RESEARCH

TRIF/EGFR signalling mediates angiotensin-II-induced cardiac remodelling in mice

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Abstract

The excessive activation of renin-angiotensin system (RAS) is one of key pathophysiological characteristics in the development of cardiac remodelling. Angiotensin (Ang) II, as a main active peptide in RAS, induces cardiac structural disorders and dysfunction. However, the molecular mechanisms are still not fully disclosed. Present study aimed to determine the role and potential mechanisms of cardiac TIR-domain-containing adapter-inducing interferon-β (TRIF) in Ang-II-mediated cardiac remodelling in mice. *In vitro* and *in vivo* studies showed Ang II and downstream aldosterone obviously increased the expression of TRIF, accompanied with cardiac structural abnormalities and functional injuries. Specific blockage of cardiac TRIF effectively decreased Ang-II/aldosterone-induced cardiac inflammation, fibrosis, hypertrophy and dysfunction in mice. Mechanistically, the TRIF triggered the activation of EGF receptor (EGFR) signalling by nuclear factor (NF)-κB transcriptional regulation and downstream EGFR ligands. Taken together, present study supported that cardiac TRIF was a potential therapeutic target for attenuating cardiac pathophysiological remodelling. The TRIF/EGFR axis partially explained the molecular mechanism of Ang-II/aldosterone-induced cardiac inflammation, fibrosis, hypertrophy and dysfunction in mice.

Key Words

- heart
- angiotensin II
- TIR-domain-containing adapter-inducing interferon-β (TRIF)
- epidermal growth factor receptor

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Introduction

Cardiovascular disease is one of leading causes of morbidity and mortality in the world (Sabatine *et al.* 2017). In this regard, the renin-angiotensin system (RAS) has remained a focus of cardiovascular research for over a century. The RAS has been shown to play a pathophysiological role in the development and progression of numerous cardiovascular-related diseases including heart failure, hypertension, chronic kidney disease and stroke (Bangalore *et al.* 2017, Ferrario & Mullick 2017). As a result, pharmacological agents targeting the RAS are increasingly used in these clinical populations (Bangalore *et al.* 2017). However, despite decades of research into underlying mechanisms, there is still rare effective optimal therapeutic approach to protect against cardiovascular disease.

The RAS is a series of enzyme-substrate interactions that generate functional peptide hormones critical to physiological and pathophysiological regulation of cardiovascular function. Among them, angiotensin (Ang) II is the most critical active peptide to stimulate the downstream signalling, including cellular

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proliferation, oxidative stress, inflammatory response, and cardiovascular dysfunction (Pan *et al.* 2014*a*, von Lueder *et al.* 2015, Jamme *et al.* 2017). In mice and human, abnormal upregulation of Ang II levels are closely associated with cardiac injuries (Snijder *et al.* 2015, Ferrario *et al.* 2016). Clinical and experimental evidence using inhibitors of Ang II production suggest that inappropriate activation of cardiac Ang II contributes significantly to the process of cardiac hypertrophy, fibrosis and inflammation (Snijder *et al.* 2015, Ferrario *et al.* 2016). Several signalling molecules have been implicated in the Ang-II-induced cardiac remodelling, including EGF receptor (EGFR), tyrosine kinases, phosphatidylinositol 3 kinase (PI3K)/protein kinase B (AKT) and mitogen-activated protein kinases (MAPKs) (Frangogiannis 2014). Among them, a major recent finding suggests that many of the mitogenic effects of Ang II are controlled via transactivation of the EGFR. Kagiyama *et al*. reported that EGFR activation was required for Ang-II-mediated hypertension and left ventricular hypertrophy, while both of which were attenuated when rats were treated with an i.v. infusion of antisense oligodeoxynucleotide to EGFR (Kagiyama *et al.* 2003). Blocking EGFR kinase activity with the selective inhibitor AG1478 also abolished Ang-II-mediated downstream signalling (Peng *et al.* 2016). These studies pointed to EGFR as an important factor in cardiac hypertrophy and dysfunction caused by Ang II.

Meanwhile, Ang II has been reported to activate toll-like receptors (TLRs) as well as its downstream signalling cascade, such as myeloid differentiation primary response (MyD) 88 and TIR-domain-containing adapter-inducing interferon-β (TRIF). In Ang-II-infused mouse models, TLRs/MyD88/TRIF pathways play critical roles in cardiac remodelling (Kenny & O'Neill 2008). However, Singh *et al*. have demonstrated that global MyD88 deficiency appositely deteriorated Ang-II-induced cardiac hypertrophy, whereas global TRIF deficiency possibly attenuated cardiac structural remodelling with slight effects on cardiac expression of inflammatory genes (Singh *et al.* 2015). On the contrast, several studies have shown that TRIF mediates inflammation and fibrosis in different tissues (Chen *et al.* 2015, Antoniak *et al.* 2017, Li *et al.* 2019). Due to wide distribution in multiple organs and cells, TRIF exhibits complicated physiological effects. Therefore, it is necessary to specifically address the pathophysiological roles of cardiac TRIF and potential molecular mechanisms in regulating cardiac biology.

To this end, present study aimed to determine the role of cardiac TRIF in Ang-II-induced cardiac remodelling and disclose the underlying molecular mechanisms. The elevated levels of cardiac TRIF mediated Ang-II-induced cardiac hypertrophy, fibrosis and inflammation. In addition, the TRIF/EGFR signalling was a potential molecular mechanism to explain the effects of Ang II on mouse cardiac remodelling.

Materials and methods

Reagents

Ang II, aldosterone, spironolactone, haematoxylin, and eosin solution were purchased from Sigma chemicals. Anti-TRIF, anti-TGF-β1, anti-phosp-IκB, anti-IκB and anti-Tubulin antibodies were purchased from Cell Signaling. Anti-phosp-EGFR and anti-EGFR antibodies were purchase from Abcam. Mouse TNF-α and IL-1β ELISA kits were purchased from R&D Systems, and mouse aldosterone ELISA kit was purchased from Enzo Life Sciences.

Mouse experiments

Male C57BL/6J mice, aged 8 weeks, were randomly divided into four groups: (1) saline-treated mice (Veh group); (2) saline-treated mice that were locally injected with lentivirus encoding *Trif* siRNA (Veh+si*Trif* group) in hearts; (3) Ang II-treated mice that were locally injected with lentivirus encoding control siRNA (Ang II+si*Ctrl* group) in hearts; (4) Ang II-treated mice that were locally injected with lentivirus encoding *Trif* siRNA (Ang II+si*Trif* group) in hearts. For Ang II treatment, mice were infused with Ang II (1 μg/kg/min, dissolved in saline) by osmatic pump for 2 or 4 weeks. The serum and cardiac tissues were collected and stored in −80°C refrigerator before further analysis. All experimental procedures were approved by the Institutional Animal Use and Care Committee at the Harbin Medical University (Harbin, China).

Cardiac histological analysis

Mouse left ventricles were collected and fixed in 4% paraformaldehyde for 24 h and embedded in paraffin. Five-micrometre paraffin sections were prepared and stained with haematoxylin and eosin solution (HE). To measure the histological changes, the cardiac images were observed under a light microscope (Nikon). To quantify the average size of cardiomyocytes, 10 fields (50 cardiomyocytes per field)/mouse were selected from left ventricular section and calculated by using Image J analysis software version 1.38e.

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Echocardiography analysis

The mice were given anaesthesia by isoflurane (0.5% in oxygen), and cardiac functional parameters were recorded by using echocardiographic examinations through the Vevo 2100 system (VisualSonics, Toronto, Canada). The thickness of left ventricular anterior wall (LVAW) and posterior wall (LVPW) and diastolic and systolic left ventricular internal dimensions (LVID) were recorded from M-mode images. Echocardiographic parameters also included ejection fraction (EF), fractional shortening (FS), left ventricular end-systolic dimension (LVESD), left ventricular end-diastolic dimension (LVESD) and left ventricular mass.

Cell experiment

Primary mouse cardiomyocytes were isolated from 3 to 5 days-old neonatal C57BL/6J mice as described in previous study (Pan *et al.* 2014*b*). In details, mouse hearts were removed and perfused with Krebs–Henseleit bicarbonate buffer containing the following (mM): 118 NaCl, 4.7 KCl, 1.2 MgSO4, 1.2 KH2PO4, 25 NaHCO3, 10 HEPES, and 11.1 glucose. Left ventricles were removed and minced. The tissues were digested with collagenase D for 10 min for three times, and the buffer was centrifuged at 500 ***g*** for 5 min. The pellets were suspended and cultured in RMPI1640 medium on 10 μg/mL gelatin-coated flask. After a 2-h incubation, the medium was transferred to a new flask to avoid the contamination of primary fibroblasts. For Ang II treatment, 1×10^6 cells were incubated with different concentration of angiotensin peptide for 6 h.

Real-time PCR analysis

The total RNA was extracted from mouse primary cardiomyocytes or hearts according to the TRIzol manufacturer's protocol. RT was performed using the Takara RT System (Takara), and real-time PCR analysis was performed using SYBR Green (Applied Biosystems). The sequence of primers are listed as following (5′-3′): *Trif*: F-AGGACAAACGCCGGAACTTTT; R-GCCGATAGTCTGTCTGTTCTAGT, *CYP11B2*: F-TGG CTGAAGATGATACAGATCCT; R-CACTGTGCCTGAAAA TGGGC, *ANP:* F-ACGCCAGCATGGGCTCCTTCTCC; R-GCTGTTATCTTCGGTACCGGAAG, *BNP:* F-AAGCTG CTGGAGCTGATAAGA; R-GTTACAGCCCAAACG ACTGAC, *TNF-α*: F-GCCACCACGCTCTTCTGTCTA; R-GATGAGAGGGAGGCCATTTG. *IL-1β*: F-AAATACCTG TGGCCTTGGGC; R-CTTGGGATCCACACTCTCCAG, *EREG:* F-AGACGCTCCCTGCCTCTTG; R-TTCTCCTGG GATGCATGATG, *AREG:* F-TTGGTGAACGGTGTGGAGAA; R-CGAGGATGATGGCAGAGACA, and *GAPDH:* F-AGGAGC GAGACCCCACTAAC; R-GATGACCCTTTTGGCTCCAC. Relative gene levels were normalized to *GAPDH* level.

Western blot analysis

Protein extracts (50 μg) from the mouse hearts or cardiomyocytes were separated using 10% SDS-PAGE, then transferred to a polyvinylidene difluoride membrane (PVDF, Amersham Biosciences). The membranes were blocked with 10% milk in PBS/0.05% Tween 20 for 1 h and incubated overnight at 4°C with primary antibodies and secondary antibodies (Cell Signaling). The protein expression was visualized using enhanced chemiluminescence reagents (Bio-Rad). The amounts of the proteins were analyzed using Image J analysis software version 1.38e.

Statistical analysis

Data were presented as mean ± S.E.M. The one-way ANOVA was used for comparing different groups. GraphPad Prism 5 (GraphPad) was used to analyze the statistical significance between sets of data. Differences were considered to be significant at $P < 0.05$.

Results

Effect of Ang II on TRIF expression in primary mouse cardiomyocytes and hearts

Previous study has demonstrated that Ang-II-mediated cardiac hypertrophy was mainly dependent on TRIF pathway in global TRIF$^{-/-}$ mice (Singh *et al.* 2015). However, it is unclear whether specific cardiac TRIF participates in the process of Ang-II-induced cardiac dysfunction. To this end, we treated primary mouse cardiomyocytes with different concentrations of Ang II peptite. As showed in Figure S1 (see section on supplementary data given at the end of this article), we successfully isolated and purified the primary mouse cardiomyocytes, which specially expressed cTnT. Then, the cells were treated with different concentrations of Ang II. As showed in Fig. 1A, Ang II dose-dependently stimulated gene level of *Trif* in cardiomyocytes. Consistently, the protein level was also obviously increased after Ang II treatment (Fig. 1B and C). Next, we infused Ang II into mice for 2 or 4 weeks to measure the changes of cardiac TRIF expression.

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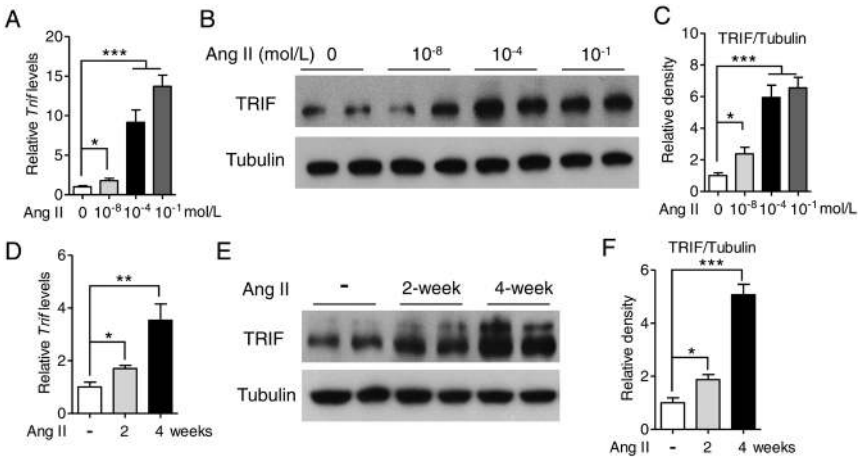

Figure 1
Induction of TRIF mRNA and protein with Ang II in primary mouse cardiomyocytes and mouse hearts. (A, B and C) 1 × 10^6 primary mouse cardiomyocytes were incubated with Ang II (10^{-8}, 10^{-4} and 10^{-1} mol/L) or control saline for 6 h. Real-time PCR analysis of *Trif* mRNA level (A) and Western blot analysis of TRIF protein level (B) in cardiomyocytes. Quantitative analysis of relative TRIF protein level (C). $n = 5$ independent experiments. (D, E and F) Male C57BL/6J mice, aged 8 weeks, were infused with Ang II (1 μg/kg/min) for 2 or 4 weeks. Real-time PCR analysis of *Trif* mRNA level (D) and Western blot analysis of TRIF protein level (E) in hearts. Quantitative analysis of relative TRIF protein level (F). $n = 6$ mice/group. Results are mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The relative *Trif* mRNA level was normalized to the expression of house-keeping gene *Gapdh*.

Ang II significantly increased mRNA level of *Trif* (Fig. 1D) and also stimulated protein level (Fig. 1E and F) after administration for 2 or 4 weeks. These findings support that cardiac TRIF was a downstream factor in Ang-II-activated signalling.

Previous studies have demonstrated that Ang II could induce the expression of aldosterone, an activator of mineralocorticoid receptor (MR), and mediate the cardiac remodelling (Schiffrin & Touyz 2003). To this end, we then measured the expression of aldosterone in Ang-II-treated mice. As shown in Fig. 2A, Ang II time-dependently increased the circulating level of aldosterone ($P < 0.05$). The cardiac level of aldosterone was also significantly upregulated (Fig. 2B, $P < 0.05$), whereas the mRNA expression of *CYP11B2*, encoding aldosterone synthase, was also increased in Ang-II-treated mice (Fig. 2C, $P < 0.05$).

To identify the potential effects of aldosterone in regulating TRIF expression, we further treated primary mouse cardiomyocytes with Ang II or aldosterone with or without a MR antagonist, spironolactone. As showed in Fig. 2D and E, spironolactone inhibited the TRIF expression in both Ang-II- and aldosterone-treated cardiomyocytes ($P < 0.01$). These results indicated that aldosterone/MR signalling could mediate the pathophysiological roles of Ang II.

Suppression of cardiac TRIF protects against Ang-II-induced mouse cardiac structural abnormalities and dysfunction

To explore the role of TRIF in Ang-II-induced cardiac modification, we then knocked down cardiac *Trif* by

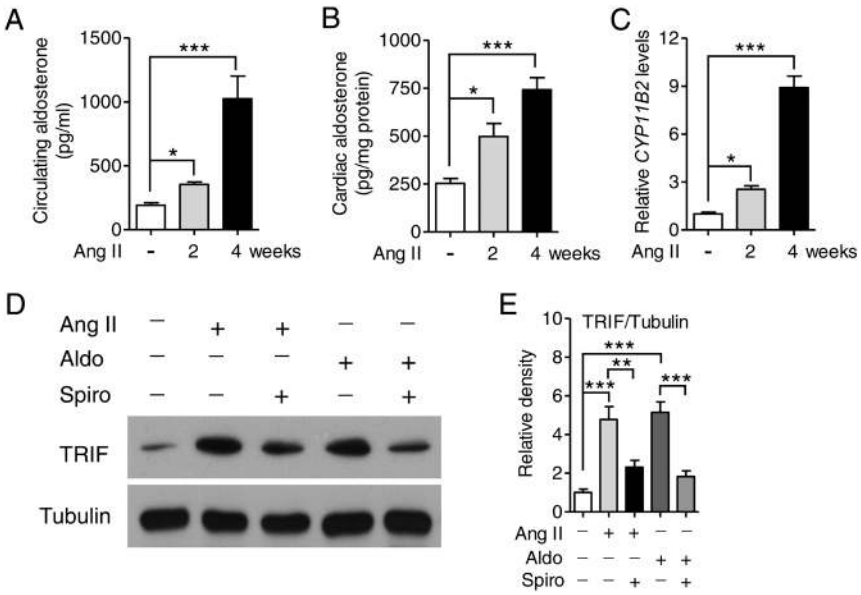

Figure 2
The induction of aldosterone increases the cardiac expression of TRIF in Ang-II-treated mice and mouse primary cardiomyocytes. (A, B and C) Male C57BL/6J mice, aged 8 weeks, were infused with Ang II (1 μg/kg/min) for 2 or 4 weeks. The aldosterone levels in circulation (A) and hearts (B) and real-time PCR analysis of cardiac *CYP11B2* mRNA level (C). $n = 6$ mice/group. (D and E) 1 × 10^6 primary mouse cardiomyocytes were incubated with 10^{-7} mol/L Ang II or 10^{-7} mol/L aldosterone with or without 10^{-6} mol/L spironolactone for 6 h. Western blot analysis of TRIF protein level (D) and quantitative analysis of relative expression (E). $n = 5$ independent experiments. Results are mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The relative *CYP11B2* mRNA level was normalized to the expression of house-keeping gene *Gapdh*.

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locally injected with lentivirus encoding *Myhc-Trif* silence RNA. As shown in Fig. 3A and B, Ang II significantly increased cardiac level of TRIF, whereas *Myhc-Trif* RNAi effectively blocked cardiac TRIF expression, but did not affect renal TRIF levels. Previous studies have already demonstrated that Ang II is a strong inducer of cardiac hypertrophy (Ferrario *et al.* 2016, Peng *et al.* 2016). Consistently, the histological staining also indicated that Ang II increased the size of cardiomyocyte to 2.5 folds than saline-treated mice (Fig. 3C and D, $P < 0.001$). Fig. 3E showed Ang II obviously increased a critical hypertrophic marker ($P < 0.001$), ratio of heart weight/tibia length (Nie *et al.* 2018). However, cardiac *Trif* deficiency decreased the Ang-II-induced cardiac hypertrophy (Fig. 3C and E, $P < 0.01$). ANP and brain natriuretic peptide (BNP) are biomarkers for pathological cardiac hypertrophy (Crowley *et al.* 2006). As shown in Fig. 3F and G, Ang II significantly upregulated mRNA of these two genes ($P < 0.001$), but si*Trif* treatment effectively decreased the expression level. Cardiac fibrosis is another parameter of heart injuries, which is largely controlled by transforming growth factor (TGF)-β signalling (Yue *et al.* 2017). Fig. 3H and I also found that Ang II obviously stimulated protein expression of TGF-β1 ($P < 0.001$), whereas silence of TRIF significantly suppressed the upregulation of TGF-β1 ($P < 0.01$). Sirius red staining of hearts found that the fibrotic collagen levels were severely increased in Ang-II-treated mice, whereas TRIF silence attenuated these accumulation (Fig. S2A and B).

Next, we utilized echocardiographic measurement to analyse cardiac functional parameters. As Supplementary Table 1 and Table 1 showed, Ang II treatment significantly damaged cardiac function, including ejection fraction (EF) and fractional shortening (FS) at 2- and 4-week treatment, as compared with saline-treated mice. Meanwhile, the value of other cardiac hypertrophic parameters, including left ventricular anterior wall thickness (LVAW), left ventricular internal dimension (LVID), left ventricular posterior wall thickness (LVPW) and LV mass, were significantly upregulated in Ang-II-infused mice. However, co-administration of *Trif* siRNA increased EF ($P < 0.05$) and FS ($P < 0.05$), but decreased the values of LVAW,

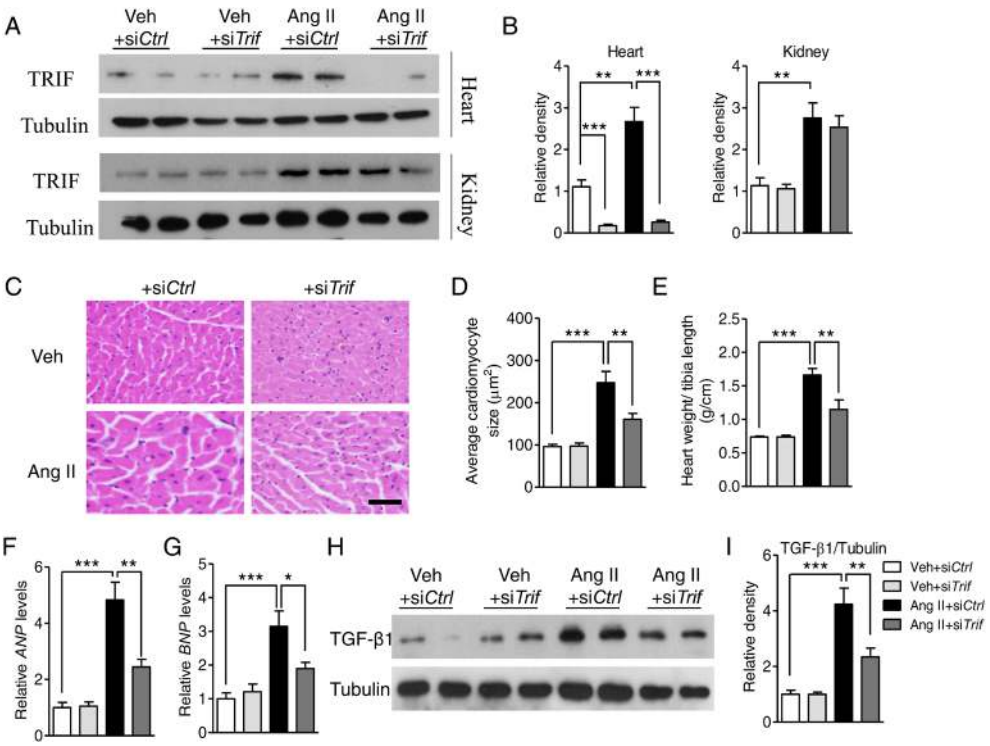

Figure 3
Silencing cardiac *Trif* attenuates Ang-II-induced mouse cardiac hypertrophy and fibrosis. Male C57BL/6J mice, aged 8 weeks, were locally infused with 1×10^{10} lentiviral particles encoding *Trif* (si*Trif*) or control siRNA (si*Ctrl*) and administrated with Ang II (1 μg/kg/min) or saline for 4 weeks. (A and B) Western blot analysis of TRIF protein in hearts and kidneys (A), and quantitative analysis of relative TRIF protein levels (B). (C) The haematoxylin & eosin (HE) staining of cardiac tissues. (D) Quantitative analysis of average cardiomyocyte size. (E) The heart weight/tibia length ratio. (F and G) Real-time PCR analysis of *ANP* (F) and *BNP* (G) mRNA levels. (H and I) Western blot analysis of TGF-β1 protein level (H) in hearts, and quantitative analysis of relative TGF-β1 protein level (I). $n = 6$ mice/group. Results are mean ± S.E.M. *$P < 0.05$, **$P < 0.01$, ***$P < 0.001$. The relative *ANP* and *BNP* mRNA levels were normalized to the expression of *Gapdh*.

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Table 1 Echocardiographic analysis of mouse cardiac parameters after infusion with Ang II for 4 weeks.

	Veh+si*Ctrl*	Veh+si*Trif*	Ang II+si*Ctrl*	Ang II+si*Trif*
Heart rate (beats/min)	465 ± 23	446 ± 18	497 ± 6	475 ± 11
EF%	80.32 ± 9.17	78.32 ± 5.43	65.54 ± 6.44[c]	74.32 ± 5.98[e]
FS%	48.65 ± 4.09	47.12 ± 3.32	37.77 ± 2.90[b]	42.56 ± 2.23[d]
LVAW;d (mm)	0.79 ± 0.11	0.82 ± 0.32	1.29 ± 0.21[c]	0.95 ± 0.32[e]
LVID;d (mm)	3.81 ± 0.07	3.69 ± 0.32	4.29 ± 0.21[a]	3.93 ± 0.19[d]
LVPW;d (mm)	0.75 ± 0.11	0.74 ± 0.19	1.05 ± 0.15[b]	0.83 ± 0.09[e]
LVAW;s (mm)	1.27 ± 0.23	1.19 ± 0.21	1.67 ± 0.32[b]	1.32 ± 0.09[e]
LVID;s (mm)	2.26 ± 0.28	2.24 ± 0.16	2.88 ± 0.27[b]	2.43 ± 0.29[d]
LVPW;s (mm)	1.17 ± 0.26	1.11 ± 0.4	1.56 ± 0.13[c]	1.32 ± 0.09[d]
LV Mass (mg)	112.17 ± 5.32	111.21 ± 14.32	168.43 ± 13.01[c]	127.32 ± 4.01[e]

Abbreviations: d, diastolic; EF, ejection fraction; FS, fractional shortening; LV, left ventricle mass; LVAW, left ventricular anterior wall thickness; LVID, left ventricular internal dimension; LVPW, left ventricle posterior wall thickness; s, systolic. Results are mean ± S.E.M. [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.001$ vs Veh+siCtrl-treated mice; [d]$P < 0.05$, [e]$P < 0.01$ vs Ang II + siCtrl-treated mice.

LVAW, LVPW and predicted LV mass. However, TRIF silence had no significant effects on Ang-II-induced hypertension, as indicated by systolic blood pressure (Fig. S2D). Previously mentioned results showed that specific silence of cardiac TRIF effectively attenuated Ang-II-mediated cardiac hypertrophy, fibrosis and dysfunction in mice.

Suppression of cardiac TRIF attenuates Ang-II-induced inflammatory response in mouse hearts

Previous studies have already reported that Ang II remarkably stimulates cardiac inflammation, including activation of NF-κB signalling and secretion of pro-inflammatory cytokines (Frangogiannis 2014, Snijder *et al.* 2015). Consistently, the phosphorylation of IκB and IκB degradation was upregulated in Ang-II-infused mouse hearts (Fig. 4A and B). Meanwhile, the downstream cytokines of NF-κB transcriptional pathway, including TNF-α, IL-1β, IL-6, and MCP-1, were decreased in Ang-II-treated mice (Fig. 4C and F). However, silence of TRIF inhibited cardiac NF-κB activation (Fig. 4A and B, $P < 0.01$) and supressed the gene levels of NF-κB transcriptional inflammatory cytokines (Fig. 4C and F), as compared with Ang-II-treated mice. The gene of F4/80, a marker of infiltrated macrophage, was also decreased after TRIF silence in Ang-II-treated mice (Fig. 4G). Furthermore, the protein levels of cardiac TNF-α and IL-1β were also significantly increased in Ang-II-treated mice, but decreased in TRIF-siRNA-treated mice (Fig. 4H and I).

TRIF/EGFR signalling mediates the effects of Ang II/MR-induced cardiac injuries

EGF receptor (EGFR) is a critical mediator of Ang-II-induced cardiac hypertrophy and dysfunction (Kagiyama *et al.* 2003, Peng *et al.* 2016). However, whether there is a potential link between TRIF and EGFR is unknown in the process of cardiac dysfunction. First, we measured the relative expression of EGFR in different cell types. As shown in Fig. S3A, primary mouse cardiomyocytes had similar EGFR expression pattern to circulating monocytes, but higher than endothelial cells. Next, we measured the changes of EGFR levels after Ang II treatment in the mice. As shown in Fig. 5A and B, administration of Ang II obviously increased the phosphorylation of EGFR in a time-dependent manner ($P < 0.001$). Interestingly, knockdown of cardiac *Trif* inhibited the activation of EGFR (Fig. 5C and D, $P < 0.01$). However, inactivation of EGFR signalling by AG1478, a specific inhibitor of EGFR phosphorylation, could not suppress Ang-II-induced TRIF expression (Fig. S3B and C). Mechanistically, Fig. 5E and H demonstrated that knockdown of cardiac *Trif* could suppress the mRNA and protein levels of two EGFR ligands amphiregulin (AREG) and epiregulin (EREG), which explained the downregulation of EGFR activation. Meanwhile, MR antagonist spironolactone suppressed the relative expression of p-EGFR/EGFR in both Ang-II- and aldosterone-treated cardiomyocytes (Fig. 5I and J, $P < 0.05$). Furthermore, the mRNA levels of *AREG* and *EREG* were also suppressed after blocking with MR antagonist (Fig. 5K and L, $P < 0.05$). All these findings supported that there existed Ang II-MR-TRIF- EGFR signalling in cardiac injuries.

Discussion

Chronic infusion of Ang II in experimental animals induces gradual vascular injuries and cardiac remodelling, including hypertrophy, fibrosis, inflammation and oxidative stress (Frangogiannis 2014). However, the molecular mechanism is still not fully disclosed. We reported here for the first time that TRIF/EGFR signalling

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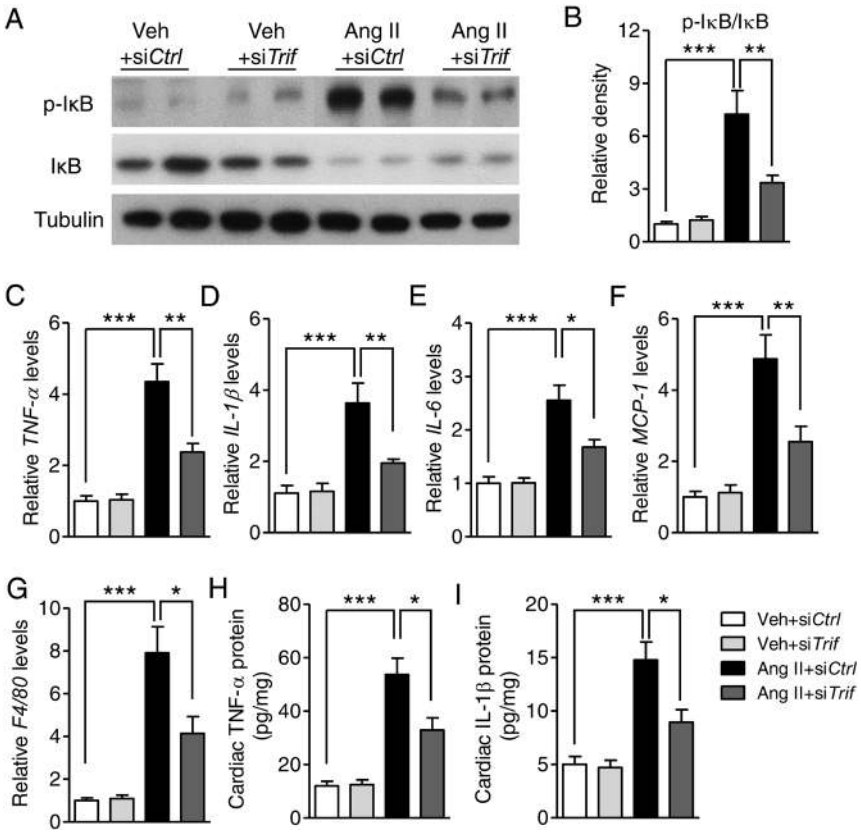

Figure 4
Silencing cardiac *Trif* decreases Ang-II-induced cardiac inflammation. Male C57BL/6J mice, aged 8 weeks, were locally infused with 1×10^{10} lentiviral particles encoding si*Trif* or si*Ctrl* and administrated with Ang II (1 μg/kg/min) or saline for 4 weeks. (A and B) Western blot analysis of phosph (p)-IκB and IκB protein levels (A), and quantitative analysis of the ratio of p-IκB/IκB (B). (C, D, E, F and G) Real-time PCR analysis of *TNF-α* (C), *IL-1β* (D), *IL-6* (E), *MCP-1* (F), and *F4/80* (G) mRNA levels. (H and I) ELISA analysis of cardiac protein levels of TNF-α (H) and IL-1β (I). $n = 6$ mice/group. Results are mean ± S.E.M. $^*P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$. The relative mRNA levels were normalized to the expression of *Gapdh*.

mediated the pathophysiological process of Ang-II-induced cardiac remodelling. Our data demonstrated that infusion of Ang II (1 μg/kg/min) over a 2- or 4-week period in mice caused a gradual elevation of TRIF that was correlated with marked cardiac injuries. Interestingly, specific blockage of cardiac TRIF attenuated Ang-II-induced cardiac hypertrophy, collagen deposit dysfunction and inflammatory response. The TRIF-dependent upregulation of EGFR ligands stimulated the activation of EGFR signalling in Ang-II-treated mice. Furthermore, aldosterone and mineralocorticoid receptor, as downstream signalling, could determine the pathophysiological effects of Ang II/TRIF/EGFR-axis-mediated cardiac remodelling. These data supported the notion that TRIF/EGFR was critical in the signalling mechanisms underlying the development of Ang II/aldosterone-induced cardiac remodelling.

The cardiac injuries and dysfunction upon abnormal activation of renin-angiotensin system (RAS) has been mainly attributed to Ang II, a key peptide playing roles in the regulation of vascular tone, blood pressure, and tissue remodelling. Ang II is known to directly damage cardiomyocytes as well as local immune cells to increase expression of inflammatory cytokines (Frangogiannis 2014). Classically, evidence obtained from a variety of experimental models indicates that Ang II appears to also act through toll-like receptor (TLR) 4 (Lv *et al.* 2009, Biancardi *et al.* 2017). Moreover, Ang II is hypothesized as a potential endogenous ligand of TLR4 (Biancardi *et al.* 2017), despite that it is unclear how Ang II interacts/activates TLR4. As one of necessary adaptors in the TLR complex, TRIF is another critical mediator of signalling cascades that directly influence multiple cellular process (Kenny & O'Neill 2008). For example, stimulation of TRIF activates NF-κB and MAPK pathways that trigger transcription of numerous inflammatory cytokines (Palova-Jelinkova *et al.* 2013). Previous studies have reported that TRIF-mediated inflammatory cascades led to oxidative stress, cardiac hypertrophy and cellular apoptosis after myocardial infarction (Chen *et al.* 2014). In global TRIF$^{-/-}$ mouse model, infusion of high dosage of Ang II only slightly increased the ratio of heart weight/body weight (Singh *et al.* 2015). The gene expression of *Tnf-a* and *Nox4* were also unchanged after administration of Ang II in global TRIF$^{-/-}$ mice. To further address the function of cardiac TRIF in Ang-II-induced cardiac hypertrophy, present study specifically deleted the cardiac *Trif* by transfected with lentivirus encoding Myhc-si*Trif*. Locally, loss of function of cardiac TRIF provided evidence that infused Ang II modulated cardiac remodelling dependent on cardiac TRIF, but not nearby secondary effects.

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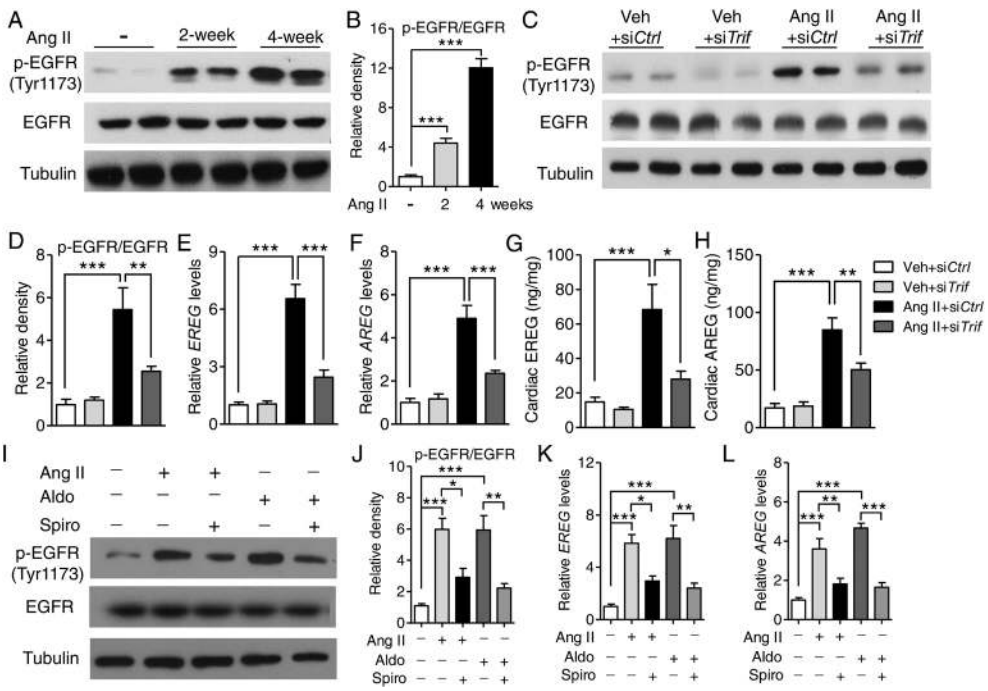

Figure 5

Silencing cardiac *Trif* inhibits Ang-II-induced activation of EGFR signalling. (A and B) Western blot analysis of p-EGFR and EGFR protein levels (A), and quantitative analysis of the ratio of p-EGFR/EGFR (B) in mice infused with Ang II (1 μg/kg/min) for 2 or 4 weeks. (C, D, E, F, G and H) Male C57BL/6J mice, aged 8 weeks, were locally infused with 1×10^{10} lentiviral particles encoding si*MyD88* or si*Ctrl* and administrated with Ang II (1 μg/kg/min) or saline for 4 weeks. Western blot analysis of p-EGFR and EGFR protein levels (C), and quantitative analysis of the ratio of p-EGFR/EGFR (D). Real-time PCR analysis of *EREG* (E) and *AREG* (F) mRNA levels. ELSA analysis of cardiac protein levels of EREG (G) and AREG (H). $n = 6$ mice/group. (I, J, K and L) 1×10^6 primary mouse cardiomyocytes were incubated with 10^{-7} mol/L Ang II or 10^{-7} mol/L aldosterone with or without 10^{-6} mol/L spironolactone for 6 h. Western blot analysis of p-EGFR and EGFR protein levels (I), and quantitative analysis of the ratio of p-EGFR/EGFR (J). Real-time PCR analysis of *EREG* (K) and *AREG* (L) mRNA levels. n = 5 independent experiments. Results are mean ± S.E.M. **P* < 0.05, ***P* < 0.01, ****P* < 0.001. The relative mRNA levels of genes were normalized to the expression of *Gapdh*.

Aldosterone, as a direct downstream factor of angiotensin signalling, was closely correlated with subsequent increasing incidence of cardiovascular injuries (Vasan *et al.* 2004). The circulating level of aldosterone was extremely higher in patients with chronic heart failure (Pitt *et al.* 1999, 2003). In patients with myocardial infarction, the plasma level of aldosterone was suddenly increased to more than two-fold and predicted the therapeutic consequences (Beygui *et al.* 2006). In animal experiments, circulating level of aldosterone was also upregulated in Ang-II- or dietary-treated mice, accompanied with severe cardiovascular complications (Luther *et al.* 2012, Huby *et al.* 2016). Genetic deficiency of aldosterone signalling protected against Ang-II-induced multiple tissue injuries (Luther *et al.* 2012). Mechanistically, mineralocorticoid receptor determines the biological effects of aldosterone in cardiovascular consequences. Using mouse model with cardiomyocyte-specific ablation of the mineralocorticoid receptor, Fraccarollo *et al.* found that cardiac fibrosis and dysfunction were suppressed after myocardial infarction (Fraccarollo *et al.* 2011). The therapeutic addition of mineralocorticoid receptor antagonists obviously reduced the incidence of cardiovascular mortality in clinical trials (Pitt *et al.* 1999). Consistent with previous reports, the present study also found that aldosterone level was remarkably increased in Ang-II-infused mice and that blockage of mineralocorticoid receptor activation could inhibit the activities of Ang II/TRIF/EGFR axis.

Another key finding of our study was to address the crosstalk between TRIF and EGFR activation in Ang-II-mediated cardiac remodelling. Although EGFR has been well demonstrated as a therapeutic target for cancer treatment in patients, emerging studies have demonstrated that EGFR also plays critical roles in cardiac remodelling. Liang *et al.* showed that EGFR mediated the pathogenesis of STZ-induced diabetic cardiac damage and remodelling via ROS generation (Liang *et al.* 2015). Specific EGFR inhibitor effectively attenuated Ang-II-induced cardiac hypertrophy, disorganization and fibrosis (Peng *et al.* 2016). Mechanistically, the elevated levels of EGFR ligands, such as amphiregulin (AREG) and epiregulin (EREG), activated EGFR and

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stimulated its phosphorylation (De *et al.* 2015). Previous studies have also found that TLR4/EGFR axis mediated lipopolysaccharide (LPS)-induced murine model of acute lung injury and inflammatory response in hepatic stellate cells (Tu *et al.* 2012, De *et al.* 2015). Besides, palmitic acid induced EGFR activation through TLR4/c-Src signalling cascade in H9C2 cells (Li *et al.* 2016). However, whether TLR4 co-adaptor TRIF participated in this EGFR-mediated cardiac remodelling is still unknown. Present finding showed that blockage of cardiac TRIF inhibited Ang-II-induced inflammatory response, including inactivation of NF-κB and downregulation of inflammatory mRNA levels. More interestingly, the levels of EGFR ligands AREG and EREG also obviously decreased in TRIF siRNA-treated heart. All these findings supported TRIF/EGFR signalling could mediate Ang II-induced cardiac remodelling.

Present echocardiographic results showed cardiac TRIF was a critical mediator in Ang-II-induced cardiac hypertrophy. As an inflammatory transducer, this finding supported the close links between excessive inflammatory response and cardiac hypertrophy. Pathological hypertrophy is often characterised as impaired myocardial vascularization, unfavorable changes in the extracellular matrix composition, and fibrosis (Weber & Brilla 1991). The abnormal inflammatory response, such as increased levels of TNF-α and soluble TNF-α receptors, serve as prognostic markers in patients with heart hypertrophy (Schonbohn *et al.* 1995). Meanwhile, the activation of NF-κB pathway further amplifies inflammatory cascade in the process of cardiac pathophysiology. In mice mutant for the TNF-α transgene and knockout of the p50 subunit of NF-κB, cardiac function and survival was significantly improved in inflammation-induced cardiac hypertrophy (Purcell *et al.* 2001). In the present study, silence of cardiac TRIF not only significantly reduced Ang II-induced inflammation, but also protected against cardiac hypertrophy.

Present study first addressed the existence of cardiac TRIF/EGFR signalling, which mediated the pathophysiological role of Ang II in cardiac remodelling. However, the detailed molecular interaction between these two targets is still unclear. Our finding supposed NF-κB and its downstream EGFR ligands were possible mediators. In the future study, it is needed to fully investigate the underlying mechanism.

In conclusion, the present study supported that cardiac TRIF was a potential therapeutic target for attenuating cardiac pathophysiological remodelling. The TRIF/EGFR axis partially explained the molecular mechanism of Ang-II-induced cardiac inflammation, fibrosis, hypertrophy and dysfunction in mice.

Supplementary materials

This is linked to the online version of the paper at https://doi.org/10.1530/JME-20-0059.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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