Title: Ca\textsuperscript{2+} overload and mitochondrial permeability transition pore activation in living δ-sarcoglycan deficient cardiomyocytes

Running head: Mitochondria, calcium and δ-sarcoglycan

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Abstract

Muscular dystrophies are often associated with significant cardiac disease that can be the prominent feature associated with gene mutations in sarcoglycan. Cardiac cell death is a main feature of cardiomyopathy in sarcoglycan deficiency and may arise as a cardiomyocyte intrinsic process that remains unclear. Deficiency of δ-sarcoglycan (δ-SG) induces disruption of the dystrophin-associated glycoprotein complex, a known cause of membrane instability that may explain cardiomyocytes cytosolic Ca\(^{2+}\) increase. In this study we assessed the hypothesis that cytosolic Ca\(^{2+}\) increase triggers cardiomyocyte death through mitochondrial Ca\(^{2+}\) overload and dysfunction in the δ-SG deficient CHF147 hamster. We showed that virtually all isolated CHF147 ventricular myocytes exhibited elevated cytosolic and mitochondrial Ca\(^{2+}\) levels by the use of the Fura-2 and Rhod-2 fluorescent probes. Observation of living cells with Mito-Tracker red leaded to conclude that ~15% of isolated CHF147 cardiomyocytes had disorganized mitochondria. Transmission electron microscope imaging showed mitochondrial swelling associated with crest and membrane disruption. Analysis of the mitochondrial permeability transition pore (MPTP) activity using Calcein revealed that mitochondria of CHF147 ventricular cells were 2 fold leakier than wild-types, whereas reactive oxygen species production was unchanged. Bax, Bcl-2 and LC3 expression analysis by Western-Blot indicated that the intrinsic apoptosis and the cell death associated to autophagy pathways were not significantly activated in CHF147 hearts. Our results lead to conclude that cardiomyocytes death in δ-SG deficient animals is an intrinsic phenomenon, likely related to Ca\(^{2+}\)-induced necrosis. In this process Ca\(^{2+}\) overload-induced MPTP activation and mitochondrial disorganization may have an important role.

**Keywords:** calcium, mitochondria, cardiac muscle, sarcolemmopathy
Introduction

Dilated cardiomyopathies (DCM) are highly prevalent pathologies that often lead to terminal heart failure (11). Even though DCM can result from various etiologies, more than 35% of DCM cases are of genetic origin (21). DCM and muscular dystrophies are very often associated, in particular when the pathology involves mutations in the genes that code for components of the dystrophin-associated glycoprotein complex (DGC), such as the sarcoglycan family (4). Mutations in the δ-sarcoglycan (δ-SG) gene are responsible for limb-girdle muscular dystrophy (LGMD) 2F, which is associated with DCM (24). The pathogenesis of this latter is still unclear but may arise from an intrinsic defect of the cardiomyocytes.

Cardiac cell death is a major feature of DCM with δ-SG mutation but the exact degenerative process remains unknown and it was even debated whether it could be due to an intrinsic cardiomyocyte defect. Disruption of the DGC occurs in all sarcoglycan-mediated LGMDs and Duchenne Muscular Dystrophy (DMD) and this disruption is thought to be an important mediator of the membrane instability. Indeed, disruption of the DGC is associated, in human, with protein leakage into the serum, such as creatine kinase and, in animal models, with an increased membrane permeability to Evans Blue Dye (1). Defects in membrane permeability may also lead to elevated intracellular calcium levels that might be responsible for progressive muscle cell degeneration (7, 34). Indeed, elevated cytosolic Ca\(^{2+}\) levels and decreased tolerance to extracellular Ca\(^{2+}\) stress have been reported in isolated cardiomyocytes from BIO 14.6 cardiomyopathic hamsters, which are deficient in δ-SG (5). In addition, treatments based on Ca\(^{2+}\) antagonists were shown to prevent cardiomyocyte death in cardiomyopathic hamsters (13, 15). Taken together, these results suggest that intracellular Ca\(^{2+}\) overload could play a primary role in the cardiac cell death observed in δ-SG deficient heart. However, the mechanism by which Ca\(^{2+}\) overload leads to cell death remains unclear.
Under physiological conditions, mitochondria participate to the intracellular Ca\(^{2+}\) homeostasis (10). In particular, an increase in mitochondrial Ca\(^{2+}\) content results in activation of ATP production allowing cells to adapt their energy production to requirements (14). However, an excess in mitochondrial Ca\(^{2+}\) uptake leads to cell death through the triggering of apoptotic signaling or necrosis (32, 34).

Interestingly, Ca\(^{2+}\) accumulation defects have been found in isolated mitochondria from cardiomyopathic hamsters (17, 19). Yet, mitochondrial Ca\(^{2+}\) levels in intact δ-SG deficient cardiomyocytes have not been investigated. In the present study we assessed the hypothesis that a cytosolic Ca\(^{2+}\) increase triggers dysfunction in freshly isolated left ventricular myocytes from δ-SG deficient CHF147 hamsters and moreover will lead to cardiomyocyte death through mitochondrial Ca\(^{2+}\) overload.
Material and Methods

Animals
Male Syrian hamsters (36 to 52 weeks old) were used for the experiments. Wild-type (wt, healthy group) animals (strain Rj : AURA) were purchased from Elevage Janvier, France. CHF147 hamsters were bred at the local animal facility of the Institut de Myologie. The investigation conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

Solutions and chemicals
A normal physiological solution (NPS) was used for cardiomyocyte perfusion during isolation or experiments. NPS contained (in mmol/L): 140 NaCl, 5 KCl, 1 MgCl₂, 10 HEPES, 10 glucose and 1.8 CaCl₂, pH 7.35.

Ventricular cardiomyocyte isolation
Hearts were excised from heparined (5,000 U/kg body weight) and anesthetized (ketamine/xylazin/midazolam) hamsters, mounted in a Langendorff perfusion apparatus, and antegradely perfused through the aorta with a Ca²⁺-free NPS at 37°C for 3 min. Perfusion was then switched to the same solution containing 75 units/ml Liberase Blendzyme 4 (Roche Diagnostics) for 20 min. The left ventricular tissue was excised, minced and gently pipette-dissociated. Collagenase activity was stopped by the addition of 4% bovine serum albumin. Cell suspension was then sequentially washed in 100, 200, 300, 600, 800 and 1200 μmol/L Ca²⁺-NPS and resuspended in 1.8 mM Ca²⁺-NPS at RT. A typical ~50% yield of rod-shaped and excitable cardiomyocytes was routinely obtained. All experiments, excepted when specified, were performed on ventricular myocytes corresponding to the following criteria: i) rod-shaped, ii) no membrane blebs, iii) no hypercontractile zones and iv) no spontaneous contraction. Isolated cardiomyocytes were used within 5 hours after isolation. All experiments were done at room temperature.
Mitochondrial Ca$^{2+}$ measurement

The acetomethyl ester (AM) form of Rhod-2 (Invitrogen) was dispensed from DMSO stock, dispersed in 20% Pluronic F127, and diluted to 2.5 µmol/L in NPS. Cardiomyocytes were incubated at RT in this solution during 45 min before washing and then let 30 min before measurement to allow complete desesterification of the probe. In these conditions, cytosolic Rhod-2 was found to be negligible. Indeed, incubation of loaded cardiomyocytes in NPS-Ca$^{2+}$ free containing MnCl$_2$ (200 µmol/L) and saponin (0.02% w/v) did not induce significant fluorescence decrease (<5% of initial after 15 min). Additionally, co-incubation of cardiomyocytes with Rhod-2 and Mitotracker Green FM (Invitrogen, 300 nmol/L) allowed confirming specific loading of Rhod-2 in mitochondria (data not shown). Rhod-2 loaded cells were excited at 546 nm and background corrected emitted fluorescence collected at 590 nm (F) was converted in [Ca$^{2+}$]$_m$ with the equation: [Ca$^{2+}$] = $K_d$ $(F-F_{\text{free}})/(F_{\text{CaSat}}-F)$ where $K_d$ (570 nmol/L) is the Rhod-2 dissociation constant; $F_{\text{free}}$ and $F_{\text{CaSat}}$ are Rhod-2 emitted fluorescence when all the dye is free of bound Ca$^{2+}$ or all the probe is bound to Ca$^{2+}$ ions, respectively. $F_{\text{free}}$ and $F_{\text{CaSat}}$ were both calculated from the $F_{\text{MnSat}}$ using the $F_{\text{MnSat}}/F_{\text{CaSat}}$ (0.24) and $F_{\text{CaSat}}/F_{\text{free}}$ (15) ratios provided by the manufacturer. $F_{\text{MnSat}}$ corresponds to the fluorescence emitted by Rhod-2-AM loaded and ionomycin-treated (10 µmol/L) cells perfused with a NPS solution in which 2 mmol/L MnCl$_2$ replaced CaCl$_2$. For practical reasons Rhod-2 fluorescence measurement and calibration were made in different cells. In order to overcome the lack of common reference between measurement and calibration we used an alternative approach by calculating ratio of emitted fluorescence of rod-shaped living cells on emitted fluorescence of ball-shaped cells acquired in the same image. These latter cardiomyocytes were skinned-like cells since partial or complete Ca$^{2+}$-withdrawal by addition of EGTA induced immediate Rhod-2 fluorescence fluctuation (data not shown). Rhod-2 fluorescence of ball-shaped cells was higher in NPS and likely represented value close to $F_{\text{CaSat}}$. Rod-shaped to ball-shaped cell
ratios of fluorescence were more reliable than absolute fluorescence values measured in rod-
shaped cells.

**Cytosolic Ca\(^{2+}\) measurement**

Cardiomyocytes were incubated in NPS containing 5 µmol/L Fura-2-AM (Invitrogen) for 1 h
at RT, rinsed twice and let 30 min before use to ensure complete desesterification. Ratiometric Fura-2 fluorescence measurements were made using an integrated IonOptix
device and excitation filters of 380 and 360 nm. Emitted fluorescence (510 nm) was
background subtracted. The [Ca\(^{2+}\)]\(_{c}\) was calculated from ratiometric measurements according
to a modified method from Grynkiewicz and collaborators (6, 8).

**Mitochondrial permeability transition pore activity measurement**

The activity of the mitochondrial permeability transition pore (MPTP) was determined using a
method adapted from Petronilli and collaborators (27). This technique is based on the
evaluation of the calcein leak from mitochondria, monitored as a decrease of emitted
fluorescence. Cardiomyocytes are loaded with calcein-AM (1 hour, 2 µmol/L calcein-AM in
NPS containing 1 mmol/L CoCl\(_{2}\)), after desesterification the dye is not able to cross the
membrane anymore and is trapped in the cell. Addition of Co\(^{2+}\) allows quenching of cytosolic
and nuclear calcein fluorescence, but not mitochondrial one, since Co\(^{2+}\) is not able to cross
mitochondrial inner membrane. Loaded cardiomyocytes were washed free of calcein just
before experiments but let in NPS-CoCl\(_{2}\) and excited at 484 nm. Emitted fluorescence was
acquired at 520 nm and at 0.1 Hz. For each cardiomyocyte, fluorescence was acquired for 2
minutes to allow calculation of the fluorescence decrease rate. Calcein leak from
mitochondria was estimated using linear regression analysis of fluorescence signal and
expressed as the decline per minute of the initial fluorescence intensity. During some
experiments cyclosporin A (CsA, 1 µmol/L) was added to assess the involvement of MPTP.
Determinations of mitochondrial morphology in isolated cardiomyocytes

Cardiomyocytes were incubated in NPS containing 300 nmol/L of MitoTracker® red (Invitrogen) for 30 min at RT and then washed. Cells were excited at 546 nm and fluorescence images were acquired at 590 nm.

In situ measurement of reactive oxygen species (ROS) production

Isolated cardiomyocytes were incubated with 10 µmol/L of the acetylated form of 2',7'-dichlorofluorescein (DCF, Invitrogen), during 30 min at room temperature and in the dark. Oxidation of DCF by intracellular ROS results in the formation of a fluorescent compound. Basal ROS production was measured as the fluorescence emitted at 520 nm under 484 nm light excitation. Experiments were conducted up to 20 min after washing.

Epifluorescence microscopy and image deconvolution

An inverted microscope (Olympus, France) was equipped for epifluorescence. The system included a mercury light source, a 12-bit digital cooled CCD camera (Photometrics), and appropriate excitation and emission filters. For all experiments, isolated loaded cells were disposed in an experimental chamber (Warner Instruments, Inc) with uncoated glass bottom.

For mitochondrial Ca\(^{2+}\) and MPTP activity measurements, fluorescence images obtained with a dry objective (Olympus, 40X and 0.6 NA) were collected over exposure times minimized to avoid phototoxicity and ranging from 1 to 10 ms. In the case of fluorescent probe localization and organelle morphology studies, fluorescence image stacks of 50 sections obtained with an oil immersion objective (Olympus, 60X, 1.42 NA) were collected at 0.3 µm spacing and over exposure times ranging from 1 to 500 ms. Images were acquired with MetaVue (Molecular Devices) and analyzed with ImageJ (NIH) softwares. Image deconvolution was performed with AutoDeblur software (AutoQuant Imaging, Inc). Volume rendering images were computed using MeVisLab software (MeVis Research GmbH, Germany).
Transmission electron microscope imaging

The heart was removed from anesthetized hamsters and fragments of left ventricle were immediately fixed during 2 hours in 2.5 % cold glutaraldehyde with 0.1 mol/L cacodylate buffer, pH 7.3. After washing with several changes of cacodylate buffer, the specimens were post-fixed in 1 % osmium tetroxide in the same buffer, dehydrated with a graded series of ethanol and propylene oxide, and embedded in Epon. Semithin sections of 0.5 µm thick were stained with alkaline toluidin blue. Ultrathin sections were stained with uranyl acetate, Reynold’s lead citrate and observed with a Philips CM 120 electron microscope at 80 kV.

LC3, Bcl-2 and Bax expression analysis by Western-Blot

Five hearts from each hamster genotype were excised and homogenized separately. Whole left ventricles were homogenized in a protein extraction buffer (20 mmol/L Tris, pH 7.5, 2 mmol/L EGTA, 1 mmol/L EDTA, 1 mmol/L DTT and inhibitor cocktails of proteases (10 µg/ml leupeptin, 25 µg/ml aprotinin, 10 µg/ml pepstatin and 10 mmol/L Pefabloc® Sc) and phosphatases (cocktails 1 and 2)). The homogenates were centrifuged at 22,000 g for 15 min at 4°C, and the supernatants were stored as the soluble cytosolic fractions. Pellets were suspended in the extraction buffer completed with 0.35 % TritonX-100, and centrifuged at 22,000 g for 15 min at 4°C. The second pellets were newly homogenized and added to the second supernatants, the mixtures were saved as solubilized membrane fractions. Both fractions were aliquoted and stored at -80°C. Homogenates were normalized for protein content (40 µg/lane) and subjected to SDS-PAGE using 12 % (Bcl-2 and Bax) or 15 % (LC3) gels. Fractioned homogenates were used to determine the localization of Bax and Bcl-2 expression. Whole homogenates were used to determine the expression ratio of Bcl-2/Bax and LC3II/LC3I proteins. Proteins were transferred to nitrocellulose membranes (Invitrogen). The relative content of muscle protein between lanes was checked by Ponceau S coloration of the resulting blots and by alpha-tubulin immunostaining (antibody dilution 1:500; A2172;
Sigma). The LC3, Bax and Bcl-2 proteins were detected using specific antibodies at dilutions of 1:1000 (Cell Signaling), 1:500 (AbCam) and 1:500 (AbCam), respectively. Subsequently, alkaline phosphatase-conjugated goat anti-mouse (Bio-Rad for Bax and Bcl-2) or anti-rabbit (Dako for LC3) were used at a dilution of 1:1000. Specific signals were detected with the ECF (enhanced fluorescence) Western blotting detection reagent (Amersham Biosciences).

Statistical analysis

Data are expressed as mean ± SEM obtained from $n$ fibers and $N$ animals. Comparisons between 2 means were based on unpaired Student’s $t$-test except when the effect of cyclosporin A was tested on calcein leak. In this latter case, paired Student’s $t$-test was used. Comparisons between more than 2 means were based on one way analysis of variance followed by Bonferroni’s $t$-test. Threshold for statistical significance was set at 0.05.
Results

Cytosolic Ca\(^{2+}\)

\([\text{Ca}^{2+}]_c\) was approximately 50 % higher in CHF147 than that measured in wt heart (Figure 1A). Gaussian fit of CHF147 \([\text{Ca}^{2+}]_c\) distribution histogram exhibited a right shift towards higher \([\text{Ca}^{2+}]_c\) (Figure 1B). Therefore, the difference in mean cytosolic Ca\(^{2+}\) values seems consecutive to an increase in \([\text{Ca}^{2+}]_c\) in all the tested CHF147 cells rather than to the appearance of a cardiomyocyte population with high \([\text{Ca}^{2+}]_c\).

Mitochondrial Ca\(^{2+}\)

We used Rhod-2 to assess the matrix mitochondrial Ca\(^{2+}\) level in wt and CHF cardiomyocytes. In our loading conditions, Rhod-2 fluorescence was not dependent on cytosolic Ca\(^{2+}\) since emitted fluorescence arose mainly from dye trapped into mitochondria (Figure 2A). Calculation from pseudoratiometric measurements showed a 3 fold higher \([\text{Ca}^{2+}]_m\) in CH147 cardiomyocytes than in wt ones (Figure 2B). In order to overcome the lack of common reference between measurement and calibration we used an alternative approach by calculating the ratio of emitted fluorescence of rod-shaped living cells to emitted fluorescence of ball-shaped cells acquired in the same image (Figure 2C). Whatever the approach used our results demonstrated that CHF147 cells exhibited high mitochondrial Ca\(^{2+}\) levels. Distribution analysis of fluorescence ratio values indicated an overall right shift leading to suggest that all \(\delta\)-SG deficient cardiomyocytes exhibited a mitochondrial Ca\(^{2+}\) overload (Figure 2D).

Mitochondrial organization in isolated ventricular myocytes

In Mitotracker Red loaded cardiomyocytes, two types of mitochondrial organizations were observed. The first one was found in wt and CHF147 cells and consisted in a homogeneous labeling showing regular arrangement of ‘mitochondrial columns’ along the myofibrils (Figure 3a-h). The other mitochondrial arrangement was seen only in CHF147
cardiomyocytes. Indeed, mitochondria appeared heterogeneously distributed (Figure 3c-3f-3i) with various size and shape in 15.7 ± 3 % of the CHF147 cells assessed (484 cells observed in 4 CH147 animals). In order to verify whether mitochondrial disorganization was not an artifact related to a higher sensitivity of δ-SG deficient cells to experimental procedures and so as to determine morphological alterations of individual mitochondria, we used transmission electron microscopy (TEM) as a complementary approach.

Mitochondrial ultrastructure

As observed in isolated living cells, CHF147 cardiomyocytes displayed variable cellular distribution of mitochondria with TEM. The majority of cells exhibited a normal mitochondrial arrangement, but the other part displayed a disorganized mitochondrial pattern. Additionally, alterations varied from one cell to another, showing dispersed mitochondria between myofibrils (Figure 4B-D) or mitochondrial aggregates (Figure 4F). At the ultrastructural level, mitochondrial impairments consisted in swelling and clarification (Figure 4G), condensation of the matrix (Figure 4H), disruption of the crests (Figure 4I), the presence of intramitochondrial residual bodies or loss of integrity of mitochondrial inner and outer membranes (Figure 4J).

Mitochondrial permeability transition pore activity

Calcein-AM was used to determine MPTP activity in hamster cardiomyocytes (Figure 5). At the beginning of experiments calcein loaded cells exhibited fluorescence arising mainly from mitochondria (Figure 5A). After the washing step with Co^{2+}-calcein free-NPS solution, fluorescence began to decrease. This reflected the leak of calcein from mitochondria to the cytoplasm through the MPTP. The mean value of fluorescence decrease rate in CHF147 cardiomyocytes was twice the one in wt (P<0.05, Figure 5B). When cyclosporin A, a potent MPTP inhibitor, was applied the rates of fluorescence decrease were reduced of more than 50 % and became similar in wt and CHF147 cardiomyocytes (Figure 5C-5D). This indicated that
calcein leak was mainly related to MPTP activity.

*Reactive oxygen species production*

DCF was used to determine reactive oxygen species (ROS) production in wt and CHF147 cardiomyocytes. Using this method we were able to measure ROS production increase under oxidative stress induced by tert-butyl hydroperoxide addition (*Figure 5E*). On the other hand, mean basal ROS level was found similar between wt and CHF147 isolated cardiomyocytes (*Figure 5F*).

*Bcl-2/Bax expression ratio and Bax subcellular location*

Western-Blot and immunostaining analysis indicated no change in the expression and in the localization of Bax, the pro-apoptotic protein being restricted in the cytosolic fraction in wt and CHF hamsters (*Figure 6A*). As expected, Bcl-2 expression was mainly detected in the membrane fraction (*Figure 6A*). Additionally, Bcl-2/Bax expression ratio value was similar in wt and CHF147 heart (*Figure 6B*). Interestingly, no apoptotic nuclei were observed under TEM even in CHF147 cells that exhibited disorganized mitochondria (*Figure 6C*).

*LC3II/LC3I expression ratio*

We used Western-Blot analysis to determine the relative levels of the microtubule-associated protein light chain 3 (LC3) form I and II that belong to the autophagic cell signaling system (22) (*Figure 6D*). The ratio of LC3II/LC3I protein level was similar between wt and CHF147 (*Figure 6E*).
Discussion

Cardiomyopathies evolve inevitably towards heart failure due to progressive cell loss and the limited regenerative capacity of the cardiac muscle. However, the exact mechanism for the vanishing contractile mass remains unclear.

Coral-Vasquez and collaborators have shown that δ-SG–null mice displayed disruption of the sarcoglycan complex in vascular smooth muscle and microvascular filling defects (3). As injection of a vascular relaxant compound prevented cardiomyopathy onset, these authors proposed that cardiomyopathy was a consequence of the vascular alterations. According to this hypothesis, only cardiomyocytes surrounding defective vessels should undergo necrosis. At variance with this postulate, our results demonstrated that cytosolic and mitochondrial Ca^{2+} overload was present in all the left ventricular cardiomyocytes. In agreement with our observation are the results presented by the group of McNally, which demonstrated that, using a δ-SG deficient mice model, the conditioned expression of a δ-SG transgene exclusively in cardiomyocytes corrected the degenerative process that led to cardiomyopathy, whereas restricted expression of the same transgene in vascular smooth muscle did not (33). These data suggest that the cardiac myolysis induced by the lack of δ-SG expression is mainly intrinsic to the cardiomyocytes and is related to calcium overload.

There is strong evidence for a link between alterations of the Ca^{2+} homeostasis and heart failure; however we still lack an integrated view on how the various events may fit together and lead from a compensated cardiomyopathy phenotype to overt heart failure. In this study, we have observed that virtually all CHF147 hamster left ventricular cardiomyocytes exhibit an elevated basal cytosolic and a high mitochondrial Ca^{2+} levels. Moreover, we also observed a two fold increase of the MPTP activity associated with some disorganization of the mitochondrial network and the swelling of individual mitochondria. Our results are in good agreement with previous observations made by different groups on the same animal model or
on mouse models. For example, high cytosolic Ca\textsuperscript{2+} level has been previously described (30).

Additionally, the involvement of MPTP in the pathogenesis of sarcolemmopathies has been shown in mouse models lacking the cyclophilin D (23, 26). Nevertheless the latter studies did not address the role of Ca\textsuperscript{2+} in the MPTP activation. Yet, this is an important issue since it has been shown that mitochondria act as Ca\textsuperscript{2+} buffers (2, 9) and that mitochondrial Ca\textsuperscript{2+} overload is the main triggering signal for MPTP opening (29). Although increased Ca\textsuperscript{2+} alone may activate MPTP, ROS are known to participate to the induction of MPTP (18). ROS production was similar in wt and CHF147 cardiomyocytes. This leads us to conclude that in CHF147 cells Ca\textsuperscript{2+} was the main activating factor of MPTP opening.

MPTP activation has been involved in necrosis and apoptosis cell deaths and cardiomyocytes loss is a feature of the cardiomyopathy induced by δ-SG deficiency (12, 18). There are three major cell death processes that have been described necrosis, apoptosis and cell death associated with autophagy (16). The two latter processes can be distinguished by biochemical events. We first excluded apoptosis activation since we observed any modification in the expression of Bcl-2 or Bax and no typical chromatin changes (16). Additionally, the LC3I and LC3II protein levels were similar between genotypes hence allowing us to conclude that, in this experimental system, cell death associated to autophagy has no role to play in the loss of cell mass. Therefore, cardiac myolysis taking place in δ-SG deficiency appears to be related to necrosis.

Based on our results, we propose the following mechanism for the pathophysiology of the sarcolemmopathy linked to δ-SG deficiency. The initial genetic membrane defect leads to an elevation of cytosolic [Ca\textsuperscript{2+}], disease progresses through mitochondrial defects and finally leading to cell death. As already proposed in the case of dystrophic skeletal muscle fibers (7), we postulate that the absence of δ-sarcoglycan leads to increased permeability of the sarcolemma that is responsible for the rise in cytosolic Ca\textsuperscript{2+}. Because of the Ca\textsuperscript{2+} buffer
function of mitochondria (2, 9), this increase will lead to the observed elevation in mitochondrial Ca^{2+}. As indicated by others (29), this latter rise leads to increased MPTP activity. Under normal conditions, acute disturbance of the sarcolemmal permeability remains reversible and mitochondrial dysfunction is not triggered. In the case of CHF147 hamster cardiomyocytes, the sarcolemmal permeability never returns to normal, Ca^{2+} content continues to rise and the increased MPTP activity, finally triggers mitochondrial dysfunction as evidenced by the disorganized mitochondria network and the swelling of isolated mitochondria. Lastly, the mitochondrial dysfunction leads to cell death through the process of necrosis.

Interestingly, myocardial ischemia-like necrotic injuries have been described in some histological studies made in δ-SG deficient mice and hamsters (3, 12, 31). It is interesting to note that the calcium cascade of events suggested by our results has been involved in the ischemia-induced necrotic process (20). Noteworthy also, the clinical work by Piot et al. gives some preliminary clues on the protective effects of cyclosporine at the time of myocardial reperfusion (28). The cardiomyopathy induced by δ-SG deficiency is progressive whereas ischemia-induced necrosis is a rapid process. Nevertheless, we propose that δ-SG deficiency triggers the activation of Ca^{2+} and mitochondrial-dependent necrosis cell death pathway similar to that observed during ischemia. Further experiments are needed to refine this hypothesis, even though a chronic *ex vivo* model is still missing to dissect further the involved mechanisms.

Taken together our results demonstrate that like in dystrophinopathies, the lack of dystrophin associated proteins lead to alteration of the calcium homeostasis, thus supporting the concept of sarcolemmopathies proposed by Ozawa and collaborators (25). Furthermore, our study stresses the role of mitochondrial Ca^{2+} disturbances in the pathophysiology of cardiomyopathies.
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Disclosure:

None


33. Wheeler MT, Allikian MJ, Heydemann A, Hadhazy M, Zarnegar S, and McNally EM. Smooth muscle cell-extrinsic vascular spasm arises from cardiomyocyte...

Legends:

Figure 1: Cytosolic calcium in wt and CHF147 cardiomyocytes. A, Each bar represents mean $[\text{Ca}^{2+}]_c \pm \text{SEM}$ value calculated in $N$ animals/in $n$ cells. *: Significantly different from wt, $P<0.001$, Student’s unpaired $t$-test. B, histogram distribution of $[\text{Ca}^{2+}]_c$ values used in A; White dotted lines are calculated Gaussian curves, centers of which are indicated and materialized by vertical dotted lines.

Figure 2: Free mitochondrial $\text{Ca}^{2+}$ concentration ($[\text{Ca}^{2+}]_m$) in wt and CHF147 cardiomyocytes. A, deconvolved image of a wt cardiomyocyte loaded with Rhod-2 showing that the localization of the dye is restricted to mitochondria; Right image is a zoomed of the area delimited by a white square in the left image. B, Each bar represents mean $[\text{Ca}^{2+}]_m \pm \text{SEM}$ value calculated in $N$ animals/in $n$ cells after calibration of Rhod-2 fluorescence. C, Each bar represents mean $\pm \text{SEM}$ values of fluorescence ratio (rhod-shaped/ball-shaped cells, see text) calculated in $N$ animals/in $n$ cells. D, Histogram distribution of fluorescence ratio values used in B; White dotted lines are calculated Gaussian curves. *: Significantly different from wt, $P<0.05$, Student’s unpaired $t$-test.

Figure 3: Cellular organization of mitochondria in wt and CHF147 cardiomyocytes. a, b and c, deconvolved images of representative optical slices obtained in MitoTracker-Red loaded cardiomyocytes and used to process three-dimensional images shown in d to i. d, e and f, overall distribution and shape of mitochondria from single cardiomyocytes shown in a, b and c, respectively. g, h and i, are zoomed images of the areas surrounded by the white squares shown in d, e and f. **CHF147 NORM**: CHF147 cardiomyocyte exhibiting mitochondria pattern as observed in wt cells. **CHF147 Dis.**: CHF147 cardiomyocyte with disorganized mitochondria, although few orderly mitochondria are still present (white arrow).
Figure 4. Mitochondrial ultrastructure in wt and CHF147 cardiomyocytes. A and C, respectively, show longitudinal and transverse section of wt hamster ventricular myocardium. Mitochondria are orderly arranged between myofibrils. B and D, respectively, are longitudinal and transverse sections of left ventricle from a CHF147 hamster. E-J, Transversal section pictures acquired in TEM from wt (A) and CHF147 (F-J) heart hamsters. E, In wt, and in most CHF147, ventricular myocytes had mitochondria with paralleled crests and dark matrix; F and G, CHF147 cells with mitochondria exhibiting paralleled crests but clear matrix, some of the crests are disrupted (G). H and I, CHF147 cells presenting mitochondria with no more crests and a clear matrix; J, CHF147 cardiomyocytes having swollen mitochondria. Scale bars: 2 µm, A-G; 1 µm, H-J.

Figure 5: Mitochondrial permeability transition pore activity and ROS production in wt and CHF147 (CHF) cardiomyocytes. A, Epifluorescence images of calcein-loaded cardiomyocytes from wt hamster. Left non-deconvolved images were acquired in cardiomyocytes loaded with calcein-AM in absence (-Co^{2+}) or in presence of 1 mmol/L Co^{2+} (+Co^{2+}). Right deconvolved images are zoomed detail from the area delimited by the white squares in left images. B, Rates of calcein fluorescence intensity decrease. C, Mean rates of calcein fluorescence intensity decrease before and after cyclosporin A (CsA) addition. D, Typical records of calcein fluorescence intensity before and after addition of CsA. E, Typical record of DCF fluorescence intensity before and after addition of TBH (tert-butyl hydroperoxide) in a wt cardiomyocyte. F, Mean DCF fluorescence intensity recorded in wt and CHF147 cardiomyocytes in basal conditions. B, C, and F, Each bar represents mean ± SEM value measured in N animals/ in n cells. *: Significantly different from wt, P<0.05, Student’s unpaired t-test. §: Significantly different from basal conditions, P<0.05, Student’s paired t-test; #: Significantly different from wt, P<0.05, ANOVA and Bonferroni’s t-test.
Figure 6: Cellular location of Bax, Bcl-2/Bax expression ratio, nucleus morphology and LC3II/LC3I level ratio in wt and CHF147 cardiomyocytes. A, Immunoblot analysis of Bcl-2 and Bax expression in fractioned heart homogenates from wt and CHF147 (CHF) hamsters. Lanes 1 and 2 were loaded with the pooled cytosolic fractions from wt and CHF hearts, respectively. Lanes 3 and 4 were loaded with the pooled membrane fractions from wt and CHF hearts, respectively. For a given homogenate fraction, immunodetection of α-tubulin was used to check protein content between wt and CHF147. B, Ratio of Bcl-2 on Bax fluorescence emitted after their respective revelation by immunodetection and chemiluminescence. C, Nuclei from wt and CHF147 cardiomyocyte observed under transmission electron microscopy. CHF147 cell shown has disorganized mitochondria. Scale bars: 2 µm. D, Representative immunoblot analysis of LC3I and LC3II level in whole heart homogenates from wt and CHF147 (CHF) hamsters. E, Ratio of LC3II/LC3I fluorescence emitted after their revelation by immunodetection and chemiluminescence. B and E, Each bar represents mean ± SEM value measured in 5 whole left ventricle homogenates (5 different animals) and at least in two different immunoblots.
Figure 1

A

[Ca^{2+}]_c (nmol/L)

B

Number of cells

wt

CHF

wt

122.5 nmol/L

150.3 nmol/L

6/267 7/361

120

80

40

0

100 200 300 400 500 600 700

[Ca^{2+}]_c nmol/L

* 7.361

250 200 150 100 50 0

CHF

wt

6267
Figure 3
Figure 5

A. Fluorescence images showing the effects of 
Co²⁺ and CsA on cell viability.

B. Bar graph showing the Calcein fluorescence decrease rate in wt and CHF conditions.

C. Bar graph showing the Calcein fluorescence decrease rate in wt CHF conditions with or without CsA.

D. Graph showing the Calcein fluorescence decrease rate over time with CsA 1 μmol/L.

E. Graph showing the DCF fluorescence increase over time with TBH 250 μmol/L.

F. Graph showing the DCF fluorescence in wt and CHF conditions with or without CsA.


Figure 6

A

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<tr>
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- α-tubulin: 52 kDa
- Bcl-2: 26 kDa
- Bax: 21 kDa

B

Bcl-2/Bax

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C

wt

CHF147

D

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LC3II/LC3I

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<tbody>
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