Experimental and Molecular Pathology 92 (2012) 97–104

Contents lists available at SciVerse ScienceDirect

Experimental and Molecular Pathology

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

# Attenuation of endoplasmic reticulum stress using the chemical chaperone 4-phenylbutyric acid prevents cardiac fibrosis induced by isoproterenol

Pedro Ayala [a], José Montenegro [a], Raúl Vivar [a], Alan Letelier [a], Pablo Aránguiz Urroz [a], Miguel Copaja [a], Deisy Pivet [a], Claudio Humeres [a], Rodrigo Troncoso [a], José Miguel Vicencio [a], Sergio Lavandero [a,b], Guillermo Díaz-Araya [a,*]

[a] FONDAP CEMC, Centro de Estudios Moleculares de la Célula, Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Santiago, Chile
[b] Instituto de Ciencias Biomédicas, Facultad de Medicina, Universidad de Chile, Santiago, Chile

ARTICLE INFO

*Article history:*
Received 28 July 2011
Available online 10 November 2011

*Keywords:*
4-Phenylbutyric acid
Endoplasmic reticulum stress
Heart
Isoproterenol
Fibrosis

ABSTRACT

Increasing evidence indicates that endoplasmic reticulum (ER) stress is involved in various diseases. In the human heart, ischemia/reperfusion has been correlated to ER stress, and several markers of the unfolded protein response (UPR) participate during cardiac remodeling and fibrosis. Here, we used isoproterenol (ISO) injection as a model for in vivo cardiac fibrosis. ISO induced significant cardiomyocyte loss and collagen deposition in the damaged areas of the endocardium. These responses were accompanied by an increase in the protein levels of the luminal ER chaperones BIP and PDI, as well as an increase in the UPR effector CHOP. The use of the chemical chaperone 4-phenylbutyric acid (4-PBA) prevented the activation of the UPR, the increase in luminal chaperones and also, leads to decreased collagen deposition, cardiomyocyte loss into the damaged zones. Our results suggest that cardiac damage and fibrosis induced in vivo by the beta-adrenergic agonist ISO are tightly related to ER stress signaling pathways, and that increasing the ER luminal folding capacity with exogenously administrated 4-PBA is a powerful strategy for preventing the development of cardiac fibrosis. Additionally, 4-PBA might prevent the loss of cardiomyocytes. Our data suggests that the attenuation of ER stress pathways with pharmacological compounds such as the chemical chaperone 4-PBA can prevent the development of cardiac fibrosis and adverse remodeling.



## Introduction

A variety of human diseases involve endoplasmic reticulum (ER) stress, a condition characterized by an accumulation of unfolded proteins in the ER lumen (Hotamisligil, 2010; Kaufman, 1999; Oyadomari et al., 2001). The activation of a mechanism known as the unfolded protein response (UPR) attempts to reduce the amount of misfolded proteins by increasing the production of the ER chaperones such as BIP/Grp78, Grp94 and PDI, which optimize protein folding (Patil and Walter, 2001). However, the persistent accumulation of misfolded proteins leads to cellular dysfunction and cell death. In the human heart, myocardial ischemia is a severe trauma for cardiac cells. As a result, ischemia causes extensive biochemical changes and one of them is stress affecting the ER (Scarabelli and Gottlieb, 2004). Several studies have correlated high levels of ER stress with myocardial damage (Belmont et al., 2010; Brodsky, 2007; Glembotski, 2008; Okada et al., 2004; Thuerauf et al., 2006), whereas others suggest that ER stress might protect the heart, and even foster the hypertrophic growth of the myocardium (Barnes and Smoak, 2000; Mao et al., 2006). Irrespective of such discrepancies, the involvement of ER stress in cardiac fibrosis in particular, has not been extensively studied.

The experimental administration of isoproterenol (ISO) constitutes a well-established model for in-vivo study of an acute hyperadrenergic state that it is accompanied by cardiac myocyte necrosis which happens to lead to tissue repair and consequent fibrosis (Benjamin et al., 1989; Grimm et al., 1998; Rona, 1985). The pathogenesis of the catecholamine-induced myocardial necrosis is multifactorial (Benjamin et al., 1989; Chagoya de Sánchez et al., 1997; Díaz-Muñoz et al., 2006; Rona, 1985). The necrosis is due to catecholamine-induced intracellular $Ca^{2+}$ overloading of cardiac myocytes, including their mitochondria. There follow the induction of oxidative stress and the opening of the mitochondrial inner membrane permeability transition pore with ensuing organellar degeneration. Myocyte necrosis follows and is replaced by collagen. An imbalance between synthesis/degradation of extracellular matrix (ECM) proteins finally results in excessive accumulation of fibrillar collagen.

High levels of type I collagen, the main fibrillar collagen found in cardiac fibrosis, stiffen the ventricles and impede both contraction

*Abbreviations:* ER, Endoplasmic reticulum; UPR, Unfolded protein response; ISO, Isoproterenol; 4-PBA, 4-Phenylbutyric acid; ECM, Extracellular matrix.

* Corresponding author at: Facultad de Ciencias Químicas y Farmacéuticas, Universidad de Chile, Sergio Livingstone 1007, Santiago 8380492, Chile. Fax: +56 2 978 2912.
*E-mail address:* gadiaz@ciq.uchile.cl (G. Díaz-Araya).

0014-4800/$ – see front matter © 2011 Elsevier Inc. All rights reserved.
doi:10.1016/j.yexmp.2011.10.012

and relaxation, impairing the electrical coupling of cardiomyocytes and the global cardiac function (Swynghedauw, 1999). The synthesis of type I collagen is tightly regulated by ER chaperones; therefore proper luminal folding of its triple helix is crucial for the generation of the cardiac ECM. The precursor procollagen is flanked by globular N- and C-terminal peptides, and a variety of different chaperone molecules are involved in its folding. Some of them are general ER chaperones (calnexin, BIP, GRP94 and PDI), whereas others are collagen-specific (HSP47 and prolyl-4-hydroxylase) (Lamande and Bateman, 1999). Therefore, cardiac fibrosis apparently is tightly related with ER folding capacity and an imbalance between synthesis and folding capacity could trigger ER stress and treatments leading to enhanced ER folding capacity might restore proper collagen ECM balance and avoid cardiac fibrosis.

Sodium 4-phenylbutyrate (4-PBA) is a low-molecular weight fatty acid and a non-toxic pharmacological compound that is currently approved for its clinical use in pathologic disorders of the urea cycle because of its properties as an ammonia scavenger (Maestri et al., 1996). In addition, 4-PBA is a weak histone deacetylase inhibitor and a transcriptional activator of β and γ globins (Perlmutter, 2002). 4-PBA displays low toxicity and provides protection against various noxious stimuli (Ozcan et al., 2006), thus it has been proposed for the treatment of cystic fibrosis, sickle cell disease and cancer (Dover et al., 1994; Goh et al., 2001; Zeitlin et al., 2002). Importantly, 4-PBA can act as a chemical chaperone in the ER, because its physicochemical properties allow the stabilization of peptide structures, improving the luminal folding capacity and the traffic of aberrant proteins (de Almeida et al., 2007; Vilatoba et al., 2005). Thus, the use of 4-PBA may provide a therapeutic approach for blocking the pathologic process induced by ISO. However, no pharmacologic approach for the treatment of cardiac fibrosis due to ER stress has been reported to our knowledge. In this work, we aimed at this hypothesis and evaluated the effect of the chemical chaperone 4-PBA upon cardiac damage, as well as the mechanisms underlying these effects in a model of cardiac fibrosis induced by ISO. We present novel and interesting data that supports the cardioprotective actions of increased buffering of misfolded proteins on cardiac fibrosis.

## Materials and methods

### *Reagents*

The following reagents were acquired from Sigma Chemical Co (St. Louis, MO, USA): trypan blue, 4-phenylbutyric acid. Trypsin/EDTA, pre-stained molecular weight standard and fetal bovine serum (FBS) 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 purchased from PerkinElmer Life Sciences, Inc (Boston, MA, USA). The primary antibody for PDI and BIP was purchased from Cell Signaling Technology (Boston, MA, USA). CHOP antibody was acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

### *Animals*

Animal handlings were conducted according to the Animal Welfare Regulations of the University of Chile. The investigation conformed to the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health (NIH publication No. 85–23, revised 1996).

### *Experimental groups*

Cardiac fibrosis was induced by ISO administration as previously described (Grimm et al., 1998). Briefly, rats received a single subcutaneous injection of (±) isoproterenol hemisulfate (ISO; Sigma, St. Louis, MO), 50 mg/kg body weight. We recognize that small and repetitive doses or isoproterenol delivery by osmotic pump is more frequently used, however, with the single doses of isoproterenol we had a rapid and strong cardiac damage. A matched group of control rats received saline injection. The chemical chaperone (4-PBA sodium salt, 80 mg/kg, was dissolved in 0.9% saline) was administrated via subcutaneous injection, 2 h before isoproterenol injection, and for 10 days after. Controls were given similar volumes of saline. Animals were killed at 9 h, 2, 5 and 10 days after ISO injection. On the day of the experiment, hearts were rapidly removed and washed in cold 0.9% saline, freed of connective tissue, and placed on ice. The left and right ventricles were separated, washed extensively with saline to remove all contaminating blood, dried, and weighed. The tissues were quick-frozen in liquid nitrogen and stored at −80 °C until analyzed for western blot. Hearts were also fixed in neutrally buffered formalin for collagen and morphometric analysis.

### *Collagen deposition*

A 2-mm thick coronal section was taken from the equator of each heart and fixed in neutrally buffered formalin. Formalin-fixed sections were dehydrated through a graded series of alcohol and xylene and embedded in paraffin. Paraffin sections (5 mm thick) were stained with hematoxylin and eosin for histological evaluation. Sections from the myocardium located at the equator of the left ventricle were stained with Masson's Trichrome for determination of interstitial collagen. Each section was viewed in its entirety by a single investigator who was unaware of the nature of the experimental groups. ISO-induced fibrosis was located primarily within the endocardium, with extensions of fibrous tissue reaching the midwall.

### *Soluble collagen quantification*

For soluble collagen quantification, heart tissue homogenized aliquots were taken and samples were treated with “soluble collagen assay” Sircol® (Biocolor, Ireland) according to the manufacturer's instructions.

### *Immunohistochemistry*

Left ventricle (LV) sample tissue was fixed with silane, dried and dewaxed to water, and cuts were washed with TBS/Tween-20 (TBS-T) and blocked with 3% horse serum in TBST. The samples were incubated with anti-BIP 1:50, anti-PDI 1:50 and developed with Vectastain PK7100 kit (Vector, California, USA). The cell nuclei are contrasted with Mayer hematoxylin, washed with water and turned to blue and the sections were dehydrated to a battery of ethanol, rinsed with xylene and permanently mounted with hydrophobic medium.

### *Preparation of total cell extract*

Left ventricle sample was homogenized in cold lysis buffer (20 mM HEPES, pH 7.5, 150 mM NaCl, 1% Triton-X100, 10% glycerol, 1 mM EDTA, 10 mM sodium diphosphate, 100 mM NaF, 17.5 mM beta-glycerophosphate, 1 mM phenylmethylsulfonyl fluoride, leupeptin 2 μg/mL; 10 mM aprotinin; 1 mM PMSF and 100 μM $Na_3VO_4$). Homogenized solutions were centrifuged at 10,000 RPM during 15 min at 4 °C. Supernatants were recovered and protein concentration was determined by a Bradford assay. Proteins were denaturized into SDS-PAGE buffer 4× (glycerol 20 mL, 2-mercaptoethanol 10 mL, SDS 5 g, Tris-base 1.51 g, bromophenol blue 0.01 g, water q. s. 100 mL, pH adjusted to 6.8). Samples were heated to 95 °C for 5 min before electrophoresis.