Aldosterone Induces Tissue Inhibitor of Metalloproteinases-1 Expression and Further Contributes to Collagen Accumulation
From Clinical to Bench Studies

Chi-Sheng Hung, Chia-Hung Chou, Che-Wei Liao, Yen-Tin Lin, Xue-Ming Wu, Yi-Yao Chang, Ying-Hsien Chen, Vin-Cent Wu, Ming-Jai Su, Yi-Lwun Ho, Ming-Fong Chen, Kwan-Dun Wu, Yen-Hung Lin, the TAIPAI Study Group*

Abstract—Aldosterone induces myocardial fibrosis. Tissue inhibitor of metalloproteinases-1 (TIMP-1) is a key factor of myocardial fibrosis. This study tested the hypothesis that aldosterone induces TIMP-1 expression and contributes to the fibrotic process. We prospectively enrolled 54 patients with primary aldosteronism, and measured plasma TIMP-1 and echocardiographic parameters. In the cell study, we investigated the possible molecular mechanisms by which aldosterone induces TIMP-1 secretion and the effects on collagen accumulation. In the animal study, we measured serum TIMP-1 levels, cardiac TIMP-1 levels, and cardiac structure in an aldosterone infusion mouse model using implantation of aldosterone pellets. In patients with primary aldosteronism, plasma TIMP-1 was correlated with 24-hour urinary aldosterone, left ventricular mass, and impairment of left ventricular diastolic function. In human cardiac fibroblasts, TIMP-1 protein and mRNA expressions were significantly increased by aldosterone through the glucocorticoid receptor/PI3K/Akt/nuclear factor-κB pathway. TIMP-1 small-interfering RNA significantly reduced aldosterone-induced collagen accumulation, and aldosterone did not alter the levels of collagen1a1 or matrix metalloproteinase-1 mRNA. The aldosterone-induced TIMP-1 expression was inversely related to matrix metalloproteinase-1 activity. Furthermore, in the animal model, the serum and cardiac levels of TIMP-1 were significantly elevated in the mice that received aldosterone infusion. This elevation was blocked by RU-486 but not by eplerenone, suggesting that the effect was through glucocorticoid receptors. In a long-term aldosterone infusion model, serum TIMP-1 was associated with serum aldosterone level, cardiac structure, and fibrosis. In conclusion, aldosterone induced TIMP-1 expression in vivo and in vitro. This increased TIMP-1 expression resulted in enhanced collagen accumulation via the suppression of matrix metalloproteinase-1 activity.

Key Words: aldosterone ■ collagen ■ fibroblasts ■ glucocorticoid receptor ■ tissue inhibitor of metalloproteinases-1

Aldosterone has been shown to increase cardiac fibrosis in animal and clinical studies in the past 3 decades, and aldosterone treatment has been shown to increase collagen synthesis in rat cardiac fibroblasts and myocardium.1,2 In our previous clinical studies, patients with primary aldosteronism (PA), a disease characterized by excessive aldosterone secretion, exhibited increased myocardial fibrosis in serum fibrosis markers,3 myocardial texture,3,4 and gadolinium-enhanced cardiac magnetic resonance imaging.5 Furthermore, in patients with heart failure and diastolic dysfunction, treatment with mineralocorticoid receptor (MR) blockade has been shown to result in improvements in collagen turnover markers5 and diastolic function.6 These data suggest that aldosterone plays an important role in the fibrotic process in myocardium, and may lead to diastolic dysfunction. The underlying mechanisms of aldosterone-induced myocardial fibrosis, although not totally clear, include reactive oxygen species activation,4,6 inhibition of B-type natriuretic peptide (BNP) and bone morphogenetic protein-4,11 and apoptosis signal-regulating kinase 1.12

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From the Telehealth Center, National Taiwan University Hospital, Taipei, Taiwan (C.-S.H., Y.-H.C., Y.-L.H.); Departments of Internal Medicine (C.-S.H., V.-C.W., Y.-L.H., M.-F.C., K.-D.W., Y.-H.L.) and Obstetrics and Gynecology (C.-H.C.), National Taiwan University Hospital, National Taiwan University College of Medicine, Taipei, Taiwan; Department of Internal Medicine, National Taiwan University Hospital Hsin-Chu Branch, Hsin-Chu, Taiwan (C.-W.L., Y.-T.L.); Department of Internal Medicine, Taoyuan General Hospital, Taoyuan, Taiwan (X.-M.W.); Division of Cardiovascular Medical Center, Far Eastern Memorial Hospital, New Taipei City, Taiwan (Y.-Y.C.); and Institute of Pharmacology, National Taiwan University Medical College, Taipei, Taiwan (M.-J.S.).
*A list of all TAIPAI Study Group is given in the Appendix.
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Correspondence to Yen-Hung Lin, Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Rd, Taipei 100, Taiwan. E-mail austinr34@gmail.com
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Tissue inhibitor of metalloproteinases (TIMPs) are a family of intrinsic inhibitors to matrix metalloproteinases (MMPs), and are thus important regulators of the extracellular matrix. Among the TIMP family, TIMP-1 has been associated with fibrosis in organs, including the liver,13,14 and the heart.15 An in vivo study showed that the overexpression of TIMP-1 in mice cardiac fibroblasts increased the proliferation of fibroblasts and myofibroblasts, which could then be suppressed by anti-TIMP-1 antibodies.16 Moreover, increasing evidence suggests that TIMP-1 may have an effect on fibrosis in addition to inhibiting MMPs.17,18 These data suggest that TIMP-1 may be a potential target to control myocardial fibrosis.

In our recent study, we found that plasma TIMP-1 level was associated with diastolic dysfunction among patients with PA.19 The direct regulation of TIMP-1 in the heart by aldosterone, however, has never been demonstrated in the literature. We hypothesized that aldosterone can induce TIMP-1 expression in cardiac fibroblasts and contribute to cardiac fibrosis. The aims of this study, therefore, were 4-fold: (1) to demonstrate that aldosterone can induce TIMP-1 expression and then enhance collagen synthesis in human cardiac fibroblasts, (2) to explore the intracellular pathway of aldosterone-induced TIMP-1 expression in human cardiac fibroblasts, (3) to investigate whether aldosterone can increase TIMP-1 secretion in vivo, and (4) to investigate clinical evidence of the associations among aldosterone, TIMP-1 and cardiac structure and function.

**Methods**

**Participants**

We prospectively enrolled 54 patients with PA between October 2006 and March 2010, all of whom were registered in the Taiwan Primary Aldosteronism Investigation (TAIPAI) database. Twenty of these 54 patients were included in 2 of our previous studies.20 All of the patients underwent thorough clinical evaluations, blood biochemical examinations, measurement of plasma TIMP-1 concentration, and echocardiography at enrollment.

The following criteria were used to diagnose PA: (1) aldosterone/renin ratio >35 or urine aldosterone amount ≥12 μg/24 hours and a TAIPAI score of >60%, and postsaline loading plasma aldosterone concentration >10 ng/dl, (the TAIPAI score was developed from our database using age, sex, plasma renin activity, body mass index, serum potassium, and estimated glomerular filtration rate to identify the patients with an area under a receiver operating characteristic curve of 0.904),11 and (2) lateralization of aldosterone secretion at adrenal venous sampling or during dexamethasone suppression NP-59 single-photon emission computerized tomography/computerized tomography,22 or (3) adenoma on a computerized tomographic scan.

**Echocardiography**

Transthoracic echocardiography was performed using a Hewlett-Packard 5500 ultrasound system with an S3 transducer (1.0–3.0 MHz). Chamber dimension, wall thickness, left ventricular (LV) ejection fraction (E-mode), and peak mitral inflow velocity (E) were measured according to the guidelines of the American Society of Echocardiography. The peak mitral annular velocities (E′) were obtained by tissue Doppler imaging at the medial margins of the mitral annulus. The LV diastolic function was assessed using the E/E′ ratio,23 and the LV mass was measured by echocardiography according to the formula of Devereux and Reichek.24

The study complied with the Declaration of Helsinki and was approved by the Institutional Review Board of National Taiwan University Hospital (Taipei, Taiwan). Informed consent was obtained from all patients before inclusion in the study.

**Preparation of Human Cardiac Fibroblasts**

Fibroblasts from human fetal hearts at passage 1 were purchased from Cell Applications (San Diego, CA). Cells were maintained in fibroblast medium and detached from the culture dish before confluence before passage 6 and used for the cell experiments. The myofibroblast content was <10% to 20% before passage 7 as previously reported.25

**Animal Model**

Eight-week-old C57Bl/6 male mice were purchased from the Animal Center of the Medical College of National Taiwan University and kept in standard animal housing conditions. The protocol of this experiment was approved by the Animal Care and Use Committee of the Medical College of National Taiwan University. Mice with a similar body weight (25–30 g) were randomly divided into 2 groups (vehicle and aldosterone, n=5 per group) or 4 treatment groups (vehicle, aldosterone, aldosterone with eplerenone [50 mg/kg per day], and aldosterone with RU-486 [50 mg/kg per day]). In the aldosterone treatment group, a 21-day continuous aldosterone release pellet (Innovative Research of America, Sarasota, FL; 0.25 mg/pellet, 21-day release, 0.11 mg/d) was implanted subcutaneously. In the control group, a placebo pellet (vehicle only) was implanted. For the continuous aldosterone infusion model over 4 months, the pellets were changed every 20 days.

**Masson Trichrome Staining and Analysis**

Masson trichrome staining was used to detect cardiac fibrosis according to the manufacturer’s instructions (Sigma-Aldrich). Sections were analyzed microscopically. To evaluate the fibrosis index of the heart tissues, 6 random heart fields per tissue section were captured at x400 magnification.

**Measurement of TIMP-1 and Aldosterone Concentration**

The concentration of human TIMP-1 in the supernatant of the cell culture was measured using a commercially available enzyme immunoassay kit (MTM100, R&D Systems). Mouse serum levels of TIMP-1 and levels of cardiac TIMP-1 were measured using a commercially available enzyme immunoassay kit (MTM100, R&D Systems). Mouse serum levels of aldosterone were measured using a commercially available enzyme immunoassay kit (KGE016, R&D Systems). Each measurement was performed in triplicate, and the average concentration was then recorded as ng/mL.

**RNA Interference**

Small-interfering RNA duplexes (siRNA) of the MR gene (sc-38836), glucocorticoid receptor (GR) gene (sc-35505), and TIMP-1 gene (sc-29505) were purchased from Santa Cruz Biotechnology (Paso Robles, CA). Negative control siRNAs (Invitrogen Corporation, Carlsbad, CA) with sequences with no similarity to any gene product were used as controls. The transfection of siRNA is described in detail in the online-only Data Supplement.

**Measurement of mRNA Expression by Real-Time Quantitative Reverse Transcription Polymerase Chain Reaction**

Total RNA was isolated from human cardiac fibroblasts or ventricle tissue using RNAzol B reagent (Biotecn Laboratories, Houston, TX). cDNA was then prepared from 2 μg of the total RNA using random hexamer primers (ImProm-II Reverse Transcription System; Promega, Southampton, United Kingdom). The expressions of TIMP-1, MMP-1, and glyceraldehyde-3-phosphate dehydrogenase mRNA were measured using a fluorescence quantitative real-time polymerase chain reaction detection system (Light Cycler DNA Master SYBR Green I; Roche Molecular Biochemicals, Indianapolis, IN). The primer pairs are described in the Data Supplement. The amount of mRNA was normalized by that of glyceraldehyde-3-phosphate dehydrogenase mRNA (ΔCt). A ΔΔCt method was then used to calculate relative gene expressions using the mean of the vehicle or day 0 as the calibrator (assigned a value of 1 or 100) to which all other samples were analyzed.
Western Blot
To confirm protein expressions, the cells were lysed using 50 mmol/L Tris, 150 mmol/L NaCl, 1% NP40, and 0.1% SDS. To measure collagen, the cell culture supernatant was concentrated 20-fold using Centricon Plus-70 (EMD Millipore). Equal concentrations of cell lysate (20 μg) were electrophoresed on 8% to 12% SDS-polyacrylamide gels, electroblotted to polyvinylidene fluoride membranes, and then probed with specific antibodies followed by a peroxidase-conjugated secondary antibody. The immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Boehringer Mannheim). Antibodies against p-Akt, Akt, nuclear factor (NF)-κB (p65), albumin, and type I collagen were purchased from Santa Cruz Biotechnology.

Chemical Inhibitors and Aldosterone
Aldosterone, eplerenone, or RU486 (dissolved in dimethyl sulfoxide). LY294002, PD98059, and SB203580 were purchased from Sigma (St. Louis, MO).

NF-κB or Activator Protein 1 Decoy Oligodeoxynucleotide
Synthetic double-stranded decoy oligodeoxynucleotide six elements were used to block the binding of NFs to the promoter of the targeted genes. The sequences of phosphorothioate oligodeoxynucleotides are described in the Data Supplement. The oligodeoxynucleotides were mixed with TransFast for 15 minutes, and then incubated with the cells in a serum-free medium.

NF-κB–Binding Site–Driven Luciferase Reporter Assay
Human cardiac fibroblasts were transfected with NF-κB–binding site–driven luciferase plasmids (BD Bioscience, Palo Alto, CA) in 6-well plates using TransFast transfection reagent (Promega). The cells were used in the indicated conditions 24 hours after transfection. In addition, the cells were cotransfected with pSVβ-galactosidase to calculate the transfection efficiency, and the data were normalized. All transient transfections were conducted using triplicate cultures.

Determination of Total MMP-1, proMMP-1, and MMP-1 Activity
Human total activity of MMP-1 and proMMP-1 in the supernatant of the cell culture was measured using commercially available enzyme immunoassay kits (DY901 and DMP100, respectively; R&D Systems). The activity of MMP-1 was measured using a Fluorokine E Enzyme Activity Assay Kit (F1M00; R&D Systems). Human total activity of MMP-1 and proMMP-1 in the supernatant were significantly increased in the 10⁻⁷ M and 10⁻⁶ M aldosterone-treated groups compared with the vehicle group (Figure 2A). To determine the time course of aldosterone-induced TIMP-1 expression, we measured the mRNA level of human cardiac fibroblasts by quantitative reverse transcription polymerase chain reaction at 0, 4, 8, 16, and 24 hours after 10⁻⁶ M aldosterone treatment. The results showed that the TIMP-1 expression significantly increased shown in Table. The plasma TIMP-1 concentration was positively correlated with the amount of 24-hour urinary aldosterone (Pearson correlation coefficient r=0.468, P=0.001), interventricular septum thickness (r=0.317, P=0.020), LV mass (r=0.271, P=0.047), and E/E′ ratio (r=0.321, P=0.018; Figure 1A–1D). There was no significant correlation between TIMP-1 and blood pressure (r=0.079, P=0.572). The correlations between plasma TIMP-1 and urinary aldosterone and E/E′ ratio remained significant after adjusting for age, sex, and mean blood pressure. The correlations between plasma TIMP-1 and interventricular septum thickness remained significant after adjusting for age and sex (Table S1).

Aldosterone-Induced TIMP-1 Expression
To test whether aldosterone induces cardiac fibroblasts to express TIMP-1, we treated human cardiac fibroblasts with 10⁻¹⁰ to 10⁻⁶ M aldosterone for 24 hours. The results revealed that TIMP-1 protein levels in the cell culture supernatant were significantly increased in the 10⁻⁷ M and 10⁻⁶ M aldosterone-treated groups compared with the vehicle group (Figure 2A). To determine the time course of aldosterone-induced TIMP-1 expression, we measured the mRNA level of human cardiac fibroblasts by quantitative reverse transcription polymerase chain reaction at 0, 4, 8, 16, and 24 hours after 10⁻⁶ M aldosterone treatment. The results showed that the TIMP-1 expression significantly increased shown in Table. The plasma TIMP-1 concentration was positively correlated with the amount of 24-hour urinary aldosterone (Pearson correlation coefficient r=0.468, P=0.001), interventricular septum thickness (r=0.317, P=0.020), LV mass (r=0.271, P=0.047), and E/E′ ratio (r=0.321, P=0.018; Figure 1A–1D). There was no significant correlation between TIMP-1 and blood pressure (r=0.079, P=0.572). The correlations between plasma TIMP-1 and urinary aldosterone and E/E′ ratio remained significant after adjusting for age, sex, and mean blood pressure. The correlations between plasma TIMP-1 and interventricular septum thickness remained significant after adjusting for age and sex (Table S1).

Results
TIMP-1 Was Associated With Aldosterone Level, LV Mass, and Diastolic Dysfunction
The mean age of the 54 patients was 46.6±11.3 years, and 23 patients (42.6%) were male. Half of the patients had adenomatous PA. The baseline characteristics of these patients are shown in Table. The plasma TIMP-1 concentration was positively correlated with the amount of 24-hour urinary aldosterone (Pearson correlation coefficient r=0.468, P=0.001), interventricular septum thickness (r=0.317, P=0.020), LV mass (r=0.271, P=0.047), and E/E′ ratio (r=0.321, P=0.018; Figure 1A–1D). There was no significant correlation between TIMP-1 and blood pressure (r=0.079, P=0.572). The correlations between plasma TIMP-1 and urinary aldosterone and E/E′ ratio remained significant after adjusting for age, sex, and mean blood pressure. The correlations between plasma TIMP-1 and interventricular septum thickness remained significant after adjusting for age and sex (Table S1).

Table. Baseline Characteristics and Echocardiographic Parameters for Patients With Primary Aldosteronism

<table>
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<tr>
<th>Characteristics</th>
<th>Patients With Primary Aldosteronism</th>
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<tr>
<td>Number</td>
<td>54</td>
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<tr>
<td>Age, y, mean (SD)</td>
<td>46.6 (11.3)</td>
</tr>
<tr>
<td>Sex, male, n (%)</td>
<td>23 (42.6%)</td>
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<tr>
<td>Body weight, kg, mean (SD)</td>
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<td>Body height, cm, mean (SD)</td>
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<tr>
<td>Body mass index, kg/m², mean (SD)</td>
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<td>Systolic blood pressure, mmHg, mean (SD)</td>
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<td>Diastolic blood pressure, mmHg, mean (SD)</td>
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<td>Creatinine, mg/dL, mean (SD)</td>
<td>0.95 (0.23)</td>
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<td>Potassium, mEq/L, mean (SD)</td>
<td>3.7 (0.8)</td>
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<td>Plasma aldosterone concentration, ng/dL, mean (SD)</td>
<td>42.7 (32.9)</td>
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<tr>
<td>Plasma renin activity, ng/mL per h, mean (SD)</td>
<td>4.0 (6.3)</td>
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<tr>
<td>Aldosterone/renin ratio, ng/dL per ng/mL per h, mean (SD)</td>
<td>1070 (2476)</td>
</tr>
<tr>
<td>Plasma TIMP-1 level, ng/mL, mean (SD)</td>
<td>76 (37.5)</td>
</tr>
<tr>
<td>Interventricular septum, mm, mean (SD)</td>
<td>11.7 (2.5)</td>
</tr>
<tr>
<td>Left ventricular ejection fraction, %</td>
<td>72.1 (7.3)</td>
</tr>
<tr>
<td>E/E′ ratio</td>
<td>10.2 (2.8)</td>
</tr>
<tr>
<td>Left ventricular mass, g, mean (SD)</td>
<td>219.3 (83.5)</td>
</tr>
</tbody>
</table>

TIMP indicates tissue inhibitor of metalloproteinase.
Hypertension
June 2016

At 8 hours after aldosterone treatment, and then decreased gradually (Figure 2B). These results suggest that aldosterone induces the TIMP-1 expression in cardiac fibroblasts via transcriptional regulation.

Aldosterone Induced TIMP-1 Expression via MR or GR

To test whether aldosterone induces TIMP-1 expression via MR or GR, we used the siRNA of these 2 receptors to block the downstream pathway. Human cardiac fibroblasts were pretreated with siRNA for 24 hours before $10^{-6}$ M aldosterone treatment, and the TIMP-1 mRNA expression was measured 8 hours after aldosterone treatment. The results showed that the aldosterone-induced TIMP-1 mRNA expression was blocked by siRNA of GR, but not by siRNA of MR (Figure 2C). A chemical inhibitor of GR, RU-486, was then tested, and the results showed that the TIMP-1 protein level was suppressed by RU-486 pretreatment (Figure 2D). These results suggest that aldosterone increases the expression of TIMP-1 via GR but not MR.

Aldosterone-Induced TIMP-1 Expression via the PI3K Pathway

We used chemical inhibitors for PI3K/Akt, MAPK/ERK (mitogen-activated protein kinases/extracellular signal-regulated kinase), and MAPK/p38 (LY294002, PD 098059, and SB 203580, respectively) to identify the intracellular pathway of aldosterone-induced TIMP-1 expression. Human cardiac fibroblasts were pretreated with chemical inhibitors 1 hour before $10^{-6}$ M aldosterone treatment (for 24 hours). The protein level of TIMP-1 was then measured 24 hours after aldosterone treatment, and the results showed that LY294002 (the PI3K/Akt inhibitor) suppressed the aldosterone-induced expression of TIMP-1 (Figure 2E).

We then measured the phosphorylated form of Akt after aldosterone treatment. Human cardiac fibroblasts were pretreated with chemical inhibitors for 1 hour before $10^{-6}$ M aldosterone treatment, and the phosphorylated protein levels of Akt were then determined. The results showed that aldosterone treatment increased the level of phosphorylated Akt. However, this increase in phosphorylated Akt was suppressed by pretreatment with RU-486 or LY294002 (Figure 2F). These results suggest that aldosterone induces Akt phosphorylation via GR.

Aldosterone Induced TIMP-1 Expression via NF-κB

NF-κB and activator protein 1 (AP-1) are critical transcriptional factors for TIMP-1 expression. To determine whether aldosterone induces TIMP-1 expression via the effect of NF-κB or AP-1, we used NF-κB and AP-1 decoy oligodeoxynucleotides to block the downstream response. Human cardiac fibroblasts were pretreated with decoy oligodeoxynucleotides before $10^{-6}$ M aldosterone treatment, and the results showed that the aldosterone-induced expressions of protein and TIMP-1 mRNA were both suppressed by NF-κB decoy oligodeoxynucleotide, but not by AP-1 decoy oligodeoxynucleotide (Figure 3A–3B). We further clarified the relationship between the GR/PI3K-Akt signal transduction pathway and NF-κB activity. NF-κB activation includes nuclear translocation and NF-κB-binding site–driven activity. The results showed that the aldosterone-enhanced NF-κB nuclear translocation (nuclear p65) was significantly inhibited by RU486 and LY294002 (Figure 3C). However, the DNA-binding activity of NF-κB that was increased by aldosterone was significantly inhibited by RU486 and LY294002 (Figure 3D). These results suggest that aldosterone regulates TIMP-1 expression via NF-κB and AP-1.
the GR/PI3K-Akt/NF-κB signal transduction pathway in human cardiac fibroblasts.

Aldosterone-Induced TIMP-1 May Enhance Collagen Accumulation via Inhibiting MMP-1 Activity

To investigate whether TIMP-1 plays an important role in aldosterone-induced collagen production, we treated human cardiac fibroblasts with TIMP-1 siRNA or control siRNA before aldosterone treatment. The cell culture supernatant was then collected and concentrated to measure the level of type I collagen (Figure 4B). It has been reported that an altered proteolytic balance between MMP-1 and TIMP-1 contributes to the accumulation of extracellular matrix.27 We therefore investigated whether aldosterone influences MMP levels, and the results showed that aldosterone did not alter the total levels of MMP-1 or proMMP-1 (Figure 4C). We then further determined MMP-1 activity and TIMP-1 expression in human cardiac fibroblast cells (Figure 4D). The results showed that MMP-1 activity was substantially decreased after high-dose aldosterone treatment, and that the level of TIMP-1 was significantly increased after high-dose aldosterone treatment. To test whether aldosterone treatment influences the expression of MMP-1 or collagen1α1 mRNA, human cardiac fibroblast cells were used to determine the...
expressions of MMP-1 and collagen1a1 mRNA after aldosterone treatment (Figure 4E). The results showed that aldosterone treatment did not change the expression of MMP-1 or collagen1a1 mRNA.

**In-Vivo Mouse Model of Serum and Cardiac TIMP-1 Expression**

To investigate whether aldosterone induces the expression of serum and cardiac TIMP-1 in vivo, we used aldosterone-release pellets implanted into mice for serial studies. Compared with the vehicle control, the levels of serum aldosterone, serum TIMP-1, and cardiac TIMP-1 were significantly higher at days 7, 14, and 21 in the aldosterone-infused mice after implantation (Figure 5A–5C). Furthermore, the effect of increasing levels of serum TIMP-1, cardiac TIMP-1 mRNA, and cardiac TIMP-1 protein after aldosterone infusion was blocked by RU-486, but not by eplerenone (Figure 5D–5F). These findings suggest that the effect was through GR.

To investigate the long-term effect of aldosterone infusion on TIMP-1 expression and cardiac structure, we used continuous aldosterone infusion for 4 months by changing the pellets every 20 days. Compared with the vehicle controls, the mice receiving aldosterone infusion had higher levels of serum aldosterone and TIMP-1 (Figure 6B and 6C). In cardiac structure analysis, the mice receiving aldosterone had a higher heart weight/body weight ratio, thicker ventricular wall, and increased interstitial fibrosis (Figure 6A and 6D–6F). The serum TIMP-1 levels were significantly correlated to serum aldosterone, heart weight/body weight ratio, thicker ventricular wall, and increased interstitial fibrosis (Table S2).

**Summary of the Intracellular Pathway of Aldosterone-Induced TIMP-1 Expression on Collagen Content**

Our results showed that aldosterone induced the expression of TIMP-1 via a genomic pathway by binding to GRs in cardiac fibroblasts. The subsequent activation of PI3K/Akt and NF-xB resulted in increased expressions of TIMP-1 mRNA and protein. TIMP-1 may therefore increase collagen content via suppressing MMP-1 activity (Figure 7).
Discussion

In this study, we demonstrated that (1) in vivo, plasma levels of TIMP-1 were correlated with aldosterone, LV mass, and diastolic function in patients with PA and (2) in vitro, aldosterone-induced TIMP-1 expression in human cardiac fibroblasts via a GR/PI3K/NF-κB pathway (Figure 6). Aldosterone-induced TIMP-1 secretion was also further confirmed in our mouse model. Furthermore, TIMP-1 increased the content of type I collagen in cardiac fibroblasts. Taken together, these results suggest that TIMP-1 may be a downstream effector of aldosterone, and that TIMP-1 may play a role in aldosterone-induced cardiac fibrosis.

TIMP-1 has been shown to be induced by inflammatory mediators and transforming growth factor-β in cardiac fibroblasts. However, to the best of our knowledge, there have been no previous reports on the direct effect of aldosterone on TIMP-1, and that this study is the first to show that TIMP-1 can be regulated by aldosterone to enhance the collagen content in cardiac fibroblasts. In our recent clinical study, we showed that among patients with PA, TIMP-1 level was associated with diastolic dysfunction and was reduced after adrenalectomy. Thus, the aldosterone-induced TIMP-1 expression may serve as a potential target to regulate myocardial fibrosis.

Our results also showed that aldosterone induced the TIMP-1 expression through GRs but not through MRs. Although aldosterone-induced myocardial fibrosis has been associated with MRs in most studies, our results demonstrated that aldosterone increased the expression of galactin-3 secretion via MRs in macrophage cell lines. In a recent study using double transgenic mice, aldosterone was found to enhance myocardial fibrosis through MR-dependent mechanisms.
namely activation of galactin-3–induced fibrosis and inhibition of BNP and bone morphogenetic protein-4.11 Aldosterone has also been shown in a few studies to act through GRs to mediate genomic or nongenomic effects.31–34 The aldosterone–GR pathway has also been observed in cardiomyocytes, and has been related to hypertrophy.35 Moreover, in a study using cardiac fibroblast-specific MR knockout mice, cardiac fibrosis was not reduced in pressure-overload conditions.36 In clinical studies, patients with PA who received adrenalectomy have been reported to have a greater decrease in LV mass than in patients who received spironolactone treatment alone.37–39 This also implies that MRs may not be the only pathway of aldosterone-induced myocardial hypertrophy. Taken together with our findings, a GR-mediated profibrotic response in cardiac fibroblasts may be an important pathway for cardiac fibrosis (Figure 7).

Our results also showed that aldosterone did not directly induce the expression of collagen mRNA in cardiac fibroblasts, which is consistent with some but not all previous studies.40,41 This discrepancy may be because of the cell type, phenotype, and duration of aldosterone treatment. The advantage of our study is the use of human cardiac fibroblasts, which may be more closely related to a human condition than the rat cardiac fibroblasts used in the previous studies. It has also been shown that aldosterone exerts a profibrotic effect on cardiac fibroblasts mainly through 2 pathways: (1) macrophage activation, which in turn induces collagen synthesis in cardiac fibroblasts and (2) the inhibition of antifibrotic factors. Azibani et al41

Figure 5. Mouse model of implanted aldosterone (Aldo) release pellets (21 days). A–C, Mice with a similar body weight (25–30 g) were randomly divided into 2 treatment groups (vehicle or aldosterone; n=5 per group). The mice were implanted subcutaneously with a 21-day continuous aldosterone release pellet (Innovative Research of America). The vehicle control mice received a placebo pellet. Whole blood of the mice was collected on days 0, 7, 14, and 21. The sera were used to determine levels of (A) aldosterone and (B) tissue inhibitor of metalloproteinases-1 (TIMP-1). ANOVA P<0.001 for both aldosterone and TIMP-1. P<0.05 compared with the vehicle group at day 0. C, Cardiac tissue level of TIMP-1 mRNA were determined by qRT-PCR. ANOVA P=0.001. *P<0.05 compared with the aldosterone group at day 0. #P<0.05 compared with the vehicle group at days 0, 7, 14, and 21. D–F, Mice with a similar body weight (25–30 g) were randomly divided into 4 treatment groups (vehicle, aldosterone, with an MR inhibitor [eplerenone, 50 mg/kg per day], and GR inhibitor [RU-486, 50 mg/kg per day], n=5 per group). The mice were implanted subcutaneously with a 21-day continuous aldosterone release pellet. The vehicle control mice received a placebo pellet. Whole blood of the mice was collected on day 14. D, Serum TIMP-1 was determined. ANOVA P<0.001. *P<0.05 compared with the vehicle group; #P<0.05 compared with the aldosterone group. E, Cardiac TIMP-1 protein was determined by EIA. ANOVA P<0.001. *P<0.05 compared with the vehicle group; #P<0.05 compared with the aldosterone group. F, Cardiac TIMP-1 mRNA was determined by qRT-PCR; ANOVA P<0.05. *P<0.05 compared with the vehicle group; #P<0.05 compared with the aldosterone group. GRI indicates glucocorticoid receptor inhibitor; and MRI, mineralocorticoid receptor inhibitor.
suggested that aldosterone increases the expression of TIMP-1 via the inhibition of BNP and bone morphogenetic protein-4. However, in a recent clinical observational study, the BNP level among patients with PA was elevated compared with patients with essential hypertension or normotensive patients, and this elevated BNP level decreased after drug or surgical treatment. Therefore, the inhibitory effect of aldosterone on BNP observed in this in vitro study may not be the major pathway in vivo. Our results clearly show that aldosterone has a direct effect on cardiac fibroblasts to increase the mRNA and protein synthesis of TIMP-1, which is more compatible with the human data.

The effect of TIMP-1 on fibrosis has conventionally been thought to occur through MMP. However, recent evidence suggests that other MMP-independent pathways exist. TIMP-1 may act as a signaling molecule to influence cell growth, cell differentiation, or miRNA-mediated post-transcriptional regulation. In a study with adenovirus-mediated TIMP-1 overexpression in cardiac fibroblasts, the overexpression of TIMP-1 was associated with increased cell proliferation but not with changes in collagen content. Our results showed that the concentration of TIMP-1 increased with an increasing concentration of aldosterone, and that this increase in TIMP-1 concentration was associated with a decrease in MMP-1 activity. However, there were no differences in MMP-1 expression and collagen mRNA expression after aldosterone treatment. This suggests that the aldosterone-induced TIMP-1 expression acts mainly through MMP-1 activity inhibition to increase net collagen content.

Myocardial fibrosis has been shown to contribute to the development of diastolic dysfunction. In a study using aldosterone infusion in mice, the group receiving aldosterone infusion was associated with a higher degree of cardiac fibrosis and diastolic dysfunction as assessed using the E/E' ratio. MR blockers have been used to treat patients with heart failure with diastolic dysfunction, and this treatment reduced diastolic dysfunction and improved exercise capacity. However, in a large clinical trial, the outcomes were not significantly improved after MR blockade treatment. Our results showed that aldosterone not only acts via MRs but also acts via GRs in cardiac fibroblasts. Whether this alternative pathway leads to the escape phenomenon in a large clinical trial warrants further study. Our findings also suggest another possible target of treatment for aldosterone-induced cardiac fibrosis.

This study has several limitations. First, we did not investigate NF-κB binding to the promoter of TIMP-1, and further studies are needed to clarify whether NF-κB directly leads to the induction of TIMP-1 or indirectly through other mediators. Second, although we demonstrated that the aldosterone-induced TIMP-1 expression in cardiac fibroblasts occurred mainly through GRs, we did not determine whether the

Figure 6. Mouse model of implanted aldosterone release pellets (4 months). A, C57BL/6 male mice were implanted subcutaneously with a 21-day continuous aldosterone release pellet or vehicle pellet (Innovative Research of America; 0.25 mg/pellet, 21-day release, 0.11 mg/d) which were reimplanted subcutaneously every 20 days for 4 months; n=5 per group. After euthanization, the hearts were weighed and the body weight (BW) ratio was calculated. B, Serum levels of aldosterone and (C) serum levels of tissue inhibitor of metalloproteinases-1 (TIMP-1) were measured. D, Histological transverse sections of each mouse ventricle were used to measure the thickness of the lateral ventricular wall. *P<0.001. E, The cardiac tissue sections were stained with Masson trichrome to determine collagen fiber content. The blue represents fibrosis and the red represents normal myocardium. F, The percentage of interstitial fibrosis of the sections was analyzed microscopically. *P<0.001. HW indicates heart weight.
downstream pathway was genomic or nongenomic. Finally, as previously reported, ≤10% to 20% of fibroblasts may convert to myofibroblasts during the preparation of experiments.

**Perspectives**

Aldosterone induces myocardial fibrosis, and TIMP-1 is a key factor of myocardial fibrosis. Our data clearly show that aldosterone induces TIMP-1 expression in vivo and in vitro. Aldosterone induces TIMP-1 expression via the GR, PI3K and NF-κB pathway in cardiac fibroblasts. This increased TIMP-1 expression results in enhanced collagen synthesis via the suppression of MMP-1 activity. Our findings suggest TIMP-1 as a possible target of treatment for aldosterone-induced cardiac fibrosis.

**Appendix: Membership of the Taiwan Primary Aldosteronism Investigation (TAIPAI) Study Group**

Che-Hsiung Wu, MD (Chi-Taz Hospital, PI of Committee); Vin-Cent Wu, MD (NTUH, PI of Committee); Yen-Hung Lin, MD (NTUH, PI of Committee); Yi-Luwn Ho, MD, PhD (NTUH, PI of Committee); Hung-Wei Chang, MD, PhD (Far Eastern Hospital, PI of Committee); Lian-Yu Lin, MD, PhD (NTUH, PI of Committee); Fu-Chang Hu, MS, ScD, (Harvard Statistics, Site Investigator); Kao-Lang Liu, MD (NTUH, PI of Committee); Shuo-Meng Wang, MD (NTUH, PI of Committee); Kuo-How Huang, MD (NTUH, PI of Committee); Yung-Ming Chen, MD (NTUH, PI of Committee); Chin-Cheng Liao, MD (NTUH, PI of Committee); Ruoh-Fang Yen, MD, PhD (NTUH, PI of Committee); and Kwan-Dun Wu, MD, PhD (NTUH, Director of Coordinating Center).

**Acknowledgments**

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**Disclosures**

None.

**References**


**Figure 7.** Summary of the intracellular pathway of aldosterone-induced tissue inhibitor of metalloproteinases-1 (TIMP-1) expression on collagen content. Aldosterone (Aldo) induced the expression of TIMP-1 via a genomic pathway by binding to glucocorticoid receptors (GRs) and the subsequent activation of PI3K/Akt and nuclear factor (NF)-κB in cardiac fibroblasts. TIMP-1 may increase collagen content via a paracrine or autocrine effect.


Novelty and Significance

What Is New?
- In patients with primary aldosteronism, plasma tissue inhibitor of metalloproteinases-1 (TIMP-1) was correlated with 24-hour urinary aldosterone, left ventricular mass, and impairment of left ventricular diastolic function.
- Aldosterone induces TIMP-1 expression in vivo and in vitro.
- Aldosterone induces TIMP-1 expression via the glucocorticoid receptor, PI3K and nuclear factor-κB pathway in cardiac fibroblasts.
- This increased TIMP-1 expression results in enhanced collagen synthesis via the suppression of matrix metalloproteinase-1 activity.

What Is Relevant?
- Primary aldosteronism is the most common cause of secondary hypertension, and patients with primary aldosteronism have increased myocardial fibrosis because of aldosterone excess.
- Our findings suggest TIMP-1 as a possible target of treatment for aldosterone-induced cardiac fibrosis.

Summary
Aldosterone-induced TIMP-1 expression. This increased TIMP-1 expression resulted in enhanced collagen accumulation via the suppression of matrix metalloproteinase-1 activity.
Aldosterone Induces Tissue Inhibitor of Metalloproteinases-1 Expression and Further Contributes to Collagen Accumulation: From Clinical to Bench Studies

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Aldosterone induces TIMP-1 expression and further contributes to collagen accumulation: from clinical to bench studies

Chi-Sheng Hung\textsuperscript{ab}, Chia-Hung Chou\textsuperscript{c}, Che-Wei Liao\textsuperscript{d}, Yen-Tin Lin\textsuperscript{d}, Xue-Ming Wu\textsuperscript{e}, Yi-Yao Chang\textsuperscript{f}, Ying-Hsien Chen\textsuperscript{a}, Vin-Cent Wu\textsuperscript{b}, Ming-Jai Sui\textsuperscript{g}, Yi-Lwun Ho\textsuperscript{ab}, Ming-Fong Chen\textsuperscript{b}, Kwan-Dun Wu\textsuperscript{b}, Yen-Hung Lin\textsuperscript{b}\textsuperscript{*}, the TAIPAI Study Group

\textsuperscript{a}Telehealth Center, National Taiwan University Hospital, Taipei, Taiwan; \textsuperscript{b}Department of Internal Medicine, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan; \textsuperscript{c}Department of Obstetrics and Gynecology, National Taiwan University Hospital and National Taiwan University College of Medicine, Taipei, Taiwan; \textsuperscript{d}Department of Internal Medicine, National Taiwan University Hospital Hsin-Chu Branch, Hsin-Chu, Taiwan; \textsuperscript{e}Department of Internal Medicine, Taoyuan General Hospital, Taoyuan, Taiwan; \textsuperscript{f}Division of Cardiovascular Medical Center, Far Eastern Memorial Hospital, New Taipei City, Taiwan; \textsuperscript{g}Institute of Pharmacology, National Taiwan University Medical College, Taipei, Taiwan.

Corresponding author

Yen-Hung Lin, MD, PhD.
Department of Internal Medicine, National Taiwan University Hospital, No. 7, Chung-Shan South Road, Taipei, 100, Taiwan
Tel: + 886-2-23123456 ext. 62152 Fax: + 886-2-23515811
E-mail: austinr34@gmail.com
Detailed Methods

RNA interference

Small interfering RNA duplexes (siRNA) of the MR gene (sc-38836), GR gene (sc-35505), and TIMP-1 gene (sc-29505) were purchased from Santa Cruz Biotechnology. Negative control siRNAs (Invitrogen Corporation, Carlsbad, CA, USA) with sequences with no similarity to any gene product were used as the controls. Lyophilized siRNA duplex was resuspended in RNase-free water at a concentration of 10 mM with 10 mM Tris–HCl, pH 8.0, 20 mM NaCl, and 1 mM EDTA buffered solution. The siRNAs (25 μM) were incubated for 15 minutes at room temperature to allow for complex formation between the siRNA and TransFast™ Transfection Reagent (Promega, Southampton, UK). Cells (1x10^5 cells in a 6-cm dish) were transfected with siRNAs in serum-free Opti-MEM (Invitrogen Corporation) at a concentration of 25 nM by incubation for 1 hour at 37°C. The culture medium of the cells was then changed, and the cells were incubated for 24 hours at 37°C prior to the experiments.

Measurement of mRNA expression by real-time quantitative reverse transcription polymerase chain reaction (PCR)

RNA extraction and reverse transcription

Total RNA was isolated from human cardiac fibroblasts or ventricle tissue using RNAzol B reagent (Biotecx Laboratories, Houston, Tex, USA). cDNA was then prepared from 2 μg of the total RNA using random hexamer primers (ImProm-II reverse transcription system; Promega, Southampton, UK). TIMP-1, MMP-1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA expressions were measured using a fluorescein quantitative real-time PCR detection system (Light Cycler DNA Master SYBR Green I; Roche Molecular Biochemicals, Indianapolis, IN, USA). The primer pairs were as follows: (human) TIMP-1, 5’-TTCGTGGGGGACACCAGAAGTCAAC-3’ and 5’-TGGACACTGTGCAGGCTTCAGTTC-3’. (human) MMP-1, 5’-CTGGCCACAAGCTGCAAGGAC-3’ and 5’-CTGCTGGGACACCAGAAGTCAAC-3’. (human) collagen 1a1, 5’-GGGAAGGTGAAGGTCGG-3’ and 5’-TGGACTCCAGATCTACG-3’. (mouse) TIMP-1, 5’-GGATTCCGGGAATGACATCTAT-3’ and 5’-CGCCTTCCCTGCAATTAGATA-3’ and GAPDH, 5’-GGGAAGGTGAAGGTCGG-3’ and 5’-TGGACTCCAGATCTACG-3’. The amplification program was one cycle of initial incubation at 61°C for 20 minutes, followed by 50 cycles of denaturation at 95°C for 10 seconds, annealing at 55–57°C for 10 seconds, and extension at 72°C for 10 seconds. The amount of mRNA was normalized to that
of GAPDH mRNA (ΔCt). A ΔΔCq method was then used to calculate relative gene expressions using the mean of the vehicle or day 0 as the calibrator (assigned a value of 1 or 100) to which all other samples were analyzed.

**NF-kB or AP-1 decoy ODN**

Synthetic double-stranded decoy oligodeoxynucleotide (ODN) cis elements were used to block the binding of nuclear factors to the promoter of the targeted genes. The sequences of the phosphorothioate ODNs were: NF-kB decoy ODN: 5’-CCTTGAAGGATTTCCCTCC-3’ and 3’-GGAACTCCCTAAAGGGAGG-5’; AP-1 decoy ODN: 5’-TGTCTGACTCATGTC-3’ and 3’-CAGACTGAGTACA-5’; scrambled decoy ODN: 5’-TTGCCGTACCTGACTTAGCC-3’ and 3’-AACGGCATGGACTGAATCGG-5’. The ODNs were mixed with TransFast for 15 minutes, and then incubated with the cells in a serum-free medium.
Supplemental Table

Table S1. The correlations between plasma TIMP-1 and urinary aldosterone concentration, interventricular septum thickness, LV mass, and E/E` ratio after the adjustment for age, sex and mean blood pressure among the 54 patients with primary aldosteronism.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Model 1*</th>
<th></th>
<th>Model 2†</th>
<th></th>
<th>Model 3‡</th>
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<tr>
<td></td>
<td>Partial correlation</td>
<td>p-value</td>
<td>Partial correlation</td>
<td>p-value</td>
<td>Partial correlation</td>
<td>p-value</td>
</tr>
<tr>
<td>Urinary aldosterone</td>
<td>0.481</td>
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<td>0.45</td>
<td>0.003</td>
<td>0.449</td>
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<tr>
<td>Interventricular septum</td>
<td>0.321</td>
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<td>0.296</td>
<td>0.033</td>
<td>0.274</td>
<td>0.051</td>
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<td>Left ventricular mass</td>
<td>0.292</td>
<td>0.034</td>
<td>0.263</td>
<td>0.059</td>
<td>0.239</td>
<td>0.091</td>
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<tr>
<td>E/E` ratio</td>
<td>0.342</td>
<td>0.014</td>
<td>0.334</td>
<td>0.018</td>
<td>0.304</td>
<td>0.034</td>
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</tbody>
</table>

*Model 1: adjusted for age
†Model 2: adjusted for age and sex
‡Model 3: adjusted for age, sex and mean blood pressure
Table S2. The Spearman’s correlations between serum TIMP-1 and serum aldosterone concentration, and heart weight to body weight ratio pressure among the mouse receiving aldosterone infusion for 4 months (n=10).

<table>
<thead>
<tr>
<th>Variables</th>
<th>Spearman’s correlation</th>
<th>p-value</th>
</tr>
</thead>
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<tr>
<td>Serum aldosterone</td>
<td>0.649</td>
<td>0.043</td>
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<tr>
<td>HW to BW ratio</td>
<td>0.879</td>
<td>&lt;0.001</td>
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<tr>
<td>Left ventricular wall thickness</td>
<td>0.734</td>
<td>0.016</td>
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<tr>
<td>Interstitial fibrosis</td>
<td>0.772</td>
<td>0.009</td>
</tr>
</tbody>
</table>

BW=body weight, HW=heart weight, TIMP-1=tissue inhibitor of metalloproteinases-1