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Ataxia telangiectasia mutated kinase plays a protective role in β-adrenergic receptor-stimulated cardiac myocyte apoptosis and myocardial remodeling

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Abstract

β-Adrenergic receptor (β-AR) stimulation induces cardiac myocyte apoptosis and plays an important role in myocardial remodeling. Here we investigated expression of various apoptosis-related genes affected by β-AR stimulation, and examined first time the role of ataxia telangiectasia mutated kinase (ATM) in cardiac myocyte apoptosis and myocardial remodeling following β-AR stimulation. cDNA array analysis of 96 apoptosis-related genes indicated that β-AR stimulation increases expression of ATM in the heart. In vitro, RT-PCR confirmed increased ATM expression in adult cardiac myocytes in response to β-AR stimulation. Analysis of left ventricular structural and functional remodeling of the heart in wild-type (WT) and ATM heterozygous knockout mice (hKO) 28 days after ISO-infusion showed increased heart weight to body weight ratio in both groups. M-mode echocardiography showed increased percent fractional shortening (%FS) and ejection fraction (EF%) in both groups 28 days post ISO-infusion. Interestingly, the increase in %FS and EF% was significantly lower in the hKO-ISO group. Cardiac fibrosis and myocyte apoptosis were higher in hKO mice at baseline and ISO-infusion increased fibrosis and apoptosis to a greater extent in hKO-ISO hearts. ISO-infusion increased phosphorylation of p53 (Serine-15) and expression of p53 and Bax to a similar extent in both groups. hKO-Sham and hKO-ISO hearts exhibited reduced intact β1 integrin levels. MMP-2 protein levels were significantly higher, while TIMP-2 protein levels were lower in hKO-ISO hearts. MMP-9 protein levels were increased in WT-ISO, not in hKO hearts. In conclusion, ATM plays a protective role in cardiac remodeling in response to β-AR stimulation.

Keywords

ATM; Apoptosis; Cardiac remodeling; p53; β1 Integrin

Introduction

Ataxia telangiectasia (A–T) is an autosomal recessive disease where the ataxia telangiectasia mutated kinase (ATM) protein is missing or inactivated. Patients suffering from A-T show a high incidence of muscular and cerebellar degeneration, immune deficiency, lymphomas, and insulin resistance [1–3]. Additional features in individuals with a heterozygous phenotype include a high incidence of cancer predisposition and susceptibility to ischemic

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heart disease [4]. ATM is a multifunctional kinase regulating cellular repair following DNA damage by activation of downstream targets [5, 6]. Among these are cell cycle regulators; p53 and Chk2 [7]. ATM was initially thought to be localized in the nucleus, affecting only proliferating cells [8]. Evidence has shown that ATM is present within the cytoplasm of post-mitotic cells and plays a direct role in other disease phenotypes such as insulin resistance and glucose intolerance [9–12]. While ATM affects multiple downstream targets in response to cellular stress or damage, the decision to activate survival or apoptotic (cell suicide) mechanisms may depend on the cell type and extent of damage [6].

Catecholamines, such as norepinephrine, are released during myocardial ischemia and accumulate in the interstitial space of the heart [13, 14]. This accumulation of catecholamines may contribute to the ischemic heart disease and heart failure [15]. Acting via β-adrenergic receptor (β-AR), catecholamines (norepinephrine and iso-proterenol) are shown to increase apoptosis in cardiac myocytes in vitro and in vivo, and play a role in myocardial remodeling associated with increased cardiac fibrosis [16–21]. Cardiac myocyte apoptosis is recognized as an important determinant of structure and function of the myocardium [22–24]. Integrins play a crucial role in the regulation of cell growth, apoptosis and hypertrophy [25]. Cardiac myocytes predominantly express β1 integrins [26]. Our laboratory has provided evidence that β1 integrin signaling protects cardiac myocytes against β-AR-stimu-lated apoptosis in vitro [27, 28] and in vivo [20]. β-AR stimulation increases expression and activity of matrix metalloproteinase-2 (MMP-2) in cardiac myocytes [29] and MMP-2 may interfere with the survival signals induced by β1 integrin [28].

The increased susceptibility of A-T heterozygous patients to ischemic heart disease and the role of ATM in myocardial remodeling following β-AR stimulation have not been examined. Here, using cDNA array analysis of apoptosis-related genes, we identified that β-AR stimulation increases expression of ATM. Using wild-type (WT) and ATM heterozygous knockout mice, we report that ATM may play an important role in β-AR-stimulated myocardial remodeling with effects on ventricular function, apoptosis and fibrosis. To gain an insight into the mechanism by which ATM affects β-AR-stimulated myo-cardial remodeling, we measured phosphorylation of p53, and expression of p53, Bax, β1-integrins, MMP-2, MMP-9 and tissue inhibitors of MMPs (TIMPs). Our findings suggest that interplay between β1 integrin and MMP-2 plays an important role in myocyte apoptosis and myocardial remodeling during ATM deficiency.

Methods

Vertebrate animals

Heterozygous knockout (hKO) and wild-type (WT) ATM mice, purchased from the Jackson Laboratory, were of 129x black Swiss hybrid background. Genotyping was performed by polymerase chain reaction (PCR) using primers suggested by the Jackson Laboratory. The absence of both ATM alleles produces a lethal phenotype at ~2 months of age mainly due to thymic lymphomas [1, 30]. Therefore, experiments were carried out using hKO mice. 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.

Isoproterenol infusion

For functional studies, L-isoproterenol (ISO; 400 μg/kg/day) was infused in age-matched (4 months old) male and female WT and hKO mice by subcutaneous implantation of mini-osmotic pumps (Alzet) as described [20].

Cell isolation and culture

Adult rat ventricular myocytes (ARVM) were isolated as previously described [31].

RNA isolation and GEArray analysis

Total RNA was extracted from the left ventricles of sham and ISO-infused (7 days) mice using the RNAqueous-4PCR kit (Ambion, Inc., Austin, TX) according to the manufacturer's instructions. Two micrograms of RNA was used as a template to generate ^{32}P-dCTP-labeled cDNA probes according to the manufacturer's instructions (SuperArray Bioscience Corp., Frederick, MD). The cDNA probes were denatured and hybridized at 60°C with a GEArray membrane, containing 96 apoptosis-related genes. After washing, the membrane was incubated with a chemiluminescent substrate. The spots were digitized with ScanAlyze software, and signal intensities were normalized to ribosomal protein L13a (Rpl13a) using the GEarray analyzer program (SuperArray Bioscience Corp., Frederick, MD).

Cell treatment and RT-PCR

Adult rat ventricular myocytes (ARVMs), cultured for 24 h, were treated with ISO (10 μM) in the presence of ascorbic acid (100 μM) for 24 h. Total RNA (2 μg) was reverse transcribed using the M-MLV RT kit (Promega Corp., Madison, WI) according to the manufacturer's instructions. Primer sequences for RT-PCR amplification were as follows: ATM; 5′-GATCTGTGGGTGTTCCGACT-3′ and 5′-CTTTGGGTGCATTCTTGGTT-3′, GAPDH; 5′-CTC ATGACCACAGTCCATGC-3′ and 5′-TTCAGCTCTGGG ATGACCTT-3′. PCR products were analyzed by gel electrophoresis on a 2% agarose gel stained with ethidium bromide.

Echocardiography

Transthoracic two-dimensional M-mode echocardiography was performed using a Toshiba Aplio 80 Imaging System (Tochigi, Japan) equipped with a 12 MHz linear transducer. Mice were anesthetized using a mixture of isoflurane (1.5%) and oxygen (0.5 l/min). The body temperature was maintained at ~ 37°C using a heating pad. Measurements were averaged from nine different readings per mouse [20, 32]. Percent fractional shortening (%FS) and ejection fractions (EF%) were calculated [20]. All echocardiographic assessments and measurements were performed by the same investigator. A second person also performed measurements on a separate occasion using the same recordings with no significant differences in interobserver variability.

Morphometric analyses

Following ISO-infusion, animals were killed and hearts were arrested in diastole using KCl (30 mmol/l) followed by perfusion fixation with 10% buffered formalin. Cross sections (4 μm thick) were stained with Masson's trichrome for the measurement of fibrosis using Bioquant image analysis software (Nashville, TN).

Apoptosis

To detect apoptosis, TUNEL staining was carried out on 4-μm thick sections as per manufacturer's instructions (cell death detection assay kit, Roche). To identify apoptosis associated with cardiac myocytes, the sections were immunostained using α-sarcomeric

actin antibodies (1:50; 5C5 clone, Sigma). Hoechst 33258 staining was used to determine the total number of nuclei. Apoptosis was calculated as the percentage of apoptotic cardiac myocyte nuclei/total number of nuclei.

Western blot analysis

LV lysates were prepared in RIPA buffer as previously described [20]. Protein lysates (75 μg) were separated by SDS-PAGE (10%) and transferred to a PVDF membrane (240 mA, 2.5 h). The membranes were incubated with antibodies against p53, p-p53 (serine-15; Cell Signaling), Bax (Santa Cruz), β1 integrin (Transduction Lab.), MMP-9 and MMP-2 (Millipore), TIMP-2 and TIMP-4 (Chemicon). The immune complexes were detected using chemilumi-nescence reagents (Pierce Biotech.). Membranes were stripped and probed with GAPDH (Santa Cruz) as a protein loading control. Band intensities were quantified using Kodak photodocumentation system (Eastman Kodak Co.).

Statistical analyses

Data are represented as mean ± SEM. Data were analyzed using student's *t* test or one-way analysis of variance (ANOVA) and a post hoc Tukey's test. Probability values of <0.05 were considered to be significant.

Results

β-AR stimulation increases ATM expression

GEArray analysis of apoptosis-related genes indicated that β-AR stimulation modulates expression of apoptosis-related genes, specifically increasing expression of ATM and BNIP-3. This analysis showed decreased expression of Bcl2 following β-AR stimulation (Fig. 1a, b). RT-PCR analyses of total RNA indicated ~2.5 fold increase in ATM mRNA following β-AR stimulation (Fig. 1c). Involvement of BNIP-3 in cardiac myocyte apoptosis and myocardial remodeling has previously been examined [33–36]. However, the role of ATM in cardiac myocyte apoptosis and myocardial remodeling has not been previously investigated. Therefore, we investigated the role of ATM in β-AR-stimulated cardiac myocyte apoptosis and myocardial remodeling.

Morphometric studies

There was no significant difference in the body weights before or 28 days after ISO-infusion between WT and hKO groups. Heart weight (HW) and HW to body weight (BW) ratios were increased in both ISO-infused groups ($P < 0.05$; Table 1) with no significant difference between the two ISO groups.

Echocardiographic studies

No differences in the echocardiographic parameters were observed between baseline ($n = 8$–9) and sham ($n = 4$) groups at any time-point. Therefore, they were grouped as sham to obtain a larger sample size. There was no difference in %FS and EF% between the two sham groups. Percent FS and EF% were increased in both ISO groups when compared to the respective sham groups. However, the increase in %FS and EF% was significantly lower in the hKO-ISO group when compared to WT-ISO ($^{*}P<0.05$ versus WT-sham and hKO-sham; $^{\#}P<0.005$ versus WT-ISO; Fig. 2). There was no difference in posterior and septal wall thicknesses between the genotypes following ISO-infusion (data not shown).

Fibrosis and apoptosis

Quantitative analysis of fibrosis using trichrome-stained sections revealed increased fibrosis in hKO-sham mice versus WT-sham ($^{\$}P < 0.01$ versus WT-sham). ISO-infusion

significantly increased the amount of fibrosis in both groups. However, the increase in fibrosis was greater in hKO-ISO group when compared to WT-ISO (*$P < 0.001$ versus WT-sham and hKO-sham; $^{\#}P < 0.01$ versus WT-ISO; Fig. 3a).

TUNEL-staining assay revealed increased cardiac myo-cyte apoptosis in the hKO-sham versus WT-sham group ($^{\$}P < 0.05$ versus WT-sham). ISO-infusion increased the number of apoptotic cardiac myocytes in both groups. However, the increase in apoptotic cardiac myocytes was significantly higher in the hKO-ISO group when compared to WT-ISO (*$P < 0.01$ versus WT-sham and hKO-sham; $^{\#}P < 0.05$ versus WT-ISO; Fig. 3b).

Phosphorylation of p53 and expression of p53 and Bax

The tumor suppressor protein p53 is phosphorylated by a number of protein kinases in response to stress stimuli. Following DNA damage, ATM phosphorylates p53 on serine-15 resulting in activation of downstream apoptotic signaling pathways [37, 38]. We therefore examined expression and phosphorylation of p53 in response to β-AR stimulation in WT and hKO hearts. Western blot analysis of LV lysates using anti-p53 antibodies showed no immunostaining for p53 in WT-sham or hKO-sham groups. ISO-infusion increased levels of p53 protein with no significant difference between the two ISO groups (Fig. 4a). Likewise, ISO-infusion increased phosphorylation of p53 (serine-15) to a similar extent in both groups (Fig. 4a). p53 is shown to transcriptionally increase expression of a pro-apoptotic protein, Bax [39]. Western blot analysis of LV lysates using anti-Bax antibodies showed that ISO-infusion increases Bax expression to a similar extent in WT and hKO hearts (Fig. 4b).

Expression of β1 integrin, MMPs, and TIMPs

Recently, our laboratory has shown that stimulation of β1 integrin signaling protects cardiac myocytes against β-AR-stimulated apoptosis in vitro and in vivo [20, 28, 40]. Western blot analysis of LV lysates using anti-β1 integrin antibodies showed a significant reduction in the levels of intact β1 integrin protein in hKO-sham versus WT-sham (Fig. 5a). ISO-infusion had no effect on the levels of intact β1 integrin and β1 integrin levels remained lower in hKO-ISO versus WT-ISO hearts. Interestingly, appearance of a β1 integrin immunoreactive band with an apparent molecular weight of ~55 kDa was observed in both ISO-infused groups. The intensity of this ~55 kDa fragment was significantly higher in hKO-ISO group ($^{\$}P < 0.05$ versus WT-sham; $^{\#}P < 0.05$ versus WT-ISO, $n = 4$ Fig. 5). Monoclonal antibodies raised against the extracellular domain of β1 integrin were used for western blot analysis. Therefore, the ~55 kDa band most likely represents the previously identified extracellular domain of β1 integrin [41].

Activation of MMP-2 may play a role in the fragmentation of β1 integrin [29]. Therefore, we next analyzed expression of MMP-2 and MMP-9 by western blot. ISO-infusion increased MMP-2 protein levels in both groups. However, the increase in MMP-2 protein was significantly higher in the hKO-ISO group (Fig. 6a). MMP-9 protein levels were increased only in the WT-ISO, not in hKO-ISO group (Fig. 6a).

TIMP-2 is suggested to inhibit MMP-2 activity [42], while TIMP-4 is predominantly expressed in the heart [43]. Western blot analysis showed increased TIMP-2 protein levels in hKO-sham when compared to WT-sham. ISO-infusion decreased TIMP-2 protein levels in the hKO, but not in the WT group (Fig. 6b). ISO-infusion and/or ATM deficiency had no effect on TIMP-4 protein levels (Fig. 6b).

Discussion

Ataxia telangiectasia mutated kinase (ATM) has been linked to various signaling pathways regulating multiple diseases which include diabetes [9, 44] and metabolic syndrome with

effects on atherosclerosis [45]. This is the first study to suggest that β-AR stimulation increases expression of ATM, and ATM plays a crucial role in β-AR-stimulated myocardial remodeling with effects on cardiac myocyte apoptosis and left ventricular fibrosis and function. ATM deficiency induces remodeling of the heart at basal levels with increased myocyte apoptosis and myo-cardial fibrosis. β-AR stimulation induced cardiac hypertrophy and increased %FS and EF% in both groups. However, the increase in %FS and EF% was lower in ATM deficient mice. β-AR stimulation increased cardiac myo-cyte apoptosis and fibrosis in both WT and ATM deficient mice. However, the increase in fibrosis and apoptosis was higher in ATM deficient mice. ATM deficiency had no effect on β-AR-stimulated increases in the expression and phosphorylation of apoptosis-related proteins (p-p53, p53, and Bax), while it negatively affected expression of β1 integrin and other extracellular matrix proteins.

In cells of non-cardiac origin, expression of ATM is shown to be regulated by radiation and growth factors [46, 47]. The ATM promoter has a number of potentially important *cis*-regulatory sequences, including a modified AP-1 site fat specific element (Fse) [48]. The Fse site is shown to bind Fos-Jun complexes, making it an alternate binding site for the AP-1 transcription factor [49]. The transcription factor AP-1 is formed by dimerization of different members of Fos and Jun protein family members. The expression of Fos and Jun is mainly regulated via the activation of ERK1/2 and JNKs, respectively. Here, we show for the first time that adult cardiac myocytes express ATM at basal levels. Stimulation of β-AR increases expression of ATM in vivo and in vitro. Previously, β-AR stimulation is shown to activate ERK1/2 and JNKs [31]. Therefore, it is likely that β-AR stimulation increases expression of ATM via the involvement of ERK1/2 and JNKs.

Ventricular hypertrophy is an important compensatory mechanism that allows the heart to maintain its output. HW/ BW ratio, a measure of hypertrophy was increased to a similar extent in both groups in response to β-AR stimulation. However, results from M-mode echocardiography revealed a decrease in %FS and EF% in ATM deficient mice in response to β-AR stimulation, suggesting compromised myocardial performance of these mice after chronic ISO stimulation. Cardiac myocyte loss due to apoptosis plays an important role in the pathogenesis of the heart [22–24]. Stimulation of β-AR increases apoptosis in cardiac myocytes in vitro and in vivo, and myocardial remodeling associated with increased cardiac fibrosis [16–19, 50]. Increased fibrosis is suggested to poorly affect myocardial performance by decreasing the elastic recoil of the heart [51]. ATM deficiency resulted in increased cardiac myocyte apoptosis at basal levels and 28 days after ISO-infusion. Basal fibrosis was higher in ATM deficient mice, and was exaggerated following chronic β-AR stimulation. Since changes in cardiac function are not an isolated event, it is possible that the greater amounts of myocardial fibrosis and cardiac myocyte apoptosis in ATM deficient mice (Fig. 3a, b) may account for the observed %FS and EF% in these mice after chronic β-AR stimulation.

The p53 tumor suppressor gene plays a key role in cell death through transcriptional regulation of downstream apoptotic targets [37, 52]. In response to DNA damage, ATM phosphorylates p53 on serine-15 resulting in stabilization of p53 [53]. Stabilization of p53 may increase the transcriptional activity of p53, ultimately leading to apoptosis [54]. Consistent with these observations, we observed increased protein levels of p53 and its phosphorylation in response ISO in the WT group with no effect in ATM deficient hearts. Downstream effects of p53 include activation of pro-apoptotic factors such as the mitochondrial protein Bax [39]. β-AR stimulation increased Bax expression in WT and hKO hearts to a similar extent. It is possible that one copy of the ATM gene is sufficient to activate p53-dependent apoptotic signaling in response to β-AR stimulation.

Previously our laboratory has provided evidence that β1 integrins play a protective role in β-AR-stimulated apop-tosis (in vitro and in vivo), and MMP-2 interferes with the β1 integrin-mediated survival signals [20, 28, 55]. Evidence has also been provided that purified TIMP-2 inhibits β-AR-stimulated apoptosis in cardiac myocytes [29]. TIMP-2 is suggested to inhibit MMP-2 activity [42]. The data presented here show decreased β1 integrin levels in ATM deficient hearts at basal levels. The levels of intact β1 integrin remained lower in ATM deficient hearts in response to ISO-infusion. Interestingly, the levels of a 55 kDa protein, a likely extracellular fragment of β1 integrin, were greatly increased in ATM deficient hearts. ATM deficient hearts also exhibited increased MMP-2 expression and decreased TIMP-2 expression. Taken together, these observations suggest (1) decreased β1 integrin expression in ATM deficient hearts; and (2) shedding of the extracellular domain of β1 integrin, possibly via the activation of MMP-2. Decreased β1 integrin expression and/or shedding of β1 integrins may ultimately affect the anti-apoptotic signaling pathway induced by β1 integrin during β-AR stimulation in ATM deficient hearts. ATM may regulate expression of MMP-2 and β1 integrin via the modulation of activities of transcription factors such as AP-1 and NF-kB [56]. A functional AP-1 binding site in the promoter region of MMP-2 has been identified in rat cardiac cells [57]. In cardiac fibroblasts, activation of NF-κB plays an important role in the regulation of MMP-2 and MMP-9 expression and activity [58]. Activation of AP-1 and NF-κB may also modulate β1 integrin expression [59]. However, further studies are needed to examine the role of ATM in the regulation of MMP-2 and β1 integrin expression.

Cardiac fibrosis is promoted by extracellular matrix remodeling (ECM) and is a common and well recognized feature of heart failure (HF) [60]. MMP-9 and MMP-2 are suggested to play a major role in ECM remodeling by continued degradation of collagen leading to left ventricular dilation and pump dysfunction [61]. Here we observed no change in MMP-9 expression but an increase in MMP-2 expression following chronic ISO-infusion in hKO mice. The up regulation of MMP-2 in the hKO group correlates with increased amounts of fibrosis. Several reports have shown increased MMP-2 expression with myocardial remodeling and that inhibition of MMP's is shown to attenuate LV remodeling in several HF models [62–64]. Doxycyline, MMP inhibitor, is shown to attenuate ISO-induced myocardial fibrosis and MMP activity in rats [65]. Also expression of active MMP-2 induced severe ventricular remodeling with replacement fibrosis and systolic dysfunction with increased left ventricular volumes in the absence of injury [66]. Replacement fibrosis (formation of scar tissue) follows an inflammatory cell response that appears at the sites of cardiac myocyte necrosis preserves the structural integrity of the myocardium [67]. This replacement fibrosis is distinct from reactive fibrosis that surrounds intramyocardial coronary arteries and may extend into the contiguous interstitial space with time. Reactive fibrosis has been observed in hypertensive animal models [68]. Apoptosis usually does not associate with inflammatory response. Therefore, increased myfocardial fibrosis observed at basal levels in ATM deficient mice and following ISO-infusion (in WT and hKO mice) may be due to reactive fibrosis. However, the involvement of necrosis and/or apoptosis in this process cannot be ruled out.

Ataxia telangiectasia mutated kinase (ATM) is a key regulator of multiple signaling pathways in response to DNA damage. The data presented here suggest that ATM has the potential to play a crucial role in β-AR-stimulated left ventricular dysfunction with effects on cardiac myo-cyte apoptosis and fibrosis. It is interesting to note that deficiency of ATM associates with decreased cardiac function and increased cardiac myocyte apoptosis. The data presented here suggest that reduced levels of intact β1 integrins with increased MMP-2 may enhance cardiac myocyte apoptosis and myocardial dysfunction during ATM deficiency. Investigation of signaling pathways that can shift the balance from cell survival

to apoptosis during chronic β-adrenergic stimulation may have important clinical implications.

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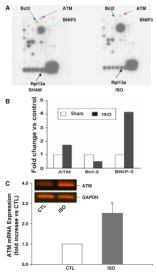

Fig. 1.

β-AR stimulation increases ATM expression. **a** Autoradiograms representing expression of apoptosis-related genes in sham and ISO-infused hearts. **b** Quantitative analysis of ATM, Bcl-2, and BNIP3 expression 7 days after ISO-infusion. The data normalized using ribosomal protein L13a (Rpl13a) gene expression as a control. **c** RT-PCR analyses. Total RNAs were reverse transcribed and resulting cDNAs were subjected to amplification of ATM and GAPDH genes. The *upper panel* depicts a 2% agarose gel of the RT-PCR. The *lower panel* exhibits the mean data from sham and ISO-treated animals normalized to GAPDH ($n = 3$, $^*P < 0.05$)

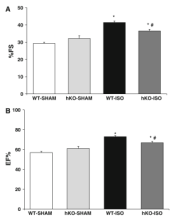

Fig. 2.

LV remodeling in ATM deficient mice following ISO-infusion. Echocardographic M-Mode measurements of WT and hKO mice 28 days following ISO-infusion. **a** Percent fractional shortening, %FS. **b** Ejection fraction, EF. Values are show as mean ± SE; (sham, $n = 12–13$; ISO, $n = 11$; $^{*}P < 0.05$ versus WT-sham and hKO-sham, $^{\#}P < 0.005$ versus WT-ISO)

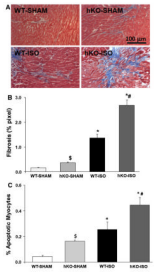

Fig. 3.

Myocardial Remodeling. **a**. Masson's trichrome-stained sections demonstrating fibrosis in WT and hKO mice 28 days after ISO-infusion. **b** Quantitative analysis of fibrosis 28 days after ISO-infusion. $^{\$}P < 0.01$ versus WT-sham, $n = 3–4$; $^{*}P < 0.001$ versus WT-sham and hKO-sham; $^{\#}P < 0.01$ versus WT-ISO; $n = 6–7$. **c** Quantitative analysis of TUNEL-stained myocytes 28 days after ISO-infusion. $^{\$}P < 0.05$ versus WT-sham; $^{*}P < 0.01$ versus WT-sham and hKO-sham, $n = 3–4$; $^{\#}P < 0.05$ versus WT-ISO; $n = 6–7$

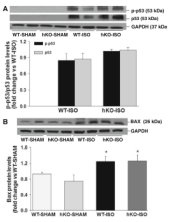

Fig. 4.

Expression of apoptosis related proteins 28 days after ISO-infusion. **a** Expression and phosphorylation of p53. **b** Expression of Bax. Total LV lysates (50 μg) were analyzed by western blot using anti-p53 and phospho-specific (serine-15) p53 antibodies or anti-Bax antibodies (*$P < 0.05$ versus respective shams). Protein loading in each lane is indicated by GAPDH immunostaining. The *lower panels* exhibit mean data normalized to GAPDH ($n = 4$)

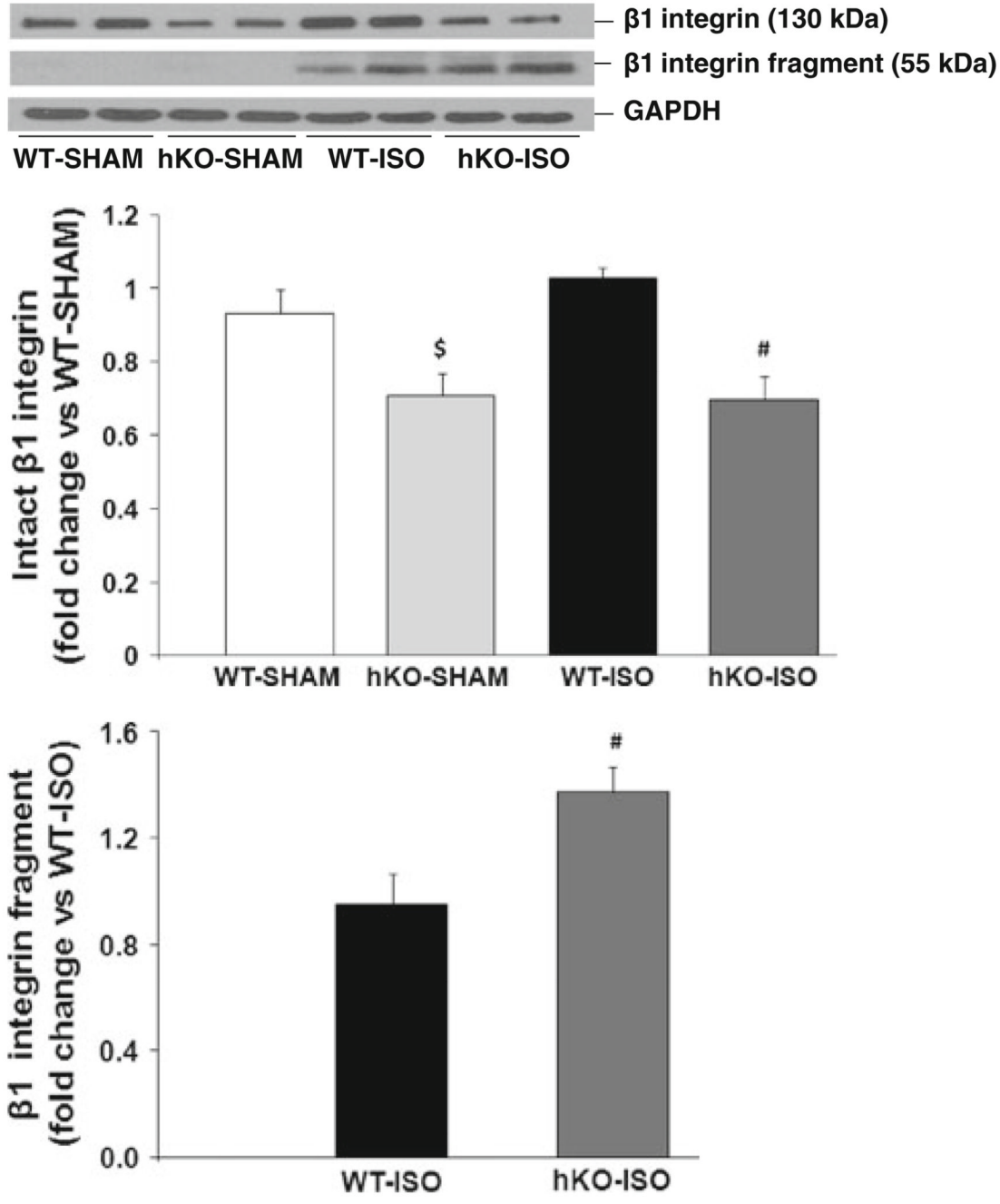

Fig. 5.

Expression of β1 integrin 28 days after ISO-infusion. Total LV lysates were analyzed by western blot using anti-β1 integrin antibodies. Autoradiograms indicating the intact 130 kDa β1 integrin and the 55 kDa β1 integrin (cytoplasmic) fragment. Protein loading in each lane is indicated by GAPDH immunostaining. The *middle panel* shows quantitative analysis of intact β1 integrin ($^{\$}P < 0.05$ versus WT-sham; $^{\#}P < 0.05$ versus WT-ISO, $n = 4$) normalized to GAPDH. The *lower panel* shows quantitative analysis of β1 integrin fragment ($^{\#}P < 0.005$ versus WT-ISO; $n = 4$) normalized to GAPDH and shown as fold increase versus WT-ISO

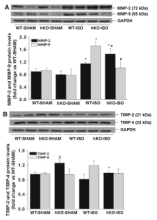

Fig. 6.

Expression of MMPs and TIMPs 28 days after ISO-infusion. **a** MMP-2 and MMP-9 expression. Total LV lysates were analyzed by western blot using anti-MMP-2 or anti-MMP-9 antibodies. The *upper panel* shows autoradiograms indicating immunostaining for MMP-2, MMP-9, and GAPDH. The *lower panel* shows quantitative analysis of MMP-2 (**P* < 0.05 versus WT-Sham and hKO-sham; $^{\#}P$ < 0.01 versus WT-ISO; *n* = 4) and MMP-9 (**P* < 0.05 versus WT-Sham and hKO-sham; $^{\#}P$ < 0.01 versus WT-ISO; *n* = 4) normalized to GAPDH. **b** TIMP-2 and TIMP-4 expression. Total LV lysates were analyzed by western blot using anti-TIMP-2 and TIMP-4 antibodies. The upper panel shows autoradiograms indicating immunostaining for TIMP-2, TIMP-4, and GAPDH. The lower panel shows quantitative analysis of TIMP-2 (**P* < 0.05 versus hKO-sham; $^{\$}P$ < 0.05 versus WT-sham; *n* = 4) and TIMP-4 protein levels (*P* = NS) normalized to GAPDH

Table 1

Morphometric measurements

	Sham (*n* = 6)		28 days (*n* = 9)		*P*
	WT-SHAM	**hKO-SHAM**	**WT-ISO**	**hKO-ISO**	
Body weight (g)	26.80 ± 1.32	31.10 ± 1.03	29.76 ± 0.54	29.09 ± 1.00	
Heart weight (mg)	133.40 ± 8.36	141.20 ± 15.22	180.19 ± 6.17*	170.35 ± 7.68*	*<0.05
HW/BW ratio (mg/g)	4.98 ± 0.18	4.56 ± 0.19	5.97 ± 0.19*	5.83 ± 0.26*	*<0.05

Values are mean ± SE;

*comparison between sham and ISO group