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Heavy Metal Scavenger Metallothionein Attenuates ER Stress-Induced Myocardial Contractile Anomalies: Role of Autophagy

Lifang Yang^{1,2,*}, Nan Hu^{2,*}, Shasha Jiang^{2,3}, Yunzeng Zou³, Jian Yang⁴, Lize Xiong¹, and Jun Ren²

¹Department of Anesthesiology, Xijing Hospital, the Fourth Military Medical University, Xi'an, China 710032

²Center for Cardiovascular Research and Alternative Medicine, University of Wyoming, Laramie, WY 82071 USA

³Institute of Biomedical Sciences, Fudan University, Shanghai, China 200032

⁴Department of Cardiac Surgery, Xijing Hospital, the Fourth Military Medical University, Xi'an, China 710032

Abstract

Endoplasmic reticulum (ER) stress increases the risk of cardiovascular morbidity and mortality although the underlying mechanism remains elusive. This study was designed to examine the impact of cardiac over-expression of metallothionein, a cysteine-rich heavy metal scavenger, on ER stress-induced changes in myocardial function and underlying mechanism involved with a focus on autophagy. Wild-type friendly virus B (FVB) and metallothionein transgenic mice were subjected to the ER stress inducer tunicamycin (1 mg/kg). Our results showed that ER stress led to compromised echocardiographic and cardiomyocyte contractile function, intracellular Ca^{2+} mishandling. Tunicamycin promoted ER stress and oxidative stress, increased left ventricular end systolic and diastolic diameter, as well as suppressed fractional shortening and whole heart contractility, the effects of which were significantly attenuated or ablated by metallothionein. Levels of the autophagy markers such as phosphorylated ULK1, Atg5, Atg7, LC3B and the autophagy adaptor p62 were significantly upregulated. These ER stress-induced changes in myocardial function, autophagy and autophagy signaling were distinctly mitigated or alleviated by metallothionein. Inhibition of autophagy using 3-methyladenine *in vitro* reversed ER stress-induced cardiomyocyte contractile defects. Meanwhile, ER stress-induced cardiomyocyte dysfunction was attenuated by the antioxidant N-acetylcysteine. Collectively, these findings suggested that metallothionein protects against ER stress-induced cardiac anomalies possibly through attenuation of cardiac autophagy.

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Correspondence should be addressed to: Dr. Jun Ren, University of Wyoming College of Health Sciences, Laramie, WY 82071, USA; Tel: (307)-766-6131; Fax: (307)-766-2953; jren@uwyo.edu.

*These two authors contributed equally.

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Keywords

ER stress; Metallothionein; Tunicamycin; Cardiac function; Autophagy

Introduction

Endoplasmic reticulum (ER) denotes an extensive intracellular membranous network, governing a cascade of biological events such as Ca^{2+} storage, Ca^{2+} signaling, glycosylation and trafficking of newly-synthesized membrane and secretory proteins. It was demonstrated that perturbations of these processes by chemical triggers such as tunicamycin might interfere with the proper functioning of ER, thus creating a condition defined as ER stress (Haynes et al., 2004; Travers et al., 2000; Yoshida, 2007). Ample of studies have revealed that ER stress prompts the activation of a complex signaling network, namely unfolded protein response (UPR). ER stress and UPR, if sustained, usually result in apoptotic cell death. Both clinical and experimental evidence has consolidated a role for ER stress in the onset and progression of a wide variety of diseases such as neurodegenerative disorders, diabetes mellitus and ischemic heart disease (Kraskiewicz and FitzGerald, 2012; Logue et al., 2013). Similar to ER stress, accumulation of reactive oxygen species (ROS) or oxidative stress, is closely associated with development of cardiovascular diseases (Dhalla et al., 2000). Oxidative stress is typically triggered by disturbed prooxidant-antioxidant balance and promotes progression of heart dysfunction. Latest evidence has shown that ER stress may be associated with ROS production (Liu et al., 2013; Menu et al., 2012). However, the precise mechanisms underscoring ER stress-induced cardiac anomalies have not been elucidated, making it difficult to develop chaperon or therapeutic intervention against ER stress-induced cardiac pathology. Recently, results from our lab as well as others had unveiled a rather complex interplay between ER stress and oxidative stress in cardiac morphological and functional defects under various disease conditions. In particular, ER stress seems to serve as both cause and consequence for production of ROS and redox deviation (Guo et al., 2009; Merksamer et al., 2008; Ozcan et al., 2006; Yoshida, 2007).

Metallothioneins belong to a family of intracellular, low molecular weight, cysteine-rich proteins. Ubiquitous in eukaryotes, metallothioneins possess unique structural characteristics to offer potent metal-binding and redox capabilities. The most widely expressed isoforms in mammals, metallothionein-1 and metallothionein-2, are rapidly induced in the liver by a wide range of metals, inflammatory mediators and drugs. Use of genetically engineered animal model has greatly improved our knowledge of the multifaceted role of metallothioneins (Kang et al., 1997) although much still remains unknown for the action of the heavy metal scavenger. To this end, the present study was designed to examine the effect of cardiac-specific over-expression of metallothionein on tunicamycin, an ER stress inducer, induced myocardial contractile dysfunction and intracellular Ca^{2+} mishandling. Generation of ROS was also monitored in an effort to better understand the mechanism of action involved in the metallothionein-elicited myocardial response against short-term tunicamycin exposure.

Materials and Methods

Experimental animals and induction of ER stress

All animal procedures described in this study were approved by the University of Wyoming Institutional Animal Use and Care Committee (Laramie, WY). In brief, 5–6-month-old adult male mice with a ten-fold cardiac-specific transgenic overexpression of the heavy metal scavenger driven by the mouse α -MHC promoter (Kang et al., 1997) and wild-type Friend virus B (FVB) mice were used. The fur color was employed as a marker to identify wild-type FVB littermates (white) and metallothionein (dark brown) mice. All mice were maintained at 22°C with a 12/12-light/dark cycle and received lab chow and water ad libitum. A cohort of FVB and metallothionein mice was given the ER stress inducer tunicamycin (1 mg/kg, i.p.) once a day for two days (Kondoh et al., 2004; Roe and Ren, 2013; Zhang et al., 2011).

Echocardiographic assessment

Cardiac geometry and function were evaluated in anesthetized (80 mg/kg ketamine and 12 mg/kg xylazine, i.p.) mice using a two-dimensional guided M-mode echocardiography (Sonos 5500) equipped with a 15–6 MHz linear transducer. Left ventricular (LV) anterior and posterior wall dimensions during diastole (LVPWd) and systole (LVPWs) were recorded from three consecutive cycles in M-mode using the methods adopted by the American Society of Echocardiography. Fractional shortening was calculated from LV end-diastolic (LVEDD) and end-systolic diameters (LVESD) using the equation of $(LVEDD - LVESD)/LVEDD$. Estimated echocardiographic LV mass was calculated as $[(LVEDD + \text{septal wall thickness} + \text{posterior wall thickness})^3 - LVEDD^3] \times 1.055$, where 1.055 (mg/mm^3) is the density of myocardium. Heart rates were averaged over 10 consecutive cycles (Ren et al., 2008).

Isolation of murine cardiomyocytes

After ketamine/xylazine sedation, hearts were removed and perfused with Krebs-Henseleit bicarbonate (KHB) buffer containing (in mM): 135 NaCl, 4.0 KCl, 1.0 MgCl_2 , 10 HEPES, 0.33 NaH_2PO_4 , 10 glucose, 10 butanedione monoxime, and the solution was gassed with 5% CO_2 /95% O_2 . Hearts were isolated using liberase enzymatic digestion for 20 min. The digested heart was then removed from the cannula and the left ventricle was cut into small pieces in the modified Tyrode solution. Tissue pieces were gently agitated and pellet of cells was resuspended. Extracellular Ca^{2+} was added incrementally back to 1.20 mM over a period of 30 min. Isolated cardiomyocytes were used for study within 8 h of isolation. Normally, rod-shaped cardiomyocytes with clear sarcomere striations were selected for mechanical studies (Li et al, 2007).

Cell shortening/relengthening

Mechanical properties of cardiomyocytes were assessed using an IonOptix soft-edge system (IonOptix, Milton, MA) as described previously (Doser et al, 2009). Cardiomyocytes were placed in a chamber mounted on the stage of an Olympus IX-70 microscope and superfused (~ 2 ml/min at 25°C) with a Krebs-Henseleit bicarbonate buffer containing 1 mM CaCl_2 .

Cardiomyocytes were field stimulated at 0.5 Hz. Cell shortening and relengthening were assessed including peak shortening (PS), indicating peak contractility; time-to-PS (TPS), indicating contraction duration; time-to-90% relengthening (TR₉₀), indicating relaxation duration; and maximal velocities of shortening/relengthening ($\pm$ dL/dt), indicating maximal pressure development and decline (Fang et al, 2005). To assess the effect of tunicamycin (3 μ g/ml for 4 hrs) on cardiomyocyte contractile function and the underlying mechanism involved, freshly isolated murine cardiomyocytes from FVB mice were pretreated with the antioxidant *N*-acetylcysteine (NAC, 500 μ M) and the autophagy inhibitor 3-methyladenine (3-MA, 3 mM) for 4 hrs.

Intracellular Ca²⁺ transients

A cohort of cardiomyocytes was loaded with fura-2/AM (0.5 μ M) for 15 min, and fluorescence intensity was recorded with a dual-excitation fluorescence photomultiplier system (Ionoptix). Cardiomyocytes were placed onto an Olympus IX-70 inverted microscope and imaged through a Fluor $\times$ 40 oil objective. Cells were exposed to light emitted by a 75-W lamp while being stimulated to contract at a frequency of 0.5Hz. Fluorescence emissions were detected between 480 and 520 nm; qualitative change in fura-2 fluorescence intensity (FFI) was inferred from the fura-2 fluorescence intensity ratio at the two wavelengths (360/380). Fluorescence decay time (single exponential) was calculated as an indicator of intracellular Ca²⁺ clearance (Doser et al, 2009).

Analysis of ROS production

Cardiomyocytes were loaded with 10 μ M non-fluorescent dye 2, 7-dichlorodihydrofluorescein diacetate (H₂DCFDA, Molecular Probes, Eugene, OR) at 37°C for 30 min. After rinsing with the Krebs-Henseleit buffer (KHB), fluorescent intensity was measured using a fluorescent microplate reader at an excitation wavelength of 480 nm and an emission wavelength of 530 nm. ROS were detected by analyzing the fluorescence intensity of the intracellular fluoroprobe H₂DCFDA in isolated cardiomyocytes. Untreated cells showed no fluorescence and were used to determine the background fluorescence. The final fluorescent intensity was normalized to the protein content in each group (Zhang et al, 2011).

Western blot analysis

Pellets of cardiomyocytes were sonicated in a lysis buffer containing 20 mM Tris (pH 7.4), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 0.1% sodium dodecyl sulfate, 2nM NaF (phosphatase inhibitor), and a protease inhibitor cocktail. Protein levels of the ER stress markers GRE78, GADD153, eIF2 α , Phosphorylated eIF2 α , IRE1 α , Phosphorylated IRE1 α ; autophagy markers ULK1, Phosphorylated ULK1, Atg5, Atg7, LC3B and P62 were examined by standard Western immunoblotting. Membranes were probed with anti-GRE78 (1:1,000), anti-GADD153 (1:500), anti-eIF2 α (1:1,000), anti-Phosphorylated eIF2 α (peIF2 α ; SER⁵¹, 1:500), anti-IRE1 α (1:500), anti-Phosphorylated IRE1 α (pIRE1 α ; ser⁷²⁴, 1:1,000), anti-ULK1 (1:500), anti-Phosphorylated ULK1 (pULK1; ser⁷⁷⁷, 1:500), anti-Atg5 (1:500), anti-Atg7 (1:500), anti-LC3B (1:1,000), anti-p62 (1:1,000) and anti- α -tubulin (1:1,000, loading control) antibodies. Antibodies were purchased from Cell Signaling

Technology (Beverly, MA) or Santa Cruz Biotechnology (Santa Cruz, CA). The membranes were incubated with horseradish peroxidase-coupled secondary antibodies. After immunoblotting, the film was scanned and intensity of immunoblot bands was detected with a Bio-Rad Calibrated Densitometer (Zhang et al., 2011).

Statistical analysis

Data were expressed as Mean $\pm$ SEM. Statistical significance ($p < 0.05$) was estimated by one-way analysis of variation followed by a Tukey test for post hoc analysis.

Results

General features and echocardiographic properties of FVB and metallothionein mice with or without tunicamycin treatment

Treatment of tunicamycin (1 mg/kg, i.p.) for 48 hrs did not overtly affect body weights in either animal group. Tunicamycin significantly increased LVESD and LVEDD as well as suppressed fractional shortening. Metallothionein transgene overtly attenuated tunicamycin-induced changes in LVESD, LVEDD and fractional shortening, with little changes in any other geometric or functional parameters tested (Fig. 1).

Mechanical and intracellular Ca^{2+} properties of cardiomyocytes from FVB and metallothionein mice

Neither tunicamycin treatment (1 mg/kg, i.p.) nor metallothionein exhibited any overt effect on cell phenotype. Resting cell length was comparable between FVB and metallothionein mice. Tunicamycin remarkably increased resting cardiomyocyte cell length in FVB but not metallothionein mice. Cardiomyocytes from tunicamycin-treated FVB mice displayed significantly depressed peak shortening (PS), maximal velocity of shortening and relengthening ($\pm \text{dL/dt}$) as well as prolonged time-to-90% relengthening (TR_{90}) along with unchanged time-to-PS (TPS). Metallothionein protected against tunicamycin-elicited cardiomyocyte mechanical anomalies without eliciting any notable effect itself (Fig. 2). To explore the potential mechanism of actions involved in metallothionein-elicited beneficial effect in cardiomyocyte mechanical responses, intracellular Ca^{2+} homeostasis was evaluated using the fluorescence dye fura-2. Our results indicated that tunicamycin significantly decreased electrically-stimulated rise in intracellular Ca^{2+} and prolonged intracellular Ca^{2+} decay without affecting basal intracellular Ca^{2+} levels, the effects of which were nullified by metallothionein transgene. However, metallothionein transgene failed to affect intracellular Ca^{2+} properties by itself (Fig. 3).

Effect of metallothionein on tunicamycin-induced ROS generation and oxidative stress

To assess the antioxidant property of metallothionein against tunicamycin-induced cardiac injury, ROS generation and oxidative stress were examined using DCF fluorescence technique. Our data depicted that tunicamycin promoted ROS generation, consolidating a state of overt oxidative stress. As expected, metallothionein effectively alleviated tunicamycin-induced ROS generation and oxidative stress (Fig. 4).

Effect of metallothionein on tunicamycin-induced changes in ER stress and autophagy protein markers

Our result revealed significantly upregulated expression of the ER stress markers GRP78, Gadd153, phosphorylated eIF2 α and phosphorylated IRE1 α in tunicamycin-treated FVB murine hearts, the effects of which were obliterated by metallothionein. The metallothionein transgene itself did not affect levels of these ER stress protein markers (Fig. 5).

To explore the possible mechanism(s) behind metallothionein-induced protection against tunicamycin, expression of the autophagy markers ULK1 (total and phosphorylated Ser⁷⁷⁷ forms), Atg5, Atg7, LC3B, p62 were examined in FVB and metallothionein mice with or without tunicamycin challenge. Our results indicated that tunicamycin significantly upregulated ULK1 Ser⁷⁷⁷ phosphorylation (absolute or normalized value), Atg5, Atg7, LC3B-II, LC3B-II/LC3B-I ratio, and the autophagy adaptor protein p62. Metallothionein transgene itself did not affect the expression of these autophagy markers, while the heavy metal scavenger significantly attenuated or ablated tunicamycin-induced elevation in phosphorylated ULK1, Atg5, Atg7, LC3B-II, LC3B-II/LC3B-I ratio and p62 (Fig. 6).

Effect of antioxidant on tunicamycin-induced cardiomyocyte contractile responses

To further examine the causal relationship between autophagy, oxidative stress and mechanical response following tunicamycin challenge, we further examined the effect of tunicamycin on cardiomyocyte contractile function *in vitro* in the absence or presence of the antioxidant *N*-acetylcysteine (NAC, 500 μ M) and the autophagosome formation inhibitor 3-methyladenine (3-MA, 3 mM) prior to assessment of mechanical properties. Resting cell length was unaffected by tunicamycin, NAC or 3-MA. Similar to *in vivo* observations, tunicamycin exposure *in vitro* significantly decreased PS, $\pm$ dL/dt and prolonged TR₉₀ without affecting TPS. Although neither NAC nor 3-MA affected mechanical indices tested, both inhibitors effectively abolished tunicamycin-induced alterations in cardiomyocyte contractile responses (Fig 7).

Discussion

The salient findings from our study revealed that the cysteine-rich heavy metal scavenger metallothionein significantly attenuated tunicamycin-induced cardiac contractile dysfunction, intracellular Ca²⁺ homeostasis, ER stress, oxidative stress, and the induction of autophagy. These data favored the notion that ER stress is capable of promoting oxidative stress and autophagy *en route* to the onset and development of myocardial dysfunction.

Compromised cardiac contractility and prolonged diastolic duration are commonly seen in cardiac pathological conditions afflicted with ER stress (Guo et al, 2009; Li et al, 2009; Minamino and Kitakaze, 2010; Okada et al, 2004; Privratsky et al, 2003). Our study revealed that short-term tunicamycin exposure significantly increased LVEDD and LVESD, as well as reduced fractional shortening. Meanwhile, other studies prompt the notion of an unfavorable role of ER stress in cardiac homeostasis (Minamino and Kitakaze, 2010; Minamino et al, 2010; Okada et al, 2004), which is supported by our current echocardiographic findings of elevated LVESD, LVESD and reduced fractional shortening.

In our hands, tunicamycin prompted ER stress as evidenced by overtly elevated ER stress markers including GRE78, GADD153, phospho-IRE1 α and phospho-eIF2 α without affecting IRE1 α and eIF2 α . ER stress has been demonstrated to play a pivotal role in the pathogenesis of a number of cardiovascular diseases such as diabetes, ischemia-reperfusion injury and alcoholic cardiomyopathy (Li and Ren, 2008; Ozcan et al, 2006; Ron and Walter, 2007). Nonetheless, evidence is still lacking with regards to the precise interplay between oxidative stress and ER stress in the heart. Earlier studies from our lab indicated that fat-diet intake induces overt ER stress while ER chaperones protect the heart against ER stress-induced hypertrophy and contractile dysfunction (Ceylan-Isik et al, 2013; Ceylan-Isik et al, 2011). Ablation of the ER stress protein GADD153 has been shown to attenuate cardiac dysfunction in mice subjected to pressure overload (Fu et al, 2010). Other study reported that blockade of the IRE1 α pathway significantly decreased autophagic activity (Hernandez-Gea et al, 2013). Our study depicted that metallothionein significantly attenuates tunicamycin-induced ER stress as evidenced by GADD153, GRE78, phospho-IRE1 α and phospho-eIF2 α , suggesting a likely role of oxidative stress in tunicamycin-induced ER stress.

ROS are highly reactive oxygen free radicals or non-radical molecules generated by multiple mechanisms. ROS serves as important multifaceted signaling molecules to regulate a number of biological events. ROS production such as by ER stress may activate autophagy and autophagic cell death (Huang et al., 2011). ER stress has been reported to trigger ROS production and redox deviation. Recently neurologic studies have revealed that ER stress or UPR is likely downstream of 6-hydroxydopamine-induced oxidative stress *en route* to cell death (Holtz et al., 2006; Yamamuro et al., 2006). ROS generation has been shown in a number of pathologic processes in the heart such as cardiac hypertrophy, ischemia-reperfusion injury, myocardial stunning and heart failure. Using DCF staining, our data favored generation of ROS in ER stress-induced cardiac anomalies, consistent with the previous notion for a pivotal role of ROS in ER stress-induced cellular damage (Merksamer et al., 2008). Other results showed that ROS production and oxidative stress were not only coincidental to ER stress, but also essential integral ER stress components that may be turned on by ER stressors to mediate the proapoptotic and proadaptive UPR signaling (Ceylan-Isik et al., 2010; Guo et al., 2009; Santos et al., 2009). Our *in vitro* experiment further supports the notion that ER stress occurs likely upstream of oxidative stress in the regulation of cardiomyocyte contractile function. Involvement of oxidative stress in tunicamycin-induced cardiac contractile anomalies was further substantiated by the observation that antioxidant *N*-acetylcysteine significantly alleviated tunicamycin-induced cardiomyocyte contractile dysfunction. Other studies from our lab have reported that NAC effectively attenuated tunicamycin-induced autophagy and cardiomyocyte contractile dysfunction, favoring a role of oxidative stress upstream of autophagy in tunicamycin-induced cardiac anomalies (Jiang et al., 2013).

Our results showed that tunicamycin triggers autophagy as evidenced by autophagy markers phosphorylated ULK1, Atg5, Atg7, LC3B-II and the autophagy adaptor protein p62. Involvement of autophagy in tunicamycin-induced cardiac contractile anomalies was further substantiated by the fact that autophagy inhibition using 3-MA (to suppress the early-stage

autophagosome formation) mitigated tunicamycin-induced cardiomyocyte dysfunction. These results collectively imply a possible role of autophagy induction in the regulation of myocardial contractile function following tunicamycin exposure and, more importantly, the therapeutic potential of antioxidants and autophagy inhibition in tunicamycin-induced myocardial dysfunction. Nonetheless, the fact that autophagy inhibition reconciled tunicamycin-induced cardiomyocyte dysfunction suggests a permissive role of autophagy in tunicamycin-induced cardiac anomalies. Autophagy usually starts with an “induction phase” that is initiated by beclin-1 as an internal stimulus, followed by ascend “formation phase” involving Atg proteins such as Atg5, Atg7, and autophagosomal membrane-specific protein LC3 or Atg8, a marker for autophagosome (Xu et al., 2013c; Xu and Ren, 2012). Eventually, autophagosome will fuse with lysosomes, using p62 as the autophagosome cargo protein, prior to the degradation by lysosomal proteases (Nemchenko et al., 2011; Xu et al., 2013a; Xu et al., 2013b). Our results strongly favor a role of autophagy in metallothionein elicited cardioprotection, which is also supported by the finding that autophagy inhibition overtly attenuated tunicamycin-induced cardiomyocyte contractile dysfunction.

In conclusion, our study revealed that the heavy metal scavenger metallothionein protects against tunicamycin-induced cardiac contractile dysfunction. Our data favor the notion that oxidative stress and autophagy may play an essential role in tunicamycin- and metallothionein-elicited cardiac contractile responses. Although in depth mechanistic scenario remains to be explored, it is becoming apparent that oxidative stress may be the main regulatory machinery for cardiac contractile and intracellular Ca^{2+} defects under ER stress. Our findings further revealed a likely role of metallothionein-induced regulation of myocardial autophagy and contractile function. These data should shed some lights toward a better understanding for the therapeutic value of antioxidants in the management of ER stress-associated cardiac dysfunction.

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Research highlights

- We examined the effect of metallothionein on ER stress-induced heart dysfunction;
- Metallothionein rescues against ER stress-induced cardiac dysfunction;
- The beneficial effect of metallothionein was related to attenuated autophagy;

Fig. 1.

Effect of heavy metal scavenger metallothionein (MT) on tunicamycin (TN)-induced echocardiographic alterations. FVB and MT mice were given the ER stress inducer TN (1 mg/kg, i.p.) once a day for two days prior to assessment of echocardiographic properties. A: Body weight; B: Fractional shortening; C: LV end-diastolic diameter (LVEDD); D: LV end-systolic diameter (LVESD); E: LV posterior wall thickness at diastole (LVPWd); and F: LV posterior wall thickness at systole (LVPWs). Mean $\pm$ SEM; n = 9-10 mice/group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.

Fig. 2.

Effect of tunicamycin (TN) on cell shortening in isolated cardiomyocytes from FVB and MT mice. A: Resting cell length; B: Peak shortening (% of resting cell length); C: Maximal velocity of shortening (+ dL/dt); D: Maximal velocity of relengthening (- dL/dt); E: Time-to-peak shortening (TPS); and F: Time-to-90% relengthening (TR₉₀). Mean ± SEM, n = 80 cells per group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.

Fig. 3.

Effect of tunicamycin (TN) on intracellular Ca^{2+} properties in cardiomyocytes from FVB and MT mice. A: Baseline fura-2 fluorescence intensity (FFI); B: Electrically-stimulated increase in FFI (Δ FFI); and C: Intracellular Ca^{2+} decay rate (single exponential). Mean $\pm$ SEM, n = 52-54 cells per group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.

Fig. 4. Effect of tunicamycin (TN) on ROS in cardiomyocytes from FVB and MT mice. A-D: Representative DCF fluorescent images depicting cardiomyocytes from FVB, FVB-TN, MT and MT-TN mice; and E: ROS production (DCF fluorescence intensity). Mean $\pm$ SEM, $n = 5-8$ isolations per group, * $p < 0.05$ vs. FVB group; # $p < 0.05$ vs. FVB-TN group.

Fig. 5.

Effect of tunicamycin (TN) challenge (1 mg/kg, i.p.) on ER stress markers in FVB and MT mice. A: Representative gel blots depicting expression of GRP78, GADD153, eIF2α, phosphorylated eIF2α, IRE1α, phosphorylated IRE1α and α-Tubulin (used as loading control); B: GRP78 expression; C: GADD153 expression; D: Phosphorylated eIF2α expression; E: eIF2α expression; F: p-eIF2α-to-eIF2α ratio; G: Phosphorylated IRE1α expression; H: IRE1α expression and I: p-IRE1α-to-IRE1α ratio. All densities were normalized to that of α-tubulin. Mean ± SEM, n = 5-7 mice per group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.

Fig. 6. Effect of tunicamycin (TN) challenge (1 mg/kg, i.p.) on autophagy markers in FVB and MT mice. A: Representative gel blots depicting expression of ULK1, Phosphorylated ULK1, Atg5, Atg7, LC3B, P62 and α-tubulin (used as loading control); B: Phosphorylated ULK1 expression; C: ULK1 expression; D: p-ULK1-to-ULK1 ratio; E: Atg5 expression; F: Atg7 expression; G: LC3B-I expression; H: LC3B-II expression; I: LC3B-II-to-LC3B-I ratio; and J: P62 expression. All densities were normalized to that of α-Tubulin. Mean ± SEM, n = 5-7 mice per group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.

Fig. 7.

Effect of tunicamycin (TN) on cell shortening in cardiomyocytes from FVB mice.

Cardiomyocytes were incubated with TN (3 $\mu\text{g/ml}$) for 4 hrs *in vitro* prior to assessment of mechanical properties. A cohort of cardiomyocytes was co-incubated with the antioxidant *N*-acetylcysteine (NAC, 500 μM) or the autophagy inhibitor 3-methyladenine (3-MA, 3 mM) alone with TN. A: Resting cell length; B: Peak shortening (% of resting cell length); (C): Maximal velocity of shortening (+dL/dt); (D): Maximal velocity of relengthening (-dL/dt); (E): Time-to-peak shortening (TPS); and (F): Time-to-90% relengthening (TR₉₀). Mean $\pm$ SEM, n = 80 cells per group, *p < 0.05 vs. FVB group; #p < 0.05 vs. FVB-TN group.