Toxicology in Vitro 28 (2014) 1443–1448

Contents lists available at ScienceDirect

Toxicology in Vitro

journal homepage: www.elsevier.com/locate/toxinvit

# 4-Phenylbutyric acid prevent cytotoxicity induced by thapsigargin in rat cardiac fibroblast

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ARTICLE INFO

*Article history:*
Received 8 April 2014
Accepted 31 July 2014
Available online 20 August 2014

*Keywords:*
Cardiac fibroblast
ER stress
Thapsigargin
4-Phenylbutyric acid

ABSTRACT

Cardiac fibroblast (CF) survival is important for the maintenance of the extracellular matrix homeostasis in the heart; providing a functional support to cardiomyocytes necessary for the correct myocardial function. Endoplasmic reticulum (ER) stress causes cellular dysfunction and cell death by apoptosis; and thapsigargin is a well-known ER stress inducer. On the other hand, the chemical chaperone, 4-phenylbutyric acid (4-PBA) had showed to prevent ER stress; however, in cardiac fibroblast both the ER stress induced by thapsigargin and prevention by 4-PBA, have not been studied in detail.

Neonate rat CF were treated with thapsigargin in presence or absence of 4-PBA, and cell viability was evaluated by trypan blue exclusion and apoptosis by flow cytometry; whereas CHOP, BIP, PDI, ATF4 and procollagen protein levels were assessed by western blot.

In CF, thapsigargin triggered the unfolded protein response detected by early increases in ATF4, CHOP, PDI and BIP protein levels as well as, the accumulation of intracellular procollagen. Thapsigargin also stimulated CF death in a time and concentration-dependent manner. ER stress, CF death and apoptosis induced by thapsigargin were prevented by 4-PBA.

Conclusion our data suggest that 4-PBA prevent ER stress, intracellular procollagen accumulation, CF death and apoptosis induced by thapsigargin.



## 1. Introduction

The accumulation of unfolded proteins in the endoplasmic reticulum (ER) evokes the unfolded protein response (UPR), which is involved in a wide variety of human disorders (Patil and Walter, 2001; Kaufman, 1999; Oyadomari et al., 2001), including cardiac diseases such as cardiac hypertrophy and fibrosis (Park et al., 2012; Ayala et al., 2011; Lenna and Trojanowska, 2012). The persistent accumulation of misfolded proteins that surpass the capacity of ER quality control causes cellular dysfunction and cell death by apoptosis (Patil and Walter, 2001). In this regard, CHOP protein levels are increased and involved in apoptosis by ER stress (Gotoh et al., 2002). However, other evidence indicates that high CHOP protein levels are associated to cell death by ER stress independent mechanisms (Sung et al., 2012; Gopalan et al., 2013). Different pathophysiological or chemical stimuli lead to the development of ER stress, including thapsigargin (inhibitor of sarco(endo)plasmic reticulum $Ca^{2+}$ ATPase, SERCA). Thapsigargin is capable of producing ER stress in primary rat islet cells (Choi et al., 2008), mouse embryonic fibroblasts (Dianhong et al., 2008) and pancreatic β-cells (Gómez et al., 2008), among others.

Cardiac fibroblasts (CF) are the most numerous cell in the heart, their main function is the secretion of extracellular matrix (ECM) proteins, growth factors and cytokines involved in the maintenance and homeostasis of the ECM, to give a functional support to cardiomyocytes, contributing to the correct myocardial function (Weber, 1997). CF plays an active role in wound healing, hypertrophy, and fibrosis (Porter and Turner, 2009). Studies in isolated CF have focused on gene regulation and secretion of various ECM proteins (Díaz-Araya et al., 2003), like collagen, which is tightly regulated by chaperones in the ER (Lamandé and Bateman, 1999; Nagata, 1998). Type I collagen is the most abundant protein in

*Abbreviations:* 4-PBA, 4-phenylbutyric acid; CF, cardiac fibroblasts; ER, endoplasmic reticulum; FBS, fetal bovine serum; FCS, fetal calf serum; PBS, phosphate buffer saline; ROS, reactive oxygen species; UPR, unfolded protein response.

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http://dx.doi.org/10.1016/j.tiv.2014.07.013
0887-2333/

cardiac tissue, and the proper folding of its triple helix is crucial for the formation of tissue matrix. As with many other proteins, a variety of different chaperone molecules appear to be involved in its folding. Some are general ER chaperones, e.g., calnexin, BiP, GRP94, and PDI; while others are collagen-specific, e.g., HSP47 and prolyl-4-hydroxylase (Lamandé and Bateman, 1999; Nagata, 1998).

On the other hand, chemical chaperones such as 4-phenylbutyric acid (4-PBA), are a group of compounds known to improve ER folding capacity and facilitate the trafficking of mutant proteins by stabilizing their conformation (Welch and Brown, 1996). 4-PBA is a nontoxic butyrate analog and by its physicochemical properties, 4-PBA stabilizes the protein structure, improving the folding protein capacity and mutant protein trafficking (de Almeida et al., 2007; Özcan et al., 2006). Moreover, there are some studies in gingival fibroblast where ER stress thapsigargin-induced is prevented by 4-PBA (Kim et al., 2012). However, CF are key players in the initiation, progression and resolution of cardiac diseases. Up to the date, there are no studies focused on ER stress thapsigargin-induced and their relationship with CF viability and collagen secretion. Understanding how CF responds to ER stress is necessary to assess their contribution to cardiac remodeling and fibrosis, and moreover, the application of this chaperone could be a promising pharmacological agent for the treatment of the aforementioned disorders.

## 2. Materials and methods

### 2.1. Reagents

The following reagents were from Sigma Chemical Co (St. Louis, MO, USA): trypan blue, 4-phenylbutiric acid and β-Tubulin antibody. 0.5% Tripsin/EDTA, pre-stained molecular weight standard, fetal bovine serum (FBS) and fetal calf serum (FCS) were purchased from Gibco BRL (Carlsbad, CA, USA). All organic and inorganic compounds were purchased from Merck (Darmstadt, Germany). The enhanced chemo-luminescence reagent was from Perkin Elmer Life Sciences, Inc (Boston, MA, USA). The sterile plastic material used for CF cultures was purchased from Corning®. The primary antibody for PDI and BIP were purchased from Cell Signaling Technology (Boston, MA, USA). CHOP and ATF4 antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Procollagen antibody was from Millipore (MA, USA), Thapsigargin was obtained from BIOMOL international Inc. (Playmouth, PA, USA) and was prepared according to the data sheet protocol.

### 2.2. Animals

All the animals were handled according to animal welfare regulations of the University of Chile. The investigation was performed in compliance with the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1996).

### 2.3. Isolation and culture of cardiac fibroblasts

The isolation and culture of CF was performed according to Díaz-Araya et al. (2003). Briefly, neonatal Sprague–Dawley rats were decapitated and heart was extracted immediately under aseptic environment. Atria were removed and ventricles were finely chopped for posterior pancreatine and colagenase II digestion. The digestion yield was precultured for 2 h at 37 °C in culture media containing 5% FBS and 10% FCS. Differential adhesion on plastic allows the isolation of CF from cardiac myocytes. The purity of the CF population was assessed through the expression of several markers. The majority of the cells expressed vimentin and DDR2, while there was poor expression of α-SMA (myofibroblast marker).

After 2 h, culture media was replaced for DMEM-F12 containing 10% FBS media, 100 μg/mL streptomycin and 100 units/mL penicilin in a humid atmosphere of 5% $CO_2$ and 95% air at 37 °C. When CF proliferated to confluence, cellular passages were performed using 0.05% trypsin/EDTA (passage 2 as maximum). After passage 2, cultures were maintained with DMEM-F12. For the experimental assays that require the use of thapsigargin, cells were incubated by 24 h with DMEM-F12, then the media was replaced and thapsigargin was added for different times. For the experiments that require the use of 4-PBA, this chemical was added 30 min before thapsigargin.

### 2.4. Cell viability

CF cells were passaged and cultured in 60 mm culture dishes at $5 \times 10^4$ cells per dish for one day. CF were treated with thapsigargin (0–10 μM), for different times in presence or absence of 4-PBA (0.1–10 mM). After stimulation period, cells were treated with 0.05% trypsin/EDTA 1× for cell detachment. Then, trypsin/EDTA action was inhibited by the addition of DMEM-F12 plus 10% FBS. After detachment, a 20 μL aliquot was mixed with 20 μL trypan blue and transferred to a Neubauer haemocytometer and the cells were counted by microscopy. The viable cell ratio was calculated as: viable cell ratio (%) = (unstained cell number/total cell number) × 100.

### 2.5. Detection of DNA fragmentation by flow cytometry

After treatment, cells were collected, permeabilized with ice-methanol for 24 h, and treated with RNAase in PBS for 2 h, and 3 μL propidium iodide (PI, 25 mg/mL). Finally, the DNA fragmentation (sub-population G1) was analyzed by flow cytometry (Becks, Dickinson). A total of 15,000 cells/sample were analyzed.

### 2.6. Western blot analysis

Protein extracts (50 μg) were separated in 10% SDS-polyacrilamide gels. Electrophoresis was performed at 70 V for 2 h. Proteins were electrotransferred to a nitrocellulose membrane (BioRad) at 350 mA for 90 min. Membranes were incubated in blocking buffer (non-fat milk 5% (w/v)/TBS-Tween-20 0.1%, 5% p/v) for 60 min at room temperature. After several wash cycles using TBS 1×, the membranes were incubated with antibodies against PDI, BIP, procollagen (1:1000 dilution) or against ATF4 and CHOP antibodies (1:2000 dilution) in TBS-tween-20 0.1% buffer containing BSA 5%/ or PBS 1× – 5% non-fat milk overnight at 4 °C. After incubation, membranes were washed three times with TBS-Tween-20 0.1% for 5 min and then incubated with secondary antibodies for 2 h at room temperature. After washing with TBS–Tween 0.01% for 5 min, membranes were exposed to ECL reagent. BioMax (Kodax) films were used for Western blot registering. Blots were quantified by laser scanning densitometry. All blots were controlled for equal loading by inmunostaining membranes with β-tubulin (1:10,000 dilution). Results were expressed as the ratio of protein present in samples following stimulation to control samples.

### 2.7. Statistical analysis

All data are presented as the mean ± SEM of *n* number of experiments. Each replicate is considered as an independent biological experiment. The differences in each parameter were evaluated by a 1-way ANOVA of the increase or decrease of each variable measured. One-way ANOVA was followed by Tukey test to compare