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Telmisartan regresses left ventricular hypertrophy in caveolin-1 deficient mice

Marta H Kreiger[+], **Annarita Di Lorenzo**[+], **Christine Teutsch**, **Katalin Kauser**, and **William C. Sessa**[*]

Vascular Biology and Therapeutics Program and Department of Pharmacology, Yale University School of Medicine, New Haven, Connecticut, USA and Boehringer-Ingelheim, Ridgefield, CT USA

Abstract

The role of angiotensin II (Ang II) in promoting cardiac hypertrophy is well known, however the role of the Ang II in a spontaneous model of hypertrophy in mice lacking the protein caveolin-1 (Cav- KO) has not been explored. In this study, WT and Cav-1 KO mice were treated with angiotensin receptor blocker (ARB), telmisartan, and cardiac function assessed by echocardiography. Treatment of Cav-1 KO mice with telmisartan significantly improved cardiac function compared to age-matched, vehicle treated Cav-1 KO mice, while telmisartan did not affected cardiac function in WT mice. Both left ventricular (LV) weight to body weight ratios and LV to tibial length ratios were also reverted by telmisartan in Cav-1 KO but not WT mice. LV hypertrophy was associated with increased expression of natriuretic peptides-A and –B, β-myosin heavy chain and TGF-β and telmisartan treatment normalized the expression of these genes. Telmisartan reduced the expression of collagen genes (Col1A and Col3A) and associated perivascular fibrosis in intramyocardial vessels in Cav-1 KO mice. In conclusion, telmisartan treatment reduces indexes of cardiac hypertrophy in this unique genetic model of spontaneous LV hypertrophy.

Keywords

angiotensin; cardiac function; caveolin-1; hypertrophy

Introduction

Several lines of experimental and clinical studies have identified a key role of the renin-angiotensin-aldosterone system (RAAS) in the pathophysiology of a number of cardiovascular diseases, such as myocardial infarction, stroke and congestive heart failure. In addition to angiotensin-converting enzyme-inhibitors, in the past few years Ang II receptor blockers (ARB) that selectively antagonize the Ang II type 1 receptor (AT1R) such

[*]Address correspondence: William C. Sessa Ph.D., Vascular Biology & Therapeutics Program, Yale University School of Medicine, Amistad Research Building, 10 Amistad Street, New Haven, CT06519, USA. Fax: 203-737-2290. william.sessa@yale.edu.
[+]Authors contributed equally to these studies

as telmisartan, became a valid alternative approach to interfere with renin-angiotensin-aldosterone axis (1). Although ARBs selectively antagonize the actions of Ang II there is still debate over the role of additional mechanisms by which ARB improve cardiac function(2) such as activation of PPAR-gamma (3) or improving endothelial function (4).

Caveolae are 50-100 nm invaginations of the plasma membrane that are decorated by caveolins (Cav) as their signature coat proteins (5, 6). There are three Cav genes in mammals, Cav-1, Cav-2 and Cav-3 and genetic knockouts of Cav-1 and Cav-3 abolish the formation of caveolae in cells that express these isoforms, whereas the loss of Cav-2 does not qualitatively influence caveolae, but leads to pulmonary hyperplasia (7-11). In the heart, Cav-1 is highly expressed in the vascular endothelium and to a lesser extent in cardiac fibroblasts and smooth muscle, whereas Cav-3 is exclusively expressed in cardiac myocytes but not in vascular cells(12). Interestingly, deletion of either Cav-1 or Cav-3 results in cardiac hypertrophy, left ventricular dilation and dysfunction and combined deletion of both genes results in severe cardiomyopathy, hypertrophy, disorganization and degeneration of the cardiac myocytes and interstitial fibrosis (13, 14). In the hearts of Cav-1 KO mice, there is evidence for increased nitrosative stress and endothelial dysfunction due to enhanced endothelial nitric oxide synthase (eNOS) activation; these effects are suppressed by pharmacological blockade of eNOS or by the genetic reconstitution of Cav-1 back into the endothelium (12, 15). However, the role of Ang II in promoting the cardiac phenotype in Cav-1 KO mice has not been examined. Thus, the goal of the present study was to investigate the effect of the ARB, telmisartan, on functional and molecular markers of cardiac hypertrophy in Cav-1 KO mice.

Methods

Treatment of Cav-1 KO Animals

Age matched 8-week-old male wild-type (WT, C57Bl6) and congenic Cav-$1^{-/-}$ mice on a C57Bl6 background (16) were treated with telmisartan (Boehringer-Ingelheim Pharmaceutical Inc., CT, USA) (1mg kg^{-1} day^{-1} in drinking water) or a vehicle during 8 weeks. The mice were anesthetized with ketamine/xylazine (100 mg kg^{-1} i.m.) and PBS perfusion of the right ventricle (RV) under physiological pressure (~20 mmHg), and their hearts were dissected under ice and used for the experiments.

Echocardiography studies

The cardiac dimensions of conscious mice were monitored in using transthoracic echocardiography (Vevo 770 VisualSonics, Toronto, Canada), both before and after the 8 weeks of treatment as described (17). In brief, left ventricular (LV) M-mode (VisualSonics) was used and all measurements were made during 3 to 6 consecutive cardiac cycles, and the average values were used for analysis. LV end-diastolic (LVDd) and end-systolic (LVDs) dimensions, as well as the thickness of the interventricular septum (IVST) and posterior wall (PWT) were measured from the M-mode tracings, and fractional shortening (FS) was calculated as (LVDd-LVDs)/LVDd. Diastolic measurements were taken at the point of maximum cavity, and systolic measurements were made at the point of minimum dimension, using the leading-edge method.

Morphometric analysis

Mice were sacrificed and their tissues harvested. Tibias were dissected and measured. The heart was stopped at diastole (KCl, 14 mm) and dissected to obtain the right ventricle (RV) separated from left ventricle plus septum (LV+S) and then were weighed. For morphometric analysis, left ventricle samples obtained from the free wall, at the level of papillary muscle, were fixed in 4% buffered formalin and embedded in paraffin, cut in 4 μm sections and subsequently stained with haematoxylin and eosin. Two randomly selected sections from each animal were visualized by light microscopy using an objective with a calibrated magnification (400x). Myocytes with visible nucleus and intact cellular membranes from areas of transversely cut muscle fibers were chosen for diameter determination. The width of individually isolated cardiomyocyte displayed on a viewing screen was manually traced, across the middle of the nuclei, with a digitizing pad and determined by a computer assisted image analysis system. Quantification of left ventricular fibrosis involved Picrosirius red staining for detection of collagen in the heart. Sections measuring 6 μm were deparaffinized, rehydrated, and stained with Picrosirius red using a modification of the method(18). Images were obtained with a computerized digital camera, and collagen was examined under a microscope (Nikon E600) using polarized light. Cardiac myocyte width and ventricular fibrosis were also measured in the free left ventricle wall using a computer-assisted morphometric system (Leica Quantimet 500, Cambridge, UK). For each animal approximately 10 visual fields were analyzed in a blinded fashion.

Quantitative reverse transcription PCR analyses

Samples from left ventricle were homogenized with TRIzol reagent (Invitrogen). Total RNA was isolated with the RNAEasy kit (Qiagen) and transcribed into complementary DNA with the use of the TaqMan protocol (Applied Biosystem). Quantitative PCRs were carried out on an iCycler (Biorad) with specific primers against the genes of interest, SYBRgreen (Biorad) ready mix. The primers sequences used were presented on Supplementary List.

Enzyme Immunosorbent Assay

A commercially available EIA kit (Spi-Bio) was used to measure plasma levels of Ang II in WT and Cav-1 KO mice treated with vehicle or telmisartan for 8 weeks as per the manufacturer’s instructions.

Statistical Analysis

The results of the experiments are expressed as the means ± SEM. A two way analysis of variance was used to evaluate the data followed by a Tukey post-test for comparison of the groups using Prism 3.0 software. A confidence level $p < 0.05$ was considered to be statistically significant.

Results

Telmisartan reverts cardiac hypertrophy in Cav-1 KO mice

To examine the contribution of Ang II to cardiac hypertrophy in Cav-1 KO mice, age matched (8 week old), male wild-type (WT, C57Bl6) and congenic Cav-1 KO mice on a

C57Bl6 background were treated with telmisartan (Telm; 1mg/kg in drinking water) or vehicle for 8weeks duration. Cardiac dimensions were monitored in conscious mice using serial echocardiography (Vevo 770), before and after 8 weeks of treatment. As seen in Fig.1 A-D (black bars), compared to WT mice, Cav-1 KO mice had indices of increased concentric cardiac hypertrophy as determined by a reduction in left ventricular end diastolic diameter (LVDd) and systolic diameter (LVDs) and an increase in intraventricular septal wall thickness (IVSW) and posterior wall thickness (PW). However, there were no changes in fractional shortening (FS) as shown in Fig. 1E since the initial phase of pathological cardiac hypertrophy is likely to be a compensative adaptive response (19, 20). These data are consistent with previous reports documenting cardiac abnormalities and dysfunction in Cav-1 KO mice (9, 13, 14). Treatment of WT mice with Telm for 8 weeks had no appreciable effects on cardiac dimensions as monitored via echocardiography. However, treatment of Cav-1 KO mice with Telm increased LVDd and LVDs and reduced both PW and IVSW thickness (Fig 1; Telm group). Improvements in cardiac dimensions were obvious via serial echocardiography (Fig 1F). Morphometric assessments of the myocyte hypertrophy in (Telm) treated mice revealed that the Cav-1 KO cardiomyocytes were wider than control myocytes and telmisartan reduced myocyte width (Table 1). Cardiac morphometry showed that left ventricular (LV) weight to body weight (BW) ratios and LV to tibial length (TL) ratios as well as right ventricular weight (RV) to that of BW and RV to TL were also reduced by telmisartan in Cav-1 KO but not WT mice (Fig 2A, B, C and D).

Telmisartan normalizes the expression of genes associated with LV hypertrophy, inflammation and cardiac fibrosis in Cav-1 KO mice

Next, we examined gene expression of natriuretic peptides-A and –B (Nppa and Nppa and Nppb) and β-myosin heavy chain (β-MHC) using total RNA isolated from LV of treated WT and Cav-1 KO mice by q-PCR. As seen in Fig. 3 A-C, the expression of all three hypertrophic markers were elevated in vehicle treated Cav-1 KO mice compared to WT mice. Treatment with Telm reduced the expression of these genes to levels observed in vehicle treated or Telm treated WT mice. Since there is evidence that cardiac chemo/cytokines may participate in hypertrophy, we assessed the mRNA levels of macrophage chemotactic peptide-1 (MCP-1) in vehicle and telmisartan treated mice. As seen in Fig 3 D, vehicle treated Cav-1 KO mice had elevated levels of MCP-1 compared to WT mice and treatment of Cav-1 KO mice reduced MCP-1 gene expression. It has been reported that p42/44 MAP kinase cascade is hyperactivated in Cav-1 KO cardiac tissue and in isolated cardiac fibroblasts, and this effect contributes to the cardiac hypertrophy (13). Telmisartan treatment did not affect the increase in MAPK phosphorylation and eNOS expression observed in the LV of Cav-1 KO compared to WT mice (Fig. 3E).

There is evidence that Cav-1 KO mice have increased cardiac and perivascular fibrosis, due to increased TGF–β mediated signaling. As seen in Fig. 4 A-C, Cav-1 KO mice had increased expression of TGF-β and the interstitial collagen α chains, Col1A and Col3A. Treatment with telmisartan reduced both Col1A and Col3A gene expression, but did not influence TGF–β levels. The reduction in gene expression was associated with less interstitial collagen and perivascular fibrosis (via picrosirius red) in intramyocardial vessels in telmisartan treated mice (Table 1; Fig. 4D).

Telmisartan increases the cardiac expression of angiotensin receptor 2 (AT2R), reduces the plasma levels of Ang II but does not affect other components of the renin-angiotensin system in Cav-1 KO hearts

To examine if hypertrophic, Cav-1 KO mice exhibited altered expression of components of the cardiac renin-angiotensin system or if Telm influenced gene expression, we quantified the expression of AT1, AT2, renin, angiotensinogen (angiogen) and angiotensin converting enzyme (ACE) in hearts from treated WT and Cav-1 KO mice. As seen in Fig 4, no intrinsic differences in gene expression were found in vehicle or Telm treated WT and Cav-1 KO mice except Telm significantly increased the levels of AT2R in hearts of Cav-1 KO mice. However, measurement of blood levels of Angiotensin II (Ang II) by revealed increased levels of Ang II in Cav-1 KO mice versus WT (4.51 ± 0.91 and 0.45 ± 0.07 fmol/ml, respectively, n= 8 mice per group). Treatment with telmisartan reduced the blood level of Ang II in Cav-1 KO mice but did not influence Ang II levels in WT mice (1.24 ± 0.26 and 1.11 ± 0.15fmol/ml, respectively).

Discussion

The central finding of this study is that the ARB, telmisartan, improves the parameters of cardiac hypertrophy in Cav-1 KO mice suggesting that the endogenous generation of Ang II contributes to cardiac hypertrophy in this model. Treatment with the AT1-receptor blocker telmisartan suppressed and reverted this abnormal remodeling, as evidenced by serial echocardiography, as well as by a reduction in myocyte dimensions, RV and LV mass. The reversal of cardiac remodeling in Cav-1KO mice with telmisartan treatment likely occurs through several mechanisms including improvement of endothelial function, reduction in inflammatory gene expression, fibrosis and blood Ang II levels, and the upregulation of AT2 expression.

There are several papers showing that the genetic deletion of Cav-1 leads to spontaneous cardiac hypertrophy. This is surprising given that in the heart, Cav-1 is primarily found in the vasculature (endothelium and smooth muscle) and cardiac fibroblasts, but not in cardiac myocytes, which express ample amounts of Cav-3. Thus, the loss of Cav-1 in vasculature and cardiac fibroblasts is sufficient to promote abnormal cardiac remodeling. Interestingly, re-expression of Cav-1 into the endothelium of global Cav-1 KO mice rescues cardiac hypertrophy suggesting that Cav-1 in the endothelium is critical for this phenotype (21). With this in mind, what is a potential endothelial dependent mechanism linking Cav-1 to hypertrophic signaling? It is well appreciated that Cav-1 serves as an inhibitory clamp on eNOS function (22, 23), and the loss of Cav-1 increases basal eNOS activity, NO release, superoxide generation and promotes nitrosative stress during conditions of inflammation. Indeed, treatment of Cav-1 KO mice with NOS inhibitors reduces cardiac hypertrophy and improves exercise tolerance in Cav-1 KO mice (15, 24, 25) and breeding of Cav-1KO mice to eNOS-KO mice reverses impaired pulmonary function in Cav-1 KO mice (26). Thus, dysregulated eNOS can explain, in part, the endothelium dependent mechanism contributing to increased cardiac hypertrophy in Cav-1 KO mice. Additionally, there are data suggesting that Cav-1 negatively regulates TGF-β signaling in cardiac fibroblasts and the loss of Cav-1 enhances TGF-β signaling leading to hypertrophy and tissue fibrosis (13, 27, 28). Also,

studies in cardiac fibroblasts have shown that Ang II increase reactive oxygen species and collagen synthesis, effects abolished by telmisartan, but not by the specific AT2-receptor antagonist P-186 (29, 30). Therefore, in the present study, the beneficial effect of telmisartan may be due to an improvement in endothelial function and/or suppression of cardiac remodeling by antagonizing AT1R in fibroblasts and reducing the circulating levels of Ang II.

Previous clinical and experimental studies have shown the beneficial effects of ARBs in the suppression of cardiac remodeling. However, these studies have shown that low doses of ARB can exert cardioprotective effects independent of blood pressure reduction. Since the dosage used in the present study (1mg kg^{-1} day^{-1} in drinking water) does not lower blood pressure in normotensive mice (31), it seems that the cardioprotective effect is independent of blood pressure. Such blood pressure independent actions of telmisartan mediating the transition from hypertrophy to heart failure, has been demonstrated previously (32, 33).

The systemic and local RAAS plays a key role in the compensatory neurohumoral response to myocardial injury. Advances in the molecular pharmacology of the AT1 receptor have led to the development of highly specific AT1R specific ARBs and these compounds clearly block AT1R and reduce cardiac fibroblast proliferation and net accumulation of fibrillar collagen, both *in vitro* and *in vivo*.[36]. In the present study, there were no differences in the local RAS in Cav-1 KO mice compared to WT mice and telmisartan treatment did not influence AT1R, renin, angiotensinogen or ACE mRNA levels but significantly increased AT2R gene expression in Cav-1 KO mice. This effect was associated to an increase of circulating levels of Ang II in Cav-1 KO, reduced upon telmisartan treatment. How this occurs is not known but is consistent with the protective role of AT2R upregulation after telmisartan treatment(34, 35). AT2R signaling has been implicated in the suppression of myocardial hypertrophy(36), fibroblast proliferation(37), and vascular cell hyperplasia(38), although little is known about the mechanisms. Previous studies have suggested the possible involvement of the bradykinin/nitric oxide (NO)/cGMP system (39)in the AT2R-mediation of the prevention of heart failure post-myocardial infarction(40), and the hypertrophic heart caused by aortic coarctation (8). Despite tremendous research efforts, the specific mechanisms of these protective actions, as well as the underlying role the local RAS in myocardial remodeling remain enigmatic.

In summary, telmisartan reverses cardiac hypertrophy initiated by the loss of Cav-1. Since Cav-1KO mice are normo- or hypotensive, the reversal of hypertrophy occurs independent of blood pressure and is likely due to an effect of the ARB on endothelial function and/or cardiac fibroblasts. Mechanistically, telmisartan reduced cardiac hypertrophy, normalized hypertrophic gene expression and reduced indices of cardiac fibrosis. Thus, it is likely that endogenous activation of AT1 receptors contributes to cardiac hypertrophy initiated by the genetic loss of Cav-1.

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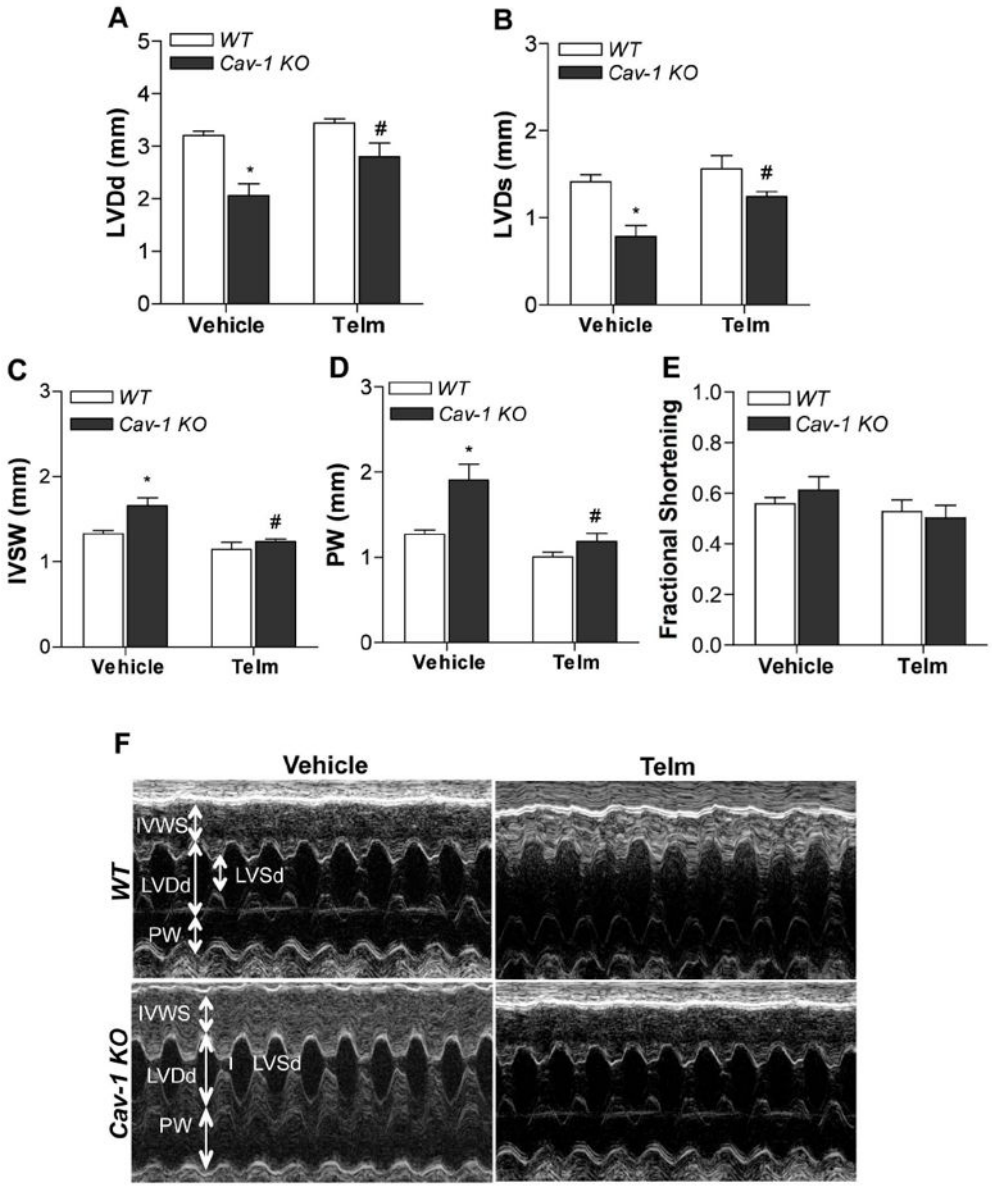

Figure 1. Telmisartan reduces cardiac hypertrophy in Cav-1 KO mice

Serial echocardiography was performed in the four groups of mice as described and the following parameters (LVDd, LVDs, PW, IVSW and FS) measured before and after treatment with Telm for 8 weeks. (D) Picture representative from ten individual records from post- 1 μM Telm comparison with Vehicle treatment transthoracic echocardiography on WT and Cav-1 KO mice. Data are mean +/- SEM with n=10 for each group tested, *$p <$ 0.05.

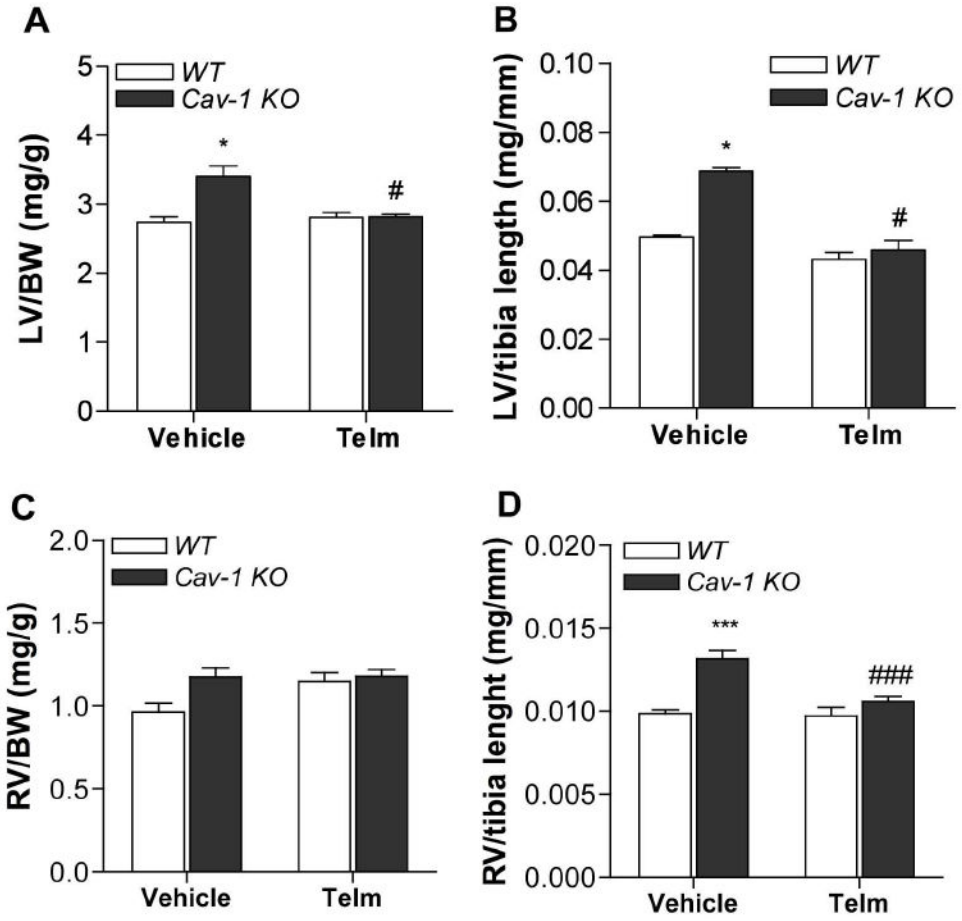

Figure 2. Telmisartan improves cardiac morphometry in Cav-1 KO mice

WT and Cav-1 KO mice were treated with Telm as above and after sacrifice cardiac morphometrics assessed. In A. Left Ventricular weight (LV)/ Body Weight (BW) in mg/g; B. LV/Tibia Length in mg/cm; C. (B) Right Ventricular (RV) per BW in mg/g and D. RV/ Tibia Length in mg/cm. N= 10 mice for each group tested, $*p < 0.05$.

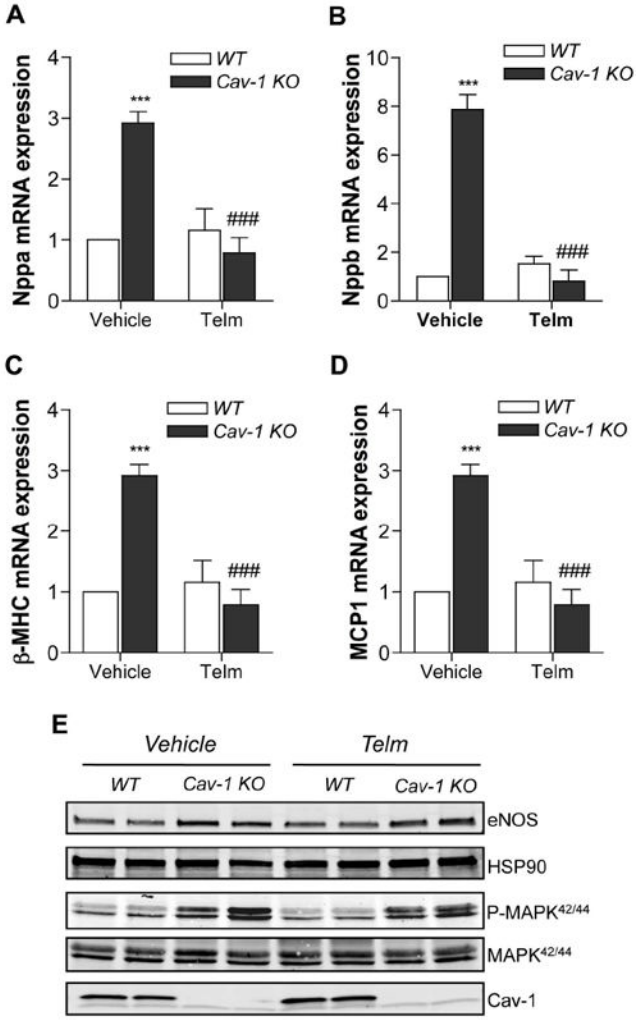

Figure 3. Telmisartan reduces hypertrophic and inflammatory gene expression in Cav-1 KO mice

In A-C, pro-natriuretic peptide type B and A (Nppb and Nppa) and β-myosin heavy chain (β-MHC) genes were measured. In D, monocyte chemoattractant protein-1 (MCP-1) expression was quantified in the four groups. E, Western blot analysis was performed in different treatment group for eNOS, P-MAPK$^{42/44}$ and MAPK$^{42/44}$, HSP90 and Cav-1. N= 6 mice for each group tested, * $p < 0.05$.

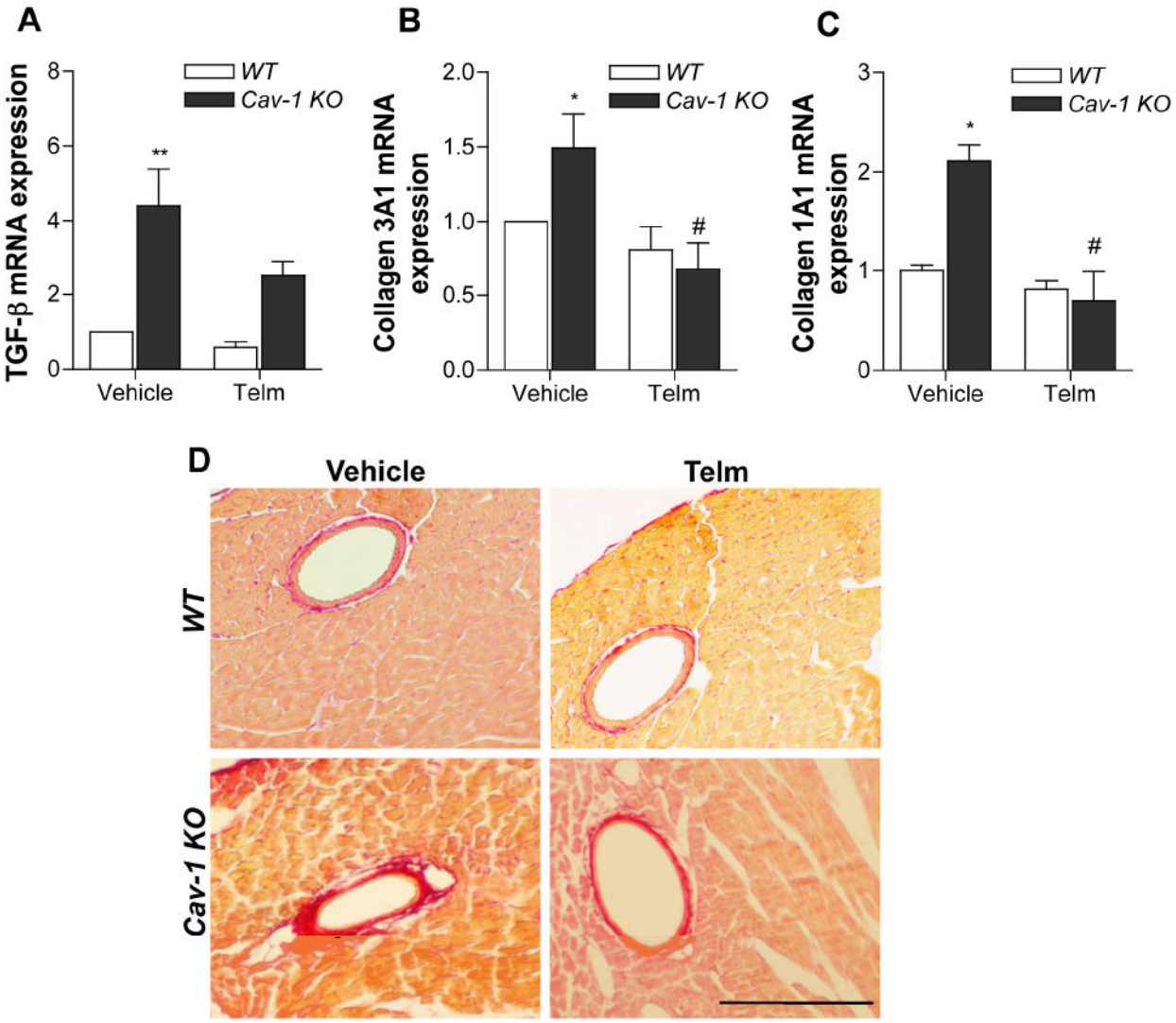

Figure 4. Telmisartan diminishes pro-fibrotic gene expression and perivascular collagen deposition in Cav-1 KO mice

A, transforming growth factor β (TGF-β), B, collagen 1A (Col1A) and C, collagen 3A (Col3A) genes were quantified in the different treatment groups. (D) Photomicrographs of picrosirius red stained sections of heart section from different treatments containing perivascular collagen. The bar represents 100 μm.

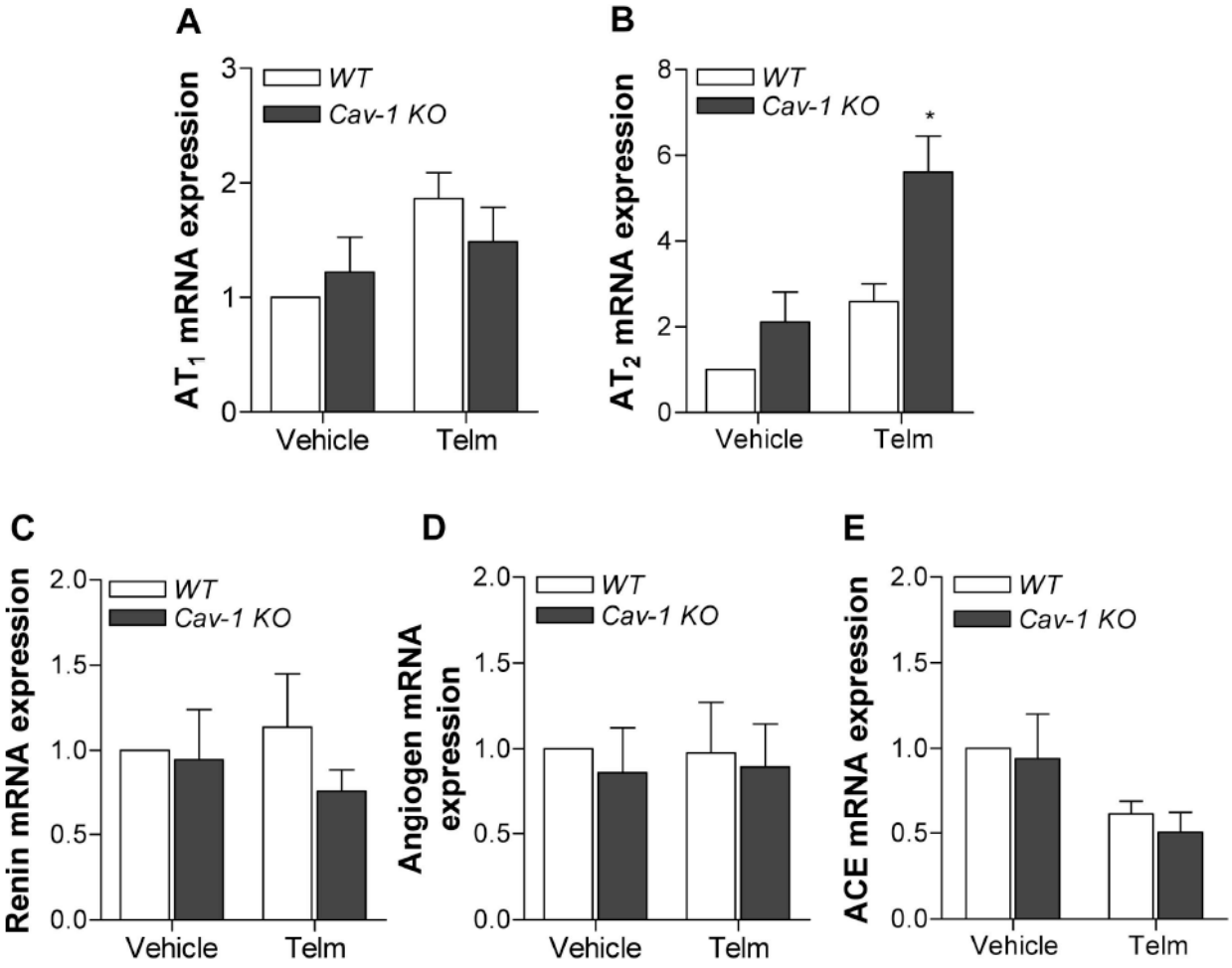

Figure 5. Telmisartan increases the expression of ATR2 in Cav-1 KO mice

ATR1, ATR2, renin, angiotensinogen (angiogen) and angiotensin converting enzyme (ACE) mRNA levels were measured in the left ventricle of vehicle and telmisartan treated mice. N= 6 for each group of mice tested, * $p < 0.05$.

Table 1

Cardiomyocyte morphology and indices of fibrosis in WT and Cav-1$^{-/-}$ mice treated with vehicle or telmisartan

Left ventricular cardiomyocyte width (μm), left ventricular collagen deposition in cardiac interstitium and perivascular regions from the 4 different groups of mice in the study.

Group	WT + Vehicle (n=6)	WT + Telmi (n=6)	Cav-1$^{-/-}$ +Vehicle (n=6)	Cav-1$^{-/-}$ + Telmi (n=6)
Cardiomyocyte width (μm)	19±0.7	18±0.5	28±0.6*	20±0.7$^{\#}$
Collagen interstitial area (%)	2.8±0.25	2.9±0.10	3.0±0.27	2.9±0.32
Collagen perivascular area (%)	1.4±0.1	1.6±0.1	4.0±0.05*	1.5±0.07#

Data are mean+/- SEM, n= 6 mice per group,

* $p < 0.05$ compared to WT mice and

$^{\#}$ $p < 0.05$ compared to Cav-1-/-.