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Lack of ATM induces structural and functional changes in the heart: Role in β -adrenergic receptor-stimulated apoptosis

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Abstract

Ataxia telangiectasia mutated kinase (ATM) is involved in cell cycle checkpoints, DNA repair and apoptosis. β -adrenergic receptor (β -AR) stimulation induces cardiac myocyte apoptosis. Here we analyzed basal myocardial structure and function in ATM knockout (KO) mice, and tested the hypothesis that ATM modulates β -AR-stimulated myocyte apoptosis. Left ventricular (LV) structure and function, myocyte apoptosis, fibrosis and expression of fibrosis-, hypertrophy- and apoptosis-related proteins were examined in wild-type (WT) and KO mice with or without L-isoproterenol treatment for 24h. Body and heart weights were lower in KO mice. M-mode echocardiography showed reduced septal wall thicknesses and LV diameters in KO mice. Doppler echocardiography showed increased ratio of early peak velocity (E wave) to that of the late (A wave) LV filling in KO mice. Basal fibrosis and myocyte cross-sectional area was greater in KO hearts. Expression of fibrosis-related genes (CTGF, PAI-1 and MMP-2) and hypertrophy-related gene (ANP) was higher in KO hearts. β -AR stimulation increased myocyte apoptosis to a similar extent in both groups. Activation of JNKs, and expression and phosphorylation of p53 in response to β -AR stimulation was only observed in WT group. Akt phosphorylation was lower in KO-sham and remained lower following β -AR stimulation in KO group. β -AR stimulation activated GSK-3 β to a similar extent in both groups. Thus, lack of ATM induces structural and functional changes in the heart with enhanced myocardial fibrosis and myocyte hypertrophy. β -AR-stimulated apoptosis in WT hearts is associated with p53- and JNKs-dependent mechanism, while decreased Akt activity may play a role in increased myocyte apoptosis in the absence of ATM.

Keywords

ATM; apoptosis; heart; AKT; p53

Introduction

Ataxia telangiectasia (A-T), a hereditary multi-systemic disease resulting from mutation of ATM (ataxia telangiectasia mutated kinase), is characterized by neuronal degeneration, immunodeficiency, genomic instability, premature aging and cancer predisposition. Individuals with an ATM mutation in one allele are spared from most of the symptoms of the disease, but are more susceptible to cancer and ischemic heart disease (Lavin *et al.*, 1995; Su & Swift, 2000). In proliferating cells, ATM facilitates cell cycle arrest and DNA

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repair in response to DNA damage induced by ionizing radiation. ATM phosphorylates an extensive array of substrates, including transcription factors such as p53, AP-1 and p73 (Khanna *et al.*, 2001; Lavin *et al.*, 1995). ATM-mediated phosphorylation of p53 on Serine-15 stabilizes p53 and enhances its transcriptional activity (Shiloh, 2001). Pro-survival regulator PKB/AKT is also identified as an ATM substrate in response to cellular stress and the maintenance of cellular homeostasis (Barlow *et al.*, 1996; Viniegra *et al.*, 2005). ATM was initially thought to be localized in the nucleus, affecting only proliferating cells (Watters *et al.*, 1999). Evidence has been provided that ATM is present within the cytoplasm of neuronal cells and plays a direct role in disease phenotypes such as insulin resistance and glucose intolerance (Boehrs *et al.*, 2007; Yang & Kastan, 2000).

Increased sympathetic activity to the heart is an early response to hemodynamic dysfunction (Fowler *et al.*, 1986; Singh *et al.*, 2000). Cardiac myocyte apoptosis is recognized as an important determinant of structure and function of the myocardium (Kajstura *et al.*, 2006; Nadal-Ginard *et al.*, 2003). Stimulation of β -AR increases apoptosis in cardiac myocytes *in vitro* and *in vivo*, and plays a role in myocardial remodeling associated with increased cardiac fibrosis (Krishnamurthy *et al.*, 2007; Singh *et al.*, 2001). Using heterozygous ATM knockout mice and isoproterenol infusion as a model of myocardial remodeling, we have provided evidence that deficiency of ATM plays a protective role in β -AR-stimulated cardiac myocyte apoptosis and myocardial remodeling (Foster *et al.*, 2011). There are no reports investigating basal structure and function of the heart in the complete absence of ATM. We report that ATM plays an important role in modulating structure and function of the heart. Lack of ATM associates with increased myocardial fibrosis and myocyte hypertrophy. β -AR stimulation increased myocyte apoptosis to a similar extent in mice with or without ATM. Decreased Akt activity, not p53 and JNKs, may be involved in increased myocyte apoptosis following β -AR stimulation in mice lacking ATM.

Methods

Vertebrate Animals

The investigation conforms to the *Guide for the Care and Use of Laboratory Animals* published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996). The animal protocol was approved by the University Committee on Animal Care. Mice were euthanized by exsanguination. Animals were anesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 l/min) and the heart was removed following a bilateral cut in the diaphragm. Heterozygous knockout (hKO) and wild type (WT) ATM mice, purchased from the Jackson Laboratory, were of 129xblack Swiss hybrid background. These ATM deficient mice were originally generated by the disruption of a 178bp exon, corresponding to nucleotides 5178-5979 in *ATM* by placing a PGK*neo* gene at position 5790 in the opposite orientation relative to *ATM* transcription (Barlow *et al.*, 1996). Homozygous mice are infertile. Therefore, hKO mice were used for breeding to obtain knockout (KO) mice. Genotyping was performed by polymerase chain reaction using primers suggested by the Jackson laboratory. Genotyping results for ATM mice are depicted in Figure 1 (Data supplement). The absence of both ATM genes produces a lethal phenotype between 2 and 4 months of age mainly due to thymic lymphomas (Barlow *et al.*, 1996; Yan *et al.*, 2002). Therefore, mice (WT and KO) were used at ~2 months of age.

Echocardiography

Transthoracic 2D M-mode echocardiograms and pulsed wave Doppler spectral tracings were performed as previously described (Foster *et al.*, 2011; Krishnamurthy *et al.*, 2007). M-mode tracings were used to measure LV wall thickness and end-systolic (LVESD) and end-diastolic dimensions (LVEDD), and calculation of percent fractional shortening (%FS) and

ejection fractions (%EF). Doppler tracings of mitral and aortic flow were acquired from apical four-chamber view. These tracings were used to measure peak velocity of the early ventricular filling (E wave), peak velocity of the late ventricular filling (A wave), peak E/A ratio, E wave deceleration time, heart rate (HR), ejection time (ET), isovolumic relaxation time (IVRT; measured from the aortic valve closure to the mitral valve opening), and isovolumic contraction time (IVCT; measured from the closing of the mitral valve to the opening of the aortic valve). M-mode and Doppler images obtained from WT and KO hearts are depicted in Figure 2 (Data supplement). LV mass was calculated using the formula = $1.05 [(LVSd + LVEDD + LVPWd)^3 - (LVEDD)^3]$, where LVSd and LVPWd are the diastolic septal and posterior wall thickness, respectively, and LVEDD is the LV internal diameter in diastole (Collins *et al.*, 2001). LV circumferential stress and fiber shortening velocity (vcf) was calculated using the formula = $LVEDD - LVESD / LVEDD \times LVET$, where LVEDD and LVESD are LV diastolic and systolic diameters, respectively, and LVET is LV ejection time (Quinones *et al.*, 1975).

Isoproterenol Treatment

Isoproterenol (ISO; 10 mg/kg, 10 μ l/g body weight; s.c.) was injected in WT and KO mice (2 months old). The mice were sacrificed 24 h after ISO- injection.

Morphometric analyses

Mice were sacrificed and hearts were arrested in diastole using KCl (30 mmol/L) followed by perfusion fixation with 10% buffered formalin. Cross sections (4 μ m thick) were stained with Masson's trichrome for the measurement of fibrosis using Bioquant image analysis software (Nashville, TN).

Immunohistochemistry

Heart sections (4 μ m thick) were deparaffinized and blocked with 10% goat serum for 1 h at room temperature. The sections were incubated with monoclonal anti-ATM (Santa-Cruz, CA) or monoclonal anti-phospho-ATM (Ser1981; Santa-Cruz, CA) antibodies (1:50 dilution) overnight at 4°C in a humidified chamber followed by incubation with secondary antibodies conjugated with Alexa fluor 555 for 1 h at 37°C. Nuclei were stained using Hoechst 33258 (10 μ M; Sigma, St Louis, MO). The sections were visualized using fluorescent microscopy (100X; Nikon) and images were recorded using Retiga 1300 color-cooled camera.

Myocyte cross-sectional area

Heart sections (4 μ m thick) were deparaffinized and stained with FITC-labeled wheat germ agglutinin (WGA). The sections were visualized using fluorescent microscopy (20X; Nikon) and images were recorded using Retiga 1300 color-cooled camera. To measure myocyte cross-sectional area, suitable area of the sections was defined as the one with nearly circular capillary profiles and nuclei.

Apoptosis

To detect apoptosis, TUNEL-staining was carried out on 4 μ m thick sections as per manufacturer's instructions (cell death detection assay kit, Roche) and previously described (Foster *et al.*, 2011).

Reverse Transcription – Polymerase Chain Reaction (RT-PCR)

Total RNA from the left ventricles (LV) was isolated as described (Foster *et al.*, 2011). Total RNA (1 μ g) was reverse transcribed using SuperScript III RT kit (Invitrogen, Carlsbad,

CA). The products were amplified by PCR using primer sequences as described (Table; Data supplement). PCR products were analyzed by agarose gel electrophoresis, stained with ethidium bromide and quantified using Carestream Gel Imaging System.

Western Blot Analyses

LV lysates (50 μ g) were separated by SDS-PAGE and transferred to PVDF membranes (Krishnamurthy *et al.*, 2007). The membranes were incubated with antibodies against p53, p-p53 (serine-15; Cell Signaling), JNK, p-JNK, Bax (Santa Cruz), Akt, p-Akt, (serine-473; Cell Signaling), GSK-3 β , p-GSK-3 β , (serine- 9; Cell Signaling), MMP-2 and MMP-9 (Millipore). The immune complexes were detected using chemiluminescence reagents (Pierce Biotech.). Membranes were stripped and probed with β -actin (actin; Chemicon) or GAPDH (Santa Cruz) as a protein loading control. Band intensities were quantified using Kodak photodocumentation system (Eastman Kodak Co.). The data are presented as fold change vs WT-sham.

Statistical Analysis

Data are represented as mean $\pm$ SE. Data were analyzed using student's t test or one-way analysis of variance (ANOVA) and a post hoc Student- Newman-Keuls test. Probability (p) values of <0.05 were considered to be significant.

Results

Immunohistochemical and morphometric Studies

Immunohistochemical analyses of WT hearts using anti-ATM and phospho-ATM antibodies demonstrated cytosolic as well as nuclear localization of ATM protein in cardiac cells (Figure 3; Data supplement). The body weight and heart weight in the ATM KO mice were significantly lower compared to the WT group. This however resulted in no difference in the heart weight to body weight (HW/BW) ratios between the WT and KO groups (Table 1).

Echocardiographic studies

At basal levels, M-mode echocardiography showed a significant reduction in LVEDD, LVESD, LVEDV, and LVESV in the KO group when compared to WT (Table 2). These changes in the structure of heart did not result in decreased % FS or EF. Septal wall thicknesses (systolic and diastolic) were significantly lower in KO mice. Posterior wall thicknesses tended to be lower in the KO group. However, the data was not found to be significant. LV mass was significantly lower in KO mice. No significant difference in Vcf was observed between the two groups. Doppler spectral tracings showed a significant decrease in A wave in KO mice, resulting in an increased E/A wave ratio. Deceleration time (Dct) for the E-wave was significantly lower in KO mice, while HR, ET, IVRT and IVCT remained unchanged between the two groups (Table 3).

Fibrosis, hypertrophy, and Apoptosis

Quantitative analysis of fibrosis using trichrome stained sections revealed increased fibrosis in the KO group when compared to WT (Fig 1A). Myocyte cross-sectional area was significantly higher in KO-sham group when compared to WT-sham ($p < 0.05$ vs WT-sham). ISO treatment increased myocyte cross-sectional area in WT, not in KO group ($p < 0.05$ vs WT-sham; Fig 1B). Analysis of cardiac myocyte apoptosis using TUNEL-staining assay revealed no change in myocyte apoptosis between the two groups (Fig 1C). ISO-treatment increased the number of cardiac myocyte apoptosis to a similar extent in both groups (Fig 1C).

Expression of fibrosis- and hypertrophy-related genes

RT-PCR analysis of expression of fibrosis-related genes showed increased expression of connective tissue growth factor (CTGF) and plasminogen activator inhibitor-1 (PAI-1) in KO-sham group as compared to WT-sham (Fig 2). ISO treatment increased CTGF expression in WT, not in KO group. Neither lack of ATM nor ISO treatment had any effect on the expression of insulin-like growth factor 1 receptor (IGF-1R; Fig 2).

Expression of sarcoplasmic reticulum Ca⁺⁺-ATPase (SERCA2a), a protein decreased during heart failure, was not different between WT and KO groups. ISO treatment had no effect on SERCA2a expression. However, expression of atrial natriuretic peptide (ANP), a marker of myocardial hypertrophy (Chien *et al.*, 1991), was significantly higher in KO-sham group when compared to WT-sham (Fig 2). ISO treatment had no effect on ANP expression.

Expression of matrix metalloproteinases (MMPs; MMP-2 and MMP-9)

Western blot analysis of LV lysates showed increased MMP-2 protein levels in the KO-sham group when compared to WT-sham. ISO-treatment increased MMP-2 protein levels in the WT, but not in the KO group (Fig 3A). There was no change in MMP-9 protein levels with or without ISO treatment (Fig 3B).

Expression and phosphorylation of apoptosis-related proteins

Western blot analysis of LV lysates using anti-p53 antibodies showed no immunostaining for p53 in WT-sham or KO-sham groups. ISO-treatment increased levels of p53 in the WT-ISO, but not in the KO-ISO group (Fig 4A; middle panel). Phosphorylation of p53 (serine-15) was only observed in the WT-ISO group (Fig 4A; upper panel). Western blot analysis of LV lysates showed no phosphorylation (activation) of JNKs in sham groups. ISO treatment activated JNKs in the WT-ISO, but not in the KO-ISO group (Fig 4B). There was a non-significant trend towards increased Bax protein levels in KO-sham group as compared to WT-sham. ISO treatment decreased Bax expression in the KO, but not in the WT group (Fig 4C).

Analysis of Akt phosphorylation (activation) by western blot showed a trend towards decreased Akt activity in KO-sham as compared to WT-sham. ISO-treatment increased Akt activity in the WT, but not in the KO group (**p*<0.01, *n*= 3-4; Fig 5A). Phosphorylation of an N-terminal serine residue (serine-9) inactivates GSK-3 β (Hardt & Sadoshima, 2002). Analysis of GSK-3 β phosphorylation using western blot demonstrated no change in GSK-3 β phosphorylation between WT-sham and KO-sham groups (Fig 5B). ISO-treatment activated GSK-3 β in both groups, as analyzed by reduced GSK-3 β phosphorylation, when compared to their respective shams (**p*<0.05 vs WT-sham and KO-sham). There was no significant difference in GSK-3 β phosphorylation between the two ISO-treated groups.

Discussion

Lack of ATM is shown to be associated with reduced body weight when compared to their WT counterparts (Barlow *et al.*, 1996). The main finding of this study is that lack of ATM induces structural and functional changes in the heart with enhanced myocardial fibrosis and myocyte hypertrophy. β -AR stimulation increased myocyte apoptosis to a similar extent in the myocardium of mice with or without ATM. However, the signaling pathway involved in myocyte apoptosis appears different in presence or absence of ATM. β -AR-stimulated apoptosis in WT hearts may occur via the involvement of p53-and JNKs-dependent mechanism, while decreased Akt activity may play a role in β -AR-stimulated cardiac myocyte apoptosis in the absence of ATM.

ATM was initially thought to be localized in the nucleus, affecting only proliferating cells (Watters *et al.*, 1999). In differentiated human neuron-like SH-SY5Y cells, ATM is shown to be present throughout the cell body (Boehrs *et al.*, 2007) (Boehrs *et al.*, 2007). Consistent with these observations, we observed presence of ATM throughout the cell body, including the nucleus. Growth retardation is described as a prominent feature during lack of ATM, suggesting a growth promoting function of ATM (Barlow *et al.*, 1996). This growth retardation may affect animal and heart size as indicated by the decreased body and heart weights. Mice lacking ATM also exhibited decreased LV mass and septal wall thickness. Increase in HW/BW ratio, myocyte cross-sectional area and ANP expression are considered as measures of myocardial hypertrophy. HW/BW ratio was not found to be significantly different between the KO and WT groups. However, myocyte cross-sectional area and ANP expression were significantly higher in mice lacking ATM when compared to WT. Therefore, decreased heart weight, LV mass and septal wall thickness are most likely due to growth retardation and/or cell cycle-regulatory defects in the complete absence of ATM. Increased cardiac myocyte hypertrophy may not be sufficient to compensate for these defects in the absence of ATM. Of note, heterozygous ATM knockout (ATM^{+/-}) mice, although investigated at 4-5 months of age, exhibit no signs growth retardation, and heart weight and septal wall thicknesses were comparable to their WT counterparts (Foster *et al.*, 2011).

M-mode echocardiography showed no change in %FS and EF in mice lacking ATM. This may be due to the fact that %FS and EF are calculated using LV diameters (Gardin *et al.*, 2002). Although ATM mice exhibit decreased LV diameters and volumes, however, the decreased diastolic and systolic diameters and volumes is proportionate, hence no change in %FS and EF. Other factors such as heart rate, myocardial fibrosis and cardiac myocyte hypertrophy may affect systolic function of the heart, leading to changes in %FS. Heart rate was not significantly different between WT and KO groups. Myocardial fibrosis and myocyte cross-sectional area were significantly greater in the KO group, suggesting that myocyte hypertrophy may play a compensatory role in maintaining %FS during increased myocardial fibrosis. Results from Doppler analysis revealed no change in IVRT and IVCT between the WT and KO groups at baseline. However, peak velocity of late LV filling was lower in KO mice. This decrease in A wave associated with increased E/A wave ratio. E wave deceleration time was shortened in the absence of ATM. These changes in Doppler parameters in mice lacking ATM are suggestive of diastolic impairment. Patients with acute myocardial infarction with pseudonormal or restrictive physiology pattern are shown to exhibit reduced A wave, increased E/A ratio and shortened E wave deceleration time (Nearchou *et al.*, 2006).

Cardiac fibrosis deposition is a well-recognized feature of heart failure (Weber *et al.*, 1994). The data presented here suggest that lack of ATM associates with increased fibrosis in the heart. Expression of CTGF and PAI-1, mediators of cardiovascular fibrosis (Samarakoon *et al.*, 2010), was significantly greater in mice lacking ATM. Cardiac-specific overexpression of IGF-1R is shown to reduce cardiac fibrosis during streptozotocin-induced diabetes (Huynh *et al.*, 2010). Expression of IGF-1R was not found to be different in the presence or absence of ATM, suggesting IGF-1R-independent mechanism of increased myocardial fibrosis in the absence of ATM. MMPs are suggested to play a significant role in the deposition of fibrosis and myocardial remodeling (Spinale, 2002). Activation of MMPs, specifically MMP-2, is suggested to decrease cardiac tissue tensile strength and cause systolic and diastolic dysfunction (Mujumdar *et al.*, 2001). Cardiac specific expression of MMP-2 induces development of cardiac contractile dysfunction in the absence of superimposed injury (Wang *et al.*, 2006). Transgenic mice expressing active MMP-2 driven by the alpha-myosin heavy chain promoter exhibited ventricular remodeling and systolic dysfunction (Bergman *et al.*, 2007). These later mice also exhibited extensive myocardial

fibrosis. On the other hand, targeted deletion of MMP-2 attenuates early rupture and improves %FS in mice post MI (Hayashidani *et al.*, 2003). Here we observed a basal increase in MMP-2, not MMP-9, in mice lacking ATM. Therefore, increased MMP-2 expression and fibrosis may reflect observed functional changes in mice lacking ATM.

Replacement fibrosis (formation of scar tissue) generally follows an inflammatory cell response that appears at the sites of cardiac myocyte necrosis and preserves the structural integrity of the myocardium (Weber, 2004). This replacement fibrosis is distinct from reactive fibrosis that surrounds intra-myocardial coronary arteries and may extend into the contiguous interstitial space with time. Apoptosis usually does not associate with the inflammatory response. The number of apoptotic myocytes was not different between WT and KO mice. Therefore, increased myocardial fibrosis observed at basal levels in mice lacking ATM is most likely due to necrosis. However, further investigations are needed to examine the involvement of necrosis and/or apoptosis in this process.

Cardiac myocyte apoptosis is suggested to play an important role in modulating the structure and function of the heart (Kajstura *et al.*, 2006; Nadal-Ginard *et al.*, 2003). Basal myocyte apoptosis and Bax expression were not different in the presence or absence of ATM. Stimulation of β -AR increases apoptosis in cardiac myocytes *in vitro* and *in vivo* (Singh *et al.*, 2001). Here, we found that β -AR stimulation increases myocyte apoptosis to a similar extent in both groups. However, differential signaling pathway/s appeared to be involved in the apoptotic process in WT and ATM KO mice. In response to DNA damage, ATM phosphorylates p53 on serine-15 resulting in stabilization of p53. Stabilization of p53 may increase the transcriptional activity of p53, ultimately leading to apoptosis (Meulmeester & Jochemsen, 2008). Consistent with these observations, we observed increased protein levels of p53 and its phosphorylation in response to 24 h ISO treatment in the WT group. JNK is not a direct ATM target. However, loss of ATM is shown to reduce JNKs phosphorylation in embryonic fibroblast following UV radiation and oxidative stress (Lee *et al.*, 1998). In cardiac myocytes, activation of JNKs plays a pro-apoptotic role via the activation of mitochondrial pathway (Remondino *et al.*, 2003). We observed increased phosphorylation of JNKs only in the WT-ISO group. These data, taken together, suggest that β -AR-stimulated apoptosis in the presence of ATM occurs via p53- and JNKs-dependent mechanisms.

Akt is a potent anti-apoptotic cell survival protein because of its ability to regulate several downstream effectors (Miyamoto *et al.*, 2009). Akt targets the mitochondria protecting cardiac myocytes against necrotic and apoptotic death (Miyamoto *et al.*, 2009). Evidence for a protective role for Akt in an ATM dependent manner was observed in insulin resistance (Viniegra *et al.*, 2005). In glioma cells, an ATM kinase inhibitor decreased basal levels of Akt phosphorylation and ATM deficient cells were impaired in the activation of Akt in response to ionizing radiation and insulin (Golding *et al.*, 2009). β -AR stimulation increased Akt phosphorylation in WT mice. However, Akt phosphorylation tended to be in the KO-sham and remained lower following β -AR stimulation. These data suggest a diminished or compromised ability of the heart to activate the Akt survival mechanism in the absence of ATM. Phosphorylation of an N-terminal serine residue (serine-9) inactivates GSK-3 β . Protein kinases, including Akt, protein kinase A, protein kinase C and integrin-linked kinase are implicated in serine-9 phosphorylation and inactivation of GSK-3 β (Hardt & Sadoshima, 2002). Using embryonic human lung fibroblasts, Kwon *et al.* provided evidence that activation of GSK-3 β may induce ATM-mediated cell apoptosis via the activation of p53 (Kwon *et al.*, 2008). Here, β -AR stimulation activated GSK-3 β to a similar extent in both WT and KO groups, despite an increased Akt activity in WT-ISO group. Therefore, it is plausible that increased GSK-3 β activity may be a secondary cellular response to β -AR stimulation independent of ATM and/or Akt.

ATM is a key regulator of multiple signaling pathways in response to DNA damage. The data presented here suggest that ATM plays a crucial role in basic structure and function of the heart, possibly by modulating myocardial fibrosis and myocyte hypertrophy. Following β -AR stimulation, activation of pro- and anti-apoptotic signaling pathways determines the cell fate in the presence of ATM in WT hearts. In the absence of ATM, decreased activation of pro-apoptotic (p53 and JNKs) and anti-apoptotic (Akt) pathways may shift the balance towards enhanced cardiac myocyte apoptosis. Investigation of signaling pathways that can shift the balance from cell survival to apoptosis during chronic β -adrenergic stimulation may have important clinical implications.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Myocardial fibrosis, myocyte hypertrophy, and apoptosis

A. Upper panel demonstrates Masson's trichrome-stained sections from WT and KO hearts. The lower panel demonstrates quantitative analysis of fibrosis; $^{\#}p < 0.05$ vs WT-sham; $n = 6-7$. B. Measurement of myocyte cross-sectional area; $^{\#}p < 0.05$ vs WT-sham; $n = 5$; $^{*}p < 0.05$ vs WT-sham; $n = 3-5$. C. Upper panel demonstrates TUNEL-stained images obtained from KO-sham and KO-ISO hearts. Yellow-green fluorescence represents apoptotic cells, while red fluorescence indicates α -sarcomeric actin staining (specific for myocytes). The lower panel demonstrates quantitative analysis of myocytes apoptosis with or without ISO-treatment; $^{*}p < 0.05$ vs WT-sham; $^{\dagger}p < 0.05$ vs KO-sham; $n = 3-4$.

Figure 2. RT-PCR analysis of fibrosis- and hypertrophy-related genes using RT-PCR
Total RNA isolated from LV was analyzed by RT-PCR using specific primer sequences CTGF, PAI-1, IGF-1R, SERCA2a and ANF. GAPDH was used as an internal control. The upper panel demonstrates ethidium bromide stained portions of the agarose gels, while the lower panel exhibits fold changes vs WT-sham; [#]p<0.05 vs WT-sham; ^{*}p<0.05 vs WT-sham; n=4.

Figure 3. Expression of MMPs

Total LV lysates (75 μ g) were analyzed by western blot using anti-MMP-2 (A) or anti-MMP-9 (B) antibodies. The upper panels show autoradiograms indicating immunostaining for MMP-2, MMP-9, and GAPDH. The lower panels exhibit quantitative analysis of MMP-2 ([#] $p < 0.05$ vs WT-sham; ^{*} $p < 0.05$ vs WT-sham) and MMP-9 normalized to GAPDH.

Figure 4. Expression and phosphorylation of p53, JNKs and Bax

A. Total LV lysates were analyzed by western blot using phospho-specific (serine-15) p53 or total p53 antibodies. Protein loading in each lane is indicated by actin immunostaining. Similar data were obtained when experiment was repeated using LV lysates from a different set of animals. B. Total LV lysates were analyzed by western blot using phospho-specific JNKs antibodies. Protein loading in each lane is indicated by total JNKs (middle panel) and actin immunostaining. Similar data were obtained when experiment was repeated using LV lysates from a different set of animals. C. Total LV lysates were analyzed by western blot using Bax antibodies. Protein loading in each lane is indicated by actin immunostaining. The lower panel exhibits the mean data normalized to actin; &p<0.05 vs KO-sham; n=4.

Figure 5. Phosphorylation of Akt and GSK-3

β. A. Total LV lysates were analyzed by western blot using phospho-specific Akt (serine-473) antibodies. Protein loading in each lane is indicated by Akt or GAPDH immunostaining. The lower panel exhibits the mean data normalized to total Akt. * $p < 0.05$ vs WT-sham; \$ $p < 0.05$ vs WT-ISO; $n = 3-4$. B. Total LV lysates were analyzed by western blot using phospho-specific GSK-3 β (serine-9) antibodies. Protein loading in each lane is indicated by GSK-3 β or GAPDH immunostaining. The lower panel exhibits the mean data normalized to total GSK-3 β . * $p < 0.05$ vs WT-sham and KO-sham; ($n = 3-4$).

Table 1**Morphometric Measurements**

Parameters	WT-sham (n=5)	KO-sham (n=5)	p value
Body weight (g)	23.8 ± 1.2	17.0±0.9 [#]	[#] <0.005
Heart weight (mg)	128.6 ± 16.4	75.8±3.2 [#]	[#] <0.005
HW/BW ratio (mg/g)	5.4±0.5	4.5±0.2	NS

HW, heart weight; BW, body weight. Values are mean±SE;

[#] comparison between genotypes. NS, not significant.

Table 2**M-mode Echocardiographic Measurements**

Parameters	WT-SHAM (n=7)	KO-SHAM (n=7)	p value
LVEDD (mm)	3.71 ± 0.08	3.36 ± 0.05 [#]	[#] <0.005
LVESD (mm)	2.81 ± 0.11	2.48 ± 0.06 [#]	[#] <0.005
LVEDV (μl)	58.6 ± 3.07	46.21 ± 1.84 [#]	[#] <0.01
LVESV (μl)	30.27 ± 2.86	22.08 ± 1.26 [#]	[#] <0.05
LVSwd (mm)	0.64 ± 0.04	0.52 ± 0.03 [#]	[#] <0.05
LVSWs (mm)	0.90 ± 0.03	0.73 ± 0.04 [#]	[#] <0.01
LVPWd (mm)	0.47 ± 0.02	0.42 ± 0.02	NS
LVPWs (mm)	0.66 ± 0.05	0.60 ± 0.05	NS
% FS	24.48 ± 1.69	25.94 ± 2.22	NS
% EF	49.11 ± 2.75	51.64 ± 3.20	NS
LV mass (mg)	63.86 ± 1.81	44.25 ± 4.05	[#] <0.05
Mean Vcf (circ/sec)	4.90 ± 0.31	4.72 ± 0.10	NS

Values are mean±SE; LVEDD, LV end-diastolic diameter; LVESD, LV end-systolic diameter; LVEDV, LV end-diastolic volume; LVESV, LV end-systolic volume; LVSws, LV septal wall thickness in diastole; LVSWs, LV septal wall thickness in systole; LVPWd, LV posterior wall thickness in diastole; LVPWs, LV posterior wall thickness in systole; %FS, percent fractional shortening; %EF, percent ejection fraction; Vcf, mean velocity of circumferential shortening.

[#] comparison between genotypes. NS, not significant.

Table 3**Doppler Measurements**

Parameters	WT-SHAM (n=7)	KO-SHAM (n=7)	p value
E (cm/s)	59.87 ± 4.06	65.54 ± 3.58	NS
A (cm/s)	35.94 ± 2.41	29.50 ± 1.02 [#]	[#] <0.05
E/A	1.67 ± 0.03	2.24 ± 0.15 [#]	[#] <0.01
DCT (msec)	34.17 ± 1.27	30.02 ± 1.39 [#]	[#] <0.05
ET (msec)	52.43 ± 2.48	52.41 ± 2.03	NS
IVRT (msec)	30.50 ± 4.64	28.22 ± 3.60	NS
IVCT (msec)	31.17 ± 3.68	30.63 ± 2.22	NS
HR	323.65±19.71	333.48±14.79	NS

Values are mean ±SE; E, peak velocity of the early ventricular filling; A, peak velocity of the late ventricular filling; DCT, E wave deceleration time; IVRT, isovolumic relaxation time; IVCT, isovolumic contraction time; HR, heart rate

[#] comparison between genotypes. NS, not significant