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Arjunolic Acid, a Peroxisome Proliferator-Activated Receptor Alpha Agonist Regresses Cardiac Fibrosis by Inhibiting Non-canonical TGF-β Signaling.

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Running Title: Antifibrotic role of Arjunolic acid as a PPARα Agonist

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Abstract:
Cardiac hypertrophy and associated heart fibrosis remain a major cause of death worldwide. Phytochemicals have gained attention as alternative therapeutics for managing cardiovascular diseases. These include the extract from the plant *Terminalia arjuna* which is a popular cardioprotectant and may prevent or slow progression of pathological hypertrophy to heart failure. Here, we investigated the mode of action of a principal bioactive *T. arjuna* compound, arjunolic acid (AA) in ameliorating hemodynamic load-induced cardiac fibrosis and identified its intracellular target. Our data revealed that AA significantly represses collagen expression and improves cardiac function during hypertrophy. We found that AA binds to and stabilizes the ligand binding domain of peroxisome proliferator-activated receptor alpha (PPARα) and increases its expression during cardiac hypertrophy. PPARα knockdown during AA treatment in hypertrophy samples, including angiotensinII treated adult cardiac fibroblasts and renal artery-ligated rat heart suggests that AA-driven cardioprotection primarily arises from PPARα agonism. Moreover, AA-induced PPARα upregulation leads to repression of TGF-β signaling, specifically by inhibiting TGF-β activated kinase 1 (TAK1) phosphorylation. We observed that PPARα directly interacts with TAK1, predominantly via PPARα N-terminal transactivation domain (AF-1) thereby masking the TAK1 kinase domain. AA-induced PPARα-bound TAK1 level thereby shows inverse correlation with phosphorylation level of TAK1 and subsequent reduction in p38 MAPK and NF-κB p65 activation, ultimately culminating in amelioration of excess collagen synthesis in cardiac hypertrophy. In conclusion, our findings unravel the mechanism of AA action in regressing hypertrophy associated cardiac fibrosis by assigning a role of AA as a PPARα agonist that inactivates non-canonical TGF-β signaling.

Introduction:
Cardiac hypertrophy is accompanied by excess deposition of collagen and other extracellular matrix (ECM) proteins in heart which leads to cardiac stiffness and eventual heart failure (1, 2).
Increased hemodynamic load in heart leads to activation of renin-angiotensin system (RAS) resulting in heightened local concentration of angiotensinII (AngII) which has been established as a principal causal factor for cardiac hypertrophy and associated fibrosis (3, 4, 5). Therefore, ameliorating AngII-induced cardiac fibrosis might be one of the possible measures for prevention of such disease progression with improved efficacy of cardiac performance.

Phytomedicines have been greatly appreciated in recent times as promising candidates towards alternative therapeutic regime in several fibrotic diseases (6, 7). *Terminalia arjuna* (arjuna), being considered as one of the most accepted beneficial plants from ancient times has also widely been known to have cardiotoxic functions (8). Preclinical studies with arjuna extracts as well as clinical trials in the form of combination therapy with standard drugs have shown to be protective in several models of cardiac ailments (9, 10).

Arjunolic acid (AA), a chiral triterpenoid is one of the principal bioactive components of arjuna extracts. Aqueous extract of arjuna bark containing AA, significantly inhibited isoprenaline-induced increase in oxidative stress and also prevented fibrosis without regression of hypertrophy or improvement of cardiac function (11). Purified AA resulted in anti-oxidant, anti-platelet, anti-coagulant, anti-apoptotic, free-radical-scavenging, anti-inflammatory, hypolipidemic or hypotensive effects in various cardiac disease models (9, 10, 12, 13). However, the precise molecular mechanism of cardioprotection by AA has still remained elusive. Further, pleiotropic effects of AA make it even harder to annotate specific molecular targets within the cellular milieu.

Thus, the objective of our study was to look into the effect of AA which is purified and crystallized from ethyl acetate and methanol extracts of the corewood of arjuna plant (14) on cardiac hypertrophy associated fibrosis and seek out its plausible mechanism of action on fibrotic signaling through identification of its specific molecular target.

Our study for the first time establishes PPARα as a molecular target of AA. It is a ligand-dependent transcription factor and the most abundant PPAR isoform in heart (15). PPARα primarily acts as a modulator of energy metabolism but also acts as anti-inflammatory agent (16, 17). Growing evidences have shown that PPARα activators are involved in integrating inflammatory and hypertrophic pathways thereby governing the pathological outcome of hypertrophy associated fibrosis (18, 19, 20) in hypertensive rats *in vivo*. Moreover, PPARα double knockout mice subjected to transverse aortic constriction (TAC) showed much heightened fibrotic and inflammatory gene expressions along with severely aggravated hemodynamic function compared to wild type mice that has undergone TAC (21).

During pressure overload hypertrophy, autocrine and paracrine actions of locally expressed AngII induce TGF-β signaling in cardiac fibroblasts (2, 3). Upon ligand binding, TGF-β receptor II (TβRII) transphosphorylates TGF-β receptor I (TβRI). In the canonical branch, active TβRI phosphorylates ‘suppressor of mothers against decapentaplegic’ (SMAD 2/3) which combines with co-SMADs (SMAD 4) for nuclear translocation. Active nuclear SMADs upregulate transcription of several genes involved in ECM synthesis (3, 22). SMAD3/- mice have shown reduced cardiac fibrosis after TAC (23). Independent of the SMAD kinase activity, non-canonical pathway is principally governed by MAPKs and TAK1 is a principal MAP3K. Phosphorylation-dependent activation of TAK1 requires interaction of its kinase domain with C terminal of TAK1 binding protein (TAB1) (24, 25). Once activated, TAK1 phosphorylates inhibitor of kappab kinase beta (IKKβ) (but not IKKa); MKK4/7 and MKK3/6 which in turn phosphorylates NF-κB; JNK and p38 MAPK respectively (25, 26). Ultimately, NF-κB and other transcription factors downstream of JNK and p38 MAPK are activated resulting in transcription of the target genes (27). Importance of non-canonical branch via TAK1 has also been well documented in cardiac hypertrophy and fibrosis (28). Dominant negative TAK1 has been found to inhibit TGF-β induced hypertrophic events in mouse cardiomyocytes and fibroblasts, including ECM production (29).

Inhibitory effect of PPAR activation upon TGF-β signaling has been extensively documented (30, 31, 32). Our work thus aims to study the role of PPARα in AA-driven modulation of TGF-β axes during cardiac hypertrophy and associated fibrosis. This work identifies PPARα::TAK1 interaction as a causal factor preventing TAK1 phosphorylation and further analyzes the relative strength of different PPARα domains contributing
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Hypertrophy was confirmed in vivo by significantly increased heart weight (in mg) to body weight (in g) ratio (HW:BW; 1.71±0.091 fold; Figure 1E), increased cardiomyocyte cross-sectional area (CSA) (627.42±21.197 μm² vis-à-vis 379.48±10.606 μm²; Figure 1F) and increased expression of atrial natriuretic factor (anf) (3.27±0.019 fold), β-myosin heavy chain (β-mhc) (2.38±0.086 fold) and skeletal alpha actin (acta1) (1.89±0.024 fold) genes (Figure 1G) in ligated rats compared to sham operated control rat heart. AA-mediated regression of hypertrophy was confirmed by significantly reduced HW:BW ratio (1.28±0.067 fold; Figure 1E), significantly decreased CSA (505.81±20.326 μm² vis-à-vis 627.42±21.197 μm²; Figure 1F) and significant downregulation in anf (1.69±0.009 fold), β-mhc (2.38±0.086 fold) and acta1 (1.57±0.017 fold) gene expressions (Figure 1G) in AA treated hypertrophy samples compared to renal artery ligated rat heart (Figure 1E-G).

Hypertrophic induction significantly reduced cardiac functional efficacy in ligated rats [fractional shortening; (%FS): 38.53±1.781 % and left ventricular internal diastolic diameter; (LvIdd): 5.65±0.273 mm] compared to sham operated control rats (%FS: 57.51±1.026 % and LvIdd: 3.56±0.062 mm) as observed by M-mode echocardiography in transthoracic parasternal short axis view at papillary muscle level. Furthermore, significant restoration of such compromised cardiac function was evident in AA-treated ligated rats showing increased %FS (46.67±1.504 %) and decreased LvIdd (4.01±0.096 mm) compared to aforementioned hypertrophy samples in vivo (Figure 1H).

The viability measured was more than 90% after AA treatment in all hypertrophied fibroblasts as checked by cell viability assay (data not shown). Effective dose of AA for both in vitro and in vivo experiments were determined by maximal regression of collagen during hypertrophy via dose-dependent study (data not shown).

AA directly interacts with PPARα:

Fluorescence and circular dichorism (CD) titrations of AA with PPARα:
Steady state fluorescence titrations of PPARα with increasing concentrations of AA showed no apparent shifts around 330 nm (λ_max), indicative of unaltered tertiary architecture. However, the corresponding hypo-chromatic shifts observed indicated binding of AA to PPARα (Figure 2A-i).
The binding was also apparent when monitored through CD analyses where similar titrations induced marginal secondary structure perturbations (near 55 μM) in this predominantly α-helical protein. (Figure 2A-ii).

Fourier transform infra-red spectroscopy (FTIR) analysis:

FTIR spectrum of AA supported previous reports (14) showing characteristic –OH, C-H and C=O stretches at 3467 cm⁻¹, 2927 cm⁻¹, 1708 cm⁻¹ positions respectively. Characteristic C-O stretching vibrations were also obtained at 475 cm⁻¹, 598 cm⁻¹, 1042 cm⁻¹ and 1456 cm⁻¹. In vitro translated and purified PPARα revealed intense absorption characteristic of amide-I and amide A bands of secondary structure of a protein (33, 34). Peak at 1642 cm⁻¹ position, represented amide-I bond. The amide-A band (at 3300-3600 cm⁻¹) representing the N-H stretching vibrations, were also obtained. Successful interaction between PPARα and AA by in vitro interaction assay was shown by the absence of peaks at 3467 cm⁻¹, 2927 cm⁻¹, 1708 cm⁻¹ in the complex compared to free AA. Peaks characteristic of free AA at 952 cm⁻¹, 1018 cm⁻¹, 1317 cm⁻¹, 1412 cm⁻¹, 1456 cm⁻¹ and those characteristic of free in vitro translated PPARα at 1642 cm⁻¹ and in the range of 3300-3600 cm⁻¹ were maintained in the complex when compared to free AA or free in vitro translated protein respectively (Figure 2B).

AA stabilizes tertiary and secondary architecture of PPARα:

Thermal melting studies:

Stabilization of the tertiary and secondary architecture of PPARα by AA was assessed by monitoring changes in melting temperature (Tm) (Figure 2C-i, ii). In fluorescence thermal shift (FTS) assays, PPARα, bound to AA showed higher Tm (59.5±0.5 °C) compared to PPARα alone (56.4±0.2 °C) (Figure 2C-i). Similar trend was observed through CD studies where changes in both θ218 (alpha-helical content, Figure 2C-ii) and θ222 (beta content, inset Figure 2C-ii) with respect to temperature clearly showed higher Tm (56.4±0.1 °C) upon AA binding compared to control (53±0.2 °C).

AA binds to the ligand binding domain (LBD) of PPARα:

Molecular modeling and molecular docking study:

*Rattus norvegicus* PPARα protein model was built and integrity of the model was confirmed through all atomistic molecular dynamics simulation. To gain atomistic details, binding mode of AA with rat-PPARα (UniProtKB-P37230) was compared to the other known agonists namely Iloprost, TIPP-703 and fenofibrate.

PPARα::AA complex obtained through docking via ‘binding site prediction and shape-based ligand matching’ (BSP-SLIM) tool showed binding score close to previously studied synthetic agonists such as Iloprost, TIPP-703, and fenofibrate. Additionally, the calculated binding free energy (~6.3 kcal/mol) matched with its experimentally determined energy obtained from fluorescence and CD titrations (~6.2 kcal/mol). Interestingly, AA was found to bind to the identical pocket (formed of Met 220, Cys 276, Ser 280, Thr 283, Phe 318, Leu 331, Ile 332, Leu 324, Tyr 334 and His 440) for which binding of all other PPARα agonists have been reported, including pre-docked complex with fenofibrate. With root mean square deviation (RMSD) of ~0.62 Å amongst all aligned structures, it showed structural superposition of AA with other agonists, confined to the same ligand binding site. This was further confirmed through tertiary structure alignment of modelled PPARα (bound to AA and fenofibrate) with known crystal structures of PPARα in complex with agonists such as Iloprost and TIPP-703 (Figure 2D, Supplementary Table 1).

AA stabilizes the LBD of PPARα:

All atomistic molecular dynamics (MD) simulations:

Molecular dynamics simulation trajectories of both free and AA-bound PPARα were analyzed for changes in backbone RMSD, gyration radius (Rg), root mean square fluctuations in Cα atoms (RMSF) as well as protein-ligand H-bond network occupancy. In simulations of PPARα alone, projection of variations in backbone RMSD versus Rg showed dual distribution of species along both components (Figure 2E-i). The projections were however dominated by the species with high RMSD and Rg compared to initial model. However in presence of AA, only moderate variations were observed along both components (Figure 2E-ii). This system showed single distribution with dominant population close to initial starting conformation (low RMSD and marginal variations in Rg). These variations were identically reflected in RMSF analysis of both systems (Figure 2E-iii). Marginal changes in Cα fluctuations were observed in residues of
DNA binding domain (DBD) of PPARα. However, the LBD showed much reduced Ca fluctuations in AA-bound PPARα complexes compared to PPARα alone, indicating stabilization of LBD by AA. H-Bond network analysis showed persistence of at least three H-bonds per time frame between AA and Thr 283, Leu 331 and Tyr 334 of PPARα (Figure 2E-iv).

**AA-induced PPARα transcriptional activation results in increased ppara gene expression in an autoregulatory loop:**

Effect of AA upon PPARα-mediated autoregulation of PPARα transcription was analyzed by Chromatin immunoprecipitation (ChIP) using anti-PPARα antibody coupled to qRT-PCR analyses of (PPAR response element) PPRE within PPARα promoter, both in vitro and in vivo. DMSO and non-specific siRNA (NS siRNA) treated hypertrophy samples showed significant downregulation in binding of PPARα upon PPRE sequence within PPARα promoter compared to DMSO and NS siRNA treated control samples (1.99±0.078 fold in vitro and 2.75±0.199 fold in vivo). However, AA treatment in NS siRNA treated hypertrophied groups showed significant fold enrichment in binding of PPARα to the PPRE (3.00±0.075 fold in vitro and 3.27±0.237 fold in vivo) compared to DMSO and NS siRNA treated hypertrophy samples. PPARα knockdown in AA treated groups showed significantly lowered binding (6.49±0.520 fold in vitro and 8.17±0.749 fold in vivo) compared to AA treated hypertrophy samples pretreated with NS siRNA as negative controls (Figure 3A). Also, qRT-PCR analyses both in vitro and in vivo indicated significant downregulation in ppara gene expression in the hypertrophy samples compared to respective control samples (1.81±0.026 fold in vitro and 3.37±0.154 fold in vivo) and significant increase in ppara gene expression in AA treated hypertrophy samples compared to respective hypertrophied groups (3.45±0.131 fold in vitro and 3.94±0.179 fold in vivo). PPARα knockdown in AA treated hypertrophied groups showed significantly downregulated ppara gene expression (5.49±0.208 fold in vitro and 7.04±0.609 fold in vivo) compared to respective AA treated hypertrophy samples were used as negative controls (Figure 3A).

**AA increases PPARα protein expression during cardiac hypertrophy:**

Significant reduction of PPARα protein expression was observed in AngII treated fibroblasts (2.05±0.088 fold in vitro) and renal artery ligated rat heart (1.98±0.059 fold in vivo) compared to respective control groups as analyzed by western blot. Whereas, AA treated hypertrophy samples showed significant upregulation in PPARα expression (2.12±0.127 fold in vitro and 2.05±0.031 fold in vivo) compared to the hypertrophy groups (Figure 3B). Successful knockdown of PPARα expression by PPARα siRNA treatment in AA treated hypertrophy samples was also confirmed by western blot (3.18±0.191 fold downregulation in vitro and 1.67±0.025 fold downregulation in vivo compared to respective AA treated hypertrophy samples) (Figure 3B).

Moreover, time point analyses showed significant upregulation of PPARα expression in AngII treated fibroblasts when treated along with AA compared to only AngII treatment at respective time points under study (Figure 3C).

**AA-mediated upregulation of PPARα results in regression of cardiac hypertrophy associated fibrosis:**

AA treated hypertrophy samples pretreated with PPARα siRNA resulted in significantly increased expression of col-1 (2.48±0.137 fold in vitro and 2.58±0.058 fold in vivo) and col-3 (3.19±0.06 fold in vitro and 3.42±0.103 fold in vivo) genes compared to respective AA treated hypertrophy groups as revealed by qRT-PCR (Figure 4A). Hydroxyproline assay also revealed significant recovery of secreted collagen content in fibroblast culture supernatant (420.7±28.943 ng/ml vis-à-vis 296.84±20.981 ng/ml of culture supernatant; Figure 4B) as well as total left ventricular collagen content (418.82±21.927 µg/g vis-à-vis 262.29±18.828 µg/g of wet tissue; Figure 4C) during PPARα siRNA treatment in AA treated hypertrophied groups compared to respective AA treated hypertrophy samples in vitro and in vivo. Collagen volume fraction (%CVF) showed significant downregulation (1.82±0.153 fold) during AA treatment in hypertrophied heart compared to ligated heart tissue. PPARα knockdown in AA treated ligated rats showed significant increase in %CVF (1.57±0.133 fold) compared to AA treated in vivo hypertrophy samples (Figure 4D).

Pretreatment of PPARα siRNA in AA treated ligated rats also showed deterioration of cardiac function as revealed by decreased %FS and
increased LvIDd compared to that of AA treated hypertrophy groups (%FS: 37.83±1.186 % vis-à-vis 49.39±1.618 % and LvIDd: 5.57±0.144 mm vis-à-vis 4.79±0.310 mm) by M-mode echocardiography (Figure 4E). Furthermore, mean values of the other echocardiographic parameters such as left ventricular anterior (LvAWD) and posterior (LvPWD) wall diameter both in diastole (d) and systole (s), left ventricular volume in diastole and systole (represented by stroke volume: SV) as well as percentage of left ventricular ejection fraction (EF), measured from all the in vivo experimental groups are summarized in Supplementary Figure S-1A with representative images of echocardiographic analyses in Supplementary Figure S-1B.

**AA-mediated upregulation of PPARα specifically targets TAK1 to inhibit TGF-β signaling during cardiac hypertrophy:**

AA treatment in AngII treated fibroblasts or renal artery ligated rats significantly repressed expressions of TβRI (1.85±0.007 fold *in vitro* and 1.94±0.017 fold *in vivo*), TβRII (1.42±0.026 fold *in vitro* and 2.09±0.016 fold *in vivo*), phospho/total SMAD 2 (1.73±0.015 fold *in vitro* and 2.15±0.025 fold *in vivo*), phospho/total SMAD 3 (1.36±0.016 fold *in vitro* and 4.06±0.034 fold *in vivo*) and phospho/total TAK1 (5.23±0.356 fold *in vitro* and 7.93±0.372 fold *in vivo*) compared to hypertrophy samples as revealed by western blot analyses (Figure 5A, Supplementary Figure S-2A). However, AA treatment in TGF-β treated cardiac fibroblasts showed no significant alterations in TβRI, TβRII phospho/total SMAD 2 and phospho/total SMAD 3 expression levels, with significantly repressed phospho/total TAK1 level (1.47±0.075 fold) compared to TGF-β treated cells (Figure 5B, Supplementary Figure S-2B). PPARα siRNA pretreatment in AA treated hypertrophied fibroblasts or renal artery ligated rats significantly restored expressions of all these proteins (TβRI, *in vitro*: 1.39±0.018 fold; *in vivo*: 2.76±0.013 fold; TβRII, *in vitro*: 2.05±0.029 fold; *in vivo*: 2.04±0.051 fold; phospho/total SMAD 2, *in vitro*: 1.53±0.058 fold; *in vivo*: 1.37±0.022 fold; phospho/total SMAD 3, *in vitro*: 1.49±0.016 fold; *in vivo*: 3.01±0.047 fold; phospho/total TAK1, *in vitro*: 4.92±0.309 fold; *in vivo*: 10.00±0.280 fold) compared to respective AA treated hypertrophy samples (Figure 5A, Supplementary Figure S-2A). However, PPARα knockdown in TGF-β treated fibroblasts during AA treatment showed significant increase in phospho/total TAK1 level (1.75±0.069 fold) with no significant change in TβRI, TβRII phospho/total SMAD 2 and phospho/total SMAD 3 expression levels compared to TGF-β infused cells treated along with AA (Figure 5B, Supplementary Figure S-2B).

**AA-mediated upregulation of PPARα inhibits non-canonical TGF-β axes during hypertrophy:**

Significant downregulation of non-canonical TGF-β pathway intermediates, downstream to TAK1 namely, phospho/total IKKβ (1.99±0.045 fold *in vitro* and 1.77±0.041 fold *in vivo*), phospho/total NF-κBp65 (4.76±0.063 fold *in vitro* and 2.21±0.096 fold *in vivo*); phospho/total p38 MAPK (1.57±0.029 fold *in vitro* and 1.30±0.025 fold *in vivo*) and phospho/total JNK (1.26±0.050 fold *in vitro* and 1.21±0.060 fold *in vivo*) were observed due to AA treatment in hypertrophied groups compared to respective hypertrophy samples as shown by western blot analyses. On the other hand, PPARα siRNA treatment showed significant recovery of phospho/total IKKβ (2.61±0.046 fold *in vitro* and 1.73±0.014 fold *in vivo*), phospho/total NF-κBp65 (3.58±0.025 fold *in vitro* and 3.18±0.119 fold *in vivo*); phospho/total p38 MAPK (2.23±0.018 fold *in vitro* and 1.46±0.045 fold *in vivo*) and phospho/total JNK (1.20±0.050 fold *in vitro* and 1.21±0.016 fold *in vivo*) level compared to AA treated hypertrophy samples *in vitro* and *in vivo* (Figure 6A, Supplementary Figure S-2C).

**Inhibition of either TAK1 or its downstream signaling intermediates results in regression of collagen gene expression in AngII treated cardiac fibroblasts:**

Significant downregulation of collagen gene expressions were observed due to siRNA mediated knock down of TAK1 (3.71±0.141 fold for *col-1* and 2.24±0.072 fold for *col-3*), NF-κBp65 (3.46±0.063 fold for *col-1* and 2.06±0.125 fold for *col-3*) or p38 MAPK (1.92±0.094 fold for *col-1* and 1.57±0.056 fold for *col-3*) in AngII treated fibroblasts compared to NS siRNA treated hypertrophied cells as revealed by qRT-PCR analyses (Figure 6B). JNK specific siRNA however had no inhibitory effect upon AngII induced collagen gene expression. Successful knockdown of TAK1, NF-κBp65, p38 MAPK and JNK by pretreatment with respective siRNAs in AngII treated fibroblasts were confirmed by
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Western blot analyses (Supplementary Figure S-3).
NF-κBp65 activity on collagen1 (coll1a1) promoter was analyzed by Dual-luciferase assay showing significant increase in luciferase activity (2.08±0.120 fold) of coll1a1 promoter in AngII treated cardiac fibroblasts compared to respective control cells. Further, NF-κBp65 driven coll1a1 promoter activity was found to be significantly downregulated (2.03±0.032 fold) by AA treatment in AngII treated cells compared to hypertrophied fibroblasts. PPARα knockdown in AA infused AngII treated fibroblasts significantly restored (1.92±0.058 fold) such promoter activity compared to AA treated hypertrophied cells. NF-κBp65 knockdown in AngII treated cells showing downregulated NF-κBp65 activity (2.14±0.081 fold) on coll1a1 promoter activity was found to be significantly increased binding between PPARα and TAK1 (2.73±0.177 fold) compared to hypertrophied cells pretreated with empty vector were used as positive control (Figure 7C).

Analyses of interaction between PPARα and TAK1:

Molecular modeling study: Rattus norvegicus TAK1 protein model was built and integrity of the model was confirmed through all atomistic molecular dynamics simulation (Supplementary Figure S-4).

Molecular docking study: For docking between PPARα and TAK1, the interfacial interacting residues between these two proteins were identified using CPoRT to limit the plausible protein-protein docking landscape. Using HADDOCK for docking, 23 clusters of the 296 predicted structures were found that represented 70.4 % of the water-refined models. The best conformation in the top cluster according to best fit HADDOCK score revealed not only that PPARα and TAK1 extensively interact via inter-protein hydrogen bonds, but also gives an insight into the possible binding interface. The model with the best HADDOCK score revealed maximum contribution of N terminal transactivation domain (AF-1) of PPARα for hydrogen bonding with TAK1 with added contribution of a few hydrogen bonds through the hinge region (H) and LBD. Only one residue at position 107 from DBD had been shown to interact with TAK1 (Figure 7A). Status of interaction between PPARα and TAK1 is summarized in Supplementary Table 2A-B.

Fluorescence resonance energy transfer (FRET) assay: FRET assay measured fluorescence recovery after photobleaching (FRAP) showing positive FRET efficiency (20.87±1.151) which revealed direct interaction between endogenous PPARα and TAK1 in control fibroblasts in vitro (Figure 7B).

Co-immunoprecipitation (Co-IP) assay: AA treatment in hypertrophied fibroblasts resulted in significant upregulation (3.63±0.334 fold) of PPARα bound TAK1 expression compared to respective hypertrophy samples in vitro as revealed by immunoprecipitation with anti-PPARα antibody followed by western blot with anti-TAK1 antibody. PPARα overexpression in AngII treated fibroblasts showing significantly increased binding between PPARα and TAK1 (2.73±0.177 fold) compared to hypertrophied cells pretreated with empty vector were used as positive control (Figure 7C).

Study of the effect of PPARα domains upon PPARα::TAK1 interaction:

His tagged plasmids with inserts of full length PPARα: ‘F’, N terminal transactivation domain: ‘AF-1’, DNA binding domain: ‘DBD’ or Hinge region along with Ligand binding domain: ‘H+LBD’ were overexpressed into AngII treated cardiac fibroblasts. Immunoprecipitation with anti-His antibody followed by western blot analyses with anti-TAK1 antibody revealed strongest binding of TAK1 with ‘AF-1’(96.97±1.829 %), a moderate interaction with ‘H+LBD’ (35.78±0.975 %) and almost negligible interaction with ‘DBD’ (10.59±0.887 %) in comparison to TAK1 interaction with ‘F’ (100 %), that was used as a positive control (Figure 8A). Thus, ‘DBD’ (9.03±0.683 fold) and ‘H+LBD’ (2.64±0.076) showed significantly lower binding with TAK1, compared to ‘AF-1’ in AngII treated cells (Figure 8A, Supplementary Figure S-5A).

Study of the effect of PPARα domains in AngII treated fibroblasts upon modulation of non-canonical TGF-β pathway and collagen synthesis:
‘F’ plasmid overexpressed AngII treated cardiac fibroblasts showed significant reduction in the level of phospho/total TAK1 (3.68±0.065 fold), phospho/total NF-kBp65 (5.13±0.109 fold) and phospho/total p38 MAPK (3.69±0.141 fold) compared to empty pCDNA6/V5 His B mammalian expression plasmid infected AngII treated cells. ‘AF-1’ and ‘H+LBD’ overexpression into AngII treated cells also showed significant regression of phospho/total TAK1 (2.07±0.144 fold for ‘AF-1’ and
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1.49±0.117 fold for ‘H+LBD’), phospho/total NF-κBp65 (5.16±0.165 fold for ‘AF-1’ and 1.36±0.066 fold for ‘H+LBD’) and phospho/total p38 MAPK (3.56±0.185 fold for ‘AF-1’ and 1.54±0.049 fold for ‘H+LBD’) level compared to AngII treated cells. Whereas, overexpression of ‘DBD’ in AngII treated cells showed no significant difference in the expression level of such proteins compared to the hypertrophied cells. Moreover, ‘AF-1’ overexpression in AngII treated cells showed significantly higher level of regression of the aforementioned proteins (1.38±0.028 fold for phospho/total TAK1, 3.79±0.199 fold for phospho/total NF-κBp65 and 2.31±0.183 fold for phospho/total p38 MAPK) compared to ‘H+LBD’ overexpressed AngII infused fibroblasts (Figure 8B, Supplementary Figure S-5B).

The qRT-PCR analyses showed significant downregulation in col-1 and col-3 gene expressions (3.48±0.17 fold for col-1 and 3.47±0.249 fold for col-3) in ‘F’ overexpressed AngII treated fibroblasts compared to AngII treated cells pretreated with empty plasmid. Similar trend of regression was also observed by ‘AF-1’ and ‘H+LBD’ overexpression in AngII treated fibroblasts (2.66±0.233 fold and 1.478±0.017 fold for col-1; 2.35±0.202 fold and 1.875±0.073 fold for col-3 respectively) compared to AngII treated cells. However, ‘AF-1’ overexpression caused significantly higher level of regression in collagen gene expression (1.77±0.081 fold for col-1 and 1.24±0.025 fold for col-3) compared to ‘H+LBD’ overexpression in hypertrophied fibroblasts. ‘DBD’ overexpression in AngII treated cells on the other hand showed no significant change in collagen gene expressions compared to hypertrophied fibroblasts (Figure 8C).

Discussion:
Phytomedicines are promising candidates for unravelling novel strategies to combat maladaptive cardiac remodeling. The present study focuses on the precise mechanism of protection conferred by one such phytochemical arjunolic acid (AA) during pressure overload cardiac hypertrophy and fibrosis. Our investigation showed that AA significantly inhibits excess synthesis of collagen and its subsequent deposition in ECM during cardiac hypertrophy both in vitro and in vivo. Cardiac functional betterment was also observed with AA treatment in renal artery ligated rats along with significant regression of collagen synthesis and redemption from hypertrophic load (Figure 1A-H, supplementary Figure S-1A-B). This encouraged us to probe into the specific molecular mechanism involved in AA-mediated amelioration of fibrosis during cardiac hypertrophy.

Arjunolic acid (2, 3, 23-trihydroxyolean-12-en-28-oic acid) is a pentacyclic triterpenoid monocarboxylic acid substituted by three hydroxy groups at position 2, 3 and 23 (CHEBI: 68381). Two hydroxyl groups and one hydroxymethyl group are attached to the “A” ring. The carboxyl group is attached at the ring junction of the cis-fused “D” and “E” rings (35, 36). Triterpenoids have long been known for their ability to suppress inflammatory pathways (37) and have been recognized to activate different PPARs (38, 39). PPARα being the predominant cardiac isoform of PPAR family (15), interaction of PPARα with AA was studied in detail.

Stabilizing interaction between AA and PPARα was confirmed by fluorescence and CD titrations and thermal shift assays (Figure 2A, 2C). FTIR spectrum of the conjugate in a cell free system also indicated successful docking via masking of the hydrogen bond forming groups of AA. However, amide-I and amide -A peaks of PPARα remained intact in the conjugate indicating maintenance of protein architecture (Figure 2B). Further evidences for AA as a potential agonist of PPARα came through molecular docking studies where the former occupied identical binding pocket as reported for established agonists such as Ilprost, TIPP-703 (40, 41) as well as fenofibrate (42) which is a well-known PPARα activator exerting its effect in ailment of cardiac fibrosis (18, 19) (Figure 2D). Moreover, higher affinity of PPARα to AA compared to the other agonists as estimated by their free energy of binding suggests that AA might act as an even better agonist (Supplementary Table 1). Simulation outcomes further confirmed AA-driven stabilization of PPARα via persistence of strong hydrogen bonding network between AA and the LBD of PPARα (Figure 2E). In many previous reports, the agonistic potential had been attributed to stabilization of helices in this region (31, 43, 44), which is also observed in our case through RMSF analysis (Figure 2E-i). Altogether, these data suggested that AA-driven stabilization of PPARα could shift the equilibrium of PPARα toward the active
configurations resulting in higher interactions with different co-regulators, thus augmenting activation of PPARα-driven transcriptional machinery (45, 46). Presence of PPRE, the putative PPARα binding site on PPARα promoter suggests that PPARα might regulate its own transcription (47). ChIP analyses coupled to qRT-PCR revealed AA-induced increased binding of PPARα upon PPRE within the PPARα promoter during hypertrophy and the resultant PPARα transcriptional activation increased PPARα mRNA and protein expression during AA treatment in hypertrophy in an autogulatory loop (Figure 3A-B). Time-dependent rise in PPARα expression in AA treated hypertrophied cells further confirmed AA-mediated PPARα agonism during hypertrophy (Figure 3C).

Significant increase in collagen transcription and its extracellular accumulation during PPARα knockdown in AA infused AngII treated fibroblasts compared to AA treated hypertrophied cells implied the importance of PPARα in modulation of collagen transcription in cardiac fibroblasts. Moreover, PPARα knockdown in AA treated ligated rats resulted in increased synthesis and accumulation of collagen with subsequent deterioration of cardiac function that altogether confirms the influence of PPARα in AA mediated cardioprotection during hypertrophy and associated fibrosis (Figure 4A-E, Supplementary Figure S-1A-B). PPARα activation during AA treatment caused inhibition of AngII induced TGF-β signaling by affecting both canonical and non-canonical branches (Figure 5A, Supplementary Figure S-2A). However, this effect might result from reduction in TGF-β level as a secondary response to minimized hypertrophic load. Therefore, fibroblasts were treated with TGF-β instead of AngII in presence or absence of AA and the results proclaimed that AA predominantly affects the non-canonical branch of TGF-β signaling pathway via selectively inactivating TAK1 and not TβRI, TβRII or SMADs. PPARα knock down in TGF-β treated fibroblasts during AA treatment restored TAK1 phosphorylation compared to AA infused TGF-β treated fibroblasts further confirming the specificity of AA towards PPARα mediated deactivation of non-canonical TGF-β signaling (Figure 5B, Supplementary Figure S-2B).

AA-mediated inactivation of TAK1 and downstream proteins namely IKKβ/NF-κBp65, p38 MAPK and JNK also suggested significant involvement of PPARα during AA treatment in hypertrophy groups (Figure 6A, Supplementary Figure S-2C). Knockdown experiments revealed remarkable contribution of TAK1 as well as NF-κBp65 and p38 MAPK, in promoting collagen transcription in AngII treated fibroblasts in vitro. JNK on the other hand showed no effect upon collagen synthesis at hypertrophic stimulus to cardiac fibroblasts in vitro (Figure 6B). Our group and also others have earlier shown the involvement of p38 MAPK (48, 49) and NF-κBp65 (50, 51) in cardiac collagen biosynthesis. In addition to that, analysis of luciferase activity of col1a1 promoter containing NF-κBp65 binding site also revealed the role of NF-κBp65 as a transcriptional activator of collagen promoter during hypertrophy which could be targeted for inhibition by AA driven PPARα activation (Figure 6C). Interestingly, PPARα-mediated downregulation of non-canonical TGF-β axes during AA treatment in hypertrophy samples was associated with decreased phosphorylation of TAK1, without any alteration in its total level (Figure 5A-B). This clearly indicates that changes in TAK1 activation during AA treatment in hypertrophy samples results from post translational modifications and is not related to PPARα-mediated regulation of gene transcription. Reports stating PPARs’ role in modulating signaling pathways by interacting with other signal intermediates (52, 53, 54) prompted us to check whether PPARα directly interacts with TAK1. Successful docking between PPARα and TAK1 as revealed by in silico analyses (Figure 7A) was further validated by FRET-FRAP assay in fibroblasts (Figure 7B). Additionally, Co-IP data showing significantly higher interaction between PPARα and TAK1 in AA treated hypertrophied cells as well as in PPARα overexpressed hypertrophied cells compared to AngII treated cells also suggested that the level of PPARα-bound TAK1 is directly proportional to the availability of PPARα (Figure 7C).

Analyses of domain specificity for PPARα and TAK1 interaction in AngII treated fibroblasts revealed that ‘AF-1’ domain of PPARα contributes maximally to the binding strength of PPARα with TAK1 when compared to that of full length PPARα (‘F’) (Figure 8A, Supplementary Figure S-5A). The best fit cluster of PPARα::TAK1 interaction model in our bioinformatic analyses also identified highest
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number of interacting amino acids from ‘AF-1’ domain of PPARα (Supplementary Table 2A). Interestingly, in silico data suggests that TAK1 interacts with PPARα via amino acids (aa) belonging to its kinase domain (36-291 aa in rat-TAK1) which also contains TAK1 phosphorylation sites (Thr 184/187) (Figure 7A, Supplementary Table 2A). This further indicates that PPARα::TAK1 interaction inactivates TAK1 possibly by preventing its phosphorylation.

Wet lab experiments confirmed that, higher the binding between different PPARα domains and TAK1 in AngII treated fibroblasts, greater is the amount of regression in TAK1 phosphorylation with subsequent downregulation of non-canonical TGF-β axes (Figure 8B, Supplementary Figure S-5B). This finding also corroborates to inactivation of non-canonical branch of TGF-β signaling (Figure 5A, 6A) during AA treatment in hypertrophy samples resulting from AA-induced PPARα upregulation (Figure 3A-B) and increased interaction between PPARα and TAK1 (Figure 7C). Degree of regression of collagen synthesis as observed by overexpression of different PPARα domains into hypertrophied fibroblasts also followed the similar trend revealing maximum regression by ‘AF-1’ overexpression (Figure 8C). An earlier report suggested that deletion of AF-1 region of PPARα did not affect its function keeping its DNA binding activity unaltered (55). However, our study shows that ‘AF-1’ region of PPARα promotes PPARα::TAK1 protein-protein interaction resulting in suppression of non-canonical TGF-β pathway and reduced collagen gene transcription during cardiac hypertrophy. H region (Helix-1) of PPAR-γ has been reported to bind with cytosolic kinases such as ERK5 (52) and PKC-α (53) in other cell types. Although significantly lower than ‘AF-1’, interaction of ‘H+LBD’ of PPARα with TAK1 also had a significant role in regression of non-canonical TGF-β pathway induced collagen synthesis during hypertrophy.

In summary, our work for the first time shows a precise mechanism of arjunolic acid (AA) action in regression of cardiac hypertrophy associated fibrosis and subsequent betterment of cardiac function. This work assigns a new role of AA as a PPARα agonist and such transcriptional activation and increased expression of PPARα during aforesaid pathophysiological condition inactivates the non-canonical TGF-β axes (Figure 9A). Specific molecular mechanism involves direct interaction of PPARα, predominantly by its N terminal ‘AF-1’ domain with TAK-1 kinase domain, leading to decreased phosphorylation of TAK1 and its downstream signal intermediates ultimately inhibiting excess collagen transcription during hypertrophy (Figure 9B).

TGF-β signaling is one of the key mediators for maintenance of normal cellular function and homeostasis of cardiac fibroblasts (56). As TAK1 resides at a moderately downstream position in TGF-β axes, inhibiting TAK1 by using AA as an anti-fibrotic agent might be therapeutically advantageous. Further detailing of PPARα binding surface in TAK1 or influence of TABs in such interaction would be necessary for better understanding of the regulation of TAK1 autoactivation and its role in ECM turnover. Not only that, transition of such a natural compound from initial screening through preclinical and clinical trials to finally marketable drug form is associated with challenging demands for the plant source. Therefore, organic synthesis of AA bio-mimetic might be beneficial considering the wide range of protective effects exerted by AA with enhanced clinical significance against such deadly disease.

**Experimental Procedures:**

**Animals used:**

28-week old male Wistar rats (Rattus norvegicus; Taxonomy ID: 10116) (n=7 per experimental group), used in this study were procured from National Institute of Nutrition, Hyderabad, AP, India. The investigation conforms with the *Guide for the Care and Use of Laboratory Animals* published by the US National Institute of Health (NIH Publication No. 85-23, revised 1996) and was also approved by the Institutional Animal Ethics Committee, University of Calcutta (Registration # 885/ac/05/CPCSEA), registered under “Committee for the Purpose of Control and Supervision of Experiments on Laboratory Animals” (CPCSEA), Ministry of Environment and Forests, Government of India.

**Isolation and culture of adult cardiac fibroblasts:**

Adult cardiac fibroblast cells were isolated from 28-week-old male Wistar rat hearts by the collagenase dispersion method. Briefly, animals were euthanized in a prefilled Carbon dioxide (CO₂) chamber with 100% concentration of CO₂, and the hearts were chopped and digested by collagenase (80 units/ml DMEM; Worthington). The cells were pelleted by centrifugation and
resuspended in fresh complete DMEM supplemented with 10% fetal bovine serum and plated in cell culture flask. Cells were maintained at 37°C with 5% CO₂ and were subsequently passaged (49).

**Generation of cardiac hypertrophy in vitro and in vivo:**
75–80% confluent serum-starved adult cardiac fibroblasts were treated with 10⁻⁸ mol/liter (Sar1)-Angiotensin II (AngII) (Bachem, Torrance, USA) for 24 hrs to generate cardiac hypertrophy model in vitro. AngII was replenished every 6 hrs throughout the incubation period (49).

*In vivo* cardiac hypertrophy model was generated by ligating right renal artery of 28-week-old male Wistar rats (250–300 g) as described earlier (49). Rats were anesthetized with an intraperitoneal (i.p) injection of a mixture of ketamine (80 mg/kg) and xylazine (12 mg/kg). Sham-operated control group underwent a similar procedure without renal artery ligation. Animals were maintained in optimum condition for 14 days and were sacrificed on the 15th day after surgery. Hearts were taken out, collected in liquid N₂ and stored in - 80°C for future use.

Hypertrophy was measured by the heart weight (HW in mg) to body weight (BW in g) ratio and activation of hypertrophy marker genes (*anf, β-mhc and actα1*) by RT-PCR analyses (57).

**Measurement of cardiomyocyte cross-sectional area (CSA):**
To analyse cardiomyocyte cross-sectional area as a marker of hypertrophy, hearts were excised, washed in 1X PBS, and fixed in Karnovsky’s fixative. Then, samples were paraffin-embedded and cut into 4–5 μm sections. Tissue sections were processed and stained with hematoxylin and eosin (H&E) and cardiomyocyte cross sectional area in each sample was quantified (57) using a computer morphometric program (ImageJ, NIH).

**Evaluation of collagen deposition:**

**Hydroxyproline Assay:**
Hydroxyproline assay was performed to measure total secreted collagen content in fibroblast culture supernatant in vitro and total left ventricular collagen content from rat heart tissues. With the help of standard curve, hydroxyproline content in the unknown samples were calculated. The amount of collagen was estimated by multiplying hydroxyproline content by a factor of 8.2 (49).

**Estimation of percentage of collagen volume fraction (%CVF):**

Heart tissues from each group of animals were taken for the analysis %CVF. Coronal sections obtained from the equator of the left ventricle were fixed and stained with Massons’ Trichrome (Sigma-Aldrich, St. Louis, MO) to analyse collagen deposition under microscope (Nikon NIS BR) (Nikon, Shinagawa, Tokyo, Japan) following standard protocol. Fifty sections were scanned and at least 10 images were captured from each section. Images were digitized and processed by ImageJ, a computer morphometric program. CVF was calculated as the sum of all collagen stained tissue areas of the coronal sections represented as percentage (%) of the total surface of the section. Colour segmentation was applied to separate the stained tissues from other nonspecific objects (58).

**Determination of cardiac function:**
Cardiac function of lightly sedated animals from sham operated control group, renal artery ligated group, AA treated hypertrophied rat group and PPARα siRNA infused AA treated ligated rat group were measured by M-mode echocardiographic analysis by a transthoracic study in a short axis view at papillary muscle level on the 15th day before euthanization. *In vivo* ventricular PPARα siRNA injection was also guided by echocardiography. Digitized images were obtained using an ultrasound system (Vivid S5 system, GE Healthcare) for the calculation of fractional shortening (%FS); left ventricular internal diastolic dimension (LVIDd); left ventricular anterior wall diameter in diastole (LVAWd); left ventricular anterior wall diameter in systole (LVAd); left ventricular posterior wall diameter in diastole (LPWd); end diastolic volume (EDV); end systolic volume (ESV); stroke volume (SV) and ejection fraction (EF) following standard guidelines (58).

**Treatment of fibroblasts with TGF-β:**
Another set of adult cardiac fibroblasts were treated with active TGF-β-2 (Life Technologies, Waltham, MA) at a dose of 8 ng/ml of serum free media for 16 hrs as described previously (59) to induce hypertrophic effect. TGF-β treated cells were pretreated with AA with or without simultaneous treatment of PPARα siRNA following standard protocol.

**Treatment with arjunolic acid (AA) in vitro and in vivo:**
Arjunolic Acid (AA) was extracted and purified from core wood of the plant *Terminalia arjuna* (14). *In vitro* hypertrophied fibroblasts were treated with 20 μM of AA (dissolved in DMSO) 6 hrs after first AngII treatment. Effective dose was chosen from a dose-dependent study (5 – 100 μM of AA) based on optimal results with minimum mortality. A group of control fibroblasts were also treated with 20 μM of AA. All other control and AngII treated cells were also treated with equivalent amount of DMSO. Time-dependent changes upon AA treatment were analyzed in AngII treated cells at 3 hrs and 6 hrs after induction with AA.

A set of TGF-β treated cells were also treated with 20uM AA 6 hrs after TGF-β treatment. *In vivo* renal artery ligated rats were treated with AA, dissolved in DMSO by intraperitoneal injection on every alternate day from day 6 upto day 14 at a dose of 10 mg/kg/day after ligation. Effective dose was chosen after a dose-dependent study (5 mg to 50 mg/kg/day as described above) based on optimal results. Untreated sham operated rats and renal artery-ligated rats were also treated with equivalent amount of DMSO. Animals were sacrificed after 14 days. Commercially available arjunolic acid (#SMB00119; Sigma-Aldrich, St. Louis, MO) was also used at similar dosage showing uniform results (data not shown).

**Cell viability assay:**
Cell viability and cytotoxicity in presence or absence of AA in AngII treated fibroblasts were determined colorimetrically by Cell titer 96® AQUeous One Solution Cell Proliferation Assay (Promega, Madison, WI) following manufacturer’s protocol.

**Administration of siRNAs:**
For *in vitro* experiments, cardiac fibroblasts were treated with 100nm of PPARα siRNA (#S130650, Silencer® select siRNA, Ambion; Thermo Fisher Scientific, Waltham, MA), TAK1 siRNA (#S10095939, Qiagen, Hilden, Germany), NF-κBp65 siRNA (#S103097885, Qiagen, Hilden, Germany), p38 MAPK siRNA (#6243, Cell Signaling Technology, Dannvers, MA), JNK siRNA (#6233, Cell Signaling Technology, Dannvers, MA) or negative control siRNA (NS siRNA) (All Stars Negative Control siRNA, #S103650325, Qiagen, Hilden, Germany) using HiPerfect transfection reagent (Qiagen, Hilden, Germany) as per manufacturer’s protocol. 18 hrs after transfection, cells were used for AngII treatment with or without AA treatment. For *in vivo* experiments PPARα siRNA (#S130650, Ambion® *In vivo* siRNA, Thermo Fisher Scientific, Waltham, MA) in RNase free sterile PBS was injected directly into ventricles of ligated animals (49) that are to be treated with AA with simultaneous echocardiographic guidance, following the manufacturer’s instructions at a concentration of 10 nmol on every alternate day from the 5th day of ligation before sacrifice. Sham-operated and renal artery ligated rats were also treated with equivalent amount of DMSO along with *in vivo* NS siRNA (#4457287, Ambion® *In vivo* Negative Control siRNA, Thermo Fisher Scientific, Waltham, MA). Ligated rats to be treated with AA were also pretreated with equivalent amount of NS siRNA.

**Structural modeling of PPARα and TAK1:**
The amino acid sequence of *Rattus norvegicus* PPARα (P37230) and TAK1 (P0C8E4) were retrieved from the UniProtKB database (http://www.uniprot.org/). The retrieved amino acid sequence of PPARα and TAK1 were subjected to a protein-protein BLAST (BLASTp) search against the protein data bank (PDB) to identify a suitable template structure for comparative modeling. The 3D structure predictions of these two proteins were then performed using I-TASSER (60). For rat-PPARα, PDB ID: 4BCR_A was selected as the best suited template with 99% identity and 45% sequence coverage (E-value 0.0). For rat-TAK1, PDB ID: 5JGA_A was selected as a suitable template with 93% identity and 58% sequence coverage (E value 5×10^-170). The best I-TASSER outputs were refined using ModRefiner (61). The I-TASSER Z-scores were analysed to check the likelihood that the domains had correct folds and correct overall tertiary structure. The final model was also checked for its quality using ProQ2 (62). Further, refined structures were subjected for MD simulation.

**All atomistic molecular dynamics (MD) simulation:**
To check for conformational stability, MD simulation was done using GROMACS 4.6.1 (63). All atomistic simulations were carried out using the CHARMM36 all-atom force field (November release) (64, 65, 66) using periodic boundary condition. The starting model was solvated in a periodic box with TIP3 water model.
9 Na\(^+\) ions were added to the solvent to neutralize electrical net charge of the protein. The system was then minimized for 50000 steps using a steepest decent algorithm with emtol of 200 kJ/mol after a minimization with emtol of 100 kJ/mol. This was followed by an equilibration run of 100ps in NVT ensemble with restraints on the protein atoms. The NPT ensemble was used for production simulation. Systems were simulated at 310K, maintained by a Berendsen thermostat with a time constant of 1 ps with the protein and non-protein molecules coupled separately. Pressure coupling was done employing a parrinello-rahman barostat using a 1 bar reference pressure and a time constant of 2.0 ps with compressibility of 4.5e\(^{-5}\) bar using isotropic scaling scheme. Electrostatic interactions were calculated using the Particle Mesh Ewald (PME) summation with 2 fs time step for each run. For simulations of PPARα::AA complex, identical parameters were employed. Topology for AA (PubChem ID: CID73641) and other parameters were assigned using SwissParam (67).

**Three dimensional structural modeling of the chemical compounds:**
The 2D structures of AA (PubChem ID: CID73641), fenofibrate (PubChem ID: CID3339) were retrieved from the NCBI PubChem database. The compounds were drawn using ChemDraw 8 software. The two-dimensional molecules were then converted to three-dimensional structures using OpenBabel (68). The energy minimization was performed using Maestro 9.8 (Schrödinger, LLC, New York).

**Molecular docking:**
Docking of AA to PPARα was performed using the BSP-SLIM tool (69). Briefly, the holo-structures with similar global topology to PPARα were identified and geometric centers of bound ligands in the holo-forms were clustered to identify putative AA-binding sites. Subsequently, shape and chemical feature comparison of multiple target ligand conformers with all negative images of binding pockets was done employing the OMEGA program. Best overlay for each ligand conformer with the negative images was carried out using OEChem toolkit v.1.7 while the chemical features of AA were assigned using the Implicit-MillsDean color force field (70). Finally, all ligand conformations were sorted by docking scores with a RMSD tolerance limit of 4 Å.

The top lowest energy binding complex was re-ranked by performing flexible docking using Molecular Operating Environment (MOE; v2009.10). The triangular matching placement method with the Affinity dG scoring function was employed to generate most favorable poses of ligand conformations by aligning ligand triplets of atoms with triplets of receptor site points. AA::PPARα Docking scores were compared to the score obtained for known agonists of PPARα i.e. Iloprost (PDB ID: 3SP6) and TIPP-703 (PDB ID: 2ZNN) and fenofibrate (Obtained through pre-docking by BSP-SLIM). On the other hand in silico binding affinities were calculated using AutoDock Vina 1.1.2 (71) extension of UCSF chimera (72). For protein-protein docking, residues forming the interface surfaces were predicted. CPORT program drew a consensus for the interface residues using PIER, cons-PPISP, SPPIDER and PINUP. The consensus residues were mapped onto the model. The predicted interface residues were submitted to the PPARα and TAK1 monomer model for protein-protein docking to build the hetero-dimer model, using HADDOCK webserver (73, 74). Molecular graphics and analyses were performed with the UCSF Chimera (72).

**Fluorescence and circular dichorism (CD) spectroscopy:**
Fluorometric titrations were carried out in LS55 Fluorescent spectrometer (PerkinElmer, Waltham, MA). At constant PPARα (in vitro translated and purified) protein concentration of 8 μM, titrations were carried with increasing concentrations of AA up to 100 μM with a pre-scan delay of 300 secs. Incubated samples were excited at 274 nm due to absence of tryptophan and emission spectra were recorded from 290 nm to 400 nm with excitation/emission slits set 10/10.

CD titrations were carried out in J-1500 Circular Dichroism Spectrophotometer (Jasco, Easton, MD). Far UV CD titrations were carried out following similar protocol in a 2 mm path length quartz cuvette and scanned from 250-200 nm at scan rate of 200 nm per minute.

**Fluorescence-based thermal shift (FTS) assay:**
FTS assay for PPARα was done to assess tertiary structure stabilization by monitoring change in Tyr emission at 330 nm over the temperature range from 25 to 90 °C with step size of 2 °C.

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Excitation and emission slits were kept at 5/10 with an equilibration time of 1 min at each step. Thermal denaturation of PPARα (8 μM) was done both in absence or presence of AA (100 μM). AA incubation with PPARα was done 30 mins prior to the experiment. To assess secondary structure stabilization of PPARα by AA, change in both ‘θ218’ and ‘θ222’ were monitored against temperature range of 25 to 90°C at ramping rate of 1°C per minute.

In vitro coupled transcription-translation and in vitro interaction assay:

Full length PPARα, cloned in pCDNA6/V5 His B mammalian expression plasmid (Invitrogen, Waltham, MA) was subjected to in vitro coupled transcription–translation using TNT® Quick coupled Transcription/Translation system (Promega, Madison, WI) according to the manufacturer’s specifications. Briefly, 2 μg circular plasmids were added to the rabbit reticulocyte lysates in 50 μl reactions for 60–90 mins at 30 °C. These 6xHis-tagged products were then purified using Ni-NTA spin kit (Qiagen, Hilden, Germany) in native condition and analyzed by SDS-poly-acrylamide gel electrophoresis (SDS-PAGE) followed by coomassie blue staining to check the purity (data not shown). Purified products were then subjected to in vitro interaction in binding buffer overnight on a rocker at 4 °C with AA (59). This complex along with the purified protein was used for further assay.

Fourier transform infra-red (FTIR) spectroscopy:

FTIR spectra were obtained with a Jasco FTIR 6300 spectrometer (Jasco, Easton, MD). The spectra presented were obtained from an average of 64 scans. The resolution of the spectra was 4 cm⁻¹. The spectra were acquired at room temperature. All data analyses were performed with the data analysis software Origin (OriginLab, Northampton, MA) provided with the FTIR instrument.

RNA isolation:

Total RNA from cells (24 hrs treatment) and tissues (on the 15th day after surgery) was isolated using TRIzol reagent (Invitrogen, Waltham, MA) following manufacturer’s instructions.

RT-PCR and qRT-PCR:

1 μg of total RNA was used to make cDNA using the cloned avian myeloblastosis virus (AMV) first-strand cDNA synthesis kit (Invitrogen, Waltham, MA). RT-PCR was done for col-1, col-3, anf, β-mhc, acta1 and ribosomal protein L32 (rpl 32). Rpl32 was used as loading control. Relative quantification of PCR-amplified products was also done by qRT-PCR with Power SYBR Green™ PCR Master Mix using Applied biosystems StepOne® Real-Time PCR System (#4369074, Applied Biosystems, Waltham, MA) for col-1, col-3, ppara and rpl32, where rpl32 was used as a reference gene to normalize expression of other genes. Relative gene expression was quantified by comparative “ct” (2–ΔΔct) method (49). Both RT-PCR and qRT-PCR primer sequences are summarized in Supplementary Table 3A-B.

Chromatin immunoprecipitation (ChIP) assay:

ChIP analysis was performed with modifications from the methodology described by Kouskouti et al (75). Briefly, cardiac fibroblasts and cardiac tissue sections were treated with 37% formaldehyde (final concentration 1%) for crosslinking proteins to DNA. Crosslinking was stopped by adding 10X Glycine (final concentration 0.125 M) after 10 mins and mixed at room temperature. Cardiac fibroblasts were now scraped out in ice cold 1X PBS with protease inhibitor cocktail (PIC) (#P8340; Sigma-Aldrich, St. Louis, MO). Cardiac tissue samples with crosslinked chromatin were also taken in ice cold 1X PBS with PIC and were thoroughly minced with Dounce homogenizer. Both in vitro and in vivo samples were now centrifuged at 1500 rpm for 5 mins at 4 °C and the pellet were resuspended in sonication buffer (50mM HEPES, 140 mM NaCl, 1 mM EDTA, 1% TritonX, 0.1% Sodium deoxycholate and 0.1% SDS; pH=7.9). The samples were now sonicated and centrifuged to obtain supernatants containing 500-800 bp sized chromatin fragments. For each reaction, 400 µg of purified chromatin was pre-cleared with protein G sepharose bead (#P3296; Sigma-Aldrich, St. Louis, MO) and immunoprecipitated overnight at 4 °C either by anti-PPARα antibody or anti-IgG antibody (used as a negative control) (antibodies listed in Supplementary Table 4). Further, antibody-bound chromatin complexes were washed using low and high salt wash buffers followed by Tris-EDTA (TE) buffer wash, all at 4 °C. Protein-DNA complexes were eluted from beads by elution buffer (50 mM NaHCO₃, 50 mM Tris, 1 mM EDTA and 1% SDS) for 10 mins at 65 °C. To separate protein from DNA, samples were treated with 21 µl of 4 M NaCl at 65 °C overnight. Further purification
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DNA was extracted from each sample for ChIP coupled qRT-PCR (primers listed in Supplementary Table 3B). Data analysis was done by “Fold Enrichment Method” where ChIP signals are normalized by the IgG signals.

Plasmid construction, transfection:
Coding DNA sequences of *Rattus norvegicus* full length PPARα (Reference Sequence: NM_013196.1) (F; 1-1406 nucleotides, 1-478 amino acids) along with its domains; N-terminal transactivation domain (AF-1; 1-297 nucleotides, 1-99 amino acids), DNA binding domain (DBD; 298-520 nucleotides, 100-173 amino acids) and hinge region followed by C terminal ligand binding domain (H+LBD; 521-1406 nucleotides, 174-478 amino acids) were amplified from extracted RNA by RT-PCR with respective primers (Supplementary Table 3C) and cloned in frame into pCDNA6/V5 HisB mammalian expression vector (Invitrogen, Waltham, MA) with C-terminal His-Tag as described previously (59). All the clones were confirmed by sequencing (Applied Biosystems™, 3730 DNA Analyzer, Waltham, MA). Plasmids were transfected to AngII treated adult cardiac fibroblasts with the help of TurboFect transfection reagent (Thermo Fisher Scientific, Waltham, MA) as per manufacturer’s protocol. Protein extracts were prepared from ventricular tissue following the procedure described previously (77).

Western Blotting:
Western blot analyses were done to study protein expressions both *in vitro* and *in vivo* as described previously (77). Briefly, 30 μg protein samples were incubated in 10–12.5% SDS-PAGE and transferred to PVDF membrane. After blocking with 5% nonfat dry milk, membranes were incubated with polyclonal antibodies to TβRI, TβRII (Santacruz biotechnology, Dallas, TX), total TAK1 (Abcam, Cambridge, UK), phospho (Thr 184/187) TAK1, phospho (Thr 180/Tyr182) and total p38 MAPK, total SMAD 3 and phospho (Thr 183/Tyr185) JNK (Cell signaling technology, Danvers, MA) ; monoclonal antibodies to PPARα (Abcam, Cambridge, UK), phospho (Ser 465/467) and total SMAD 2, phospho (Ser 423/425) SMAD 3, phospho (Ser 536) and total NF-κBp65, total JNK, GAPDH (Cell signaling technology, Danvers, MA), phospho (Tyr 199) and total IKKβ (Abcam, Cambridge, UK) and Horse radish peroxidase (HRP) conjugated secondary antibodies (Pierce, Waltham, MA). Specifications of the antibodies used are summarized in Supplementary Table 4. Immunoreactive bands were visualized using Immobilon™ Western chemiluminescence HRP substrate (Millipore, Billerica, MA). List of antibodies are summarized in Supplementary Table 4. The blots were scanned and quantitated using GelDoc XR system with Quantity One® software version 4.6.3 (Bio-Rad, Hercules, CA).

Co-immunoprecipitation (Co-IP) assay:
Co-IP was done following manufacturer’s protocol (Pierce Co-IP Kit, Thermo Fisher Scientific; Waltham, MA). Briefly, proteins were incubated with fast flow protein G or protein A sepharose beads and centrifuged to eliminate...
nonspecifically bound proteins (59). 200 µg of precleared protein were immunoprecipitated using primary antibody. Immunoprotein complex was again incubated with protein G (#P3296, Sigma-Aldrich, St. Louis, MO) or protein A (#P3391; Sigma-Aldrich, St. Louis, MO) sepharose beads. Attached proteins were then eluted from beads in 1% SDS buffer followed by immunoblotting. In this study, immunoprecipitation was done using primary antibodies for anti-PPARα or anti-His (antibodies are listed in Supplementary Table 4). For immunoprecipitation with anti-His, proteins were purified in nickel column, as described previously for in vitro protein purification above. Western blotting was done using monoclonal antibody against TAK1. Normalization was done by immunoblotting using the same antibodies used to immunoprecipitate the proteins.

Fluorescence recovery after photobleaching (FRAP):

For endogenous PPARα and TAK1 interaction, cells were treated with PPARα-TRITC and TAK1-FITC. As TRITC and FITC work as a donor-acceptor in FRAP assay, TRITC was subjected to 50% bleaching and FITC intensity in a single cell was measured. Fluorescence resonance energy transfer (FRET) efficiency was calculated in confocal microscope (Olympus FV1200, Olympus, Singapore) (76).

Statistical analysis:

All the results were expressed as mean ±S.E. of three independent experiments. Data were analyzed by independent sample t-test by SPSS (Version 14.0). For multiple comparison experiments, one way analysis of variance (ANOVA) was conducted followed by Tukey's post-hoc test. Results with p value <0.05 were considered significant.

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Conflict of Interest: None declared.

Author contributions: SS1 conceived the idea of the project. TB1 designed and conducted most of the experiments, analyzed the results and wrote the paper with SS1. EC1 assisted in most experiments with animals. JS2 and BK2 did most of the bioinformatic and biophysical studies and analysed the data. AR3 conducted protein modeling and protein-protein docking and analysed the data with SS3. VT4 did extraction and purification of arjunolic acid used in the study.

References:

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Footnotes: The abbreviations used are,
Peroxisome Proliferator-Activated Receptor Alpha, PPARα; Transforming Growth Factor beta, TGF-β; TGF-β Activated Kinase 1, TAK1; Angiotensin II, AngII; Arjunolic acid, AA; Mitogen Activated Protein Kinase, MAPK; Mitogen Activated Protein Kinase Kinase, MKK; Nuclear Factor kappa B, NF-kB; Renin-Angiotensin System, RAS; Transverse Aortic Constriction, TAC; TGF-β Receptor-I , TβRI; TGF-β Receptor-II , TβRII; Suppressor of Mothers Against Decapentaplegic, SMAD; TAK1 Binding Protein, TAB1; Inhibitor of kappab Kinase, IKK; c-Jun N terminal Kinase, JNK; Extracellular matrix, ECM; collagen-1, col-1; collagen-3, col-3; ribosomal protein L32, rpl32; atrial natriuretic factor, anf; beta myosin heavy chain, β-mhc; skeletal alpha actin, acta1; Percentage of Fractional Shortening, %FS; Left ventricular Internal Diastolic dimension, LvIDd; Left ventricular Anterior Wall Diameter in diastole, LvAWDd; Left ventricular Anterior Wall Diameter in systole, LvAWDs; Left ventricular Posterior Wall Diameter
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in diastole, LvPWDd; Left ventricular Posterior Wall Diameter in systole, LvPWDs; End Diastolic Volume, EDV; End Systolic Volume, ESV; Stroke Volume, SV; Ejection Fraction, EF; Protein Data Base, PDB; Dimethyl Sulphoxide, DMSO; Non Specific siRNA, NS siRNA; Collagen Volume Fraction, CVF; Fluorescence resonance energy transfer, FRET; Fluorescence recovery after photobleaching, FRAP; Full length PPARα, F; N terminal transactivation domain, AF-1, DNA Binding Domain, DBD; Hinge region, H; Ligand binding Domain, LBD; Fourier transform infrared spectroscopy, FTIR; PPAR Response Element, PPRE; Dulbecco’s Modified Eagle Medium, DMEM; Carbon dioxide, CO₂; Melting temperature, Tₘ; Binding Site Prediction and Shape-based Ligand Matching, BSP-SLIM; Root Mean Square Deviation, RMSD; Gyration radius, Rg; Root Mean Square Fluctuation, RMSF; Chromatin Immunoprecipitation, ChIP; Reverse Transcription-Polymerase Chain Reaction, RT-PCR; Quantitative Real time Reverse Transcription-Polymerase Chain Reaction, qRT-PCR; Nitrogen, N₂; Hematoxylin and eosin, H&E; Basic Local Alignment Search Tool, BLAST; Sodium Dodecyl Sulfate-Poly-Acrylamide Gel Electrophoresis, SDS-PAGE; Ethylene-Diamine-Tetra-Acetic acid, EDTA; Phosphate Buffered Saline, PBS; Tris(hydroxymethyl)aminomethane, Tris; 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid, HEPES.

Figure Legends:

Figure 1: Arjunic acid (AA) regresses collagen expression and improves cardiac function during cardiac hypertrophy:
A. RT-PCR analyses showing significant increase in collagen-1(col-1) & collagen-3(col-3) gene expressions in AngII treated adult cardiac fibroblasts in vitro compared to control fibroblasts. AA infused AngII treated cells showed significant decrease in col-1 and col-3 gene expressions compared to hypertrophied fibroblasts. Rpl-32 was used as internal loading control. Control cells treated with either DMSO or AA yielded similar results. AngII treated cells were also treated with equivalent amount of DMSO. n=10 for each experimental group. All the results were expressed as ±S.E. of three independent experiments. Representative graphs showing relative alterations in collagen gene expressions among different experimental groups. ***, p<0.001 with respect to DMSO treated control cells; ###, p<0.001 with respect to AngII treated cells.

B. RT-PCR analyses showing significant increase in col-1 and col-3 gene expressions in right renal artery ligated rat heart compared to sham operated control rat group. AA treatment in ligated animals showed significant decrease in col-1 and col-3 gene expressions compared to ligated animals. Rpl-32 was used as internal loading control. Sham operated control animals and renal artery ligated animals were also treated with equivalent amount of DMSO. n=7 for each experimental group. All the results were expressed as ±S.E. of three independent experiments. Representative graphs showing relative alterations in collagen gene expressions in different in vivo experimental groups. **, p<0.01 with respect to sham operated control rat group; ***, p<0.001 with respect to sham operated control rat group; ##, p<0.01 with respect to renal artery ligated rat group; ###, p<0.001 with respect to renal artery ligated rat group.

C. Graphical representation of in vitro hydroxyproline assay showing significantly increased collagen content in AngII treated fibroblast culture supernatant compared to control cells. AA treatment in AngII treated fibroblasts showing significant decrease in the collagen content in the culture supernatant compared to AngII treated cells. Control cells treated with either DMSO or AA yielded similar results. AngII treated cells were also treated with equivalent amount of DMSO. n=10 for each experimental group. Results were expressed as ±S.E. of three independent experiments. *, p<0.05 with respect to control cells; #, p<0.05 with respect to Ang II treated cells.

D. Graphical representation of in vivo hydroxyproline assay showing significantly increased total left ventricular collagen content in ligated rat hearts compared to sham operated control hearts. AA treatment in ligated rat hearts showed decreased total left ventricular collagen content compared to ligated rat heart. Equivalent amount of DMSO were administered into sham operated and renal artery ligated rat group. n=7 for each experimental group. Results were expressed as ±S.E. of three independent experiments. **,
p<0.01 with respect to sham operated control rat group; #, p<0.05 with respect to renal artery ligated rat group.

E. Graphical representation of heart (HW/BW in mg/g) ratio showing significant increase in renal artery ligated rat group compared to sham operated control rat group. AA treatment in ligated animals showed significant downregulation of HW/BW ratio compared to hypertrophied rat group. Sham operated and renal artery ligated rat group were also treated with equivalent amount of DMSO. n=7 for each experimental group. Results were expressed as ±S.E. of three independent experiments. **, p<0.01 with respect to sham operated control rat group; ##, p<0.01 with respect to renal artery ligated rat group.

F. Graphical representation of CSA measured from H&E stained heart tissue sections showing significant upregulation in renal artery ligated rat heart compared to sham operated control rat group. AA treatment in ligated animals showed significant downregulation of CSA compared to ligated rat group. Sham operated and ligated rats were also treated with equivalent amount DMSO. n=7 for each experimental group. Results were expressed as ±S.E. of three independent experiments. ***, p<0.001 with respect to sham operated control rat group; ###, p<0.001 with respect to renal artery ligated rat group.

G. RT-PCR analyses showing significant increase in hypertrophy marker gene expressions namely anf, β-mhc and acta1 in renal artery ligated rat hearts compared to sham operated control hearts. AA treatment in ligated animals showed significant downregulation of all these gene expressions compared to ligated rat hearts. Sham operated control and renal artery ligated animals were also treated with equivalent amount of DMSO. Rpl-32 was used as internal loading control. n=7 for each experimental group. Results were expressed as ±S.E. of three independent experiments. Representative graphs showing relative alterations in the hypertrophy marker gene expressions among different experimental groups. ***, p<0.001 with respect to sham operated control rat group; **, p<0.01 with respect to sham operated control rat group; ###, p<0.001 with respect to renal artery ligated rat group; ##, p<0.01 with respect to renal artery ligated rat group.

H. M-mode echocardiographic analyses from parasternal long axis view at papillary muscle level graphically representing decreased %FS and increased LVIDd in renal artery ligated rat group compared to sham operated control rat group. AA treatment in ligated animals showed restored %FS and lowered LVIDd in ligated rats compared to the hypertrophied rat group. Sham operated control and renal artery ligated animals were also treated with equivalent amount of DMSO. n=7 for each experimental group. Results were expressed as ±S.E. of three independent experiments. **; p<0.01 with respect to sham operated control rat group, ##; p<0.01 with respect to renal artery ligated rat group; #; p<0.05 with respect to renal artery ligated rat group.

Figure 2: Analyses of interaction between AA and PPARα:
A. Fluorescence and CD titrations of AA with PPARα. (i) Titration of 8 μM PPARα with increasing AA concentrations up to 55 μM, monitored by fluorescence spectroscopy. (ii) Titration of 8 μM PPARα with increasing AA concentrations up to 55 μM, monitored by CD spectroscopy. All titrations were carried out in 20 mM Na3(PO)4, 150 nM NaCl, pH 8.0 buffer at 298 K.

B. Overlaid FTIR spectra of free arjunolic acid (AA), free PPARα, and AA::PPARα complex.
C. Thermal melting study: (i) FTS assay to determine melting temperature of PPARα alone (Black) and in presence of AA (Red). (ii) CD Thermal melting of PPARα alone (black) and in presence of AA (Red) probed by monitoring changes θ218 (Alpha helical transitions) and θ222 (Inset, Beta transitions). Normalized data is plotted as percent unfolding induced by temperature increments. All experiments were carried out in 20 mM Na3(PO)4, 150 nM NaCl, pH 8.0 buffer at 298 K.

D. Tertiary structure alignment of PPARα (modelled, Black), bound to AA (Red), Iloprost (Green), TIPP-703 (Blue) and fenofibrate (Purple). Zoomed part shows superposition of AA with other agonists occupying identical ligand binding pocket in PPARα.

E. Projections of MD simulations and thermal melting profiles for PPARα alone (Black) and in bound state with AA (Red). (i-ii) Distributions of Backbone RMSD and Rg averaged over whole 100 ns trajectory for PPARα alone (i) and in presence of AA (ii). Arrows indicate major areas of distribution over two components. (iii) RMSF analyses of PPARα alone and in presence of AA averaged over last 10 ns of trajectory. (iv) H-bond network for whole trajectory showing persistence of H-bonds between PPARα and AA. Inset shows three H-bonds between AA and Thr 283, Leu 331 and Tyr 334 residues.
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Figure 3: AA treatment during hypertrophy increases PPARα expression in an autoregulatory loop:

A. ChIP assay with anti-PPARα antibody followed by qRT-PCR analyses of the PPRE showing relative binding of PPARα to the PPRE within PPARα promoter among different experimental groups both in vitro and in vivo are represented graphically on logarithmic scale. Hypertrophy samples showed significantly downregulated fold enrichment in binding of PPARα to the PPRE within PPARα promoter compared to respective controls. AA treatment in hypertrophy samples further showed significantly increased fold enrichment of the same, compared to hypertrophy samples. PPARα siRNA treatment in AA treated hypertrophy groups showing downregulated fold enrichment in binding of PPARα to the PPRE compared to AA treated hypertrophy samples were used as negative controls. Control and hypertrophy samples in vitro and in vivo were treated with equivalent amount of DMSO and Non-Specific (NS) siRNA. AA treated hypertrophy samples were treated with equivalent amount of NS siRNA. Chromatin from each experimental group immunoprecipitated with anti-IgG antibody were used for normalization. n=3 both in vitro and in vivo. Results were analyzed by one way analysis of variance (ANOVA) followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. **, p<0.01 with respect to control samples; #, p<0.05 with respect to hypertrophy samples; ##, p<0.01 with respect to AA treated hypertrophy samples; ††, p<0.01 with respect to AA treated hypertrophy samples in vitro and/or in vivo.

B. Western blot analyses revealed significant decrease in PPARα protein expression during hypertrophy compared to respective control groups that again showed significant recovery in AA treated hypertrophy samples compared to respective hypertrophy groups in vitro and in vivo. Successful knockdown of PPARα protein expression was also confirmed by PPARα siRNA pretreatment in AA treated hypertrophy samples. GAPDH was used as internal loading control. Control and hypertrophy samples were treated with equivalent amounts of DMSO and NS siRNA. AA treated hypertrophy samples were also treated with NS siRNA. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=10 in vitro, n=7 in vivo for each experimental group. Representative graphs showing relative changes in expression of PPARα among different experimental groups on logarithmic scale. **, p<0.01 with respect to control samples; #, p<0.05 with respect to hypertrophy samples; ##, p<0.01 with respect to hypertrophy samples; ††, p<0.01 with respect to AA treated hypertrophy samples in vitro and/or in vivo.

C. Western blot analyses showing time dependent increase in PPARα expression in AA treated hypertrophied fibroblasts compared to AngII treated cells at respective time points under study. GAPDH was used as internal loading control. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=5 for each experimental group. Representative graphs showing relative changes in PPARα expressions at respective time points among different groups under study. C, control fibroblasts; A, AngII treated fibroblasts at different time points; A+AA, AngII treated fibroblasts at different timepoints, treated along with AA. C and A cells were also treated with equivalent concentration of DMSO. Pb<0.01 with respect to “A” samples at 3hr time point; øø<0.01 with respect to “A+AA” samples at 3hr time point.

Figure 4: AA-mediated upregulation of PPARα results in regression of cardiac fibrosis and betterment of cardiac function:

A. Graphical representation of qRT-PCR data showing significant downregulation of col-1 and col-3 gene expressions in AA treated hypertrophy samples compared to hypertrophy groups. PPARα siRNA pretreated hypertrophied groups treated along with AA showed significant recovery in these gene expressions compared to AA treated hypertrophy samples both in vitro and in vivo. Control and hypertrophy samples were treated with equivalent amount of DMSO and NS siRNA. AA treated hypertrophy samples were also treated with equivalent amount of NS siRNA. Rpl-32 was used as internal
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Reference control. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=10 in vitro, n=7 in vivo for each experimental group. **, p<0.01 with respect to control samples; ***, p<0.001 with respect to control samples; ##, p<0.01 with respect to hypertrophy samples; †, p<0.05 with respect to AA treated hypertrophy samples; †††, p<0.001 with respect to control cells; #, p<0.05 with respect to AngII treated cells; ††, p<0.01 with respect to AA treated cells treated along with AA.

B. Graphical representation of in vitro hydroxyproline assay showing significantly restored collagen content in the culture supernant of PPARα siRNA pretreated hypertrophied fibroblasts treated along with AA compared to AA treated hypertrophied cells in vitro. Control and AngII treated cells were treated with equivalent amount of DMSO and NS siRNA. AA treated hypertrophied cells were also treated with equivalent amount of NS siRNA. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=10 for each experimental group. **, p<0.01 with respect to control cells; #, p<0.05 with respect to AngII treated cells; †, p<0.05 with respect to AA treated hypertrophy samples in vitro and/or in vivo.

C. Graphical representation of in vivo hydroxyproline assay showing significant recovery in total left ventricular collagen content in PPARα siRNA pretreated AA infused hypertrophied heart compared to AA treated renal artery ligated rat heart in vivo. Sham operated control rats and renal artery ligated rats were also treated with equivalent amount of DMSO and NS siRNA. AA treated ligated rats were also treated with equivalent amount of NS siRNA. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=7 for each experimental group. **, p<0.01 with respect to sham operated control rat group; ##, p<0.01 with respect to renal artery ligated rat group; †, p<0.05 with respect to AA treated renal artery ligated rat group.

D. Micrographs of Massons' Trichrome staining showing significantly decreased %CVF in AA-treated hypertrophy samples compared to renal artery ligated rat heart, which again showed significant recovery in AA treated ligated samples pretreated with PPARα siRNA. S; Sham operated control group, L; Ligated rat group, L+AA; AA treated Ligated rat group, L+AA+PPARα si; PPARα siRNA infused AA treated ligated rat group. S and L groups were treated with equivalent amount of DMSO and NS siRNA. L+AA group animals were also treated with equivalent amount of NS siRNA. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=7 for each group. (Scale Bar=40uM, Magnification=60X). **, p<0.01 with respect to sham operated control samples; ##, p<0.01 with respect to renal artery ligated hypertrophy samples; ††, p<0.01 with respect to renal artery ligated samples treated with AA.

E. M-mode echocardiographic analyses of PPARα siRNA pretreated ligated rats treated with AA showing significant decrease in %FS and significant increase in LVIDd compared to AA treated ligated rats. Sham controls and renal artery ligated rats were treated with equivalent amount of DMSO and NS siRNA. AA treated ligated rats were also treated with equivalent amount of NS siRNA. n=7 for each group. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. **, p<0.01 with respect to sham operated control rat group; ##, p<0.01 with respect to renal artery ligated rat group; ††, p<0.01 with respect to AA treated renal artery ligated rat group.

Figure 5: AA-mediated upregulation of PPARα specifically targets TAK1 for regression of TGF-β signaling during cardiac hypertrophy:

A. Western blot analyses showing significantly reduced TβRI, TβRII, phospho/total levels of SMAD 2, SMAD 3 and TAK1 in AA treated hypertrophy samples compared to hypertrophy groups both in vitro and in vivo. Significantly restored levels of all of these proteins were observed in AA treated hypertrophy groups pretreated with PPARα siRNA compared to AA treated hypertrophy samples. Control and hypertrophy samples were treated with equivalent amount of DMSO and NS siRNA. AA treated hypertrophy samples were also treated with equivalent amount of NS siRNA. GAPDH was used as internal loading control. n=10 in vitro, n=7 in vivo for each experimental group.

B. Western blot analyses showing significantly decreased phospho/total TAK1 level during AA treatment in TGF-β treated cardiac fibroblasts compared to only TGF-β treated cells. PPARα knockdown in TGF-β and AA infused cells showed significant recovery in phospho/total TAK1 level compared to AA treated fibroblasts pretreated with TGF-β. Expressions of TβRI, TβRII and phospho/total levels of SMAD 2 and SMAD 3 remained unaltered in these experimental groups. Control and TGF-β treated fibroblasts...
were also treated with equivalent amount of DMSO and NS siRNA. AA treated fibroblasts pretreated with TGF-β were also treated with equivalent amount of NS siRNA. GAPDH was used as internal loading control. n=5 for each group.

Figure 6: AA-mediated regression of collagen gene expression involves PPARα-dependent inactivation of non-canonical TGF-β signaling:
A. Western blot analyses showing significantly reduced phospho/total levels of IKKβ, NF-κBp65, p38 MAPK and JNK in AA treated hypertrophy samples compared to hypertrophy groups both in vitro and in vivo. Significantly restored levels of all these proteins were observed during PPARα knockdown in AA treated hypertrophied groups compared to AA treated hypertrophy samples. Control and hypertrophy groups were also treated with equivalent amount of DMSO and NS siRNA. AA treated hypertrophy samples were treated with equivalent amount of NS siRNA. GAPDH was used as internal loading control. n=5 for each group.

B. Graphical representation of qRT-PCR analyses showing significant downregulation of col-1 and col-3 gene expressions in AngII induced fibroblasts during knockdown of either TAK1 or NF-κBp65 or p38 MAPK via specific siRNA treatments compared to hypertrophied fibroblasts. JNK specific siRNA treatment in AngII treated fibroblasts showed no significant regression of collagen gene expression compared to AngII treated cells. Control and AngII treated cells were also treated with equivalent amount of NS siRNA. Rpl32 was used as internal reference control. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=5 for each group. **, p<0.01 with respect to control cells; ***, p<0.001 with respect to control cells, #; p<0.05 with respect to AngII treated cells, ##; p<0.01 with respect to AngII treated cells.

C. Dual-luciferase assay showing significant increase in col1a1 promoter activity in AngII treated fibroblasts compared to control fibroblasts. AA treatment in hypertrophied fibroblasts showed significant reduction in col1a1 promoter activity compared to AngII treated fibroblasts that again showed significant restoration in AA treated hypertrophied fibroblasts pretreated with PPARα siRNA. Reduced col1a1 promoter activity shown in NF-kBp65 siRNA pretreated hypertrophied cells compared to AngII treated cells was used as negative control. Control and AngII treated cells were also treated with NS siRNA with or without DMSO. AA treated hypertrophy samples were also treated with equivalent amount of NS siRNA. Results were normalized by Renilla luciferase activity in all the treatment groups. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=5 for each group. **, p<0.01 compared to DMSO and NS siRNA treated control cells; ##, p<0.01 compared to NS siRNA infused AngII treated cells; ¶, p<0.01 with respect to AA treated hypertrophied cells pretreated with NS siRNA; iii, p<0.01 with respect to AngII treated cells pretreated with NS siRNA.

Figure 7: Analyses of interaction of PPARα with TAK1:
A. Overall schematic representation of the docking simulation between predicted structures of full length rat TAK1 (Silver) and different domains of rat-PPARα (Cyan: AF-1; Orange: DBD; Green: H+LBD) based on the best fit HADDOCK score.
B. FRAP analysis showed a positive FRET efficiency between endogenous PPARα and TAK1 in Cardiac fibroblasts. Cells were probed for endogenous PPARα and TAK1 expressions with respective primary antibodies and stained with PPARα-FITC (green) and TAK1-TRITC (Red). TRITC was subjected to 50% photobleaching. n=5. i, Prebleach donor; ii, postbleach donor; iii, delta donor; iv, prebleach acceptor; v, postbleach acceptor; vi, FRET efficiency.
C. Co-IP experiments were done by immunoprecipitating proteins with anti-PPARα antibody followed by immunoblotting with anti-TAK1 antibody in vitro. PPARα overexpressed AngII treated fibroblasts were used as a positive control. Normalization was done by western blot with anti-PPARα antibody in the same samples. Control and AngII treated cells were also treated with either DMSO or empty pCDNA6/V5-HisB vector yielding similar results. n=5 for each group. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. Graph showing relative changes in the level of interaction between PPARα and TAK1 between different experimental groups. C, Control fibroblasts; A, AngII treated fibroblasts; A+AA, AA co-treated AngII infused fibroblasts; A+pOV, PPARα overexpressed AngII treated fibroblasts. **, p<0.01 with respect to control fibroblasts, ##, p< 0.01 compared to AngII treated fibroblasts
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**Figure 8:** Study of the effect of different PPARα domains upon PPARα::TAK1 interaction and their roles in modulation of non-canonical TGF-β pathway induced collagen synthesis in hypertrophied fibroblasts:

A. Co-IP experiments were done by immunoprecipitating proteins with anti-His antibody followed by immunoblotting with anti-TAK1 antibody *in vitro*. Normalization was done by immunoblotting with anti-His antibody. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. n=5 for each group. E, Empty pCDNA6/V5 HisB vector; F, Full length PPARα plasmid; AF-1, PPARα N terminal transactivation domain plasmid; DBD, PPARα DNA binding domain; H+LBD, PPARα combined Hinge region+ C terminal Ligand binding domain plasmid. All the plasmids were transfected into AngII treated fibroblasts. n=5 for each group.

B. Western blot analyses showing alterations in expression levels of phospho/total TAK1, NF-κBp65 and p38 MAPK in AngII treated cells transfected with full length or individual domains of PPARα plasmids compared to AngII treated fibroblasts. GAPDH was used as internal loading control. C, Control fibroblasts; A, AngII treated fibroblasts; A+ F, Full length PPARα transfected AngII treated fibroblasts; A+ AF-1, PPARα N terminal transactivation domain transfected AngII treated cells; A+ DBD, PPARα DNA binding domain transfected AngII treated cells; A+ H+LBD, PPARα combined Hinge region+ C terminal Ligand binding domain transfected AngII treated cells. C and A cells were also treated with empty pCDNA6/V5 HisB vector. n=5 for each group.

C. Graphical representation of qRT-PCR analyses showing changes in levels of *col-1* and *col-3* gene expressions in AngII treated cells transfected with full length or individual domains of PPARα plasmids compared to AngII treated fibroblasts. *Rpl-32* was used as internal loading control. Results were analyzed by ANOVA followed by Tukey's post-hoc test and expressed as ±S.E. of three independent experiments. C, Control fibroblasts; A, AngII treated fibroblasts; A+ F, Full length PPARα transfected AngII treated cells; A+ AF-1, PPARα N terminal transactivation domain transfected AngII treated cells; A+ DBD, PPARα DNA binding domain transfected AngII treated cells; A+ H+LBD, PPARα combined Hinge region+ C terminal Ligand binding domain transfected AngII treated cells. C and A cells were also treated with empty pCDNA6/V5 HisB vector. n=5 for each group. **, p<0.01 with respect to control cells; ***, p<0.001 with respect to C; ##, p<0.01 with respect to A; ###, p<0.001 with respect to A; ††, p<0.01 with respect to A+ F; †††, p<0.001 with respect to A+ F.

**Figure 9:** Schematic representation of the molecular mechanism of AA action upon cardiac hypertrophy associated fibrosis:

A. During cardiac hypertrophy TGF-β signaling pathway action is promoted leading to excess collagen synthesis with downregulated PPARα expression. Treatment with AA in hypertrophy samples increases PPARα expression in an autoregulatory loop leading to increased binding of PPARα to TAK1 thereby ameliorating TAK1-driven non-canonical TGF-β axes with subsequent regression of collagen synthesis. B. Schematic representation of different PPARα domains interacting with TAK1 and the role of PPARα::TAK1 interaction in prevention of phosphorylation dependent activation of TAK1 for subsequent regression of collagen synthesis in AngII treated adult cardiac fibroblasts.
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Figure-1
Figure-2
Antifibrotic role of Arjunolic acid as a PPARα Agonist

Figure-3
Antifibrotic role of Arjunolic acid as a PPARα Agonist

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**in vitro**

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Figure-4
Antifibrotic role of Arjunolic acid as a PPARα Agonist

<table>
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<th>Treatment</th>
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<tr>
<td>NS siRNA</td>
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<tr>
<td>AA</td>
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<td>Sham</td>
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<tr>
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<td>Ligated</td>
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Figure 5

Table A: In vitro and in vivo treatment conditions.

Diagram A: Western blot analysis showing protein expression levels for various markers under different treatment conditions.

Table B: In vitro treatment conditions.

Diagram B: Western blot analysis showing protein expression levels for various markers under different treatment conditions.
### Figure-6

#### A

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<td>AA</td>
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#### B

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<tr>
<td>TAK1 si</td>
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<tr>
<td>NF-kBp65 si</td>
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<td>JNK si</td>
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#### C

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Antifibrotic role of Arjunolic acid as a PPARα Agonist

**Figure-7**
Antifibrotic role of Arjunolic acid as a PPARα Agonist

Figure-8
Antifibrotic role of Arjunolic acid as a PPARα Agonist

A  Cardiac Hypertrophy
    TGF-β pathway
    Arjunolic acid (AA) treatment
    TAK1
    Prevention of phosphorylation and kinase activity
    p38 MAPK
    NF-κB p65
    Promotion of fibrosis
    Regression of fibrosis
    Compromised cardiac function
    Improvement of cardiac function

B  PPARα

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Figure-9
Arjunolic Acid, a Peroxisome Proliferator-Activated Receptor Alpha Agonist Regresses Cardiac Fibrosis by Inhibiting Non-canonical TGF- β Signaling.
Trisha Bansal, Emeli Chatterjee, Jasdeep Singh, Arjun Ray, Bishwajit Kundu, V. Thankamani, Shantanu Sengupta and Sagarthirtha Sarkar

J. Biol. Chem. published online August 18, 2017

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