and downstream hypertrophy markers, which together could act as effectors of TACE in agonist-induced cardiac hypertrophy.
agonist-induced cardiac hypertrophy (in Ang II–infused mice, as well as SHR models) were primarily attributed to the downregulation of TACE. In all of the models, the degree of TACE knockdown, albeit modest, was significant, ~40% from baseline measured by either immunoreactivity or proteolytic activity and 25% as measured by quantitative RT-PCR. Potential off-target effects, eg, signaling through the toll-like receptor/interferon-γ pathway,24 may not be major mechanisms of protective effects of TACE siRNA because administration of an siRNA to a nonmammalian gene (Luc) by 2 different routes of administration (ie, infusion through minipump and injection via jugular vein) had no protective effect on Ang II–induced hypertension, cardiac hypertrophy, and fibrosis, and no upregulation of interferon-γ was observed; these in vivo findings are in agreement with a recent report using the same siRNA sequence on myoblast cultures.10

TACE siRNA had no long-term protective effect on hypertension in either SHRs or Ang II–infused mice. However, we cannot exclude that TACE siRNA treatment could have a short-term or transient protective effect that was nonetheless insufficient to prevent the development of hypertension in both SHRs and Ang II–infused mice. Because of the complexity of the effects of TACE inhibition on the transcription of multiple genes, dedicated studies are warranted to further dissect the mechanism of the short-term roles of TACE in the regulation of blood pressure of hypertension. However, our long-term data are consistent with previous research showing that the pharmacological inhibition of TACE (with TAPI-2) does not decrease systolic blood pressure in Ang II–infused mice.12 Our long-term data are also in agreement with previous research showing that pharmacological blockade (with KB-R7785) of ADAM-12 (which we found to be downstream of TACE) does not protect mice from agonist-induced hypertension.7

Previous studies have found that TACE, ADAM-12, and MMP-2 are all upregulated in human hypertrophic cardiomyopathy16,21 and that ADAM-12 may mediate agonist-induced cardiac hypertrophy.7 Recently, a novel role for ADAM-12 was reported in facilitating activation of transforming growth factor-β signaling through Smads, which is the main pathway mediating the development of agonist-induced fibrosis. This action of ADAM-12 is mediated via protein-protein interactions, independent of ADAM-12 protease activity.25 Similarly, MMP-2 has been shown to promote myocardial hyper-
by previous research,9,10,21 TACE involvement in these processes has not been demonstrated in vivo. Accordingly, the activated expression of TACE regulates the development of cardiac hypertrophy and fibrosis processes7–9,12,19,20 (Figure S5).

Despite extensive research,7–9,12,19,20 an interaction among the pathways of TACE, ADAM-12, and MMP-2 has never been identified. Therefore, a novel finding of this research has been the observation that baseline gene expression levels of TACE and ADAM-12 are transcriptionally connected, although further research is necessary to dissect the transcriptional pathways linking these metalloproteinases. Together with previously reported agonist-induced posttranscriptional signaling events,7–9,12,19,20 the TACE/ADAM-12 signaling axis may regulate the development and progression of hypertrophy and fibrosis, which are hallmarks of agonist-induced cardiovascular remodeling (Figure S5).

Furthermore, it is possible that metalloproteinases form a highly regulated signaling network (as opposed to acting in isolation). Certain metalloproteinases (eg, TACE) may act like primary mediators of cardiovascular (hypertrophic) growth and fibrosis processes, whereas other metalloproteinases (eg, MMP-2 and ADAM-12) are downstream effectors. Interestingly, we found recently that MMP-7 may exert a transcriptional regulation of ADAM-12 in cardiac hypertrophy, similar to that demonstrated here for TACE.27

Perspectives
These current findings have therapeutic potential in hypertensive heart disease. Our data suggest that TACE, MMP-2,
and ADAM-12 may define a novel signaling axis in agonist-induced cardiac hypertrophy and fibrosis processes and that these processes can be disrupted by targeting TACE. This notion is supported by our studies both in mice with agonist-induced cardiac hypertrophy and in SHR, a model where cardiac hypertrophy is likely signaled by multiple agonists. Together with our previous studies on MMP-7 and ADAM-12, it may be possible to counter different hypertensive cardiac diseases and specific complications thereof by targeting one or more nodes in the emerging network of interconnected metalloproteinases, which includes TACE, MMP-7, MMP-2, and ADAM-12.

Future studies should investigate the emerging notion of metalloproteinase networks as mediators of agonist-induced cardiovascular hypertrophy and fibrosis processes and the dynamics of this network in various models and stages of hypertension and cardiac remodeling.

**Sources of Funding**

This work was supported by research grants of the Alberta Heritage Foundation for Medical Research (block grant), the Natural Sciences and Engineering Council and the Canadian Institutes of Health Research (to C.F.-P., who is also a Canadian Institutes of Health Research New Investigator). This work was also supported by Canadian Institutes of Health Research research grants to Z.K. and G.D.L.

**Disclosures**

None.

**References**

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Hypertension. published online July 6, 2009;
Hypertension is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 0194-911X. Online ISSN: 1524-4563

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TACE is a key regulator of agonist-induced cardiac hypertrophy and fibrosis

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Short title: TACE and cardiac hypertrophy, fibrosis
Supplement Materials and 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. C57BL/6 mice (12 or 20-week old) were purchased from The Jackson Laboratory (Bar Harbor, ME). Already-hypertensive (22-week old) spontaneously hypertensive rats (SHR) were purchased from Charles River Laboratories Inc. (Wilmington, MA). All animals were anesthetised by 3% isoflurane through inhalation before and during the surgical procedures. Unless specifically indicated, n=4 animals were used in each group in the experiments.

Generation of TACE knock-down models using siRNA The animal (mouse and rat) model of TACE expression knock-down was generated using a previously validated TACE siRNA\(^1\) (for siRNA design, please see Figure 1) synthesized by Sigma-Aldrich (Paris). Luciferase siRNA (antisense: 5’-GUAUCUCUUCAUAGCCUUAdTdT) was used as control. The first two nucleotides of each strand were 2’-O methylated to increase siRNA stability. The siRNAs were dissolved in PBS prior to use.

siRNA studies in rats  siRNAs (9 nmol/kg/d, i.e., 0.12 mg/kg/d) or PBS were infused for 14 days into spontaneously hypertensive rats (SHR) through subcutaneously implanted ALZET osmotic minipumps (DURECT Corporation, Cupertino, CA) in the back of the animals.

siRNA studies in mice  siRNAs (30 nmol/kg/d, i.e., 0.4 mg/kg/d) or PBS were infused for 14 days into C57bl/6J mice through subcutaneously implanted osmotic minipumps. Another group of mice received siRNA (15µg/mouse, i.e., 0.45mg/kg) or PBS by injection via jugular vein. The injection was conducted every 5 days to maintain the knockdown effects.

Mouse model of angiotensin II-induced hypertension  Male C57BL/6 mice were infused with angiotensin II (1.4 mg/kg/d) through subcutaneously implanted osmotic minipumps implanted on the backs for 12 days.

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). All animals were trained by being placed into the restrainer for 2-3 times before their blood pressure was measured.

Echocardiography  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 x 0.8 x [(LVID;d + LVPW;d + IVS;d)\(^3\) – (LVID;d)\(^3\)] on diastole (d). In the formula, ID is internal diameter (in mm), PW 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. 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 µM NaF, 6.25 µg/ml leupeptine, 312.5 µM 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 µg/well) were subsequently subjected to zymography or western analysis.

Substrate zymography To determine MMP-2 levels by quantitative substrate zymography, tissue lysates were subjected to electrophoresis on SDS-PAGE gels co-polymerized with gelatin (2 mg/mL), 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 CaCl₂, 142 mM NaCl, 0.5 mM NaN₃, 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 TACE (1:250) (Santa Cruz), p-ERK-1/2 (1:1000) (Sigma-Aldrich), ERK-1/2 (1/1000) (Cell Signaling), MMP-2 (1/500) (Calbiochem) followed by incubation in corresponding secondary antibodies.

TACE proteolytic activity assay TACE activity was determined using a fluorogenic TACE substrate IV Abz-LAQAVRSSR-Dpa (Calbiochem). The substrate is cleaved by TACE at the Ala-Val amide bond. The reaction was monitored by using light of 320 nm for excitation and a 420 nm filter to detect light emitted by the substrate following cleavage by TACE. To further ensure selectivity, the assay was conducted in the absence and presence of a TACE pharmacological inhibitor (TAPI-2) and results were corrected by subtracting the TAPI-2 resistant activity (~40% across all samples). Equal amount of protein was used as determined by Bradford assay.

Cryosectioning Hearts and aortas from SHR treated with PBS, or TACE siRNA 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 with cold acetone.

Immunofluorescence Slides with 6µm thick sections of frozen mouse hearts were stained with commercially available antibodies for actin (Sigma) and TACE (Santa Cruz) diluted 1:250. Slides were imaged using a spinning disk laser confocal microscope (Confocal Imagine Core facility, University of Alberta) and analyzed with Improvision Velocity® software.

WGA-FITC staining and cardiomyocytes cross-sectional area quantification Slides with 10 µm thick sections of frozen mouse hearts. Slides were washed in PBS-T and incubated for 2 hrs in 50 µg/mL Wheat Germ Albumin (WGA) conjugated with FITC (Invitrogen) and DAPI solution then washed again in PBS-T and mounted with a coverslip. Confocal microscope images were taken with a spinning disk laser confocal microscope (Confocal Imagine Core
facility, University of Alberta) and analyzed with ImageQuant 5.1 to determine cardiomyocyte cross-sectional area (>100 cells per heart were counted).

**Collagen staining with picrosirius red** Slides with 10 µm thick mouse heart frozen sections were brought back to water and stained for 1 hr in PSR staining solution (1 g/L Direct Red 80 in saturated picric acid solution), washed in acidified water and dehydrated. Slides were photographed by a DCM500 camera digital camera on a Kyowa Medlux-12 light microscope and viewed using ScopePhoto (NonLinear Dynamics). Area covered by collagen was determined by converting the image to a grayscale image showing measurement of red versus blue+green colour in Adobe Photoshop and then using ImageQuant 5.1 to measure the area of Picrosirius red staining.

**Interferon-γ Measurement** The level of interferon γ (IFN γ) in siRNA treated mice or SHR was measured using VeriKine™ Mouse IFN-γ sandwich ELISA kit (Ebiosciences) as per manufactures instructions.

**RNA expression analysis by TaqMan RT PCR** Total RNA was extracted from flash-frozen tissue using Trizol (Invitrogen), and cDNA was generated from 1 µg RNA 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 previously2.

**Data analysis** Results were analyzed using one-way ANOVA or t-test (Jandel SigmaStat 3.5 statistical software) as appropriate. 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.
**Supplement Figures**

**Figure S1**

**TACE**

<table>
<thead>
<tr>
<th>SHR #</th>
<th>PBS</th>
<th>Non-TACE siRNA</th>
<th>TACE siRNA</th>
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<td>1</td>
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<td></td>
</tr>
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<td>2</td>
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</table>

**Immunoreactivity (%) of PBS group**

- **PBS**
- **Non-TACE siRNA**
- **TACE siRNA**

Figure S1 TACE immunoreactivity in the left ventricle of SHR treated with TACE siRNA vs. SHR treated with either an unrelated siRNA or vehicle (PBS). Signals were quantified by densitometry and normalized to SDS-PAGE gel loading control. (*): $p < 0.05$ vs. PBS. Results are means ± sem of n=4 rats in each study group.
Figure S2

Western blot of phosphorylated ERK (p-ERK)-1/2 and total ERK-1/2 in the left ventricle of SHR treated with TACE siRNA vs. SHR treated with vehicle (PBS). Quantitative analysis of the ratio between p-ERK-1 and ERK-1 showed decreased phosphorylation level of ERK in the left ventricle of SHR treated with TACE siRNA. (*): $p < 0.05$ vs. PBS. Results are means ± sem of $n=4$ rats in each study group.
Figure S3 Knockdown of cardiac TACE in response to jugular vein injection of TACE siRNA

siRNA (15 µg) or PBS was injection into mice via their jugular veins (on day 0, day 5 and day 10), angiotensin II (Ang II, 1.4 mg/kg/d) was delivered through a subcutaneously implanted osmotic minipump (from day 5 to day 16). The mice were euthanized on day 16.

A, left panel TACE siRNA inhibited the increase in cardiac TACE expression induced by Ang II infusion as detected by immunofluorescence of TACE-FITC in representative left ventricle frozen sections (6 µm) surrounding cardiac arteries (indicated by arrows). Immunofluorescence of actin-Texas Red (TR) was also shown. A, right panel TACE siRNA reduced the increase in cardiac TACE activity induced by angiotensin II infusion as detected by TACE activity assay using a commercially available fluorogenic TACE substrate. B, C, Decrease in TACE expression also inhibited the increased expression of MMP-2 (B, detected by western blot) and ADAM-12 (C, measured by quantitative RT-PCR) in left ventricle induced by the angiotensin II infusion.

(*): p < 0.05 vs. PBS group. (+): p < 0.05 vs. (PBS + Ang II) group.
Results are means ± sem of n=2-4 mice for each study group.
Data supplement figure legends
TACE and cardiac hypertrophy, fibrosis
Corresponding author: Fernandez-Patron, C.

**Figure S4**

A. Gross pathology

<table>
<thead>
<tr>
<th>Condition</th>
<th>HW / BW (mg / g)</th>
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<tr>
<td>PBS</td>
<td>0.5 ± 0.1</td>
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<tr>
<td>Luc siRNA</td>
<td>0.7 ± 0.2</td>
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<tr>
<td>TACE siRNA</td>
<td>0.8 ± 0.3</td>
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<tr>
<td>PBS + Ang II</td>
<td>1.0 ± 0.5</td>
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<tr>
<td>Luc siRNA + Ang II</td>
<td>1.1 ± 0.6</td>
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<tr>
<td>TACE siRNA + Ang II</td>
<td>1.2 ± 0.7</td>
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</table>

B. WGA-FITC immunostaining

C. Hypertrophy markers

<table>
<thead>
<tr>
<th>Condition</th>
<th>Cardiomyocyte cross-sectional area (arbitrary units)</th>
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<tbody>
<tr>
<td>PBS</td>
<td>1000 ± 100</td>
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<tr>
<td>TACE siRNA</td>
<td>1500 ± 200</td>
</tr>
<tr>
<td>PBS + Ang II</td>
<td>2000 ± 300</td>
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<tr>
<td>TACE siRNA + Ang II</td>
<td>2500 ± 400</td>
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D. Picrosirius red staining

E. Collagen type I

<table>
<thead>
<tr>
<th>Condition</th>
<th>Area covered by collagen (%) of tissue above staining threshold</th>
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</thead>
<tbody>
<tr>
<td>PBS</td>
<td>5 ± 1</td>
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<tr>
<td>TACE siRNA</td>
<td>10 ± 2</td>
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<tr>
<td>PBS + Ang II</td>
<td>15 ± 3</td>
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<tr>
<td>TACE siRNA + Ang II</td>
<td>20 ± 4</td>
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</table>
**Figure S4 Knockdown of TACE blocked cardiac hypertrophy and fibrosis induced by angiotensin II infusion**

siRNA (15 µg) or PBS was injection into mice via their jugular veins (on day 0, day 6 and day 10), angiotensin II (Ang II, 1.4 mg/kg/d) was delivered through a subcutaneously implanted osmotic minipump (from day 5 to day 15). The mice were euthanized on day 15.

A-C, Pre-treatment with TACE siRNA, but not luciferase (Luc) siRNA or PBS, prevented Ang II-induced left ventricular hypertrophy as evidenced by the ratio of heart weight (HW) to body weight (BW) (A), size of cardiomyocytes (B), detected by average area of cardiomyocytes cross-section following WGA-FITC staining, >100 cells per heart were counted, and expression of hypertrophy markers: brain natriuretic peptide (BNP), α-skeletal actin and β-myosin heavy chain (β-MHC) (C, measured by quantitative RT-PCR).

D, E, Pre-treatment with TACE siRNA, but not PBS, prevented Ang II-induced-cardiac fibrosis, as evidenced by the picrosirius red staining (D) and type I collagen expression (E, measured by quantitative RT-PCR).

(*): $p < 0.05$ vs. PBS group. (+): $p < 0.05$ vs. (PBS + Ang II) group.

Results are means ± sem of n=2-4 mice for each study group.
Previous work by many groups has demonstrated that vasoconstrictive GqPCR agonists trigger the post-transcriptional activation of multiple metalloproteinases, including TACE, MMP-2 and ADAM-12. The activated metalloproteinases next transactivate growth factor receptor and downstream MAPKs including ERK-1/2. Findings from this research indicate that metalloproteinases may establish novel transcriptional relationships among each other. Agonist-induced transcriptional pathways mediated by TACE may link sustained GqPCR stimulation to gene expression of other metalloproteinases including MMP-2 and ADAM-12 and developmental genes and extracellular matrix proteins. Cardiovascular hypertrophy and fibrosis processes may be signaled by a metalloproteinase network involving short-term (post-transcriptional) and long-term (transcriptional) mechanisms. The current model could explain, at least in part, the apparent functional redundancy and the concurrence of multiple metalloproteinases from the ADAM and MMP families in signaling of cardiovascular hypertrophy and fibrosis processes.
References


