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TRAF3IP2 Mediates Aldosterone/Salt-Induced Cardiac Hypertrophy and Fibrosis

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Abstract

Aberrant activation of the renin-angiotensin-aldosterone system (RAAS) contributes to adverse cardiac remodeling and eventual failure. Here we investigated whether TRAF3-interacting Protein 2 (TRAF3IP2), a redox-sensitive cytoplasmic adaptor molecule and an upstream regulator of nuclear factor- κ B (NF- κ B) and activator protein-1 (AP-1), mediates aldosterone-induced cardiac hypertrophy and fibrosis. Wild type (WT) and TRAF3IP2-null mice were infused with aldosterone (0.2mg/kg/day) for 4 weeks along with 1%NaCl in drinking water. Aldosterone/salt, but not salt alone, upregulated TRAF3IP2 expression in WT mouse hearts. Aldosterone elevated blood pressure to a similar extent in both WT and TRAF3IP2-null groups. Importantly, *TRAF3IP2* gene deletion attenuated aldosterone/salt-induced (i) p65 and c-Jun activation, (ii) extracellular matrix (collagen I α 1 and collagen 3 α 1), matrix metalloproteinase (MMP2), lysyl oxidase (LOX), inflammatory cytokine (IL-6 and IL-18), chemokine (CXCL1 and CXCL2), and adhesion molecule (ICAM1) gene expression in hearts, (iii) IL-6, IL-18, and MMP2 protein levels, (iv) systemic IL-6 and IL-18 levels, and (iv) cardiac hypertrophy and fibrosis. These results indicate that TRAF3IP2 is a critical signaling intermediate in aldosterone/salt-induced myocardial hypertrophy and fibrosis, and thus a potential therapeutic target in hypertensive heart disease.

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Declaration of the Interest

Authors have no conflicts of interest.

Keywords

RAAS; aldosterone; TRAF3IP2; CIKS; Act1; cardiac fibrosis; cardiac hypertrophy

Introduction

Aldosterone regulates various cellular functions through classical mineralocorticoid receptor (MR)-mediated gene regulation, and by MR-independent non-genomic actions (Connell and Davies, 2005). It is a key regulator of blood pressure and electrolyte balance. Aldosterone, in the presence of high salt, has been shown to induce cardiac fibrosis in animal models (Nakamura et al., 2009; Robert et al., 1995). However, the molecular mechanisms underlying these pathological changes have not been fully investigated.

TRAF3-interacting protein 2 (TRAF3IP2), also known as CIKS (connection to I kappa B kinase and stress-activated protein kinase/c-Jun N-terminal kinase) and Act1 (NF- κ B activator 1), is a cytoplasmic, redox-sensitive adaptor molecule, and is known to activate NF- κ B and AP-1 via I kappa B kinase (IKK) and c-Jun N-terminal kinase (JNK), respectively (Leonardi et al., 2000; Li et al., 2000). Its causal role in various inflammatory and autoimmune disorders is well described (Debniak et al., 2014; Doyle et al., 2012; Kang et al., 2013; Perricone et al., 2013; Qian et al., 2007). However, its causative role in cardiovascular diseases has not been fully investigated. In the heart, both cardiac fibroblasts and cardiomyocytes express TRAF3IP2 (Valente et al., 2012; Valente et al., 2013a; Valente et al., 2013b). The proinflammatory cytokine interleukin (IL)-18 induces cardiac fibroblast migration and differentiation *in vitro* in part via TRAF3IP2 (Valente et al., 2012). TRAF3IP2 also contributes to advanced oxidation protein products (AOPPs)-induced cardiomyocyte death (Valente et al., 2013b). Recently, we also demonstrated the fundamental role of TRAF3IP2 in angiotensin (Ang) II-induced cardiac hypertrophy and fibrosis. In those studies, induction of TRAF3IP2 led to the activation of NF- κ B and AP-1, and upregulation of various proinflammatory and pro-fibrotic genes, implicating a causal role for TRAF3IP2 in the pathogenesis of cardiovascular diseases (Valente et al., 2013a; Valente et al., 2013b). However, its role in aldosterone-induced cardiac hypertrophy and remodeling has not been investigated.

Previous studies have reported that aldosterone activates NF- κ B and AP-1 in the heart (Doi et al., 2008; Okoshi et al., 2004; Rebsamen et al., 2004; Rude et al., 2005; Sun et al., 2002). Since TRAF3IP2 is an upstream activator of both NF- κ B and AP-1, we hypothesized that TRAF3IP2 mediates aldosterone-induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation *in vitro*. Supporting our hypothesis, silencing TRAF3IP2 blunted aldosterone-induced pro-growth, pro-mitogenic and pro-migratory effects in cultured cardiomyocytes and cardiac fibroblasts (Somanna et al., 2015). To confirm our *in vitro* observations, we investigated whether TRAF3IP2 mediates aldosterone-induced cardiac hypertrophy and fibrosis *in vivo*, and determined whether blood pressure plays a role in this phenotype.

Materials and Methods

Animals

This investigation conformed to the *Guide for the Care and Use of Laboratory Animals*, published by the National Institutes of Health. All protocols were approved by the Institutional Animal Care and Use Committee of Tulane University, New Orleans, LA. TRAF3IP2-null mice on the C57Bl/6 background were previously described (Claudio et al., 2009). The absence of TRAF3IP2 expression was confirmed by immunoblotting using left ventricular (LV) tissue (Figure 1A). Wild type C57Bl/6 mice (WT; The Jackson Laboratory, ME) served as controls.

Aldosterone treatment

Twelve to thirteen week-old male mice were used for the study. As salt intake is a requisite for the development of cardiac fibrosis in aldosterone treated mice, both WT and TRAF3IP2-null mice were maintained on 1% (w/v) NaCl in drinking water. Aldosterone was dissolved in 5% ethanol (Sigma-Aldrich), and continuously infused at a rate of 0.2 mg/Kg/day using subcutaneously implanted osmotic minipumps (model 2004; Alzet Durect Corp) for 4 weeks. The vehicle, 5% ethanol, was infused in control mice. Dosage and duration of aldosterone infusion were derived from previous studies (Nakamura et al., 2009). Blood pressure was monitored weekly by tail-cuff plethysmography (BP 2000 Blood Pressure Analysis System, Visitech) and measurements were made between 9.00 and 11.00AM. After 4 weeks of infusion, mice were anesthetized, blood was collected for serum, vessels perfused with saline, and the hearts excised and weighed. A thin midsection of heart was collected for histological analysis. LV tissue was used for protein and mRNA expression.

Echocardiography, hypertrophy, and histology

Prior to sacrifice, transthoracic echocardiography was performed under isoflurane anesthesia using a Vevo 770 high-resolution ultrasound system (VisualSonics, Toronto, ON) with a 30-MHz frequency real-time microvisualization scan head (RMV707). M-mode tracings were taken. LV anterior and posterior wall thickness and internal diameters were measured at both systole and diastole. Fractional shortening was measured as an indicator of left ventricular function. Heart and body weights were recorded. A thin midsection of heart was fixed overnight in 4% paraformaldehyde, embedded in paraffin and 5 μ m-thick sections were stained with H&E for cardiomyocyte size determination and Masson's trichrome for collagen deposition. For cardiomyocyte size, 50 cells were measured for each mouse (4 mice/group) using Image J software.

mRNA expression

Total RNA was isolated from frozen LV tissue using Trizol reagent (Sigma), and 0.5 g of total RNA was reverse transcribed into cDNA using a reverse transcription kit (Agilent). mRNA expression was analyzed by RT-qPCR using TaqMan® probes (Applied Biosystems): Atrial natriuretic peptide (ANP; Assay ID: Mm01255748), collagen, type 1, α 1 (Col1 α 1; Assay ID: Mm00801666), Angiotensin II receptor, type 1 (AGTR1A; Assay

ID: Mm01957722), collagen, type III, $\alpha 1$ (Col3 $\alpha 1$; Assay ID: Mm1254476), Matrix metalloproteinase 2 (MMP2; Assay ID: Mm01253621), Interleukin 6 (IL-6; Assay ID: Mm00446191), Interleukin 18 (IL-18; Assay ID: Mm00434226), Intercellular adhesion molecule 1 (ICAM1; Assay ID: Mm01175876), chemokine (C-X-C motif) ligand 1 (CXCL1; Assay ID: Mm00433859), chemokine (C-X-C motif) ligand 2 (CXCL2; Assay ID: Mm00436450), and Lysyl oxidase (LOX; Assay ID: Mm00495386). Data were analyzed using the 2^{-C_t} method. 18S (Assay ID: Hs99999901) served as the endogenous invariant control gene, and all data were normalized to corresponding 18S levels.

Immunoblotting

LV tissue homogenization, electrophoresis, and immunoblotting were all described previously (Valente et al., 2012). α -tubulin was used as loading control. Phospho-p65 (Ser536; # 3031), p65, phospho-c-Jun (Ser63, # 9261), c-Jun and α -tubulin antibodies were obtained from Cell Signaling Technology, Inc. (Beverly, MA). Anti-TRAF3IP2 antibodies were purchased from IMGENEX (#IMG-563; San Diego, CA). Mouse IL-6 affinity-purified polyclonal antibodies (AF-406-NA) were from R&D Systems. Anti-IL-18 antibodies that specifically detect the mature form of IL-18 (210-401-323S) were from Rockland. Anti-MMP2 antibodies (NB200-114G) that detect both pro and active forms were from Novus Biologicals (Littleton, CO).

Serum cytokine levels

The concentration of IL-6 in serum was analyzed by ELISA (Mouse IL-6 Quantikine ELISA Kit, #M6000B, R&D System) according to the manufacturer's instructions. The sensitivity of assay was 1.8 pg/ml. Serum IL-18 levels were also analyzed by an ELISA (Mouse IL-18 Platinum ELISA, #BMS618/2, Affymetrix eBioscience, San Diego, CA).

Statistics

All data are expressed as mean $\pm$ SE. Statistical significance was determined by one-way analysis of variance followed by Tukey's post-hoc test. Differences are considered significant if the *P* value is less than or equal to 0.05.

Results

Effect of TRAF3IP2 on aldosterone-induced blood pressure

Under basal conditions, no significant differences in blood pressure were observed between WT and TRAF3IP2-null mice during the 4-week study period (Figure 1B). Further, 1% NaCl (salt) alone failed to alter blood pressure in either group. However, aldosterone treatment for 4 weeks significantly increased blood pressure in both WT and TRAF3IP2-null mice to a similar extent (Figure 1B).

Effect of TRAF3IP2 on aldosterone-induced myocardial NF- κ B and AP-1 activation

While salt treatment alone failed to alter cardiac TRAF3IP2 expression in WT mice (Supplemental Figure 1), aldosterone/salt treatment markedly upregulated cardiac expression of TRAF3IP2 in WT mice (Figure 2A). Further, aldosterone increased

myocardial NF- κ B activity in WT mice, as evidenced by increased phospho-p65 levels (Figure 2B). Similarly, aldosterone increased AP-1 activity in the LV tissue of WT mice, as seen by increased levels of phospho-c-Jun levels (Figure 2C). However, levels of both phospho-p65 and phospho-c-Jun were markedly attenuated in aldosterone-treated TRAF3IP2-null mice (Figure 2B and 2C). These results support our hypothesis that aldosterone induced NF- κ B and AP-1 activities in the heart are mediated partly via TRAF3IP2.

Effect of TRAF3IP2 on aldosterone-induced cardiac hypertrophy

Aldosterone significantly increased cardiac hypertrophy in WT mice as evidenced by the increased heart to body weight ratio (Figure 3A). Although hypertrophy was observed in TRAF3IP2-null mice, it was significantly less than that of WT mice (Figure 3A). These *post mortem* data were consistent with the echocardiography measurements obtained prior to sacrifice. LV posterior wall thickness at diastole and systole was significantly increased (57% and 68%) by aldosterone treatment in WT mice, but not in TRAF3IP2-null mice (3% and 4.5%) (Figure 3B & Figure 4A). Similar findings were observed in LV anterior wall dimensions in WT and TRAF3IP2-null mice after aldosterone treatment (Figure 3B & Figure 4B). Cardiomyocyte size (cross-sectional area) was increased significantly (by 35%) in aldosterone treated-WT mice but not in TRAF3IP2-null mice (Figure 3C). Supporting these results, expression of atrial natriuretic peptide (ANP), a marker for cardiac hypertrophy, was increased 7-fold in WT mice, whereas its levels increased by only 2.5-fold in TRAF3IP2-null mice (Table 1). The cardiac function did not appear to be altered significantly by aldosterone treatment in either WT or TRAF3IP2-null mice, as evidenced by insignificant changes in percent fractional shortening (Figure 4D). These results demonstrate that TRAF3IP2 mediates aldosterone-induced cardiac hypertrophy.

Effect of TRAF3IP2 on aldosterone-induced cardiac fibrosis and ECM-related gene expression

Increased cardiac hypertrophy observed in aldosterone-treated WT mice was associated with a marked increase in perivascular, but not interstitial, fibrosis (Figure 5). However, fibrosis was less pronounced in TRAF3IP2-null mice (Figure 5). In line with the histological observations, the mRNA expression of ECM proteins, Col1 α 1, Col3 α 1, LOX and MMP2 was increased by 2.5-, 2.8-, 2.0- and 2.4-fold respectively in aldosterone-treated WT mice, but no such changes were observed in TRAF3IP2-null mice (Table 1). These results indicate that TRAF3IP2 mediates aldosterone-induced cardiac fibrosis by inducing pro-fibrotic gene expression.

Effect of TRAF3IP2 in aldosterone-induced proinflammatory, adhesion molecule and AGTR1 (angiotensin II receptor, type 1) gene expression

We next analyzed the expression levels of NF- κ B- and AP-1-responsive proinflammatory mediators that also play a role in the development and progression of cardiac hypertrophy and fibrosis. Aldosterone infusion increased cardiac interleukin-6 (IL-6) and IL-18 mRNA levels (4.5- and 2.1-fold respectively) in the WT mice, but not in TRAF3IP2-null mice (Table 1). Similarly, intercellular adhesion molecule 1 (ICAM1), chemokine (C-X-C motif) Ligand 1 and 2 (CXCL1 & CXCL2) mRNA levels showed a strong trend towards an

increase in the aldosterone-treated WT mouse hearts, but not in TRAF3IP2-null mice (Table 1). Interestingly, AT1 (AGTR1A) mRNA expression was also increased (~2.9-fold) in aldosterone-treated WT mice, but not in TRAF3IP2-null mice (Table 1).

Effect of TRAF3IP2 in aldosterone-induced IL-6, IL-18 and MMP2 protein expression

As *TRAF3IP2* gene deletion blunted aldosterone-induced IL-6, IL-18 and MMP2 mRNA expression, we next determined whether their protein expression follows a similar trend in aldosterone-infused TRAF3IP2-null mice. In fact, while aldosterone significantly increased IL-6, IL-18 and MMP2 protein levels in hearts of WT mice, their induction was attenuated in the TRAF3IP2-null mice (Figure 6).

Effect of TRAF3IP2 in aldosterone-induced systemic IL-6 and IL-18 levels

IL-6 and IL-18 are secreted proteins. Our results show that aldosterone increases their serum levels in WT mice. Although their levels were also increased in the serum of TRAF3IP2-null mice, these were much lower compared to WT mice (Figure 7).

Discussion

The renin-angiotensin-aldosterone system (RAAS) is a critical regulator of blood pressure and water balance under normal physiological conditions. However, aberrant activation of the RAAS can contribute to cardiac hypertrophy, fibrosis, and eventual failure. Here we investigated the role of TRAF3IP2 as a potential mediator of aldosterone/salt-induced cardiac hypertrophy and fibrosis *in vivo*. We demonstrated that aldosterone-induced cardiac hypertrophy and fibrosis are associated with increased TRAF3IP2 expression and enhanced NF- κ B/p65 and AP-1/c-Jun activation in LV tissues of WT mice, whereas *TRAF3IP2* gene deletion markedly attenuated aldosterone-induced NF- κ B and AP-1 activation, and cardiac hypertrophy and fibrosis, in a manner that is independent of increased blood pressure. Further, the protective effects of *TRAF3IP2* gene deletion were associated with reduced expression of aldosterone-induced extracellular matrix proteins and their regulators (Col1 α 1, Col3 α 1, LOX, and MMP2), proinflammatory cytokines (IL-6 and IL-18), chemokines (CXCL1 and CXCL2), and the adhesion molecule ICAM1. Thus, TRAF3IP2 is a critical signaling intermediate in aldosterone-induced adverse cardiac remodeling.

Increased systemic aldosterone levels are strongly associated with left ventricular hypertrophy (LVH). It is also an important regulator of cardiac collagen turnover in man (Iraqi et al., 2009; MacFadyen et al., 1997; Matsumura et al., 2006; Nakahara et al., 2007; Rossi et al., 1996; Stowasser et al., 2005; Zannad et al., 2000). Further, aldosterone has been shown to induce hypertrophy and re-expression of the fetal gene ANP in isolated cardiomyocytes (Lopez-Andres et al., 2008; Okoshi et al., 2004), and its continuous infusion induces cardiac hypertrophy and fibrosis in animal models (Nakamura et al., 2009). Aldosterone has also been shown to induce activation of NF- κ B and AP-1 and proinflammatory cytokine expression in hearts (Doi et al., 2008; Okoshi et al., 2004; Rebsamen et al., 2004; Rude et al., 2005; Sun et al., 2002). Consistent with these *in vitro* and *in vivo* reports, our study showed that aldosterone induced LVH, elevated ANP expression, increased phosphorylated p65 (NF- κ B) and c-Jun (AP-1) levels, and enhanced

the expression levels of the extracellular matrix proteins Col1 α 1 and Col3 α 1 in WT mice. Aldosterone also enhanced the expression levels of IL-6 and IL-18 and activation of MMP2 in WT mice hearts. ICAM1 expression was also markedly increased in WT mice, indicating that aldosterone infusion is associated with enhanced inflammation- and remodeling-associated gene expression in the heart.

We have previously demonstrated that TRAF3IP2 physically associates with IKK and JNK, and activates both NF- κ B and AP-1 in cardiomyocytes (Valente et al., 2012). We also reported that aldosterone induces hypertrophy of isolated cardiomyocytes in part via TRAF3IP2-dependent NF- κ B and AP-1 activation (Somanna et al., 2015). Supporting these *in vitro* observations, here we show that *TRAF3IP2* gene deletion blunts aldosterone-induced NF- κ B and AP-1 activation, and cardiac hypertrophy and fibrosis in an animal model

IL6, *IL18*, and *MMP2* are NF- κ B and/or AP-1 responsive genes. One possible mechanism by which *TRAF3IP2* gene deletion blunts the development of LVH may be by inhibiting the induction/upregulation of aldosterone-induced IL-6, IL-18 and MMP2 expression, as these mediators are known to contribute to adverse cardiac remodeling and dysfunction (Melendez et al., 2010; Wang et al., 2006; Xing et al., 2010). Recently, we also reported that TRAF3IP2 mediates aldosterone-induced IL-18 expression and cardiac fibroblast proliferation and migration in vitro (Somanna et al., 2015). Thus, the decreased expression of IL-18, the reduced levels of collagens I and III, and the reduced activation of LOX and MMP2 might have contributed to reduced cardiac fibrosis in aldosterone-treated TRAF3IP2-null mice. Along with reduced local (cardiac) levels, the systemic levels of IL-6 and IL-18 levels were also reduced, possibly blunting their autocrine and paracrine signaling, resulting ultimately in diminished hypertrophy and fibrosis in the TRAF3IP2-null mice.

Interestingly, aldosterone has been shown to induce cardiac dysfunction in part via Ang II, since AT1 receptor antagonists partially blocked aldosterone-induced cardiac hypertrophy and fibrosis (Robert et al., 1999). Recent studies also reported that AT1 mediates aldosterone-induced profibrotic gene expression in isolated cardiomyocytes (Tsai et al., 2013). Since AGTR1 (AT1) is an AP-1 responsive gene (Herzig et al., 1997), it is possible that decreased Ang II signaling might have contributed to reduced hypertrophy and fibrosis in aldosterone-treated TRAF3IP2-null mice. In support of this hypothesis, our results show a marked reduction in AT1 expression in aldosterone-treated TRAF3IP2-null mice compared to their WT controls.

In addition to IKK/NF- κ B and JNK/AP-1 activation, TRAF3IP2 has also been shown to regulate activation of C/EBP β , p38 MAPK, and ERK1/2, all of which contribute to the development of cardiac hypertrophy (Bostrom et al., 2010; Zepp et al., 2011; Zhang et al., 2003). Of note, p38 and ERK1/2 play a role in aldosterone-induced cardiomyocyte hypertrophy and cardiac fibroblast proliferation, respectively (Lopez-Andres et al., 2008; Stockand and Meszaros, 2003). Therefore, reduced activation of these signaling molecules/pathways might have also contributed to decreased cardiac hypertrophy and fibrosis in aldosterone-treated TRAF3IP2-null mice.

Mechanisms by which aldosterone activates TRAF3IP2 are not well understood. TRAF3IP2 mediates IL-17 signaling by binding to the IL-17 receptors through an SEFIR (similar expression to fibroblast growth factor genes and IL-17R) domain (Zhang et al., 2013). However, no SEFIR domains are identified in MR, thus ruling out the possibility of a direct interaction between TRAF3IP2 and MR. Further, analysis of *TRAF3IP2* 5'-flanking sequence revealed no obvious classical MR-binding sequences, thus ruling out a direct role for MR in *TRAF3IP2* transcription. However, we previously demonstrated that oxidative stress induces *TRAF3IP2* gene expression (Valente et al., 2012; Valente et al., 2013a; Valente et al., 2013b), and it is well known that aldosterone is a potent inducer of oxidative stress in cardiomyocytes (Sun et al., 2002) (Takimoto and Kass, 2007). In our recent study, we also demonstrated that Nox4/ROS play a critical role in aldosterone-induced TRAF3IP2 expression in cardiomyocytes (Somanna et al., 2015). Thus, it is highly likely that aldosterone-induced oxidative stress contributed to TRAF3IP2 upregulation in the WT mouse heart.

In conclusion, we show for the first time a potential causal role for TRAF3IP2 in aldosterone-induced cardiac hypertrophy and fibrosis *in vivo*. As clinical studies provided the evidence that aldosterone antagonists offer additional beneficial effects when given along with AT1 blockers or ACE inhibitors in heart failure subjects, our study highlights TRAF3IP2 as a promising therapeutic target in heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

Act1	activator of NF- κ B
Ang II	angiotensin II
ANP	atrial natriuretic peptide
AOPPs	advanced oxidation protein products
AP-1	activator protein-1
C/EBP	CCAAT/enhancer-binding protein
CIKS	Connection to IKK and SAPK/JNK
IKK	I κ B kinase
Col1α1	collagen I α 1

Col3a1	collagen III α 1
CXCL1	chemokine (C-X-C motif), ligand 1
CXCL2	chemokine (C-X-C motif) ligand 2
ICAM1	inter cellular adhesion molecule 1
IκB	inhibitory kappa B
IL	interleukin
JNK	c-Jun amino-terminal kinase
LOX	Lysyl Oxidase
MMP	matrix metalloproteinase
MR	mineralocorticoid receptor
NF-κB	nuclear factor κ B
ROS	reactive oxygen species
SAPK	stress-activated protein kinase/Jun kinase
SBP	systolic blood pressure
SEF	similar expression to fibroblast growth factor genes
SEFIR	SEF-IL-17 receptor
TRAF	TNF Receptor Associated Factor
TRAF3IP2	TRAF3 interacting protein 2

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Highlights

- Aldosterone induces cardiac TRAF3IP2 levels in WT mice
- TRAF3IP2-null mice are resistant to aldosterone-induced cardiac hypertrophy
- TRAF3IP2-null mice are resistant to aldosterone-induced cardiac fibrosis
- TRAF3IP2 mediates aldosterone-induced pro-hypertrophic gene expression
- TRAF3IP2 mediates aldosterone-induced pro-inflammatory and fibrotic gene expression

Figure 1. Effect of aldosterone on blood pressure in wild type and TRAF3IP2-null mice
 (A) Immunoblot of TRAF3IP2 in WT and TRAF3IP2-null mice hearts (n=4/group). (B)
 Blood pressure in WT and TRAF3IP2-null mice treated with salt alone and aldosterone +
 salt combination. Values are mean $\pm$ SE (n = 6-8). ‘#’ and ‘*’ indicates statistical
 significance ($P < 0.05$) when compared between salt and aldosterone + salt-treated wild type
 and TRAF3IP2-null mice respectively.

Figure 2. Effect of aldosterone treatment on cardiac TRAF3IP2 protein expression, and NF-κB, and AP-1 activation

(A) Immunoblot of TRAF3IP2 in WT mice treated with salt alone or aldosterone + salt. (B) Immunoblot analysis of phosphorylated and total p65 levels. Densitometric analysis of the immunoreactive bands is shown on the left. (C) Immunoblot analysis of phosphorylated and total c-Jun levels. Densitometric analysis of the immunoreactive bands is shown on the left. Values are mean $\pm$ SE (n = 3). ‘*’ indicates $P < 0.05$ when compared between salt and aldosterone + salt-treated groups within the same strain. ‘#’ indicates $P < 0.05$ when compared between aldosterone + salt-treated WT and TRAF3IP2-null mice.

Figure 3. Effect of aldosterone treatment on cardiac hypertrophy in wild type and TRAF3IP2-null mice

(A). Heart to body weight ratio. (B). Echocardiographic images (M mode). (C).

Cardiomyocyte size. Values are mean $\pm$ SE of 6-8 mice for the heart to body weight ratio and for echocardiography, whereas 4 mice for cardiomyocyte size determination. * P 0.05, ** P 0.01; *** P 0.001 when compared between salt and aldosterone + salt-treated-treated groups within the same strain; # P 0.05, ## P 0.01, ### P 0.001 when compared between aldosterone + salt-treated WT and TRAF3IP2-null mice.

Figure 4. Effect of aldosterone treatment on echocardiographic parameters in WT and TRAF3IP2-null mice

(A). Left Ventricular Posterior wall dimensions at systole and diastole (LVPWs, LVPWd). (B). Left Ventricular Anterior wall dimensions at systole and diastole (LVAWs, LVAWd). (C). Left Ventricular Internal diameter at systole and diastole (LVIDs, LVIDd). (D). Fraction shortening (FS). Values are mean $\pm$ SE (n = 6-8). * P 0.05, ** P 0.01, *** P 0.001 when compared between salt and aldosterone + salt-treated groups within the same strain; # P 0.05, ## P 0.01; ### P 0.001 when compared between aldosterone + salt-treated WT and TRAF3IP2-null mice.

Figure 5. Effect of aldosterone treatment on cardiac fibrosis in wild type and TRAF3IP2-null mice

Representative photomicrographs of cardiac sections stained with Masson's trichrome (magnification, X100) for collagen deposition (stained blue). WT: wild type; Aldo/salt: aldosterone/salt treatment.

Figure 6. Effect of aldosterone treatment on cardiac IL-6, IL-18 and MMP2 expression
 (A) Immunoblot analysis of IL-6. Densitometric analysis of the immunoreactive bands is shown on the right. (B). Immunoblot analysis of IL-18. Densitometric analysis of the immunoreactive bands is shown on the right. (C). Immunoblot analysis of MMP2. Densitometric analysis of the immunoreactive bands is shown on the right.

Figure 7. Effect of aldosterone treatment on systemic IL-6 and IL-18 levels in wild type and TRAF3IP2-null mice

(A) Serum IL-6 and (B) IL-18 levels were measured by ELISA. WT: wild type; Aldo/salt: aldosterone/salt treatment.

Table 1

Effect of aldosterone treatment on cardiac gene expression in WT and TRAF3IP2-null mice

Target	WT		TRAF3IP2-null	
	Salt	Aldo/salt	Salt	Aldo/salt
ANP	1.00±0.45	7.05±3.18 [*]	1.23±0.24	2.52±0.37 ^{*#}
Col1α1	1.00±0.25	2.50±0.30 ^{**}	1.50±0.4	1.40±0.30
Col3α1	1.00±0.35	2.80±0.30 ^{**}	1.60±0.60	1.80±0.50
MMP2	1.00±0.30	2.40±0.16 ^{**}	1.10±0.08	1.20±0.20 [#]
IL-6	1.00±0.12	4.50±0.80 ^{***}	1.20±0.30	2.00±0.30 [#]
IL-18	1.00±0.15	2.10±0.60 [*]	1.10±0.45	1.50±0.30
CXCL1	1.00±0.20	1.80±0.40	1.10±0.10	1.00±0.10
CXCL2	1.00±0.20	1.40±0.30	0.70±0.10	1.00±0.20
ICAM1	1.00±0.10	1.50±0.30	0.90±0.10	1.20±0.10
LOX	1.00±0.16	2.0±0.55 ^{**}	0.79±0.25	0.55±0.05 [#]
AT1A	1.00±0.27	2.96±0.26^{**}	1.65±0.25	1.75±0.35[#]

WT, wild type; Aldo, aldosterone. 18S rRNA served as an internal control. Values are mean ± SE (n = 3-5).

^{*} *P* 0.05^{**} *P* < 0.01 and^{***} *P* < 0.001 when compared between salt and Aldo/salt-treated groups within the same strain.[#] *P* 0.05 when compared between Aldo/salt-treated WT and TRAF3IP2-null mice.