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Endoplasmic Reticulum Stress Sensor IRE1 alpha Preserves Function of the Stressed Myocardium

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Endoplasmic Reticulum Stress Sensor IRE1 alpha Preserves Function of the Stressed Myocardium

A dissertation submitted in partial satisfaction of the requirements for the degree Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology

By

DeAnna Lee Steiger

2013

ABSTRACT OF THE DISSERTATION

Endoplasmic Reticulum Stress Sensor IRE1 alpha Preserves Function of the Stressed Myocardium

By

DeAnna Lee Steiger
Doctor of Philosophy in Molecular, Cellular, and Integrative Physiology
University of California, Los Angeles, 2013
Professor Yibin Wang, Chair

Many diseases and insults to the heart disrupt homeostasis in the endoplasmic reticulum (ER) and cause ER Stress, leading to activation of the ER stress response, or Unfolded Protein Response (UPR) signaling pathway. The UPR from the endoplasmic reticulum is emerging to play a vital role in health and disease. The most ancient member of this signaling pathway, IRE1 alpha, has been reported to induce both protective UPR and apoptotic downstream signaling events in various tissues, but the role for IRE1 alpha in heart is unknown. We aimed to characterize the specific contribution of IRE1 alpha in heart in health and in response to stress.

We generated a mouse model with inducible, heart-specific IRE1 alpha overexpression in order to investigate a role for IRE1 alpha in heart under baseline and stressed conditions. We observed that IRE1 alpha did not induce a detrimental phenotype in the absence of stress. Moreover, IRE1 alpha overexpression preserved heart function in response to pressure overload. Adaptive UPR signaling was enhanced

while inflammatory and fetal gene program members were blunted. Also, IRE1 alpha activation and downstream signaling was transient in cardiac myocytes in vitro. Inflammatory cytokine expression was reduced following IRE1 alpha expression, recapitulating observations made in vivo.

IRE1 alpha induces adaptive, transient signaling in heart. We conclude that the UPR signaling repertoire includes unknown, heart-specific endogenous regulatory mechanisms. To our knowledge, this is the first report of a specific and protective role for IRE1 alpha in heart and provides new evidence for the integration of ER stress and inflammatory signaling.

The dissertation of DeAnna Lee Steiger is approved.

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2013

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I also wish to acknowledge Dr. Eric J. Steiger for his unwavering support and patience. Without my study buddy, this work would not have been possible. I also want to thank my dear Frederick, for sharing his sheer joy for life with Eric and me.

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DeAnna Lee Steiger

PROFILE

Business savvy scientist with solid comprehension of the Pharmaceutical and Healthcare Industries. High performance professional with advanced presentation and interpersonal skills. Team player with initiative, discipline, and consistency. Proven leader with a relentless focus on the clinical application of scientific discoveries.

EXPERTISE

Heart failure physiology and treatment, animal models of heart failure, molecular biology and pharmacology. Building relationships, communication of scientific data to diverse audiences, and building consensus across functional areas.

EDUCATION

NIGMS Pharmacology & Translational Biology 2007
University of California San Diego

- Pharmacology, animal models and imaging of heart failure and Cardiovascular disease

MS, Entomology and Applied Ecology 2006
University of Delaware

BS, Biology 2002
Pacific Lutheran University

PROFESSIONAL EXPERIENCE

Licensing Associate, External Research & Development, Amgen 2013-

- Evaluate licensing opportunities based on scientific rigor for medical pharmaceutical company
- Drive cross-functional review process
- Direct communications between internal and external stakeholders

Founder and Chair, Advancing Women in Science & Engineering (AWiSE) 2012-2013

- Identified an unmet need, formally proposed and received funding for a new program to develop women leaders in the biotech and pharmaceutical industries
- Established an advisory board and mentor network of key opinion leaders and high-level industry professionals
- Forged partnerships with key organizations to accelerate buy-in and maximize impact
- Worked within a team to organize a successful kickoff event with 90 attendees and to launch a mentoring program

Marketing & Industry Outreach Coordinator, UCLA Business of Science Center 2011-2012

- Wrote marketing summaries of emerging medical technologies, promoted licensing of university discoveries
- Established and maintained strategic partnerships with 25 biotech and pharmaceutical industry leaders
- Led outreach efforts, acted as point of contact for external stakeholders, increased event attendance by 200%

Student Consultant, UCLA Business of Science Center 2010-2013

- Assessed commercialization potential of 40 emerging medical technologies
- Identified intellectual property to license, target markets, and potential competitors for a biomedical device startup company
- Worked within multi-disciplinary team to generate specific recommendations for the company's business plan

TECHNICAL EXPERIENCE

Predoctoral Research Fellow, University of California Los Angeles 2007-2013

- Awarded NIH T32 Fellowship in Molecular, Cellular, and Integrative Physiology
- Awarded NIA F31 Fellowship for Novel Mechanisms of ER Stress Regulation in Aging Heart
- Designed and executed research projects, troubleshot and optimized experimental conditions, and led two researchers to identify cell signaling events protective against heart failure
- Employed cutting-edge imaging methods to advance translation of research findings into clinically relevant conclusions
- Secured multiple federal grant awards totaling more than $100,000
- Presented findings at seminars, American Heart Association national conferences, and in journals (total of 11 presentations, 1 abstract, and 1 manuscript)

Graduate Student Researcher, University of Delaware 2004-2006

- Designed, prototyped, and optimized a device to measure insect susceptibility against Bt insecticide

TEACHING EXPERIENCE

Teaching Assistant, University of California Los Angeles 2007-2008

- Taught endocrine physiology to 150 senior pre-medical undergraduate students

Assistant Language Teacher, Shimane Board of Education, Izumo City, Japan 2002-2004

- Collaborated with Japanese teachers of English to generate curriculum and teach 400 senior high school students

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CHAPTER 1

Introduction

A. Cardiovascular Disease and Heart Failure

Cardiovascular diseases are the number one cause of mortality in the United States and worldwide (1). Heart failure comprises 35% of cardiovascular diseases and is one of the most significant diseases facing people today. It affects more than five million people in the United States alone.

Heart failure is most simply defined as the disease that results from the heart being unable to pump sufficient blood to the body. Heart failure is a progressive disease with a spectrum of symptoms and pathologies including hypertrophy, compensated heart failure where the heart enlarges but is able to maintain function, and decompensated heart failure where the heart ventricle walls thin and function is diminished. Effective treatments remain elusive due to our limited understanding of the many causes, clinical manifestations, and underlying disease mechanisms. Advancing the understanding of underlying mechanisms will provide additional treatment strategies and novel therapeutic targets.

B. Intracellular Signaling Networks and Adaptation to Stress

The heart is a dynamic organ with constant mechanical activity that provides the body with a consistent supply of freshly oxygenated blood. The heart is constantly challenged by stresses. Insults from external stimuli, hormonal input, exercise, and

disease all stress the heart. Appropriate responses to stress stimuli occur as the result of cellular signaling activity. Intracellular signaling networks bring about structural and functional changes that allow the heart to adapt to various stresses and meet increasing demands. Challenges and injuries cause a wide variety of cellular disruptions, ranging from calcium dysregulation, changes in redox status, alteration of nutrient requirements, mechanical stress, and increased hormonal input (2). Many of these insults disrupt homeostasis in the endoplasmic reticulum (ER), causing a state termed ER stress. ER stress is increasingly being appreciated as a critical factor underlying heart function and failure (3).

Protein maturation and integrity are critical factors affecting cellular signaling which are now gaining the attention of researchers and clinicians alike. Precise cellular signaling communication requires structural integrity of signaling molecules. Protein function is highly dependent upon protein structure. Protein structure is achieved by complex folding arrangements and conformations. Thus, misfolded proteins may not function correctly and take on new activities. If cellular signaling molecules take on rogue activity, the resultant cellular and tissue outcomes can have drastic implications for health and disease (4). Therefore, preceise protein folding and maturation are critical for maintaining correct cellular signaling integrity. During ER stress, protein maturation is disrupted, causing activation of cell signaling pathways to restore ER homeostasis.

C. Intracellular Signaling Maintains Protein Homeostasis

The endoplasmic reticulum is the site of protein folding for all membrane and secreted proteins as they are generated. Polypeptides are co-translationally transported

from the ribsome into the lumen of the ER where a reducing environment allows peptides to be folded into corect protein conformations (5-7).

Because cell identities, cell to cell interactions, and extracellular communications are achieved through membrane protein interactions, correct membrane protein folding and modificiation in the ER lumen is critical for cell homeostasis and viability. Similarly, secreted proteins must achieve their precise conformations in order to cause intended endocrine and paracrine effects, further requiring precise protein folding in the ER (8). In recent years, it has become apparent that peptide folding and maturation is impaired in many diseases (9-12).

Homeostasis within the ER lumen is monitored and preserved by highly conserved quality control mechanisms (Figure 1-1) (4). Any type of disruption to ER homeostasis, including altered redox status, calcium buffering, aggregation or accumulation of client peptides is said to produce ER stress (8, 13-14). During ER stress, a specific ER stress response, also known as Unfolded Protein Response (UPR), becomes activated to convey information from the ER lumen to the nucleus (15, 16). This culminates in activation of transcription factors to upregulate target molecules that will restore ER homeostasis (17). The three ER stress signaling molecules act in concert to restore optimal protein folding conditions and enhance protein folding capacity, reduce the client protein folding load, and degrade unfolded peptides.

Misfolded and damaged proteins in the ER trigger a cascade of highly conserved signaling events to restore ER homeostasis by attenuating de novo protein synthesis, enhancing protein folding capacity, restoring ER lumen reducing environment, and promoting targeted peptide degradation (14). This integrated response has several

major components, including ER stress sensors/ signal transducers and target proteins as shown in Figure 1-1 (18). Three ER stress sensors are located on the ER membrane and include Inositol Requiring 1 (IRE1α), Pancreatic eukaryotic initiation factor 2 kinase-like Endoplasmic Reticulum Kinase (PERK) and Activating Transcription Factor 6 (ATF6). In metazoans, the three ER stress sensors and signal transducers collectectively orchestrate cellular signaling in response to ER stress.

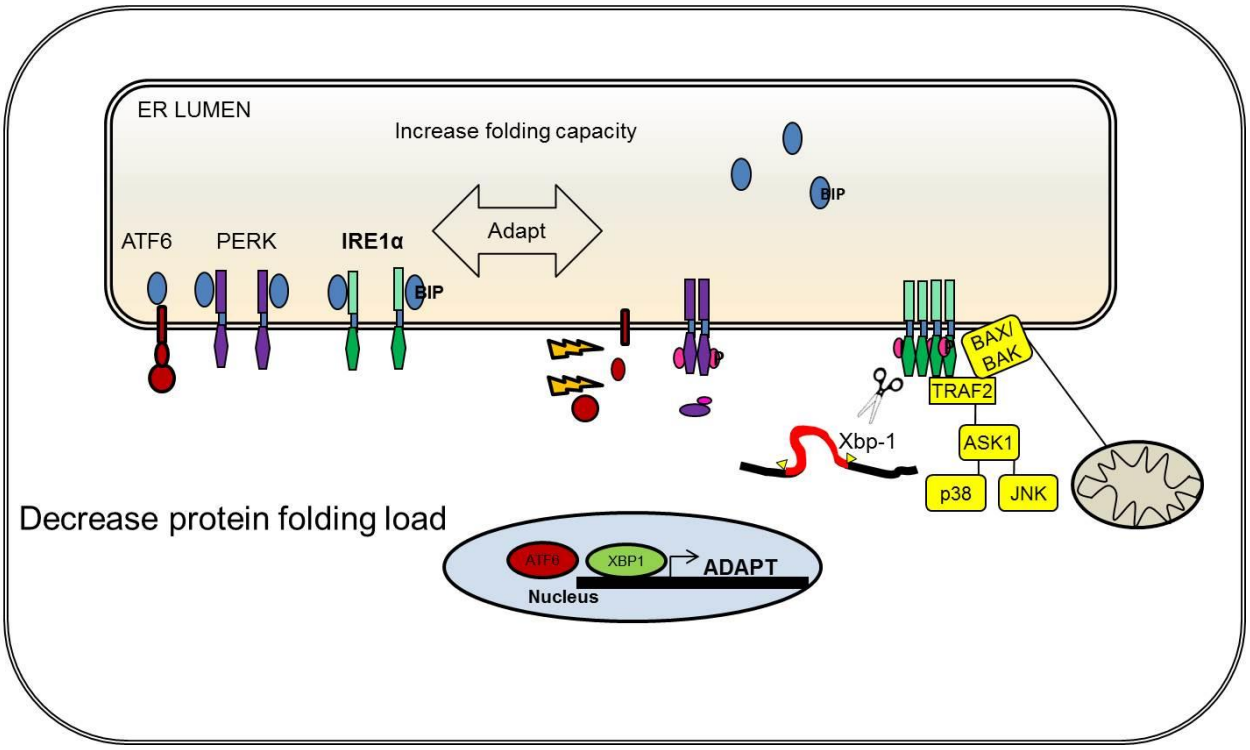

Figure 1-1. The Unfolded Protein Response Restores ER Homeostasis. ATF6, PERK and IRE1α monitor conditions in the ER lumen and become activated by BiP release upon accumulation of unfolded proteins. Activation of the three stress sensors and signal transducers leads to upregulation of adaptive protein folding chaperones and concurrent reduction in protein folding load. If ER stress is not resolved, IRE1α can induce apoptosis signaling through MAPK cascades or mitochondrial apoptotic pathways.

IRE1α and PERK are serine/ threonine protein kinases specifically localized on the ER membrane. Dimerization motifs extend into the ER lumen where they are masked by ER specific chaperone BiP/ Grp78/ Hspa5, rendering them inactive under basal conditions (18 -20). ATF6 is a member of the bZIP transcription factor family and

is retained on the ER membrane under basal conditions where golgi localization signaling sequences are also masked by BiP (20, 21).

Anytime peptide folding or protein maturation in the ER lumen is compromised, unfolded proteins accumulate in the ER lumen. BiP preferentially binds to exposed hydrophobic regions of unfolded peptides (22) and, thus, can be competitively recruited away from ER stress sensors any time unfolded peptides accumulate (19). Sequestration of BiP causes a release of inhibition on the three stress sensors. ATF6 translocates to the golgi apparatus for further processing, then is shuttled to the nucleus (21, 23). IRE1α and PERK homo-oligomerize and trans-phosphorylate at serine/ threonine sites in the cytosolic domains, conferring conformational changes required for activation (24-26). Phosphorylation of IRE1α activates its intrinsic RNase activity, inducing specific removal of a non-classical intronic sequence from the X-box Binding Protein-1 (Xbp1) mRNA, leading to efficient expression of the functional XBP1 protein as a potent nuclear transcription factor (27-29). Active transcription factors XBP1 and ATF6 induce UPR gene expression of heat shock proteins, antioxidant proteins, and ER-associated protein degradation proteins. Meanwhile, activated PERK phosphorylates elongiation initiation factor 2 (eIF2α) and inhibits protein synthesis (30). Therefore, a coordinated UPR reduces protein folding load and increases protein folding, reducing, and disposal capacity in order to resolve ER stress and restore ER homeostasis. UPR signaling is critical to normal cellular function (17).

D. IRE1 is Regulated by Multiple Mechanisms

IRE1 is the most ancient ER stress sensor and is conserved from yeast to mammals (31). IRE1 has two isoforms, IRE1α and IRE1β, differing in expression pattern. IRE1α has ubiquitous expression whereas IRE1β is expressed exclusively in the gut (16, 31). Although IRE1α and PERK share strong homology in both luminal and kinase domains (32), IRE1α may be regulated by additional mechanisms. The current dogma of IRE1α regulation is that, like ATF6 and PERK, IRE1α is held in an inactive state by BiP in the ER lumen (19, 33- 34). Upon BiP release, IRE1α is allowed to dimerize and oligomerize within the ER membrane, bringing kinase domains within proximity for trans-autophosphorylation (35). Following initial activation by phosphorylation, IRE1α undergoes a conformational change which results in a RNase domain platform being created to which Xbp1 unspliced transcript is recruited (36). The IRE1α RNAse splices 26 nucleotides from unspliced Xbp1 transcript to produce a spliced transcript isoform which, because of the frameshift, is efficiently translated on the ribosome (27-28). This unconventional cytosolic splicing event produces a highly active transcription activator (29, 37).

E. ER Stress Signaling Promotes Cell Death

ER stress signaling can induce apoptosis directly through multiple paths and also indirectly by intersection with other signaling pathways. Signal integration between different branches of the UPR and other signaling pathways is highly complex and may be cell type specific. There are many ways by which ER stress can induce apoptosis and there are many nodes where these signaling pathways intersect.

If adaptive UPR signaling does not restore ER homeostasis, cell death signaling pathways can become activated through IRE1α and PERK pathways (6, 38, 39). During severe ER stress, IRE1α binds to TRAF2 and activates Mitogen Activated Protein Kinase (MAPK) signaling cascades including ASK1, JNK, and p38 activation and apoptosis (40- 41). Mitochondria-initiated apoptosis may also be activated by IRE1α by direct interaction with BAX and BAK (42). Alternatively, IRE1α and TRAF2 interactions with JNK may also activate autophagy in order to promote cell survival during ER stress (43).

Unliike IRE1α, PERK has specific kinase activity toward eIF2α, leading to global attenuation of protein translation and reducing protein folding load on the ER (30). At the same time, this inhibition frees ribosomal machinery for translation of eIF2α-independent transcripts, including ATF4 (44- 45). ATF4 regulates transcription of Gadd153/ CHOP, a key signal integrating ER stress with mitochondrial-mediated apoptosis (46). CHOP expression can also be enhanced by p38 MAPK, suggesting additional nodes where the PERK and IRE1α branches of the UPR intersect with MAPK signaling pathways.

F. ER Stress Signaling in Heart; friend or foe?

The specific role of ER stress signaling in heart is not clear. ER stress signaling is activated in response to ischemia (47- 48), pressure overload (49), and hypoxia (13, 50-51) but it is unclear whether ER stress signaling is protective or detrimental to heart in these settings. Some studies suggest ER stress induction contributes to myocyte

apoptosis and heart failure (47, 50, 52- 53) while others report it is cardioprotective (48, 54).

Protective signaling during the ER stress reponse activates transcription of molecules to restore the specialized ER luminal environment (redox or calcium status), increase the size of the ER so to increase the peptide folding capacity of the ER, upregulate expression of protein folding chaperones, or enhance protein degradation of misfolded peptides. ATF6 has been identified as a highly protective signaling pathway (48). Overexpression of ATF6 in heart induced expression of two protein folding chaperones, BiP and GRP94, and provided protection against ischemia/ reperfusion injry including reduced necrosis and apoptosis. Similarly, XBP1, downstream of IRE1α activation, was found to be protective against hypoxia and myocardial infarction also by inducing BiP (54). BiP can inhibit the apoptotic signal CHOP and reduce apoptosis in cardiomyocytes (55). Therefore ATF6 and IRE1α promote adaptation to ER stress and restoration of homeostasis.

Detrimental effects of ER stress signaling by PERK and IRE1α have also been identified. Angiotensin II, along with tunicamycin and thapsigargin, can induce both adaptive protein folding chaperones and apoptotic signal CHOP, which is downstream of the PERK branch of ER stress signaling (49). ER stress can also induce hypertrophy signals Atrial Natriuretic factor (ANF) and Brain Natriuretic factor (BNF), suggesting that ER stress could underly remodeling of the heart. Cancer patients using Imatinib developed heart failure, which was found to induce ER stress and apoptosis in a mouse model (52). Imatinib induced JNK activation, cytochrome c release from mitochondria leading to cell death, left ventricular dysfunction and heart failure. IRE1α has been

reported to activate JNK, and act in complex with TRAF2 and ASK1 (apoptotic signaling molecules) indicating a highly detrimental outcome from IRE1α signaling in heart (40). Therefore, both PERK and IRE1α downstream signaling can promote apoptosis in cardiac myocytes.

Several investigations have uncovered intersections between inflammation and ER stress signaling (56). In the heart, deletion of CHOP can blunt apoptosis, inflammation, and injury in response to ischemia/ reperfusion (57). Activation of p38 MAPK in heart led to increased expression of inflammatory cytokines TNFα and IL6, interstitial fibrosis, abundant extracellular matrix, and impaired heart function whereas inhibition of p38 reversed these negative effects (58). Further investigation into the intersection between inflammation and ER stress signaling will fill an important gap in our understanding of the biology of heart failure.

The specific role of ER stress signaling in heart is not clear. ER stress signaling is activated in response to acute and chronic stresses in heart (13, 48, 49, 51). ATF6 was reported to protect heart against ischemia/reperfusion injury, while PERK and downstream CHOP is apoptotic (46, 57). A role for IRE1α in heart is completely unknown. IRE1α has been reported to induce adaptive signaling through XBP1 or apoptotic signaling by interaction with TRAF2, ASK1 and JNK. Therefore, IRE1α is poised to be a critical decision maker for life or death decisions in cardiomyocytes.

G. The Role of IRE1α in Heart is Unknown

ER stress signaling is emerging to be an important signaling pathway for health and disease. ATF6, PERK, and IRE1α are three ER stress sensors and signal

transducers that monitor the status of protein folding in the lumen of the ER and are activated when ER homeostasis is disrupted. This Unfolded Protein Response activates a transcriptional program to re-establish efficient protein folding homeostasis in the ER. The role of UPR signaling in heart is poorly understood. IRE1α has been shown to promote either apoptotic signaling activities through JNK, TRAF2 (59) or adaptive signaling through XBP1 (27, 29). ATF6 has been shown to maintain normal heart physiology and also protect against ischemia/ reperfusion injury (48). PERK signaling, on the other hand, promotes apoptotic signaling pathways (59). The role of IRE1α in heart is completely unknown. Advancing the understanding of IRE1α in heart will fill an important gap in knowledge of heart biology. Additional characterization of ER stress signaling in heart may provide important insight into underlying disease mechanisms and also novel therapeutic strategies.

In order to investigate the specific contribution if IRE1α in heart, we generated an animal model with heart specific, tamoxifen inducible IRE1α overexpression. IRE1α overexpression did not lead to any detrimental phenotype at baseline conditions. Under stress, however, IRE1α was cardioprotective, preserving heart function following pressure-overload. ER stress signaling was preserved, and fetal gene program was inhibited in IRE1α mice, suggesting intersection between ER stress and fetal gene program regulation. Inflammatory cytokines TNFα and IL-6 mRNA expression were also blunted after pressure overload, suggesting IRE1α mediated regulation of inflammatory cytokines in heart. Thus, IRE1α may have protective activities specific to heart. In vitro characterization studies revealed that IRE1α has adaptive and transient UPR signaling activity in cardiomyocytes, suggesting that heart possesses cardiomyocyte-specific

regulatory mechanisms that remain to be characterized. Further, we identified signal integration between the UPR and inflammatory signaling pathways. This highlights the potential for heart-specific IRE1α activities that are achieved by integration between multiple pathways.

CHAPTER 2

Materials and Methods

A. Animal Models and Surgical Procedures

The investigation conforms with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1985). All procedures were performed in accordance with the University of California, Los Angeles animal welfare guidelines.

IRE1α was cloned into a vector for generation of transgenic animals with cre-regulated expression of the transgene of interest (60). Transgenic animals were generated in C57/Bl6 background through collaboration with the UCLA Molecular Genetics Technology Center. Founder animals were identified by PCR with transgene specific primers.

Animals with heart-specific, inducible IRE1α overexpression were generated by crossing transgenic founder animals with previously established αMHC-Mer-Cre-Mer (MCM) transgenic mice (61, 62). IRE1α transgene overexpression was induced by intraperitoneal injection of Tamoxifen Citrate Salt (Sigma) 20mg / kg body weight/ day for five days (62). Wild-type and floxed single transgenic littermate animals treated with tamoxifen or double transgenic flox-GFP/ CRE animals treated with vehicle were also used as controls. Both male and female mice age 12-16 weeks were included in this study.

Transverse Aortic Constriction (TAC) was performed as previously described with modifications (63). Mice were anesthetized with ketamine (80mg/ kg) / xylazine (20mg/ kg) by i.p. injection. Respiration was provided by mechanical ventilation with 95% O_2 (tidal volume 0.5 mL, 130 breaths per minute). Left parasternal thoracotomy was performed to access the transverse aorta, which was tied with 5-0 nylon suture on a 27 gauge needle. The needle was removed, leaving in place a 65-70% constriction of the aortic lumen. Constriction of the aorta was confirmed by measuring differential blood flow through the right and left carotid arteries one week after surgery.

Animals were continuously anesthetized with 1.5% isoflurane and 95% oxygen. VisualSonics Vevo 770 and Vevo 2100 imaging systems and 30mHz scanhead (Toronto, Ontario, Canada) was used to collect short and long axis B-Mode and M-Mode views. Reported values refer to short axis measurements and calculations.

B. Histology

Hearts were perfused and fixed in 10% formalin prior to embedding in paraffin. All short axis sections were prepared from mid-ventricle. Sections of 4 μm heart were deparaffinized and rehydrated prior to staining by hematoxylin and eosin (H&E) or Masson trichrome and Verhoeff's Van Gieson. Stained tissue sections were recorded as digital images by Aperio XT whole slide scanning system and snapshot images were taken using the ImageScope software.

C. Cell culture

293 cells were maintained in DMEM supplemented with 10% RBS and 1% pen/strep. Lipofectamine reagent (Life Technologies) was used according to the manufacturer's protocol to achieve overexpression of the flox-GFP-IRE1α construct. INS-1 cells were cultured in RPMI1640 according to published methods (64). Neonatal Rat Ventricular Myocytes (NRVM) were harvested from 1-3 day old Sprague-Dawley rat pups as described previously (65) and cultured in serum-free DMEM supplemented with 1% pen/strep and ITS. NRVM were infected with adenovirus for IRE1α tagged with –Myc and incubated for two days before additional treatment with 5g/mL TM for 4 hours, 100 nM TG for 4 hours or 10μM H_20_2 for 30 minutes. Experiments with prolonged IRE1α expression were incubated for five days before RNA or protein analysis.

D. Western Blot

Cells were harvested for protein analysis with standard lysis buffer containing 1% Triton-X 100, 1mM β-glycerophosphate, 2.5mM $Na_4P_2O_7$, 20mM NaF, 1mM Na_3VO_4, 1mM PMSF and protease inhibitor cocktail (Roche). Proteins were boiled for 5 minutes in LDS loading buffer containing 0.1% β-mercaptoethanol and separated on a 4-12% Bis-Tris SDS-PAGE (Life Technologies). Specific proteins were detected with antibodies directed against p-IRE1α (Novus Bio), IRE1α, Actin, (Santa Cruz Biotechnology), BiP/Grp78 (Stressgen), p-p38, p38, p-JNK, JNK, p-IEF2α, eIF2α, GFP, and CHOP (Cell Signaling Technologies).

E. RNA and RT-PCR Analysis

Total RNA was isolated from heart or cells with TRIzol (Life Technologies). For animal studies, cDNA was prepared using iScript Reverse Transcription Supermix and amplified with SsoFast EvaGreen Supermix on a CVX96 thermal cycler (all Bio-Rad). For cell studies, cDNA was prepared using Superscript II (Invitrogen) and amplified with SYBR green supermix on a MyIQ system (Bio-Rad). Primer sequences are shown in Table 1.

Table 1. Primers used for RT-PCR experiments

Primers for mouse		
TARGET	FORWARD	REVERSE
Xbp1 Total	TGGACTCTGACACTGTTGCC	CTCTGGGGAAGGACATTTGA
sXbp1	CAGTGGTCGCCACCGTCCATC	TGCCGCGCCCAGCCTTTCTA
Xbp1 splicing	GTTCCAGAGGTGGAGGCCA	CATGACAGGGTCCAACTTGTCC
CHOP	TATCTCATCCCCAGGAAACG	GGGCACTGACCACTCTGTTT
BiP	GAGGCTGTAGCCTATGGTGC	TTTGTTAGGGGTCGTTCACC
Rps26	GCCTCTTTACATGGGCTTTG	GCCATCCATAGCAAGGTTGT
Primers for mouse/ rat		
IRE1α	ACGGTGGACATCTTTTCGC	TGGGGATCCATAGCAATCAT
IRE1α MYC	TCAGGAGACGCTGGGCTCCATC	AGAGATCAGCTTCTGCTCGCCTC
ANF	CTGATGGATTTCAAGAACCTGCT	CTCTGGGCTCCAATCCTGTC
βMHC	CTCAACTGGGAAGAGCATCCA	CCTTCAGCAAACTCTGGAGGC
TNFα	CTCTTCAAGGGACAAGGCTG	TGGAAGACTCCTCCCAGGTA
GAPDH	TCCTGCACCACCAACTGCTTAG	GATGACCTTGCCCACAGCCTTG
Primers for rat		
RatXBP1	CTCAGAGGCAGAGTCCAAGG	ACAGGGTCCAACTTGTCCAG
sXBP1	TCTGCTGAGTCCGCAGCAGG	CTCTAAGACTAGAGGCTTGG
uXBP1	CAGACTACGTGCGCCTCTGC	CTTCTGGGTAGACCTCTGGG
CHOP	CCTTCACTACTCTTGACCTGC	CGCTCGTTCTCTTCAGCAAG
BIP	TTCCGCTCTACCATGAAACC	CTTATTGTTACGGTGGGCT

F. IRE1α RNase Activity Assay

IRE1α RNase activity toward Xbp1 mRNA was monitored by semiquantitative PCR. Both unspliced and spliced Xbp1 mRNA was amplified with primers targeting the region surrounding the IRE1α splicing site (Forward 5'GTTCCAGAGGTGGAGGCCA3', Reverse 5'CATGACAGGGTCCAACTTGTCC3'). Products were amplified with the cycling protocol of 95℃ 0:30 followed by 35 cycles of 95℃ for 0:30, 60℃ for 0:30 and 72℃ for 0:25 followed by 72℃ for 10:00. PCR products were separated on 4% agarose gel.

G. Statistical Analysis

Data are presented as mean ± 1 standard deviation. Means of two groups were compared by two-tailed Student t-test. Means of more than two groups were compared by ANOVA. Differences between groups were considered statistically significant when $p<0.05$. Significant differences compared to control genotypes are indicated by # and significant differences compared to baseline within the same genotype group are indicated by *.

CHAPTER 3

IRE1α Protects Myocardium from Heart Failure Induced by Pressure Overload

A. Generation of IRE1α Transgenic Animals

In order to elucidate a specific role for IRE1α in heart, we generated an animal model with IRE1α overexpression. Because IRE1α overexpression has been reported to be detrimental to several cell types, we employed a strategy where IRE1α expression would be both inducible and restricted to the heart (Figure 3-1A). To that end, cDNA encoding IRE1α tagged with Myc was inserted following a floxed GFP cassette so that, in the absence of cre, the ubiquitous promoter drives GFP expression (66). In the presence of cre, however, the floxed GFP cassette (which contains a stop codon) is removed, allowing IRE1α-Myc expression. Efficient cre-dependent IRE1α protein overexpression was first achieved in vitro, confirming the strategy (Figure 3-1B-C). Flox-GFP-IRE1α-Myc single transgenic animals were identified by PCR and western blot, where GFP protein expression was confirmed (Figure 3-1D). Single transgenic founder animals were then crossed with αMHC-MCM animals where cre expression is restricted to heart and activity is tamoxifen dependent (61, 62, 67). Four genotype groups; wild type, αMHC-MCM, flox-GFP-IRE1α and αMHC-MCM/flox-GFP-IRE1α were represented in offspring litters in ratios expected from the breeding pair (data not shown). Double transgenic flox-GFP-IRE1α-Myc/αMHC-MCM animals treated with tamoxifen had significant induction of IRE1α mRNA expression ($p=0.01$) (Figure 3-1E-F).

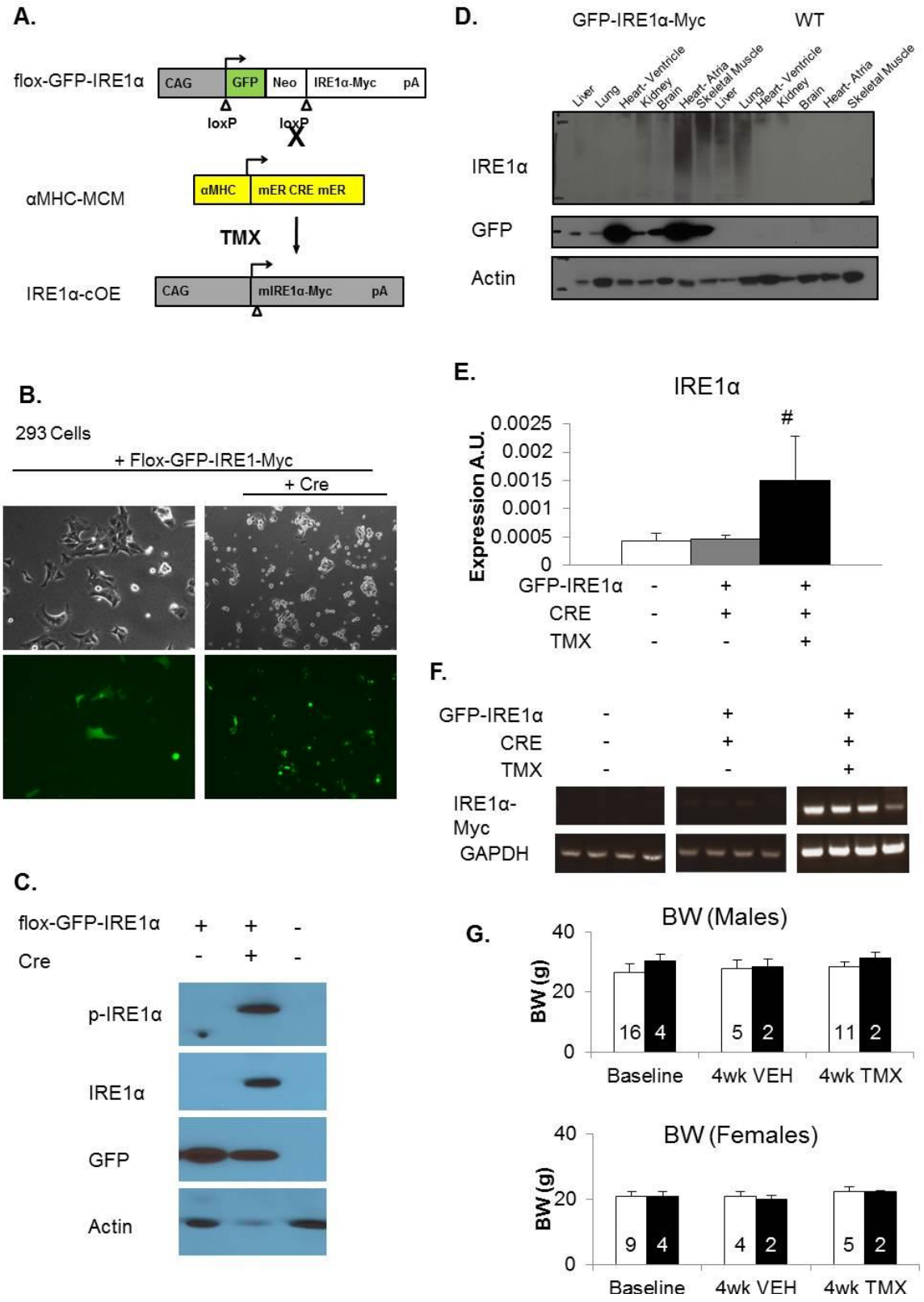

Figure 3-1. Transgenic Strategy of Tamoxifen Regulated IRE1α Overexpression. A. Schematic of transgenic strategy where IRE1α-Myc follows constitutive GFP expression in the absence of Cre recombinase. Crossing with transgenic

animals positive for heart specific (αMHC promoter) tamoxifen-mediated cre, shown as modified estrogen receptor (mER) CRE mER produces double transgenic animals that, when provided with tamoxifen, have heart specific overexpression of IRE1α-Myc. B. Validation of IRE1α conditional overexpression strategy in vitro. Brightfield (top) and fluorescent images (bottom) of 293 cells expressing flox-GFP-Ire1α or Cre + Flox-GFP-IRE1α. Note that cells with IRE1α expression are irregular in shape and detached from the culture dish. C. Western blot from cells treated in B where IRE1α is overexpressed and activated in the presence of Cre recombinase. D. Ubiquitous GFP protein expression in all tissues tested in flox-GFP-IRE1α single transgenic animals. E. IRE1α mRNA from hearts of wildtype or double transgenic animals treated with vehicle or tamoxifen was measured by quantitative RT-PCR. F. Exogenous IRE1α-Myc mRNA amplified by PCR. G. IRE1α does not cause adverse phenotype in the absence of stress. Gross outward health was monitored by weekly measurement of body weight over the course of four weeks following treatment as indicated.

B. IRE1α Does Not Induce Detrimental Phenotype in Heart in the Absence of Stress

No obvious phenotype was observed in adult IRE1α animals four weeks after transgene induction (Figure 3- 2). No adverse heart functional or structural changes were observed, though LIVDd was slightly increased compared to control animals (wildtype and single transgenic litermates) (Figure 3- 2A-C). Tissue structure and organization in histological sections was also indistinguishable between control and IRE1α transgenic animals (Figure 3-2D). Heart weight/body weight ratio was also indistinguishable between control and IRE1α transgenic animals (Figure 3-2E). Together, these observations indicate that IRE1α did not lead to any detrimental phenotype upon overexpression in adult heart.

IRE1α overexpression can induce activation and downstream UPR signaling in the absence of ER stress in vitro (68). In order to determine if this was also the case in

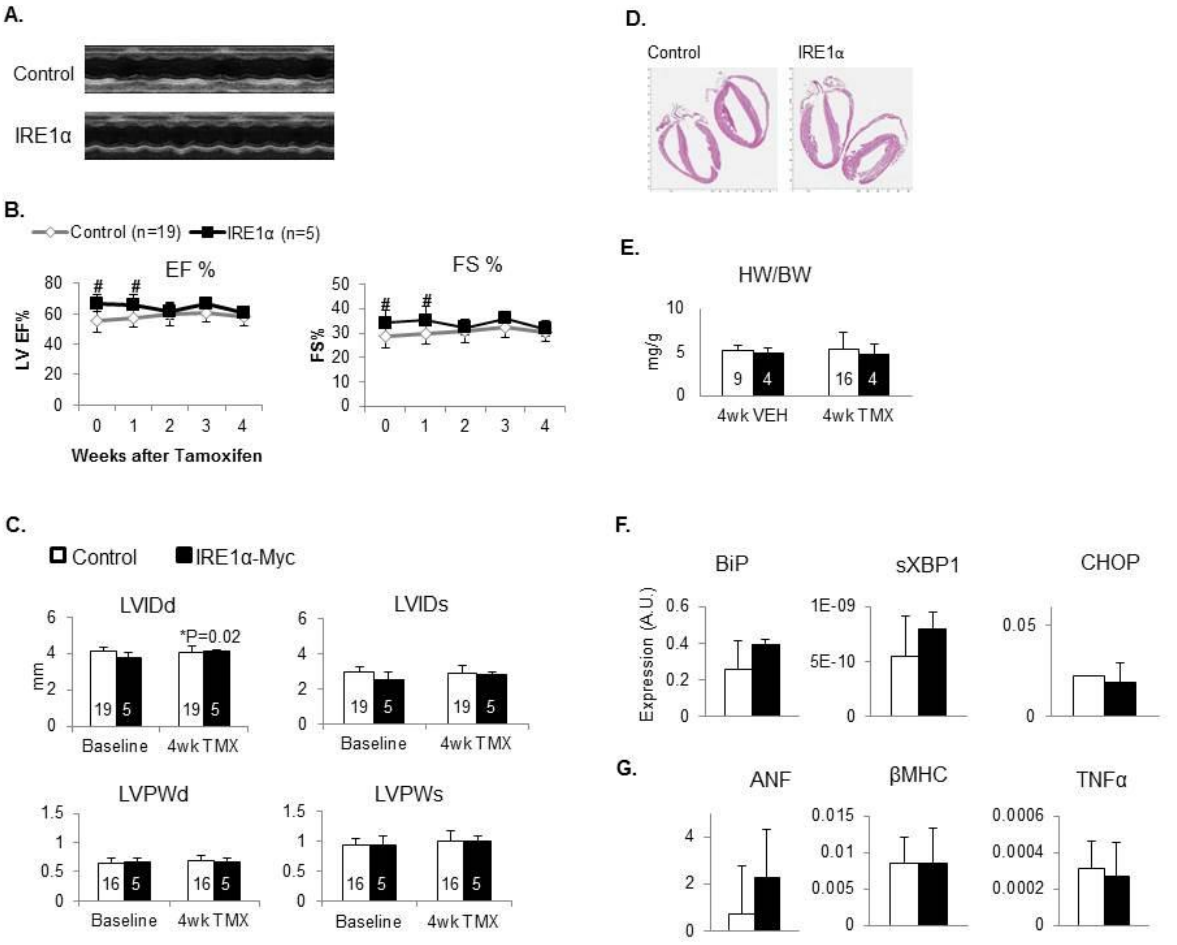

Figure 3-2. IRE1α Does Not Induce Phenotype in Heart in the Absence of Stress. A. Representative M-Mode images viewing the short axis of the left ventricle of control and IRE1α animals. B. Left ventricular function, measured by ejection fraction and fractional shortening, were calculated from short axis M-mode images collected weekly for four weeks following tamoxifen treatment. C. Left ventricular internal diameter (LVID) and Left ventricular posterior wall (LVPW) was measured during diastole and systole from short axis M-Mode images. All dimensions are reported in millimeters (mm). Error bars represent standard deviation. Comparisons were found to be significant where indicated (# denotes comparison to control genotype and * indicates comparison to same genotype group baseline treatment where $p<0.05$). D. Long axis (four chamber) sections collected from control or IRE1α hearts four weeks after treatment as indicated and stained with hematoxylin and eosin. E. Heart weight to body weight (HW/BW) ratio was calculated from hearts collected four weeks after treatment as indicated. Both male and female animals were included in this data. F-G. RT-PCR was used to measure UPR (F) and fetal gene program and inflammatory signaling (G) after 4 weeks of IRE1α overexpression in heart. Expression is indicated as arbitrary units (A.U.) and was calibrated against Rps26S.

heart, we measured mRNA of sXbp1, Bip, and CHOP (Figure 3-2F). IRE1α overexpression led to slight increases in sXbp1 and Bip expression though neither target reached statistical significance ($p=0.33$ and $p=0.1$, respectively). Apoptotic factor CHOP, on the other hand, was slightly decreased ($p=0.61$). The expression of ANF

(p=0.42), β-MHC (p=0.27), and TNFα (p=0.18) were all unchanged following IRE1α overexpression, supporting the observation that IRE1α did not cause any detrimental phenotype to heart (Figure 3- 2G).

C. IRE1α Preserves Heart Function After Pressure Overload

UPR preconditioning has been shown to protect against acute tissue injuries (69). Because no detrimental phenotype was observed following IRE1α transgene induction, and adaptive UPR signaling was slightly increased, we hypothesized that IRE1α overexpression could protect against stress-induced injury in a similar fashion. In order to test this hypothesis, control and IRE1α transgenic animals were treated with pressure-overload by transverse-aortic constriction (TAC) (63, 70). IRE1α preserved heart function after TAC (Figure 3-3A). Pressure overload did not cause a detrimental change to heart function or structure in IRE1α animals (Figure 3-3B-C). After four weeks, ejection fraction and fractional shortening were both significantly higher in IRE1α animals than in control animals (Figure 3-3B) and LVID at diastole and systole were unchanged over the course of the study (Figure 3- 3C). TAC caused hypertrophy in both IRE1α and control animals (Figure 3-3D-E).

Because IRE1α preserved heart function, we hypothesized that protective UPR signaling would be enhanced, and detrimental signaling would be dampened in response to TAC. We used RT-PCR to measure UPR, fetal gene program, and inflammatory signaling and western blot to measure stress signaling activation after TAC. IRE1α preserved adaptive UPR signaling and inhibited markers of the fetal gene program, inflammation, and heart failure signaling. sXbp1 and BiP mRNA were

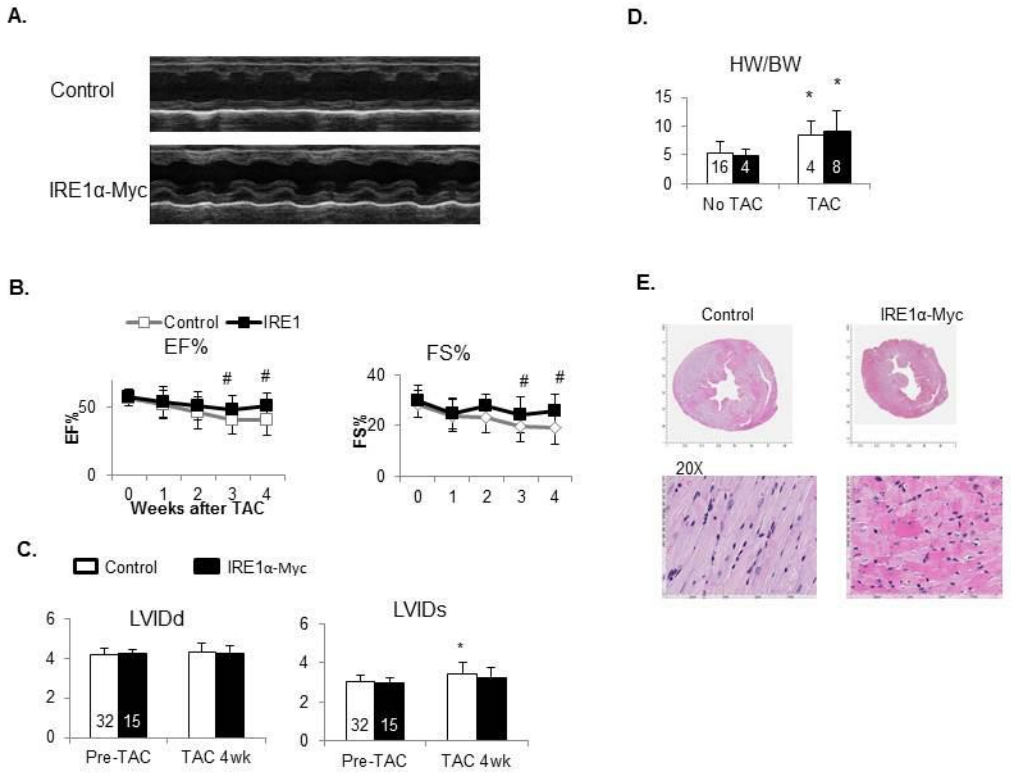

Figure 3-3. IRE1α Preserves Heart Function in Response to TAC. A. Representative short axis M-Mode images from left ventricle of Control and IRE1α-Myc animals, respectively, four weeks after TAC. B. Left ventricle ejection fraction and fractional shortening were calculated from short axis M-Mode images weekly after tamoxifen and TAC and compared between control and IRE1α. C. Left ventricular dimensions were measured from short axis M-Mode images during weekly echocardiography after TAC and compared between IRE1α and controls. D. Heart weight to body weight ratio (HW/BW) was calculated four weeks after TAC and is presented as mg/g. C. Upper: mid-ventricular cross sections of heart four weeks after TAC stained by H&E. Lower: 20X magnification representative images from free wall of LV. In all data, error bars represent 1 standard deviation and statistical significance is indicated where compared to control (# where p<0.05), or compared to Pre-TAC (* where p.<0.05).

increased in IRE1α animals after TAC (Figure 3-4A-B). CHOP, which was slightly lower in IRE1α animals at baseline, increased four weeks after TAC (Figure 3- 4B). ER stress marker ATF4, which is a transcription factor selectively translated following eIF2α-mediated translational inhibition (44), was significantly lower in IRE1α animal hearts at baseline, but increased in IRE1α animals in response to TAC (p=0.04). BiP protein expression was increased at baseline and after TAC (Figure 3-4C). From these data,

we concluded that IRE1α overexpression enhanced adaptive UPR signaling and preserving heart function in response to TAC.

In addition to enhancing adaptive UPR signaling, IRE1α expression blunted the molecular shift to the fetal gene program and activation of stress signaling after TAC. Both ANF and βMHC mRNA expression were unchanged in response to TAC (Figure 3-4D). Moreover, stress signaling was not activated. Activated p38 was not increased in IRE1α hearts four weeks after TAC (Figure 3-4E).

ER stress signaling by IRE1α and TRAF2 has been reported to activate inflammatory cytokines (71). We measured inflammatory cytokine expression by RT-PCR and fibrotic dispositions by EVG/Trichrome staining of heart tissues. In animals with IRE1α overexpression, both TNFα and IL-6 were significantly reduced after TAC compared to wildtype ($p=0.03$) and compared to baseline ($p=0.001$) (Figure 3-4F). IRE1α animals had less myocardial fibrosis than control animals (Figure 3- 4G).

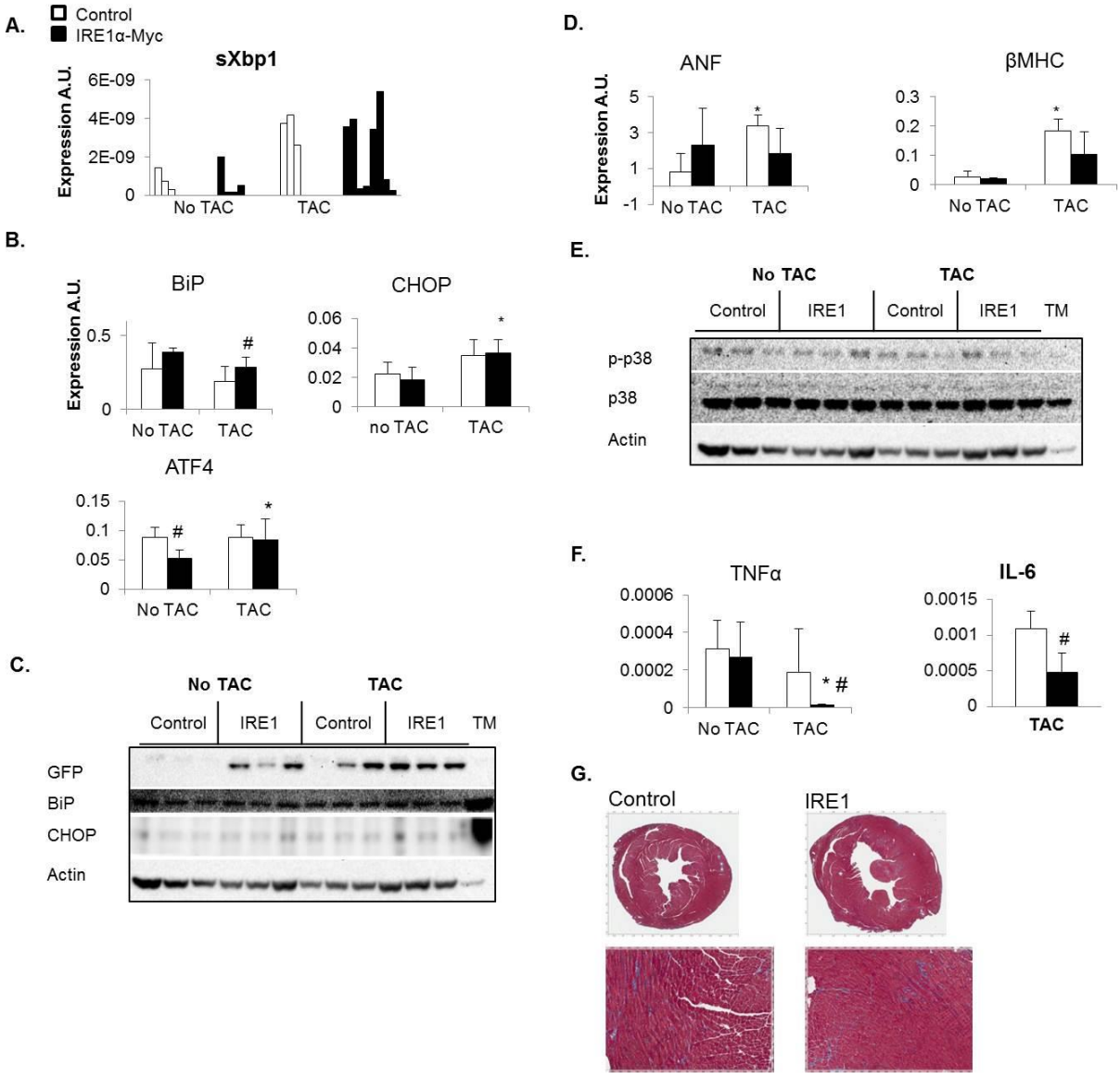

Figure 3-4. Molecular Profile of IRE1α Mice After Four Weeks of TAC. A. Quantitative RT-PCR of sXbp1 from animals treated with tamoxifen or tamoxifen and four weeks TAC. Each bar represents mRNA expression from a single animal. B. Quantitative RT-PCR measuring mRNA expression of ER stress signaling four weeks after tamoxifen or tamoxifen and TAC treatment. C. Western blot measuring protein expression of UPR signaling in hearts from genotypes and treatment groups as indicated D. Quantitative RT-PCR as in B, measuring mRNA expression of fetal gene program induction after tamoxifen or tamoxifen and TAC. E. Western blot measuring p38 MAPK activation in hearts from control and IRE1α groups at baseline and four weeks after TAC as indicated. F. RT-PCR measuring inhibition of inflammatory cytokine TNF and IL-6 after TAC. G. Trichrome/ EVG staining for fibrosis in short axis sections from mid-ventricle of heart after four weeks TAC. In all RT-PCR experiments, # denotes significant difference between control and IRE1α animals within the treatment group and * denotes significant difference within the same genotype between No TAC and TAC treatments.

CHAPTER 4

IRE1α Induces Adaptive and Transient Unfolded Protein Response Signaling in Cardiac Myocytes

A. IRE1α Induces Stress Signaling in Various Cell Types

IRE1α has been reported to induce cell death in CHO, COS, and 293 cells (31). In order to establish a robust system for investigation of IRE1α signaling in heart in vitro, IRE1α was first expressed in 293 cells or INS-1 cells. After 2 days, both 293 and INS-1 cells with overexpressed IRE1α were irregular in shape, rounded up, and detached from the culture dishes (Figure 3-1A, 4-1A).

In order to investigate UPR activation following IRE1α overexpression in INS-1 cells, RNA and protein were collected from cells two days after treatment. IRE1α kinase and RNase were both found to be activated following IRE1α overexpression. Spliced Xbp1 mRNA was amplified by PCR in samples with IRE1α overexpression in a dose-dependent fashion (Figure 4-1B). Phospho-eIF2α was increased with IRE1α, indicating parallel activation of PERK (Figure 4-1C). BiP protein was also increased following IRE1α expression.

Besides adaptive UPR signaling, IRE1α has been shown to activate MAPK signaling, including p38 and JNK (40). Therefore, we investigated downstream MAPK signaling by IRE1α in INS-1 cells (Figure 4-1D). IRE1α overexpression led to a dose-dependent increase in p-JNK, but not p-p38. Therefore, we concluded that IRE1α

overexpression in INS-1 cells led to activation of all three branches of UPR as well as the detrimental p-JNK signaling pathways independent of true ER stress.

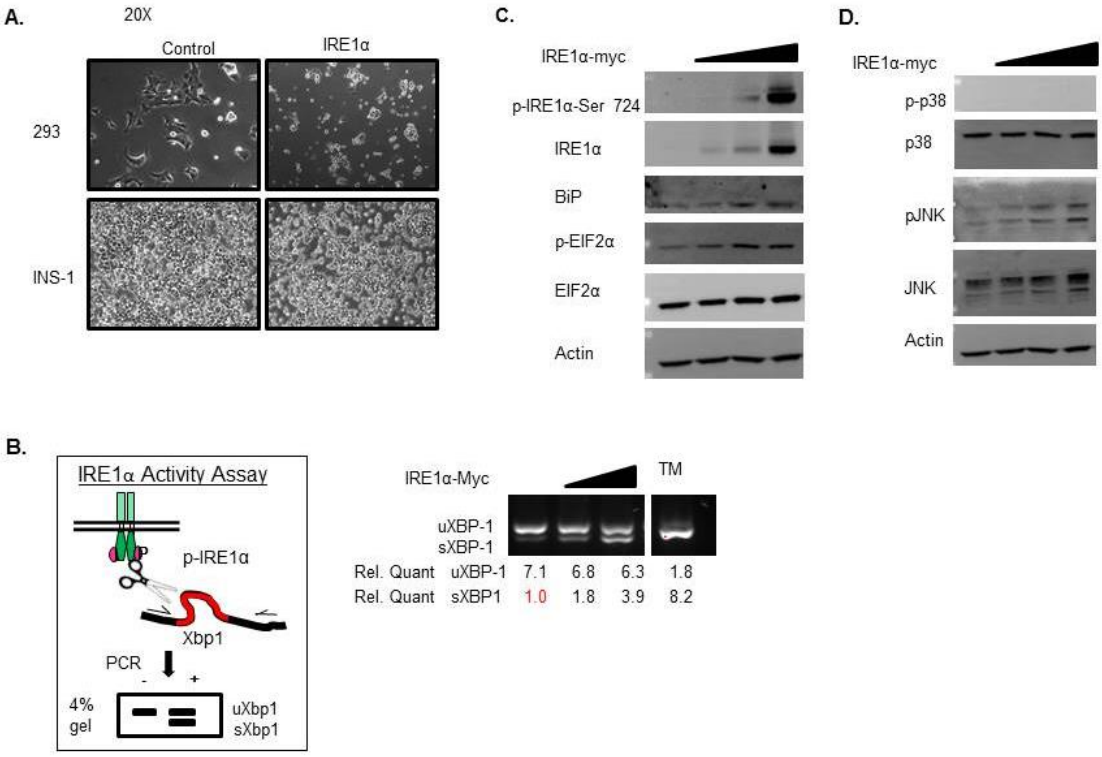

Figure 4-1. IRE1α Activates UPR and Stress Signaling in INS-1 Cells. A. Brightfield images of 293 (top) and INS-1 (lower) cells two days after treatment as indicated. B. Schematic of IRE1α RNase activity assay and amplification of sXBP-1 and uXBP-1 by PCR with IRE1α or TM treatment. C. Western blot measuring activation of UPR in INS-1 cells IRE1α overexpression. D. As in C, but monitoring MAPK stress signaling.

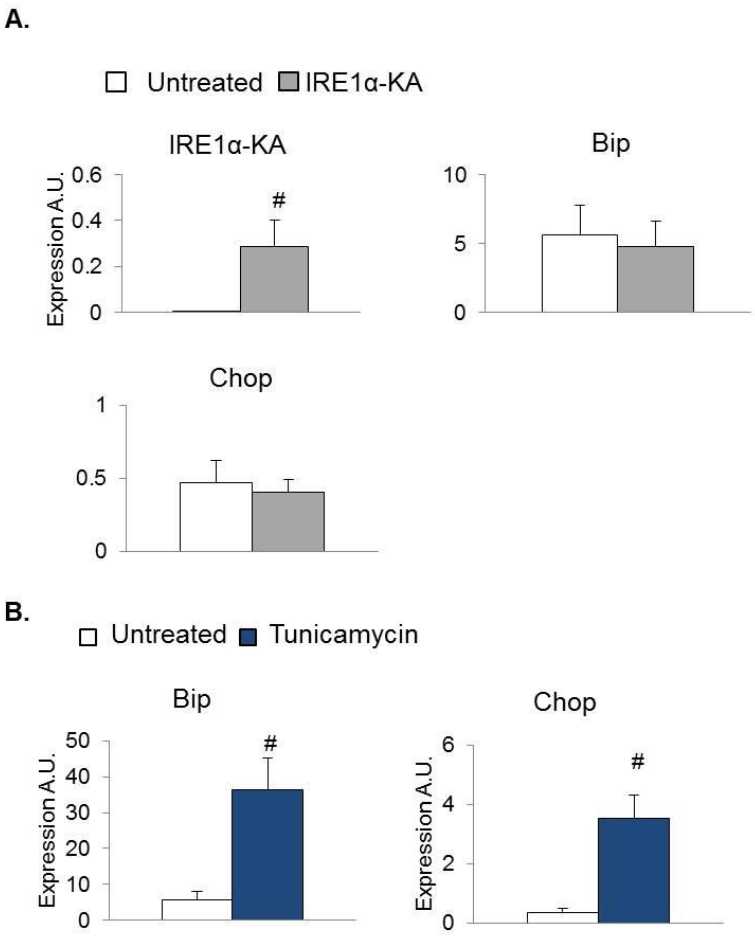

Figure 4-2. NRVMs Are Sensitive to ER Stress. A. RT-PCR to measure IRE1-KA overexpression after 2 days in NRVMs and UPR signaling members CHOP and BiP. B. RT-PCR to measure UPR induction after TM-mediated ER stress in NRVMs. In all RT-PCR experiments, # denotes significant difference between control and IRE1α animals within the treatment group and * denotes significant difference within the same genotype between No TAC and TAC treatments

B. IRE1α Induces Adaptive and Transient UPR Signaling in Cardiac Myocytes

In order to uncover the molecular mechanism underlying cardioprotection by IRE1α in vivo, we further investigated IRE1α-mediated UPR signaling in NRVMs. We first established whether or not virus treatment would activate endogenous UPR signaling. Treatment with kinase dead IRE1α adenovirus did not activate UPR signaling (Figure 4-2A). Neither BiP (p=0.64) nor Chop (p=0.53) expression were induced in response to IRE1α overexpression. Treatment with true ER stress by Tunicamycin,

however, activated UPR as indicated by RT-PCR measurements of increased BiP ($p=0.004$) and CHOP ($p=0.002$) expression (Figure 4-2B).

In order to next investigate UPR signaling by IRE1α, IRE1α-Myc was overexpressed by adenovirus and gross cell morphology, activation, activity, and downstream signaling were assessed. After two days, NRVM with IRE1α overexpression were indistinguishable from untreated controls (Figure 4-3A) whereas 293 and INS-1 cells with the same treatment were highly irregular in shape and detached from the culture dish (Figure 3-1A and Figure 4-1A). IRE1α-Myc protein expression and kinase and RNase activation in NRVM were confirmed by western blot and PCR, respectively (Figure 4-3B-C). Adaptive protein chaperone Bip was significantly upregulated following IRE1α overexpression ($p=0.03$). Apoptotic molecule Chop, on the other hand, was not ($p=0.09$). Expression of TNFα was reduced with IRE1α expression ($p=0.03$), recapitulating the observations of blunted inflammatory cytokine expression in vivo. We did not observe activation of either adaptive (Bip) or apoptotic (Chop) downstream UPR signaling following IRE1α-KA expression, further indicating that the adaptive signaling we observed downstream of IRE1α expression was specific to IRE1α and not an artifact of viral infection (Figure 4-2A). Additionally, we confirmed that true ER stress elicited an UPR in cardiac myocytes (Figure 4-2B).

We next sought to uncover the contribution of chronic IRE1α overexpression in NRVMs. Prolonged p-IRE1α activation (5 days) did not cause sustained RNase or downstream UPR gene expression (Figure 4-3E-G). Bip expression was not significantly greater in IRE1α- expressing cells ($p=0.25$). Even after sustained IRE1α expression, CHOP mRNA expression was not induced ($p=0.76$) compared to control.

This result suggested that IRE1α kinase activation was sustained, but RNase activity and UPR signaling was transient in NRVMs in the absence of ER stress.

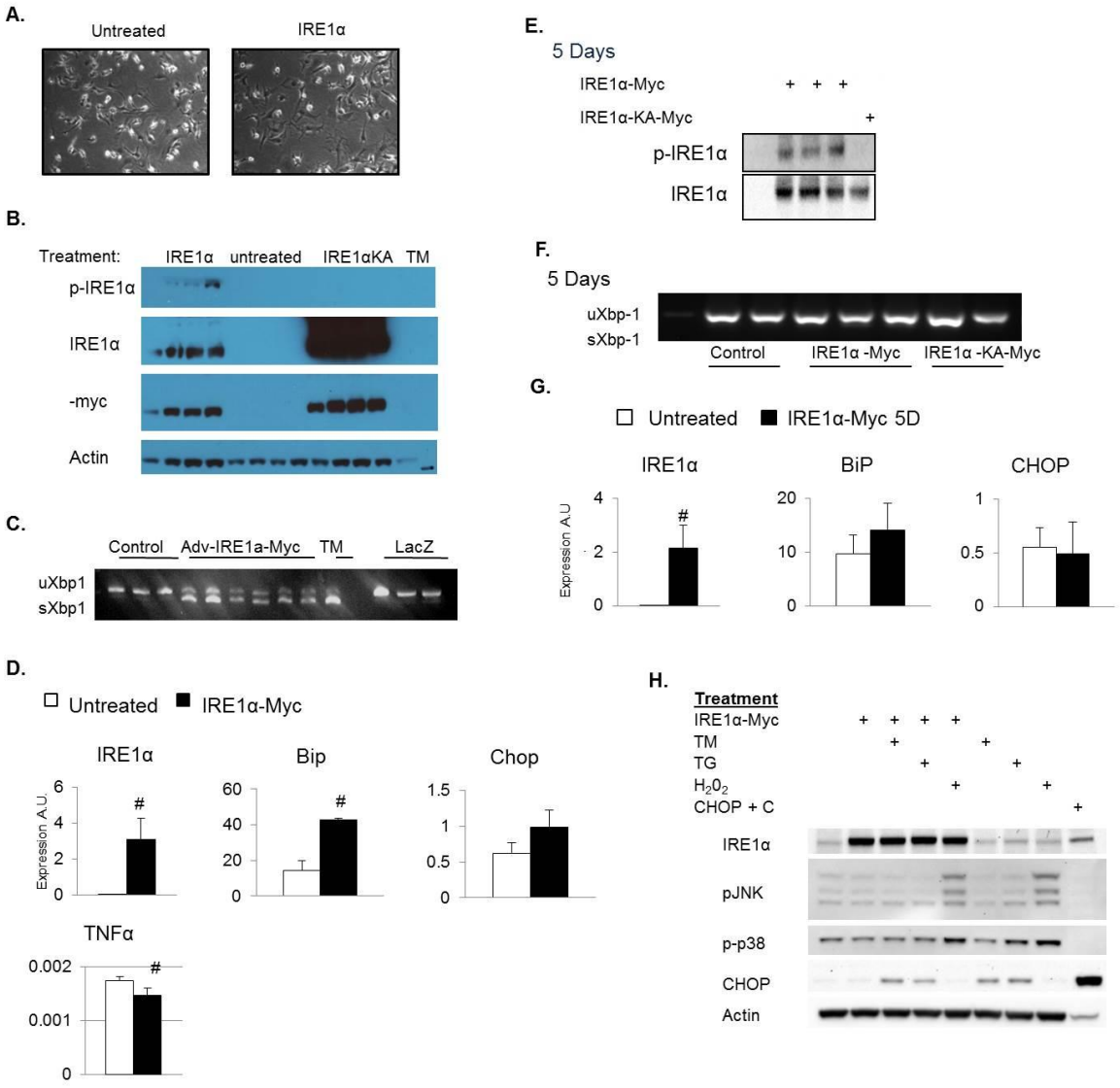

Figure 4-3. IRE1α Induces Adaptive UPR Signaling in NRVM. A. Representative images of NRVMs viewed at 20X magnification two days after treatment. B. Western blot of IRE1α expression and kinase activation (p-IRE1α Ser724). C. Xbp1 PCR splicing assay to assess IRE1α RNase activation in NRVMs. D. RT-PCR to measure IRE1α expression and adaptive and apoptotic downstream UPR signaling. Significant differences ($p<0.05$) compared to control are indicated by #. E. NRVMs were treated with Adv-IRE1α-Myc, nothing, or Adv-IRE1α-KA-Myc for five days before harvesting. Western blot of IRE1α protein expression and sustained activation by phosphorylation. F. Xbp1 splicing PCR assay. NRVMs were treated as in A and total RNA was collected after five days. G. RT-PCR measuring UPR induction after five days of IRE1α overexpression in NRVMs. H. IRE1α does not induce stress signaling or full UPR in the absence of stress. Western blot of total protein from NRVMs treated as indicated. TM, tunicamycin 5ųg/mL 4 hours, TG, thapsigargin 100nM 4 hours, H_20_2, 10 ųM 30 minutes.

In order to understand if IRE1α could protect against specific ER stress insults, we treated cells with various forms of ER stress in the presence of absence of IRE1α expression (Figure 4-3H). We measured MAPK signaling activation by western blot. IRE1α did not activate any stress signaling on its own. Cells with IRE1α had reduced activation of JNK when challenged with ER stress, whereas p-p38 activation and CHOP were not markedly reduced.

CHAPTER 5

Discussion

The Unfolded Protein Response is an important cellular response to various insults and is activated in human heart failure (49), ischemic heart disease (47) and heart failure in response to the cancer drug imatinib (52). Recent experimental models have recapitulated these observations (48-49) but it is unclear whether ER stress signaling contributes to protective (48, 54-55) or pathological signaling to heart (50, 52, 57, 73).

UPR signaling can be cardioprotective (48) or deleterious (55, 57). An animal model with constitutive activation of ATF6 had strong protection against a model of ischemia reperfusion. CHOP, on the other hand, is a strong inducer of apoptosis in cardiac myocytes as CHOP deletion protected mouse hearts against apoptosis in ischemia/reperfusion injury (57). Chop deletion in NRVMs was also protective against apoptosis in the setting of ER stress by proteasome inhibition (55). IRE1α is capable of both protective and apoptotic signaling through XBP1 or TRAF2 and MAPK signaling cascades, respectively. IRE1α preserved heart function and adaptive ER stress signaling in response to pressure overload insult. Here we report the molecular events in vitro that may underlie the protective phenotype in vivo.

We sought to uncover a role for IRE1α in whole heart and so generated a mouse model with heart-specific, temporally regulated IRE1α overexpression. Like others, we observed that IRE1α overexpression in vitro caused cell death in several cell lines (31).

In whole heart, IRE1α overexpression did not lead to change in heart structure or function and induced slight upregulation in adaptive molecule BiP. This led us to hypothesize that, in cardiac myocytes, IRE1α protective signaling may be preferentially activated in the absence of true ER stress and that IRE1α may prevent cardiac injury.

Preconditioning has been shown to improve outcomes in response to acute injury in kidney (74), eye (75), neurons (76), and heart (48). Here, we demonstrate that IRE1α protects the heart against pressure-overload injury. We observed that IRE1α mice had preserved heart function and myocardial structure in response to pressure-overload. Fibrosis and inflammatory signaling are known to be significant contributors to left ventricular remodeling. We tested whether expression levels of inflammatory cytokines were reduced. Indeed, both TNFα and IL-6 mRNA expression was strongly reduced in IRE1α animals following TAC. TNFα signaling is induced by p38 in heart (58). We were unable to detect an increase in p-p38 in IRE1α hearts after TAC, suggesting this signaling pathway is not preferentially activated downstream of IRE1α in our model. Thus, our results reveal a new intersection between IRE1α and inflammatory signaling pathways. Integration between ER, inflammation, and MAPK signaling networks is not fully characterized.

In order to uncover the molecular mechanism of IRE1α-mediated cardioprotection, we investigated IRE1α signaling in NRVMs. IRE1α specifically induced downstream adaptive UPR signaling, including activation of Xbp1 splicing and Bip mRNA expression. These observations were reconstituted in diverse cell types, including 293 and INS-1. On the other hand, apoptotic signaling pathways were never induced by IRE1α, even though NRVMs were sensitive to ER stress. Even sustained

IRE1α kinase activation did not lead to morphological abnormality (data not shown) or induction of apoptotic Chop.

The data presented here suggest a protective role for IRE1α. In heart, IRE1α was cardioprotective against a pressure overload. It is unknown if IRE1α will provide a similar protective role in acute injuries, such as ischemia/reperfusion or myocardial infarction. Moreover, this study highlights the need for greater understanding of integration between UPR, MAPK, and inflammatory signaling pathways. Also, the data presented herein suggests the presence of cardiac specific IRE1α regulatory mechanisms that are yet to be characterized.

CHAPTER 6

Future Directions

ER stress is gaining attention as an important factor underlying many diseases, including heart diseases (10, 78). Understanding the biology of ER stress signaling may identify novel therapeutic targets (59). ER stress signaling has been described as protective (48, 54, 55) or detrimental to heart (50, 52, 57, 73). Stress signaling from the Endoplasmic Reticulum is a critical component of cellular homeostasis.

Unfolded Protein Response signaling by IRE1α is highly conserved from yeast to mammals and can have drastic protective or apoptotic effects. IRE1α overexpression was detrimental to many cell types, but not NRVM. IRE1α-mediated cardioprotection to chronic pressure-overload suggests that signaling activities and regulation specific to cardiac myocytes may exist and are not yet identified.

Additional characterization of IRE1α-mediated UPR signaling in cardiomyocytes is needed in order to advance the understanding of ER biology. Investigations of highest priority are to identify heart-specific IRE1α interaction partners. Because endogenous IRE1α expression is very low and not measurable by standard western blot, an aproach where IRE1α is overexpressed in cardiac myocytes in vitro would be required. IRE1α-Myc would be overexpressed in vitro and IRE1α-Myc protein complex pulled out by -Myc antibody-directed immunoprecipitation. Alternatively, this experiment could be conducted using adult cardiac myocytes from animals where IRE1α is overexpressed, assuming that the process by which the myocytes are collected from the heart does not

activate ER stress signaling pathways. Proteins from the IRE1α-Myc complex would then be separated by electrophoresis and specific proteins would be identified by mass spectrometry. This approach has already been accomplished by members of our laboratory to identify IRE1α interacting partners in 293 cells, providing the reagents, technical strategy, and also the dataset to compare IRE1α interacting partners in 293 cells versus cardiac myocytes. This investigation would be critical for identifying the molecular signaling that contributes the heart-specific role for IRE1α.

The current investigation identified a new, protective role for IRE1α in heart. Unlike in 293 and INS-1 cells, NRVMs did not have detrimental phenotype following IRE1α overexpression. In search of the molecular mechanisms underlying this observation, we discovered that during extended overexpression, IRE1α remained activated by phosphorylation independent of the RNase. Even though abundant phospho-IRE1α could be detected, no spliced Xbp1 could be observed, suggesting that IRE1α kinase and RNase activities could be uncoupled. Alternatively, this result could be indicative of an unknown cytosolic regulator of IRE1α RNase. The experiment described above to characterize IRE1α interacting partners may provide insight into the cytosolic regulation of IRE1α RNase activity.

It is also possible that IRE1α has activity toward mRNA targets in addition to Xbp1 in NRVMs. Future investigations into IRE1α regulation would test these hypotheses. It is likely that no single mechanism fully explains the diverse cellular behaviors that are observed.

IRE1α overexpression in vitro was reported to cause cell death in several cell lines (reported here and reference (31)). We sought to uncover a role for IRE1α in whole heart. In pursuit of this, we generated a mouse model with heart-specific, temporally regulated IRE1α overexpression. We did not observe any adverse phenotype following IRE1α overexpression in heart in adult mice. This led us to hypothesize that IRE1α protective signaling may be preferentially activated in the absence of true ER stress in heart. We found that downstream BiP expression was slightly higher in IRE1α animals, and thus may act as preconditioning against cardiac injury.

Preconditioning, or priming, specific cell signaling pathways has been shown to be cardioprotective against ischemia/reperfusion injury in mouse models (79). Preconditioning by activating UPR pathways has been shown to protect neurons, kidney, and retina against acute injury (74-76). We found that IRE1α overexpression provided protection against a chronic pressure overload stress model in a manner similar to preconditioning. Future studies into cardioprotection by IRE1α will address the question of whether this protective effect is also observed in an acute injury such as ischemia/ reperfusion. Data reported here indicates that even in the setting of acute ER stress in vitro, IRE1α did not induce detrimental signaling; suggesting that the cardioprotected phenotype observed following chronic pressure overload may also be seen in response to acute injury.

IRE1α is capable of both protective and apoptotic signaling, either through XBP1 or TRAF2 and MAPK signaling cascades, respectively. XBP1, with ATF6, upregulates BiP in response to ER stress thereby providing adaptation to ER stress and protection

from CHOP mediated cell death (55). In contrast, IRE1α interactions with TRAF2 allow complex formation with JNK in vitro (40). Roles for TRAF2 and JNK in the heart have been interrogated (independent of IRE1α), but it is unclear how the volatile and diverse activities of JNK contribute to IRE1α downstream signaling. It is also unknown if the reported interactions of various molecules with IRE1α, including JNK, are recapitulated in heart in vitro or in vivo.

Although the molecular mechanisms of IRE1α activation have been known for many years, many questions remain. Endogenous IRE1α protein expression is reported to be very low, and is not detected by standard western blot (19). Because of this, characterization of the molecular identity and interaction partners of IRE1α requires model systems where IRE1α is overexpressed. IRE1α overexpression can drive activation in the absence of ER stress, thus potentially forcing interactions in vitro which do not occur in vivo.

Additional cytosolic activation states have been reported, but it is unknown if these activities occur in vivo. IRE1α may possess additional RNase activity directed toward a library of mRNAs for degradation (80). It has been proposed that this mRNA degradation is part of the adaptive ER stress response and represents an additional mechanism by which IRE1α relieves ER stress by reducing protein folding load. Alternatively, this could also be part of the cell death program where self-destructive activities disrupt cell homeostasis so that apoptosis pathways will become activated. It is unknown if these pathways exist. These conflicting hypotheses are yet to be investigated.

Molecular characterization of IRE1α activation has been limited by inherent properties of IRE1α. Unique properties of IRE1α, such as having an N-terminus within the highly specialized ER lumen environment, a transmembrane domain, and an RNase domain within the cytosol creates a technical challenge that, so far, has prevented accurate crystallization. Partial structures have been reported with the cytosolic domain in complex with small molecules but arrive at conflicting conclusions due to the different IRE1α partial peptides that are used for crystallization. This underscores the structural complexity of this conserved stress sensor. Until full-length, native IRE1α is characterized, the true molecular nature of IRE1α activation and regulation will not be fully understood. Further, a full- length IRE1α crystal structure would allow investigation into the interaction between BiP and IRE1α within the ER lumen and provide additional information about IRE1α activation and inactivation in response to ER stress.

Currently, experimental limitations prevent true understanding of ER stress within the ER lumen. Experimental manipulations to investigate properties of the ER lumen inherently disturb the highly specialized environment and, thus, present a fundamental challenge. New imaging and calcium sensor tools can be used to visualize properties of the ER lumen, but because they require overexpression of recombinant fluorescent proteins, do not actually portray a physiologic state. Therefore, innovative research techniques and strategies will be required to advance understanding of true ER biology.

Fibrosis and inflammatory signaling is known to be a significant contributor to left ventricular remodeling. Because we saw preserved function in IRE1α animals, we tested whether inflammatory cytokine expression was reduced. Indeed, both TNFα and IL-6 mRNA expression was strongly reduced in IRE1α animals following TAC compared

to controls or IRE1α animals at baseline. P38 has been reported to induce TNFα signaling in heart. We were unable to detect any increase in p-p38 in IRE1α hearts after TAC, suggesting this signaling pathway may not be activated downstream of IRE1α in our model. Further investigations into signal integration between UPR and inflammatory pathways will identify additional intersections and regulatory mechanisms.

Although countless investigations have characterized the highly conserved UPR signaling pathway, many questions remain. Molecular, cellular, and integrative biology of the ER contributes to health and disease. Advancing the understanding of UPR signaling from the ER will likely drive development of new therapeutic strategies for new drugs to treat many diseases, including heart failure.

CHAPTER 7

References

1. Roger VL, Go AS, Lloyd-Jones DM, Benjamin EJ, Berry JD, Borden WB, et al. Heart disease and stroke statistics--2012 update: a report from the American Heart Association. Circulation. 2012 Jan 3;125(1):e2-e220.

2. Mudd JO, Kass DA. Tackling heart failure in the twenty-first century. Nature. 2008 Feb 21;451(7181):919-28.

3. Glembotski CC. Endoplasmic reticulum stress in the heart. Circ Res. 2007 Nov 9;101(10):975-84.

4. Ellgaard L, Helenius A. Quality control in the endoplasmic reticulum. Nat Rev Mol Cell Biol. 2003 Mar;4(3):181-91.

5. Freedman RB, Dunn AD, Ruddock LW. Protein folding: a missing redox link in the endoplasmic reticulum. Curr Biol. 1998 Jun 18;8(13):R468-70.

6. Groenendyk J, Michalak M. Endoplasmic reticulum quality control and apoptosis. Acta Biochim Pol. 2005;52(2):381-95.

7. Bulleid NJ. Disulfide bond formation in the mammalian endoplasmic reticulum. Cold Spring Harb Perspect Biol. 2012 Nov;4(11).

8. Ron D, Harding HP. Protein-folding homeostasis in the endoplasmic reticulum and nutritional regulation. Cold Spring Harb Perspect Biol. 2012 Dec;4(12).

9. Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. Cell Death Differ. 2006 Mar;13(3):385-92.

10. Marciniak SJ, Ron D. Endoplasmic reticulum stress signaling in disease. Physiol Rev. 2006 Oct;86(4):1133-49.

11. Zhang K, Kaufman RJ. The unfolded protein response: a stress signaling pathway critical for health and disease. Neurology. 2006 Jan 24;66(2 Suppl 1):S102-9.

12. Kim I, Xu W, Reed JC. Cell death and endoplasmic reticulum stress: disease relevance and therapeutic opportunities. Nat Rev Drug Discov. 2008 Dec;7(12):1013-30.

13. Janero DR, Hreniuk D, Sharif HM. Hydrogen peroxide-induced oxidative stress to the mammalian heart-muscle cell (cardiomyocyte): lethal peroxidative membrane injury. J Cell Physiol. 1991 Dec;149(3):347-64.

14. Schroder M, Kaufman RJ. ER stress and the unfolded protein response. Mutat Res. 2005 Jan 6;569(1-2):29-63.

15. Mori K, Kawahara T, Yoshida H, Yanagi H, Yura T. Signalling from endoplasmic reticulum to nucleus: transcription factor with a basic-leucine zipper motif is required for the unfolded protein-response pathway. Genes Cells. 1996 Sep;1(9):803-17.

16. Tirasophon W, Welihinda AA, Kaufman RJ. A stress response pathway from the endoplasmic reticulum to the nucleus requires a novel bifunctional protein kinase/endoribonuclease (Ire1p) in mammalian cells. Genes Dev. 1998 Jun 15;12(12):1812-24.

17. Shen X, Zhang K, Kaufman RJ. The unfolded protein response--a stress signaling pathway of the endoplasmic reticulum. J Chem Neuroanat. 2004 Sep;28(1-2):79-92.

18. DuRose JB, Tam AB, Niwa M. Intrinsic capacities of molecular sensors of the unfolded protein response to sense alternate forms of endoplasmic reticulum stress. Mol Biol Cell. 2006 Jul;17(7):3095-107.

19. Bertolotti A, Zhang Y, Hendershot LM, Harding HP, Ron D. Dynamic interaction of BiP and ER stress transducers in the unfolded-protein response. Nat Cell Biol. 2000 Jun;2(6):326-32.

20. Lee AS. The ER chaperone and signaling regulator GRP78/BiP as a monitor of endoplasmic reticulum stress. Methods. 2005 Apr;35(4):373-81.

21. Wang Y, Shen J, Arenzana N, Tirasophon W, Kaufman RJ, Prywes R. Activation of ATF6 and an ATF6 DNA binding site by the endoplasmic reticulum stress response. J Biol Chem. 2000 Sep 1;275(35):27013-20.

22. Kaufman RJ, Pipe SW, Tagliavacca L, Swaroop M, Moussalli M. Biosynthesis, assembly and secretion of coagulation factor VIII. Blood Coagul Fibrinolysis. 1997 Dec;8 Suppl 2:S3-14.

23. Haze K, Yoshida H, Yanagi H, Yura T, Mori K. Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. Mol Biol Cell. 1999 Nov;10(11):3787-99.

24. Nikawa J, Yamashita S. IRE1 encodes a putative protein kinase containing a membrane-spanning domain and is required for inositol phototrophy in Saccharomyces cerevisiae. Mol Microbiol. 1992 Jun;6(11):1441-6.

25. Shamu CE, Walter P. Oligomerization and phosphorylation of the Ire1p kinase during intracellular signaling from the endoplasmic reticulum to the nucleus. EMBO J. 1996 Jun 17;15(12):3028-39.

26. Koumenis C, Naczki C, Koritzinsky M, Rastani S, Diehl A, Sonenberg N, et al. Regulation of protein synthesis by hypoxia via activation of the endoplasmic reticulum kinase PERK and phosphorylation of the translation initiation factor eIF2alpha. Mol Cell Biol. 2002 Nov;22(21):7405-16.

27. Yoshida H, Matsui T, Yamamoto A, Okada T, Mori K. XBP1 mRNA is induced by ATF6 and spliced by IRE1 in response to ER stress to produce a highly active transcription factor. Cell. 2001 Dec 28;107(7):881-91.

28. Calfon M, Zeng H, Urano F, Till JH, Hubbard SR, Harding HP, et al. IRE1 couples endoplasmic reticulum load to secretory capacity by processing the XBP-1 mRNA. Nature. 2002 Jan 3;415(6867):92-6.

29. Lee K, Tirasophon W, Shen X, Michalak M, Prywes R, Okada T, et al. IRE1-mediated unconventional mRNA splicing and S2P-mediated ATF6 cleavage merge to regulate XBP1 in signaling the unfolded protein response. Genes Dev. 2002 Feb 15;16(4):452-66.

30. Harding HP, Zhang Y, Ron D. Protein translation and folding are coupled by an endoplasmic-reticulum-resident kinase. Nature. 1999 Jan 21;397(6716):271-4.

31. Wang XZ, Harding HP, Zhang Y, Jolicoeur EM, Kuroda M, Ron D. Cloning of mammalian Ire1 reveals diversity in the ER stress responses. EMBO J. 1998 Oct 1;17(19):5708-17.

32. Liu CY, Schroder M, Kaufman RJ. Ligand-independent dimerization activates the stress response kinases IRE1 and PERK in the lumen of the endoplasmic reticulum. J Biol Chem. 2000 Aug 11;275(32):24881-5.

33. Okamura K, Kimata Y, Higashio H, Tsuru A, Kohno K. Dissociation of Kar2p/BiP from an ER sensory molecule, Ire1p, triggers the unfolded protein response in yeast. Biochem Biophys Res Commun. 2000 Dec 20;279(2):445-50.

34. Kimata Y, Kimata YI, Shimizu Y, Abe H, Farcasanu IC, Takeuchi M, et al. Genetic evidence for a role of BiP/Kar2 that regulates Ire1 in response to accumulation of unfolded proteins. Mol Biol Cell. 2003 Jun;14(6):2559-69.

35. Welihinda AA, Kaufman RJ. The unfolded protein response pathway in Saccharomyces cerevisiae. Oligomerization and trans-phosphorylation of Ire1p (Ern1p) are required for kinase activation. J Biol Chem. 1996 Jul 26;271(30):18181-7.

36. Aragon T, van Anken E, Pincus D, Serafimova IM, Korennykh AV, Rubio CA, et al. Messenger RNA targeting to endoplasmic reticulum stress signalling sites. Nature. 2009 Feb 5;457(7230):736-40.

37. Uemura A, Oku M, Mori K, Yoshida H. Unconventional splicing of XBP1 mRNA occurs in the cytoplasm during the mammalian unfolded protein response. J Cell Sci. 2009 Aug 15;122(Pt 16):2877-86.

38. Kadowaki H, Nishitoh H, Ichijo H. Survival and apoptosis signals in ER stress: the role of protein kinases. J Chem Neuroanat. 2004 Sep;28(1-2):93-100.

39. Kim R, Emi M, Tanabe K, Murakami S. Role of the unfolded protein response in cell death. Apoptosis. 2006 Jan;11(1):5-13.

40. Urano F, Wang X, Bertolotti A, Zhang Y, Chung P, Harding HP, et al. Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science. 2000 Jan 28;287(5453):664-6.

41. Zhang C, Kawauchi J, Adachi MT, Hashimoto Y, Oshiro S, Aso T, et al. Activation of JNK and transcriptional repressor ATF3/LRF1 through the IRE1/TRAF2 pathway is implicated in human vascular endothelial cell death by homocysteine. Biochem Biophys Res Commun. 2001 Dec 7;289(3):718-24.

42. Hetz C, Bernasconi P, Fisher J, Lee AH, Bassik MC, Antonsson B, et al. Proapoptotic BAX and BAK modulate the unfolded protein response by a direct interaction with IRE1alpha. Science. 2006 Apr 28;312(5773):572-6.

43. Ogata M, Hino S, Saito A, Morikawa K, Kondo S, Kanemoto S, et al. Autophagy is activated for cell survival after endoplasmic reticulum stress. Mol Cell Biol. 2006 Dec;26(24):9220-31.

44. Blais JD, Filipenko V, Bi M, Harding HP, Ron D, Koumenis C, et al. Activating transcription factor 4 is translationally regulated by hypoxic stress. Mol Cell Biol. 2004 Sep;24(17):7469-82.

45. Zhang K, Kaufman RJ. Signaling the unfolded protein response from the endoplasmic reticulum. J Biol Chem. 2004 Jun 18;279(25):25935-8.

46. Oyadomari S, Mori M. Roles of CHOP/GADD153 in endoplasmic reticulum stress. Cell Death Differ. 2004 Apr;11(4):381-9.

47. Azfer A, Niu J, Rogers LM, Adamski FM, Kolattukudy PE. Activation of endoplasmic reticulum stress response during the development of ischemic heart disease. Am J Physiol Heart Circ Physiol. 2006 Sep;291(3):H1411-20.

48. Martindale JJ, Fernandez R, Thuerauf D, Whittaker R, Gude N, Sussman MA, et al. Endoplasmic reticulum stress gene induction and protection from ischemia/reperfusion injury in the hearts of transgenic mice with a tamoxifen-regulated form of ATF6. Circ Res. 2006 May 12;98(9):1186-93.

49. Okada K, Minamino T, Tsukamoto Y, Liao Y, Tsukamoto O, Takashima S, et al. Prolonged endoplasmic reticulum stress in hypertrophic and failing heart after aortic constriction: possible contribution of endoplasmic reticulum stress to cardiac myocyte apoptosis. Circulation. 2004 Aug 10;110(6):705-12.

50. Terai K, Hiramoto Y, Masaki M, Sugiyama S, Kuroda T, Hori M, et al. AMP-activated protein kinase protects cardiomyocytes against hypoxic injury through attenuation of endoplasmic reticulum stress. Mol Cell Biol. 2005 Nov;25(21):9554-75.

51. Stadtman ER. Role of oxidant species in aging. Curr Med Chem. 2004 May;11(9):1105-12.

52. Kerkela R, Grazette L, Yacobi R, Iliescu C, Patten R, Beahm C, et al. Cardiotoxicity of the cancer therapeutic agent imatinib mesylate. Nat Med. 2006 Aug;12(8):908-16.

53. Cook AR, Bardswell SC, Pretheshan S, Dighe K, Kanaganayagam GS, Jabr RI, et al. Paradoxical resistance to myocardial ischemia and age-related cardiomyopathy in NHE1 transgenic mice: a role for ER stress? J Mol Cell Cardiol. 2009 Feb;46(2):225-33.

54. Thuerauf DJ, Marcinko M, Gude N, Rubio M, Sussman MA, Glembotski CC. Activation of the unfolded protein response in infarcted mouse heart and hypoxic cultured cardiac myocytes. Circ Res. 2006 Aug 4;99(3):275-82.

55. Fu HY, Minamino T, Tsukamoto O, Sawada T, Asai M, Kato H, et al. Overexpression of endoplasmic reticulum-resident chaperone attenuates cardiomyocyte death induced by proteasome inhibition. Cardiovasc Res. 2008 Sep 1;79(4):600-10.

56. Zhang K. Integration of ER stress, oxidative stress and the inflammatory response in health and disease. Int J Clin Exp Med. 2010;3(1):33-40.

57. Miyazaki Y, Kaikita K, Endo M, Horio E, Miura M, Tsujita K, et al. C/EBP homologous protein deficiency attenuates myocardial reperfusion injury by inhibiting myocardial apoptosis and inflammation. Arterioscler Thromb Vasc Biol. 2011 May;31(5):1124-32.

58. Li M, Georgakopoulos D, Lu G, Hester L, Kass DA, Hasday J, et al. p38 MAP kinase mediates inflammatory cytokine induction in cardiomyocytes and extracellular matrix remodeling in heart. Circulation. 2005 May 17;111(19):2494-502.

59. Toth A, Nickson P, Mandl A, Bannister ML, Toth K, Erhardt P. Endoplasmic reticulum stress as a novel therapeutic target in heart diseases. Cardiovasc Hematol Disord Drug Targets. 2007 Sep;7(3):205-18.

60. Liao P, Georgakopoulos D, Kovacs A, Zheng M, Lerner D, Pu H, et al. The in vivo role of p38 MAP kinases in cardiac remodeling and restrictive cardiomyopathy. Proc Natl Acad Sci U S A. 2001 Oct 9;98(21):12283-8.

61. Petrich BG, Molkentin JD, Wang Y. Temporal activation of c-Jun N-terminal kinase in adult transgenic heart via cre-loxP-mediated DNA recombination. FASEB J. 2003 Apr;17(6):749-51.

62. Sohal DS, Nghiem M, Crackower MA, Witt SA, Kimball TR, Tymitz KM, et al. Temporally regulated and tissue-specific gene manipulations in the adult and embryonic heart using a tamoxifen-inducible Cre protein. Circ Res. 2001 Jul 6;89(1):20-5.

63. Wang Y, Huang S, Sah VP, Ross J, Jr., Brown JH, Han J, et al. Cardiac muscle cell hypertrophy and apoptosis induced by distinct members of the p38 mitogen-activated protein kinase family. J Biol Chem. 1998 Jan 23;273(4):2161-8.

64. Ota A, Wang Y. Cdc37/Hsp90 protein-mediated regulation of IRE1alpha protein activity in endoplasmic reticulum stress response and insulin synthesis in INS-1 cells. J Biol Chem. 2012 Feb 24;287(9):6266-74.

65. Iwaki K, Sukhatme VP, Shubeita HE, Chien KR. Alpha- and beta-adrenergic stimulation induces distinct patterns of immediate early gene expression in neonatal rat myocardial cells. fos/jun expression is associated with sarcomere assembly; Egr-1 induction is primarily an alpha 1-mediated response. J Biol Chem. 1990 Aug 15;265(23):13809-17.

66. Ishikawa TO, Herschman HR. Conditional knockout mouse for tissue-specific disruption of the cyclooxygenase-2 (Cox-2) gene. Genesis. 2006 Mar;44(3):143-9.

67. Hayashi S, McMahon AP. Efficient recombination in diverse tissues by a tamoxifen-inducible form of Cre: a tool for temporally regulated gene activation/inactivation in the mouse. Dev Biol. 2002 Apr 15;244(2):305-18.

68. Back SH, Lee K, Vink E, Kaufman RJ. Cytoplasmic IRE1alpha-mediated XBP1 mRNA splicing in the absence of nuclear processing and endoplasmic reticulum stress. J Biol Chem. 2006 Jul 7;281(27):18691-706.

69. Kolattukudy PE, Niu J. Inflammation, endoplasmic reticulum stress, autophagy, and the monocyte chemoattractant protein-1/CCR2 pathway. Circ Res. 2012 Jan 6;110(1):174-89.

70. Rockman HA, Ross RS, Harris AN, Knowlton KU, Steinhelper ME, Field LJ, et al. Segregation of atrial-specific and inducible expression of an atrial natriuretic factor transgene in an in vivo murine model of cardiac hypertrophy. Proc Natl Acad Sci U S A. 1991 Sep 15;88(18):8277-81.

71. Hu P, Han Z, Couvillon AD, Kaufman RJ, Exton JH. Autocrine tumor necrosis factor alpha links endoplasmic reticulum stress to the membrane death receptor pathway through IRE1alpha-mediated NF-kappaB activation and down-regulation of TRAF2 expression. Mol Cell Biol. 2006 Apr;26(8):3071-84.

72. Balakrishnan B, Sen D, Hareendran S, Roshini V, David S, Srivastava A, et al. Activation of the cellular unfolded protein response by recombinant adeno-associated virus vectors. PLoS One. 2013;8(1):e53845.

73. Isodono K, Takahashi T, Imoto H, Nakanishi N, Ogata T, Asada S, et al. PARM-1 is an endoplasmic reticulum molecule involved in endoplasmic reticulum stress-induced apoptosis in rat cardiac myocytes. PLoS One. 2010;5(3):e9746.

74. Inagi R, Kumagai T, Nishi H, Kawakami T, Miyata T, Fujita T, et al. Preconditioning with endoplasmic reticulum stress ameliorates mesangioproliferative glomerulonephritis. J Am Soc Nephrol. 2008 May;19(5):915-22.

75. Li J, Wang JJ, Zhang SX. Preconditioning with endoplasmic reticulum stress mitigates retinal endothelial inflammation via activation of X-box binding protein 1. J Biol Chem. 2011 Feb 11;286(6):4912-21.

76. Hayashi T, Saito A, Okuno S, Ferrand-Drake M, Chan PH. Induction of GRP78 by ischemic preconditioning reduces endoplasmic reticulum stress and prevents delayed neuronal cell death. J Cereb Blood Flow Metab. 2003 Aug;23(8):949-61.

77. Iwawaki T, Akai R, Yamanaka S, Kohno K. Function of IRE1 alpha in the placenta is essential for placental development and embryonic viability. Proc Natl Acad Sci U S A. 2009 Sep 29;106(39):16657-62.

78. Groenendyk J, Sreenivasaiah PK, Kim do H, Agellon LB, Michalak M. Biology of endoplasmic reticulum stress in the heart. Circ Res. 2010 Nov 12;107(10):1185-97.

79. Amsterdam EA, Schaefer S. Ischemic preconditioning in coronary heart disease: a therapeutic golden fleece? J Am Coll Cardiol. 2004 May 5;43(9):1515-6.

80. Hollien J, Lin JH, Li H, Stevens N, Walter P, Weissman JS. Regulated Ire1-dependent decay of messenger RNAs in mammalian cells. J Cell Biol. 2009 Aug 10;186(3):323-31.