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Angiotensin II signaling up-regulates the immediate early transcription factor ATF3 in the left but not the right atrium

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Abstract

The atria respond to various pathological stimuli including pressure and volume overload with remodeling and dilatation. Dilatation of the left atrium is associated with atrial fibrillation. The mechanisms involved in chamber-specific hypertrophy are largely unknown. Angiotensin II is hypothesized to take part in mediating this response. ATF3 is an immediate early gene found at the receiving end of multiple stress and growth stimuli. Here we characterize ATF3 as a direct target gene for angiotensin II. ATF3 expression is regulated by angiotensin receptor-mediated signaling in vivo and in vitro at the transcriptional level. ATF3 induction is mediated by cooperation between both the AT_{1A} and AT₂ receptor subtypes. While AT₂R blocker (PD123319) efficiently blocks ATF3 induction in response to angiotensin II injection, it results in an increase in blood pressure indicating that the effect of angiotensin II on ATF3 is independent of its effect on blood pressure. In contrast to adrenergic stimulation that induces ATF3 in all heart chambers, ATF3 induction in response to angiotensin II occurs primarily in the left chambers. We hypothesize that the activation of differential signaling pathways accounts for the chamber-specific induction of ATF3 expression in response to angiotensin II stimulation. Angiotensin II injection rapidly activates the EGFR-dependent pathways including ERK and PI3K-AKT in the left but not the right atrium. EGF receptor inhibitor (Gefitinib/Iressa) as well as the AKT inhibitor

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(Triciribine) significantly abrogates ATF3 induction by angiotensin II in the left chambers. Collectively, our data strongly place ATF3 as a unique nuclear protein target in response to angiotensin II stimulation in the atria. The spatial expression of ATF3 may add to the understanding of the signaling pathways involved in cardiac response to neuro-hormonal stimulation, and in particular to the understanding of left atrial-generated pathology such as atrial fibrillation.

Keywords

Angiotensin; Signal transduction; ATF3; Left atrium

Introduction

The chambers of the mammalian heart are specialized to handle differing physiological conditions from embryonic through adult life. Each chamber is therefore composed of cells with unique functional, structural, metabolic, and electrophysiological characteristics. Moreover, each chamber has a different genetic expression profile [43]. It is therefore possible that hypertrophy and hypertrophic pathways differ between the atria and ventricles. Despite intensive research directed at elucidating the pathways involved in ventricular hypertrophy, the mechanisms involved in atrial hypertrophy remain largely unstudied. This may be due to the fact that the atria are relatively small organs and were believed to follow the stretch and hypertrophic signals of the ventricles [13, 30].

Several lines of evidence suggest that the transcription complex activating protein 1 (AP-1) plays a major role in myocardial hypertrophy [9, 21, 31, 36]. AP-1 is composed of dimeric complexes of the Jun and Fos basic leucine zipper (bZIP) protein family [27, 40]. Their involvement in cardiac function is indicated by the up-regulation of their gene expression or activity, and the regulation of hypertrophy genes by the AP-1 complexes. Both pro- and anti-hypertrophy genes can be regulated, depending on the AP-1 complex formed on the promoter and on the signal exerted. In addition, knockout (KO) mice deficient in JunD were shown to have enhanced cardiomyocytes apoptosis upon pressure overload, indicating a protective role of JunD [21].

The above Jun/Fos dimers primarily function as transcription activators. However, two bZip proteins—JDP2 [2, 24] and ATF3 [16] are primarily transcriptional repressors as homodimers and are known to bind to the AP-1 binding sites. Their roles in cardiac function were investigated by the generation of transgenic mice ectopically expressing them in the heart [25, 26, 33]. These mice experience massive bi-atrial dilatation and display increased mortality and atrioventricular conduction defects. Genes encoding JDP2 and ATF3 differ significantly in their mode of regulation. Whereas JDP2 is ubiquitously expressed, ATF3 is an immediate early gene that responds to various stress and growth stimuli [16]. Previously, we reported that administration of angiotensin II (Ang II) to mice results in the up-regulation of ATF3 [26]. In the present study, we focussed our attention on the up-regulation of ATF3 by acute angiotensin II stimulation, specifically in different chambers of the heart. We also examined the signaling pathways that mediate the up-regulation of ATF3 and found that ATF3 is induced by angiotensin II in the left chambers in an angiotensin receptor-dependent manner mediated by the EGFR and AKT signaling pathways.

The induction of ATF3 in the left atrium correlates with atrial physiology and pathology, suggesting that the ATF3 protein may play an important role in mediating atrial signaling, function and pathology.

Materials and methods

Chemicals

Angiotensin II (Sigma A-9525); isoproterenol (sigma cat#15627); phenylephrine (Sigma P6126); tricitiribine, AKT inhibitor V (Calbiochem cat#124012); gefitinib, EGFR inhibitor (Iressa, LC laboratories G-4408); PD123319, AT₂R antagonist (Tocris Bioscience 1361); losartan, AT₁R antagonist, (50 mg tablets, MSD, Haarlem, Netherlands) were diluted in drinking water containing 5% sucrose at 1.25 mg/ml.

Mice

All studies involving mice and rats were performed according to the protocol approved by the Technion Animal Inspection Committee. The Technion holds an NIH animal approval license, number A5026-01. The animals were fed standard rat chow containing 0.5% NaCl and tap water ad libitum. C57Bl/6 background strain mice and male Munich Wistar rats were used in this study.

Mice injections

C57Bl/6 were injected intraperitoneally with 0.15/1.5, 2.5, 2.5 mg/kg of angiotensin II, isoproterenol, phenylephrine, respectively. Gefitinib was injected 18 h and 1 h prior to angiotensin II injection (DMSO, 100 mg/kg). AKT inhibitor V, was injected 30 min prior to Ang II (10% DMSO, 1 mg/kg). PD123319 (20 mg/kg) was injected 30 min prior to angiotensin II injection. At the indicated time following injection the mice were anesthetized using Ketamine and Xylazine and heart chambers were separated and flush-frozen in liquid nitrogen until further extraction.

Rat injections and blood pressure measurement

Rats weighing ~300 g was anesthetized with Nembutal (40 mg/kg, i.p.) and prepared for blood pressure measurements. Specifically, after tracheotomy, polyethylene tubes (PE50) were inserted into the carotid artery, for continuous online blood pressure monitoring with a pressure transducer (model 1050.1, UFI, Morro Bay, CA, USA). Angiotensin II injections were performed at 0.15 mg/kg and PD123319 15 mg/kg as described for mice protocol.

Western blot analysis

Organs derived from two mice (unless otherwise indicated) were pooled and homogenized in RIPA buffer containing a protease inhibitors cocktail (P-8340, Sigma-Aldrich) and subjected to SDS-PAGE followed by Western blotting [26]. The primary antibodies used were: ATF3 (C-19), GAPDH (FL-335) from Santa-Cruz, anti- α -tubulin (T-9026, Sigma-Aldrich) pERK (M9692, Sigma-Aldrich), ERK (#9102, Cell Signaling), AKT (#9272, Cell Signaling Ltd.), and pAKT (#9271, Cell Signaling Ltd.).

Immunohistochemistry

Tissue was fixed using 4% buffered formaldehyde solution for 2 days and subsequently embedded in paraffin. Staining was performed using an automated stainer ventana IVIEW DAB detection kit (Ventana medical systems, Tucson Az 85737) with primary anti-ATF3 antibody.

mRNA

Frozen tissue was either processed immediately or stored in RNAlater solution (Ambion AM7020). RNA was purified using RNeasy fibrous tissue mini kit (Qiagen 74704) according to the manufacturer's protocol. mRNA from HEK-293-AT₁R cell line was

prepared using Tri-reagent (T9424, Sigma-Aldrich) following the manufacturer's instructions.

Realtime PCR

cDNA was synthesized using a Verso™ cDNA kit (Thermo scientific AB-1453/A) according to the manufacturer's instructions. RT-PCR was conducted using Rotor-Gene Q (Qiagen Inc.) equipment using absolute blue SYBR green ROX mix (Thermo scientific AB4162/B). Primers used were:

mATF3 F	GAGGATTTTGCTAACCTGACACC
mATF3 R	TTGACGGTAACTGACTCCAGC
hATF3 F	AAGAACGAGAAGCAGCATTGTGAT
hATF3 R	TTCTGAGCCCGGACAATACAC
mB2m F	TTCTGGTGCTTGTCTCACTGA
mB2m R	CAGTATGTTTCGGCTTCCCATTC
hGAPDH F	ATGGGGAAGGTGAAGGTCG
hGAPDH R	GGGGTCATTGATGGCAACAATA
mHb-EGF F	CGGGGAGTGCAGATACCTG
mHb-EGF R	TTCTCCACTGGTAGAGTCAGC

Angiotensin II receptor subtypes expressed in the atria were performed by qRT-PCR Taqman gene expression assay (Applied Biosystems Inc.) according to the manufacturer recommended protocol. The primers used were Agtr1A # Mm 00616371_m1, Agtr1b # Mm 017071115, Agtr2 # Mm 01341373_m1 and b2 m # Mm 00437762_m1.

Cell culture

HEK-293-AT₁R cells were kindly provided by Dr. Gaetan Guillemette [3]. Cell lines were grown in DMEM with 10% FBS supplemented with antibiotics. Cells were incubated in serum-free medium for 24 h before the addition of angiotensin II (1 μM).

Statistical analysis

Analysis was performed with Microsoft Excel software. A one-tailed *t* test was used to determine statistical significance.

Results

Cardiac ATF3 expression following neuroendocrine stimuli

We have previously showed that ATF3 protein is induced in the heart following acute angiotensin II stimulation [26]. To further explore whether or not ATF3 is induced by other neuroendocrine hormones, we examined angiotensin II and either α or β adrenergic stimuli. Mice were injected intraperitoneally with pharmacological doses of the α adrenergic agonist, phenylephrine (2.5 mg/kg) the β agonist, isoproterenol (2.5 mg/kg) and two concentrations of angiotensin II (0.15 and 1.5 mg/kg). Three hours following injections, ventricles were harvested and lysates were separated by SDS-PAGE followed by Western blotting. Western blot analysis with anti-ATF3 antibodies revealed that ATF3 expression level is greatly elevated following both the α and β adrenergic stimuli (Fig. 1a). In addition, angiotensin II injection resulted in a significant increase in ATF3 expression (Fig. 1a).

Increasing the injected concentration of angiotensin II from 0.15 to 1.5 mg/kg did not result in a significant further increase in ATF3 induction (Fig. 1a right panel).

We next focussed on angiotensin II-dependent increase in ATF3 expression. We first sought to examine whether or not ATF3 induction in response to angiotensin II occurs in other organs as well. Toward this end, Western blot analysis was performed with different organs derived from mice injected with angiotensin II (Fig. 1b). As previously shown, ATF3 expression is highly induced in the heart (ventricles) following angiotensin II injection. In contrast, no induction of ATF3 expression in response to angiotensin II injection is observed in the other organs tested (Fig. 1b). Interestingly, the lung is the only organ that displays basal ATF3 expression independent of angiotensin II exposure (Fig. 1b lanes 7, 8). We next examined whether ATF3 expression in response to angiotensin II stimulation in the heart occurs in both the atria and the ventricles. To this end, mice were injected with either saline control or angiotensin II and ATF3 expression was analyzed in cell lysate derived from atria and ventricles by Western blot analysis with anti-ATF3 antibody. Cell lysates derived from either the atria or the ventricles of angiotensin II-injected mice display ATF3 induction 3 h following injection (Fig. 1c lane 3).

To examine whether ATF3 induction following angiotensin II injection is mediated by the angiotensin II type 1 receptor (AT₁R), mice were pretreated with losartan, a selective AT₁R antagonist. Losartan was provided in the drinking water for 5 days prior to angiotensin II injection. Pretreatment with losartan alone resulted in no increase in ATF3 expression in lysate derived from both the atria and ventricles (Fig. 1c lane 2). Significantly, losartan pretreatment strongly suppressed ATF3 induction following angiotensin II injection (Fig. 1c lane 4 and right panel). Thus, this experiment suggests that an angiotensin receptor-dependent signaling mediates the increase in ATF3 expression. Therefore, ATF3 expression may represent a direct nuclear target gene found at the receiving-end of the AT₁R signaling pathway [29]. Alternatively, angiotensin II injection results in an increase in blood pressure leading to the activation of AT₁R through mechanical stretch [49] or through other indirect mechanisms. Losartan was already found to inhibit the stretch-induced alterations in gene expression and activation such as for MMP 2/9 and membrane type MMP [37].

Angiotensin II mediates ATF3 induction in vitro

To examine whether ATF3 response to angiotensin II is a cell autonomous process, we used a human embryonic kidney cell line (HEK-293) with stable ectopic expression of AT₁R (HEK-293-AT₁R) and followed ATF3 expression at different time points following the addition of angiotensin II to the medium. Whole cell lysate derived from these cells was subjected to SDS-PAGE and Western blotting with anti-ATF3 antibody (Fig. 2a). Indeed, ATF3 expression is induced within 30 min following angiotensin II addition and remains high for 24 h (Fig. 2a lower panel). To examine whether the increase in ATF3 protein level correlates with an increase in ATF3 mRNA level, we used quantitative real-time PCR (qRT-PCR). We examined ATF3 mRNA levels after various exposure periods to angiotensin II stimulation in HEK-293-AT₁R cells (Fig. 2b). This analysis revealed that ATF3 mRNA is induced as early as 15 min after angiotensin II addition. Thereafter, ATF3 mRNA level peaks at 30 min–1 h and persists at twofold higher level even up to 24 h following angiotensin II exposure (Fig. 2b).

Collectively, the data strongly suggest that angiotensin II stimulation results in an angiotensin II receptor-mediated increase in mRNA steady state level. Regulation of the ATF3 expression can be provoked in vitro independent of physiological effects.

Chamber-specific response to angiotensin II

To compare the angiotensin II response between the atria and the ventricles, we separated atria and ventricles and examined the kinetics of ATF3 induction by angiotensin II using Western blot analysis with anti-ATF3 antibody (supplementary Fig. 1a). Atria and ventricles display similar kinetics. In saline control-treated mice ATF3 protein is expressed at a very low basal level. Following angiotensin II injection ATF3 induction initiates at 30 min in both atria and ventricles. ATF3 protein reaches maximal levels 2 h following angiotensin II injection in both tissues. While ATF3 expression is relatively low by 5 h in the atria, the expression persists in the ventricles (supplementary Fig. 1a).

We then compared ATF3 response to angiotensin II stimuli in the four heart chambers. To this end, mice were injected with either saline control or angiotensin II (1.5 mg/kg). Three hours following injection the four heart chambers were separated and cell lysate was subjected to SDS-PAGE and Western blotting with anti-ATF3 antibody. The cardiac expression of ATF3 in saline control-injected mice is barely detectable (Fig. 3a, lane 1). Following angiotensin II injection, a robust increase in ATF3 expression is observed in the left chambers (Fig. 3a lane 2). While in the right atrium, ATF3 expression is undetectable, a modest increase in ATF3 expression level is observed in the right ventricles (Fig. 3a lower panel). Interestingly, mice injected with either the α agonist, phenylephrine (2.5 mg/kg) or the β agonist, isoproterenol (2.5 mg/kg) displayed induced ATF3 expression in both the left and the right atria (Fig. 3b). Consistently, quantitative real-time PCR from mice injected with angiotensin II revealed that ATF3 mRNA transcript is low in saline control-injected mice (Fig. 3c). Following angiotensin II injection ATF3 mRNA level remains low in the right atrium. However, a significant potentiation of ATF3 mRNA level is observed in the left atrium and ventricles (Fig. 3c). Next we investigated the subset of cells that expresses ATF3 within the atrium. Atria derived from angiotensin II-injected and saline-injected control mice were fixed with formaldehyde and atrial sections were stained with anti-ATF3 antibody (Fig. 3d). In the control mice few cardiomyocytes in the left and right atrial specimen display low level of ATF3 nuclear staining. Following angiotensin II injection, nuclear ATF3 staining is observed in the cardiomyocytes derived from the left atrium section but only low background staining is observed in sections derived from the right atrium (Fig. 3d, middle panel). In contrast, isoproterenol injection resulted in an equivalent increase in ATF3 nuclear staining in sections derived from both left and right atria (Fig. 3d, bottom panel). Collectively, cell staining is consistent with the results obtained by Western blot analysis and qRT-PCR, suggesting that cardiomyocytes in the left atrium but not the right atrium respond to angiotensin II stimuli by increasing ATF3 expression, whereas adrenergic stimulation potentiates ATF3 expression in both the left and the right atria to a similar extent.

Angiotensin II receptor 1 and 2 are required for angiotensin II-dependent activation of ATF3

Two receptor isoforms mediate angiotensin II actions AT₁R and AT₂R [44]. Angiotensin II type 1 receptor (AT₁R) consists of subtypes, 1A and 1B. To reveal which one of the subtypes is expressed in the atria, mRNA derived from the atria was subjected to qRT-PCR with subtype-specific primers. While subtype AT_{1B}R expression is below the detectable level in both atria (data not shown) AT_{1A}R is expressed to similar level in both atria (Fig. 4a). Similar analysis for AT₂R revealed that it is expressed in the right atrium at fourfold higher level as compared with the left atrium (Fig. 4a, $P < 0.05$). We showed that the AT₁R blocker, losartan, completely suppressed ATF3 expression in response to angiotensin II stimulation (Fig. 1c). To examine the role of AT₂R in ATF3 response to angiotensin II, mice were injected with the AT₂R blocker (PD123319) 30 min prior to angiotensin II injection and the expression of ATF3 in the different heart chambers was examined by Western blot

analysis with anti-ATF3 antibody (Fig. 4b). Whereas cell lysate derived from mice injected with angiotensin II alone displayed efficient expression of ATF3 in left chambers, lysate derived from mice treated with the AT₂R blocker either alone or AT₂R blocker followed by angiotensin II injection display no ATF3 induction (Fig. 4b). Thus, AT₂R blocker completely abrogates the induction of ATF3 expression in response to angiotensin II. This data suggest that both AT₁R and AT₂R are required to mediate angiotensin II response to ATF3.

AT₂R was shown to mediate cardiac hypertrophy [19], vasodilatation and decrease in blood pressure in response to angiotensin II stimulation [23]. To examine the effects of blocking the AT₂R on both blood pressure and ATF3 induction, we decided to switch to a rat model in which blood pressure can be accurately monitored. We first tested whether or not the chamber-specific ATF3 induction in response to angiotensin II occurs also in rats. Rats were injected with angiotensin II (0.15 mg/kg) and ATF3 expression level was examined by Western blot analysis (supplementary Fig. 2a). Consistent with the results in mice, chamber-specific expression of ATF3 was observed in rats as well. We next examined the effect of AT₂R inhibition on ATF3 induction by Ang II. Rats were pretreated with AT₂R antagonist 30 min prior to Ang II injection and ATF3 expression level was examined by Western blot analysis. Similar to mice, AT₂R antagonist (PD123319) completely abrogated Ang II-dependent ATF3 induction (supplementary Fig. 2b). Thus, the chamber-specific induction of ATF3 in response to Ang II is suppressed by AT₂R inhibition in both mice and rat models. We next sought to measure the blood pressure in rats in the presence or absence of AT₂R antagonist. The basal blood pressure measured in non-injected rats is around 130 mmHg. Following injection of either AT₂R blocker or Ang II a significant increase in blood pressure is observed (Fig. 4c, $P < 0.01$). Pretreatment of rats with AT₂R blocker for 30 min followed by Ang II injection potentiated the increase in blood pressure by Ang II by 7% (Fig. 4c, $P < 0.1$). Collectively, the data suggest that Ang II-dependent increase in blood pressure is not sufficient to explain the left chamber-specific induction of ATF3. Although, we cannot exclude the possibility that higher pressure in the left chambers facilitates the activation of signal transduction pathways in the left atrium, we hypothesize that unique mechanisms are involved in Ang II signaling pathways in the left chambers leading to ATF3 induction.

The left and right atria respond differently to angiotensin II stimulation

ATF3 expression level is typically induced by either growth factors or various stress stimuli [17, 18]. The angiotensin receptor is known to activate multiple signaling pathways in the heart that may include both stress and growth stimuli [29]. To reveal the angiotensin II receptor-mediated signaling pathway involved in ATF3 expression, we examined the epidermal growth factor receptor (EGFR) transactivation. A case in point is Hb-EGF. Hb-EGF participates as a downstream component mediating the angiotensin II receptor signaling [39]. Hb-EGF is released upon angiotensin II stimulation by metalloprotease and binds to the EGFR. Thus, we examined the expression of Hb-EGF in response to angiotensin II injection by qRT-PCR (Fig. 5a). Whereas Hb-EGF is induced by twofold at the right atrium, it is induced by sevenfold in the left atrium. Hb-EGF binding to the EGFR is proposed to induce growth and proliferation signaling pathways such as the Ras-ERK signaling pathway, mTOR translation signaling and the PI3K-AKT pathway [41]. To examine whether EGFR is activated in response to angiotensin II, we followed EGFR activation by Western blot analysis with anti-phospho-ERK (Fig. 5b) and anti-phospho-AKT (Fig. 6a). Whereas, rapid ERK phosphorylation occurs already 10 min following Ang II injection in the left atrium (Fig. 5b lane 2), no induction of phospho-ERK is observed in the right atrium (Fig. 5b lane 4). Interestingly, cell lysate derived from mice injected with β -agonist, isoproterenol, resulted in ERK phosphorylation in both left and right atria (Fig. 5c). This is consistent with the finding that ATF3 is induced by β -agonist in both atria (Fig. 3d).

To determine whether ERK phosphorylation occurs in an EGFR-dependent manner, we pretreated mice with an EGFR inhibitor, Gefitinib (Iressa) (Fig. 5d). Whereas mice injected with Ang II display a significant increase in phospho-ERK level, mice pretreated with Gefitinib exhibit a significantly lower level of phospho-ERK activation, similar to the level obtained in untreated mice (Fig. 5d).

EGF signaling pathway is required for ATF3 induction by angiotensin II

To examine whether EGFR inhibitor and suppression of the EGFR signaling affect ATF3 induction, mice were either pretreated with Gefitinib or left untreated followed by Ang II injection. Three hours following Ang II injection mice were sacrificed and hearts were separated. Tissue lysate derived from the left chambers was analyzed by Western blot with anti-ATF3 antibodies (Fig. 5e). Left atria lysate derived from mice pretreated with Gefitinib alone showed a mild increase in ATF3 expression level. Both atria and ventricles lysate derived from mice injected with Gefitinib followed by Ang II failed to induce ATF3 expression. Thus, suggesting that activation of the EGFR signaling pathway is necessary for ATF3 induction in the left chambers. To examine the role of the PI3K pathway in ATF3 induction by Ang II, we examined PI3K activation by the use of anti-phospho-AKT (Fig. 6a). Indeed, phospho-AKT level is significantly elevated following Ang II injection specifically in the left atrium (Fig. 6a, $P < 0.05$). Pretreatment of mice with AKT inhibitor V for 30 min prior to Ang II injection partially inhibited ATF3 induction by 60% (Fig. 6b, $P < 0.01$).

Collectively, we suggest that the right and left atrium display differential signaling pathways including a differential pattern of gene expression (ATF3 and HB-EGF). The EGFR signaling pathway through the activation of the ERK and PI3K-AKT pathways is essential for the ATF3 induction in the left atrium following acute angiotensin II stimulation.

Discussion

Atrial remodeling and hypertrophy is a process that is crucial for the accommodation of pressure and volume overloads and, therefore, represents an important clinical aspect of human cardiac physiology. A case in point is the enlargement of the left atria. Left atrial hypertrophy is a known risk factor for the development atrial fibrillation which is the most common sustained arrhythmia in the clinic [5, 7, 46]. Therefore, revealing the signaling pathways as well as the mechanisms responsible for left atrial enlargement is important for the development of novel treatments. Although much is known regarding the signaling pathways resulting in ventricular pathology, little information is currently available concerning the mechanisms involved in atrial dilatation. Angiotensin II is a key hormone that affects the function of many organs with beneficial and pathological effects. Acute stimulation with angiotensin II regulates salt/water homeostasis and vaso-constriction and modulates blood pressure. Chronic angiotensin II stimulation promotes hyperplasia and hypertrophy in the heart [29]. Identifying the components involved in angiotensin II signal transduction pathway is of great importance, since it is well accepted that angiotensin II plays a role in mediating morbidity and mortality in coronary disease, heart failure, atrial fibrillation, and stroke.

Cardiac expression of bZIP repressor proteins such as CREB [12], ATF3 [33], and JDP2 [26] were shown to be sufficient to induce atrial hypertrophy. In addition, transgenic mice with cardiac expression of AT₁R and ACE also display massive enlargement of the atria [20, 48]. It was initially suggested that the observed phenotypes may be due to the early α -MHC expression of the transgene in the atria during development. However, using a tetracycline inducible JDP2 transgene, we demonstrated that the atrial phenotype is independent of any developmental process [25, 26] and represents a genuine atrial signaling pathway. Indeed,

data is accumulating on differing atrial and ventricular responses. A recent study described how atrial fibroblasts behave differently from ventricular fibroblasts over a range of in vivo and in vitro paradigms [6]. In the current study, we focussed on angiotensin II stimulation and examined the induction of ATF3 expression in the different heart chambers. ATF3 is highly induced in the left chambers and to much lower extent in the right ventricle while no induction is observed in the right atrium.

ATF3 is an important adaptive response gene. Its expression contributes to the modulation of cell death and/or cell cycle machineries [16]. Previous reports identified cardiac ATF3 induction in response to various stimuli. For example, both ischemia and ischemia-reperfusion induce ATF3 expression in rat ventricles [8]. In isolated cardiomyocytes, ATF3 is induced by adenylyl cyclase VI overexpression and is postulated to suppress phospholamban expression [14]. We have previously showed that both JDP2 and ATF3 cardiac expression may suppress MLC2a and connexin40 expression [26]. Here we also demonstrate that ATF3 is a target of beta-agonists (Fig. 1c); therefore, its role in heart physiology and pathology may be wider than initially thought.

We partially characterized the mechanism of induction of the nuclear factor ATF3 in response to angiotensin II stimulation. ATF3 expression is mainly regulated at the mRNA and protein levels. ATF3 response to angiotensin II is receptor mediated, cell-autonomous and independent of physiological effects of angiotensin II such as increasing blood pressure. We provide three lines of evidences to suggest that blood pressure elevation is not sufficient to explain the difference in ATF3 induction between the left and right atria. First, we show that the AT₂R blocker potentiates increase in blood pressure but suppresses ATF3 expression in response to Ang II (Fig. 4b, c and supplementary Fig. 1b). Second, the α agonist, phenylephrine, known to promptly increase blood pressure, induces ATF3 in both the left and right atria (Fig. 3b). Third, ATF3 induction in response to Ang II occurs in vitro in HEK-293-AT₁R cells (Fig. 2a). Nevertheless, we cannot completely exclude the possibility that higher mechanical stretch in the left chambers potentiates specific expression of ATF3 in response to Ang II.

Although HEK-293-AT₁R system successfully mimics ATF3 induction by angiotensin II, it does not represent the signal transduction pathway that occurs in the left atrium. Unlike in mice, both the EGFR and AT₂R blockers were unable to abrogate ATF3 expression in response to angiotensin II in HEK-293-AT₁R cell line (data not shown). An additional difference of the in vitro system is that in mice the increase in ATF3 expression is transient (supplementary Fig. 1a), whereas in HEK-293-AT₁R cells, ATF3 expression persists for up to 24 h (Fig. 2). This lasting effect in vitro can be explained by the lack of down-regulation mechanism in tissue culture cell lines.

Interestingly, our data suggest that both angiotensin receptor subtypes, AT₁R and AT₂R, are required for angiotensin II signaling for ATF3, since pharmacological blockade by either losartan or PD123319 abrogates the increase in ATF3 expression level in response to angiotensin II. The distribution of the angiotensin II receptors differs between ventricles and atria but no selective expression was suggested between left and right chambers [47]. Using qRT-PCR, we show that the right atrium displays higher level of mRNA for the AT₂R isoform. It was believed for many years that the physiological role of the AT₂R is to antagonize the effects of the AT₁R [1, 29]. However, AT₂R KO mice display reduced hypertrophy. Therefore, AT₂R is mediating hypertrophic signals on one hand [10, 22], but on the other hand, AT₂R is able to antagonize other AT₁R functions [32, 35, 42]. Our results support the notion that AT₂R complements AT₁R activity. Namely, both angiotensin II receptor subtypes mediate angiotensin II signaling to ATF3. It might be that the ratio between the two angiotensin II receptor isoforms is crucial to facilitate signaling and the fact

that the right atrium displays excess of the AT₂R isoform may result in suppression of the Ang II signaling.

Most striking is the differential response of ATF3 to angiotensin II in the left versus the right atrium. Although it is well established that the left and right atria develop from different embryonal origin and have a dissimilar pattern of gene expression [43], this is for the first time, to our knowledge, that a differential signaling response between atria has been documented. We identified Hb-EGF as an angiotensin II target gene with higher mRNA levels at the left atrium as compared to the right atrium. EGF receptor transactivation plays a central role in cardiac hypertrophy in response to angiotensin II [39]. In addition, cardiac EGFR activation occurs in an angiotensin II-independent manner following mechanical stretch [34]. Here, we identified that the activation of both EGFR and AKT signaling plays a role in the cardiac expression of ATF3 in an angiotensin II-dependent manner. Further studies are required to explore the post-receptor signaling initiated at the angiotensin II receptors to the receiving-end of nuclear bZIP transcription factor, ATF3.

In patients, left atrial dilatation has been connected with adverse sequel including atrial fibrillation [15]. The effects of blocking angiotensin II stimulation on prevention of atrial fibrillation is a subject of debate. Whereas some clinical studies suggest that angiotensin II inhibition reduces the occurrence of atrial fibrillation [4, 7, 28, 45], other studies show no significant reduction in atrial fibrillation reoccurrence or improvement in quality of life [11, 38]. We suggest that ATF3 expression in the atria is a general mediator of neuroendocrine stimuli that may play an important role in structural and functional atrial remodeling [26, 33]. The precise ATF3 target genes are yet to be identified.

In summary, the data presented strongly place the nuclear bZIP transcription factor, ATF3, at the receiving-end of various neuroendocrine signaling pathways. Specifically, ATF3 displays a unique expression confined to the left heart chambers following acute angiotensin II stimulation. The angiotensin II receptor subtypes, AT₁R and AT₂R, cooperatively mediate the elevation of ATF3 expression level in an EGFR and AKT-dependent manner. ATF3 may represent a novel nuclear target in heart pathology and a potential drug target to reduce the mortality and morbidity due to atrial-associated pathology.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Fig. 1.

Neuroendocrine stimuli induce ATF3 expression in the heart. **a** Representative Western blot analysis (*left panel*) of cell lysate derived from ventricles of C57Bl/6 mice injected 3 h before sacrifice with saline (–), angiotensin II (Ang II), isoproterenol (Iso.) and phenylephrine (Phe.) at the indicated dose (mg/kg). The expression level of ATF3 (*top panel*) and α -tubulin loading control (*bottom panel*) are shown. Densitometry analysis (*right panel*) of ATF3 expression in response to angiotensin II injection was performed for the indicated dose. ATF3 expression was normalized with α -tubulin level. ATF3 level following Ang II injection was calculated relative to the level obtained in saline-injected mice. The results represent the mean and SEM of four independent experiments. *Asterisk* indicates P value < 0.01. **b** Representative Western blot analysis of cell lysate derived from the indicated tissues; heart (ventricles), kidney, liver, lung, skeletal muscle (muscle) and spleen. Mice were injected with either angiotensin II (1.5 mg/kg, +) or with saline control (–) 3 h prior to tissue harvesting. The expression levels of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) is shown. **c** Representative Western blot analysis (*left panel*) of cell lysate derived from atria and ventricles of mice injected with either angiotensin II (Ang II +, 1.5 mg/kg) or saline control (–). Losartan at 1.25 mg/ml was dissolved in water containing 5% sucrose. Losartan (Los. +; *lanes 2 and 4*) or sucrose alone (–, *lanes 1 and 3*) were provided in the drinking water for 5 days prior to Ang II injection. Mice were sacrificed 3 h following Angiotensin II injection. The expression levels of ATF3 (*top panel*) and α -tubulin (*bottom panel*) are shown. Each *lane* represents a lysate prepared from a pool derived from two mice. Densitometry analysis (*right panel*) of ATF3 expression level derived from *lanes 3 and 4*. ATF3 expression was normalized with α -tubulin expression level. Normalized ATF3 expression level of Ang II-injected control mice (sucrose treated) in the atria and the ventricles were determined as 1 and ATF3 expression level of losartan-treated mice was calculated relatively for each chamber. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01

Fig. 2.

Angiotensin II-dependent increase in ATF3 expression is cell autonomous. **a** Representative Western blot analysis of whole cell extracts derived from HEK-293-AT₁R cells treated with either angiotensin II (Ang II, 1 μ M) or saline control (saline, –) for the indicated time points. The expression levels of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) are shown. Densitometry analysis of normalized ATF3 expression level derived from three independent experiments described in **a**. ATF3 expression level was normalized with GAPDH expression level. Normalized ATF3 expression level of saline-treated cells was determined as 1. The expression level of ATF3 at different time points (h) following Ang II addition was calculated relatively. The mean and SEM of ATF3 expression is shown. Asterisk indicates P value < 0.05 . **b** qRT-PCR of cDNA derived from mRNA as described in **a**. The data represent the mean ratio of ATF3 to GAPDH expression and SEM from three independent experiments. Asterisk indicates P value < 0.05 .

Fig. 3.

Angiotensin II induction of ATF3 expression is confined to the left chambers. **a** Representative Western blot analysis (*upper panel*) of cell lysate derived from: left atrium (LA), right atrium (RA), left ventricle (LV) and right ventricle (RV). Lysate was prepared from a pool of six mice injected with either saline control (-) or angiotensin II (+). The expression level of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) is shown. Densitometry analysis (*lower panel*) of ATF3 expression in response to angiotensin II injection was performed. ATF3 expression level was normalized with GAPDH level and was calculated relative to the level obtained in the left atria of saline-injected mice (set as 1). **b** Representative Western blot analysis (*upper panel*) of cell lysate from left atrium (LA) and right atrium (RA) derived from a pool of five mice injected with saline, angiotensin II (Ang II 1.5 mg/kg) isoproterenol (Iso. 2.5 mg/kg) and phenylephrine (Phe. 2.5 mg/kg) 3 h prior to mice sacrifice. The expression levels of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) is shown. Densitometry analysis (*lower panel*) of ATF3 was performed. ATF3 expression level was normalized with GAPDH level and was calculated relative to the level obtained in the left atria of saline-injected mice (set as 1). **c** qRT-PCR with cDNA derived from mRNA of either separated atrium or ventricles of mice either saline injected or Ang II injected (1.5 mg/kg) 3 h prior to mice sacrifice. The mRNA used for cDNA preparation was pooled from three mice. The ratio between ATF3 expression level divided by $\beta 2$ micro globulin expression level was calculated. The results represent the

mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01 . **d** Immuno-histochemistry of tissue sections derived from left and right atrium of mice injected as described in **b**. Tissue sections were probed with anti-ATF3 antibody followed by peroxidase and haematoxylin staining. Representative nuclear ATF3 staining is indicated by *arrows* in the relevant panels

Fig. 4.

AT₂R is required for ATF3 response to angiotensin II. **a** qRT-PCR with cDNA derived from mRNA of separated atria. The mRNA used for cDNA preparation was pooled from three mice. The AT_{1A}R (Agtr1a) and AT₂R (Agtr2) expression levels were normalized to the level obtained for the housekeeping gene β 2 micro globulin. The expression level of the corresponding Ang II receptor subtype in the left atrium was considered as 1 and the expression in the right atrium was calculated relatively. The results represent the mean and SEM of three independent mRNA pools. Asterisk indicates P value < 0.05 . **b** Representative Western blot analysis (*upper panel*) of cell lysate derived from the left chambers of mice injected with either vehicle control or AT₂R antagonist (PD123319; 20 mg/kg) 30 min prior to injection with either saline or angiotensin II (0.5 mg/kg Ang II). The expression levels of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) is shown. Densitometry analysis (*lower panel*) of ATF3 expression of cell lysate derived from left atria and left ventricles shown in *lanes 3 and 4*. ATF3 expression level was normalized with GAPDH expression level. Normalized ATF3 expression level of Ang II-injected control mice (in either left atria or left ventricles of saline-treated mice) was determined as 1 and ATF3 level

of PD123319-treated mice was calculated relatively. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01 . **c** Continuous blood pressure of anesthetized rats was measured from the carotid artery. Rats were injected with Ang II (0.15 mg/kg), PD123319 (15 mg/kg) or PD123319 30 min prior to Ang II. The number on *top* of each *bar graph* represents the maximal blood pressure reached for each treatment. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01 relative to blood pressure obtained in non-injected rats. *Asterisks* represents P value < 0.1 when comparing the maximal blood pressure measured from Ang II-treated rats with PD123319 + Ang II-treated rats

Fig. 5.

Differential signaling response of the left and right atrium to angiotensin II. **a** qRT-PCR with cDNA derived from mRNA of either left atrium (LA) or right atrium (RA) of mice 3 h following angiotensin II injection. The mRNA was pooled from three mice. The ratio between the expression following angiotensin II injection and the basal expression is shown for both left and right atria. The expression level of Hb-EGF and ATF3 are normalized with the expression of $\beta 2$ micro globulin. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01 when comparing left and right atria response. **b** Representative Western blot analysis (*upper panel*) of cell lysate derived from left atria (LA) and right atria (RA) of control saline-injected mice (–) and mice injected with Ang II (+, 1.5 mg/kg) 10 min prior to sacrifice. The expression levels of phospho-ERK (pERK 1/2, *top panel*) and total ERK loading control (*bottom panel*) is shown. Densitometry analysis (*lower panel*) of ERK phosphorylation was normalized with total ERK expression level. The extent of normalized phospho-ERK expression level in the left atrium of saline-injected mice was determined as 1 and all other phospho-ERK expression levels were calculated relatively. The results represent the mean and SEM of five independent experiments. *Asterisk* indicates P value < 0.01 . **c** Same as described in **b** except that isoproterenol (2.5 mg/kg) was injected 10 min prior to mice sacrifice. *Asterisk* indicates $P < 0.05$. P value is calculated for the left and right atria separately. **d** Representative Western

blot analysis (*upper panel*) of cell lysate derived from the left atrium (LA) of mice injected with either angiotensin II (+) or saline control (–). Mice were pretreated with Gefitinib (*lane 3*, 100 mg/kg) 18 h and one hour prior to Ang II injection. Left atria (LA) were harvested 10 min following angiotensin II injection. The expression levels of phospho-ERK (*top panel*) and total ERK (*bottom panel*) are shown. Densitometry analysis (*lower panel*) of three independent experiments is shown. The extent of ERK phosphorylation was normalized with total ERK expression level. The normalized phospho-ERK level in the left atrium of non-injected mice was determined as 1 and all other phospho-ERK expression levels were calculated relatively. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.05. **e** Representative Western blot analysis (*upper panel*) of cell lysate derived from left atria (LA) and left ventricles (LV) as described in **d**. Mice were sacrificed 3 h following Ang II injection (1.5 mg/kg). The expression levels of ATF3 (*top panel*) and GAPDH loading control (*bottom panel*) are shown. Densitometry analysis (*lower panel*) of ATF3 expression level of *lanes 3* and *4*, respectively. ATF3 expression level was normalized with GAPDH expression level. Normalized ATF3 expression level of Ang II-injected control mice in left atria and left ventricles were determined as 1 and ATF3 expression level of Gefitinib-injected mice was calculated relatively. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01

Fig. 6.

ATF3 response to angiotensin II is AKT dependent. **a** Representative Western blot analysis (*upper panel*) of cell lysate derived from left atria (LA) and right atria (RA) of mice injected with either saline control (–) or angiotensin II (Ang II +, 1.5 mg/kg). Hearts were harvested 10 min following angiotensin II injection. The expression level of phospho-AKT (pAKT, *top panel*) and total AKT as loading control (*bottom panel*) are shown. Densitometry analysis (*lower panel*) of protein level is shown. The extent of phospho-AKT level was normalized with total AKT expression level. Normalized phospho-AKT level in the left atrium of saline-injected mice was determined as 1 and all other phospho-AKT expression levels were calculated relatively. The results represent the mean and SEM of three

independent experiments. *Asterisk* indicates P value < 0.05 . **b** Representative Western blot analysis (*upper panel*) of cell lysate derived from left atria (LA) of mice injected with either saline control (–) or Ang II (+, 1.5 mg/kg). Mice were either injected with AKT inhibitor V (AKT in., 1 mg/kg) or saline 30 min prior to either saline injection or angiotensin II. Left atria (LA) were harvested 3 h following Ang II injection. Western blot analysis with anti-ATF3 and GAPDH is shown. Densitometry analysis (*lower panel*) of *lanes 3* and *4* is shown. ATF3 expression level was normalized with GAPDH expression level. Normalized ATF3 expression level of Ang II-injected control mice in left atria was determined as 1 and ATF3 expression level of AKT inhibitor V-injected mice was calculated relatively. The results represent the mean and SEM of three independent experiments. *Asterisk* indicates P value < 0.01