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ER stress induces cardiac dysfunction through architectural modifications and alteration of mitochondrial function in cardiomyocytes

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SUMMARY

Aims: Endoplasmic reticulum (ER) stress has recently emerged as an important mechanism involved in the pathogenesis of cardiovascular diseases. However, the molecular mechanisms by which ER stress leads to cardiac dysfunction remain poorly understood.

Methods and results: In the present study, we evaluated the early cardiac effects of ER stress induced by tunicamycin (TN) in mice. Echocardiographic analysis indicated that TN-induced ER stress led to a significant impairment of the cardiac function. Electron microscopic observations revealed that ultrastructural changes of cardiomyocytes in response to ER stress manifested extensively at the level of the reticular membrane system. Smooth tubules of sarcoplasmic reticulum in connection with short sections of rough endoplasmic reticulum were observed. The presence of rough instead of smooth reticulum was increased at the interfibrillar space, at the level of dyads and in the vicinity of mitochondria. At the transcriptional level, ER stress resulted in a substantial decrease in the expression of the major regulator of mitochondrial biogenesis PGC-1 α and of its targets NRF1, Tfam, CS and COXIV. At the functional level, ER stress also induced an impairment of mitochondrial Ca²⁺ uptake, an alteration of mitochondrial oxidative phosphorylation and a metabolic remodeling characterized by a shift from fatty acid to glycolytic substrate consumption.

Conclusion: Our findings show that ER stress induces cytoarchitectural and metabolic alterations in cardiomyocytes and provide evidences that ER stress could represent a primary mechanism that contributes to the impairment of energy metabolism reported in most cardiac diseases.

INTRODUCTION

Heart failure is a major global health problem and a leading cause of mortality. In response to cardiac stress, several adaptations occur such as hypertrophy and metabolism modifications as an initial compensatory mechanism required for maintenance of cardiac output. However, in the long term these processes may become maladaptive and ultimately lead to cardiac decompensation and heart failure. Despite the considerable attention focused on pathological development of cardiac diseases, the understanding of the transition from adaptive to maladaptive response is still limited.

The rough endoplasmic reticulum (rER) is the site for synthesis and folding of secreted and membrane proteins. Disruption of ER homeostasis leads to accumulation of unfolded/misfolded proteins and ER stress. As a compensatory pro-survival response, the unfolded protein response (UPR) is activated to restore normal ER function. The canonical UPR is initiated by activation of three sensors, the kinase PERK, the transcription factor ATF6 and the kinase/endoribonuclease IRE1, which are normally kept inactive through the interaction with the ER-resident chaperone GRP78/BiP¹. Upon activation, these sensors induce the transcription of UPR target genes (e.g. *atf4*, *xbp1* and *chop*) resulting in the downregulation of protein synthesis, upregulation of ER chaperone expression, and upregulation of proteins involved in ERAD (ER Associated protein Degradation). However, in the case of prolonged or severe ER stress, where ER homeostasis cannot be restored, the mitochondrial apoptotic pathway is triggered to eliminate damaged cells². ER stress-dependent apoptosis was reported to be mediated mainly by the pro-apoptotic transcription factor CHOP, by pro-apoptotic members of the Bcl-2 family and by direct calcium transfer from ER to mitochondria³⁻⁵. Thereby, acute activation of ER stress response is protective while prolongation of the response typically leads to apoptosis and contributes to the maladaptive response.

Recently, ER stress has been linked to both physiological and pathological conditions in the cardiovascular system including oxygen and fuel starvation, myocardial infarction, ischemia, dilated cardiomyopathy, pressure overload, hypertrophy, and heart failure⁶. It has been suggested that the ER stress response is cardioprotective as part of the beneficial compensatory mechanism activated in the heart during a stress, while inhibition of this response is deleterious. For example, extinction of the central regulator of the ER stress response GRP78 aggravates insults upon

ischemia⁷. Protein disulfide isomerase (PDI), a major component of the UPR, is upregulated in peri-infarct myocardial region in humans and this increase is inversely correlated with the apoptotic rate and the presence of heart failure⁸. However, in the long term, ER stress response triggers apoptosis, which is part of the heart's maladaptive response and contributes to cardiac decompensation⁹. Thus, extinction of the ER stress-activated pro-apoptotic factor CHOP has been shown to reduce the cardiac dysfunction induced by pressure overload in mice¹⁰. Besides, treatment with the chemical chaperones 4-phenyl butyric acid (4-PBA) or TUDCA, which alleviate ER stress, protects the heart against isoproterenol- and anthracycline-induced cardiotoxicity and against obesity-induced myocardial contractile dysfunction¹¹⁻¹³.

Although understanding the pathophysiological role of ER stress in cardiovascular diseases has progressed in recent years, the exact physiological effect of ER stress on cardiomyocytes is still poorly understood. In the present study, we evaluated the effect of tunicamycin-induced ER stress on cardiac function, cardiomyocyte ultrastructure and mitochondrial function. Transmission electron microscopy analysis revealed that ER stress-induced cardiac dysfunction is associated with important ultrastructural modifications that manifest extensively at the level of the endomembrane system. Skinned cardiac fibers present slower mitochondrial oxidation rate and a shift from fatty acid to glucose utilization. Taken together, our results show that alteration of the cardiomyocyte ultrastructure and metabolism contributes to the maladaptive response of the heart to severe ER stress.

METHODS

Animals and treatments

To study ER stress *in vivo*, mice were given i.p. injection of TN 2 mg/kg body weight or the equivalent volume of vehicle (Dextrose 150 mM). Echocardiographic parameters were assessed 72 h after injection and hearts were harvested for molecular studies. Mice were euthanized by cervical **dislocation**. This procedure was carried out in conformity with the European Community guiding principles in the care and use of animals (Directive 2010/63/EU of the European Parliament). Authorizations to conduct animal experiments were obtained from the French Ministère de l'Éducation Nationale, de l'Enseignement Supérieur et de la Recherche (n° B9201901, November 3rd, 2015).

Echocardiography

Transthoracic echocardiography was performed using a 15 MHz transducer under 2.5% isoflurane gas anesthesia. Two-dimensional-guided (2D) M-mode echocardiography was used to determine wall thickness and left ventricular chamber volume at systole and diastole and contractile parameters, such as fractional shortening (FS) or ejection fraction (EF).

Biochemical analysis

Frozen tissues were homogenized with Precellys 24 Bertin technologies in a buffer (50 mg/mL) containing (in mM) HEPES 5, EGTA 1, DTT 1 and 0.1% Triton X-100 and were incubated for 1 h at 0°C for complete enzyme extraction. Activities of total creatine kinase (CK), citrate synthase (CS), hydroxyacyl-CoA dehydrogenase (HADHA) and mitochondrial cytochrome c oxidase (COX) were measured using standard spectrophotometric assays.

Western blot analysis

Frozen tissues were homogenized with Precellys 24 Bertin technologies in RIPA lysis buffer plus a cocktail of protease and phosphatase inhibitors (Roche), and PMSF for 30 min at 4 °C. Proteins (30 µg) were separated by SDS-PAGE and transferred to PVDF membranes (Merck Millipore). Membranes were incubated overnight at 4°C with the following antibodies: anti-GRP78 (**#3183**), anti-phospho-eIF2α (**#3597**), anti-eIF2α (**#2103**), anti-MCU (**#14997**), anti-phospho-DRP1 (**#6319**), anti-DRP1 (**#14647**), anti-OPA1 (**#80471**) and anti-CHOP (**#2895**) from Cell Signaling,

anti-IP3R (**ab5804**), anti-VDAC (**ab14734**), anti-MFN1 (**ab126575**), anti MFN2 (**ab124773**), anti-COXIV (**ab14744**) and anti-OXPHOS (**ab110413**) from Abcam, anti-Actin (**sc-47778**), anti-FIS1 (**sc-376447**), anti-SEC61 α (**sc-393182**), anti-RIBOPHORIN I (**sc-48367**), anti-RPS6 (**sc-74459**) and anti-GADD34 (**sc-8327**) from Santa Cruz. Proteins were detected on a ChemiDoc XRS (BioRad) by using the ECL method according to the manufacturer's instructions (Merck Millipore). To calculate the relative density (RD), ImageJ software was used and the intensity of each protein was normalized to actin. The data obtained were then expressed as the ratio of the intensity of the protein in treated mice to that of the corresponding protein in vehicle-treated mice (RD: relative density). The level of eIF2 α and DRP1 phosphorylation was expressed as the ratio of the phosphorylated protein vs the total protein.

RNA isolation and RT-PCR

RNA was isolated from cultured cells using Zymo Research Quick RNA MiniPrep according to the manufacturer's instructions. Total RNA (1 μ g) was reverse transcribed using BioRad iScript reverse transcription kit. cDNA was amplified by the “two-step” SsoFast EvaGreen supermix (BioRad), heated at 95°C for 30 s, followed by 50 cycles of denaturation at 95°C during 2 s and hybridization/elongation at 60°C during 5 s. The PCR primers were obtained from Eurofins and are listed in table S1. The results were quantified according to the Cq value method, where Cq is defined as the quantification cycle of PCR at which the amplified product is detected. The ratio $(1+E_{\text{target gene}})^{-(Cq_{\text{sample}}-Cq_{\text{control}})}$ target gene / $(1+E_{\text{reference gene}})^{-(Cq_{\text{sample}}-Cq_{\text{control}})}$ gene was calculated, where E represents the efficiency of the quantitative PCR reaction. With this calculation, expression of control was equivalent to one.

Electron Microscopy

Left ventricular papillary muscles were isolated from 3 control and 3 tunicamycin-treated (TN) mouse hearts, fixated with 2% glutaraldehyde in cacodylate buffer (in mM: 150 Na-Cacodylate, 2CaCl₂, pH 7.3) for 1 h, postfixated by 1% osmium tetroxide in cacodylate buffer for 30 min and stained with 1% aqueous solution of uranyl acetate. After dehydration in graded ethanol series and acetone, the tissue was embedded in Durcupan (ACM Fluka).

Preparation of sections

Ultrathin (58-60 nm) longitudinal sections were cut using an ultramicrotome (Power-Tome MT-XL, RMC/Sorvall, Tucson, USA). The sections were mounted on formvar-coated copper grids, contrasted with lead citrate and examined with a JEM 1200 electron microscope (Jeol, Tokyo, Japan) at 80 kV. Random images of cardiomyocytes were recorded with a CCD camera (Gatan DualVision 300 W) at a magnification of 15,000 \times and analyzed using Graphic Cell Analyzer¹⁴ (see below).

Stereological Analysis

Estimation of volume densities (V_v)

Random images of random samples of papillary muscle tissue were analyzed by the point integration method¹⁵. The size of the point test grid generated by the program Graphic Cell Analyzer¹⁴ was calibrated to the image scale and the point distance set to preferred resolution. Relative V_v of organelles of interest was estimated as the fraction p/P , where p represents the number of test points falling on the structure of interest and P represents the total number of test points falling on profiles of cardiomyocytes in the evaluated image. Altogether, we analyzed 152 images of control and 161 images of TN-treated mice

Estimation of mitochondrial environment.

The mitochondrial environment was estimated according to Novotova et al.¹⁶ by the cycloidal intersection method implemented in the Graphic Cell Analyzer¹⁴. The intersections of cycloidal curves with the mitochondrion outer surface were counted and classified according to the nearest organelle present at the mitochondrion surface at the point of intersection. For evaluation of mitochondrial environment, we used the relation $E = I_s/I$, where I_s corresponds to the number of intersections pertinent to individual nearest organelle at a distance of 20 nm or less and I represents total number of intersections with all mitochondria in the analyzed image. The total number of classified intersections with mitochondrial surface was 10150 (4962 for control vs. 5188 for TN-treated cardiomyocytes). The final image magnification for analysis was 50000 \times .

Quantification of mitochondrial surface

EM quantification of individual mitochondrial surface in CTL and WT hearts was performed using Image J software on 20 slices (randomly chosen, magnification 15,000 \times) per animal as

previously described¹⁷. The mean value of individual mitochondrial surface was obtained on the whole population of mitochondria (for each group), and the distribution of these mitochondrial surfaces calculated for each animal in each group.

Analysis of *in situ* mitochondrial function

Mitochondrial respiration was studied *in situ* in saponin-skinned fibers, as previously described¹⁸. Briefly, fibers were separated from the endocardial surface of the left ventricle in skinning (S) solution (see below) at 4°C and permeabilized with 50 µg/ml of saponin for 30 min. Permeabilized fibers were rinsed in (R) solution (see below) for 10 min and transferred into a 3 ml oxygraphic cell (Strathkelvin Instruments, Glasgow, UK). Solutions R and S contained (in mM): 2.77 CaK₂EGTA, 7.23 K₂EGTA (100 nM free Ca²⁺), 1 free Mg²⁺, 20 taurine, 0.5 DTT, and imidazole (pH 7.1). Solution S also contained (in mM) 50 potassium methanesulfonate, (160 mM ionic strength), 5.7 Na₂ATP, 15 phosphocreatine, while solution R contained 10 glutamate, 4 malate, 3 phosphate, 90 potassium methanesulfonate, 10 sodium methanesulfonate (ionic strength 160 mM) and 2 mg/ml fatty acid-free bovine serum albumin. Experiments were performed at 22°C under continuous stirring. Different experimental protocols were used based on substrate utilization pathways. One protocol determined mitochondrial respiration using fatty acids (0.1 mM Palmitoyl-Coenzyme A) or glycolytic substrate (1 mM pyruvate) in the presence of 2 mM ADP, 4 mM malate. Another protocol was intended to determine the dependency of respiration on external [ADP] in the presence or absence of 20 mM creatine with glutamate and malate. Fibers were exposed to increasing [ADP], to determine the apparent Km for ADP and maximal mitochondrial respiration (V_{max}). The acceptor control ratio (ACR), an index of oxidation phosphorylation coupling, was calculated as V_{max}/V_o. Succinate (20 mM) was then added to measure respiration involving complex I and II, amobarbital (2 mM) was added to inhibit complex I and to measure respiration on complex II. Finally, TMPD (0.5 mM) and ascorbate (2 mM) were added to give electrons to complex IV and sodium azide (5 mM) was added to inhibit complex IV. Respiration for complex IV was then calculated as TMPD/ascorbate respiration cut off the remaining azide respiration.

Analysis of *in situ* mitochondrial calcium uptake

Mitochondrial calcium content was studied *in situ* in isolated cardiac fibers. Fibers were separated from the endocardial surface of the left ventricle in skinning (S) solution (see analysis of mitochondrial function) at 4°C and then incubated in solution I with 200 nM MitoTracker Green (Life Technologies, France) and 5 µmol/L of the mitochondrial calcium indicator Rhod-2-acetoxymethyl ester (Rhod-2-AM; Life Technologies, France) for 60 min at 4°C, and then for 30 min at 37°C^{19, 20}. Solution I contained (in mM): 10 K₂EGTA, 1 free Mg²⁺, 3 K₂HPO₄, 20 taurine, 0.5 DTT, 54.2 potassium methanesulfonate, (160 mM ionic strength), 3.6 Na₂ATP, 12 phosphocreatine 10 glutamate, 4 malate, 60 Bes (pH 7.1) and 6% dextran. With this cold loading/warm incubation procedure, Rhod-2-AM localized to mitochondria. Colocalization of Rhod-2-AM and Mitotracker Green was evaluated with an inverted confocal laser scanning microscope Leica TCS SP8 (Leica, Germany) using a HC PL APO CS2 63x/1.40 oil immersion objective lens and quantified with the Fiji plugin Coloc 2. Rhod-2 (λ excitation: 552 nm, λ emission: 581 nm) and Mitotracker Green (λ excitation: 490 nm, λ emission: 516 nm) fluorescence was acquired in 96-well plates at 37°C in a spectrofluorimeter (TECAN infinite M200, TECAN, Austria).

Public data analysis

Analysis of gene expression using public repository data was performed using BioGPS webtools (<http://biogps.org/#goto=welcome>), a free scientific web resource²¹. We analyzed the microarray of wildtype mice from the dataset E-GEOD-19322. One-way ANOVA test with Benjamini and Hochberg false discovery rate (FDR) correction was applied to the comparison between the conditions, using a 0.05 threshold for statistical significance. Only C57BL/6J data were used.

Statistical analysis

All data are presented as mean±SEM. Data were analyzed using Sigma Stat (Sigma Stat, version 3.0, Systat Software, San Jose, CA, USA). Differences were assessed by Student's t-test when the data follow a normal distribution. Differences were assessed by Mann–Whitney U test when the data distribution was not normal. Differences between groups were considered significant if the P value was less than 0.05. *: P<0.05; **: P<0.01; ***: P<0.005 vs CTL. Microarray analysis was conducted using One-way ANOVA test with Benjamini and Hochberg false discovery rate (FDR)

correction, using a 0.05 threshold for statistical significance. Only C57BL/6J data were used. Correlation coefficient and significance were calculated using Spearman correlation test.

RESULTS

ER stress induces cardiac dysfunction in mice

To investigate the effects of ER stress on cardiac function, ER stress was induced in mice by intraperitoneal injection of tunicamycin (2 mg/kg), which inhibits N-linked glycosylation of proteins in the ER. This model is known to elicit activation of the UPR leading to cardiomyocyte apoptosis and development of cardiomyopathy^{22, 23}. TN injection provoked sustained ER stress and activation of the three arms of the UPR, as demonstrated by increased transcriptional expression of *Grp78*, *Grp94*, full-length *Xbp1* (*Xbp1u*), spliced *Xbp1* (*Xbp1s*) and *Chop* (Figure 1A), upregulation of GRP78, CHOP and GADD34 protein levels and increase in eIF2 α phosphorylation (Figure 1B). At the anatomical level, 72 h of ER stress challenge induced a loss of body weight and liver weight (Table S2) but did not overtly affected the weight of other organs. TN-induced ER stress led to a significant impairment of cardiac function as judged by the decrease in ejection fraction and fractional shortening (Figure 1D-E) induced by TN relative to vehicle-treated mice. In contrast, heart rate (Figure 1C), left ventricular internal diameters in diastole and systole or wall thickness were not affected (Table S3). Together, these data indicate that tunicamycin injection is a valuable model to study ER stress-induced cardiac dysfunction.

ER stress induces ultrastructural alterations in cardiomyocytes

To characterize the effects of ER stress induction in heart, ultrastructural analysis of cardiomyocytes was carried out by transmission electron microscopy. Application of vehicle did not induce any ultrastructural changes in control cardiomyocytes. Myofibrils were aligned in the longitudinal direction and sarcomeres were in register. Sub-sarcolemmal as well as intermyofibrillar mitochondria were regular in shape and size and formed columns grouped along myofibrils (Figure 2A). ER stress induction led to modification of the ultrastructure of cardiomyocytes. While some cardiomyocytes were similar to control, we also observed cardiomyocytes with areas characterized by enlarged cytosol (Figures 2D, E, F and S2) and the presence of mitochondrial clusters (Figures 2B and S2).

Ultrastructural alterations of the reticular membrane system

The endomembrane system in control cardiomyocytes consists of a prevailing network of smooth sarcoplasmic reticulum (SR) occurring near the sarcomeres (Figure 2C, 4C), whereas rough endoplasmic reticulum is observed near nuclei and to a lesser extent in regions of I-bands, near the Z-line. ER stress greatly influenced the composition and distribution of the reticular membrane system in cardiomyocytes. Generally, increased amount of the network of rER was observed. Areas presenting enlarged cytosol (Figure 2D) were rich in ribosomes, polyribosomes and branches of rER. These regions were similar by their composition to the growing areas in developing myocytes. The network of smooth SR was maintained in some regions of the A-band, but in other regions of the A-band, network of rER or branches of rER dominated (Figure 2E). Interestingly, the network of SR and rER sometimes shared their lumen (Figure 2F), indicating continuation of their inner volumes. From the morphological point of view, the character of reticular membrane system has been changed. An increased occurrence of rER in the area of I-band and A-band in TN-treated cardiomyocytes led to substantial ultrastructural changes at the level of dyadic microdomains (Figure 3). Dyads of control cardiomyocytes consist of a central t-tubule and of a SR cistern (Figure 3A). Remodeling of dyadic microdomains in TN-treated hearts was manifested by the appearance of ribosomes and polyribosomes on the reticular membranes occurring in a close proximity to the membrane of the central t-tubule (Figure 3B, D) and on the cistern of SR (Figure 3C). The expansion of rER network was also evidenced by the TN-induced increased expression of the ribosomal protein RBP6, the ribosome-binding protein of the ER Ribophorin I and the translocon protein Sec61 α (Figure 3E). These observations reveal for the first time a plasticity of the reticulum network in the heart. It seems that the reticulum evolved from a SR to an ER morphology in response to ER stress, probably leading to important modifications of Ca²⁺ cycling.

Ultrastructural alterations of mitochondrial population

Mitochondria of control cardiomyocytes had a typical cylindrical shape extended along the axis of the cell. In comparison to control (Figure 4A), the mitochondria of the intermyofibrillar region of TN-treated cardiomyocytes appeared to be heterogeneous in shape and size (Figure 4B). Inner mitochondrial membrane of some mitochondria was changed, and fragmentation of mitochondrial cristae was observed (Figure 4E). Moreover, quantification of mitochondrial surface revealed a significant increase of the proportion of small mitochondria (<0.2 μm^2)

associated with a decrease of large mitochondria ($>0.5 \mu\text{m}^2$, Figure 4G). To investigate whether a more fragmented network would be triggered by increased profission or reduced profusion signals, we quantified the expression level of the main regulators of these processes. While the transcriptional expression of *Mfn2*, *Opa1* and *Fis1*, the protein levels of MFN1, OPA1 and FIS1 and the phosphorylation of DRP1 were not modified by ER stress, we observed an increase of the total expression of DRP1 associated with a decrease of MFN2 protein level (Figure S1A and 4H-I).

ER stress induces mitochondrial dysfunction

We reasoned that changes in mitochondrial architecture induced by ER stress may produce changes in the oxidative capability of the heart. We therefore measured mitochondrial respiration rates and substrate preference *in situ* in permeabilized fibers. Basal respiration in the presence of substrates but without ADP in the respiratory medium (V_o) and thus without ATP production, was not modified by TN treatment. In contrast, maximal coupled respiration rate with ADP in the medium was decreased in the presence of glutamate (complex I), succinate (complex I+II), amytal (complex II) and TMPD/ascorbate (complex IV) (Figure 5A). Consequently, the coupling between oxidation and phosphorylation estimated by the ratio of maximal respiration on basal respiration was decreased following ER stress induction (Figure 5B). This low respiratory efficiency was associated with reduced enzymatic activity of citrate synthase (CS) and cytochrome c oxidase (COX) (Figure 5C, D). The reduced mitochondrial capability to generate ATP was accompanied by a dramatic inhibition of mitochondrial biogenesis as the transcriptional expression of the master coordinator of mitochondrial biogenesis *Pgc-1 α* and its targets *Nrf1*, *Tfam*, *Cs*, *CoxIV* were profoundly decreased by ER stress (Figure 5E). The decrease of mitochondrial mass suggested by the decrease of CS activity was also evidenced by reduced protein levels of the subunits of complex I, II, IV and V of the respiratory chain (Figure 5F), the mitochondrial calcium uniporter (MCU) and the porin VDAC (Figure S1C).

Stereological analysis revealed a significant decrease of the volume density of lipid droplets in cardiac muscle tissue in TN-treated mice (Table S4), while no significant change in other components of cardiomyocytes were found. The volume of lipid droplets significantly decreased from $1.25 \pm 0.38 \%$ of the unit cell volume in controls to $0.87 \pm 0.18 \%$ in TN-treated

cardiomyocytes. This result suggests a modification of the lipid metabolism by ER stress. Mitochondrial respiration was decreased when fueled by palmitoyl-Coenzyme A or by glutamate and succinate (V_{max}) but not changed when fueled with the glycolysis-derived substrate pyruvate (Figure 6A). Consequently, in TN-treated mice the relative oxidation of fatty acids (FA) was significantly decreased, whereas the relative oxidation of pyruvate was increased (Figure 6B). Accordingly, the activity of the lactate dehydrogenase involved in glycolysis was increased, whereas the activity of the fatty acid β -oxidation enzyme HADHA was decreased (Figure 6C, D). Moreover, mRNA level of the master regulator of FA oxidation PPAR α , as well as the β -oxidation enzyme MCAD, were markedly decreased after TN injection (Figure 6E). The transcriptional expression of mitochondrial FA transporters CPT1, CPT2 and CD36 remained unchanged, suggesting that the decreased oxidation rate of FA induced by ER stress is not the result of FA transport alterations but is rather due to a global decrease of mitochondrial β -oxidation (Figure 6E).

In cardiomyocytes, a localized crosstalk between energy producing and energy consuming sites is required to ensure proper cardiac contractility. This phosphotransfer is achieved by creatine kinase much more efficiently than by simple diffusion of nucleotides through the bulk cytosol²⁴. We thus assessed the functionality of the creatine kinase shuttle. We first measured creatine kinase enzymatic activity and we reported a decrease after TN injection (Figure 6F). We next analyzed the mitochondrial respiration regulation by ADP in the presence or absence of creatine to estimate the K_m to ADP. Whereas K_m to ADP was not modified in the absence of creatine, TN injection induced an increase of the K_m after creatine addition (Figure 6G), showing a decreased efficiency of mitochondrial creatine kinase. These results suggest that ER stress induces an alteration of the creatine kinase energy transfer function that could contribute to ER stress-mediated metabolic alterations in cardiomyocytes.

Mitochondrial environment

We then sought to determine the mechanism that led to mitochondrial dysfunction in response to ER stress. As we observed substantial cytoarchitectural modification in response to TN treatment, we first performed a morphometric analysis of the mitochondrial environment, which corresponds to the presence of organelles at a distance of 20 nm or less from mitochondrial

surface. As shown in Table S5, the most common structures close to mitochondria in control and TN-treated cardiomyocytes were myofibrils (41.3 ± 9.2 % vs. 36.5 ± 2.2 %, respectively) and neighboring mitochondria (29.9 ± 6.1 % vs. 35.0 ± 6.9 %, respectively). The remaining environment of mitochondria was represented by SR, rER and mixed smooth and rough reticulum, ribosomes and polyribosomes, dyads and transversal tubules, cytoplasm, plasma membrane and lipid droplets (Table S5). Among the different organelles found in the mitochondrial environment, only the proportion of rER that lies in close contact with mitochondria, the so-called mitochondria-associated-membranes fraction (MAM), was increased by ER stress induction from 0.7 ± 0.2 in control to 2.1 ± 0.6 while the total occurrence of reticular network (smooth and rough) near mitochondria was not changed.

Mitochondrial calcium homeostasis

Given that MAM plays a key role in the transport of calcium from the SR/ER to mitochondria, the mitochondrial calcium content in response to ER stress was examined. Assessment of specific mitochondrial calcium loading with Rhod-2-AM was performed on isolated cardiac fibers. With our staining protocol, fluorescent dye showed a specific mitochondrial staining, overlapping with the mitochondrial marker MitoTracker Green (Manders coefficient $>97\%$, Figure S1B). First, we observed that when cardiac fibers isolated from control mice were treated with TN ($40 \mu\text{g/ml}$), a fast and progressive increase of the Rhod-2-AM signal was induced, indicating that acute ER stress induces a rapid mitochondrial calcium uptake (Figure 6H). Second, when cardiac fibers were isolated from mice challenged for 72 h with vehicle (CTL) or with TN, we observed that the mitochondrial calcium content, normalized to mitochondrial mass (MitoTracker Green), was unchanged (Figure 6I-J). Calcium fluxes between ER and mitochondria were then monitored upon histamine stimulation (IP3R-dependent ER Ca^{2+} efflux activator), in order to release calcium from ER through the IP3R. Mitochondrial Ca^{2+} uptake induced by histamine was significantly reduced in TN-treated mice by comparison with CTL (Figure 6I-K). The alteration of Ca^{2+} flux between ER and mitochondria was associated with unchanged IP3R1 protein level but decreased VDAC and MCU contents (Figure S1C). Altogether, these results demonstrate that 72 h after ER stress, the Ca^{2+} transfer between SR/ER and mitochondria is impaired.

ER stress and cardiac disease

The important alteration of mitochondrial function should represent a major pathological event contributing to cardiac dysfunction. Therefore, we reasoned that ER stress could contribute to the alteration of mitochondrial function reported in cardiac diseases. To support these results, we conducted a search of public gene expression repositories on BioGPS (<http://biogps.org/#goto=welcome>) to identify a possible link between ER stress and mitochondrial function.²¹ Using microarray analyses of the dataset E-GEOD-19322²⁵, we reported an activation of the ER stress response in a mouse model of myocardial infarction (MI) (Figure 7A). Interestingly, the expression profile of genes of the ER stress response was heterogeneous. The increased expression of some genes was transient and reached a maximum 1 day after MI and returned to normal level 5 days after MI (e.g. *Chop*, *Grp78*, *Xbp1*, *Atf3* and *Atf4*), whereas the expression of other genes increased progressively (e.g. *Edem1*, *P58ipk*, *Pdia4* and *Calreticulin*) (Figure 7A). Given that the expression of all members of the PGC-1 α pathway were dramatically decreased upon TN injection, we paid a specific attention to PGC-1 α expression in the context of MI. PGC-1 α expression varies highly among the different conditions of the dataset (Sham and MI, infarct and border region, 1 day or 5 days after MI) and in agreement with our data, a dramatic decrease of PGC-1 α mRNA in the stressed region 1 day and 5 days after MI was observed (Figure 7B). Consequently, the expression of citrate synthase, (*Cs*), *Tfam* and *CoxIV* were decreased. Moreover, a strong negative correlation between the expression of genes of the UPR and *Pgc-1 α* was observed (Figure 7C-M). Interestingly, the negative correlation between the IRE1 and ATF6 branches of the ER stress response and *Pgc-1 α* is strong and highly significant (Figure 7C-I), whereas the correlation with the PERK branch of the UPR is less (*Atf4* and *Atf3*, Figure 7J, K) or not (*Gadd34* and *Chop*, Figure 7L, M) significant. This suggests that a member of the IRE1 or ATF6 pathway could directly regulate *Pgc-1 α* expression.

Altogether, these results demonstrate that ER stress induces a robust alteration of mitochondrial function and biogenesis and suggest that the well described decrease of PGC-1 α expression during cardiac disease could be induced by ER stress.

DISCUSSION

Different studies have demonstrated the importance of ER stress in the development of cardiac diseases, but the lack of a reliable model raises the difficulty to more specifically delineate the effects of ER stress *per se* from other pathophysiological processes. Because the ER stressor tunicamycin specifically affects N-linked glycosylation of proteins in the ER, intraperitoneal injection of TN constitutes an interesting model to study the functional effects of ER stress in the heart but was never precisely characterized. Here, we provide evidence that TN injection corresponds to a valuable model and reveal undescribed early adaptations of cardiomyocytes to ER stress. **A limitation of this *in vivo* model is that the changes induced by TN treatment may be compensatory responses secondary to cardiac dysfunction and not due to ER stress. However, our model was designed to minimize secondary side effects, and *in vitro* experiments on isolated fibers support our conclusions that most modifications reported here are direct responses to ER stress induction.** We demonstrated that ER stress induces an important alteration of the ultrastructure of cardiomyocytes that presented as early signs of cardiomyopathy phenotype, accompanied by a reorganization of the endomembrane system and modifications of mitochondrial structures. ER stress induced a profound inhibition of mitochondrial biogenesis pathway, an inhibition of mitochondrial respiration and a decrease in mitochondrial fatty acid utilization. These metabolic alterations may play an important role in the ER stress-induced cardiac dysfunction.

One purpose of this study was to evaluate the effect of acute ER stress in the heart *in vivo*. Our results demonstrate that TN-induced ER stress triggers a rapid phosphorylation of eIF2 α and an increase in the expression of the ER stress markers GRP78, CHOP and GADD34 in cardiomyocytes. Furthermore, TN injection resulted in the reduction of cardiac contractility after 72 h and thus represents a valuable tool to evaluate the early effects of ER stress in cardiomyocytes. In a previous study, it was reported that ER stress impairs intracellular Ca²⁺ homeostasis and excitation-contraction coupling in cardiomyocytes²⁶. However, the mechanisms underlying these defects were not investigated. Here, we show that early effects of ER stress are accompanied by a reorganization of the reticulum network. Importantly, we evidenced the presence of rough instead of smooth reticulum at the interfibrillar space. The presence of tubules covered by ribosomes/polyribosomes in the vicinity of myosin myofilaments (A band) in TN-

treated mice shows the possible changes of the properties of the endomembrane network in adult cardiomyocytes and could be an adaptive response to ER stress. The increased occurrence of rER in TN-treated cardiomyocytes at the level of dyads suggests higher protein synthesis in these areas and reorganization of the dyadic microdomains. Our findings suggest that structural alterations of the excitation-contraction coupling apparatus could be involved in ER stress-mediated cardiac dysfunction and that ER stress could contribute to the depressed Ca^{2+} cycling observed in cardiac diseases.

In the heart, the SR has been widely considered as just a different name for the ER, but recent biochemical and ultrastructural analysis support the concept of functionally distinct SR and ER compartments.⁶ In agreement, we observed in control mice distinct SR and ER systems. In response to ER stress, an increase in the amount of rough endoplasmic reticulum was observed in the intermyofibrillar region. This observation might appear surprising because a hallmark of the UPR via the PERK/eIF2 α axis is translational arrest to prevent overloading of ER with nascent proteins. In fact, as a consequence of UPR induction, a continued association, rather than release, of ER-bound ribosomes has been reported²⁷. In addition, the authors showed that when UPR is elicited, mRNA translation is sustained in the ER compartment, whereas it is suppressed in the cytosol. Therefore, the increased rER and ribosome content after TN treatment observed in cardiomyocytes could be considered as a compensatory mechanism to cope with ER stress. We also observed ultrastructural continuity between rough and smooth reticulum that were shown to share their lumen, indicating that the endomembrane system may form one continuous network. These observations reveal that the reticular network not only is a dynamic membrane system but also may have a high plasticity evolving from smooth to rough morphology to adapt to cellular conditions.

Optimal mitochondrial ATP production is essential in normal cardiac function and it is now established that mitochondrial biogenesis and function is altered in numerous cardiac diseases including heart failure^{28, 29}. However, the pathological mechanisms associated with alteration of the mitochondrial function in cardiac diseases remain not fully understood. In this study, we demonstrate that ER stress induces important alterations of the mitochondrial oxidative function. In functional experiments on permeabilized cardiac fibers, alteration of the mitochondrial respiration induced by ER stress is not specific to a respiratory complex as values for complex I,

I+II, II and IV dependent-respiration are similarly decreased. Remarkably, this global alteration of mitochondrial function is only visible in phosphorylating state suggesting a decrease in ATP production. This is accompanied by an increase in mitochondrial network fragmentation as suggested by the increased occurrence of small mitochondria, associated with an increase of the profission factor DRP1 and a decrease of the profusion factor MFN2, although it cannot be excluded that small mitochondria represent protrusions of large mitochondria. These results suggest that ER stress could contribute to the development of cardiac diseases by impairing mitochondrial structure and functions.

It has been demonstrated that cardiac dysfunction is associated with a shift from fatty acid to glucose utilization for ATP generation^{30, 31}. The implication of this metabolic shift in the pathogenesis of cardiac diseases is still debated as both activation and inhibition of fatty acid oxidation are considered as potential therapeutic strategies.³² In any cases, the decrease of fatty acid oxidation is interpreted as an oxygen-sparing mechanism, but the mechanism that triggers this shift is still poorly understood. We demonstrate here that ER stress induced a shift from fatty acid oxidation to glucose utilization as evidenced by an increase of the lactate dehydrogenase activity and a decrease of β -oxidation capacity after TN treatment. ER stress could thus represent a new mechanism underlying the metabolic shift observed in cardiac disease.

One other important finding of this study is the dramatic ER stress-induced decrease in expression of proteins involved in mitochondrial biogenesis that are regulated by the co-activator PGC-1 α . This alteration of PGC-1 α levels could represent a major insult in the pathological development of the disease. This hypothesis is supported by the bioinformatic study that we conducted on a mouse model of myocardial infarction²⁵ that revealed a similar alteration of PGC-1 α expression. Importantly, we found strong negative correlations between several members of the IRE1 and ATF6 branches of the UPR and PGC-1 α expression, suggesting that one member of these pathways may be a direct regulator of PGC-1 α expression at the transcriptional level. The high similarity of the activation IRE1 and ATF6 pathways probably reflect their interconnection through XBP1. These results also evidenced that the PERK pathway is less connected to the other branches and present its own kinetic of activation. Interestingly, a decrease of PGC-1 α expression was reported upon induction of ER stress in liver³³, suggesting that this alteration is not organ specific. A link between CHOP and PGC-1 α has also been recently evidenced in diabetic

embryopathy³⁴. However, additional studies are needed to confirm the close relationship between the UPR and PGC-1 α expression in cardiac diseases. Although mitochondrial biogenesis was decreased upon ER stress, the short onset of apparition of mitochondrial dysfunction associated with the fact that in the heart, mitochondria are stable organelles with a half-life of 5.6 to 6.2 days, suggest that mitochondrial alteration could not be entirely explained by a mitochondrial self-renewal defect and that other mechanisms may underlie this process.

The main physiological role of Ca²⁺ uptake in mitochondria is to increase the activity of rate-limiting enzymes of the Krebs cycle and ATP production⁹. Oppositely, mitochondrial Ca²⁺ overload uncouple oxidations from phosphorylation and triggers opening of the mitochondrial Permeability Transition Pore (mPTP) leading to apoptotic cell death³⁵. Several studies have shown that alterations in organelles' functional tethering is a common hallmark in different pathologies, such as metabolic syndrome, cancer and neurodegenerative diseases³⁵. Indeed, Ca²⁺ transfer between ER and mitochondria are altered in diabetic mice and the restoration of this flux improves hepatocytes insulin sensitivity³⁶. Similarly, the restoration of this interorganelle Ca²⁺ transfer improves muscle contractility in a mouse model of Duchenne muscular dystrophy³⁷. Altered ER-mitochondria associations are also observed in various neurodegenerative diseases including Alzheimer, Parkinson and amyotrophic lateral sclerosis and it is thought that correcting damaged ER/mitochondria association may correct damage to other neurodegenerative disease-linked features³⁸. In the heart, Ca²⁺ crosstalk between SR/ER and mitochondria has been involved in myocardial reperfusion injury³⁹ and the inhibition of SR/ER/mitochondria interactions protects cells against reperfusion injury⁴⁰. Here, we demonstrated that acute ER stress challenge provokes a rapid mitochondrial calcium uptake, as previously described in other cell types⁴¹. Intriguingly, despite increased contact between rER and mitochondria, a prolonged ER stress (72 h) leads to a decrease of mitochondrial Ca²⁺ uptake in response to histamine-induced rER calcium release. We reasoned that acute ER stress induces mitochondrial Ca²⁺ overload leading to a reduction of mitochondrial membrane potential that in turn, reduces mitochondrial Ca²⁺ uptake. This hypothesis is in agreement with the demonstration that ER stress slows intracellular Ca²⁺ clearance in cardiac cells²⁶. Decreased mitochondrial calcium content and membrane potential may thus explain decreased respiration rates. Besides, we measured a decrease of MFN2 expression after 72 h of ER stress induction. Although the role of MFN2 in the ER-mitochondria

tethering is still debated^{42,43}, its decrease could explain the alteration of Ca^{2+} transfer between the two organelles.

It was recently showed that the three subsets of heart mitochondria (i.e. intermyofibrillar, subsarcolemmal and perinuclear mitochondria) are morphologically different and respond differently to ischemic stress⁴⁴. In our model of ER stress induction, the reduction of mitochondrial size was detected only in the intermyofibrillar region, in which the occurrence of rER as well as rER-mitochondria interactions was increased. Our observations that TN induces a rapid mitochondrial Ca^{2+} uptake and a decrease of mitochondrial size, suggest that increased Ca^{2+} transfer from rER to mitochondria can participate in mitochondrial fragmentation, as reported for other cells⁴⁵.

In conclusion, we identified ultrastructure modifications of cardiomyocytes in response to ER stress with reticulum network disorganization. An important finding was that after 72 h, ER stress reduces mitochondrial Ca^{2+} uptake and triggers an alteration of all components of cardiac energy metabolism, from the substrate utilization to the oxidative phosphorylation and the transport of energy to the myofibrils. These results provide evidence that ER stress could represent an early mechanism that contributes to the impaired energy metabolism reported in most cardiac diseases.

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CONFLICT OF INTEREST

No potential conflicts of interest were disclosed by the other authors.

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FIGURE LEGENDS

Figure 1. Tunicamycin increases ER stress markers and induces cardiac dysfunction.

(A) Mice were injected intraperitoneally with TN (2 mg/kg) or with vehicle (150 mM dextrose) for 72 h and the expression of cardiac *Grp78*, *Grp94*, *Xbp1u*, *Xbp1s* and *Chop* were quantified by qPCR and expressed as fold change over untreated controls. Each genes expression is normalized on the geometrical mean of three references genes (n=6). (B) Cardiac content of the ER stress markers GRP78, CHOP, GADD34 and phosphorylation of eIF2 α were analyzed by western blot and expressed as fold change over untreated controls. Actin and total eIF2 α were used as loading controls (n=6). (C, D, E) Following 72 h of TN treatment, cardiac function was assessed by echocardiography (n=8). Results are represented as mean \pm SEM. *P<0.05, **P<0.01, ***P<0.005 vs. control (Student's t-test).

Figure 2. Ultrastructural alterations of the reticular membrane network induced by ER stress in cardiomyocytes.

Electron micrographs of control cardiomyocytes (A, C) and tunicamycin-treated cardiomyocytes (B, D, E, F) are presented. (A) Longitudinal section of cardiomyocyte from a control heart with regular arrangement of myofibrils (mf) and mitochondria (m). (B) Cardiomyocyte from tunicamycin-treated heart with myofibrillar irregularities (mf) and cluster of mitochondria (m) in the central part of the cell. (C) Area of the plasma membrane of control hearts. Myofibrils (mf) and network of smooth SR (arrow) are located just beneath plasma membrane (short arrow), m – mitochondrion. (D) Cardiomyocyte from TN-treated hearts. The space between the plasma membrane (short arrow) and myofibrils (mf) is filled with cytosol containing ribosomes or polyribosomes (*), short branches of rER (arrowheads), nascent myofibrils and cluster of mitochondria (m). (E) Area of cardiomyocytes in intermyofibrillar space with presence of the smooth (arrow) and rough reticular network (arrowhead), m - mitochondria. (F) Area of TN-treated cardiomyocytes with longitudinally oriented branches of smooth SR (arrows), arrowhead - branches of rER.

Figure 3. ER stress induces alteration of the dyadic microdomains in cardiomyocytes.

The micrographs present the dyadic microdomains in control (A) and TN-treated mice (B-D). (A) Dyadic microdomain in control cardiomyocyte, t – t-tubule, arrows - terminal cisternae of SR. (B) Dyadic microdomain in TN-treated cardiomyocyte; arrow – lumen of smooth SR, arrow head - section of the rER, t - central t-tubule of dyadic microdomain, m - mitochondrion. (C) Dyadic microdomain in cardiomyocyte after TN treatment, t – t-tubule, arrow - terminal cisterna of SR, arrowhead - polyribosomes localized at the face opposite to that facing the t-tubule membrane. (D) Longitudinal reticular membrane near the myosin myofilaments covered with ribosomes, arrow - rough reticular membrane, t – t-tubule of the longitudinal oriented dyadic microdomain. (E) Cardiac content of RPS6, RIBOPHORIN I and SEC61 α were analysed by western blot and expressed as fold change over untreated controls. Actin was used as loading controls (n=6). Results are represented as mean \pm SEM. *P<0.05, **P<0.01 vs. control (Student's t-test for RPS6 and SEC61 α quantification, Mann–Whitney U test for RIBOPHORIN I quantification).

Figure 4. Ultrastructural alteration of the mitochondrial environment induced by ER stress.

The mitochondrial environment in control cardiomyocytes (A, C) and TN-treated cardiomyocytes (B, D, E, F) are presented. (A) Longitudinal section of cardiomyocyte from a control heart. Intermyo-fibrillar mitochondria (m) have regular shape and regularly arranged mitochondrial cristae (arrow). (B) Heterogeneity of mitochondrial population (m) after TN treatment. (C) Transversal section through the I-band area in control cardiomyocyte with the presence of smooth SR vesicles (arrows), m – mitochondrion, L – lipid droplet. (D) Transversal section through the I-band area in TN-treated cardiomyocyte, arrow – vesicle of smooth SR, arrowheads - reticular membrane with ribosomes, m – mitochondrion. (E) Mitochondria (m) in close contact with rER in TN-treated cardiomyocyte, short arrows – fragmentation of mitochondrial cristae, arrowheads - branches of rER. (F) Area with mitochondria (m) and Golgi apparatus (GA) in TN-treated cardiomyocyte. (G) Distribution of individual mitochondrial surface between small (<0.2 μm^2), intermediate (between 0.2 and 0.5 μm^2) or large (>0.5 μm^2) mitochondria evaluated on 20 slices per mouse (\geq 500 mitochondria quantified per mouse). (H-I) Cardiac levels of OPA1, phospho-DRP1, DRP1, MFN1, MFN2 and FIS1 were analyzed by western blot and expressed as fold

change over untreated controls. Actin or total DRP1 were used as loading controls (n=6). Results are represented as mean±SEM. *P<0.05 vs. control (Student's t-test).

Figure 5. Cardiac energy metabolism of control and TN-treated mice.

(A) Oxygen consumption rate of skinned ventricular fibers in the absence (V_o) or presence of 2 mM ADP with 10 mM glutamate and malate (complex I, CxI), 15 mM succinate (both CxI and complex II, CxI+CxII), after complex I inhibition with 250 μ M amytal (CxII), in the presence of TMPD (0.5 mM) and ascorbate (2 mM) (CxIV). (B) Mitochondrial coupling (Ratio of Complex I respiration on V_o). (C) Citrate synthase activity. (D) Cytochrome c oxidase activity (COX). (E) mRNA levels of factors involved in mitochondrial biogenesis. Results are expressed as fold change over untreated controls. Each gene expression is normalized on the geometrical mean of three references genes. (F) Cardiac levels of ATP5A (CxV), SDHB (CxII), NDUFB8 (CxI) and COXIV-1 (CxIV) were analysed by western blot and expressed as fold change over untreated controls. Actin was used as loading controls (n=6). Values represent mean±SEM. *P<0.05, **P<0.01, ***P<0.005 vs. control (Student's t-test except for CI-NDUFB8 quantification, Mann–Whitney U test).

Figure 6. Cardiac lipid metabolism and energy transfer of control and TN-treated mice.

(A) Oxygen consumption rate without (V_o) or with 2 mM ADP and 4 mM malate and 0.1 mM octanoyl-carnitine (FA), 250 μ M pyruvate (pyruvate), 10 mM glutamate and 15 mM succinate (V_{max}). (B) Relative substrate utilisation in percent of V_{max} for octanoyl-carnitine (%FA) and for Pyruvate (%Pyruvate). (C) Lactate dehydrogenase activity (LDH). (D) Hydroxyacyl-CoA dehydrogenase activity (HADHA). (E) mRNA levels of protein involved in lipid utilization. Results are expressed as fold change over untreated controls. Each gene expression is normalized on the geometrical mean of three references genes. (F) Total creatine kinase activity (CK). (G) Apparent K_m of oxygen consumption for ADP with (+Cr) or without (-Cr) 20 mM creatine. (H) Mitochondrial calcium accumulation in cardiac fibers stimulated or not with TN (40 μ g/ml). (I) Mitochondrial calcium accumulation in cardiac fibers isolated from CTL and TN-treated animals (72 h) challenged with histamine (100 μ M). (J-K) Basal Ca^{2+} (J) and maximal mitochondrial Ca^{2+} peak fluorescence (K) determined from (I). Values represent mean±SEM (n=6). *P<0.05, **P<0.01, ***P<0.005 vs. control (Student's t-test except for G and J, Mann–Whitney U test).

Figure 7. ER stress negatively regulates *Pgc-1 α* expression in a mouse model of myocardial infarction.

(A-F) Data from the datasets E-GEOD-19322 were analyzed with BioGPS webtools (<http://biogps.org/#goto=welcome>). (A) The expression of UPR target genes in the heart of surgically infarcted wild-type mice (1 day and 5 days after infarction) were compared to SHAM wild-type animals. Results were expressed as fold change over SHAM animals. (B) The expression of *Pgc-1 α* , *Citrate Synthase (CS)*, *Tfam* and *Coxiv* in the heart of surgically infarcted wild-type mice (1 day and 5 days after infarction) were compared to SHAM wild-type animals. Results were expressed as fold change over SHAM animals. (C-M) The correlation between the expression of *Pgc-1 α* and *Xbp1* (C), *Edem1* (D), *Calreticulin* (E), *P58ipk* (F), *Grp78* (G), *Grp94* (H), *Pdia4* (I), *Atf4* (J), *Atf3* (K), *Gadd34* (L) and *Chop* (M) were calculated using Spearman's correlation test. (A-B) Values represent mean \pm SEM (n=3 for SHAM and n=4 for MI 1 d and MI 5 d). For correlation analysis (C-M), values include samples from SHAM wild-type animals in the same region as infarct tissue (**D0 infarct**, n=3), in the same region as non-infarct tissue (**D0 non-infarct**, n=3) and samples from MI wild-type animals in the infarct and non-infarct region 1 d and 5 d post MI (**D1 infarct**, **D1 non-infarct**, **D5 infarct**, **D5 non-infarct**, n=4 for each group). One-way ANOVA test with Benjamini and Hochberg false discovery rate (FDR) correction was applied to the comparison between the conditions, using a 0.05 threshold for statistical significance. Only C57BL/6J data were used. *P<0.05, **P<0.01, ***P<0.005 vs. control (One-way ANOVA test with Benjamini and Hochberg false discovery rate (FDR) correction).

Supplementary Figures

Supplementary Figure 1. ER stress, mitochondrial dynamics and Ca²⁺ homeostasis.

(A) mRNA expression of fission and fusion proteins. Results are expressed as fold change over untreated controls. Each gene expression is normalized on the geometrical mean of three references genes. (B) Cardiac fibers were loaded with the Ca²⁺ indicator Rhod-2-AM and MitoTracker Green. Merge signal revealed a mitochondrial pattern. **Scale bars represent 10 μm.** The quantitative pixel-by-pixel analysis measured with Fiji software (plugin Coloc 2) showed strong correlation between Rhod-2-AM and MitoTracker Green (Manders coefficient >97%). (C) Cardiac levels of IP3R1, MCU and VDAC were analysed by western blot and expressed as fold change over untreated controls. Actin was used as loading controls (n=6). Values represent mean±SEM. *P<0.05 vs control (Student's t-test).

Supplementary Figure 2: Ultrastructural alterations induced by ER stress in cardiomyocytes.

Electron micrographs of control cardiomyocytes (S2A-B) and tunicamycin-treated cardiomyocytes (S2C-F) are presented at low magnification (1 μM). t - t-tubules, m - mitochondrion, L - lipid droplet, mf- myofibrils, cy – cytosol.

Supplementary Tables

Table S1. Sequence of qPCR primers used in this study

Table S2. Anatomical parameters in response to ER stress.

Mice were injected i.p. with TN (2 mg/kg) or with vehicle (150 mM dextrose) for 72 h and anatomical parameters were recorded. BW: body weight. Initial BW: body weight before TN treatment, final BW: body weight after TN treatment, BW loss: difference between initial and final body weight, HW: heart weight. HW/BW: heart weight to body weight ratio, Lung/BW: Lung weight to body weight ratio, Liver/BW: Liver weight to body weight ratio, Kidney/BW, Kidney weight to body weight ratio. Results are represented as mean±SEM. n=8. ***P<0.005 vs. control values (Student's t-test).

Table S3: Echocardiographic parameters in response to ER stress.

Mice were injected i.p. with TN (2 mg/kg) or with vehicle (150 mM dextrose) for 72 h and transthoracic echocardiography was performed. EF: ejection fraction; FS: fractional shortening; HR: heart rate; LViDd: left ventricular internal dimension (diastole); LViDs: left ventricular internal dimension (systole); LVAWd: left ventricular anterior wall thickness (diastole); LVAWs: left ventricular anterior wall thickness (systole); LVPWd: left ventricular posterior wall thickness (diastole); LVPWs: left ventricular posterior wall thickness (systole); TWTd: total wall thickness (diastole); TWTs: total wall thickness (systole). Results are represented as mean±SEM. n=9. **P<0.01, ***P<0.005 vs. control values (Student's t-test).

Table S4: Quantification of the cardiomyocyte composition.

Mice were injected i.p. with TN (2 mg/kg) or with vehicle (150 mM dextrose) for 72 h and stereological analysis were performed at the test grid point distance of 280 nm to quantify the cardiomyocyte composition. The volume density of organelles is presented as mean percentage ± SEM. n=3 (152 images of control and 161 images of TN-treated mice were analyzed). *P<0.05 vs control (Mann–Whitney U test).

Table S5: Environment of mitochondria in control and TN-treated mice.

Mice were injected i.p. with TN (2 mg/kg) or with vehicle (150 mM dextrose) for 72 h and stereological analysis were performed to determine the mitochondrial environment (organelles at a distance of 20 nm or less from mitochondrial surface). Results are represented as mean percentage \pm SEM. n=3. The total number of classified intersections with mitochondrial surface was 10150 (4962 for control vs. 5188 for TN-treated cardiomyocytes) [#]P<0.1 vs control (Mann–Whitney U test).