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Cardiomyocyte-GSK-3 α promotes mPTP opening and heart failure in mice with chronic pressure overload

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Abstract

Chronic pressure-overload (PO)- induced cardiomyopathy is one of the leading causes of left ventricular (LV) remodeling and heart failure. The role of the α isoform of glycogen synthase kinase-3 (GSK-3 α) in PO-induced cardiac remodeling is unclear and its downstream molecular targets are largely unknown. To investigate the potential roles of GSK-3 α , cardiomyocyte-specific GSK-3 α conditional knockout (cKO) and control mice underwent trans-aortic constriction (TAC) or sham surgeries. Cardiac function in the cKOs and littermate controls declined equally up to 2 weeks of TAC. At 4 week, cKO animals retained concentric LV remodeling and showed significantly less decline in contractile function both at systole and diastole, vs. controls which remained same until the end of the study (6 wk). Histological analysis confirmed preservation of LV chamber and protection against TAC-induced cellular hypertrophy in the cKO. Consistent with attenuated hypertrophy, significantly lower level of cardiomyocyte apoptosis was observed in the cKO. Mechanistically, GSK-3 α was found to regulate mitochondrial permeability transition pore (mPTP) opening and GSK-3 α -deficient mitochondria showed delayed mPTP opening in response to Ca²⁺ overload. Consistently, overexpression of GSK-3 α in cardiomyocytes resulted in elevated Bax expression, increased apoptosis, as well as a reduction of maximum respiration capacity and cell viability.

Taken together, we show for the first time that GSK-3 α regulates mPTP opening under pathological conditions, likely through Bax overexpression. Genetic ablation of cardiomyocyte GSK-3 α protects against chronic PO-induced cardiomyopathy and adverse LV remodeling, and preserves contractile function. Selective inhibition of GSK-3 α using isoform-specific inhibitors could be a viable therapeutic strategy to limit PO-induced heart failure.

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Keywords

GSK-3 α signaling; Mitochondria; Bax; mPTP; Cardiomyopathy; Ventricular Remodeling; Heart Failure

Introduction

Increased cardiac pressure overload (PO), due to hypertension and/or aortic stenosis, often leads to hypertrophic cardiomyopathy, left ventricular dysfunction and heart failure [1, 2]. Pathological insult leads to cardiac maladaptation and induces the heart to undergo morphological changes and hypertrophic enlargement [3]. Cardiomyocyte hypertrophy is a complex process and characterized by changes that include increased synthesis of sarcomeric proteins, as well as altered fetal gene expression and metabolic remodeling [4]. Progressive heart failure due to hypertrophy often involves cardiomyopathy which is usually accompanied by extracellular matrix deposition and interstitial fibrosis [5]. For more than two decades the mechanisms underlying cardiac hypertrophy have been the focus of intense investigation though it is still not well defined how hypertrophy contributes to these phenotypic manifestations of heart failure.

Glycogen synthase kinase-3 (GSK-3), a Ser/Thr kinase, has been extensively studied and reported to be involved in many human pathological conditions including different types of cancer, neurological, inflammatory, metabolic, and cardiovascular disorders [6, 7]. Unlike most protein kinases, GSK-3 is largely active in resting cells and becomes inhibited following certain normal or pathological stimuli [8, 9]. GSK-3 isoforms have been reported to regulate a number of cellular processes including cell proliferation, differentiation and apoptosis [7]. GSK-3 has two highly similar (~97% homology within their kinase domain) isoforms, α and β , the activity of which is primarily regulated through phosphorylation at Ser21 and Ser9, respectively. Both isoforms have selective as well as overlapping functions and one cannot completely compensate for the loss of other. Germline global deletion of GSK-3 β results in embryonic lethality [10]; however, global deletion of GSK-3 α results in viable animals [11] that develop age-related pathologies [12].

The β isoform has largely been the focus of the GSK-3 studies because of studies in *Drosophila* suggested that GSK-3 β was dominant due to its ability to rescue the loss of fruit fly orthologue, ZesteWhite3/Shaggy more efficiently. This resulted potentially from the trivial reason that GSK-3 α has a GC-rich 5' region and is not translated as well as GSK-3 β [13, 14]. Several studies have since explored and reported the role of GSK-3 β in the regulation of several cardiac pathological conditions [10, 15–19]. In contrast, potential roles of GSK-3 α are ill-defined, specifically in PO-induced cardiac remodeling and heart failure. Reports of global knock-out (KO) and knock-in (KI) mouse models showed opposing roles of GSK-3 α in pathological cardiac remodeling under conditions of PO [8, 20, 21]. Studies using constitutively active GSK-3 α KI mice have shown that inhibition of Ser21 phosphorylation (gain-of-function) promotes PO-induced cardiac hypertrophy, fibrosis and heart failure [8]. In contrast, a similar phenotype of increased cardiac hypertrophy and fibrosis was observed in GSK-3 α global KO mice post-PO [21]. Further, a different

phenotype of heart failure was described in a distinct study which employed cardiac-specific GSK-3 α transgenic mice [20] in which GSK-3 α overexpression limited PO-induced cardiac hypertrophy but was accompanied by elevated levels of cardiac fibrosis and apoptosis.

Accumulating data support a critical role for mitochondrial permeability transition pore (mPTP) opening in the modulation of cardiac cell apoptosis in a variety of cardiac pathological conditions [22–25]. Cellular stressors including elevated levels of mitochondrial matrix Ca²⁺, changes in intracellular pH, mitochondrial membrane potential or reactive oxygen species are known to modulate mPTP opening. Although several components of the mPTP complex and a variety of signaling pathways have been implicated in the regulation of the pore [22], the precise mechanism(s) controlling mPTP opening remain undefined. GSK3 β has been implicated in the regulation of mitochondrial dynamics [26, 27], however whether GSK-3 α similarly, or distinctly, regulates mPTP opening or mitochondrial bioenergetics is unknown.

In the present study, we employed cardiomyocyte-specific conditional KO mice to assess the impact of GSK-3 α on PO-mediated cardiac pathogenesis. We report that induced genetic loss of GSK-3 α in adult cardiomyocyte protects the heart from PO-induced adverse cardiac remodeling and hypertrophic cardiomyopathy. We observe, for the first time, that GSK-3 α regulates mPTP opening, most likely through up-regulating the Bcl-2-associated X protein (Bax), and modulates mitochondrial bioenergetics. Thus, selective inhibition of GSK-3 α represents a novel therapeutic strategy for preventing PO-induced maladaptive cardiac remodeling.

Methods

Mice

The generation and characterization of cardiomyocyte-specific conditional deletion of GSK-3 α model was previously described [9]. Briefly, the *GSK3A^{flox/flox} (fl/fl)* mouse [28] was crossed with α -myosin heavy chain (α -MHC) promoter-driven, tamoxifen (Tam)-inducible *Mer-Cre-Mer* mouse for two generations to generate *GSK3A^{fl/fl}Cre+/-* mice. Both *GSK3A^{fl/fl}* and *MerCreMer* animals strains were on C57BL/6 background. At 12 weeks of age, all male mice were treated with a tamoxifen chow diet (400mg/kg) for 15 days. *GSK3A^{fl/fl}Cre+/-* /*Tam* mice were denoted as conditional knockout (cKO), whereas littermates *GSK3A^{fl/fl}Tam* littermates were used controls. The Institutional Animal Care and Use Committee of Vanderbilt University Medical Center approved all animal procedures and treatments.

Trans-aortic constriction

Trans-aortic constriction or sham surgeries were performed as described previously [29, 30]. Briefly, post-tamoxifen treatment, a group of cKO and littermate control mice were anesthetized through intraperitoneal injection of ketamine (50 mg/kg) and xylazine (2.5 mg/kg). The anesthetized mice were intubated, and an incision was made over the cervical midline to expose the trachea. A blunt 20-gauge needle was connected to a ventilator and inserted into the trachea to supply oxygen at a rate of 1L/min. The aortic arch area was

cleaned and a 27-gauge needle was placed over it and constriction performed by tying the needle and aortic arch with a 7–0 nylon suture. The needle was quickly removed to generate a constriction of approximately 0.4 mm in diameter. The pressure gradient was measured in each mouse post-TAC through vevo2100 echocardiography (color Doppler imaging) to ensure that all the mice, including cKO and control groups, experience a similar range of pressure. Any mice found to have a loose knot and/or lesser aortic pressure were excluded from the study.

Echocardiography

Echocardiography was performed as described previously [11]. In brief, transthoracic two-dimensional motion mode-echocardiography was performed at 0, 2, 4 and 6 week post-TAC with a 12-mHz probe (visualSonic, Vevo 2100) on mice anesthetized by inhalation of isoflurane (1.5%). LV end-systolic interior dimension (LVID;s), end-diastolic interior dimension (LVID;d), ejection fraction (EF) and fractional shortening (FS) values were analyzed using the Vevo2100 program. The pressure gradient was measured in mice post-TAC, through vevo2100 color Doppler imaging.

Histochemistry

Histochemistry was performed using a protocol described previously [9, 11]. Briefly, after 6 weeks of TAC or sham surgeries, whole heart was excised from anesthetized mice and fixed in 4% paraformaldehyde, dehydrated through increasing concentrations of ethanol, and then embedded in paraffin. Heart sections (5µm) were stained using Masson's trichrome kit (Sigma-Aldrich# HT15–1KT). A 20X objective of an Nikon Eclipse 80i microscope was used to capture five images from the LV region of heart sections and images were analyzed using NIS Elements software.

Cardiomyocyte circumference measurement

Post-6 week TAC heart sections were subjected to Masson's trichrome staining. Imaging was performed using a 20X objective and 5 images/sections were taken from the LV region. Cardiomyocyte cross-sectional area was measured using NIS Element software. Only myocytes that were round (i.e., were cut in cross section) were included in the analysis. Circumference measurement and analysis were performed in a blinded manner.

Determination of myocardial apoptosis

The level of apoptosis in post-TAC hearts was assessed using in situ Cell Death Detection kit, TMR red (Roche# 12156792910) through terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL). Post-TAC heart sections were treated according to the manufacturer's instructions and co-stained for the cardiomyocyte-specific marker α -actinin (Sigma# A7811, 1:200 dilution) and 4,6-diamidino-2-phenylindole (DAPI). Slides were treated with mounting media and covered with a glass cover. A Nikon Eclipse 80i fluorescence microscope was used to visualize TUNEL-positive cardiomyocytes, and NIS Elements software was used to capture the images. Quantification of TUNEL-positive cardiomyocytes was done by taking 5 images (using 20X objective) from the LV region of each longitudinal heart section. The percentage of TUNEL-positive

cardiomyocytes was calculated by quantifying the total number of cardiomyocytes present in a 20X area. All imaging and quantification was done in a blinded manner.

Sample preparation and Immunoblotting

LV tissue was homogenized in 1X lysis buffer (Cell Signaling# 9803) with a protease and phosphatase inhibitor cocktail. After homogenization, samples were centrifuged at 15,000 *g* for 15 minutes at 4°C and supernatant transferred to fresh tubes. Protein concentration in the supernatant was quantified with the bicinchoninic acid protein assay (Pierce# 23225). Equal amounts of proteins were subjected to SDS-PAGE and subsequently were transferred to nitrocellulose membranes. A primary antibody incubations was performed at 1:1,000 dilution for anti-GSK-3 α (Cell Signaling# 5676), phospho-GSK-3 α (Cell Signaling #9316), Bax (Cell Signaling #2772), Bcl-xL (Cell Signaling #2764), VDAC (Cell Signaling #4661), 1:100 for Cyclin E (Santa Cruz #sc-481) and phospho-Cyclin E1 (Santa Cruz sc-12917-R) and 1:10,000 for GAPDH (Fitzgerald #10R-G109a). All incubations were done at 4°C, overnight. The secondary antibody used was Alexa Fluor 680 (Molecular Probes), at 1:3,000 dilution for 1 hour at room temperature. Membranes were scanned with the Odyssey Infrared Imaging System (LI-COR).

Mitochondria isolation from mouse heart and mitochondrial Ca²⁺ uptake assay

Mitochondria were isolated from GSK-3 α global KO and control mice hearts as described previously [31, 32]. The batches of 8–10 week old littermate mice were euthanized through CO₂ asphyxiation and heart tissues were quickly excised. The tissues were placed in ice-cold buffer A (225mmol/L Mannitol, 70mmol/L Sucrose, 1mmol/L EGTA, 10mmol/L HEPES, pH 7.4) and washed to remove residual blood. The aorta and other connective tissue were removed and the ventricular tissues were chopped into ~2–5 mm size pieces and placed in a beaker containing buffer A with 5mg proteinase A (5mg/10ml in buffer A). The beaker was placed on stirrer with stirrer bar set at 400rpm for 8 minutes to digest the tissue and then BSA added to 0.02g/mL final concentration to quench the proteolytic reaction. The tissue was homogenized using a Teflon-Potter disruptor at 800rpm on ice. The suspension was transferred to an ice-cold centrifuge tube and spun at 1000g for 4 minutes at 4°C. The supernatant was filtered using a 100 μ m nylon membrane filter and the pellet homogenized again in Buffer A. The suspension was centrifuged at 1000g for 4 minutes at 4°C and filtered through a 100 μ mesh. The suspension (containing crude mitochondria) obtained from both filtrations was combined and centrifuged at 10733g for 10 minutes at 4°C. The resultant pellet was resuspended in buffer B (225mmol/L Mannitol, 70mmol/L Sucrose, 10mmol/L HEPES, pH 7.2) and centrifuged again at for 10 minutes at 4°C. The resultant pellet was resuspended in 400 μ l buffer B. The protein levels in mitochondrial suspension were quantified using the Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific Inc. USA). Equal mitochondrial protein were used to perform each Ca²⁺ uptake assay at room temperature. To perform the assay respiration buffer (70mmol/L Mannitol, 25mmol/L Sucrose, 20mmol/L HEPES, 120mmol/L KCl, 5mmol/L KH₂PO₄, 0.5mmol/L EGTA, L-Glut acid 5mmol/L, Malic acid 5mmol/L) containing 0.1 μ mol/L Calcium Green (Invitrogen) was added to a quartz cuvette and the baseline set to zero. Mitochondria were added at 0.73mg/mL and then 36 μ mol/L of free calcium was added every 2 min. Measurements (excitation 503nm/ emission 535nm) were obtained using a fluorimeter (Delta RAM, PTI).

Neonatal rat cardiomyocyte isolation

The neonatal rat cardiomyocytes (NRCMs) isolation and culture were performed as described previously [33]. Briefly, 1–2 day-old Sprague-Dawley rat pups were purchased from Charles River (Connecticut, USA). Pups were rinsed quickly with 75% ethanol for surface sterilization and decapitated using sterile scissors. The chest was opened along the sternum to allow access to the chest cavity and heart. Hearts were harvested and transferred immediately to the dish containing a mixture of Hanks' Balanced Salt Solution (Life Tech 14185–052) with 25 mM HEPES, pH 7.4) without Ca^{2+} and Mg^{2+} , and with Collagenase-II (0.75mg/mL). Lung, atrial lobes and blood were removed to clean the tissue. Ventricle tissues were transferred to another petri-dish containing fresh HBBS and Collagenase-II mixture and chopped using a sterile scalpel to facilitate digestion. The tissue suspension was transferred to a beaker and placed in a 37°C shaking water bath with closed lid and incubated for 5 minutes. The beaker was tilted for 2 minutes and supernatant was removed carefully without disturbing the pellet. Collagenase-II enzyme solution was added to the beaker and this process was repeated three times to digest the maximum amount of tissue. The supernatant collected from all four rounds was combined and centrifuged at 1000rpm for 5 minutes. The pellet was resuspended in fetal bovine serum (FBS) and centrifuged again at 1000rpm for 5 minutes. Supernatant was discarded and the pellet resuspended in complete F-10 medium (GIBCO 11550–043). The cell suspension was strained through a 70µm filter and pre-plated for 1 hour. The cell suspension was removed from the plate and transferred to a new plate and cultured accordingly.

TUNEL assay *ex vivo*

NRCMs were plated on laminin-coated glass coverslips and infected with Ad-GSK-3α or Ad-LacZ at 100 MOI of. Medium was changed after 24 hours of infection and cells further incubated up to 72 hours. Staurosporine (0.5 µM) was used as a positive control. Post-treatment, staining for apoptotic cells was done by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL; kit from Roche #12156792910) according to manufacturer's instructions. Images were captured using Zeiss AxioPlan2 microscope with AxioVision software. The number of apoptotic cells was counted with ImageJ software.

Cell viability assay

Isolated NRCMs were seeded in 96 well plates at 40,000 cells per well in F-10 Ham (Gibco) supplemented with 10% FBS, 2 mmol/L L-glutamine, and 1% penicillin—streptomycin, allowed to adhere overnight and then incubated for 24 hours in serumfree media. The following day cells were infected with 100 MOI of Ad-GSK-3α or Ad-LacZ. Post 24 hours of infection, the medium was changed and cells were further incubated up to 72 hours. Staurosporine (0.5 µM) was employed as a positive control. Cell viability was determined using the Cell Titer-Glo 2.0 assay kit (Promega #G9241) and the results were represented as background-subtracted relative luminescence normalized to untreated control for 5 independent studies.

Mitochondrial oxygen consumption rate

The mitochondrial oxygen consumption rate (OCR) was measured as described previously [34]. Briefly, isolated NRCMs were plated in poly-L-lysine coated 96 well Seahorse culture plates (Agilent Inc. USA). The cells were infected with 100 MOI of Ad-GSK-3 α or Ad-LacZ adenovirus for 72 hours. The OCR was measured at 37°C in an Seahorse XFe96 extracellular flux analyzer (Agilent Inc. USA) by sequentially exposing the cells to mitochondrial respiratory complex inhibitors oligomycin (1 μ M), Carbonyl cyanide-4-(trifluoromethoxy)phenylhydrazone (FCCP) (2 μ M), and rotenone (1 μ M) plus antimycin A (1 μ M). The OCR in cells were quantified and plotted as described earlier [35].

Statistics

Differences between data groups were evaluated for significance using One-way ANOVA followed by Turkey's post-hoc test for multiple comparisons and unpaired *t*-test for single comparison (Graph Pad Prism Software Inc., San Diego, CA). Data are expressed as mean $\pm$ SEM. For all tests, a *P* value <0.05 was considered for statistical significance.

Results

Chronic pressure overload inhibits GSK-3 α in the heart

We first evaluated whether pressure-overload itself modulated the activity of GSK-3 α in the heart. A group of control mice was subjected to TAC or sham surgery and heart tissue was harvested 2 and 3 weeks post-TAC. The LV lysates were prepared and the levels of GSK-3 α expressions and phosphorylation (on Ser21) were assessed through western blotting. The phosphorylation of GSK-3 α was comparable between sham and 2 wk post-TAC hearts, however a significantly elevated level of phosphorylation was identified in 3 wk post-TAC vs. sham hearts (Fig. 1A–B). The expression level of GSK-3 α post TAC was found unchanged (Fig. 1A, C). These results suggest that TAC ultimately leads to phosphorylation/inhibition of GSK-3 α and, attenuated GSK-3 α activity more likely required to prevent adverse cardiac remodeling in chronic pressure overload.

Cardiomyocyte GSK-3 α signaling aggravates adverse cardiac remodeling and dysfunction following TAC

The potential role of GSK-3 α in pressure overload-induced cardiac remodeling is unclear and reported studies from global KO and KI mice have shown contradictory results for GSK-3 α . Herein, to identify the specific roles of GSK-3 α in PO-induced cardiac remodeling and dysfunction, we employed inducible cKO mice which generated by crossing GSK-3 α floxed mice with MerCreMer (tamoxifen inducible α -MHC promoter-driven Cre) mice as described previously [9].

To assess the possible role of GSK-3 α in PO-induced adverse cardiac remodeling and dysfunction a group of GSK-3 α cKO and littermate control animals underwent sham or TAC surgery. The mortality was monitored for 6 weeks post-TAC which showed 100% survival in the sham operated group and a insignificantly higher mortality, specifically in the remodeling phase, was observed in the control vs. cKO group underwent TAC (Suppl Fig. 1). Cardiac function was assessed by serial motion mode (two-dimensional)

echocardiography at 2, 4 and 6 wks post-TAC. The ventricular chamber dilated equally in both cKO as well as littermate controls until 2 wks of TAC, however chamber dilatation, both in systole and diastole, was significantly less in the cKO vs. controls at 4 wk of TAC (Fig. 1D–E). Consistent with reduced remodeling and preserved LV interior dimension, a significantly preserved ejection fraction (EF) and fractional shortening (FS) was observed in the cKO mice at 4 wk of TAC (Fig. 1F–G). The observed protection in cardiac remodeling and contractile functions in the cKO persisted throughout the study (6 wks of TAC). These data suggest that GSK-3 α has a critical role in promoting cardiac remodeling and dysfunction under pathological conditions such as chronic pressure overload.

Cardiomyocyte-specific loss of GSK-3 α mitigates pressure overload-induced cardiac hypertrophy and heart failure

Chronic PO leads to pathological hypertrophy of the cardiac muscle, and often leads to heart failure. To investigate if GSK-3 α regulates PO-induced cardiac hypertrophy and heart failure, 6 wks post-TAC heart and lung tissues were harvested from anesthetized mice and morphometric analysis was performed at 6 weeks post-TAC. Heart weight (HW) to tibial length (TL) and lung weight (LW) to TL ratios were measured. The HW/TL ratio was significantly lower in the cKO mice vs. controls which suggests attenuated cardiac hypertrophy in the cKO (Fig. 2A). Consistent with this, a lower LW/TL ratio in the cKO vs. control mice demonstrated significant protection against heart failure (Fig. 2B). Moreover, histological assessment of the heart tissues showed a thicker LV wall with dilated chambers in the control which was not seen in the cKO (Fig. 2C). Irrespective of genotype, circumference measurements indicated a larger size of cardiomyocytes post-TAC vs. sham. However, a significantly smaller size of cardiomyocytes were observed in the cKO in comparison to control (Fig. 2D), indicating an attenuated cellular hypertrophy. These observations further confirm the attenuated cardiac hypertrophy in the cKO hearts post-TAC. Taken together, these findings suggest that GSK-3 α potentiates PO-mediated adverse cardiac hypertrophy and remodeling and that deletion of GSK-3 α in the cardiac myocyte protects against ventricular hypertrophy and adverse remodeling.

Cardiomyocyte-GSK-3 α deficiency attenuates hypertrophic cardiomyopathy and myocardial fibrosis post-TAC

Chronic pressure overload leads to adverse cardiac remodeling which is often accompanied by cardiomyopathy and excessive cardiac fibrosis thus we investigated whether GSK-3 α plays a role in these processes. To probe a role in regulation of cardiomyopathy, we performed terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining along with α -actinin (a cardiac cell-specific marker) using 6-week post-TAC heart sections. Cardiomyocytes co-localizing with TUNEL and DAPI were quantified. Irrespective of genotype, a significantly increased number of TUNEL-positive cardiomyocytes were observed post-TAC. However, the number of apoptotic cardiomyocytes was significantly lower in hearts from cKO animals (Fig. 3A–B). To assess the level of fibrosis, we performed Massons' trichrome staining on 6 wk post-TAC cKO and control heart sections followed by quantification of the fibrotic area in the LV regions. Analysis revealed a significantly lower percentage of fibrillar collagen deposition (fibrotic area) in the cKO compared to control

hearts (Fig. 3C–D). These data support the notion that GSK-3 α promotes cardiomyocyte death and resulting cardiac fibrosis in response to pressure overload.

GSK-3 α promotes mPTP opening and remodels mitochondrial bioenergetics in heart

Emerging evidence suggests that mPTP opening regulates cardiac cell death not only in ischemic stress but also in other cardiac pathologies including arrhythmias, pressure overload and even in age-related pathologies [23, 36, 37]. Chronic PO-induced pathological hypertrophy and adverse LV remodeling is associated with gradual loss of cardiomyocytes. It is well documented that dysregulation of calcium handling occurs under such conditions [38, 39] and might lead to mitochondrial calcium overload and mPTP opening. Therefore, we sought to delineate the molecular mechanism that may account for the protective phenotype and to decipher the potential role of GSK-3 α in the modulation of mPTP opening. In this series of experiments, mitochondria were isolated from 8 week old GSK-3 α knockout hearts and were subjected to a Ca²⁺ retention capacity assay by challenging them with a Ca²⁺ pulse load. Our *in vivo* studies showed that GSK-3 α strongly regulates mPTP opening. Mitochondria from cardiomyocytes lacking GSK-3 α hold more Ca²⁺ as indicated by an increased number of Ca²⁺ uptake peaks and, showed delayed pore opening in comparison to control mitochondria (Fig. 4A). Quantification showed significantly increased levels of Ca²⁺ uptake by mitochondria from GSK-3 α deficient heart tissue (Fig. 4B).

Having observed the involvement of GSK-3 α in regulation of mPTP and mitochondrial Ca²⁺ retention, we sought to evaluate the role of GSK-3 α in mitochondrial bioenergetics. To this end, we adapted a gain-of-function model and employed an adenovirus-mediated overexpression system of GSK-3 α in primary NRCM (Fig. 4C). GSK-3 α overexpressing and control cells were subjected to oxygen consumption analysis using a Seahorse XF analyzer. Consistent with our mPTP data, overexpression of GSK-3 α resulted in reduction of maximum respiration capacity of the mitochondria (Fig. 4D–E), suggesting that mitochondria from gain-of-GSK-3 α function cell are not as functionally competent as their control counterparts and may thus be more susceptible to cell death. Next, we monitored ATP production in GSK-3 α overexpressing NRCM and found that total ATP production was not compromised in these cells (Suppl Fig. 2A). However, there was a shift to increased glycolysis-mediated ATP production and reduced OXPHOS ATP production (Suppl. Fig. 2b–C). These findings are consistent with the observed protective phenotypes in the cKOs and indicate that GSK-3 α acts to remodel mitochondrial bioenergetics which may influence cardiomyocyte survival.

GSK-3 α overexpression promotes apoptotic cell death and decreases cardiomyocyte viability

To further explore the role of GSK-3 α in promoting cardiomyocyte apoptosis and viability in an acute setting, NRCMs were treated with either wildtype Ad-GSK-3 α or control Ad-LacZ viruses. To assess the levels of cardiomyocyte death in these groups, TUNEL labelling was performed and positive cardiomyocytes were quantified. A separate population of NRCMs were treated with Staurosporin which served as a positive control. As expected, an elevated number of TUNEL-positive cardiomyocytes was observed in Staurosporine treated vs. control cells. Consistent with our *in vivo* studies from cKO animals, a significantly

increased number of apoptotic cardiomyocytes were observed in GSK-3 α overexpressing cells vs. cells infected with Ad-LacZ (Fig. 5A).

Furthermore, a CellTiter-Glo 2.0 assay was performed to assess the viability of cardiomyocyte overexpressing GSK-3 α . Similar to the TUNEL-labeling experiment, a group of NRCMs was treated with Staurosporine and included as a positive control. As expected, the fraction of viable cardiomyocytes was dramatically decreased in the Staurosporine treated vs. untreated control group. Overexpression of GSK-3 α significantly reduced cardiomyocyte viability when compared with either untreated or Ad-LacZ infected groups (Fig. 5B). These findings are consistent with the data that deletion of GSK-3 α is protective *in vivo*, and show that gain of GSK-3 α function exacerbates cardiomyocyte apoptosis and reduces viability.

GSK-3 α signaling regulates Bax expression but not mPTP components

Our data implicate GSK-3 α as a critical regulator of mitochondrial energetics, mPTP opening and cardiomyocyte apoptosis. Hence, we next evaluated downstream signaling pathways that are known to regulate mPTP opening and cell death. Previously, we have reported that deletion of GSK-3 α up-regulates cyclin E1 levels in the injured heart [8, 9]. Hence, as a positive control we tested whether over-expression of GSK-3 α in NRCMs acted to suppress cyclin E1. Consistent with our previous study, Cyclin E1 expression and phosphorylation levels were found to be significantly reduced upon overexpression of GSK-3 α in NRCMs (Suppl. Fig. 3A–C). Next, we assessed whether overexpression of GSK-3 α in cardiomyocyte modulates the anti-apoptotic B-cell lymphoma-extra large (Bcl-xL) and BCL2-associated X protein (Bax) ratio, a hallmark of apoptotic cell death. NRCMs were treated with Ad-GSK-3 α or Ad-LacZ and Bax and Bcl-xL protein levels assessed via immunoblotting. The Bax/Bcl-xL ratio was found to be significantly elevated in GSK-3 α overexpressing NRCMs vs. controls (Fig. 5C–D). We also evaluated whether GSK-3 α modulated the expression of major mitochondrial pore complex components including Cyclophilin D (CyP-D) and Voltage-dependent anion channel (VDAC). Our analysis revealed no change in expression of either component in response to GSK-3 α overexpression (Fig. 6A–C). Taken together, these findings show that GSK-3 α critically regulates Bax expression in the cardiomyocytes.

Discussion

In this study, we have determined the impact of cardiomyocyte-specific GSK-3 α in a clinically important PO model of heart failure. We identified that cardiomyocyte-specific deletion of GSK-3 α in adult mice limits PO-induced cardiac hypertrophy, cardiomyocyte apoptosis, fibrosis, adverse remodeling and attenuates functional deterioration of heart. Mechanistically, we have identified that GSK-3 α regulates mPTP opening in pathological (Ca²⁺ overload) conditions and modulates mitochondrial bioenergetics (Fig. 7).

Previous studies using global KO, KI and transgenic mice have shown a differential role of GSK-3 α in PO-mediated cardiac hypertrophy and adverse remodeling, and the role of GSK-3 α from these reports is unclear and contradictory [8, 20, 21]. Studies employing transgenic mice have shown that GSK-3 α negatively regulates cardiac growth [20]. These

findings revealed that cardiac-specific overexpression of GSK-3 α suppressed cardiac growth but potentiated cardiomyocyte apoptosis and fibrosis at baseline. Moreover, in response to TAC, overexpression of GSK-3 α in transgenic mouse model demonstrated less severe cardiac hypertrophy with markedly reduced cardiac function which was accompanied by enhanced apoptosis and fibrosis [20]. Interestingly, another study using constitutively active GSK-3 α KI mice revealed that inhibition of GSK-3 α phosphorylation promoted hypertrophy, fibrosis and heart failure post-TAC [8]. The protective phenotypes in our study using cKO mice are in complete agreement with the findings derived from the constitutively active GSK-3 α KI model [8]. However, our results are only partly consistent with the phenotypes observed in the transgenic model as no changes in cardiomyocyte apoptosis and fibrosis were seen at baseline in the cKO. This differential observation in the cKO vs. transgenic mice at baseline could be due to the differences in the models. For example, the transgenic model was germline, however the cKO model provided conditional gene deletion in cardiomyocyte specifically in the adult mice.

In contrast, animals with germline global deletion of GSK-3 α displayed detrimental cardiac phenotypes post-TAC, which included increased hypertrophy, cardiac dysfunction and fibrosis [21]. As with the TAC model, GSK-3 α global KO and cKO mice demonstrated opposing phenotypes in a myocardial infarction (MI) model as well, specifically, global KO was detrimental and cKO was protective, post-MI [9, 11]. Germline global gene deletion and transgenesis often has developmental, confounding and compensatory effects and is therefore not ideal to study the specific roles of a protein under pathological conditions [40]. Our study of the cKO model focusses on the specific role of GSK-3 α as a positive regulator of cardiac hypertrophy and cardiomyopathy post-TAC.

The β isoform of GSK-3 has also been reported to critically regulate cardiac hypertrophy under pathological conditions [6]. *In vitro* studies using isolated cardiomyocytes [41] and *in vivo* studies using a cardiac-specific, constitutively active GSK-3 β KI model [42] have shown that GSK-3 β negatively regulates cardiac hypertrophy under pathological stress. Surprisingly, cardiomyocyte-specific conditional KO animals displayed no role for GSK-3 β in the development of cardiac hypertrophy in response to PO [15]. Furthermore, our studies using cardiomyocyte- and cardiac fibroblast-specific KO mice model show an opposing role of GSK-3 β in ischemia-induced cardiac remodeling and dysfunction [15, 17]. Moreover, our recent report showed that suppressing both GSK-3 α/β in the heart leads to fatal dilated cardiomyopathy and fibrosis at baseline [43, 44]. Hence, targeting either GSK-3 β or both GSK-3 α/β is not feasible as chronic inhibition may induce cardiomyopathy, fibrotic remodeling and dysfunction. All currently available small molecule inhibitors of GSK-3 inhibit both isoforms equally [45] and, irrespective of clinical presentation, chronic use of these inhibitors might place patients at risk of cardiomyopathy and cardiac fibrosis. Consistent with the observation that targeting GSK-3 α is protective in TAC and MI (current study) [9, 11], our preliminary studies in cardiac fibroblast-specific GSK-3 α KO animals show that targeting GSK-3 α in the cardiac fibroblast is also protective, limits adverse cardiac remodeling and fibrosis and, ultimately, preserves cardiac function post-MI [46].

Mechanistically, we identified for the first time that GSK-3 α potentiates mPTP opening, which is a critical regulator of cardiomyocyte death during pathological conditions in which

Ca²⁺ dysregulation and overload occurs. The mPTP is crucial for the heart as mitochondria constitute a larger volume of an adult cardiomyocyte, primarily to support the myocardial high energy demand and Ca²⁺ handling [23]. Although Cyclophilin-D has been reported to be a critical component and regulator of mPTP [23], the molecular nature of mPTP and mechanism of pore opening is largely unknown. We observed that GSK-3 α over-expression does not regulate the mPTP components CycP-D and VDAC. These findings suggest that GSK-3 α may not have a direct effect on mPTP and most likely acts through an intermediate signaling molecule(s). Previous reports have shown that Bax interacts with mPTP components and promotes pore opening through inducing permeability of the outer mitochondrial membrane [47–49]. Studies have further shown that Bax-deficient mitochondria are resistant to mPTP opening and interestingly, this was reversed by reconstituting the Bax-deficient cells with apoptosis/oligomerization-impaired Bax mutants [47, 50]. Furthermore, loss of Bax was found to be protective against cardiac ischemia-reperfusion injury and no additional protection was observed when tested in a model of combined deficiency of Bax and CycP-D[47, 50].

Importantly, we previously showed that loss of GSK-3 α in cardiomyocytes leads to a decrease in Bax expression and cardiomyopathy post-MI [9]. Consistently, our current study shows that GSK-3 α deficiency leads to delayed mPTP opening and attenuated cardiomyopathy (Fig. 4A–B). Moreover, GSK-3 α overexpression in the cardiomyocyte promotes Bax expression and apoptotic cell death (Fig. 5A, C–D). These findings are consistent and strongly suggest that GSK-3 α likely modulates mPTP opening through upregulation of Bax.

In contrast to GSK-3 α , the role of GSK-3 β in cardiomyocyte death has been extensively explored [22, 50–53]. Studies utilizing kinase-dead GSK-3 β mutant mice have shown that inhibition of GSK-3 β is required for cell protection in post-conditioning. Another study using RNA silencing approaches in NRCM showed that suppression of GSK-3 β but not GSK-3 α , mediated mPTP protection signaling [24], a finding that is contradictory to our results. This difference may relate to the efficiencies of the approaches used to inactivate GSK-3 α . Recently, we reported that cardiomyocyte-specific loss of both GSK-3 α and GSK-3 β isoforms (double knockout, DKO) lead to fatal dilated cardiomyopathy due to cell cycle dysregulation and mitotic catastrophe [43]. However, surprisingly, morphological and physiological assessment of mitochondria from DKO hearts showed significantly increased oxygen consumption rates and mitochondrial membrane potential [44]. The improved mitochondrial functions in DKO are consistent with our findings that targeting GSK-3 α leads to beneficial effects through modulating the mPTP opening.

In summary, our studies reveal the specific roles of GSK-3 α in chronic PO- induced cardiac remodeling. We have shown that cardiomyocyte-specific loss of GSK-3 α attenuates cardiac hypertrophy, cardiomyopathy, myocardial fibrosis and LV remodeling, and attenuates cardiac contractile dysfunctions post-TAC. Moreover, we have demonstrated for the first time that GSK-3 α regulates mPTP opening. As mentioned above, irrespective of cardiac cell type, GSK-3 α inhibition is protective and, considering a recent report that showed the feasibility and efficacy of selectively targeting GSK-3 α with small molecule inhibitors [54,

55], we believe this represents an attractive novel treatment strategy to limit the PO-pathological cardiac remodeling and heart failure.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1; GSK-3α potentiates ventricular remodeling and cardiac dysfunction post-TAC. (A) Immunoblot images and (B) quantification show GSK-3α phosphorylation (inhibition) and (C) protein expression levels in sham and, 2 and 3 week post-TAC control hearts. Echocardiography was performed in controls and cKO at 0, 2, 4 and 6 weeks post TAC or sham surgeries. Left ventricle end-diastolic dimension (LVID;d) (D) and end-systolic dimension (LVID;s) (E) were comparable at 2 wks and significantly preserved in the cKO at 4 and 6 wks post-TAC. (F) LV ejection fraction (LVEF) and (G) LV fractional shortening (LVFS) were significantly better in the cKO at 4 and 6 wk post-TAC. Data presented as

mean $\pm$ SEM and *P* values shown are for the comparison between *GSK3A^{fl/fl}* vs. *GSK3A^{fl/flCre}* mice subjected to TAC. n=10–14 for TAC and n=4–5 for sham groups. TAC, trans-aortic constriction; ns, not significant; **p*<0.05; ***p*<0.005.

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Figure 2; GSK-3 α promotes pressure overload-induced cardiac hypertrophy and heart failure; Post-6 wks of TaC, cardiac tissues were harvested for morphometric analysis. **(A)** Heart weight (HW) to tibia length (TL) and **(B)** lung weight to tibia length ratios were comparable in the sham group. However, these ratios were significantly lower in cKO mice vs. controls subjected to TAC. n=6–9 for TAC and n=3 for sham groups. **(C)** Representative Masson's trichrome stained heart images show better preserved LV chamber in the cKOs vs. Controls, n=6–9. **(D)** Bar diagram shows increased cardiomyocyte (CM) circumference in the WT vs. cKO, n=7–12. ns; not significant; ***P<0.0001, **P<0.005, *P<0.05.

Figure 3; Cardiomyocyte-specific loss of GSK-3 α limits hypertrophic cardiomyopathy post-TAC; (A) Representative images of TUNEL positive (apoptotic) cardiomyocytes in cKO and littermate control hearts 6wks post-TAC. (B) Quantification shows comparable numbers of apoptotic cardiomyocytes in the sham group and significantly lower numbers of apoptotic cardiomyocytes were identified in the cKOs vs. WT subjected to TAC, n=6 each for TAC and n=4 each for sham groups. (C) Representative images of Masson's trichrome stained LV from 6 wk TAC cKO and control mice. Scale bar, 20 μ m. (D) Quantification of fibrotic areas showed comparable fibrosis in the sham group and significantly less fibrosis in the cKOs vs. controls subjected to TAC. n=7–12 for TAC and n=3 each for sham groups. ns, not significant; ***P<0.0001.

Figure 4. GSK-3α promotes mitochondrial permeability transition pore (mPTP) opening and reduces the mitochondrial respiration capacity.

Representative traces from three independent experiments show (A) delayed mPTP opening by mitochondria isolated from GSK-3α global KO vs control hearts. The arrow shows mPTP opening. (B) The bar diagram shows significantly increased Ca²⁺ uptake by mitochondria from cells lacking GSK-3α. n=5 each, ***P<0.0001. (C) Standardization of the adenovirus-based overexpression system for the GSK-3α. NRCMs were infected with increasing Multiplicity of Infection (MOI) of wildtype GSK-3α adenovirus for 72 hours and

subjected to immunoblotting to measure GSK-3 α expression levels. GSK-3 β and GAPDH serve as a protein loading controls. **(D)** Representative traces for oxygen consumption rate are from three independent experiments and show reduced mitochondrial maximal respiration in GSK-3 α overexpressing NRCMs. **(E)** Bar diagram shows a significant decrease in maximal respiration in GSK-3 α overexpressing cells, ***P<0.0001. Oligo, Oligomycin; Rot, Rotenone; AntA, Antimycin A; FCCP, carbonyl cyanide 4-(trifluoromethoxy)phenylhydrazone; OCR, oxygen consumption rate.

Figure 5. Gain of GSK-3α function promotes Bax expression, apoptosis, and decreases cardiomyocyte viability:

(A) NRCMs were treated with Ad-GSK-3α or Ad-LacZ and post-72 hours of treatment, cells were stained for apoptosis using TUNEL. A group of cardiomyocytes treated with Staurosporine was included as a positive control. A significantly higher percentage of TUNEL positive cardiomyocytes was observed in both Staurosporine and Ad-GSK-3α treated groups vs. controls. (B) Assay of cell viability revealed cardiomyocyte viability was significantly decreased in GSK-3α overexpressing or Staurosporine-treated groups vs. controls. (C) Representative immunoblot of GSK-3α and GSK-3β, Bax and Bcl-xL levels from three independent experiments. (D) Blot quantification shows an increased Bax/Bcl-xL ratio in GSK-3α overexpressing cardiomyocytes in comparison to controls. UT, untreated; SSPN, Staurosporine; ns; not significant; ****p<0.0001; ***p<0.001; **p<0.005.

Figure 6. GSK-3α signaling in regulation of mitochondrial pore components in cardiomyocytes: (A) Representative blots from three independent experiments show GSK-3α and GSK-3β, Cyclophilin D, and Voltage-dependent anion channel (VDAC) levels in Staurosporin, Ad-GSK-3α and Ad-LacZ treated NRCMs groups. (B) Quantification of immunoblots show no change in Cyclophilin D and (C) VDAC expression levels in ad-GSK-3α vs. ad-LacZ treated cells. UT, untreated; SSPN, Staurosporine, ns; not significant.

Figure 7. Schematic representation illustrating the regulation of Bax levels and mPTP opening by GSK-3α in cardiomyocytes:

Diagram shows increased Bax levels and mitochondrial permeability transition pore (mPTP) opening in the control heart. GSK-3α in cardiomyocytes limits Bax expression, mPTP opening and cardiac remodeling, and attenuates cardiac function deterioration in chronic pressure-overloaded cKO heart.