Activating Transcription Factor 3 Protects against Pressure-Overload Heart Failure via the Autophagy Molecule Beclin-1 Pathway

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ABSTRACT
Activating transcription factor 3 (ATF3), a cAMP response element-binding protein/ATF family transcription factor member, has been implicated in the cardiovascular and inflammatory system and is rapidly induced by ischemic-reperfusion injuries. We performed transverse aortic banding (TAB) experiments using ATF3 gene-deleted mice (ATF3−/−) and wild-type (WT) mice to determine what effect it might have on heart failure induced by pressure overload. Compared with the WT mice, ATF3−/− mice had more numbers of terminal deoxynucleotidyl transferase-mediated digoxigenin-deoxyuridine nick-end labeling-positive cells and higher levels of activated caspase-3, as well as more apoptosis. Restoration of ATF3 expression in the heart of ATF3−/− mice by adenovirus-induced ATF3 treatment significantly improved cardiac contractility after TAB. The results from molecular and biochemical analyses, including chromatin immune-precipitation and in vitro/in vivo promoter assays, showed that ATF3 bound to the ATF/cAMP response element of the Beclin-1 promoter and that ATF3 reduced autophagy via suppression of the Beclin-1–dependent pathway. Furthermore, infusion of tert-butylhydroquinone (tBHQ), a selective ATF3 inducer, increased the expression of ATF3 via the nuclear factor erythroid 2–related transcriptional factor, inhibited TAB-induced cardiac dilatation, and increased left ventricular contractility, thereby rescuing heart failure. Our study identified a new epigenetic regulation mediated by the stress-inducible gene ATF3 on TAB-induced cardiac dysfunction. These findings suggest that the ATF3 activator tBHQ may have therapeutic potential for the treatment of pressure-overload heart failure induced by chronic hypertension or other pressure overload mechanisms.

Introduction
In response to stress from neurohumoral activation, hypertension, or other myocardial injuries, the heart initially compensates with an adaptive hypertrophic response, which increases cardiac mass. Under prolonged stress, however, the heart undergoes irreversible changes, resulting in chamber dilatation and diminished heart performance (Feihl et al., 2008). Autophagy can be induced by starvation, hypoxia, intracellular stress, hormones, or developmental signals (Klionsky and Emr, 2000; Levine and Klionsky, 2004). Autophagy, which can be defined as a self-eating process, is a mechanism through which cells degrade their own components and recycle amino acids and other building blocks that can eventually be reused (Yorimitsu and Klionsky, 2005). Depending on the pathway through which cellular components are delivered to lysosomes, three types of autophagy can be distinguished: macroautophagy, microautophagy, and chaperone-mediated autophagy (Dutta et al., 2012).

ABBREVIATIONS: AAV, adeno-associated virus; ADV, adenovirus; ATF3, activating transcription factor 3; CREB, cAMP response element-binding protein; EMSA, electrophoretic mobility shift assay; FS, fractional shortening; HDAC, histone deacetylase; LV, left ventricle; MHC, myosin heavy chain; Nrf2, nuclear factor erythroid 2–related factor; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PKC, protein kinase C; TAB, transverse aortic banding; tBHQ, tert-butylhydroquinone; TUNEL, terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxyuridine nick-end labeling; WT, wild-type.
Macrophagy, which primarily occurs in the heart, may be either beneficial or deleterious under pressure-overload conditions, depending on the circumstances under which it is induced, the extent to which it is stimulated, and the signaling pathways that are activated (Zhu et al., 2007; Cao and Hill, 2011).

Activating transcription factor 3 (ATF3), a member of the cAMP response element-binding protein (CREB)/ATF family of transcription factors (Chen et al., 1994), is rapidly induced by ischemic-reperfusion injuries in the heart or kidney (Chen et al., 1996; Li et al., 2010). ATF3 can repress or activate target genes by forming homomeric or heteromeric complexes (Hsu et al., 1992). Heart of α-miosin heavy chain (α-MHC)-driven ATF3 transgenic mice have been observed to have atrial enlargement and as well as atrial and ventricular hypertrophy accompanied with reduced contractility and aberrant conduction (Okamoto et al., 2001). Although autophagy is known to play an important role in the adaptive hypertrophic response in the heart, it is unclear whether ATF3 is involved in pressure overload–induced cardiac remodeling or heart failure.

In the present study, ATF3 gene-deleted (ATF3−/−) mice were found to have loss of normal hypertrophic remodeling in response to transaortic banding (TAB) with early pressure overload–induced heart failure. Beclin-1, the mammalian homolog of a proautophagic gene, was found to be targeted by ATF3. ATF3 re-expression by adeno-associated virus (AAV) infection in ATF3−/− mice or supplementation with the ATF3-specific inducer tert-butylhydroquinone (tBHQ) reduced the progression of pressure overload–induced heart failure in TAB mice. These findings suggest that ATF3 plays a crucial role in hypertrophic cardiac remodeling and that an ATF3 analog or inducer potentially may be used in the treatment of patients with chronic high blood pressure diseases.

Materials and Methods

Animal Model for Cardiac Transverse Aortic Banding and tBHQ Injection. The ATF3−/− mice were provided by Dr. Tsionwin Hai (Ohio State University). The ATF3−/− allele was backcrossed into C57BL/6 mice for at least seven generations before the cardiac TAB procedure was performed. Male mice (weighing 25–30 g) were anesthetized by intraperitoneal injection of chloral hydrate (125 mg/kg). The TAB procedure was performed as in the description by Hu et al. (2003). tBHQ therapy was performed at the 4th week after TAB or sham surgery. Mice were given intraperitoneal injections of 1 mg/kg weekly of tBHQ (Sigma-Aldrich, St. Louis, MO) for 7 weeks. The control group was treated with 0.1 ml of saline (instead of tBHQ) for 7 weeks. Echocardiography was performed at weeks 4 and 10 after TAB or sham surgery. After therapy, the mice were sacrificed by an overdose of sodium pentobarbital (200 mg/kg i.p.), and the hearts were rapidly excised for further experiments. All surgical procedures were performed according to the protocols approved by the Institutional Animal Care and Utilization Committee, Academia Sinica, Taipei, Taiwan.

Cell Cultures. Cardiomyocyte cell line H9c2, human embryonic kidney cells, and kidney epithelial cell line (NRK-52E cells) were maintained in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cytoplasmic and Nuclear Protein Extraction. Cells and heart tissues were separated into cytoplasmic and nuclear parts using a ProteoJET protein extraction kit (Fermentas Life Sciences, Vilnius, Lithuania).

Western Blot Analysis. Extracts of heart and cells were separated on an SDS-PAGE and subjected to Western blot analysis using the protocol of ECL kit (Thermo Scientific Pierce, Rockford, IL). The following antibodies were used: anti-ATF3 (1:500; Santa Cruz Biotechnology, Santa Cruz, CA), anti–Lamin A/C (1:1000; Genetex, Inc., Irvine, CA), anti–Beclin-1 (1:1000; Santa Cruz Biotechnology), anti–glyceraldehyde-3-phosphate dehydrogenase (1:10,000; BD Pharmingen, Franklin Lakes, NJ), anti–caspase 3 (1:1000; Cell Signaling Technology, Beverly, MA), anti–β-actin (1:10,000; Millipore, Darmstadt, Germany), and anti–LC3B (1:500; Cell Signaling Technology).

Immunohistochemistry. Tissue slides were dewaxed and then treated with epitope retrieval buffer (Thermal Scientific, Inc., Dallas, TX) in 95°C for 30 minutes. Then slides were quenched with 3% hydrogen peroxide, blocked by Power Block (BioGenex Laboratories, Fremont, CA), and incubated for 10 minutes at room temperature. The sections were then incubated with rabbit anti-ATF3 (1:100; Santa Cruz Biotechnology) for 18 hours at 4°C and Washed five times with phosphate-buffered saline (PBS) containing 0.1% Tween 20. Anti-rabbit antibody (biotinylated, 1:200; Vectastain kit; Vector Laboratories, Burlingame, CA) were applied to slides for 1 hour at room temperature. Then slides were incubated with avidin-biotin complex reagent (Vectastain kit) at room temperature for 30 minutes and washed twice with PBS containing 0.1% Tween 20. The nuclei were stained with 3,3-diaminobenzidine (Vectastain kit) for microscopic studies.

Real-Time Quantitative Polymerase Chain Reaction and Reverse-Transcription Polymerase Chain Reaction. Total RNA was extracted from the heart using Trizol (Invitrogen, Carlsbad, CA). Real-time quantitative polymerase chain reaction (PCR) analysis was performed using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems, Grand Island, NY). For reverse-transcription PCR, total RNA was extracted from the heart using Trizol. cDNA was prepared with the Super Script kit (Invitrogen) from 3 µg of total RNA with Oligo (dT) for analysis. Primers used are listed in Table 1.

In Vitro Protein Synthesis and Electrophoretic Mobility Shift Assay. For electrophoretic mobility shift assay (EMSA), the in vitro translated ATF3 was performed using the T7 polymerase-based TNT quick coupled transcription/translation kit (Promega, Madison, WI) and then incubated with the Beclin-1 DNA probes containing a consensus ATF3 binding site. Probes used are listed in Table 1. EMSA analysis was performed as described previously (Lin et al., 2008). Briefly, the binding reactions were performed in 20-µl reaction mixtures with the in vitro translated products. Reactions were loaded on a 5% polyacrylamide gel and run at 100 V at 4°C in 0.5× TBE. The gel was dried and exposed to a phosphoimager screen (Molecular Dynamics, Inc., Sunnyvale, CA).

Chromatin Immunoprecipitation Assay. After adenovirus infection, H9c2 cells were fixed in 1% formaldehyde and chromatin immunoprecipitation (performed using the Upstate protocol (Millipore)). Chromatin was immunoprecipitated using anti-ATF3 antibody (Santa Cruz Biotechnology). The purified DNA was detected using standard PCR. Primers used are listed in Table 1.

In Vitro Promoter Assay. For in vitro promoter assay, ATF3/CRE binding sites from the Beclin-1 promoter (~1 to ~1.4kb) were inserted into upstream of the luciferase reporter in pGL-4 vector (Promega) to create pGL4-Beclin-1. NRK-52E cells were transfected with or without pGL4-Beclin-1 and pCDNA3-ATF3 for 24 hours before harvesting for luciferase assay. Firefly luciferase activity was determined and normalized to Renilla luciferase activity. Data shown represent averages and S.E.M. acquired from three independent experiments.

Echocardiography Studies. For echocardiography, mice were anesthetized with phenobarbital (40 mg/kg of body weight i.p.), and then measurements were taken with an ultrasound equipment (ATL Philips IE33; Ultrasound Machine System, Orange, CA) using a 15-MHz probe. The left ventricular posterior wall thickness, interventricular septum thickness, and left ventricular lumen diameter at both end-systolic and end-diastolic phases were measured digitally on the M-mode tracings and averaged for three cardiac cycles. Fractional shortening (FS) and ejection fraction were then calculated.
Histology. Hearts from all the treated groups were fixed in 4% paraformaldehyde overnight at 4°C and processed with paraffin fixation. Sections (5 μm) were stained with hematoxylin and eosin and Masson’s trichrome.

Myocardial Injection of Plasmid DNA. C57/B6 and ATF3−/− mice were anesthetized with phenobarbital (40 mg/kg of body weight i.p.), intubated with an 18-gauge angiocath, and mechanically ventilated (model 697 ventilator; Harvard Apparatus, Holliston, MA). Body temperature was maintained at 37°C by using a heating pad plus a heating lamp. A midline sternotomy was performed under sterile technique. Then 5 μl of plasmid DNA in 30 μl of PBS or the same volume of PBS alone was injected into the anterolateral wall of the left ventricle using a 50-gauge needle. Plasmid DNA containing a promoter-less P luc (pGL4 basic vector) as a negative control or pGL4-Beclin-1 was injected. Firefly luciferase activity was determined and normalized to Renilla luciferase activity. Data shown represent averages, and S.E.M. from each of three independent experiments was done in triplicate.

Terminal Deoxynucleotidyl Transferase–Mediated Digoxigenin-Deoxuridine Nick-End Labeling Assay. Mice were perfused with 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.4) and hearts were dissected and fixed in 4% paraformaldehyde overnight at 4°C, embedded in paraffin, and sectioned (5 μm). Apoptosis in heart tissues was identified by terminal deoxynucleotidyl transferase–mediated digoxigenin-deoxuridine nick-end labeling (TUNEL) assay with an in situ Cell Death Detection kit (Roche Applied Science, Indianapolis, IN) according to the manufacturer’s instructions.

Production of Recombinant Adeno-Associated Virus Carrying ATF3. Full-length ATF3 was obtained by PCR amplification from a human complementary DNA library, flanking the XbaI and HindIII restriction cutting sites, and was cloned into the pAAV-MCS vector. To produce the AAV virus, a three-plasmid cotransfection method was used (Xiao et al., 1998). The plasmids used in transfection were as follows: 1) the AAV-CMV-ATF3 plasmid with the gene driven by the CMV promoter C57/B6 and ATF3−/− mice was significantly increased (Fig. 2B). We also observed increased numbers of apoptotic cells with TUNEL-positive staining (Fig. 2C) and increased expression of active form of caspase-3 expression in the ATF3−/− mice compared with that of WT mice (Fig. 2B). Together, our findings suggest that ATF3 plays an important protective role in pressure-overload cardiac remodeling induced by TAB.

Restoration of ATF3 Expression Rescued TAB-Induced Pressure-Overload Heart Failure in the ATF3−/− Mice. As a complementary approach to our knockout mouse studies, we performed phenotype rescue studies in which ATF3−/− mice received AA8V-mediated gene transfer of ATF3 (AAV8-ATF3) (Wang et al., 2005). Three weeks after AA8V-ATF3 intravenous injection, the expression of ATF3 in the hearts of ATF3−/− mice was significantly increased (Fig. 3A), LV contractility was recovered (Fig. 3B), and chamber dilation was attenuated (see Supplemental Fig. 2). In addition, TAB-induced cardiac fibrosis was attenuated in the ATF3−/− mice.
after in vivo AAV8-ATF3 therapy (Fig. 3C). Taken together, our results suggest that ATF3 is a key regulator of TAB-induced cardiac remodeling and heart failure.

**Inhibition of Beclin-1 Activity by ATF3.** Because the presence of the ATF3 binding sites ATF/CRE on the promoter region of Beclin-1 can upregulate Beclin-1 via reactive oxidative stress–mediated ischemic-reperfusion injury in the heart (Ma et al., 2012), it is possible that increased ATF3 during cardiac hypertrophic remodeling may be regulated through Beclin-1 signaling. To test this hypothesis, we first examined whether the expression of Beclin-1 could be regulated by ATF3 in cardiomyocytes (H9c2) cells. As shown in Fig. 4A, Beclin-1 expression was high in the HPGK vector–infected H9c2 cells under both normal and starved conditions, whereas its expression was decreased in ATF3 vector-infected H9c2 cells under starvation conditions, suggesting that ATF3 might downregulate Beclin-1 expression after stress. Consistent with the preceding results, under either sham-operated conditions or 3 days after TAB procedures, the stress induced by TAB induced Beclin-1 expression in both ATF3/−/− and WT mice, with higher Beclin-1 and LC3II levels observed in the ATF3/−/− mice compared with the WT group (Fig. 4, B and C).

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**Fig. 1.** Increased ATF3 expression was found in the heart of cardiac hypertrophic mice induced by TAB. (A) Quantitative reverse-transcription PCR analysis: *P < 0.05. (B) Immunohistochemical analysis of cardiac ATF3 expression in the sham-operated and TAB-treated mice (n = 5 for each group). Arrows indicate translocation of ATF3 into the nucleus. (C) Translocation of ATF3 was confirmed by Western blot analysis. Nuclear and cytosolic extracts were probed with specific antibodies against ATF3. Anti–β-actin and anti–lamin A/C served as loading control.

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**Fig. 2.** Loss of normal cardiac hypertrophic remodeling with increased fibrosis and apoptosis in TAB-treated ATF3/−/− mice. (A) Real-time reverse-transcription PCR analysis on cardiac tissue for hypertrophic markers α-MHC, β-MHC, atrial natriuretic factor (ANF), and brain natriuretic peptide (BNP) at 2 weeks after TAB treatment (n = 5 each group). The mean normalized value for the expression of each gene in sham-operated hearts is defined as 1. *P < 0.05. (B) Masson’s trichrome staining for cross-sectioned heart and morphometric quantitation of fibrosis area are shown on both (WT) and ATF3/−/− mice subjected to TAB treatment. Bar, 2 mm. *P < 0.05. Data reflect measurements from at least four animals per group. (C) Detection of apoptotic heart cells using in situ TUNEL staining. Diagram for the TUNEL-positive cells represents data from five independent experiments. Bar, 100 μm. *P < 0.05. (D) A representative expression of cleaved, active caspase-3 in the WT and ATF3/−/− mice is shown. Experiments were repeated twice with similar results.
To further identify whether the regulation of Beclin-1 expression by ATF3 was dependent on the translocation or binding of ATF3 to the ATF/CRE element, we performed an EMSA DNA-binding assay using ATF/CRE binding sequence located in the Beclin-1 promoter (−164 to −156 bp and −182 to −175 bp) from mouse DNA. ATF3 DNA-binding activity was observed in the nuclear extracts, but this activity was markedly suppressed in cells pretreated with unlabeled cold primer (Fig. 4D lane 5 versus lane 6). Furthermore, gel retardation was abolished by ATF3 antibody when this antibody blocked the DNA binding domain of ATF3 (Fig. 4D, lane 5 versus lane 7). A chromatin immunoprecipitation assay was conducted to further examine whether ATF3 was located in the Beclin-1 promoter (Fig. 4E). Epson’s trichrome staining of cross-sectioned heart and morphometric quantitation of fibrosis area in WT and ATf3−/− mice with or without AAV-ATF3 therapy. Bar, 2 mm; *P < 0.05. EF, ejection fraction; GFP, green fluorescent protein.

**Fig. 3.** Recovery of left ventricular contractility and attenuation of cardiac fibrosis in TAB-treated ATf3−/− mice after AAV8-ATF3 therapy. (A) Western blot of cardiac ATF3 expression in ATf3−/− mice with or without AAV-ATF3 injection. (B) Echocardiographic measurements were performed 3 weeks after TAB procedures in WT and ATf3−/− mice with or without AAV-ATF3 therapy. (C) Masson’s trichrome staining of cross-sectioned heart and morphometric quantitation of fibrosis area in WT and ATf3−/− mice with or without AAV-ATF3 therapy. Bar, 2 mm; *P < 0.05. EF, ejection fraction; GFP, green fluorescent protein.

In this study, TAB-induced pressure-overload heart failure with cardiac fibrosis and cell apoptosis was first found to be more severe in the ATf3−/− mice than in WT mice. Re-expression of the ATf3 protein in the heart via a viral infection (AAV-ATF3) was found to ameliorate TAB-induced cardiomyopathy in the ATf3−/− mice through involvement of the Beclin-1–dependent autophagy signaling pathway. Our in vivo experiments demonstrated that by use of the ATf3 specific inducer tBHQ could rescue long-term TAB-induced cardiac dysfunction. Considered together, these results suggest that ATf3 plays a prominent regulatory role in pressure overload–induced heart failure and that supplementation with an ATf3-specific inducer such as tBHQ might potentially be useful in the treatment of future pressure overload–induced cardiomyopathy.

To further substantiate the binding activity of ATF3 on the ATF/CRE binding region of Beclin-1, luciferase assay was performed in NRK-52E cells. When luciferase expression vectors were transfected with mouse Beclin-1 promoter plasmid, the addition of ATF3 significantly suppressed Beclin-1 luciferase activity (Fig. 4F, lane 2 versus lane 3). The inhibition of Beclin-1 luciferase activity by ATF3 was also observed under starvation conditions in these cells (Fig. 4G, lane 5 versus lane 6). Finally, to characterize whether ATF3 could suppress Beclin-1 transcriptional expression in vivo, we used methods suggested by a prior study (von Harsdorf et al., 1997) to inject luciferase-containing Beclin-1 promoter plasmid into the hearts of both WT and ATf3−/− mice. Increased luciferase activity was observed after the pGL4-Beclin-1 promoter injection in the WT mice (Fig. 4G), and decreased activity was observed 3 days after TAB treatment (Fig. 4G, lane 2 versus lane 4). However, the luciferase activity was significantly higher in the ATf3−/− mice after TAB treatment compared with the former group (Fig. 4G, lane 4 versus lane 6). Together, our findings suggest that ATF3 can repress Beclin-1 protein level via transcriptional regulation in vivo.

An ATf3-Specific Inducer, tBHQ, Can Attenuate TAB-Induced Pressure-Overload Heart Failure in Mice. Prior studies have reported that tBHQ, an ATf3-specific inducer, can inhibit cell death or exert an anti-inflammatory effect (Kim et al., 2010; Hoetzenecker et al., 2012). Therefore, we were interested in elucidating the role of tBHQ in protecting against TAB-induced heart failure in mice. Initially, using varying doses of tBHQ in the administration of single injections, we found a 4- to 6-fold increase in cardiac ATF3 expression at days 1 and 7 after injection (Fig. 5A). Although the higher doses appeared more effective in ATf3 induction, no positive correlation was found between the dosage and cardiac ATf3 expression. To minimize possible side effects, we applied (via intraperitoneal injection) small doses (1 mg/kg) of tBHQ weekly to TAB mice and evaluated its long-term effect on pressure-overload heart failure (experimental design, Fig. 5B). The weekly administrations of tBHQ (1 mg/kg) for 7 weeks after TAB reduced the LV chamber dilatation and increased LV wall thickness, thus restoring the normal LV contractility (FS 44% versus FS 32%, respectively; P < 0.01) compared with the TAB-treated group (Fig. 5C). In addition, TAB-induced cardiac apoptosis was also attenuated after tBHQ treatment (Fig. 6). These results suggest that the specific ATf3 inducer, tBHQ, could potentially serve as an effective therapeutic molecule to prevent TAB-induced pressure-overload heart failure.

**Discussion**

In this study, TAB-induced pressure-overload heart failure with cardiac fibrosis and cell apoptosis was first found to be more severe in the ATf3−/− mice than in WT mice. Re-expression of the ATf3 protein in the heart via a viral infection (AAV-ATF3) was found to ameliorate TAB-induced cardiomyopathy in the ATf3−/− mice through involvement of the Beclin-1–dependent autophagy signaling pathway. Our in vivo experiments demonstrated that by use of the ATf3 specific inducer tBHQ could rescue long-term TAB-induced cardiac dysfunction. Considered together, these results suggest that ATf3 plays a prominent regulatory role in pressure overload–induced heart failure and that supplementation with an ATf3-specific inducer such as tBHQ might potentially be useful in the treatment of future pressure overload–induced cardiomyopathy.

Accumulating evidence has suggested that nuclear transcription factors from the basic leucine zipper (ZIP) family play an important role in cardiac development and function. This basic ZIP family includes the CREB/ATF family of transcription factors, which include CREB, cAMP response
Fig. 4. ATF3 inhibited autophagy by a Beclin-1–dependent pathway. (A) Protein level of Beclin-1 was inhibited by adenovirus-induced ATF3 (Adv-ATF3) overexpression in H9c2 cells under starvation conditions. (B) Western analysis of Beclin-1 in WT and ATF3−/− mice after TAB. (C) The protein expression of LC3B in WT and ATF3−/− mice after TAB was confirmed by Western blot analysis. Anti–glyceraldehyde-3-phosphate dehydrogenase (GAPDH) served as a loading control. (D) Binding activity of ATF3 on the ATF/CRE binding site of Beclin promoter. Results of EMSA with the ATF/CRE probe in the nuclear extracts of in vitro transcription translation and in the presence of 100 μM concentration of unlabeled competitor probe (cold probe). (E) Chromatin was isolated from Adv-ATF3–treated or untreated H9c2 cells. Chromatin immunoprecipitation experiments were carried out with ATF3–specific antibody and primers to amplify −164 to −156 of the beclin locus, which contained one predicted ATF/CRE binding site in mouse. The data are representative of two to four independent experiments. (F) ATF3 suppressed Beclin-1 promoter activity through ATF/CRE binding site in 293T cells,
element modulator, ATF, and the related AP-1 and C/EBP families (Kehat et al., 2006). Our study and a prior study by Zhou et al. (2011) have shown that ATF3 mRNA levels are upregulated 1 day after TAB procedure and that loss of the ATF3 gene can aggravate pressure overload–induced cardiomyopathy in mice. In addition, we observed that although the nuclear translocation of ATF3 occurred for only a short duration (1 day after TAB procedure), ATF3 protein remained which were transiently cotransfected with indicated plasmids. Cells were collected and assayed for luciferase activity in both stress (starvation) and nonstress conditions; *P < 0.05. (G) Luciferase activity on PGL4-beclin promoter expression in heart tissue of both WT and ATF3−/− mice 3 days after TAB treatment. HPGK, human phosphoglycerate kinase; IP, immune precipitation; TK, thymidine kinase.

Fig. 5. Treatment with ATF3 activator tBHQ attenuated TAB-induced LV dilatation and increased contractility in mice. (A) Real-time PCR analysis on cardiac ATF3 expression at 1 day or 7 days after different dosages of tBHQ therapy. (B) Experimental timeline for TAB and tBHQ therapy. Mice were treated with intraperitoneal tBHQ (1 mg/kg) weekly from 4 to 10 weeks after TAB procedure. (C) Echocardiographic measurements were performed on mice before (week 4) and after (week 10) tBHQ therapy in both the TAB procedure group and the nonprocedure group. Parameters, including interventricular septum thickness at diastole (IVSd) and systole (IVSs), left ventricular internal diameter at diastole (LVIDd) and systole (LVIDs), left ventricular posterior wall thickness at diastole (LVPWd) and systole (LVPWs), fractional shortening of left ventricle (FS), and ejection fraction of left ventricle (EF), and heart rate (HR) are shown; *P < 0.05. GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
continuously generated for at least 1 week after TAB induction (Fig. 1, A and B). The ATF3 protein that was continuously presented in the cytoplasm 6 days after TAB may be an alternatively spliced form, an ATF3 δ Zip lacking the leucine zipper domain. ATF3 δ Zip has been found to stimulate transcription, presumably by sequestering inhibitory cofactors (Chen et al., 1994). Although the exact mechanism of how ATF3 δ Zip or cytosolic ATF3 induce cardiac hypertrophy is still unknown, they may modulate gene expression by recruiting ATF4 from the cytosol into the nucleus to affect histone deacetylase (HDAC) activity under stress conditions induced by amino deprivation (Li et al., 2010; St. Germain et al., 2010).

ATF3 is transcriptionally upregulated and present in the nucleus at day 1 (Fig. 1C) and reduced at day 3–7 after TAB, whereas Beclin-1 increased from days 1–3 (Fig. 4B), with highest levels on day 3 and declined at day 7 after TAB. The nuclear decrease of ATF3 levels and the increase of Beclin-1 levels in days 1–3 after TAB suggested that ATF3 in the nucleus affected the Beclin-1 promoter via direct binding and repressed its expression. In addition, ATF3 may have also affected cell autophagy by its localization and interaction with other transcription factors in the cytoplasm. Cardiac hypertrophy can induce endoplasmic reticulum stress, in that eIF2 can be phosphorylated by pancreatic eIF2 kinase and induce the expression of ATF4, further upregulating ATF3 and other stress genes (Jiang et al., 2004). ATF4 could promote the transcription of Atg12, resulting in increased autophagy. Endoplasmic reticulum stress can also activate inositol-requiring enzyme-1, leading to subsequent phosphorylation of Beclin-1 and prevent its binding to Beclin-1, thus activating Beclin-1 thereby promoting autophagy (Pattingre et al., 2005; Erlich et al., 2007). Cytoplasmic localization of ATF3 has been reported by others in some breast carcinomas (Wang et al., 2008; and Stat1 knockdown hepatocytes (Kim et al., 2009), although the biologic significance of this phenomenon needs further elucidation.

Although signal transduction pathways for cardiac hypertrophy are inherently complex and abundant, studies using animal models have revealed important mediators of cardiac hypertrophy. These mediators can be divided into the following categories: mitogen-activated protein kinase signaling pathway, calcineurin-nuclear factor of activated T-cell signaling pathway, insulin-like growth factor 1-phosphatidylinositol 3-kinase–AKT/protein kinase B–mammalian target of rapamycin signaling pathway, and endothelin-1 or angiotensin II–induced calcium-calmodulin-protein kinase C (PKC)-class II HDACs signaling pathway (Heineke and Molkentin, 2006). Our study showed that ATF3 could inhibit both the hypertrophic signaling, which is mediated through the mitogen-activated protein kinase–signaling pathway (Fig. 2A), and the endothelin-1 or angiotensin II–associated pathway (see Supplemental Fig. 3), suggesting that ATF3 might also inhibit hypertrophy via the calcium-calmodulin-PKC-class II HDACs signaling pathway. However, it remains unclear whether ATF3 can directly regulate PKC gene regulation (Buganim et al., 2006). Recent evidence shows that cardiac apoptosis, although at a low level, is present in pressure-overload cardiac hypertrophy (Teiger et al., 1996; Li et al., 1997). It has been proposed that chronic low-level cardiac myocyte apoptosis may contribute to the pathogenesis of heart failure. In addition to the cardiomyocytes, the TUNEL-positive cells that were observed in high percentages in both WT and ATF3−/− (30% versus 50%, respectively) after TAB may include both endothelial and interstitial cells, suggesting that ATF3 may play a role in inflammatory signaling.

The precise role of autophagy in the progression of heart failure remains unclear; however, it is possible that autophagic activity performs different functions depending on disease staging and severity (Wang et al., 2010). In our ATF3−/− mice with TAB stress, amplification of the autophagic molecule Beclin-1 led to abnormal hypertrophic remodeling with early heart failure (Fig. 4B). Our results suggest that enhanced Beclin-1 activity has pathologic consequences, potentially achieved by promoting autophagic cell death or cardiomyocyte necrosis. As Beclin-1 has been involved in detrimental autophagic signaling induced by cardiac ischemic/reperfusion injury, our findings further showed that Beclin-1, under ATF3 regulation, is involved in cardiac hypertrophic remodeling signaling (Matsui et al., 2007). Beclin-1 can be regulated by microRNA-30a in cancer cells (Zhu et al., 2009). One recent study demonstrated that the downregulation of microRNA-30a could
aggravate pressure overload–induced cardiomyocyte hypertrophy via activating autophagy signaling (Yin et al., 2013). Whether Beclin-1 can be repressed by microRNA-30a in the heart to attenuate pressure overload–induced cardiac hypertrophy requires further investigation.

In addition to Beclin-1, another molecule involved in autophagy, ATG5, was regulated by ATF3, as indicated by our gel retardation data showing an ATF3/CRE binding site located on the upstream promoter region of ATG5 (data not shown). However, ATG5 deletion in the heart resulted in aggravated ventricular enlargement and cardiac dysfunction after hemodynamic overload, implying that autophagy induced by ATG5 is a compensatory mechanism during heart failure (Nishida et al., 2008). On the contrary, Beclin-1 was shown to be regulated by autophagy, ATG5, was regulated by ATF3, as indicated by further investigation.

Whether Beclin-1 can be repressed by microRNA-30a in the heart to attenuate pressure overload has not been performed in this study. Therefore, it is worth exploring whether ATF3 has a positive or negative influence on the regulation of this conflicting autophagic signaling between ATG5 and Beclin-1 in pressure overload–induced heart failure.

ATF3 can be rapidly induced by various stimuli, in which the nuclear factor erythroid 2–related factor (Nrf2) can specifically mediate ATF3 induction by acting on the consensus ATF3 response element on the ATF3 promoter (Kim et al., 2010; Hoetze, 2012). Therefore, for the induction of ATF3 in our experiments, we chose to use the Nrf2 inducer tBHQ, which has antioxidative and anti-inflammatory effects and is used widely as a food preservative (Nishizono et al., 2000; Koh et al., 2009). The administration of tBHQ 4 weeks after the TAB procedure improved cardiac contractility in mice with prolonged pressure overload–induced heart failure in our study. The beneficial effects offered by tBHQ supplementation may be explained based on both the inhibitory effect of Beclin-1–related detrimental autophagy and the inhibitory effect on hypertension or angiotensin II-induced cardiac fibrosis and inflammation via the ATF3 pathway. A high dosage of the tBHQ (5 mg/kg), although it is reported to offer a protective effect on neurons (Koh et al., 2009), may lead to a high death rate (approximately 50–70%). Therefore, we used a comparatively low dosage of tBHQ (1 mg/kg) to avoid such severe effects. Recently, several Nrf2 activators, including sulforaphane and bardoxolone and many medicinal plants mixtures (Bai et al., 2009; Jin et al., 2011), may lead to a high death rate in mice subjected to pressure overload–induced heart failure.

In conclusion, our study showed that overexpression of ATF3 or therapy using the ATF3 inducer tBHQ can prevent or rescue cardiac dysfunction induced by pressure overload by inhibiting Beclin-1–associated detrimental autophagic activity. Future therapy using the ATF3-specific inducer tBHQ or other Nrf2 activators may be developed for possible use in the treatment of human chronic hypertension or other pressure-overload heart failures.

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Authorship Contributions


References


Hu P, Zhang D, Swenson L, Chakrabarti G, Abel ED, and Litwin SE (2003) Minichromosome maintenance (MCM) proteins are regulated by the autophagy-related factor (Nrf2) can specifically mediate ATF3 induction by acting on the consensus ATF3 response element on the ATF3 promoter (Kim et al., 2010; Hoetze, 2012). Therefore, for the induction of ATF3 in our experiments, we chose to use the Nrf2 inducer tBHQ, which has antioxidative and anti-inflammatory effects and is used widely as a food preservative (Nishizono et al., 2000; Koh et al., 2009). The administration of tBHQ 4 weeks after the TAB procedure improved cardiac contractility in mice with prolonged pressure overload–induced heart failure in our study. The beneficial effects offered by tBHQ supplementation may be explained based on both the inhibitory effect of Beclin-1–related detrimental autophagy and the inhibitory effect on hypertension or angiotensin II-induced cardiac fibrosis and inflammation via the ATF3 pathway. A high dosage of the tBHQ (5 mg/kg), although it is reported to offer a protective effect on neurons (Koh et al., 2009; Jin et al., 2011), may lead to a high death rate (approximately 50–70%). Therefore, we used a comparatively low dosage of tBHQ (1 mg/kg) to avoid such severe effects. Recently, several Nrf2 activators, including sulforaphane and bardoxolone and many medicinal plants mixtures (Bai et al., 2013; Reuland et al., 2013; Ruiz et al., 2013), have been reported to protect cardiomyocytes against high glucose or oxidative stress. However, additional studies are required to determine whether consistent results can be obtained from either an ATF3 inducer or an Nrf2 activator after pressure overload–induced heart failure.

In conclusion, our study showed that overexpression of ATF3 or therapy using the ATF3 inducer tBHQ can prevent or rescue cardiac dysfunction induced by pressure overload by inhibiting Beclin-1–associated detrimental autophagic activity. Future therapy using the ATF3-specific inducer tBHQ or other Nrf2 activators may be developed for possible use in the treatment of human chronic hypertension or other pressure-overload heart failures.

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