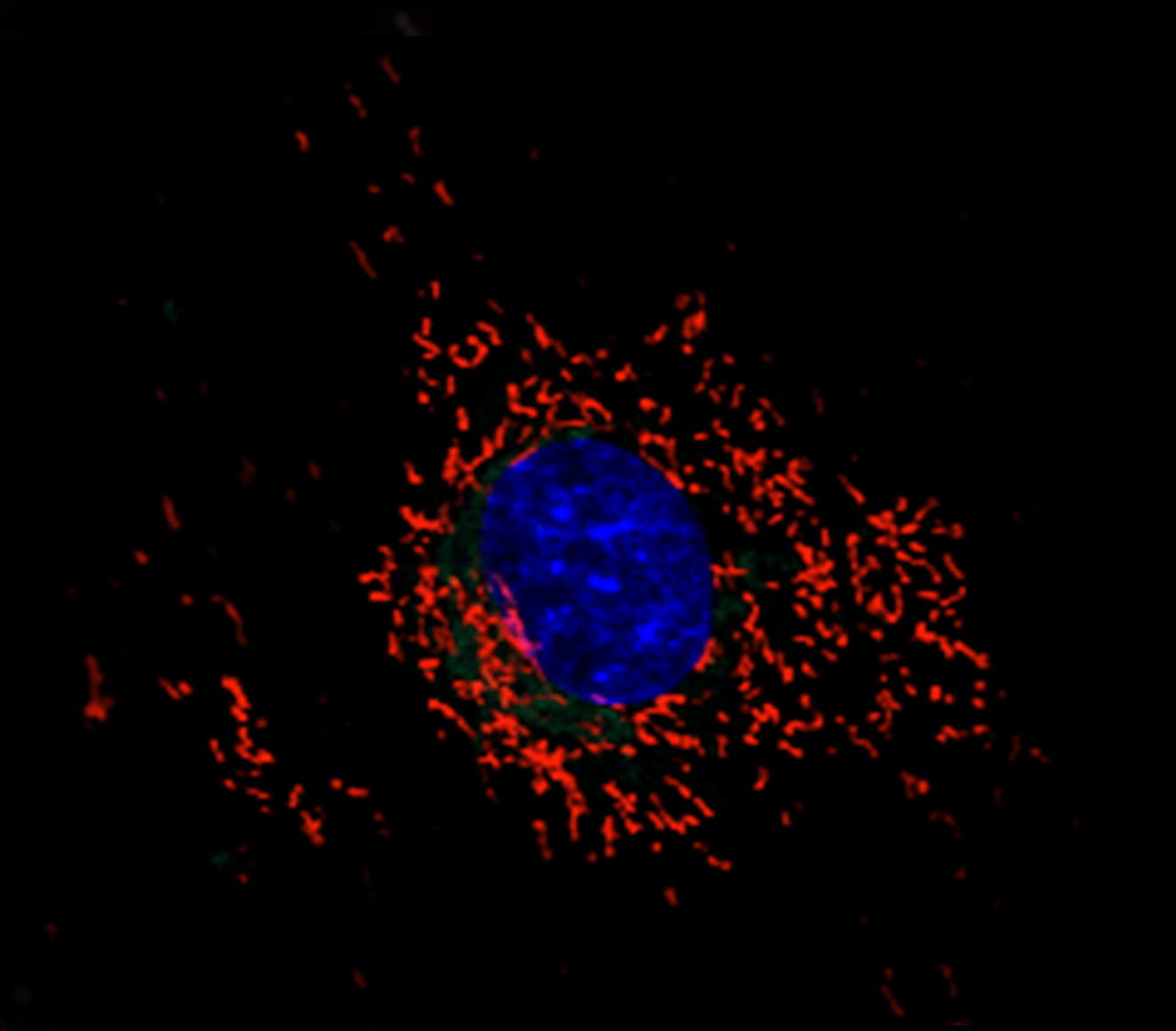
Impact of H9c2 Cardiomyoblast Differentiation on Isoproterenol Toxicity: Different Modulation of Signaling Pathways



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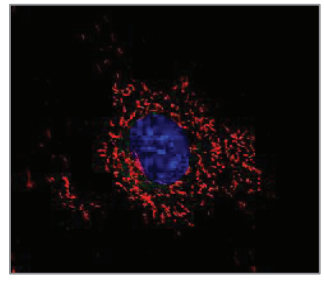

Confocal microscopy of H9c2 myoblast showing the mitochondrial network (red, labeled with Mitotracker Red), the nucleus (blue, labeled with Hoechst 33342) and intracellular calcium (green, labeled with Fluo-4-AM)

Impact of H9c2 Cardiomyoblast Differentiation on Isoproterenol Toxicity: Different Modulation of Signaling Pathways

Influência do Estado de Diferenciação de Cardiomioblastos H9c2 na Toxicidade Induzida pelo Isoproterenol: Diferente Modulação de Vias de Sinalização.

Dissertation presented to the Faculty of Sciences and Technology of the University of Coimbra in partial fulfillment of the requirements for Doctor of Philosophy (PhD) graduation in Biology, Specialization in Cellular Biology.

Dissertação apresentada á Faculdade de Ciências e Tecnologia da Universidade de Coimbra, com vista á obtenção do grau de Doutor em Biologia, especialidade em Biologia Celular.

This work was conducted at the Center for Neurosciences and Cell Biology, University of Coimbra, under the supervision of Dr. Paulo Oliveira and Dr. Maria Sancha Santos; at the Department of Anatomy, Microbiology and Pathology, University of Minnesota Medical School, Duluth, MN, USA under the local guidance of Dr. Jon Holy; at the Nencki Institute of Experimental Biology, Warsaw, Poland under the local guidance of Dr. Mariusz Wieckowski and in the Spanish National Center for Cardiovascular Research (CNIC), Madrid, Spain under the local overview of Dr. Susana Gónzalez López. This thesis was supported by a Ph.D. fellowship from the Portuguese Foundation for Science and Technology (SFRH/BD/41384/2007), co-funded by Fundo Social Europeu (FSE) in the scope of Programa Operacional Potencial Humano (POPH) of QREN, and a research grant from FCT to Dr. Paulo Oliveira (project PTDC/QUI/64358/2006)

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“If we knew what it was we were doing,
it would not be called research,
would it?" Albert Einstein

Scientific papers

Most of the work presented in this dissertation is published in international peer-reviewed scientific journals, as follows:

- **Branco AF**, Sampaio SF, Moreira AC, Holy J, Wallace KB, Baldeiras I, Oliveira PJ, Sardão VA. Differentiation-Dependent Doxorubicin Toxicity on H9c2 Cardiomyoblasts. Cardiovascular Toxicology, 2012 Jun 29.

- **Branco AF**, Pereira SL, Moreira AC, Holy J, Sardão VA, Oliveira PJ. Isoproterenol Cytotoxicity is Dependent on the Differentiation State of the Cardiomyoblast H9c2 Cell Line. Cardiovascular Toxicology, 2011 Sep;11(3):191-203.

I certify that I have obtained a permission from the co-authors of the present publications to include the above material in my thesis.

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À Susana Pereira. Mal eu sabia que aquela viagem que começou com meia hora de atraso e 6 doses de xarope no estômago ia criar este laço. Obrigada pelos momentos divertidos, pela amizade e pelos conselhos.

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List of contents

List of Figures

List of Tables

List of Abbreviations

A

AC: adenylate cyclase
AIF: Apoptosis-inducing factor
AKT/PKB: Protein Kinase B
-AM: Acetoxymethyl ester
ANOVA: Analysis of variance
ANT: Adenine nucleotide translocase
Apaf-1: Apoptotic protease-activating factor 1
ARC: Apoptosis repressor with caspase recruitment domain
ATCC: American Type Culture Collection
ATP: Adenosine triphosphate

B

β-AR: β-adrenergic receptor
Bad: Bcl-2-associated death promoter
Bax: Bcl-2-associated X protein
Bid: BH3 interacting domain death agonist
Bcl-2: B-cell lymphoma 2
BrdU: Bromodeoxyuridine
BSA: Bovine serum albumin

C

Ca^{2+}: Calcium
cAMP: Cycle adenosine monophosphate
CHAPS: 3-[(3-Cholamidopropyl)dimethylammonio]-1-propanesulfonate
CypF: Cyclophilin-F
Cytc: Cytochrome *c*
CM-H_2DCFDA: Chloromethyl derivate of dichlorodihydrofluorescein diacetate

D

DAPI: 4',6-diamidino-2-phenylindole
DHE: Dihydroethidium
DISC: Death-inducing signaling complex
DMEM: Dulbecco´s modified Eagle´s medium
DMSO: Dimethyl sulfoxide
DNA: Deoxyribonucleic
DTT: Dithiothreitol
Drp-1: Dynamin-related protein 1

E

ECF: Eosinophil chemotactic factor
EDTA: Ethylenediaminetetraacetic acid
Endo G: Endonuclease G
ERK: Extracellular signal–regulated kinase
ETC: Electron transport chain

F

FADD: Fas-associated death domain protein
FBS: Fetal bovine serum
FL1: flow cytometer green fluorescence detector
FL2: flow cytometer orange fluorescence detector

G

G_i: inhibitory G protein
G protein: guanosine trisphosphate protein
G_s: stimulatory G protein
GSH: Reduced glutathione

H

HCL: Hydrochloric acid
HEPES: 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

I

IAP: Inhibitor of apoptosis protein
IgG: Immunoglobulin G
ISO: Isoproterenol

J

JNK: c-Jun N-terminal kinase

K

kDa: kilodalton

M

MAPK: Mitogen-Activated Protein (MAP) Kinase
MFN1: Mitofusin 1
MFN2: Mitofusin 2
MIM: Mitochondrial Inner Membrane
MnSOD: Manganese Superoxide Dismutase
MOM: Mitochondrial Outer Membrane
mPTP: mitochondrial Permeability Transition Pore
mRNA: messenger RNA

N

NAC: N-Acetylcysteine
NDUFB8: NADH dehydrogenase [ubiquinone] 1 beta subcomplex subunit 8

O

Opa1: Optic atrophy 1

P

P53: Tumor protein 53
PARP: Poly(ADP-ribose) polymerase-1
PBS: Phosphate Buffered Saline
PBS-T: Phosphate Buffered Saline with 0.1% Tween
PI3K: Phosphoinositide 3-kinase
PKA: protein kinase A
PMSF: Phenylmethylsulfonyl fluoride
p-NA: p-Nitroanilide
PVDF: Polyvinylidene fluoride

Q

QIAshredder: homogenizes cell or tissue lysates to reduce viscosity

R

RA: All-trans retinoic acid
RLT: buffer for lysis of cells and tissues before RNA isolation
RNA: Ribonucleic Acid
ROS: Reactive Oxygen Species
RyR: Ryanodine Receptor

S

Smac/Diablo: Second mitochondria derived activator of caspases/direct IAP binding protein with low pI
SDS: Sodium dodecyl sulfate
SEM: Standard error of the mean
SERCA: Sarco/Endoplasmic-reticulum-Ca^{2+}-ATPase
SRB: Sulforhodamine B
SV40 large T: Simian Vacuolating Virus 40 T

T

tBid: Truncated Bid
TBST: Tris-Buffered Saline Tween-20
TMRM: Tetramethyl Rhodamine Methyl Ester
TNFα: Tumor necrosis factor-α
TNF: Tumor Necrosis Factor

U

UQCRC2: Ubiquinol-cytochrome c reductase core protein 2
UCP: Uncoupling protein

V

VDAC: Voltage-Dependent Anion Channel

X

XIAP: X-chromossome linked IAP

Z

z-vad-fmk: benzyloxycarbonyl-valine-alanine-aspartate fluoromethylketone

Abstract

The chronic exposure of cardiomyocytes to catecholamines is associated with pathologic alterations. Stress conditions and tissue insults trigger the activation of neurohormonal systems and catecholamine synthesis is consequently increased. The binding of circulating catecholamines to adrenergic receptors normally incurs in physiological responses that comprises increasing contractile force, heart rate, metabolic activity, among other effects that affect cardiac performance. However, hyperadrenergic stimulation, resulting from sustained activation of β-adrenergic receptors (β-AR), is thought to be directly involved in cardiomyocyte apoptosis. Over-activation of specific signaling pathways results in several cellular processes which lead to progressive myocardium deterioration and contribute toward decompensated heart failure.

In this regard, the present work addresses the mechanisms by which cardiac cells respond to an overstimulation of β-AR according to their differentiation state (i. e. embryonic/adult). For this objective, we used the rat myoblastic H9c2 cell line which can be differentiated in adult skeletal or cardiac muscle cells. The synthetic catecholamine isoproterenol (ISO) was used to stimulate β-AR since its specificity represents an advantage when compared with endogenous catecholamines.

Our hypothesis is that β-AR over-stimulation results in different cell fates depending on the differentiation stage of the cell. This approach is relevant since most studies are conducted in H9c2 cells in their undifferentiated state (embryonic) raising questions on its applicability to cardiotoxicity studies.

Initial characterization of H9c2 differentiation was performed concerning differences in terms of intrinsic cell defenses and signaling pathways which may render cells more or less susceptible to ISO. The alteration in cellular physiology during differentiation was strengthened by gene array analyses showing up-regulation of mitochondrial metabolism genes and genes encoding calcium transporters and differentiation markers, in retinoic acid-differentiated H9c2 cells.

In what concern ISO-induced toxicity, the present work demonstrates that undifferentiated cells have increased resistance to ISO, which can be

explained by increased anti-apoptotic proteins including Bcl-2 and Bcl-xL and intrinsic defense capacity such as MnSOD. Several stress responses were increased in differentiated cells, including p66Shc, p53 and Bax, at the same with higher mitochondrial degeneration. This was particularly seen by mitochondrial depolarization, loss of respiratory complexes and oxidative stress, which was mostly observed in differentiated H9c2 cells. Moreover, after ISO treatment, differentiated H9c2 cells showed increased cAMP, cytosolic calcium accumulation, cleavage of PARP and activation of caspase 3. Alterations in calcineurin and p38-MAPK in undifferentiated and differentiated cells can also account for the larger toxicity of ISO in the latter. Moreover, the increase in pro-apoptotic proteins was not accompanied in increased anti-apoptotic counterparts, as occurred in undifferentiated myoblasts (Bcl-xL, Bcl-2). We conclude that H9c2 cells present differential regulation of stress responses during their differentiation which impact the toxicity of several agents.

In conclusion, the present thesis demonstrates that undifferentiated and differentiated H9c2 cells possess different susceptibility to the β-AR agonist ISO in which the balance between pro- and anti-apoptotic pathways are involved. The results are relevant from a basic toxicology point of view, since this cell line is very used as a model for cardiac cells. Furthermore, the data supplies evidence that the H9c2 cell line can be used as a framework to investigate developmental cardiotoxicity.

Keywords: isoproterenol, β-adrenergic receptors, apoptotic pathways, mitochondria, H9c2 myoblasts, differentiation.

Sumário

A exposição crónica de cardiomiócitos a catecolaminas está associada a alterações patológicas.

Condições de stress e lesões no tecido cardíaco activam sistemas neurohormonais que induzem a consequente síntese de catecolaminas. A interacção de catecolaminas com receptores adrenérgicos induz respostas fisiológicas como o aumento da força de contracção muscular, aumento da actividade metabólica, entre outros efeitos que afectam o desempenho cardíaco.

Contudo, a estimulação adrenérgica resultante de uma activação prolongada de receptores β-adrenérgicos (β-AR) pode estar directamente envolvida na apoptose de cardiomiócitos contribuindo para insuficiência cardíaca descompensada. A sobre-activação de vias de sinalização específicas resulta na estimulação de vários processos celulares que conduzem á deterioração progressiva do miocardio. Neste sentido, o presente trabalho pretende investigar os mecanismos pelos quais células cardíacas respondem à sobre-activação de β-AR segundo o seu estado de diferenciação (embrionário/adulto). Com este objectivo, usamos a linha celular mioblastica de rato H9c2 que tem a capacidade de ser diferenciada em células musculares esqueléticas e cardíacas. A catecolamina sintética isoproterenol (ISO) foi usada no sentido de estimular β-AR já que a sua especificidade representa uma vantagem quando comparada com o uso de catecolaminas endógenas.

A nossa hipótese de trabalho é que a sobre-activação de β-AR resulta em diferentes respostas dependendo do estado de diferenciação celular. Dado que a maior parte dos estudos conduzidos em células H9c2 usa esta linha celular no seu estado indiferenciado (embrionário), questões relativas á sua aplicabilidade em estudos cardiotóxicos levantam algumas dúvidas.

A caracterização inicial do processo de diferenciação de H9c2 foi realizada tendo em conta diferenças em termos de defesas celulares intrínsecas e vias de sinalização que poderão induzir maior ou menor sensibilidade das células ao ISO. A alteração de processos fisiológicos nas células foi ainda investigada através de análise genética mostrando que genes envolvidos no metabolismo mitocondrial, na differenciação

celular e na codificação de transportadores de cálcio, estão sobre-expressos em células H9c2 diferenciadas em presença de ácido retinóico.

O presente trabalho demonstra que células indiferenciadas apresentam maior resistência ao ISO, o que pode ser explicado pelo aumento de proteinas anti-apoptóticas tais como Bcl-2 e Bcl-xL, e na capacidade de defesa intrínseca tais como MnSOD. Múltiplas respostas a condições de stress encontram-se aumentadas em células diferenciadas, incluíndo proteinas p66Shc, p53 e Bax bem como degeneração mitocondrial. Esta última, identificada através da despolarização mitocondrial, perda de complexos respiratórios e stress oxidativo, que foi maioritariamente observado em celulas H9c2 diferenciadas. Após tratamento com ISO, em células H9c2 diferenciadas verifica-se um aumento de cAMP, acumulação de cálcio no citosol, clivagem da PARP e activação de caspase-3. As alterações nas proteínas calcineurina e p38-MAPK nas células indiferenciadas e diferenciadas podem também contribuir para uma maior toxicidade ao ISO nas últimas. O aumento de proteínas pro-apoptóticas não foi acompanhado por um concumitante aumento de próteinas anti-apoptóticas, como verificado em células indiferenciadas (Bcl-xL e Bcl-2). Concluímos que as células H9c2 apresentam uma regulação diferencial em resposta a condições de stress durante a sua diferenciação o que influencia a toxicidade de agentes cardiotóxicos.

Em conclusão, o presente trabalho demonstra que células H9c2 indiferenciadas e diferenciadas possuem diferente susceptibilidade ao agonista de β-AR, ISO, sendo neste processo, o balanço entre proteínas pro- e anti-apoptóticas de grande importância. Os resultados são relevantes do ponto de vista toxicológico básico já que esta linha celular é normalmente utilizada como modelo para células cardíacas. Os resultados obtidos evidenciam que a linha celular H9c2 poderá ser utilizada em estudos que visam investigar a resposta cardiotóxica a fármacos, no entanto, há que ter presente que diferentes estados de diferenciação poderão influenciar diferentes susceptibilidades a vários agentes. Além disso, os resultados evidenciam que a linha H9c2 pode ser usada (como ferramenta) para investigar a cardiotoxicidade durante o desenvolvimento.

Palavras-chave: isoproterenol, receptores β-adrenérgicos, vias de sinalização apoptóticas, mitocôndria, mioblastos H9c2, diferenciação.

General Introduction

1. General Introduction

1.1 Cardiovascular disease as a public health problem

According to the World Health Organization (WHO), heart disease remains the number one cause of death and disability worldwide accounting to about one-third of all human mortality [1].

Cardiovascular disease (CVD) is a generic term to designate all pathologic alterations that affect the heart and blood vessels. The term cardiovascular disease collectively includes diverse disorders such as coronary heart disease (commonly known as heart attack), cerebrovascular disease (stroke), hypertensive heart disease, atherosclerosis (accumulation of fat, including cholesterol deposits on blood vessels walls), congenital heart disease and congestive heart failure. Usually, the latter is the culmination of long-standing diseases such as hypertension and ischemia resulting from atherosclerosis [2]. Heart failure represents one of the fastest-growing diseases and has been extensively studied as well. Heart failure is caused by any event, such as damage or overload, which compromises heart function. Interestingly, all these diseases not only have in common the fact that they occur in the cardiovascular system but also that can act together as a cause or consequence of each other as shown by sustained overload-induced heart failure.

Cardiac risk factors may include lifestyle habits such as unhealthy diet, lack of regular physical activity, tobacco smoking, excessive alcohol consumption, high blood pressure and high serum cholesterol levels as well as uncontrolled risk factors such as age, gender and genetic background [1].

Due to lifestyle habits, blood vessels can get hardened reducing both the wall diameter and the flow rate. To compensate for the decrease in blood

flow, increased sympathetic nervous system (SNS) activity can, initially, contribute to increase ventricle contractility in order to sustain ejection performance. However, over time, the neuro-hormonal stimulation of heart beating rate initiates a process of dynamic and morphological alterations [3, 4]. Often, the left ventricle develops hypertrophy associated with a complex set of alterations in the expression of cellular proteins. Also, several alterations lead to the progression of apoptotic cell death and loss of cardiomyocytes [5] (Fig. 1). If a part of the heart muscle becomes starved of oxygen or nutrients, development of localized lesions can occur.

Before the industrial revolution, the incidence of death resulting from heart diseases was lower. Modern technologies brought a sedentary lifestyle and later, processed dairy foods, further increased that tendency [6]. Living in a modern world implies also increasing deadlines and worries. In fact, stressor factors can differently interact with each individual, depending on their vulnerability to adversity which difficult the full understanding of the link between stress and heart disease. Psychological stress is well recognized by the scientific community as having an important role in cardiovascular diseases by acting as a trigger to potentiate acute heart problems [7, 8]. Several published works suggest that the release of epinephrine or norepinephrine to the bloodstream may contribute to the increase of fatty acids in the blood which can then deposit in the arteries, contributing to the development of atherosclerosis [9, 10]. The blockage of an artery can lead to stroke or myocardial infarction [11].

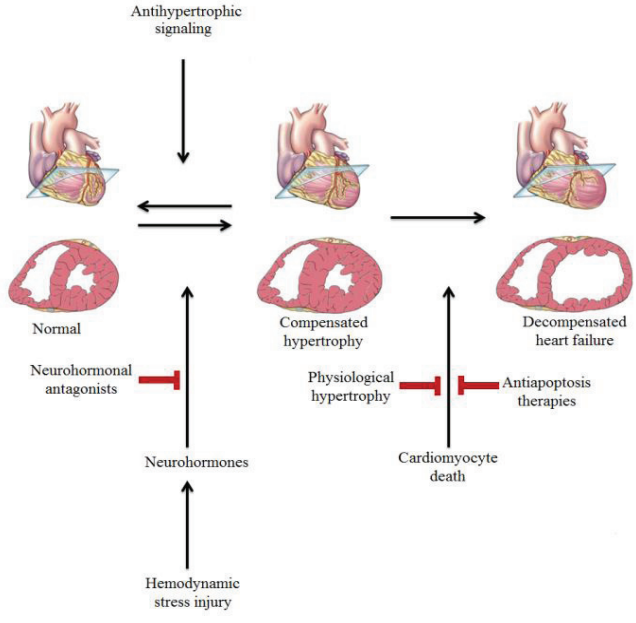


Figure 1 - Representative scheme of morphologic alterations induced by a stressor stimulus in the heart. Terminally differentiated cardiomyocytes adapt to increased work or hemodynamic stressors through an increase in heart rate. To compensate for the new cardiac output demand, neurohormonal and cellular signaling cascades are activated, resulting in ventricular remodeling as increased myocardial mass and ventricular walls thickness. Ultimately, myocardial hypertrophy leads to a progressive loss of cardiomyocyte which is the causal role of the transition to an end-stage of the disease. (Figure adapted from [12, 13]).

Evidences concerning the mechanisms by which cardiac myocytes undergo apoptotic cell death show that mechanical conditions and elevated levels of neurohumoral factors are stronger contributors [12]. In an experimental model of mechanical overload performed on cardiac papillary muscle, an increase of apoptotic cardiomyocytes were measured, demonstrating the link between ventricular stretch and apoptosis [14]. More details on the mechanisms of cardiac cell apoptosis will be given in chapter 1.3.2.

Fortunately, pharmacological treatments have improved in the last decades and are actively counteracting the progression of the disease due, in part, to extensive investigation on antihypertensive therapies and high blood cholesterol lowering agents [15].

1.2 Catecholamine synthesis and secretion as a stress response

Catecholamines are a group of organic compounds that include the hormones dopamine, norepinephrine and epinephrine which participate in different types of behavior, physiological conditions and diseases.

The scientific community has long investigated this family of compounds, especially due to the fast and relatively easy way to measure endogenous hormones in the fluids as well as their metabolites. Throughout scientific history, many Nobel Prizes in Physiology or Medicine resulted, at least in part, from research on the catecholaminergic system as evidenced by studies on the neurotransmission of nerve impulses, hypothalamus influence on emotional behaviors, neurotransmitters storage, release and reuptake mechanisms, among others [16].

In response to a stressful event, two main systems are activated: the hypothalamic–pituitary–adrenocortical (HPA) system, for long term responses, and the sympatho-adrenomedullary (SAM) pathway, for short term responses [17, 18]. When a stress reaction is triggered, the HPA and SAM systems are activated and, from the paraventricular nucleus of the hypothalamus, signals aimed at stimulating the pituitary gland to produce adrenocorticotropic hormone (ACTH) [18]. Sympathetic preganglionic neurons release ACTH towards the adrenal gland which, in turn, modulates the secretion of cortisol (from the adrenal cortex) and catecholamines (from the adrenal medulla) to maintain body homeostasis

regarding glycemic status and blood pressure (Fig. 2). Both result into an adequate response to body requirement [19]. The synthesis of catecholamines occurs in the center of the gland, the adrenal medulla, where chromaffin convert the amino acid tyrosine to the precursor of all catecholamines, DOPA, by hydroxylation.

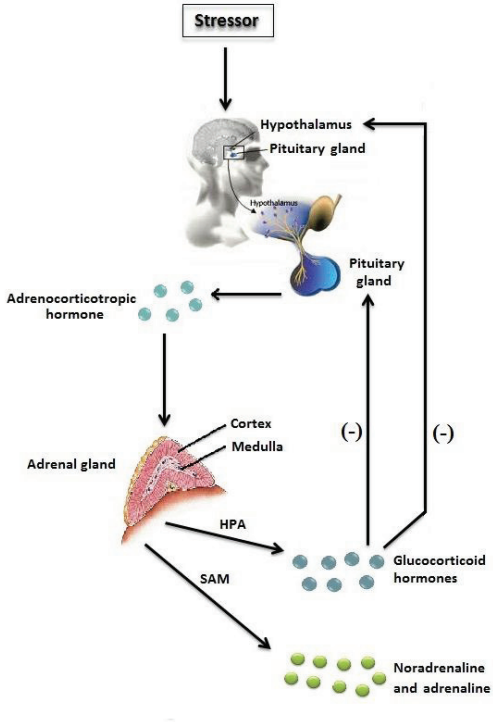


Figure 2 - Schematic representation of the components of HPA and SAM pathways triggered by stressor stimulation. A stressor stimulus activates the SNS that signals the body to be prepared to new requirements. The corticotropin-releasing hormone (CRH) released from the hypothalamus, stimulates pituitary gland to produce adrenocorticotropic hormone which in turn acts on adrenal gland where cortisol, epinephrine and norepinephrine are produced and released by the activation of the HPA and SAM axis, respectively. Ultimately and simultaneously both the modulation of body function and a negative feedback response occur to reproduce and cease the signal, respectively.

Dopamine can be synthesized by using DOPA as a precursor. Dopamine can then suffer metabolic modifications, resulting in the production of norepinephrine and epinephrine [20]. Glucocorticoids also act as a negative feedback on both the hypothalamus and pituitary gland to suppress the release of CRH and ACTH [21].

As referred before, a stressor condition can result from many different events and usually, under experimental conditions, social stressors can be distinguished from physical stressors. However, both types activate the HPA and SAM axis and increase cortisol and catecholamine secretion to modify heart rate and mobilize energy. There is evidence that positive social interactions increase levels of hypothalamus-released oxytocin that, in turn, depress HPA activity and contribute to physiological functions such as wound healing [22, 23] . There is also evidence that in patients with heart failure, SAM activity is increased which is confirmed by the increased epinephrine serum concentration [24].

1.3 Cardiovascular effects of catecholamines - Cardiomyocyte apoptosis triggered by overstimulation of β-adrenergic receptors

When cardiac performance undergoes an underloading condition, mechanisms are readily activated to stimulate the heart to fill new workload demands. Myocardial function is then highly stimulated by the SNS and released catecholamines have important effects on cardiomyocytes. During heart failure, the release of catecholamines occurs in an attempt to restore homeostasis; however, when persistent, this act as an insult driving stress to the heart. Commonly, catecholamine over-signaling originating from β-adrenergic receptors (β-AR) stimulation leads to cardiomyocyte death [25]. Stimulation of cardiac β-AR by norepinephrine or its synthetic analog isoproterenol induces apoptosis *in vivo* and *in vitro* [26]. During congestive heart failure, norepinephrine´s increase in the plasma does not only result from decreased re-uptake but also from an increase in hormone release [24].

1.3.1 Cardiomyocyte structure and behavior

The majority of the volume of the adult mammalian heart is occupied by cardiomyocytes. During fetal life, progenitor cells and early cardiomyocytes proliferate fast. Although a last round of DNA synthesis and nuclear mitosis without cytokinesis occurs after birth, cardiomyocytes soon exit the cell cycle [27]. In rats, the majority of cardiomyocytes are binucleated, while in humans this number is estimated to be around 57% [28]. Although not well studied, it has been suggested that the generation of twice the RNA for protein synthesis can be advantageous in metabolic active cells as muscles [29]. Cardiomyocytes are terminally differentiated cells which are presumed not to reenter the cell cycle even in response to mitogen or physiological stress activators. Despite this, when exposed to growth stimulus such as hormones, neuroendocrine factors or mechanical load, adult cardiomyocytes can increase their size without a concomitant cellular division undergoing hypertrophic growth.

Cardiomyocytes have three major components that drive the contractile force: a) the sarcolemma and T-tubules for impulse conduction; b) the sarcoplasmic reticulum that stores and releases calcium needed for activation of contractile proteins; and c) contractile elements including actin and myosin [30].

In cardiomyocytes, mitochondria constitute about 30% of cell mass and are strategically located due to their importance in ATP production critical for cell homeostasis and contraction reflecting the dependence of cardiac muscle on aerobic metabolism [31]. Mitochondria are the nexus of oxidative phosphorylation machinery aimed at ATP production. Besides the well-known function of mitochondria regarding the generation of energy, these organelles have other important roles including being the site of multiple metabolic pathways such as the β-

oxidation of fatty acids, the tricarboxylic acid (TCA) and the urea cycle [32]. Mitochondria also regulate reactive oxygen species (ROS) production, oxygen sensing, calcium homeostasis and thermogenesis [32].

Mitochondria are a complex and dynamic network. Apart from regulating mitochondrial function during cellular death, some proteins play a critical role on remodeling events in the mitochondrial membrane [33]. Altered mitochondrial morphology and fragmentation of mitochondrial network upon an apoptotic insult have been observed in heart disease [34, 35]. At the molecular levels, these processes have been reported to be mediated by three dynamin-family GTPases (GTPases), namely mitofusins 1 and 2 (Mfn1 and Mfn2) and the optic atrophy type 1 (OPA1), involved in fusion processes; and the dynamin-related protein 1 (DRP-1) and FIS1, which regulate mitochondrial fission processes [36].

1.3.2 Cell death mechanisms in cardiomyocyte degeneration

All cell death mechanisms, apoptosis, necrosis, and autophagy occur in cardiac myocytes during heart disease [37]. Evidences demonstrate that the inhibition of each mechanism improves cardiac function in heart disease [38-40].

Briefly, autophagy is a quality control process where dysfunctional organelles and long-lived proteins are sequestered to the interior of double-membrane vesicles, called autophagosomes, where they are degraded upon fusion with lysosomes. In this lysosomal pathway, cell constituents can be used as energy substrates. Although the mechanisms are not fully understood, excessive digestion of cellular components can also trigger cell death [41].

Although the borderline is sometimes blurry, it is considered that apoptosis is distinct of necrosis in several aspects. Necrosis is an accidental form of cell death and results in distinct morphological alterations including plasma membrane rupture, depletion of intracellular ATP stores and organelle swelling. The release of cytoplasmic content to the extracellular space evokes a detrimental inflammatory response [42].

The cardiac tissue has limited regenerative capacity which explains why cell death is not abundant. However, when the heart is challenged by a stressful condition, the incidence of apoptosis increases [5]. In fact, apoptosis has an important role in stress-induced pathogenic adaptations in several cardiovascular diseases, since it leads to the loss of terminally differentiated myocytes. A connection between chronic heart failure and apoptosis has been established. Patients with chronic heart failure have higher percentage of myocytes apoptosis than normal subjects, as determined by TUNEL-positive cells [43, 44]. In this context, cardiomyocyte apoptosis is a promising therapeutic target and has been extensively studied in clinical and experimental heart failure [45, 46]. Physiologically, apoptosis occurs as an essential and highly regulated mechanism, triggering a self-renewal progress and controlling the proliferation of aberrant cells. The cell death program can be originated by several stimuli that can include cytokines, oxidative stress and DNA damage [47]. The recognition of an apoptotic signal is a very controlled mechanism and results ultimately in the recognition of the apoptotic cell. Cellular membranes are asymmetrically composed by phospholipids and proteins. The exposure of the phospholipid phosphatidylserine on the cell surface is recognized by specific receptors in phagocytes as a signal to initiate degradation of the target and removal of cellular remnants [48].

In 1972, John Kerr made use, for the first time, of the term apoptosis to describe a morphologically different type of cellular death in which membrane blebbing, chromatin condensation and nuclear fragmentation is observed [49]. Later evidences suggested an essential role of a family of proteases termed caspases. Because proteolytic activation of the caspase cascade is a key element of the apoptotic signaling pathway, therapies that prevent caspase activation are usually noteworthy. Caspases are cysteine-specific proteases and can be subdivided in two groups: the inflammatory ones (e.g. caspase-1, 4 and 13) and caspases involved in programmed cell death, as referred above [50]. The activity of caspases can be suppressed by a group of inhibitory proteins, called X-linked inhibitor of apoptosis protein (XIAP) [51]. Downregulation of the unspecific caspase inhibitors, XIAP, in the failing myocardium contributes to increased cardiac myocyte apoptosis [52]. Therefore, understanding the mechanisms that initiate proteolytic activation of caspases is a crucial step in defining targets that allow for the modulation of apoptotic cell death.

Two distinct apoptosis signaling pathways exist in vertebrates. Both are reported to occur during heart failure: the extrinsic and intrinsic pathways. The extrinsic pathway or "death receptor pathway" is initiated by extracellular ligands, which bind to membrane receptors at the cell surface, such as the tumor necrosis factor (TNF) superfamily receptors. The interaction ligand-receptor leads to the recruitment of adapter proteins (e.g. Fas-associated death domain (FADD)) that triggers the assembly of the death-inducing signaling complex (DISC). Evidences demonstrate that apoptotic signaling induced by Fas Ligand (FasL) and TNF-α is dependent on caspase activation [53]. Upon proteolytic activation, caspase 8 is then released from the DISC to the cytosol, where it cleaves other effector caspases such as caspase 3 and mediates

the cleavage of Bid to its truncated form (tBid), triggering the intrinsic pathway (Fig. 3) [54]. Multiple studies with mice over-expressing TNF-α demonstrated that the activation of the TNF receptor can trigger congestive heart failure [47], while others suggest that the levels of circulating TNF-α are intimately related with the severity of cardiac disease [55].

The intrinsic (or mitochondrial) pathway of apoptosis is initiated upon an internal apoptotic stimulus that leads to permeabilization of the outer mitochondrial membrane (OMM). The permeabilization of the OMM can originate from two distinct events: a) opening of the mitochondrial permeability transition pore (mPTP) in the inner mitochondrial membrane (IMM) or b) by the translocation to the OMM of pro-apoptotic proteins [56]. The opening of the mPTP can be potentiated by augmented calcium and oxidative stress and can lead to membrane potential loss and matrix swelling that eventually causes rupture of the OMM [57]. Outer mitochondrial membrane permeabilization can also result from DNA damage or multiple cellular signals that initiate the translocation of pro-apoptotic Bcl-2 family components, such as Bax and Bak proteins to mitochondria, which allows, without alteration of membrane potential, the release of pro-apoptotic factors from the intermembrane space to the cytoplasm [58, 59]. Mitochondrial released pro-apoptotic factors may include cytochrome c, the apoptosis-inducing factor (AIF), Smac/diablo and Endonuclease G (Endo G) (Fig. 3). The AIF is biologically required for mitochondrial homeostasis and for the stabilization of respiratory complex I [60] while the nuclear-encoded mitochondrial Endo G is determinant for mitochondrial biogenesis, mitochondrial gene transcription and also in maladaptive cardiac hypertrophy [61]. While AIF and Endo G have the capacity to translocate to the nucleus and mediate chromatin condensation and DNA

fragmentation by a caspase-independent cell death, cytochrome *c* participates in the formation of the apoptossome complex, by binding to Apaf-1, which recruits pro-caspase 9, making it active, and initiates the caspase cascade [62]. The release of Smac /Diablo enhances caspase activity by antagonizing endogenous inhibitors of apoptosis, such as the XIAP, as mentioned above. Since apoptosis is involved in organ homeostasis, this process is highly controlled and mitochondria are a central component in apoptosis regulation [63]. These organelles have life-preserving and death-inducing components in their membranes, ready to be released upon membrane permeabilization. Once disruption of OMM occurs, mitochondrial function will be irreversibly impaired and the viability of the cell without functional mitochondria is energetically not possible.

As described above, the intrinsic pathway can be further activated by the extrinsic pathway, depending on the proteolysis of Bid to t-Bid by caspase 8, which further mediates the recruitment of Bax that permeabilizes OMM [64]. Bax belongs to the Bcl-2 family of proteins, which is divided in anti-apoptotic (e.g. Bcl-2 and Bcl-xL) and pro-apoptotic members (e.g. Bax, Bak, Bcl-xS, Bad and Bid). While Bak is located at the OMM, Bax is located in the cytosol undergoing a conformation change that induces its translocation to the mitochondria [65]. Although both have the ability to form pores in the mitochondrial membrane. The translocation of Bax to mitochondria can also be related with increased p53 expression, which transcriptionally regulates Bax. The p53 tumor-suppressor gene acts as a linkage between DNA damage detection and apoptosis initiation [66]. Bcl-2 regulates proteins translocation from the mitochondrial intermembrane space to the cytosol, counteracting the effect of pro-apoptotic proteins, as exemplified by the blockage of cytochrome *c* release in a Bcl-2-

overexpressed model [67]. Moreover, the apoptosis repressor with caspase recruitment domain (ARC) is also able to inhibit both extrinsic and intrinsic pathways by interacting with caspase 8 and components of the DISC as well as by regulating the mitochondrial translocation of Bax, respectively [68]. Another important player in apoptosis is the phospholipid cardiolipin that is almost exclusively located within inner mitochondrial membrane. This mitochondrial specific phospholipid has critical roles in mitochondrial production of energy [69]. However, oxidized cardiolipin is believed to contribute to outer membrane permeabilization and to cytochrome *c* release [70].

Oxidative phosphorylation is the source of almost all energy required for cardiac myocyte function [31]. As mentioned above, the blockage of blood flow to the myocardium compromises oxygen supply to the tissue, decreasing ATP stores. In this situation, both autophagic pro-survival mechanisms and apoptotic cell death pathways can be activated, highly involving mitochondria signaling pathways. In response to multiple stresses, cardiac mitochondria, strategically, increase their content on pro- and anti-apoptotic members of the Bcl-2 family in an attempt to regulate the intrinsic pathway. Promising assays by using the Bcl-2 family protein, Nix, demonstrated that its overexpression results in cardiomyopathy while the inhibition of the protein results in a normal cardiac phenotype [71, 72]. Investigators suggest that pressure overload-induced cardiomyocyte apoptosis is the cause of the transition from compensated hypertrophy to decompensated heart failure and that Nix has a pivotal role sensitizing myocyte to apoptosis [73]. The discovery that Bad, a proapoptotic Bcl-2 family protein, can be phosphorylated by Akt provided the first direct link between a growth factor signal pathway and apoptosis regulatory proteins [74]. In turn, the interaction between phosphorylated Bad and the membrane-associated Bcl-xL weakens,

potentiating the translocation of Bad from the intracellular membrane fraction to the cytosol [75].

Several studies are focused on cellular checkpoints that provide 'rescue' opportunities for cardiomyocytes. The control of pro-apoptotic regulatory mechanisms protects cells from complete execution of the apoptotic program. Studies in experimental animals have shown that Bcl-2 provided strong anti-apoptotic action and overexpression of this protein provided cardioprotection [76]. Similar results were also obtained in studies using the addition of exogenous oxidants scavengers, demonstrating that oxidative stress may be, at least partially, involved [77]. Calcium and ROS are known to be important mediators in cytochrome *c* release from mitochondria since they potentiate the opening of the mPTP [78]. Heart mitochondria have the capacity to store large amounts of calcium without triggering of the mPTP [79]. However, when heart tissue suffers an insult such as during cardiac reperfusion, oxidative stress largely increase and lower mitochondrial calcium concentrations are now needed to disrupt mitochondria. During mitochondrial energy production, a small amount of leaked electrons bind to oxygen and produce ROS [80]. As a by-product of metabolism, mitochondria-derived ROS have important signaling functions in cardiac cells since it mediates critical growth and differentiation processes, among others. Matsuyama *et al*. demonstrated that by lowering ROS levels during fetal-neonatal transition, the differentiation development of cardiac cells is impaired, with commitment of binucleated mammalian cardiomyocytes formation [81]. Also ROS-induced p38-MAPK activation is essential for the arrest of proliferation and formation of binucleated cells [81]. However, once detoxification through endogenous antioxidant defenses is overwhelmed by increased rate of ROS production, deleterious effects on cellular structures and

mitochondrial dysfunction occur [82]. Studies confirm that overstimulation of β_1-AR increases ROS formation while down-regulating cytosolic superoxide dismutase [83].

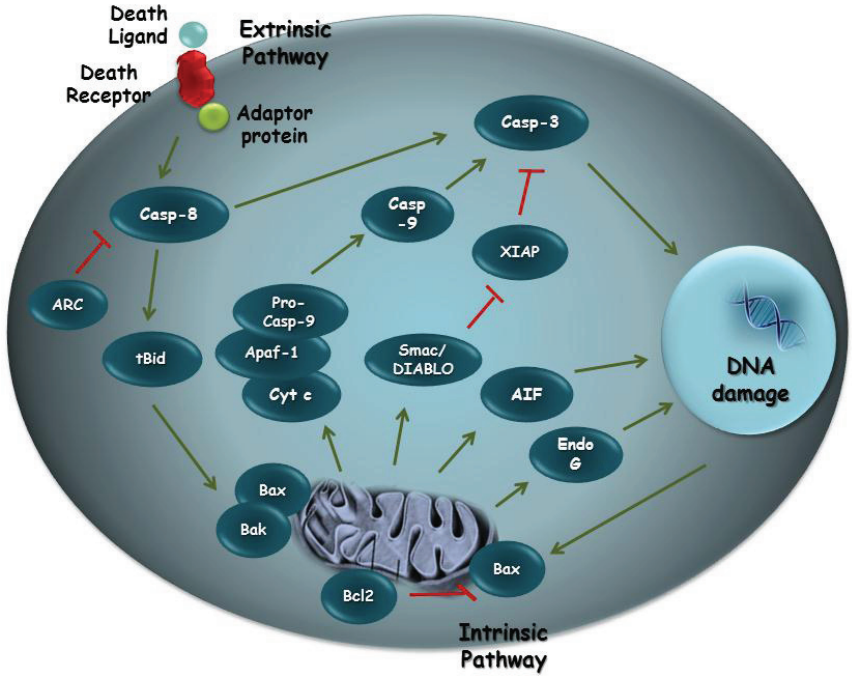


Figure 3 - Representation of apoptotic events and the main components involved. Extrinsic pathway of apoptosis activates caspase cascade via death receptors while the intrinsic pathway triggers cell death by releasing mitochondrial components. Moreover, the two pathways are linked and molecules in one pathway can influence the other. In all cases, the target is the nucleus where DNA suffers fragmentation and nuclear chromatin suffers condensation. Abbreviations: Casp- (caspase), cyt *c* (cytochrome *c*), tBid (truncated Bid), Endo G (endonuclease G), AIF (apoptosis inducing factor) SMAC/DIABLO (second mitochondrial activator of caspase/ direct IAP-binding protein with low pI).

1.3.3 β-Adrenergic Receptors – Characterization of signaling pathways

Adrenergic receptors link the cardiovascular system with the SNS. Catecholamine binding to adrenergic receptors promotes the activation of several pro- and anti-apoptotic pathways depending on the hormone and the intensity of the binding. When excessive, over-activation can lead to hypertrophy and apoptosis [24]. Several mechanisms have been forwarded to explain the progressive deterioration of the contractile system during heart failure such as loss of β-AR responsiveness, dysregulation of excitation-contraction coupling, and activation of aberrant signaling pathways [84, 85].

The ratio between β_1- and β_2-AR in cardiac cells depends on the species, age and developmental stage, but in general, β_1-AR seems to be the most predominant [86]. Evidences suggest that the β_1 subtype seems to be downregulated in human failing ventricles and in this situation β_2 is up-regulated in contrast to human non-failing ventricles [87]. Stress responses mediated by adrenergic receptors commonly incur in its desensitization which can explain the down-regulation of β_1-AR during heart disease. Receptor phosphorylation, uncoupling from G proteins, receptor internalization and degradation are some of the mechanisms involved in the desensitization of β-AR [88]. Despite this, results from genetic manipulation of β-AR content are not yet conclusive [89], and sometimes even controversial. Experiments should take in consideration the fluctuations on intensity/duration of the stimulation occurring in a physiologic situation.

Upon ligand binding, β-ARs are physically coupled to heterotrimeric G proteins, which are classified as stimulatory (Gs) or inhibitory (Gi) [90]. Both β_1- and β_2-AR are coupled via the "stimulatory" G protein (Gs) to the effector enzyme adenylyl cyclase (AC), which converts the substrate

Mg-ATP to the second messenger cAMP (Fig. 4). In studies carried out with transgenic mice and rat with cardiac overexpression of β_1 receptors Gαs, cardiomyopathic phenotype as well as increased basal rates of cardiomyocyte apoptosis were observed [91].

It was observed in heart failure rats that the combination of β1-AR antagonist and β2-AR agonist further improved the cardiac function and prevented cardiomyocyte apoptosis than the administration of a β1-AR antagonist alone. The authors suggest that the observed downregulation of bax and upregulation of bcl-2/bax expressions might contribute to the beneficial therapy effects [92]. Several studies have proposed the anti-apoptotic effects of β_2-AR receptors via coupling to the "inhibitory" G protein (Gi) which is able to inhibit the signaling axis AC-cAMP-PKA [87, 93, 94]. Moreover, studies have reported also that this survival signaling pathway via β_2-AR involves activation of phosphatidylinositol 3-kinase (PI3K) and Akt [95] (Fig. 4). Overexpression of an activated form of PI3K catalytic subunit resulted in cardiac hypertrophy, while forced expression of a dominant negative PI3K produced smaller hearts and individual fibers [96]. It has been also reported that activation of this pro-survival signaling pathway attenuates myocardial cell death after ischemia/reperfusion injury [97]. Evidences demonstrated also that although commonly involved in pro-apoptotic pathways [98], a number of studies have shown that activation of p38-MAPK via β_2 adrenergic signaling promotes cell survival. This fact confirms that downstream effects of β-adrenergic signaling cannot be considered completely beneficial or prejudicial, due to the complex mechanisms of molecular interaction. In fact, β-AR stimulation activates the three subgroups of MAPKs phosphorylation cascades from which the p38-MAPK, the c-Jun NH2-terminal kinases (JNK) and the extracellular signal regulated kinases (ERK) are components [99]. A critical role of p38-MAPK in

negatively regulating the mammalian cardiomyocyte proliferation and in the potentiation of a terminally differentiation state has been demonstrated [100]. Studies confirm that the human heart also express β_3-AR which exerts its effects by Gi-induced signaling pathways as well [101].

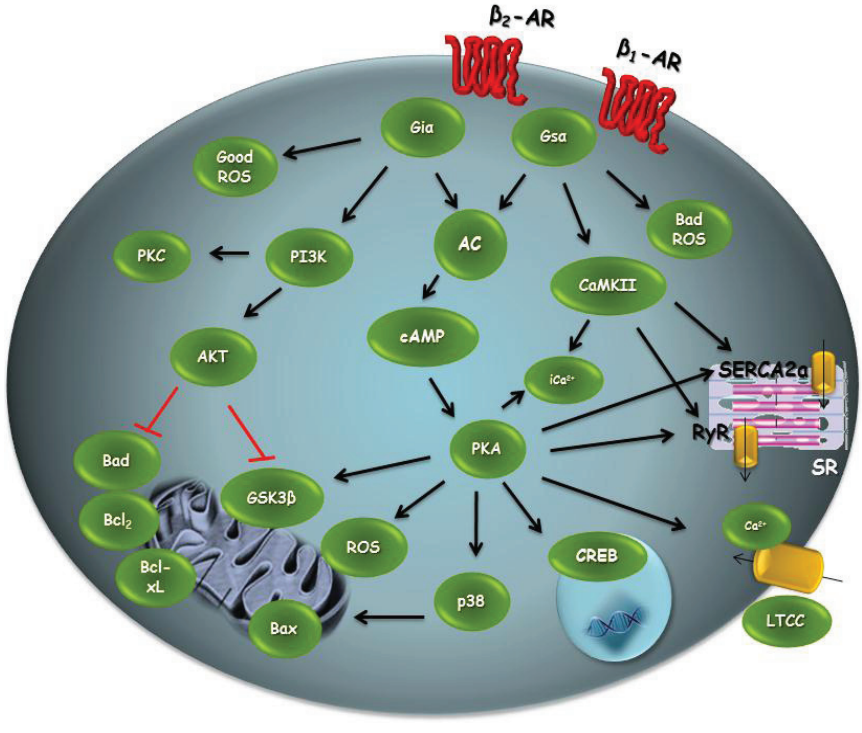


Figure 4 - Representation of some of the signaling pathways triggered by β-adrenergic receptors. β_1- and β_2-AR modulate cellular behavior via several signaling pathways. In this highly regulated system, maintenance of physiologic calcium levels and prevention of up-regulated pro-apoptotic elements is critical for cell death control. β1-AR is reported as a death sensor as it is coupled to a stimulatory G protein that activates PKA via adenylate cyclase and cAMP. While β2-AR signals via both the stimulatory and the inhibitory G protein, the latter one, related to anti-apoptotic pathways such as PI3K/Akt, alters the levels of pro-apoptotic proteins. Abbreviations: ROS (reactive oxygen species), PI3-K (phosphoinositide 3-kinase), PKC (protein kinase C), ATP (adenosine triphosphate), Gi (guanine nucleotide-binding protein inhibitory subunit), Gs (guanine nucleotide-binding protein stimulatory subunit), AC (adenylate cyclase), cAMP (cyclic adenosine monophosphate), CaMKII (Ca^{2+}/calmodulin-dependent protein kinase), Ca^{2+} (calcium), PKA (protein kinase A), p38 (p38 mitogen-activated protein kinases), GSK3β

(glycogen synthase kinase 3 beta), Bad (Bcl-2-associated death promoter), Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), Bax (Bcl-2-associated X protein), LTCC (L-type calcium channel), SR (sarcoplasmic reticulum).

1.3.4 Beta-blockers – Improving the failing heart

It is known that β-AR blockers improve cardiac contractility and reduce mortality in patients with heart failure [102-104]. But it is still unclear how blocking a pathway that increases contractility of normal hearts can improve the function of a failing heart.

Although poorly understood, it is suggested that the competition for norepinephrine-binding receptors may attenuate cardiomyocyte oversignaling where calcium plays a pivotal role [103]. As described, β-AR stimulation activates both AC-cAMP-PKA and calcium /calmodulin-dependent protein kinase II (CaMKII) pathways [105]. As in PKA signaling, CaMKII are upregulated in failing hearts [106]. Both PKA and CaMKII modulate the activity of a wide range of components involved in calcium handling such as sarcoplasmic/endoplasmic reticulum calcium-ATPase (SERCA) and its regulator, phospholamban (PLB), ryanodine receptor RyR2 (sarcoplasmic reticulum calcium release channel), and sarcolemmal L-type calcium channels (LTCC) [107].

Calcium that enters into cardiomyocytes via LTCC causes the release of a much larger amount of calcium from the sarcoplasmic reticulum (SR) storage compartment by activating the RyR2 and thereby causing contraction [108] (Fig.5). The hyperactivation of RyR2 appears to be one of the causes for impaired cardiac function in heart failure [109, 110] since it alters the gate increasing ion flux.

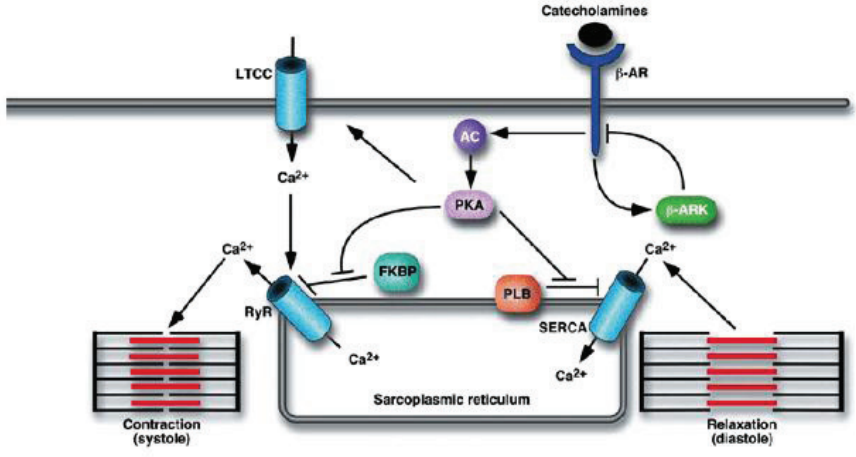


Figure 5 - Calcium transients by activation of PKA. Stimulation of β-AR induced the activation of the PKA signaling. This protein initiates the influx of calcium by activating the L-type calcium channels (LTCC) in cell membranes. The increase in intracellular calcium together with the concomitant stimulation of the RyR by PKA, lead to the release of calcium from the sarcoplasmic reticulum which stimulate cardiac contraction. PKA is also involved in the activation of SERCA which contribute to muscle relaxation by reuptake calcium from the cytosol. (From [111]).

The subsequent reuptake of released calcium is mediated by SERCA2 and by the sarcolemmal sodium/calcium exchanger (NCX) [112] which determine the strength of contraction in a PKA-dependent manner. However, if calcium influx is persistently activated or if calcium removal systems are persistently compromised, as during heart failure, calcium overload can occur. In fact, human heart failure is characterized by increased LTCC phosphorylation [113]. Besides, SERCA expression and activity is depressed during heart failure resulting in the accumulation of cytosolic calcium. This findings support studies in which calcium handling contributes to the dysfunction of the heart [114]. In fact, SERCA overexpression improves cardiac function in rodent models of heart failure [115]. In this context, there is an interest in introducing a negative form of the repressor of SERCA, phospholamban

[116]. Moreover, even when cardiomyocytes are isolated from the overstimulated heart, the ability to efflux the calcium from the cytosol during diastole is reduced [117].

By using a knockout mice for type 5 AC (AC5KO), a major cardiac isoform, it was demonstrated that a long-term isoproterenol stress (7 to 14 days) did not result in a further increase of left ventricular ejection fraction (LVEF) as observed in control condition, but instead resulted in a greater degree of AC signaling downregulation, an improvement on myocyte viability and increased Bcl-2 protein and Akt/GSK signaling, potentially elucidating a novel approach to the therapy of heart failure [118].

1.3.5 Beta-adrenergic signaling in the developing heart

As the embryonic heart underlies several modifications during developmental maturation it is expected that alterations on cellular signaling occur. The role of β-adrenergic signaling in cardiac differentiation and in the normal development of the heart is poorly understood.

To identify in [119] which developmental period the modulation of the β-adrenergic system is more evident, Kudlacz *et al.* administered the β-antagonist, propranolol, during fetal period and observed that a delay on cellular development occurs at birth showing that catecholamines may play a critical role in cardiac development [120]. In another study, Lipshutz *et al.* demonstrated that 2- to 2.5 day old chicken embryos lack β-adrenergic responsiveness to epinephrine. However, when an extract of 11-day embryo was incubated with 2- to 2.5 day old cells in culture, these cells developed sensitivity to epinephrine and increasing cAMP levels [119]. As showed by the same study, basal cAMP levels of

unresponsive cells with 2- to 2.5 day old are higher than older cells, suggesting a role for cAMP in developing heart. In respect to the contractile capacity, L-type Ca^{2+} channels from mouse embryos in early-stage (day-11 to -13) lack a response to either isoproterenol or cAMP which is reversed in later-stage (day-17 to -19) [121]. In embryonic stem cell-derived cardiomyocytes (ESC-CMs) addition of exogenous β-adrenergic agonists enhanced cardiac differentiation via p38-MAPK signaling [122], while inhibition of β-adrenergic signaling reduced the efficiency of cardiac differentiation. Retinoic acid (RA) plays an important role mediating adrenergic action during embryonic heart development [123]. Knockout of the dopamine β-hydroxylase results in the loss of mouse embryos caused by heart failure where dysfunctional retinoic acid synthesis and transport were observed [124]. Also, changes in receptor number and signaling are known to suffer alterations during fetal development. Importantly, mRNA and protein levels of β_1-AR and β_2-AR suffer alterations during cardiac differentiation [122]. The expression of β_1-AR increases and reaches the highest level at day 14 although, β_2-AR expression remain higher before and after the differentiation process suggesting that β_2-AR is predominant at early stages, while β_1-AR may be the predominant subtype for the later stages of cardiac differentiation [122]. In mouse and human cardiac progenitor cells (CPC) β_1-AR expression is induced by differentiation stimuli while β_2-AR is present from an early stage promoting proliferation and survival [125].

Interestingly, an elegant study demonstrated that repeated administration of ISO to neonatal rats did not result in cardiac hypertrophy, whereas the same treatment did produce hypertrophy in adult rats [126], suggesting that ISO downstream signaling pathways in the neonatal rat are different from the older, adult animal. Catecholamine-induced cardiac toxicity

during heart development was previously demonstrated by Iwasaki et al. [127], which demonstrated that ISO administration to pregnant rats resulted into disproportionate septal hypertrophy and frequent inter- and intra-cellular disarray in the offspring. This result clearly indicates that β-adrenergic over-stimulation during rapid cardiac development in the womb can result into later profound cardiac alterations in the offspring, which can be explained by signaling remodeling during cell differentiation.

1.4 *In vitro* models to investigate cardiac β-adrenergic signaling and cardiovascular toxicology

One important limitation in investigating signaling pathways and drug-induced toxicity responses has been the lack of adequate models to truly reproduce *in vitro* what is observed in the heart in different stages of development. In this respect, some cellular experimentally models that can be used are below described and we point out the advantages and disadvantages inherent to each model.

1.4.1 Neonatal Ventricular Cardiomyocytes

Neonatal primary cardiomyocytes have been one of the most widely used models to study morphologic and biochemical features of cardiomyocytes as well as hypertrophic mechanisms *in vitro*. Most of the biochemical and gene transcription data have largely been obtained by the use of primary cultures of 1-3 days-old mice neonatal cardiomyocytes [128]. However, these cells are neither fully differentiated adult cells neither maintain infinite proliferative capacity in culture [129]. Primary cultures can be maintained in culture for days or weeks and neonatal isolated cardiomyocytes phenotype is well

conserved in culture [130]. However, on the other hand, cardiomyocyte isolation techniques are not yet entirely established, in particular those concerned to the purification of cardiomyocyte population. In comparison with adult cardiomyocytes, neonatal have the advantage of being easily isolated from the heart since it does not require aorta cannulation and perfusion and, unlike adult cardiomyocytes, they are less sensitive to calcium alterations in the medium during the isolation procedure [131, 132]. Furthermore, both techniques require the sacrifice of laboratory animals, which has become a concern. Versus animal models, isolated cells constitute also an advantage for experiments aimed at visualizing molecular responses as imaging techniques are often limited to thick tissue. By preserving the *in vivo* integrity of cardiomyocytes, data obtained by primary cultures may be combined with experimental findings from living organisms due to the proximity of this model to an intact tissue.

1.4.2 HL-1 cells

Mouse HL-1 cells are derived from a primary established cell line, the AT-1, and were cultivated specifically to allow the maintenance of specific characteristics. The most prominent features of this cell line are related to the presence of spontaneous contractile activity and the maintenance of several characteristics of an adult cardiomyocyte phenotype such as alpha-MHC, alpha-cardiac actin and connexin43 [133]. As an immortalized cell line, HL-1 cells have some features of such unlimited proliferation and also the capacity to be conserved and recovered from liquid nitrogen storage [133]. HL-1 cells are widely used in studies aimed at investigating cardiac biology such as myocardial growth and hypertrophy.

AT-1 cells are derived from an atrial tumor that was formed in a transgenic mouse by the specific expression of the oncogenic virus SV40 Large T protein. The SV40 Large T protein gene [134] was specifically designed to be regulated by the atrial natriuretic factor (ANF) promoter, an atrial specific protein, to induce a tumor growth in that tissue [133, 135]. Genome codification of SV40 Large T protein avoids the normal function of retinoblastoma and p53 proteins disrupting the normal operation of the cell cycle.

One disadvantage is that HL-1 cells have an atrial origin, hence their use as ventricle cell model is not entirely accurate.

1.4.3 P19-derived cardiomyocytes

P19 are mouse embryonal carcinoma cells that have a pluripotent behavior [136]. From these cells, a wide spectrum of adult cells such as cardiomyocytes can be obtained by exposing them to DMSO or neurons by using retinoic acid [137, 138]. Because preparations of differentiated cells are far from be homogenous, special attention should be applied. However, these cells are still derived from a tumoral cell line and although differentiated, they are not considered completely adult cells. Cultured cells grow exponentially and, similarly to the HL-1 cell line, P19 cells are also able to have contractile activity under specific conditions [139, 140]. P19 cells are commonly used to investigate regulatory differentiation mechanisms of pluripotent cells that determine the cell fate. In respect to our study, it is worth to note that these cells present β-AR which respond to catecholamines [140]. To this regard, several cardiac markers and L-type calcium channels are present in cardiomyocyte-rich culture [141].

1.4.4 ESCs-derived cardiomyocytes

Embryonic stem cells (ESCs) are derived from the inner cell mass of pre-implantation embryos [142]. Cells proliferate *in vitro* and are easily differentiated in a wide range of cell types including cardiomyocytes expressing several markers of cardiac cells [143]. For this reason, engraftment of ESCs constitutes a well appreciated therapeutic intervention in failing hearts [144].

Several studies evidence the formation of teratomas followed by ESC injection into the myocardium [145]. However, a number of other studies have also delivered undifferentiated ESCs and failed to report teratoma formation [146, 147]. Animals receiving ESCs or ESC-derived cardiomyocytes following experimental cardiac injury exhibited superior heart function [146]. Thus, the formation of teratomas and the engraftment of ESC into the heart are technical problems that will be solved with more research.

Collectively, the studies show evidences that ESC-derived cells constitute a promised model for the treatment of heart disease, and specifically for the replacement of lost cardiomyocytes, plus being a promising *in vitro* tool for different toxicological studies.

1.4.5 H9c2 (2-1) cell line

The H9c2 (2-1) cell line was isolated from the ventricular part of a BDIX rat heart [148]. Thirteen days after fecundation, cells were isolated and, by a complex procedure, immortalized. In this stage, cells are not fully differentiated into adult cardiomyocytes but are already predestinated, which confer already the appearance of several cardiomyocytes markers. By a selective serial passage, the different adhesion kinetics of the heterogeneous isolated fraction was used to

separate the components in the culture dish. It is important to state that these cells do not present contractile activity, even when differentiated [149]. However, it is also worth to note that H9c2 cells and isolated neonatal cardiomyocytes respond similarly to hypertrophy [150].

The H9c2 cell line is not considered as a pure and homogeneous sample as it includes both embryonic skeletal (with slowly L-type calcium channels) and cardiac (with fast L-type calcium channels) muscle cells [149, 151]. Menard et al. confirmed that the H9c2 cell line is not only able to differentiated into adult cells (skeletal and cardiac muscle) by withdrawing the serum from the culture medium but also can be differentiated into a predominantly cardiac phenotype, characterized by the overexpression of the alpha-1 subunit of the L-type calcium channels, by adding RA daily to the culture media [149]. Embryonic myoblasts fuse to originate multinucleated differentiated myotubes. Cells get an elongated shape and positioned in a parallel way [152]. One limitation to the *in vitro* differentiation process is that it drives further stress to the cells [153, 154]. Some studies document that the survival of cardiomyocytes is committed by its culture in the absence of serum [155]. But it should also be taken in consideration that stress-induced cardiac differentiation is an *in vivo* feature and, as described, an essential mechanism to trigger cell differentiation to an adult stage.

Table 1 - Schematic representation of molecular, functional and culture behavior between neonatal cardiomyocytes, HL-1 cell line, P19, ESC and H9c2 (2-1) cell line. Abbreviations: Dif. (already differentiated); MHC (Myosin heavy chain), MLC2v (Myosin light chain 2 ventricular), BNP (brain natriuretic peptide), cTpC (cardiac troponin C), ANF (atrial natriuretic factor), TNNT2 (cardiac troponin T 2) [139, 141, 156-161]

	Markers of Differentiation	Proliferation capacity	Potential to differentiate into cardiomyocytes	Beating	Maintenance in culture in adult stage
Neonatal Cardio-myocytes	α-actinin, Troponin-T, β-myosin heavy chain and connexin43	No	Dif.	Yes	Short
HL-1	atrial natriuretic factor, alpha-cardiac myosin heavy chain, alpha-cardiac actin and connexin43	Yes	Dif.	Yes	High
P19	GATA-4, BNP, cTpC and αMHC	Yes	Yes	Yes	Short
ESC	αMHC, ANF, troponin-T, alpha actinin, MYH6, MYL4, TNNT2	Yes	Yes	Yes	High
H9C2	MLC-2v, myogenin, troponin T, and MyoD, α-MHC	Yes	Yes	No	Short

1.5 General Aims

In order to investigate further details into the mechanisms linking β-adrenergic overstimulation to cell death, the cardiomyoblastic cell line H9c2 was used in this study.

Since this cell line has the advantage of being differentiated to a more adult phenotype [149] this feature was used to investigate whether the differentiation status of the cell interfere with the final outcome of β-adrenergic stimulation. Also, H9c2 have functional β-AR [162], which is an essential condition to be used in the present study.

Norepinephrine signals via its interaction with α- and β- adrenergic receptors. However, several studies document that its cytotoxicity seems to be mediated by only β-adrenergic receptors. For that reason, in our study, isoproterenol (ISO; Fig. 6), a synthetic catecholamine which is a nonselective agonist of β-AR [163] was used to investigate signaling pathways involved in cardiac cell death.

In fact, ISO has been recognized as a golden-standard agent that causes cardiac injury [164, 165], which usually involves cardiac hypertrophy [166].

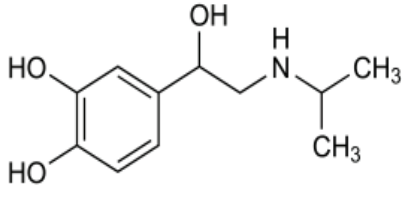


Figure 6 - Chemical structure of Isoproterenol. Systematic name: 4-[1-hydroxy-2-(propan-2-ylamino) ethyl] benzene-1,2-diol

The present thesis investigates cell and mitochondrial stress responses mediating the link between β-AR overstimulation and cell death.

Different specific aims were addressed in the present study, including:

a) Are H9c2 cells differently susceptible to the toxicity of β-AR overstimulation by ISO when in different stages of differentiation?

b) If so, is this due to altered β-adrenergic signaling or to different maturation of anti-oxidant / pro and anti-apoptotic pathways? In this context, alterations in cytosolic and mitochondrial stress responses to ISO were studied.

Regarding the second specific aim, we measured not only alterations of stress/toxicity responses to ISO, but also basal differences existing in the different groups of cells (undifferentiated vs. differentiated) which may render them differently susceptible to ISO cytotoxicity.

Materials and Methods

2. Material and Methods

2.1 Common Reagents

Bovine serum albumin (BSA), *all-trans* retinoic acid (RA), ammonium persulfate (APS), Bradford reagent, brilliant blue G, bromodeoxyuridine (BrdU), calcium chloride ($CaCl_2$), dimethyl sulfoxide (DMSO), DL-Dithiothreitol (DTT), Dulbecco's-modified eagle's medium (DMEM), ethylenediaminetetraacetic acid (EDTA), ethylene glycol-bis (2-aminoethylether)-N,N,N´,N-tetraacetic acid (EGTA), glycerol, glycine, heat-inactivated horse serum, isoproterenol, L-glutamine, β-mercaptoethanol 98%, metaprolol, mouse anti-β-Actin, M199 medium, N-acetylcysteine, pancreatin, pan-caspase inhibitor Z-Val-Ala-dl-Asp-fluoromethylketone (z-vad-fmk), phenylmethylsulfonyl fluoride (PMSF), protease inhibitor cocktail (containing 1mg/ml of leupeptin, antipain, chymostatin and pepstatin A), sucrose, sodium azide, sodiumchloride (NaCl), sodium dodecyl sulfate (SDS), sulforhodamine B sodium salt (SRB), trizma-base, tris pH 8.8, tris pH 6.8 and trypan-blue solution were obtained from Sigma (St. Louis, MO, USA). Collagenase and Claycomb medium were purchased from Worthington Biochemical Corporation (Lakewood, NJ, USA) and from SAFC Biosciences, respectively. Acetic acid, ethanol, hydrochloric acid (HCl), magnesium chloride ($MgCl_2$), methanol, perchloric acid, potassium chloride (KCl), potassium phosphate monobasic (KH_2PO_4), sodium hydrogenocarbonate ($NaHCO_3$), sodium sulfate ($NaSO_4$) and sodium hydroxide (NaOH) were obtained from Merck (Whitehouse Station, NJ, USA). Hank´s Balanced Salt Solution (HBSS), penicillin, streptomycin, fetal bovine serum (FBS) and Trypsin-EDTA were purchased from Gibco-Invitrogen (Grand Island, NY). Acrylamide, Laemmli buffer, polyvinylidene difluouride (PVDF) membranes, Ponceau solution and N,N,N´,N´-tetramethylethylenediamine (TEMED) were obtained from

BioRad (Hercules, CA, USA). The ECF detection system was obtained from Healthcare Life Sciences (Buckingamshire, UK). The fluorescent probes Hoechst 33342, 4',6-diamidino-2-phenylindole (DAPI), dihydroethidium (DHE), tetramethylrhodamine methyl ester (TMRM), 5-(and 6)-chloromethyl- 2', 7'-dichlorodihydrofluorescein diacetate (CM H_2DCFDA), fluo4-AM, Mitotracker Red CMXROS, MitoSOX, calcein-AM and the anti-fading reagent SlowFade® Gold were obtained from Invitrogen-Molecular Probes (Eugene, OR, USA). PI3 kinase inhibitor LY294002 was purchased from Cell Signaling (Beverly, MA, USA). Caspase 3 substrate, fibronectin, pifithrin-alpha and p-nitroanilide (p-NA) were purchased from Calbiochem (San Diego, CA, USA). Cyclic AMP XP^{TM} assay kit was purchased from Cell Signaling Technology (Danvers, MA, USA). All reagents used in this work were of the greatest degree of purity commercially available. To prepare solutions, ultrapure distilled water filtered by the Milli Q from a Millipore system was used in order to minimize contamination with metal ions.

2.2 Cell Culture

2.2.1 H9c2 Cell Culture and Differentiation Process

The H9c2 cell line purchased from America Tissue Type Collection (Manassas, VA; catalog # CRL – 1446) was originally derived from embryonic rat heart tissue using selective serial passages and were cultured in DMEM medium supplemented with 1.5 g/L sodium bicarbonate, 10% fetal bovine serum, 100 U/ml of penicillin and 100 µg/ml of streptomycin in 75 cm2 tissue culture flasks at 37°C in a humidified atmosphere of 5% CO2. Cells were fed every 2 – 3 days, and sub-cultured once they reached 70 – 80% confluence in order to prevent the loss of differentiation potential.

H9c2 myoblasts differentiation process was conducted by reducing media serum or by reducing the media serum followed by the addition of retinoic acid [149].

H9c2 cells were plated at a density of 35,000 cells/ml in 75cm^2 tissue culture flasks and cultured for one day in high serum in order to allow for cell attachment. In one experimental group, the concentration of media serum was reduced to 1% (low-serum medium) and cells were cultured for 5 more days. In the remaining experimental groups, addition of retinoic acid (RA, 10 nM or 1 μM) to low serum media was performed daily in the dark for 5 days. All-trans-RA was prepared in DMSO and stored at -20°C in the dark. In the fifth day of differentiation treatment (low serum media and low serum media plus RA), cells were passaged and plated at a density of 35,000 cells/ml. Cells were maintained in the respective differentiation media during the different experiments. Isoproterenol was prepared using mostly plastic specula to avoid oxidation and was directly added to the culture media at the described concentrations. Isoproterenol and RA were maintained in the media until the end of the experiments. Most of the experiments were thus conducted in four experimental groups: a) undifferentiated myoblasts in 10% serum, b) differentiated muscle cells in 1% serum, and differentiated cells in 1% serum plus c) 10 nM RA or d) 1μM RA.

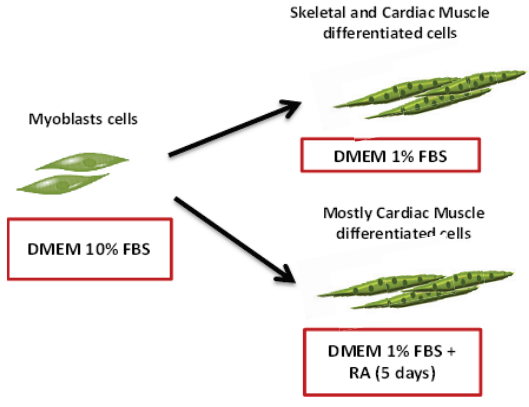


Figure 7 - Schematic representation of the differentiated process of H9c2 myoblasts. Upon reduction of serum in the medium to 1% cells differentiate in both skeletal and cardiac muscle cells, while supplementing the same medium with RA induce the differentiation toward a mostly cardiac phenotype.

2.2.2 Culture of Mouse Neonatal Ventricular Cardiomyocytes

Ventricular part of one day-old mice neonates hearts was excised and stored in a Ads solution (500 ml of 17g NaCl, 11.9g HEPES (Sigma H6147), 0.3g NaH_2PO4, 2.5g glucose, 1g KCl, 0.25g $MgSO_4$, pH 7.35) kept on ice. Ventricular myocardium was cut into small pieces (about 1-2 mm^3), and then subjected to a sequential digestion by an enzyme solution of collagenase and pancreatin, in a horizontal shaking water bath at 37°C, in order to digest the extracellular matrix and release cells into the supernatant, as follow. Heart pieces were ressuspended several times in Ads buffer, allowed to precipitate, supernatant removed carefully and pancreatin/collagenase solution added for starting the first digestion phase on the shaker. After 5 minutes, the supernatant was transferred to a tube containing FBS for the inactivation of the enzymes, centrifuged at 1000xg during 5 minutes at 25°C, mixed with 4 ml of FBS and stored in a CO_2 incubator at 37°C. The remaining tissue was then ressuspended again with Ads buffer, the supernatant removed carefully and pancreatin/collagenase solution added to initiate a new digestion phase in the shaker. Afterwards, the supernatant was transferred to a tube, mixed with FBS and centrifuged. Cells supernatants of the five digestion steps were pooled together for a final centrifugation and the

pellet plated on a gelatinized (1%) culture dishes containing medium solution (DMEM with 4.5 g/l glucose and medium M199 supplemented with 10% heat-inactivated horse serum, 5 mM HEPES, pH 7.4, 2 mM L-glutamine and 1x penicillin-streptomycin). Cells were passed through a 100µm nylon filter and allowed to attach for 45 minutes. Taking the advantage of having different kinetics of cellular attachment, non-myocytes cells (mainly cardiac fibroblasts), fast attachment cells, were separated from slowly attaching cells (mainly cardiomyocytes). Medium were taken and transferred to a new gelatinized culture dishes to allow adherence of cardiac myocytes. Bromodeoxyuridine (BrdU) was added to the culture media in order to prevent proliferation of surviving fibroblasts.

The earlier the protocol is performed after birth, the higher the number of viable cells is obtained. As an example, adult cells which are obtained by day 3 do not readily attach to gelatinized culture dishes. As earlier as this protocol is established after birth, more viable cardiac cells will be obtained, as in day 3 a much higher percentage of cells exhibit an adult phenotype that do not attach to gelatinized dishes. Before the first contact of each pipette with the resuspending tissue, pipettes should be pre-wetted in Ads buffer in order to diminish the sticking of cells to the plastic wall.

Table 2 - Volume of enzyme solution and the respective time exposure used at each digestion step in isolation of neonatal cardiomyocytes procedure.

Enzyme (ml)	10	10	8	8	6	6
Time (min)	5	10	15	15	5	5

2.2.3 HL-1 Cellular Culture

The HL-1 cell line (was a gentle gift from Dr. David Sanz Rosa) originated from adult mouse atrial tissue was cultured in Claycomb medium which is supplemented with 100 μM norepinephrine, 10% FBS, 2 mM L-glutamine and penicillin/streptomycin in 150 cm^2 tissue culture coated flasks. Flasks were coated with a salt solution supplemented with gelatin and fibronectin and then stored at -20°C. Cells were allowed to attach in an incubator at 37°C with a humidified atmosphere of 5% CO_2. Cells were sub-cultured once they reached 90% confluence.

2.3 Common Methods

2.3.1 Analysis of Drug Cytotoxicity

Evaluation of cell density was conducted using the sulforhodamine B (SRB) assay in order to perform toxicity assays and observe putative drug effects on H9c2 cell number cultured either in high- or in low-serum medium, following established protocols [167, 168]. H9c2 cells were seeded in 6- or 24-well plates (35000 cells/ml), allowed to attach for 1 day and pre-incubated individually with the specific compounds: 30 minutes with 30 μM of pifithrin-alpha, 1 hour with 50 μM of LY294002, 1 hour with 50 μM of z-vad-fmk or 2 hours with 100 μM NAC, always prior to ISO treatment (24, 48 or 96 hours). The concentration of the compounds used was the maximum amount that did not cause toxicity in preliminary toxicity assessment. Vehicle controls were also performed.

After treatment, the incubation media were removed, cells were rinsed with PBS and fixed in 1% acetic acid in ice-cold methanol for at least 30 min. Cells were then incubated with 0.5% (wt/vol) SRB in 1% acetic acid for 1 hour at 37°C. Unbound dye was removed by washing with 1%

acetic acid. SRB labeled to cell proteins was extracted with 10 mM Tris base solution, pH 10, and the optical density of the solution was determined in a spectrophotometer at 540 nm. The amount of released dye is proportional to the number of cells present in the sample.

2.3.2 Western Blotting

In order to obtain total cellular extracts, H9c2 cells were harvested by trypsinization and washed once with PBS. Floating cells were also collected and combined with adherent cells by performing two centrifugation steps for 5 minutes at 1000 xg. The cell pellet was resuspended in lysis buffer (20 mM HEPES/NaOH, pH 7.5, 250 mM sucrose, 10 mM KCl, 2mM $MgCl_2$, 1 mM EDTA) supplemented with 2mM DTT, 100mM PMSF and a protease inhibitor cocktail. Extracts were sonicated and stored at -80°C until used. Protein contents were determined by the Bradford method using bovine serum albumin as standard. After denaturation at 95°C for 5 min in laemmli buffer, equivalent amounts of total protein (25 µg) were separated by electrophoresis in a 8, 12 or 14% SDS-polyacrylamide gels (SDS-PAGE) and electrophoretically transferred to a polyvinylidene difluoride (PVDF) membrane. After blocking with 5% milk or BSA, depending on antibody information, in TBST (50mM Tris-HCl, pH 8; 154mM NaCl and 0.1% tween 20) for 2 hours at room temperature, membranes were incubated overnight at 4°C with the specific antibodies (listed in Table 3).

Table 3 – Primary and secondary antibodies used in Western Blotting assay. Abbreviations: poly(ADP-ribose) polymerase (PARP), cardiac myosin light chain-2 (Mlc2v), superoxide dismutase 2 (SOD2), voltage-dependent anion channel (VDAC), phosphorylated (P-).

Name	Dilution	Host Species	Brand
PARP	1:1000	Rabbit	Santa Cruz
Akt	1:1000	Rabbit	Cell Signaling
Bax	1:6000	Rabbit	Cell Signaling
Bcl-2	1:1000	Rabbit	Cell Signaling
Bcl-xL	1:1000	Rabbit	Cell Signaling
β-Actin	1:8000	Mouse	Sigma
β_1-AR	1:200	Rabbit	Santa Cruz
β_2-AR	1:200	Rabbit	Santa Cruz
β_3-AR	1:200	Goat	Santa Cruz
Calcineurin	1:500	Rabbit	Cell Signaling
Cardiac troponin T	1:1000	Mouse	Abcam
Caspase 3	1:1000	Rabbit	Cell Signaling
Cleaved-caspase 3	1:1000	Rabbit	Cell Signaling
Cyclophilin F	1:1000	Mouse	Abcam
Mlc2v	1:1000	Mouse	Synaptic Systems
OXPHOS	1:1000	Mouse	MitoSciences
P-p38 MAPK	1:500	Rabbit	Cell Signaling
P-p66Shc	1:1000	Mouse	Calbiochem
p66Shc	1:1000	Rabbit	BD Biosciences
P-Akt (ser473)	1:1000	Rabbit	Cell Signaling
SOD2	1:1000	Rabbit	Cell Signaling
P-troponin I	1:1000	Rabbit	Cell Signaling
VDAC	1:500	Rabbit	Abcam
Rabbit anti-goat[&]	1:5000	-	Jackson IR Lab
Goat anti-rabbit[&]	1:5000	-	Jackson IR Lab
Goat anti-mouse[&]	1:5000	-	Jackson IR Lab

* Mouse monoclonal OXPHOS cocktail comprises 5 different antibodies: CI subunit NDUFB8, CII-30kDa SDHB, CIII-Core protein 2 UQCRC2, CIV subunit I MTCO1 and CV alpha subunit ATP5A.

[&] Secondary alkaline phosphatase-conjugated antibodies

Membranes were then washed with TBST and incubated with respective secondary alkaline phosphatase-conjugated antibodies for 2 hours at room temperature.

Membranes were incubated with the ECF detection system and visualized with the Versa Doc imaging system (Bio-Rad, Barcelona, Spain). Densities of each band were calculated with Quantity One Software (Bio-Rad). Membranes were stained with Ponceau reagent before blocking to confirm equal protein loading in each lane. This experimental strategy was preferred over the use of housekeeping proteins as the ones normally found in the literature can be theoretically affected by the differentiation process.

2.3.3 Vital fluorescence of H9c2 cells

2.3.3.1 Tetramethylrhodamine methyl ester (TMRM) Hoechst 33342 and Calcein-AM

After the differentiation process, H9c2 cells grown on glass-bottom dishes (35,000 cells/ml) were treated with isoproterenol (ISO) for the desired time exposure and then, incubated with TMRM (100 nM), Hoechst 33342 (1 mg/ml) or calcein-AM (300 nM) at 37°C in the dark, 45 minutes prior the end of ISO incubation time. Media was then replaced by Krebs buffer (1 mM $CaCl_2$; 132 mM NaCl; 4 mM KCl; 1.2 mM Na_2HPO_4; 1.4 mM $MgCl_2$; 6 mM glucose; 10 mM HEPES, pH 7.4) supplemented with 10% FBS. Epifluorescence was obtained using a Nikon Eclipse TE2000U microscope and were acquired using Metamorph software (Universal Imaging, Downingtoen, PA). Confocal microscopy images were collected using a Zeiss LSM 510Meta confocal microscope and analyzed using the vendor software. Images were obtained by using LSM Image Browser.

2.3.3.2 Mitotracker Red and DAPI

H9c2 cells were seeded in 6-well plates with a glass coverslip per well at a density of 35,000cells/ml. After 1 day of cell attachment, cells were incubated with 50 μM of LY294002 (phosphoinositide 3-kinases-PI3Ks inhibitor) for 1 hour prior to the incubation with 50 or 100 μM of ISO for 24 (in the case of differentiated H9c2 cells) or 48 hour (in the case of undifferentiated cells). 30 minutes prior the end of the exposure, cells were incubated with the probe Mitotracker Red (125 nM) at 37°C in the dark, washed with cold PBS, fixed with ice-cold absolute methanol and stored at -20°C until use. A mounting solution (the anti-fading reagent conjugated with 4',6-diamidino-2-phenylindole (DAPI) was used in order to label nuclei and to detect apoptotic chromatin condensation. Cells were observed by epifluorecence microscopy using a Nikon Eclipse TE2000U microscope. The UV filter was used for DAPI imaging and the rhodamine filter for Mitotracker Red fluorescence imaging. Images were obtained using Metamorph software (Universal Imaging, Downington, PA).

2.3.4 Flow cytometry analysis of intracellular calcium, mitochondrial transmembrane potential and mitochondrial superoxide anion

Subconfluent cells originated from the 3 groups of H9c2 used in this study and cellular preparations after ISO treatment were harvested by trypsinization and centrifuge. The pellet was ressuspended in 500μl of a buffer solution (120mM NaCl; 3.5mM KCl; 0.4mM KH_2PO_4; 20mM HEPES; 5mM $NaHCO_3$; 1.2mM $NaSO_4$ and 10mM pyruvate at pH 7.4, 1.2 mM $MgCl_2$, 1.3mM $CaCl_2$ and 5% FBS) plus the respective probe (2 μM Fluo-4 AM, 100 nM TMRM or 5μM MitoSOX Red) in order to

evaluate intracellular calcium concentration, mitochondrial transmembrane potential ($\Delta\Psi m$) and mitochondrial superoxide anion respectively. Cells were incubated at 37°C in the dark for 30 minutes before the end of treatment with ISO. Cells were then analyzed in a FACScalibur Flow cytometer, using the FL1 or the FL2 filter sets (Mitosox e TMRM) and counted using the cytometer Cell Quest software package. Generation of mitochondrial superoxide anion was increased by incubating cells with the complex I inhibitor rotenone during 1 hour, with this group used as a positive control.

2.3.5 Measurement of cytosolic superoxide and H_2O_2 production by using the probes DHE and CM-H2DCFDA

Undifferentiated and differentiated H9c2 cells seeded in 12-well plates and incubated 48 hours with ISO were incubated for 20 min at 37°C in the presence of 0.5 μM dihydroethidium (DHE) in PBS containing 5 mM glucose. The cells were washed twice with PBS, and the fluorescence was recorded in a microplate reader at 535 nm excitation and 635 nm emission wavelengths. To detect H_2O_2 production, 2 μM CM-H2DCFDA were incubated with cells and results obtained by using a microplate reader during 30 minutes at 495 nm excitation and 520 nm emission wavelengths.

2.3.6 Caspase-3-like colorimetric activity assay

Total cellular extracts were collected by trypsinization and centrifuged twice at 1,000 xg, 4°C during 5 minutes. Floating cells were also collected. The pellet was resuspended in collecting buffer (20 mM HEPES/NaOH pH 7.5, 250 mM sucrose, 10 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA) supplemented with 2 mM DTT, 100 μM PMSF and a

protease inhibitor cocktail. Protein concentration was determined by the Bradford assay. To measure caspase 3-like activity, aliquots of cell extracts containing 25µg of protein were incubated in the reaction buffer, containing 25 mM HEPES (pH=7.5), 10% sucrose, 10 mM DTT, 0.1% CHAPS and 100µM of caspase substrate Ac-DEDV-pNA, for 2h at 37°C. Caspase 3-like activity was determined by following the detection of the chromophore p-nitroanilide (p-Na) after cleavage from labeled substrate Ac-DEDV. The method was calibrated with known concentrations of p-nitroanilide (p-NA). The optical density of the solution was determined in a spectrophotometer at 405 nm.

2.3.7 Determination of intracellular cAMP content

Cyclic AMP content was measured by using a commercial kit (cyclic AMP XP™ assay kit) and by following vendor´s instructions (cell signaling). Cells were seeded in 96-well plates at a density of 40,000 cells/well and allowed to attach for one day. Cells were then incubated with 50 or 100 µM of ISO during 48h. 1 hour prior the end of the treatment, cells were incubated with 20 µM of forskolin in order to increase cAMP levels (alasbahi 2012). Cells were washed twice with cold PBS and incubated 10 minutes on ice with a lysis buffer solution (supplemented with PMSF), provided inside the kit. 50 µl of the lysed sample plus 50 µl of the HRP-linked cAMP solution were added to a coated 96-well plate that contained already an anti-cAMP XP™ mAb and incubated for 3 hours in a horizontal shaker. Cells were washed 4 times with washing buffer provided by the kit and incubated with 100 µM of TMB substrate for 30 minutes. The stop solution was added to each well and optical density was measured at 450nm in a spectrophotometer. cAMP intracellular levels were determined by following the detection of a gradient of known concentrations of cAMP.

2.3.8 Total RNA Extraction

Collection of total RNA extracts was performed following the vendor's instructions (RNeasy Mini Handbook from QIAGEN). About $1x10^6$ cells were collected, and disrupted in 350µl of Buffer RLT from QIAGEN (RNeasy Protect Mini Kit) supplemented with 3.5µl β-mercaptoethanol. The suspension was homogenized, added to QIAshredder Spin Columns and centrifuged for 2 min at the highest velocity. The supernatant was ressuspend with 350µl of ethanol 70% and transferred to a RNeasy Mini Spin Columns. A sequence of centrifugations was performed after following washing steps. Total RNA bound to the membrane and was then eluted in RNase-free water. Concentration and purity of the sample were verified using Agilent Technologies, Eukaryote Total RNA Nano assay and degradation was analyzed performing an agarose gel.

2.3.9 Microarray Gene Expression Analysis

H9c2 cells, HL-1 cells and mouse ventricular neonatal cardiomyocytes were collected as described previously. Experiments were done in triplicate. After RNA extraction and concentration determined, statistical outliers criteria were performed to further analyze sample purification. The microarray hybridization was carried at the Genomic Department of the National Center for Cardiovascular Investigation (CNIC) in Madrid, Spain, and further analyzed in the Bioinformatic Unit from the same Institution. Based on the Bioconductor package "ArrayQualityMetrics" there was no evidence of any outlier among the samples. Total rat and mouse transcriptome, with about 42,000 transcripts from the Agilent 4x44K chip were identified using Agilent Rattus norvegicus / Mus musculus genome microarrays of and, respectively. Raw signals were

thresholded to 1 and quantiles normalization [169] was performed using the software GeneSpring.

Data were considered in the log2 scale. Default flags were considered as absent, except saturated points that were flagged as marginal.

2.3.10 Statistical analysis

Data analysis was performed by using GraphPad Prism 4.0 program (GraphPad Software, Inc.) and data were expressed as mean±SEM for the number of experiments indicated in the legends of the figures. Multiple comparisons were performed using one-way analysis of variance (ANOVA) followed by Bonferroni multiple comparison post-hoc test and the t-test in proliferation studies to compare two groups. Significance was accepted when p value < 0.05.

Results

3. Results

3.1 Characterization of H9c2 Myoblast Differentiation

3.1.1. Abstract

Despite the wide use of the H9c2 cell line in the undifferentiated state, a cardiac-like phenotype can be obtained after differentiation in a low FBS (1%) and retinoic acid (RA)-supplemented media. Because the adult heart tissue is composed by differentiated cardiomyocytes, toxicological studies can have different outcomes depending on the developmental state of the biological model used. In this chapter, we aim to characterize the H9c2 differentiation process to obtain a cardiomyocyte-like phenotype, which will help understanding future results involving the possible differential toxicity of isoproterenol on H9c2 cells in different differentiation stages. Alterations in H9c2 cells were found during the differentiation process towards a cardiac-like phenotype: myoblasts fuse to a multinucleated and larger cells, proliferative activity is reduced, the content in specific markers of cardiomyocytes (e.g. troponin T, troponin I and Mlc2v) is increased and, finally, alterations in some proteins involved in pathways of the β-adrenergic signaling and in stress responses/cell protection were found (e.g. increased P-p38MAPK, VDAC, Bcl-xL, SOD2 and decreased calcineurin, β_1- and β_3-AR). Also, differences in the transcriptional map were obtained when comparing undifferentiated and differentiated H9c2 cells. The results hint at a higher content in protective mechanisms in undifferentiated H9c2 cells, which may impact the toxicity of cardiotoxicants. Finally, we also compared the transcriptional map of the adult HL-1 cell line with isolated neonatal cardiomyocytes, which are also widely used as models for cardiac cell lines.

3.1.2. Background and Objective

Cardiac tissue contains mostly terminally differentiated cells without proliferative activity, although cardiac muscle progenitors in specific niches have been found [170, 171]. Despite the fact that these undifferentiated cells are a clear minority in the tissue, toxicology studies should address the different susceptibility of terminally differentiated vs. undifferentiated cardiac cells to cardiotoxicants. The H9c2 myoblast cell line, originated from rat ventricular tissue, has been used *in vitro* as a model for skeletal and cardiac muscle due their morphological features and electrical/hormonal signaling properties [148, 151]. One important feature of this embryonic cell line is its ability to differentiate from mono-nucleated myoblasts to myotubes when cultured in a low serum concentration media. In the differentiation process, cells obtain mostly a skeletal muscle phenotype, as evidenced by specific markers of skeletal muscle differentiation such as myogenin, troponin T and MyoD [149]. Ménard et al. demonstrated that daily additions of *all-trans* retinoic acid (RA) in a low-serum media lead to increased content of cells with an adult cardiac muscle phenotype. As described, the great majority of studies involving H9c2 myoblasts as a model to evaluate the cardiotoxicity of different agents are performed with cells in the undifferentiated state, raising questions on the relevance of the results when compared to the use of adult cardiomyocytes.

The differentiation potential of H9c2 myoblasts makes this cell line an attractive tool for investigating the cardiotoxicity of several agents. Concerning this, only a few investigations have applied this knowledge in experimental studies [172-175]. Since one major question in this thesis is whether the differentiation stage of H9c2 cells impacts ISO toxicity, we aimed in this chapter to better characterize the different cell populations obtained in each differentiation protocol.

3.1.3. Results

3.1.3.1. Morphological, Proliferative and Molecular Characterization of Undifferentiated and Differentiated H9c2 cells

With the objective of characterizing the differentiation process in the H9c2 cell line, a simple visual assessment by microscopy was performed in the different groups of cells investigated in this study: H9c2 myoblasts growing on medium supplemented with 10% of FBS (growth-promoting medium) and differentiated H9c2 cells maintained in medium supplemented with 1% of FBS (in presence and in the absence of RA). Because of the controversy found in the literature [99, 176] regarding the optimal concentration of RA to induce cardiac cell differentiation, two different concentrations of RA were used in some experiments (10nM and 1μM).

When cultured in high-serum medium, H9c2 cells are commonly mononucleated and exhibit spindle-to-stellate shape (Fig. 8, top panels). However, 5 days in low serum medium, both in the present and absence of RA, induce H9c2 myoblasts to fuse and form multinucleated cells with higher dimensions (Fig. 8, bottom panels), which may be morphologically considered indication of H9c2 differentiation. In order to verify if H9c2 differentiation promotes alterations in cellular viability and mitochondrial morphology, cells were incubated with the mitochondrial-selective probe TMRM and with the fluorescent probes Hoechst 33342 and Calcein-AM. As shown in Fig. 8 (right panels), both undifferentiated and differentiated myoblasts present a large density of filamentous mitochondria.

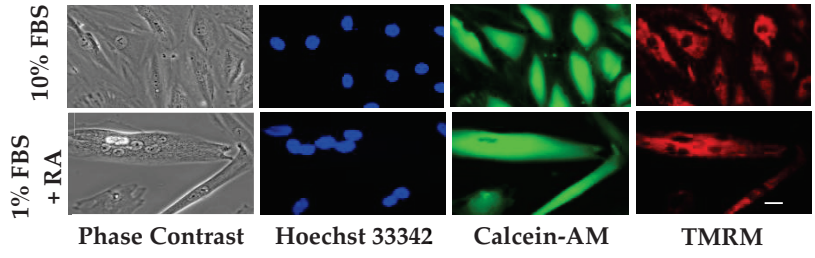


Figure 8 - Effect of serum withdrawal on H9c2 cells morphology. Morphologic alterations were observed using the fluorescent probes Hoechst 33342 (nucleus, blue), Calcein-AM (cell viability, green) and TMRM (mitochondria, red). H9c2 cells cultured in 10% of FBS in the medium (top panels) showed a stellar shape, in the most cases, with one nucleus per cell. Upon culture in a medium containing 1% of FBS (bottom panels) daily supplemented with RA differentiated H9c2 cells showed an elongated shape with several nuclei. Epifluorescent microscopy images are representative of 4 different cell preparations. White bar represents 30µm.

To assess the effect of serum withdrawal on H9c2 cells proliferation, we measured the cell mass increase during 6 days in culture by using the colorimetric SRB method. Results obtained demonstrate a reduction in the cell proliferation rate when H9c2 cells were cultured in low serum medium being the difference evident in the exponential phase of the growth (Fig. 9A).

An additional study using the same method investigated whether previously differentiated H9c2 cells were able to re-start proliferation when transferred to a high-serum medium (Fig. 9B). Results demonstrate that differentiated H9c2 cells increase gradually the proliferation activity when exposed again to a growth-promoting medium.

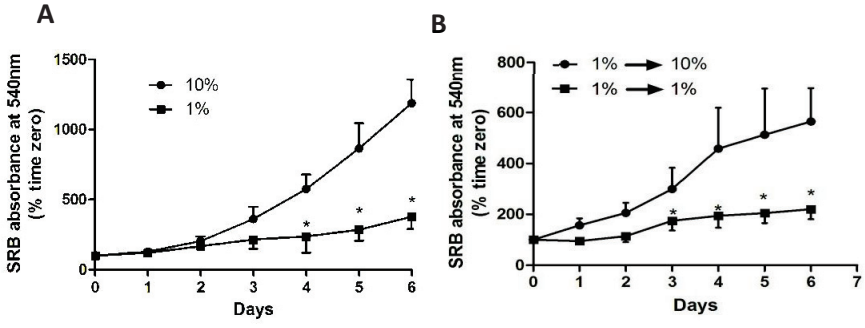


Figure 9 - Alteration on proliferative rate by modulating the percentage of serum in the medium. H9c2 cells were seeded at a density of 5,000 cells/well and allowed to proliferate during 6 days. Results were analyzed by using the SRB assay. A) Undifferentiated H9c2 cells incubated in a high-serum medium proliferate fast, while incubation in a low-serum medium during 5 days promotes cell cycle arrest. B) Studies were conducted after 5 days in a low serum media to allow cell differentiation. When differentiated cells are exposed to a proliferation-promoting medium, H9c2 cells re-entry the cell cycle, initiating proliferative activity. Statistical analysis: * $p < 0.05$ vs 10% FBS.

We then investigated whether RA-driven cell differentiation results in the increase of specific markers for cardiac cells [173, 177]. Higher protein content in phosphorylated-troponin I, cardiac troponin T and ventricular myosin light chain were observed when H9c2 cells were differentiated in a low serum media supplemented with RA (Fig. 10A-C), confirming that RA treatment drives H9c2 cells to a cardiac-like phenotype.

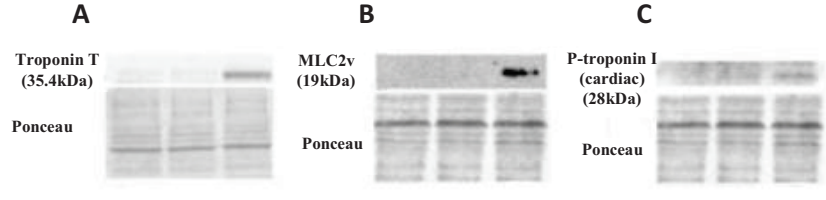


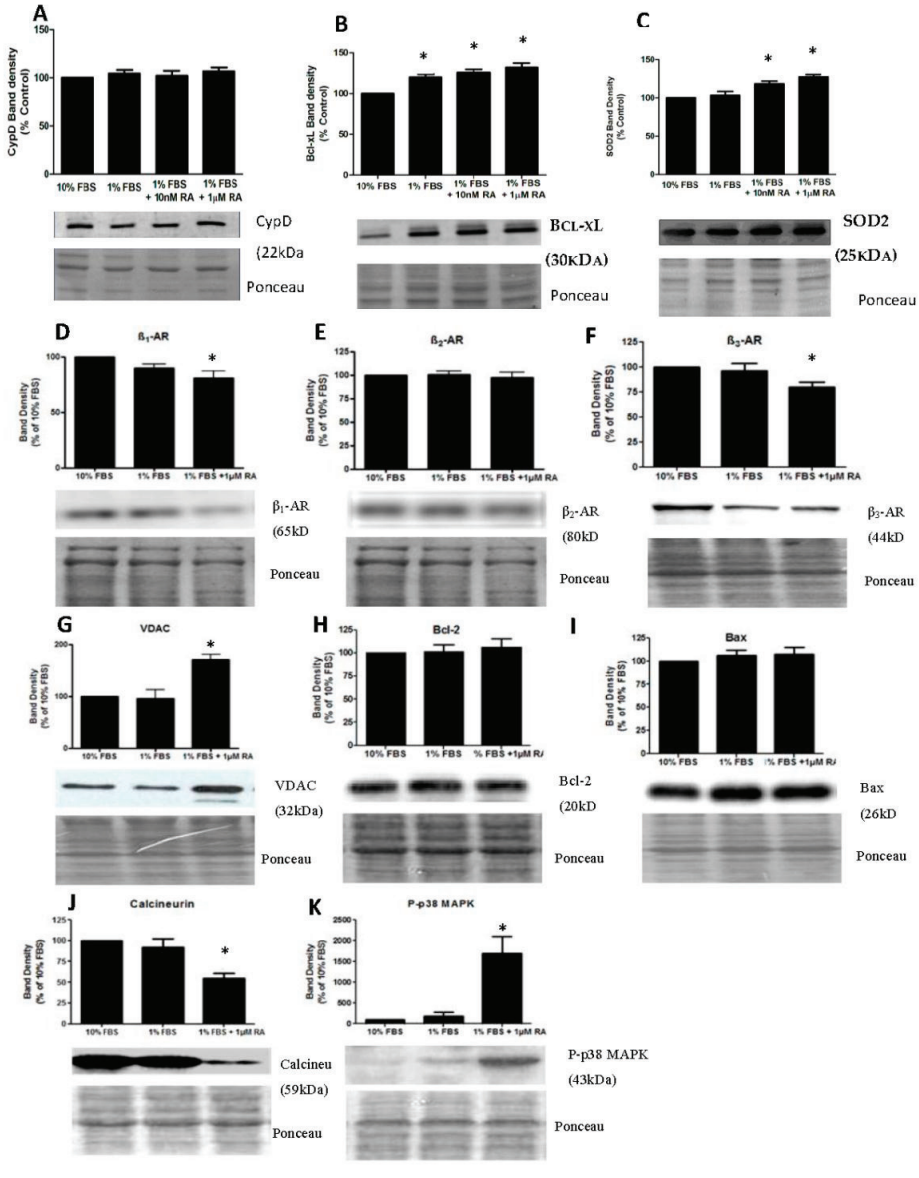
Figure 10 - Cellular content on specific cardiac markers in different cell populations. The content of cardiac troponin T increases as cells are differentiated in a low serum media in the presence of 1 μM RA (left panel). By using Western Blotting, it was also possible to confirm that RA-differentiated

cells have increased cardiac myosin light chain-2 (Mlc2v) (middle panel) and cardiac phosphorylated-troponin I (P-troponin I) (right panel), two recognized cardiac markers. The images are representative of 3 different experimental studies which developed the same result. Control loading was performed by using Ponceau labeling.

3.1.3.2. Alterations on signaling protein content during H9c2 differentiation

Since cardiomyocyte regulation of cell death by β-adrenergic stimulation is associated with specific signaling pathways [178], several proteins were analyzed in the context of apoptotic signaling alterations during H9c2 differentiation to identify whether the results would impact how the different groups of cells react to the toxicity caused by ISO over-stimulation. Evaluation by Western Blotting of the three subtypes of β-adrenergic receptors (AR) (Fig. 11D-F) was performed. While differences regarding β_2-AR between cells in different states of the differentiation process were not found, β_1-AR and β_3-AR were significantly decreased in H9c2 cells that were differentiated in the presence of RA. To investigate downstream mechanisms by which stimulated β-AR by ISO may signal to induce cell death, alterations in the cellular content of calcineurin and phosphorylated p38-MAPK in H9c2 cells subjected to the different differentiation processes were performed. Both proteins have been demonstrated to be involved in β-adrenergic regulation of cardiomyocyte death [179, 180]. While calcineurin decreased in H9c2 RA-differentiated cells (Fig. 11J), phosphorylated p38-MAPK was increased in the same group (Fig. 11K). Basal levels of the pro-apoptotic protein Bax and the anti-apoptotic protein Bcl-2 were not altered during the differentiation process (Fig.11H and 11I). The mitochondrial voltage-dependent anion channel (VDAC) was also analyzed, since this protein has also been described as

being involved in the mitochondrial apoptotic pathway [181]. H9c2 cells differentiated in the presence of RA have increased VDAC content compared with the other experimental groups (Fig. 11G). Basal content of some important proteins involved in intrinsic apoptotic signaling pathway such as Bcl-xL and cyclophilin F were evaluated by Western Blotting (Fig. 11A and 11B). We found the same content in terms of cyclophilin F, a mPTP component [179], in the four groups of H9c2 studied. A higher content in Bcl-xL, a protector of mitochondrial integrity [182], was found on both groups of differentiated cells. Mitochondrial superoxide dismutase 2 (SOD2) protein was also evaluated due its role in mitochondrial anti-oxidant defense [178, 183]. Differentiated H9c2 cells in present of RA were found to have significantly higher content of this ROS scavenging enzyme than undifferentiated cells (Fig. 11C).


A
CypD Band density (% Control)
10% FBS
1% FBS
1% FBS + 10nM RA
1% FBS + 1μM RA
CypD
(22kDa
Ponceau
B
Bcl-xL Band density (% Control)
BCL-XL
(30KDA)
Ponceau
C
SOD2 Band Density (% Control)
SOD2
(25KDA)
Ponceau
D
ß1-AR
Band Density (% of 10% FBS)
10% FBS
1% FBS
1% FBS +1μM RA
β1-AR
(65kD
Ponceau
E
ß2-AR
β2-AR
(80kD
Ponceau
F
ß3-AR
β3-AR
(44kD
Ponceau
G
VDAC
VDAC
(32kDa)
Ponceau
H
Bcl-2
Bcl-2
(20kD
Ponceau
I
Bax
Bax
(26kD
Ponceau
J
Calcineurin
Calcineu
(59kDa)
Ponceau
K
P-p38 MAPK
P-p38 MAPK
(43kDa)
Ponceau

(Legend →)

Figure 11 - Determination of specific proteins content in undifferentiated and differentiated H9c2 cells. The content of cyclophilin F (A), Bcl-xL (B), SOD2 (C), β_1-AR (D), β_2-AR (E), β_3-AR (F), VDAC (G), Bax (H), Bcl-2 (I), calcineurin (K), and phosphorylated p38-MAPK (J) in total cellular extracts was identified by Western Blotting analysis as bands with the corresponding molecular weight. Blots are representative from different cell preparations. Also shown is the respective Ponceau labeling. Graphics are expressed as mean±SEM of 7 (Bax, Bcl-2), 6 (β_1-AR, β_2-AR, β_3-AR) and 4 (cyclophilin F, Bcl-xL, SOD2, calcineurin, P-p38MAPK and VDAC) separate experiments. $p<0.05$ compared with 10% FBS.

3.1.3.3.Morphological and transcriptional comparison of H9c2 cells with neonatal cardiomyocytes and HL-1 cells.

Several cell line models have been used in cardiovascular studies instead of primary cultures due to the several limitations. Thus, in this work we further analyzed morphological differences between the HL-1 cell line, H9c2-differentiated cells and primary cultures of cardiomyocytes from neonatal mice.

Differentiated H9c2 cells are morphologically more elongated and present a fusiform shape comparing with the other groups (Fig. 12). However, similarly to preparations of HL-1, mostly differentiated H9c2 cells present several nuclei per cell, which is a characteristic from adult cardiomyocytes. Neonatal cardiomyocytes are mononucleated, presenting a smaller size. Similar to HL-1 cells, neonatal cardiomyocytes also present contractile activity under special culture conditions [128]. It is worth to note that H9c2 cells are isolated from rat and HL-1 and neonatal cardiomyocytes were collected from mice, although in different ages.

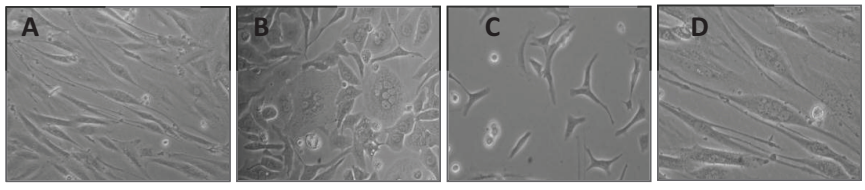


Figure 12 - Representative images of differentiated H9c2 cells, adult HL-1 cells and neonatal cardiomyocytes. Microscopy images show morphological differences between groups of cells. (A) H9c2 differentiated cells present an elongated shape and are mostly multinucleated, (B) mostly adult HL-1 cells present several nucleus per cell, (C) isolated cardiomyocytes from neonatal mice are smaller and are mostly mononucleated cells. A, B and C images represent a magnification of 200x. D corresponds to a magnification of 400x of (A) to better identification of multinucleated cells.

To obtain an alternative snapshot of the alterations suffered by H9c2 cells during the differentiation process as well as differences between HL-1 cells and isolated neonatal cardiomyocytes, we performed a comparative gene expression analysis to identify differences in gene transcription. After correction of the data and identification of significant differences on gene regulation (based on the adjusted p value < 0.05), we found several transcriptional alterations. The mentioned genes are intrinsically related and are involved in differentiated processes, proliferative activity and apoptosis signaling.

Genes present in the treemap diagram of the Fig. 13 were chosen based on the selectivity to the tissue, e.g. whether they represent a specific protein that would be mostly found on the correspondent model. The results from microarrays expression suggest that differentiation of H9c2 cells to a cardiac phenotype led to an expected increase in cardiac specific mRNAs.

Figure 13 - Treemap diagram of gene expression in RA-differentiated cells comparing with undifferentiated H9c2 cells. H9c2 cells were differentiated for 5 days in a media containing 1% serum and supplemented with RA, as described in Materials and Methods section. Data represent mRNA fold-change of selected genes by using the Treemap© version 4.1.2. The size of each square is similar in all genes and had no significance on diagram interpretation. Colors represent fold-change in the expression of the transcript. Genes differentially expressed (adjust $p<0.05$) were filtered and ordered by the respective FC. Data represent different genes whose expression was up-regulated in 10% FBS (red) and up-regulated in RA-differentiated cells (light green). Data correspond to three separate experiments. Abbreviations are explained in Table 4.

RA-differentiated H9c2 cells had increased expression of genes related with calcium transporters (such as the sarcoplasmic reticulum calcium ATPase - SERCA), those coding for cardiac sarcomeric proteins such as troponin T and troponin C and differentiation markers such as myogenin, an essential transcription factor for the terminal differentiation into myocytes [184]. With relevance for cell bioenergetics, increased

expression of genes relevant for mitochondrial energy production including mitochondrial creatine kinase, cytochrome *c* oxidase subunits COX6a2, COX8b and COX11, uncoupling proteins 2 and 3 and Complex I subunit NADH dehydrogenase (ubiquinone) Fe-S protein 4, was identified in RA-differentiated cells. The expression of genes involved in signaling pathways for cellular death such as FasL, cyclin-dependent kinase inhibitor 2B and Bcl2l11, which are involved in the inhibition of cell cycle progress and on apoptosis, were also augmented in RA-differentiated H9c2 cells. Interesting, we also found that RA-differentiated cells have increased oxidative stress growth inhibitor 1 (Osgin1) gene expression. Undifferentiated myoblasts show increased gene expression of pro-survival proteins such as Bcl-2, increased transcripts for the mitochondrial inner membrane translocase (TIM). Also, genes playing important roles in the regulation of cell cycle, including gene encoding cyclin A, cell cycle regulator required for the onset of DNA replication, and encoded or related genes were also up-regulated in undifferentiated myoblasts. Regarding related genes of the retinoblastoma-like 1, undifferentiated myoblasts had also increased expression on E2F transcription factor 1, HDAC1, BRCA1 associated RING domain 1, prohibitin-2 and mybl2. Moreover, the phosphodiesterase 4A (PDE4A), cAMP-specific, the mitogen-activated protein kinase, the B-cell CLL/lymphoma 2 (Bcl2) are also up-regulated in undifferentiated cells.

The results indicate that the differentiation of H9c2 cells with serum regulation and RA addition led to a transcriptional up-regulation of genes involved in cardiac differentiation, which again demonstrates the validity of using this cell system.

Table 4 - Groups of gene expression explored in H9c2 undifferentiated myoblasts and RA-differentiated H9c2 cells. H9c2 cells were differentiated for 5 days in a low serum concentrated medium daily supplemented with RA. Both groups, undifferentiated and RA-differentiated cells, were collected and analyzed as described in Material and Methods section. FC>2 corresponds to, at least, two-fold up-regulation of the gene, FC<2 corresponds to, at least, two-fold down-regulation of the gene. Data correspond to three separate experiments and differences are also represented in Fig. 13.

1% FBS + RA vs 10% FBS		
Gene	**Involvement**	**FC**
Chrna1	Cholinergic receptor, nicotinic (muscle)	33.66
Myom2	Myomesin 2 - sarcomeric protein	32.62
Ckmt	Creatine kinase - important in tissues that consume ATP faster	27.50
Ampd1	Adenosine monophosphate deaminase-decrease cAMP levels	22.61
Atp2a1	ATPase, Ca^{2+} transporting, cardiac muscle (SERCA1)	14.43
Faslg	Fas ligand - trigger apoptosis	17.01
Osgin1	Oxidative stress induced growth inhibitor	12.12
UCP3	Uncoupling protein 3 (mitochondrial, proton carrier)	10.97
Cdkn2b	Cyclin-dependent kinase inhibitor	9.58
Myog	Myogenin - essential for development of skeletal muscle	9.45
Cox6a2	Cytochrome c oxidase assembly protein 6	6.41
Tnnc2	Troponin C (fast)	4.60
Tnnt2	Troponin T (cardiac specific)	4.48
Pln	Phospholamban – inhibitot of SERCA2	3.63
Myl2	Light myosin specific for cardiac muscle; triggered by calcium to induce contraction	2.87
Atp2a2	Ca^{2+} transporting, cardiac muscle (SERCA2)	2.76
Camk2b	Calcium/calmodulin-dependent protein kinase II	2.54
Cox11	Cytochrome c oxidase assembly protein 11	2.23
Cpt1a	Carnitine palmitoyltransferase 1a	2.14
Ndufs4	NADH dehydrogenase (ubiquinone) Fe-S protein 4	2.10
Bcl2l11	BCL2-like 11 - apoptosis facilitator	2.07
UCP2	Uncoupling protein 2 (mitochondrial, proton carrier)	2.06
Hdac1	Histone deacetylase 1	0.50
Pde4a	Phosphodiesterase 4A, hydrolyzes the cAMP	0.45
Gata2	Promotes cellular proliferation	0.45
Rbbp8	Retinoblastoma binding protein	0.43
Timm17a	Translocase of inner mitochondrial membrane	0.38
Bcl2	Anti-apoptotic protein	0.36

Nfatc2	Nuclear factor activated by calcineurin	0.34
E2Ftf1	E2F transcription factor-mediate both cell proliferation and apoptosis	0.26
Gpx1	Glutathione peroxidase 1	0.20
Top2a	Topoisomerase (DNA)	0.15
Adcy10	Adenylate cyclase 10	0.12
Wisp2	WNT1 inducible signaling pathway	0.12
Bard1	BRCA1 - regulate cell growth	0.10
Chrm2	Cholinergic receptor, muscarinic	0.10
Mybl2	Myeloblastosis oncogene-involved in cell cycle progression	0.09
Ccna2	Cyclin A	0.08

We also performed genotyping studies comparing isolated neonatal cardiomyocytes with the HL-1 cell line. Neonatal cardiomyocytes presented up-regulation of non-differentiated cell markers such as the GATA6, a gene that is required for a proper maturation of cardiac mesoderm, but also in BMP4 which level expression are maintained by GATA6 [2, 185]. Overexpression of GATA6 in cardiac cells in the declined period of it expression (before terminally differentiated cells) results in arrest of cardiac differentiation [2].

Caveolin-2 and caveolin-3 are up-regulated in isolated cardiomyocytes. However, evidences demonstrated that these proteins increase with age but also when isolated cardiomyocytes are cultured *in vitro* [2], which was the case in this study.

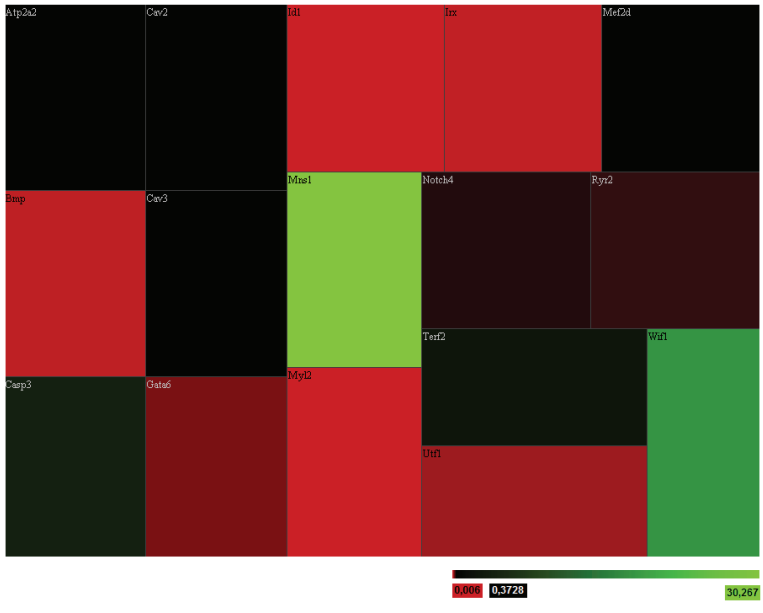


Figure 14 - Treemap diagram of gene expression of isolated neonatal ventricular cardiomyocytes compared with HL-1 cell line. Isolation and culture of ventricular cardiomyocytes was performed as described in Materials and Methods section. Cells were collected after 24 hours in culture. Data represent mRNA fold-change of selected genes by using the Treemap© version 4.1.2. The size of each square is similar in all genes and had no significance on diagram interpretation. Colors represent the fold-change in the expression of the transcript as identified by the fold-change scale. Genes differentially expressed (adjust $p<0.05$) were filtered and ordered by the respective FC. Data represent different genes whose expression was up-regulated (green) in HL-1 cells and up-regulated (red) in isolated cardiomyocytes. Data correspond to three separate experiments. Abbreviations are explained in Table 5.

Table 5 - Groups of gene investigated in isolated neonatal cardiomyocytes and HL-1 cells. Cellular preparations were collected and analyzed as described in Material and Methods section. FC>2 corresponds to, at least, two-fold up-regulation of the gene on HL-1 cells, FC<2 corresponds to, at least, two-fold up-regulation of the gene on neonatal cardiomyocytes. Gene corresponds to those represents in Fig. 14. Data correspond to three separate experiments.

Neonatal Cardiomyocytes vs. HL-1 cell line		
Gene	**Involvement**	**FC**
Mns1	Meiosis-specific nuclear structural protein	30.267
Wif1	Wnt inhibitory factor	17.744
Casp3	Effector caspase	3.923
Terf2	Telomeric repeat binding factor	2.315
Cav3	Caveolin 3	0.473
Cav2	Caveolin 2	0.448
Mef2d	Required for cardiac development	0.453
Atp2a2	ATPase, Ca2+ transporting, cardiac muscle	0.382
Notch4	Involved in cell fate decisions	0.22
Ryr2	Ryanodine receptor 2, cardiac	0.196
Gata6	Related with embryonic cardiac development	0.108
Bmp	Involved in heart development	0.022
Irx	Transcription factor required for cardiac development	0.018
Utf1	Undifferentiated embryonic cell transcription factor	0.065
Myl2	Myosin, light polypeptide 2, cardiac	0.006
Id1	Inhibitor of DNA binding	0.0063

3.1.4. Discussion

The rat H9c2 cell line is an established *in vitro* cellular model for cardiac cells in a wide variety of experimental designs [2, 31]. It has been described that H9c2 cells are an excellent model to investigate mechanisms and outcomes of cardiac hypertrophy signaling, which increases the interest in this cell model [150]. Despite the ability of H9c2 myoblasts to differentiate in adult muscle cells, all toxicological studies involving this cell line are performed with undifferentiated myoblasts. In fact, the traditional use of this cell line in its undifferentiated state may not be the best approach to use as a cardiac cell surrogate. This raises an important question: does the differentiation state of H9c2 cells interfere with their sensitivity to cardiotoxicants? In this regard, at least some discussion should be carried out on the validity of this model. The important finding that H9c2 myoblasts have the capacity to differentiate into skeletal and cardiac muscle cells increases the uses of this cell model for developmental toxicology studies, including those using cardiac stem cells. For example, a similar approach, although with a different model, demonstrated that doxorubicin cardiotoxicity involves the depletion of the cardiac stem cell pool [43].

Muscle cell differentiation, *in vivo*, is commonly accompanied by specific processes, including cell cycle arrest and morphological features such as formation of multinucleated myotubes. In this context, morphological alterations observed in H9c2 cells, after the induction of differentiation by serum withdrawal were initially examined by epifluorescence microscopy. The results obtained in this study confirm that upon reduction of serum in the culture media to 1%, H9c2 myoblasts fuse and form multinucleated cells (Fig. 8), as previously described [44]. A decrease in cell proliferation was another consequence of decreasing serum media, which is more evident in the exponential

phase of the growth (Fig. 9A). It has been previously shown that the differentiation process initiates a cell cycle arrest, which decreases cellular proliferation [60]. Cellular proliferation depends on the existence of growth factors in the culture media, hence it is expected that the reduction in the concentration of those factors will induce cell growth arrest. According to the literature [66, 70] the addition of retinoic acid (RA) drives the differentiation towards a cardiac phenotype. Interestingly, the fact that proliferation re-starts after moving cells back to a high-serum media (Fig. 9B) show that cell cycle arrest is reversible.

Although the differentiation protocols yield heterogeneous populations [31], Fig. 10 shows that RA-differentiated cells present higher content in troponin T, phosphorylated troponin I and Mlc2v, markers of cardiac differentiation [82] although it must be stressed that the relative yield of differentiation into a population showing cardiac markers appears to be very dependent on the initial cell confluence. One open question in this regard is if data from Western Blotting reflect large differences in protein expression in a small subset of cells that differentiate, or instead smaller changes in protein expression in a large majority of cells that suffer differentiation. Answering this question, evaluation of gene arrays of the entire cell genome showed that expression of multiple markers of cardiomyogenesis occurs, including the gene encoding for the cardiac troponin T protein.

During muscle cell differentiation, specific signaling pathways are activated and muscle protein synthesis patterns are altered. Modulation of signaling proteins during cell differentiation can cause a higher susceptibility of a specific developmental stage of the tissue to a cardiotoxic agent. Of particular importance, we found several proteins that may be responsible for a possible different cellular response to a toxicant. One of the objectives of the present work was to verify if

alterations in cellular levels of β-adrenergic receptors (β-AR) were observed during the differentiation process as overstimulation of different β-AR subtypes results in different outcomes: β_1- subtype is commonly associated to a pro-apoptotic signaling pathway whereas the β_2-subtype is reported to be involved in an anti-apoptotic process [83]. The present study demonstrates that β_1-AR content in undifferentiated H9c2 cells is higher than in differentiated cells (Fig. 11D), being significantly different when compared with differentiated cells in presence of RA. In fact, not only β-ARs are involved in a large variety of vital processes including intracellular oxygen availability [86], but also β_1-AR can become desensitized after prolonged stimuli [89]. Similar results were obtained with β_3-AR where differentiated cells in presence of RA have lower levels of this receptor. The physiological significance of this fact is not immediately clear. β_3-ARs have different properties from the two other cardiac subtypes. β_3-AR up-regulation during disease processes and resistance to desensitization suggests an important role for this subtype of receptors, especially because it has been described that β_3-AR can protect the myocardium against adverse effects of excessive catecholamine stimulation [88]. Also, the present work does not distinguished between membrane/active receptors and receptors that may exist in other intra-cellular compartments.

Also, proteins involved in intracellular pro and anti-apoptotic proteins were evaluated. No alterations were found in the basal content in Bax and Bcl-2, showing that cell differentiation does not alter the status of these two important proteins (Fig. 11H and 11I). Interestingly, the gene array analysis confirmed increased expression of Bcl-2, a pro-survival protein, on undifferentiated cells (Fig. 13). Both Bcl-xL and SOD2 protein content is increased in differentiated cells which can constitute a higher degree of protection of this cellular group. Interestingly, the

mitochondrial voltage-dependent anion channel (VDAC), which has been suggested to form/regulate the mPTP and to be involved in apoptosis [87] is increased in cells that were differentiated in presence of RA (Fig. 11G). The increased amount of VDAC in this experimental group can be important to explain differences on the susceptibility of cells to mitochondrial-mediated cell death.

The decrease in calcineurin, a calcium/calmodulin-dependent phosphatase that is activated by sustained elevations in intracellular calcium [94] and is inactivated by oxidative stress [87], and the increase in p38-MAPK during the differentiation process can contribute to the increased susceptibility of differentiated H9c2 cells to a cardiotoxicant agent. This is a particularly interesting observation since it has been observed that transfection of H9c2 cells with calcineurin adenovirus resulted in increased oxidative stress [94]. The decrease of calcineurin in RA-differentiated cells may act to suppress oxidative stress resulting from increased mitochondrial activation during differentiation [87]. In fact, also both decreased mitochondrial membrane potential and mitochondrial dysfunction were observed after calcineurin up-regulation [94], two results that were not verified in our study after H9c2 differentiation, suggesting that a decrease in this protein may contributes to the regular mitochondrial function toward to a mature energetic efficiency state. Moreover, we also found that the gene encoding for the nuclear transcription factor NFAT, which is regulated by calcineurin and calcium, was found increased in undifferentiated cells. Once activated, NFAT translocate to the nucleus where modulate the expression of multiple other genes [93]. Differences observed in the content of phosphorylated p38-MAPK are interestingly opposite to what was observed with calcineurin, with RA-differentiated cells having a very large increase in that protein (Fig. 11). As previously described by Lim

et al., p38-MAPK stimulates H9c2 cellular differentiation even in presence of a proliferative medium [93]. A few studies also document the key role of p38-MAPK during cardiomyogenesis involving both MyoD and myocyte enhancer binding factor 2 (MEF2) [93, 94]. Moreover, p38 MAPK has an opposite influence on NFAT/calcineurin-activated nuclear function by inducing NFAT export back to cytoplasm which suggest that the amount of calcineurin/p38MAPK during differentiated process, which is opposite, although not well understood, must be supported for an unknown reason [93].

Differentiated markers specific for cardiac myocytes were found increased upon differentiation of H9c2 cells confirming results obtained by Western Blotting. In this regard, also the genes Myl2, Myog, and Myom2 detail a clear transition to a more mature and adult muscle.

Furthermore, this transcriptional analysis reveals that RA-differentiated myoblasts have increased expression of genes related with mitochondrial function, including respiratory chain complexes and uncoupling proteins, confirming our previous results that H9c2 cell differentiation with RA towards a more cardiac-like phenotype involves remodeling and up-regulation of mitochondrial function [94]. Higher expression of uncoupling proteins 2 and 3 in RA-differentiated H9c2 cells may be a compensatory response to an increase in mitochondrial activity after cell differentiation. As previously described, uncoupling proteins have important roles in fatty acid β-oxidation and mitochondrial oxidative stress [87]. In fact, when fatty acid delivery to mitochondria exceeds oxidative capacity, the expression of UCP3 increases leading to reduced storage of non-esterified fatty acid anions [87]. Interestingly, the Cpt1 gene that encodes for carnitine palmitoyltransferase 1, was also increased in RA-differentiated cells. This shuttle system transports fatty acyl-CoA esters from the cytosol into the mitochondria, where they

undergo β-oxidation metabolism [93]. Still concerning energy production, creatine kinase, Ckmt, plays a pivotal role in muscle function since it works to transfer a "high energy phosphate" from mitochondria to the cytosolic carrier, creatine. Therefore, this enzyme is highly expressed in tissues that have a highly energy demand. In our study we found that RA-differentiated cells highly expressed this mitochondrial enzyme when compared with undifferentiated cells. Reticoic acid-differentiated H9c2 cells had also increased expression of genes encoding calcium transporters, such as the sarcoplasmic reticulum calcium ATPase – SERCA1 and 2, as well as cytochrome c oxidase subunits COX6a2, COX8b and COX11 and complex I subunit NADH dehydrogenase (ubiquinone) Fe-S protein 4.

Cyclic nucleotide phosphodiesterase (PDE) constitute also a mechanism of great importance that safeguard the specificity of GPCR/cAMP signaling in the control of cAMP transients via its degradation. The PDE4 is responsible for cAMP hydrolysis and its inhibition increase intracellular cAMP levels and L-type Ca^{2+} current increasing Ca^{2+} release at baseline during β-adrenergic stimulation. This mechanism can contribute to sensitize differentiated cells to β-adrenergic stimulation dependent of cellular Ca^{2+} influx [186].

Genes involved in cell cycle arrest and in death signaling pathways such as FasL, the cyclin-dependent kinase inhibitor 2B and the Bcl2l11, which is able to induce apoptosis, were also increased in RA-differentiated H9c2 cells (Fig. 13). As expected, genes encoding for proliferative mechanisms were highly expressed in undifferentiated cells as well as some genes related with pro-survival signaling pathways.

By working with different cellular models such as HL-1, H9c2 and isolated neonatal cardiomyocytes, especially from a toxicology perspective, different results are expected. This work confirms the use of

H9c2 cell line as a proper model to investigate developmental cardiac toxicity (myoblasts vs adult cells) or to perform a basic assessment of cardiac toxicity.

3.2 Role of β-adrenergic Signaling Stress Responses on Isoproterenol Cytotoxicity on H9c2 Cells in Different Differentiation Stages

3.2.1 Abstract

H9c2 cells are used as a surrogate for cardiac cells in several toxicological studies, which are usually performed with cells in their undifferentiated state, raising questions on the applicability of the results to adult cardiomyocytes. Since H9c2 myoblasts have the capacity to differentiate into skeletal and cardiac muscle cells under different conditions, the hypothesis of the present work was that cells in different differentiation states differ in their susceptibility to toxicants. In order to test the hypothesis, the effects of the cardiotoxicant isoproterenol (ISO) were investigated. The present work demonstrates that differentiated H9c2 cells are more susceptible to ISO toxicity. After ISO treatment, the pro-apoptotic protein Bax increase in all experimental groups, although only undifferentiated myoblasts up-regulate the anti-apoptotic proteins Bcl-2 and Bcl-xL. Mitochondrial SOD2 is also increased in the undifferentiated groups after ISO treatment. Isoproterenol-induced toxicity results in cellular calcium overload, loss of mitochondrial respiratory complexes, mitochondrial membrane depolarization and increased superoxide anion in differentiated cells. Isoproterenol also caused increased activation of the p66Shc pathway in differentiated H9c2 cell, as well as an increase in p53 protein, caspase-3 activation and PARP cleavage. Calcineurin is decreased in differentiated H9c2 cells, which suggests an important role against ISO-induced cell death.

The results show that differentiation of H9c2 cardiomyoblasts impact stress responses to cardiotoxicants, which can be relevant in the context of developmental cardiotoxicity.

3.2.2 Background and Objective

The release of catecholamines by the sympathetic nervous system under stress conditions leads to increased heart rate through its action on β-AR. The magnitude of β-adrenergic stimulation plays an important role in cardiomyocyte stress responses, which may develop to apoptosis through the activation of several signaling pathways. Indeed, the control of myocyte cell loss through the suppression of cell death pathways represents an ideal strategy to prevent many cardiodegenerative conditions. Beta-receptor activation on cardiac cells links with several signaling pathways such as mitogen-activated protein kinases (MAPK) [99], adenylyl cyclase (AC)/cAMP/ protein kinase A (PKA) [176] or phosphatidylinositol 3-kinase (PI3K)/Akt [187]. Excessive activation of these pathways originating upstream from β-adrenergic over-stimulation, leads to increased cytosolic calcium overload and production of reactive oxygen species (ROS) [178] and often to activation of apoptotic signaling [180].

Mitochondria are key regulators of cellular metabolism. However, once mitochondrial function is affected, critical ATP-dependent mechanisms are compromised and mitochondria-induced apoptotic cell death is activated [179]. During oxidative phosphorylation, the reduction of O_2 to H_2O is also accompanied by side reactive oxygen species production [181]. Reactive oxygen species are continuously produced in eukaryotic cells and play an important role in the regulation of multiple stress and toxicity responses [182]. Assessing stress responses in the cardiomyocyte is critical in the understanding of the mechanisms of heart degeneration during different pathologies and to design proper therapies. Different *in vitro* cell systems have been used to explore mechanisms of cellular stress responses to different toxicants, including P19-derived cardiomyocytes [183], HL-1 cells [178, 188], neonatal and

adult primary cardiomyocytes [184, 185] and H9c2 cardiomyoblasts [189]. The latter cell line has been used for several *in vitro* cardiotoxicity studies. However, the vast majority of studies have only been conducted with cells in their undifferentiated and proliferative state [190]. The fact that this cell line has the ability to differentiate into adult skeletal and cardiac muscle cells [149] make it a more attractive tool for investigating toxic effects of several agents during heart development. Since differences are likely to exist regarding metabolic state [191] and signaling pathway fluxes, as described in 3.1, the differentiation state of the cells presumably impacts the outcome of xenobiotic toxicity. H9c2 cells have been previously shown to express β-adrenergic receptors in their undifferentiated state [162], which provides validity to the possible use of H9c2 cells to study the role of β-adrenergic over-stimulation on cardiomyocyte dysfunction and heart failure [192, 193]. Isoproterenol (ISO), a synthetic catecholamine, has been used to address the cytotoxicity associated with hyperadrenergic states in different studies [194]. We investigated here the susceptibility of H9c2 cells in various states of differentiation to the toxicity of supra-physiological concentrations of the β-adrenergic agonist ISO and correlated the toxicity with alterations in pro-apoptotic and β-adrenergic signaling pathways, as well as with cellular and mitochondrial stress responses. Supra-physiological ISO concentrations induce cardiomyocyte cell death, with β_1-adrenergic receptors signaling a predominantly pro-apoptotic role, while β_2-adrenergic receptor activation appears to mostly inhibit cell death [105]. The hypothesis of the present work is that H9c2 myoblasts cultured under different differentiating conditions are differently affected by ISO toxicity, which is explained by different modulation of stress responses. The objective of this work is to change the paradigm that H9c2 cells can be used irrespective of their differentiation state to investigate the toxicity of several agents; a

possible clinical implication is allowing a better understanding how cardiac cells in different stages of differentiation (e.g. in the adult heart or during fetal development) are differently affected by the toxicity of β-adrenergic agonists.

3.2.3 Results

According to previously published works, four daily additions of all-trans RA contribute to H9c2 cell differentiation towards a cardiac phenotype [149]. Although 10 nM RA is commonly reported in the literature to induce H9c2 cardiac differentiation [149, 195], other RA concentrations have also been used, including 1 μM [191, 196, 197]. In our hands, both 10 nM and 1 μM RA added to a low serum media have similar effects in terms of inhibition of cell proliferation, decreased DNA synthesis, cell morphology and troponin T content. In the present work, 1 μM RA in low serum media was used, since previous data on apoptosis induction on H9c2 differentiated cells using this differentiation protocol is available [198].

3.2.3.1 Isoproterenol Affect Differently H9c2 Cell Line Depending on the Differentiation State of the Cells

The toxicity of ISO on undifferentiated (10% FBS) and differentiated cells (1% cells, no RA addition) was initially investigated. Cells were incubated with different supra-physiological concentrations of ISO (10, 20, 50, 100 and 150μM) for 24 and 48 hours. Fig. 15 shows the results obtained using the SRB colorimetric assay. A decrease in cell numbers present after exposure to 100 and 150 μM of ISO for both 24 hours (Fig. 15A) and 48 hours (Fig. 15B) was observed in differentiated but not in undifferentiated cells. For 96 hours of incubation, 50μM ISO caused

approximately the same amount of cell loss than 100μM for 48 hours (Fig. 15C). At least for up to 300 μM ISO, no effects on undifferentiated cells were observed. The next question regarded the introduction of RA in the differentiation process and its relevance for ISO toxicity. For this experiment, one time point (24 hours) was chosen to evaluate the effects of cell differentiation achieved with two RA concentrations. Despite the fact that RA addition to low serum media drives the differentiation of H9c2 cells towards a cardiac-like phenotype [149], our data indicates that the toxicity of ISO for 24 hours was equivalent in the presence and absence of both 10 nM and 1 μM RA (Fig. 15C), which indicates that the three differentiated cell populations are equally susceptible to ISO, as opposed to undifferentiated cells (Fig. 15D). A longer exposure (48 hours) to the same ISO concentrations yielded similar results (data not shown). Since the toxicity of ISO was independent of the RA concentration used to differentiate H9c2 cells, further experiments were all performed with the highest concentration of RA used.

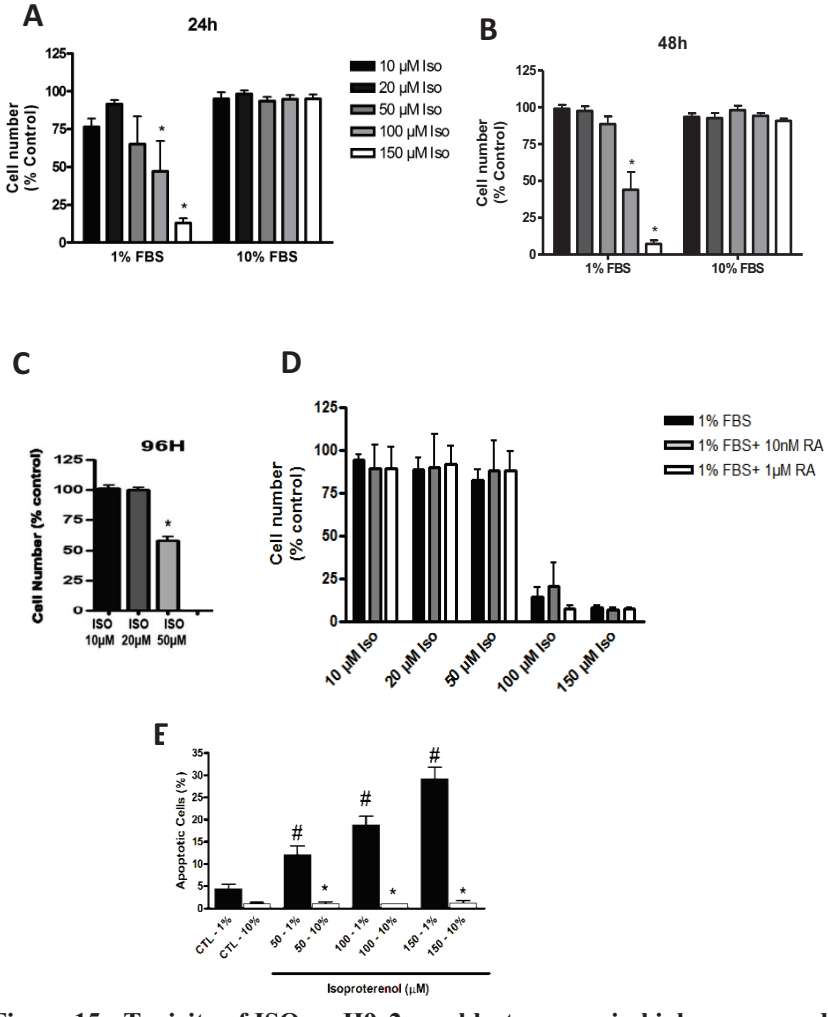
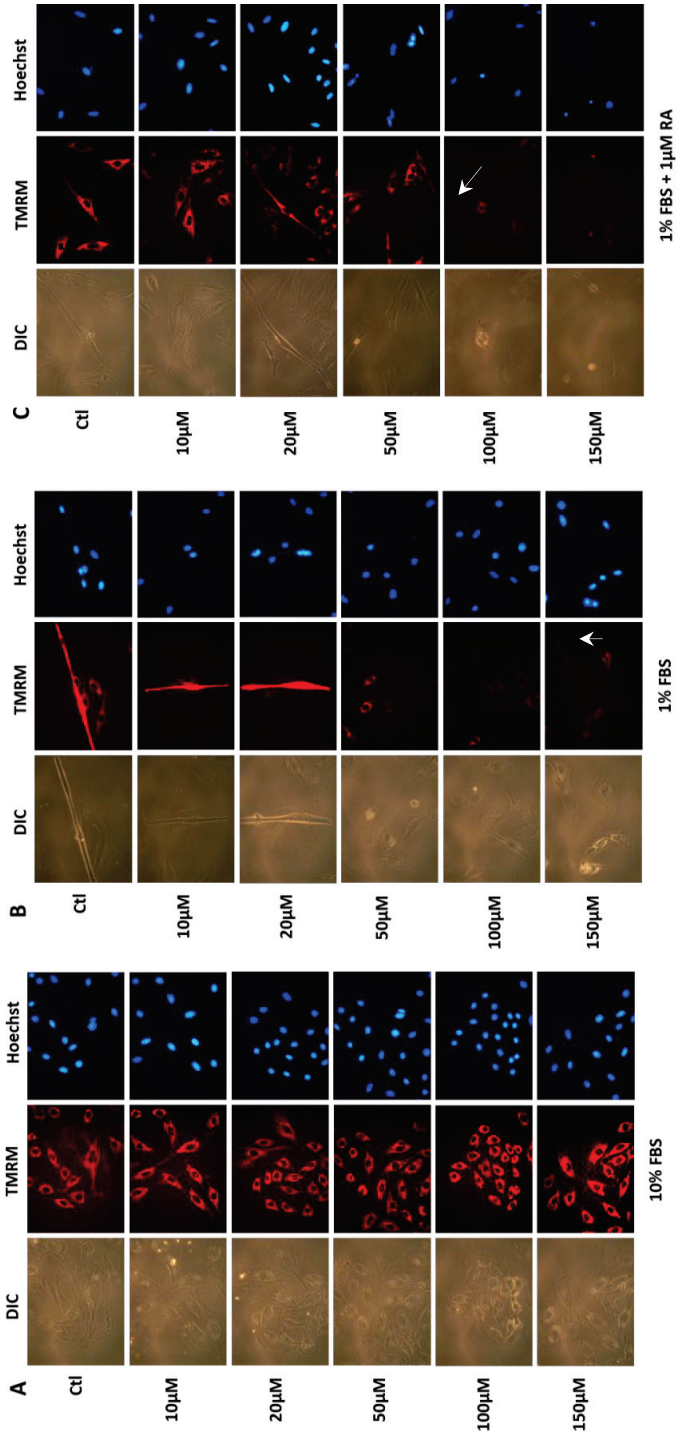


Figure 15 - Toxicity of ISO on H9c2 myoblasts grown in high-serum media (undifferentiated) and low serum media (generating differentiated adult muscle cells). Undifferentiated control cells were maintained in 10% serum medium as described in the materials and methods section. The differentiation of H9c2 myoblasts was promoted by reducing the media serum concentration from 10% to 1%. Cells were incubated with 10, 20, 50, 100 and 150µM of ISO for 24 (A), 48 (B) and 96 hours (C). Cells number was analyzed by using the SRB colorimetric assay. Data represent the mean ±SEM of 3-9 different cell preparations. Statistical analysis: * $p < 0.05$, 1% FBS vs. 10% FBS for the same ISO concentration. D) Differentiation of H9c2 cells in the presence of 10nM or 1µM RA does not alter the susceptibility of differentiated cells to ISO. H9c2 cells in the several differentiation states were incubated with 10, 20, 50, 100 and 150µM of ISO during 24 hours. The results were very similar for 48h (not shown). Results were analyzed by SRB assay as described in methods section. Data represent the mean±SEM of 3 different cell preparations. No

differences existed between the three experimental groups for each ISO concentration. E) Also shown is a graph with the number of apoptotic nuclei in H9c2 cells in high (10%) vs. low (1%) FBS media, after ISO incubation for 24 hours (data not shown) similar to those obtained for 48 hours of incubation (E). # $p<0.05$ vs control, * $p<0.05$ vs 1% FBS of 5 separate experiments.

The number of apoptotic nuclei in 1% FBS vs. 10% FBS-grown H9c2 cells was determined and confirming previous results as an increased number of apoptotic nuclei was observed in low serum media grown cells incubated with ISO for 24 hours and 48 hours (Fig. 15E). Daily additions of RA to low serum media yielded the same result as in low serum media alone, confirming earlier results. Of particular note is that caution is required when counting apoptotic nuclei/cells in differentiated H9c2 cells, since one single apoptotic cell may present several nuclei with apoptotic features. By labeling cells with the mitochondrial probe TMRM and the DNA dye Hoechst 33342, it was possible to observe that the toxicity of ISO on differentiated H9c2 cells (1% FBS and 1% FBS + RA) involved mitochondrial depolarization, as observed by loss of TMRM mitochondrial labeling (Fig. 16B and 16C). ISO did not cause any increase in cell apoptosis or mitochondrial depolarization in undifferentiated myoblasts (Fig. 16A), although it was interesting to observe that 100 and 150 μM ISO induced fragmentation of the mitochondrial network in undifferentiated cells (Fig. 17), which was not accompanied by loss of cell viability or other noticeable alterations in cell morphology. When differentiated H9c2 cells were treated with 100 or 150 μM ISO, the number of multi-nucleated cells present in the preparation was very low, which can be an indirect indication that this type of cells was predominantly affected by ISO.


A
B
C
DIC
TMRM
Hoechst
Ctl
10µM
20µM
50µM
100µM
150µM
10% FBS
1% FBS
1% FBS + 1µM RA

(Legend →)

Figure 16 - Cellular alterations observed in H9c2 cells after exposure to ISO for 48 hours (A, B and C). H9c2 cells were cultured in high-serum media (10% FBS), low serum media (1% FBS) and low serum media daily supplemented with RA (1% FBS + 1μM RA). H9c2 cells were incubated with 10, 20, 50, 100 and 150 μM of ISO and labeled with Hoechst 33342 (nuclei, blue fluorescence) and TMRM (mitochondria, red fluorescence). Epifluorescence microscopy images are representative from 3 different cell preparations. White arrows indicate cells with apoptotic nuclei (Hoechst column) or cells with depolarized mitochondria (TMRM column).

One source of experimental artifact could be a possible binding of ISO to serum proteins, thus reducing the effective amount of the compound acting on undifferentiated myoblasts, which are maintained in higher serum levels. In order to test this possibility, undifferentiated H9c2 cells were incubated with ISO in a media with 10% or 1% FBS, while cells differentiated in 1% FBS were incubated with ISO in a high (10%) and low (1%) serum media. After 48h of ISO treatment, the results suggest that ISO binding to serum proteins is not the major cause for the different susceptibility of undifferentiated vs. differentiated H9c2 cells, although there appears to be a small effect caused by altering serum concentration (Fig. 18). Interestingly, H9c2 differentiated cells exposed to a proliferation-promoting media undergo changes typical of an undifferentiated state, including an increase on cell proliferation rate (Fig. 9).

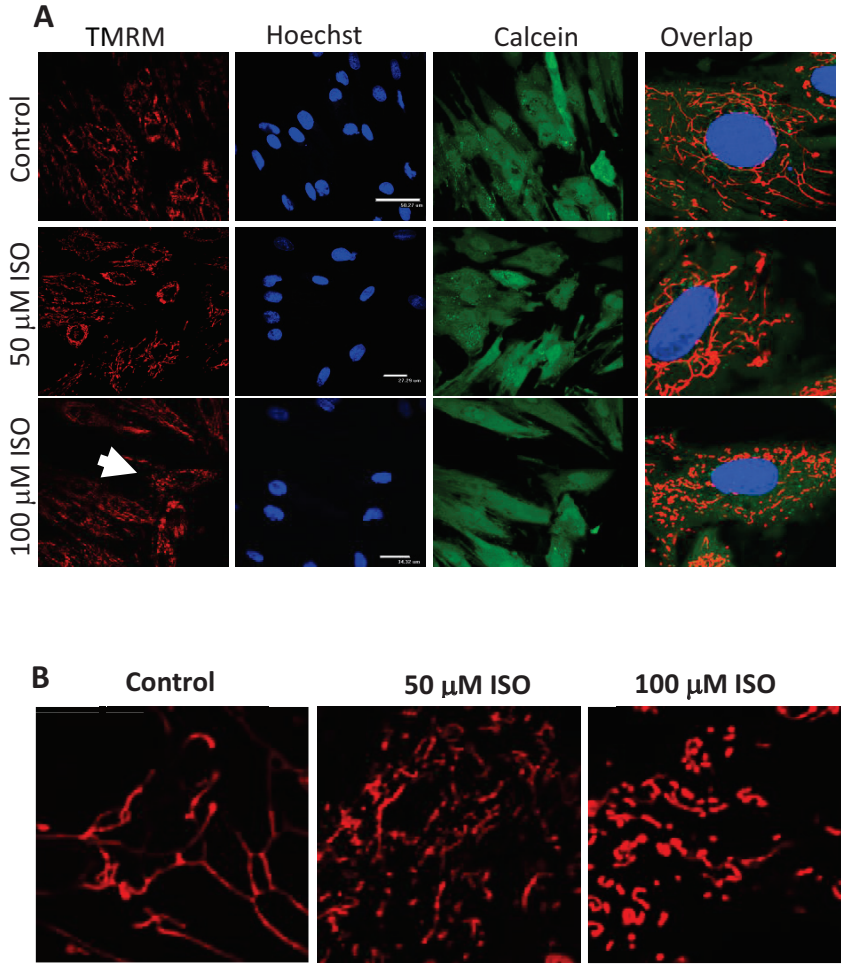


Figure 17 - Undifferentiated H9c2 cells present morphological markers of mitochondrial fragmentation. Although mitochondria are not dysfunctional, undifferentiated cells show fragmentation of the mitochondria membrane network as seen by confocal microscopy (arrow). Cells were incubated with ISO for 24 hours and labeled with the probes as described in methods section. White bar represents 30μm.

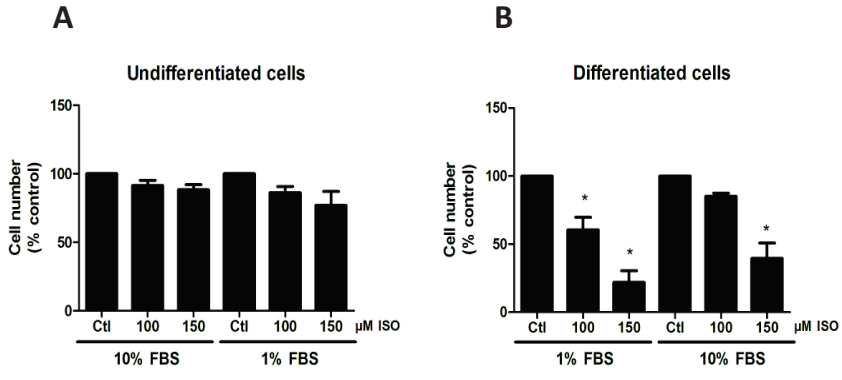


Figure 18 - Possible effects of ISO binding to serum proteins on ISO toxicity outcome. Cell numbers after ISO treatment were determined by using the colorimetric SRB method.A) Undifferentiated H9c2 cells (in 10% FBS) were incubated with 100 and 150 µM ISO for 48 hours in media containing 10% or 1% FBS. B) Differentiated H9c2 cells (in 1% FBS) were incubated with 100 and 150 µM ISO for 48 hours in media containing 10% or 1% FBS. Results showed no differences on cell death between 10% and 1% FBS-grown cells when the highest ISO concentration was used Data are expressed as mean±SEM of 5-7 different experiments. * $p < 0.05$ vs. control value (100%).

3.2.3.2 Isoproterenol-induced signaling pathway alterations

The effects of 50 and 100 µM ISO in some selected proteins of the different experimental groups was investigated by Western Blotting (Fig. 19). Cellular proteins were evaluated after 48 hours incubation with ISO. To investigate whether ISO causes apoptosis, the cleavage of poly (ADP-ribose) polymerase (PARP), an enzyme involved in programmed cell death, was measured. Fig. 19F shows that the band corresponding to the full length form of the enzyme (around 116 kDa) decreases with ISO treatment in differentiated cells (1% and 1%+RA) but not in undifferentiated cells (10%). The antibody used in the present study was not able to detect cleaved products of the enzyme. ISO treatment induced a significant increase in the pro-apoptotic protein Bax in both undifferentiated myoblasts (10% FBS) and RA-differentiated cells (Fig.

19B). Interestingly, treatment-related differences regarding Bax content were not statistically significant in cells differentiated in media containing 1% FBS, despite an apparent increase in the respective Western Blotting band after ISO treatment. What is clear, however, is that an increased amount of the anti-apoptotic protein Bcl-2 was only observed in undifferentiated myoblasts. For 100 μM ISO, a decrease in Bcl-2 content was measured for RA-differentiated cells, although the variation was not consistent for all the samples, and hence did not achieve statistical significance (Fig. 19A). The results for phosphorylated p38-MAPK were dissimilar between undifferentiated myoblasts and differentiated cells. Phosphorylated p38-MAPK concentration increased with ISO treatment in undifferentiated myoblasts and decreased in differentiated cells, although the difference was only statistically significant for RA-differentiated cells. The results were similar for calcineurin, although for this protein both populations of differentiated cells had a significant decrease in calcineurin content. Interestingly, the amount of cardiac troponin T was decreased in RA-differentiated cells exposed to 100 μM ISO.

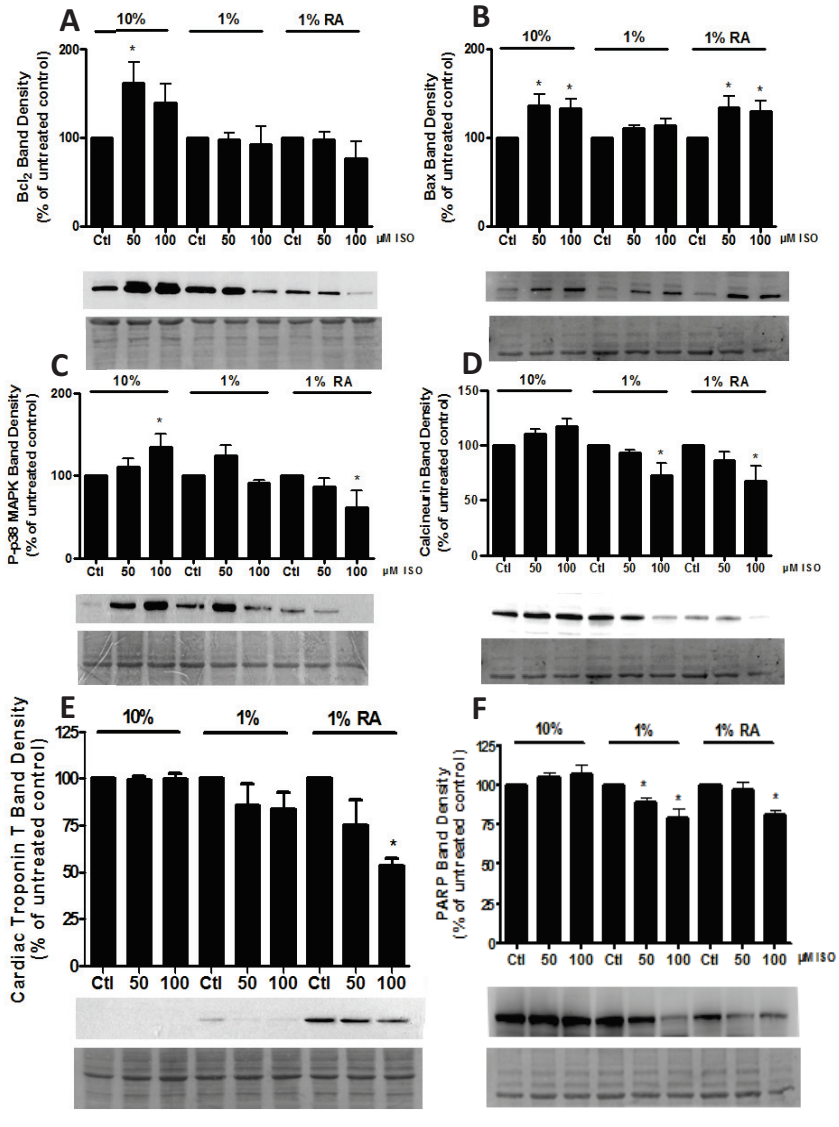


Figure 19 - Effects of 48 h ISO treatment on cell content in Bcl-2 (A), Bax (B), phosphorylated p38-MAPK (C), calcineurin (D), cardiac Troponin T (E) and full length PARP protein (F). Protein content in total cellular extracts were identified by Western Blotting analysis as bands with 26, 20, 43, 59, 35 and 116 kDa, respectively. Total cellular extracts from the three different groups of H9c2 cells were collected after treatment with 50 and 100 µM ISO during 48 hours, as described in methods section. Also shown is the respective Ponceau labeling for each Western Blotting as a loading control. The graphs represent densitometric analysis of bands, expressed as % of each differentiation control. Data are expressed as mean±SEM of four different

experiments for each protein. Statistical analysis: * $p < 0.05$ compared with respective control.

3.2.3.3 Calcium Overload in Differentiated H9c2 Cells after ISO Treatment

One of the most important mediators of ISO-induced cell cytotoxicity is the activation of calcium channels, leading to an overload of cytosolic calcium [199]. One possibility is that the cytosolic calcium levels is distinct in untreated undifferentiated vs. differentiated cells, which would create an already differential threshold for calcium-induced signaling. To verify this, we measured intracellular calcium by flow cytometry using the fluorescent probe Fluo-4 AM in the 3 experimental groups studied under basal conditions: undifferentiated H9c2 myoblasts, differentiated H9c2 cells in low-serum media (1%) and differentiated H9c2 cells in low-serum media daily supplemented with 1 μM of RA. We observed that the differentiation process of H9c2 myoblasts is accompanied by an increase in cytosolic calcium, with a statistically significant difference found for cells differentiated in the presence of RA (Fig. 20A, left), which was previously confirmed by our group as expressing cardiomyocyte-specific markers [191]. Since basal calcium levels were already different, the next experimental question was whether incubation with ISO also resulted in dissimilar cytosolic calcium accumulation. After incubation of the 3 cellular groups with two concentrations of ISO (50 and 100 μM) during 48 hours, we observed statistically significant higher amount of calcium in the two groups of differentiated H9c2 cells when incubated with the highest concentration of ISO. No differences regarding cytosolic calcium were found on non-differentiated cells incubated with ISO.

Since intracellular levels of cAMP and cellular calcium content are related in several ways, the basal levels of cAMP were also evaluated (Fig. 20B, left). A significant decrease of cAMP intracellular content on RA-differentiated H9c2 cells was found, when compared with the undifferentiated group (10% FBS). Interestingly, after incubation of 50 μM ISO for 48 hours, an increase of cAMP was observed in both differentiated cell groups (Fig. 20B, right).

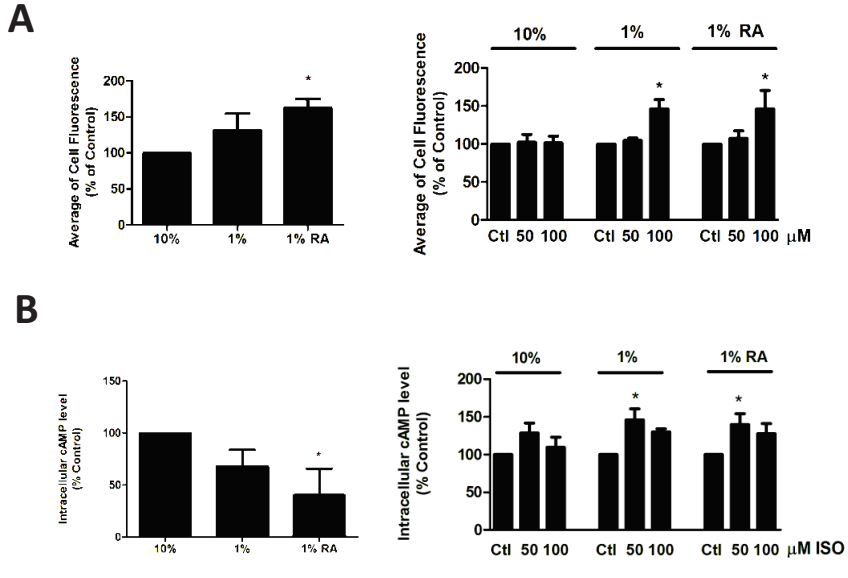


Figure 20 - Alteration of cytosolic calcium and cAMP after Isoproterenol incubation. Evaluation of intracellular calcium on undifferentiated and differentiated cells was performed by flow cytometry in basal (A, left panel) and after 48h of ISO incubation (A, right panel). Calcium was evaluated by using 2μM of Fluo-4-AM, as described in the Materials and Methods section. The data shows the means ± SEM, (*) significant difference vs. cells in 10% FBS (left) or to the respective control (right) ($p < 0.05$). (B) Evaluation of intracellular cAMP levels as described in Methods section. The data shows the means ± SEM, (*) significant difference vs. cells in 10% FBS (left) or to the respective control (right) ($p < 0.05$).

3.2.3.4 Isoproterenol toxicity involves loss of mitochondrial respiratory complexes, depolarizes mitochondria and increases mitochondrial superoxide anion in RA-differentiated cells

We have identified differentiation-specific protein content related with cell survival mechanisms including VDAC, Bax and Bcl-2 which control the apoptotic process at the mitochondrial level [198]. The next step was to determine if ISO toxicity also results in mitochondrial alterations in the different groups of cells, including loss of proteins involved in oxidative phosphorylation, mitochondrial depolarization and oxidative stress.

To investigate this, we initially used an antibody cocktail directed at several proteins involved in energy production including ATP synthase subunit alpha, NADH dehydrogenase (ubiquinone) 1 beta subcomplex subunit 8 (NDUFB8), a subunit from mitochondrial complex I and a 30 kDa subunit of mitochondrial complex II (Fig. 21). A significant decrease in complex II 30kDa subunit and in the nuclear-encoded NDUFB8 subunit in RA-differentiated H9c2 cells after treatment with ISO were found (Fig. 21B and 21C). Although no differences were found in undifferentiated cells or differentiated cells in absence of RA, it is noteworthy that a tendency for an increase in all three subunits was found in the former group, with the opposite being present in the latter, except for the ATP synthase subunit investigated (Fig. 21A).

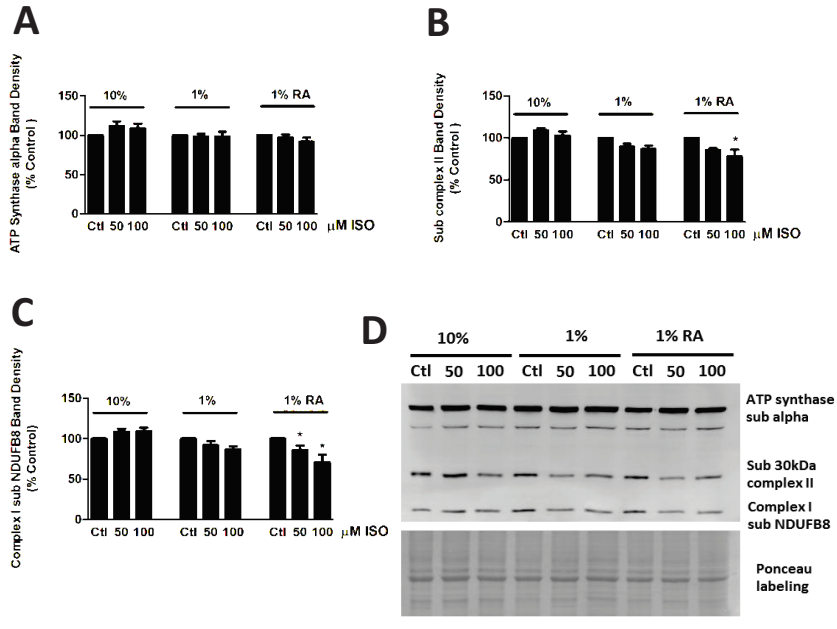


Figure 21 - Evaluation of mitochondrial alterations after ISO treatment. Mitochondrial oxidative phosphorylation components were identified by Western Blotting after undifferentiated and H9c2 differentiated cells (in presence and in absence of RA) treated with 50 and 100 µM of ISO. The antibodies used detected the proteins NDUFB8 (subunit of complex I), 30kDa subunit (complex II) and ATP synthase subunit alpha. Ponceau labeling was used as loading control. The results are representative of the means ± SEM of 4 separate assays. (*) Significant different to the respective control ($p < 0.05$).

We next investigated mitochondrial membrane potential by using the fluorescent probe TMRM under both basal conditions (no ISO treatment, Fig. 22A, left) as after ISO treatment (Fig. 22A, right). No statistically significant alterations in basal mitochondrial membrane potential were found between groups when cell TMRM fluorescence was measured by flow cytometry. When differentiated H9c2 cells were exposed to the lower concentration of ISO, increased TMRM fluorescence was measured, which may result from increased mitochondrial transmembrane electric potential when comparing to untreated cells.

When exposed to the higher ISO concentration, RA-differentiated cells suffered a large decrease of mitochondrial TMRM accumulation.

Mitochondrial superoxide anion production that escapes antioxidant scavenging was detected by using the fluorescent probe MitoSox. We found an increase in mitochondrial superoxide anion after cellular differentiation into a cardiac-like phenotype (Fig. 22B, left). Also, the RA-treated group was the only one where ISO treatment caused a further increase in MitoSox fluorescence, suggesting increased superoxide anion (Fig. 22B, right). The increase in cell fluorescence after ISO treatment found in the other two groups was not statistically significant. A positive control to investigate cell loading conditions was performed by using rotenone, which stimulates anion production.

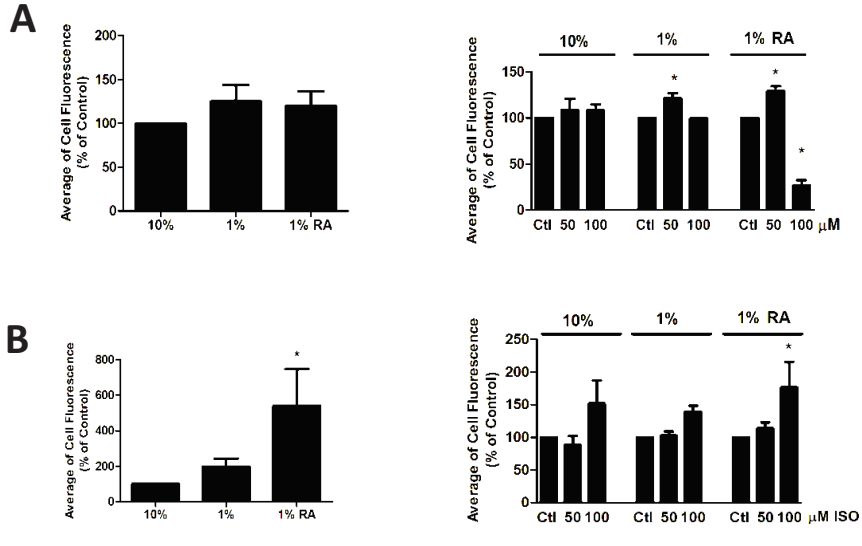


Figure 22 - Evaluation of mitochondria polarization and superoxide anion in H9c2 cells. Quantitative evaluation of TMRM (A) and MitoSox-labeling (B), as measured by flow cytometry. Undifferentiated and differentiated cells in the presence or absence of RA were treated with ISO for 48 hours, as described in Methods section. Data are means of 3 (for TMRM-labeling assay) or 5 (for MitoSox-labeling assay) independent experiments, plus SEM. (*) $p < 0.05$ vs. 10% FBS cells (left panels) or respective controls (right panels).

We also used the fluorescent probes 2',7'-dichlorofluorescein-diacetate (H_2DCFDA), a general marker of oxidative stress and dihydroethidium (DHE), a more specific marker of cellular superoxide anion production to investigate ISO-induced oxidative stress. The results indicate an increase in cell fluorescence for both probes when using the highest ISO concentration in the two differentiated cell groups (Fig. 23)

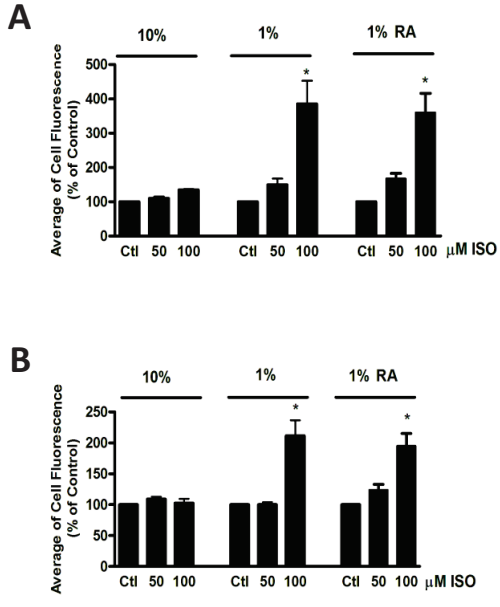


Figure 23 - Fluorescent analysis of intracellular oxidative stress. Undifferentiated and differentiated H9c2 cells incubated with 50 and 100 μM of ISO for 48 hours were stained with the probes 2',7'-dichlorofluorescein-diacetate (H_2DCFDA) (A), an indicator of oxidative stress and dihydroethidium (DHE) (B), an indicator of intracellular superoxide anion production. Cell fluorescence was determined by using a multiplate reader as described in Material and Methods section. The data shows means ± SEM, (*) significant difference to the respective control ($p< 0.05$).

3.2.3.5 Cell Differentiation Impacts Isoproterenol–induced Alterations in Survival/Death Signaling Pathways

Mitochondrial alterations, including increased macromolecular oxidative stress can trigger cell death [200] or induce protective pathways in order to rescue cells. We analyzed three proteins that have an important role on mitochondrial survival/death decisions, including SOD2, the mitochondrial manganese form of the antioxidant enzyme superoxide dismutase, cyclophilin F, a matrix chaperone involved in the mitochondrial permeability transition [201] and Bcl-xL, a Bcl-2 family protein, which inhibits mitochondrial-mediated cell death [202].

By using Western Blotting, we have previously demonstrated a higher content of both Bcl-xL and SOD2 in differentiated cells (Fig 11). We found also that cyclophilin F content is similar in all three differentiation groups (Fig. 11). Our present data shows that ISO (50 and 100 μM), when incubated with H9c2 cells in different stages of differentiation causes an up-regulation of all three proteins in the undifferentiated cell group, while the content in Bcl-xL decreases in the RA-differentiated group (Fig. 24 A-C).

The intracellular content of the multi-role transcription factor p53 was also investigated after incubation with ISO (Fig. 24D), since p53 is also involved in mitochondrial cell death signaling [203, 204]. Not unexpectedly, the highest ISO concentration used caused a statistically significant increase only in the differentiated cell groups. No basal alterations existed when comparing the three cell groups.

P66Shc is an adapter protein considered as a molecular sensor for cellular stress. Phosphorylation of p66Shc on serine 36 by protein kinase C increases during cellular stress and results in amplifying mitochondrial oxidative stress and apoptotic signaling [205]. With this in mind, we

investigated whether p66Shc phosphorylation on serine 36 increases in cells treated with ISO. The results (Fig. 24E) show that ISO increases the ratio between Ser36-phosphorylated p66Shc and the total p66Shc content in whole fractions of the two groups of differentiated cells, as opposed to undifferentiated myoblasts where a small, non-significant increase was observed. It is worth mentioning that the total amount of p66Shc was not altered during treatment.

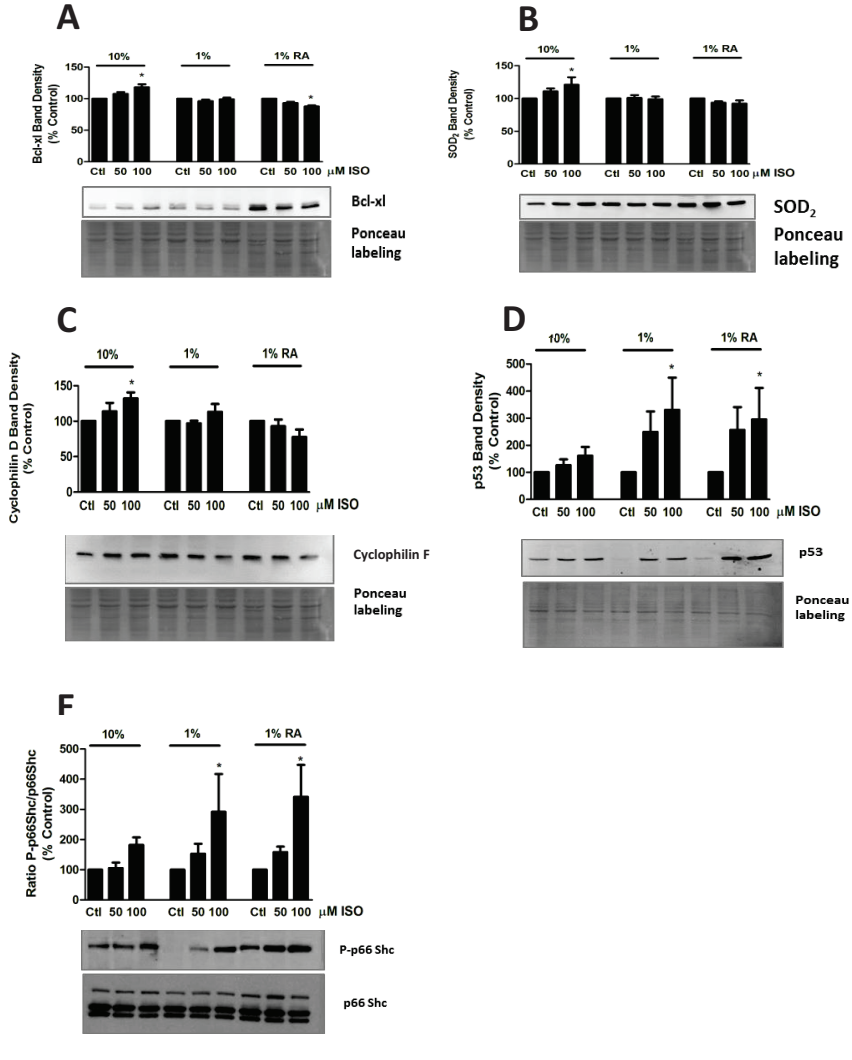


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Figure 24 - Isoproterenol induces stress responses in H9c2 cells. Bcl-xL (A), SOD2 (B), cyclophilin F (C), p53 (D) and total and phosphorylated-p66Shc (E) proteins content were measured by Western Blotting (see Materials and Methods) and identified as 30, 25, 22, 53 and 66 kDa bands, respectively. H9c2 cells in the different treatment groups were incubated with ISO for 48hours and total cellular extracts were collected. Ponceau labeling and non-phosphorylated p66Shc labeling represent the loading control and were used to normalize data. Data represent the mean±SEM of 5 independent experiments (*) $P<0.05$ versus respective control for the same differentiation group.

3.2.3.6 p53 Inhibition and Antioxidant Supplementation Have Different Effects on ISO Cytotoxicity on Undifferentiated vs. Differentiated H9c2 Cells

Since p53 is increased after ISO treatment in the two differentiated cell groups (Fig. 24D), the question was whether p53 inhibition by using pifithrin-alpha [206], prior to ISO treatment, prevented cell death. When pre-incubated with pifithrin-alpha, ISO toxicity was prevented in the two differentiated cell groups (Fig. 25A, middle and right panel). In order to confirm possible pifithrin-alpha effects on ISO incubation with undifferentiated cells, we extended ISO incubation to 72 hours in order to increase cytoxicity. This was due because in normal conditions with no inhibitor, no loss of cells was observed for shorter incubation time points of incubation. Interestingly, the addition of pifithrin-alpha (pre-incubated for 30 minutes, 30 μM) previous to ISO increased cell loss induced by the latter.

We have also investigated the role of oxidative stress on ISO-induced toxicity on H9c2 cells in different stages of differentiation. H9c2 cells in the undifferentiated state were incubated with ISO for 96 hours, while differentiated cells were incubated for 48 hours. N-acetylcysteine (NAC), a precursor of GSH [207] was pre-incubated for 2 hours (100 μM) with cells before ISO treatment. The anti-oxidant prevented the loss of cells on both groups of differentiated cells but not on undifferentiated

myoblasts, although this last group was incubated for longer periods (Fig. 25B).

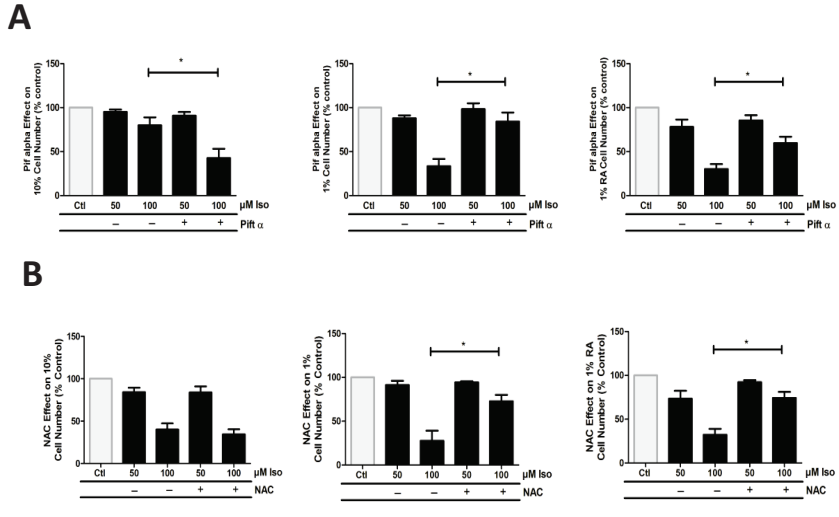
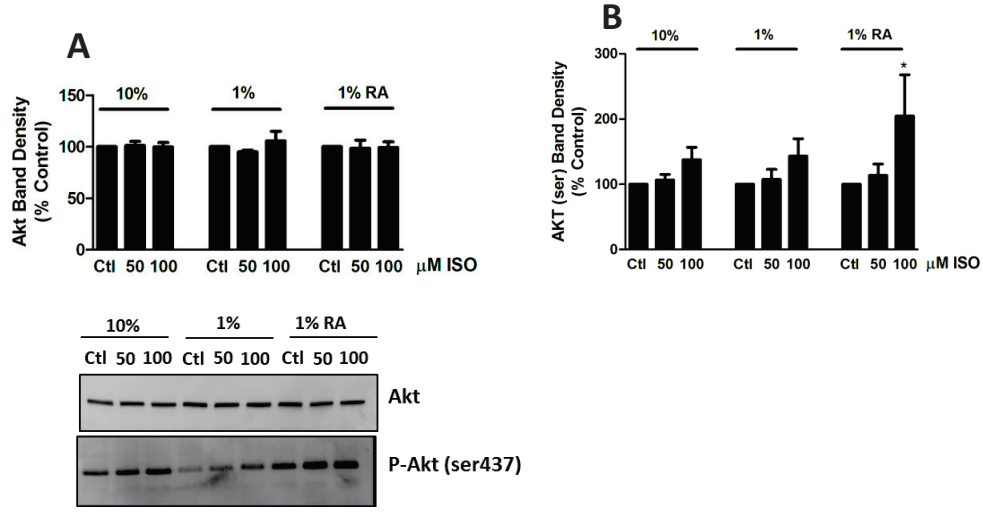


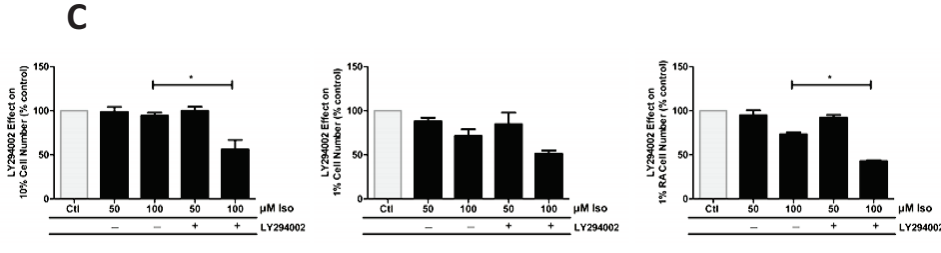
Figure 25 - p53 and oxidative stress involvement during ISO toxicity on H9c2 cells. The sulforhodamine B assay was performed in investigate if the p53 inhibitor pifithrin-alpha (A) or the antioxidant NAC (B) prevent H9c2 cell death caused by ISO. Undifferentiated myoblasts were incubated with ISO for 3 (A) or 4 (B) days, while differentiated cells (in presence or absence of RA) were incubated with ISO for 48 hours. Cells were pre-incubated for 30 minutes with 30 µM of pifithrin alpha or for 2 hours with 100 µM NAC. Data represent the mean ± SEM of 4 (for NAC) and 5 (for pifithrin-alpha) independent experiments (*) $P<0.05$ undifferentiated cells vs. the same concentration used on differentiated cells.

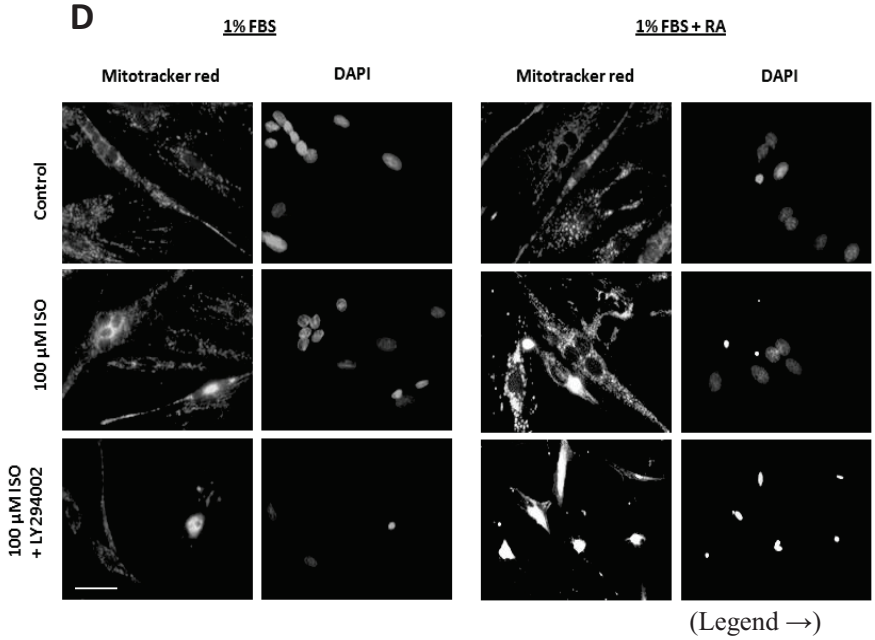
3.2.3.7 Inhibition of PI3K/Akt pathway increases ISO cytotoxicity in all differentiation and non-differentiation groups

To evaluate the significance of differential PI3K/Akt signaling pathway on ISO-induced toxicity in the three groups of H9c2 cells, intracellular Akt content and its phosphorylated forms were evaluated by Western Blotting. The results showed no differences on total Akt protein between

the three experimental groups (Fig. 26A). However, the serine 473-phosphorylated (active) form of Akt was found increased in RA-differentiated cells when incubated with 100μM of ISO. The role of PI3K/Akt-activated pathway on ISO-induced cell death was further confirmed by the cytotoxicity SRB assay. We incubated the 3 experimental groups with the specific inhibitor of the PI3K/Akt pathway, LY294002 [208] for 1 hour prior to ISO treatment. The inhibition of PI3K/Akt pathway by LY294002 on H9c2 cells increased the toxicity of ISO in all three groups (Fig. 26C and 26D) being evident for lower periods of incubation (48 hours for the undifferentiated group and 24 hours for the two differentiated groups)


A
Akt Band Density (% Control)
10%
1%
1% RA
Ctl 50 100 Ctl 50 100 Ctl 50 100 µM ISO
B
AKT (ser) Band Density (% Control)
10%
1%
1% RA
Ctl 50 100 Ctl 50 100 Ctl 50 100 µM ISO
Akt
P-Akt (ser437)


C
LY294002 Effect on 10% Cell Number (% control)
LY294002 Effect on 1% Cell Number (% control)
LY294002 Effect on 1% RA Cell Number (% control)
Ctl 50 100 50 100 µM Iso
– – + + LY294002


D
1% FBS
1% FBS + RA
Mitotracker red
DAPI
Mitotracker red
DAPI
Control
100 µM ISO
100 µM ISO + LY294002

(Legend →)

Figure 26 - PI3K/Akt pathway role on ISO-induced toxicity. (A) H9c2 undifferentiated and differentiated cells were incubated with ISO and samples were collected as described in Materials and Methods section. Total Akt and the phosphorylated form in serine 437 were identified by Western Blotting. Ponceau labeling was used as loading control. Data represent the mean±SEM of 4 independent experiments (*) $P<0.05$ versus respective control for the same differentiation state group. (C) Undifferentiated and differentiated H9c2 cells were pre-incubated with the PI3K/Akt pathway inhibitor LY294002 1 hour prior the addiction of 50 or 100μM of ISO. Differentiated cells number was analyzed 24 hours later and undifferentiated cells were analyzed 48 hours after exposure to ISO, by using the SRB assay method. Data represent the mean±SEM of 4 independent experiments (*) $P<0.05$ of undifferentiated cells vs. the same concentration used on differentiated cells (D) Microscopy images of differentiated H9c2 cells in the presence and in absence of RA incubated with ISO after pre-incubation with LY294002 by using the same protocol as in (C). Preparations were incubated with Mitotracker Red (to label mitochondria) and DAPI (to label nuclei) and images were obtained by confocal microscopy. White bar corresponds to 100μm.

3.2.3.8 Activation of Caspase 3 During ISO-induced H9c2 Cell Death.

The above results showed that toxic concentrations of ISO induce poly(ADP-ribose) polymerase (PARP) cleavage which is a specific marker of cell death (Fig. 19F). Since PARP is a target for caspase 3 [209], we confirmed the involvement of this caspase by evaluating the content of the active form (Fig. 27A) and activity (Fig. 27B). For the time point measured (48 hours of ISO treatment), a statistically significant increase in caspase 3 active fragment was detected in cells differentiated with serum reduction only. No differences were found in the two other groups (Fig. 27A). In accordance, caspase-3-like activity was significantly increased in the group differentiated with 1% serum alone, although a non-significant increase was also observed in the RA-differentiated group. Finally, we used the pan-caspase inhibitor z-vad-fmk to inhibit the loss of cells caused by ISO. In this case, only the two differentiated groups were used since previous results showed alterations

on caspase-3 active form only in these cells. With surprise, z-vad-fmk was not able to inhibit ISO-induced loss of cells (Fig. 27C).

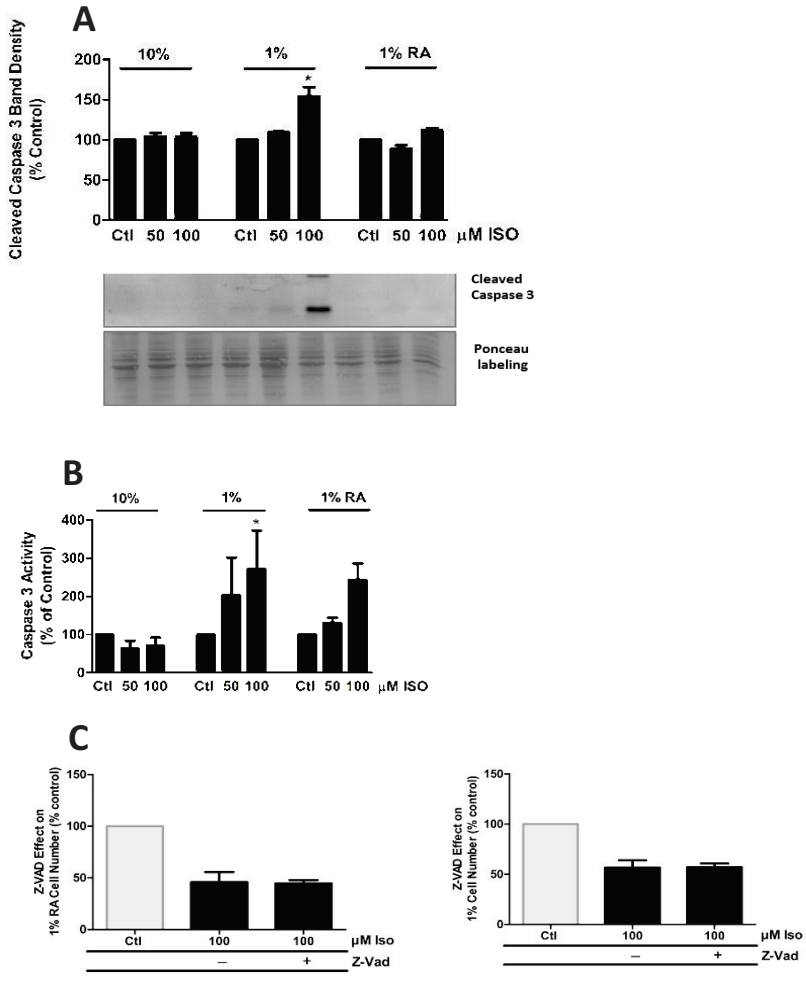


Figure 27 - Caspase 3-like activity in H9c2 cells exposed to ISO and effect of caspase inhibition. (A) The full length caspase 3 and caspase 3-resulting fragments were determining by Western Blotting analysis and identified as bands with 35 and 19/17 kDa, respectively. Ponceau labeling was used as loading control and to normalize the results of cleaved-caspase 3. Data represent the mean±SEM of 3 independent experiments (*) $p<0.05$ versus respective control for the same differentiation state group. (B) By using a colorimetric assay, undifferentiated and differentiated H9c2 cells exposed to ISO were analyzed in order to measure caspase 3-like activity. Data represent the mean ± SEM of 4 independent experiments (*) $p<0.05$ versus respective control for the same differentiation state group. (C) Differentiated H9c2 cells

without RA (left panel) or with RA (right panel), were pre-incubated with the pan-caspase inhibitor z-vad-fmk 1 hour prior to the treatment with 100μM of ISO and cell mass was measured by using the SRB method. The results were compared with a preparation in which no inhibitor/ISO was added. Data represent the mean±SEM of 3 independent experiments (*) $P<0.05$ vs. control.

3.2.4 Discussion

Several *in vitro* and *in vivo* models [163] showed that ISO-induced β-adrenergic overload leads to cardiac cell dysfunction being a good example of chemically-induced heart toxicity and failure. The use of the H9c2 cell model in different differentiation stages also allows elucidating cell signaling mechanisms that underlie possible altered susceptibility of the developing heart to β-adrenergic agonists.

In this work, it is demonstrated that differentiated H9c2 cells are more susceptible to the toxic effects of supra-physiological concentrations of the β-adrenergic agonist ISO than non-differentiated myoblasts (Fig. 15). By using 100 and 150 μM of ISO, a significant loss of differentiated cells (with or without RA) occurred after ISO treatment while undifferentiated myoblasts were not affected. An interesting study in neonatal and adult rat cardiomyocytes demonstrated that stimulation with 2 μM of NE leads to hypertrophic responses while a higher dose (100 μM) induces an apoptotic response [210].

Isoproterenol-induced selective toxicity on differentiated cells was further confirmed by multiple end-points including increased apoptotic chromatin condensation (Fig. 15E) and by mitochondrial membrane depolarization (Fig. 16). Cardiac troponin T (TnT) has been reported as a specific biomarker for cardiac tissue, being used to detect myocardial injury in a variety of experimental settings [211]. *In vivo*, degradation of the contractile structure during cardiac pathologies also involves degradation of cardiac troponin T, which allows it to be one possible

marker of cardiac damage in patients with chronic heart failure [212]. Our results (Fig. 19E) showed that exposure of RA-differentiated H9c2 cells to 100μM of ISO leads to a decrease in the amount of cardiac troponin T. Along with a loss of troponin T due to cell death, the result may also be interpreted as removal of cardiac-differentiated cells due to ISO toxicity, as differentiation results in a heterogeneous sample.

One important feature on β-adrenergic activation is the consequent calcium influx mediated by cAMP/PKA activation [213]. Calcium is the main regulatory component of the contractile system, which explains the development of calcium-sensitive signaling mechanisms during differentiation to cardiomyocytes [214]. Nevertheless, under pathological conditions, cytosolic calcium overload can also have deleterious effects in the myocardium including the induction of the mPTP [215-217] and consequent pro-apoptotic release of cytochrome c. Our results show that not only differentiated cells have a higher basal intracellular calcium content (significant results for RA-differentiated cells only, Fig. 20A), as both differentiated groups had higher cytosolic calcium content when measured 48 hours after ISO treatment. The increased basal calcium, which is associated with cell differentiation, has been previously described in several models including mouse embryonic stem cells (ES) [218], human induced pluripotent stem cells (hiPSCs) [219] and rat neonatal cardiomyocytes [220]. Despite the fact that an increase in cytosolic calcium was observed 48 hours after ISO treatment in the differentiated cell populations, we cannot distinguish between ISO/ β-adrenergic stimulation of calcium uptake which is upstream of several signaling/stress pathways or a downstream consequence of cellular disruption processes, including cell death. Further experiments must be performed with shorter ISO incubation times. Nevertheless, it is known that β-receptor activation can modulate the activity of L-type

calcium channels, the activity of ryanodine receptors (RyR) (the major sarcoplasmic reticulum calcium release channels in cardiac and skeletal muscles) and the activity of the sarcoplasmic reticulum (SR) Calcium-ATPase (SERCA2a), contributing to transient levels of calcium inside the cell [187, 213, 221]. A decrease in calcium reuptake by the SR is a central feature in several cardiovascular diseases [222]. Some studies report that membrane preparations from failing myocardium demonstrate a significantly lower SR ATPase activity when compared with nonfailing myocardium. Consequently, the SR calcium uptake rate is considerably reduced. In the specific case of the cell line here used, Menard *et al.* demonstrated that during the differentiation process, H9c2 cells increase the expression of cardiac/α_{1C} L-type voltage-dependent calcium channels (LTCCs) which gives specific conductance to cardiac calcium channels [149] and is regulated by PKA. More expressed/active calcium channels in differentiated H9c2 cells may act as a factor of susceptibility to agents that further increase cytosolic calcium and could inclusively be an important component of the different ISO toxicity observed in the experimental groups. Through adenylate cyclase (AC) signaling, β-receptors can increase intracellular levels of cAMP and cAMP-dependent kinases, such as PKA, which then activates L-type calcium channels and overphosphorylates RyR channels during hyperadrenergic states, increasing calcium entry into the cytosol, leading to cellular inotropy [223]. In this work, we have also measured increased levels of cAMP in H9c2 differentiated cells after ISO treatment (Fig. 20B), suggesting more active cAMP/PKA pathway which can explain the observed increase on intracellular calcium levels. According with Pagano *et al.*, H9c2 myoblasts, when differentiated during 6 days, gradually decrease both cAMP and AC activity, as confirmed by our results. Concomitantly, an artificial increase on cAMP levels inhibits muscle differentiation as well as morphologic alterations and increased

myogenin expression [224]. This phenomenon is still controversial but some studies associate this with an increase of PKA activity (stimulated by cAMP) which modulates MyoD effects on the transcription of myogenin and other differentiation specific proteins [224] preventing myoblast differentiation. [225]. Since mitochondria act as a calcium buffer in cardiac cells sequestering rapidly that cation during cytosolic calcium transients, cAMP/PKA hyperactivation can have deleterious effects on its function. [226]. Under normal conditions, calcium stimulates [227] mitochondrial metabolism leading to enhanced energy production [227]. However, calcium overload in mitochondria leads to organelle dysfunction, including formation of the mPTP and cell death [228]. It is likely that increased mitochondrial superoxide anion generation (Fig. 22B), decreased mitochondrial membrane potential (Fig. 16 and 22A) and decreased content in two oxidative phosphorylation-related proteins (Fig. 21) can be associated with excessive calcium accumulation after ISO treatment or be a consequence of the disruption of different intra-cellular structures by oxidative stress, which we also confirmed in the entire cell by using two fluorescent probes (Fig. 23). It is noteworthy that most of these alterations were found in the RA-differentiated group only, confirming that cell populations which tend to have a cardiac-like phenotype are more susceptible to ISO-induced mitochondrial alterations. Two other results are noteworthy: a) the intermediate concentration of ISO (50 μM) results in what appears to be mitochondrial hyperpolarization (higher TMRM accumulation) in the two differentiated groups and b) higher mitochondrial superoxide anion was detected in RA-differentiated cells under basal conditions. About the former result, although we do not have evidence to support this, the presumptive hyperpolarization can be related with mitochondrial alterations that result in a temporary increase in mitochondrial membrane potential during the initial stages of cell death. As for the

higher detection of basal mitochondrial superoxide anion, the result is in agreement with studies documenting the requirement of redox signaling alterations on the differentiation of myoblasts to a more mature state [229]. In fact, mitochondria function and generation of ROS play a fundamental role on the establishment of metabolic mechanisms that drive undifferentiated myoblasts to a cardiac-specific energetic requirement during cardiomyogenesis [153, 230]. In what undifferentiated H9c2 cells are concerned, they appear to be morphologically healthy after ISO exposure, even for the higher concentration of ISO used, although we identified what appeared to be fragmentation of the mitochondrial network (Fig. 17). Although it is unclear at the moment what may have caused an apparent fragmentation of an otherwise filamentous mitochondrial network, it is noteworthy consider two possible mechanisms: a) increased oxidative stress that causes mitochondrial fission or b) formation of Bax pores in the mitochondrial outer membrane. Although statistically non-significant, we found a small increase in mitochondrial superoxide anion content when the highest concentration of ISO was used in undifferentiated cells (Fig. 22B). Despite the important role of oxidative stress on differentiated H9c2 cells confirmed by the protection afforded by NAC, a GSH up-regulating agent, the same agent was unable to prevent ISO toxicity for longer periods on undifferentiated cells. Because a primary increase in global oxidative stress can potentiate the formation of more ROS in multiple cell sites, and because the oxidative stress markers we measured only occurred for the highest concentration of ISO used in differentiated cells (Fig. 22 and 23), we cannot truly answer if the result obtained is due to a primary event regarding a cellular response to an hyperadrenergic state caused by ISO or as a consequence of cell death signaling, which is already occurring for this late time point and ISO concentrations.

Another possible explanation for mitochondrial fragmentation involves the pro-apoptotic protein Bax. An increase in Bax is reported to be related with mitochondrial fragmentation [198] however, simultaneous increase in Bcl-2 when undifferentiated cells were exposed to 50μM of ISO suggests that this anti-apoptotic protein acts to prevent ISO-induced cell death. Regarding differentiated cells, ISO also up-regulates Bax similarly, however, Bcl-2 and Bcl-xL, which we hypothesize as both having an important role protecting cells from ISO-induced death, are not elevated in differentiated cells upon ISO treatment (Fig., 19A, 19B and 24A). Since an increase in Bax content was not accompanied with a concomitant increase in Bcl-2 or Bcl-xL it suggests that the ratio between pro- and anti-apoptotic proteins may play an important role on mitochondrial membrane physiology. The increase in the transcription factor p53 (Fig. 24D) may suggest that a nuclear stressor stimulus can be triggering the up-regulation of Bax. In this case, anti-apoptotic proteins such as Bcl-2 or Bcl-xL may alter the balance between anti- and pro-apoptotic proteins and determine cell survival or death [231]. Confirming the role of p53 protein, pifithrin-alpha, a p53 inhibitor [206] was able to decrease ISO toxicity on differentiated cells, although increased this same toxicity on undifferentiated cells, when those were incubated for longer periods (Fig. 25A). It is interesting to note that p53 can act as a pro-survival or pro-death signaling agent [204].

In addition to be more resistant to cell death induced from hyper-adrenergic induction by ISO, undifferentiated cells do not show other signs of toxicity including ROS production, calcium uptake or depolarization of mitochondrial membranes and still show increased MnSOD upon ISO treatment, showing enhanced cell and mitochondrial protection. Despite increased mitochondrial cyclophylin D content is often associated with increased induction of the mPTP [232], the fact

that this phenomenon only occurred in undifferentiated cells suggests a toxicity response which, together with others already described, may have contributed to protect cells (Fig. 24C).

When incubated with ISO, both differentiated H9c2 groups show a decrease in calcineurin while undifferentiated myoblasts show an increase (Fig. 19D). Although calcineurin has been reported to be implicated in a wide range of cellular processes such as cell-cycle regulation, stress response and apoptosis [233], how calcineurin contributes to the decision of cellular protection or commitment to cell death is still largely unknown and debatable [234]. Nevertheless, it has been reported that transgenic mice expressing a constitutively active mutant of calcineurin show increased resistance to ischemia-reperfusion-induced apoptosis [235]. These results suggest that calcineurin signaling imparts a degree of protection against cell death in the heart, which is consistent with the results in the present work, in which differentiated H9c2 cells that are more susceptible to ISO present at the same time decreased levels of calcineurin. Interestingly, it has been shown that β-adrenoceptor stimulation produces dephosphorylation of p38-MAPK in embryonic chick hearts [236], which is in agreement with the decrease in phosphorylated p38-MAPK after ISO treatment in RA-differentiated H9c2 cells (Fig. 19C). Further work is still needed to understand the link between the decreased amount in p38-MAPK and ISO toxicity.

Another stress marker was found to be only increased in differentiated cells after ISO treatment: p66Shc. Isoproterenol treatment resulted in increased activation of the Ser36-phosphorylated form of p66Shc (Fig. 24E), a protein that regulates oxidative stress responses and apoptosis [237], but only in differentiated cells. Activation of this signaling pathway has been described for several toxicants [238] and is considered a good end-point for intracellular stress responses. The pathway was not

activated in undifferentiated cells, demonstrating once more the higher intrinsic signaling protection or that an upstream activating pathway (e.g., calcium overload) was not activated.

Facing the role of PI3K/Akt signaling pathways on cell survival [239], we confirmed that inhibition of this pathway by a specific inhibitor leads to increased cytotoxicity, including on undifferentiated cells (Fig. 26). This pathway has clinical interest as it has also the capacity to inhibit pro-apoptotic Bcl-2 family members such as Bax and Bad, with several studies reporting a functional cooperation between both proteins. PI3K inhibition on differentiated cells has deleterious effects, suggesting the importance of the activation of this pathway on cell survival. However, in the other hand, differentiated cells do not have increased levels of anti-apoptotic proteins from the Bcl-2 family such as Bcl-xL and Bcl-2 which can compromise the cooperative action between both PI3K/Akt and Bcl-xL/Bcl-2 pathways.

Finally, we measured increased caspase 3 activation in differentiated cells after ISO treatment (Fig. 27), which demonstrates that apoptotic pathways are activated. Furthermore, its substrate, PARP, showed a decreased content of its full length form (Fig. 19F). Nevertheless, a pan-caspase inhibitor had no effect on ISO toxicity, suggesting that loss of cells after ISO treatment may occur through another pathway that subsists even after inhibition of caspases. Cellular death is a redundant event that includes several independent signaling pathways, which can occur simultaneously [240]. One possibility to explain the fact that z-vad-fmk did not protect from death in differentiated H9c2 cells is that mitochondrial disruption may result into the release of pro-apoptotic caspase-independent factors including the AIF. It was reported that staurosporin-induced H9c2 death, although involving caspase activation, was not inhibited by z-vad-fmk [241]. In another study, Youn *et al*.

showed that doxorubicin-induced toxicity on H9c2 cells was not prevented by pretreatment with the caspase inhibitor z-vad-fmk, in contrast with cell death induced by H_2O_2 treatment [242].

It is noteworthy that one possibility for the results would be binding of ISO to serum, which explain why cells in 10% FBS are less susceptible to ISO. Data in Fig. 18 suggest that differential ISO serum binding does not explain the results obtained, although the data may suggest that a small effect due to ISO bonding to serum may exist. Nevertheless, one has to have caution when analyzing this data and possible variations. The 48 hours period in which ISO was incubated with cells, may be enough for undifferentiated cells to start differentiating in 1% FBS media, or to differentiated cells (in 1% FBS) to re-start proliferating when exposed to a higher FBS concentration. In fact, data present in Fig. 9B show that 1% FBS-differentiated H9c2 cells re-started proliferating earlier after being placed in culture media with 10% FBS, showing biochemical alterations that may alter the susceptibility to ISO.

In conclusion, the present research describes a differential susceptibility of H9c2 myoblasts in different differentiation states to ISO toxicity and confirms that hyperadrenergic states have different outcomes on undifferentiated and differentiated muscle cells. Our findings show that the maturity of H9c2 cardiac cells is critical on their responses to β-adrenergic agonists. Identification of the molecular mechanisms that mediate stress/survival/death responses in muscle cells in different developmental stages is critical to understand how cardiac resistance to β-adrenergic stimulation varies from the womb to adulthood. In fact, several pieces of evidence demonstrate *in vivo* distinct responses to catecholamine/ISO in rats of different ages or even during heart development *in utero* [126, 127].

Conclusions

4. Conclusions

Sympathetic activation has not only an important role on the compensatory regulation of the contractility activity but is also deleterious when chronically activated since it contributes to the progressive deterioration of the myocardial structure and function. It is well established that apoptosis of cardiomyocytes contributes to a complex myocardium remodeling process [243]. Stress-activated signaling pathways are mediated by catecholamine-induced activation of G-protein-coupled receptors [244]. Manipulation of adrenergic signaling using transgenic mouse models and β-blockers has also been contributing to additional knowledge on its role in cardiac remodeling and apoptosis [117]. The suppression of cell death pathways to control myocyte loss represents an ideal strategy to prevent many heart diseases. In this context, we proposed to study the involvement of β-AR signaling on cardiac cell death during different stages of the differentiation process in H9c2 cells.

One major objective in this thesis regards the potential use of H9c2 cells as a surrogate for cardiac cells during toxicological studies (which becomes more important as the use of animals in experimental research is being limited) due to its ability to differentiate into cardiac-like cells with molecular and morphological features of a cardiomyocyte. Further characterization on the transcriptome of H9c2 cell upon differentiation process showed that expression of genes related with proliferative activity were tendentiously repressed on differentiated cells, and with RA-differentiated cells present increased expression of genes related with the differentiation process such as the oxidative stress growth inhibitor 1, the cyclin-dependent kinase inhibitor 2B, the FasL, calcium transporters (such as the sarcoplasmic reticulum calcium ATPase), cardiac sarcomeric proteins and genes with related mitochondrial energy

production such as mitochondrial creatine kinase, oxidative phosphorylation subunits, as well as uncoupling proteins. We also performed genotyping studies with isolated cardiomyocytes and with HL-1 cell line in which we found neonatal cardiomyocytes upregulation on yearly development markers such as the Gata6 gene.

The results from this thesis suggest that myoblast differentiation, for example during fetal development, results into increased toxicity to β-adrenergic agonists. Our work confirms that embryonic tissue appear to be more resistant to ISO than adult cardiac cells. Some hypotheses have been forwarded to explain the differences: one regards the intrinsic cellular defenses and, second may involve the capacity of each cell group to efficiently respond to a deleterious stimulus. In this context, basal content on some anti-apoptotic proteins can represent an advantage. Also, the fact that undifferentiated cells are metabolic more active [191], with possibly autophagic processes being constitutively active than in differentiated cells, may also contribute to a faster and more positive response to an insult.

Based on the fact that ISO impacts and triggers different mechanisms in undifferentiated and differentiated H9c2 cells we performed studies on the β-AR signaling elements to detect downstream events that may explain the base of such difference. In addition to supplying cellular energy, mitochondria are also related with calcium homeostasis, oxidative stress and apoptotic signaling. In this context, we found mitochondria alterations that can further explain protection mechanisms increased in undifferentiated cells. We confirmed that the mitochondrial phosphorylative system appears to be less affected by ISO treatment in undifferentiated cell, although we have identified early alterations of mitochondrial network. On the other hand, calcium uptake, ROS production, loss of the mitochondrial membrane potential, commitment

of mitochondrial complexes, decreased superoxide dismutase, decreased Bcl-2 and Bcl-xL increased Bax, increased cAMP, release of troponin T (a standard marker for cardiac injury), increased p53 protein and p66Shc, activation of caspase-3 and cleavage of PARP were some of the molecular alterations found in differentiated cells in the presence of ISO (Fig. 28 and 29).

Although many others stress/survival pathways could have been investigated, it appears clear that cells in an early stage of differentiation have more intrinsic defenses, which may have evolved from the need to protect the developing structure *in utero* from intrinsic and extrinsic aggressions. It is also interesting to postulate that mitochondrial maturation during development and remodeling towards a more oxidative metabolism impacts how toxicants affect cellular viability. Modulation of oxidative stress and calcium overload by intrinsic defenses during xenobiotic toxicity is apparently also a life or death decision to the cell.

The results obtained in this thesis are relevant from a toxicological point of view, especially when investigating developmental cardiovascular toxicology.

4.1 Final Remarks:

Based on the results presented in this study, we propose to highlight the following main conclusions (see Fig. 28, 29 for a summary of results):

- The differentiation of H9c2 cells led to a transcriptional up-regulation of genes involved in cardiac differentiation, diminished cellular proliferative rate and induced the formation of multinucleated cells, which demonstrates the validity of using this cell system.

- Differentiated H9c2 cells exposed to ISO present increased calcium uptake and cAMP levels, oxidative stress, depolarization of the mitochondrial membrane potential, deterioration or loss of mitochondrial complexes, decreased Bcl-2 and Bcl-xL, increased Bax, released of troponin T, increased p53 protein and p66Shc, activation of caspase-3 and PARP cleavage.
- Undifferentiated H9c2 cells exposed to ISO present mitochondrial network fragmentation, increased Bcl-2 and Bcl-xL proteins which counteract increased Bax content, as well as increased superoxide dismutase 2 enzyme.

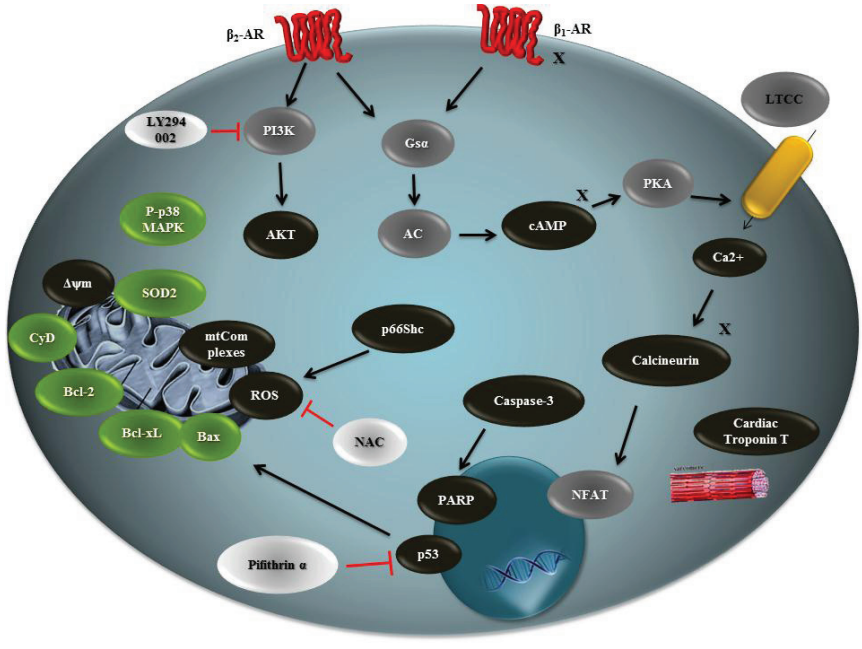


Figure 28 - Schematic representation of β-AR downstream pathways altered by ISO in undifferentiated H9c2 cells. Grey represents non-studied proteins although of importance for the signaling pathway; Green represents up-regulated proteins upon stimulation of β-AR by ISO; Red represents down-regulated proteins upon stimulation of β-AR by ISO; Brown represents protein levels that had no alterations upon stimulation of β-AR by ISO. X represents proteins that are over-expressed after the differentiation process. Abbreviations: ROS (reactive oxygen species), PI3-K (phosphoinositide 3-kinase), Gi (guanine nucleotide-binding protein inhibitory subunit), Gs (guanine nucleotide-binding protein stimulatory subunit), AC (adenylate cyclase), cAMP (cyclic adenosine monophosphate), Ca^{2+} (calcium), PKA (protein kinase A), p38 (p38 mitogen-activated protein kinases), Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), Bax (Bcl-2-associated X protein), SOD2 (superoxide dismutase 2), mΨ (mitochondrial membrane potential), mtComplexes (mitochondrial complexes subunits), cyD (cyclophilin F), PARP (cleaved from of poly(ADP-ribose) polymerase-1), LTCC (L-type calcium channel).

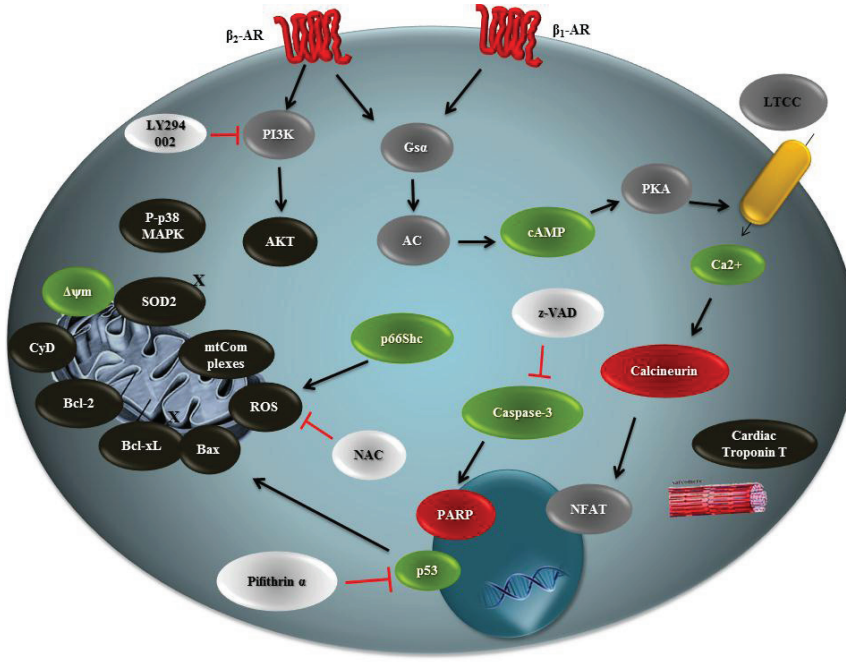


Figure 29 - Schematic representation of β-AR downstream pathways altered by ISO in differentiated H9c2 cells by reducing the concentration of serum in the medium. Grey represents non-studied proteins although of importance for the signaling pathway; Green represents up-regulated proteins upon stimulation of β-AR by ISO; Red represents down-regulated proteins upon stimulation of β-AR by ISO; Brown represents protein levels that had no alterations upon stimulation of β-AR by ISO. X represents proteins that are over-expressed after the differentiation process. Abbreviations: ROS (reactive oxygen species), PI3-K (phosphoinositide 3-kinase), Gi (guanine nucleotide-binding protein inhibitory subunit), Gs (guanine nucleotide-binding protein stimulatory subunit), AC (adenylate cyclase), cAMP (cyclic adenosine monophosphate), Ca^{2+} (calcium), PKA (protein kinase A), p38 (p38 mitogen-activated protein kinases), Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), Bax (Bcl-2-associated X protein), SOD2 (superoxide dismutase 2), mΨ (mitochondrial membrane potential), mtComplexes (mitochondrial complexes subunits), cyD (cyclophilin F), PARP (cleaved from of poly(ADP-ribose) polymerase-1), LTCC (L-type calcium channel).

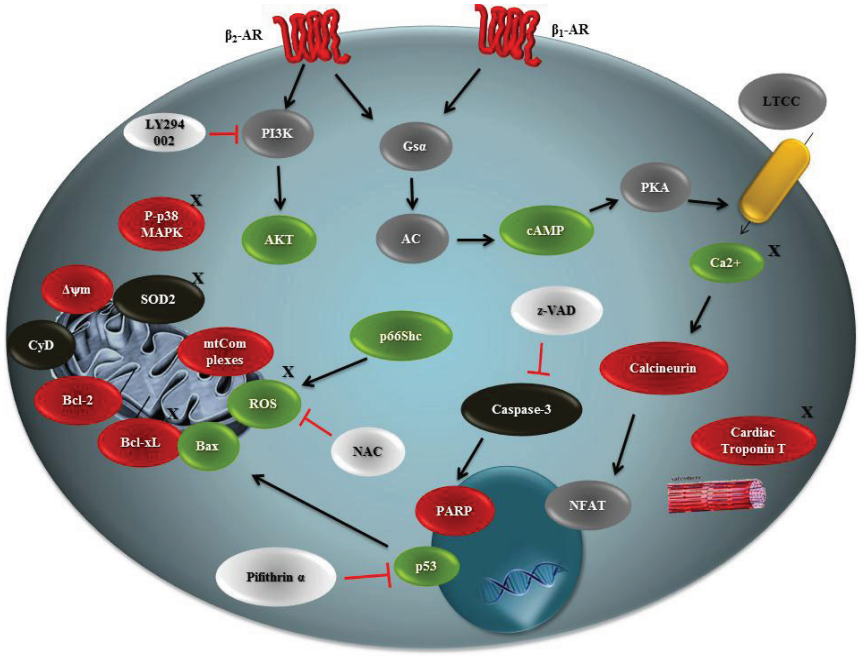


Figure 30 - Schematic representation of β-AR downstream pathways altered by ISO in RA-differentiated H9c2 cells. Grey represents non-studied proteins although of importance for the signaling pathway; Green represents up-regulated proteins upon stimulation of β-AR by ISO; Red represents down-regulated proteins upon stimulation of β-AR by ISO; Brown represents protein levels that had no alterations upon stimulation of β-AR by ISO. X represents proteins that are over-expressed after the differentiation process. Abbreviations: ROS (reactive oxygen species), PI3-K (phosphoinositide 3-kinase), Gi (guanine nucleotide-binding protein inhibitory subunit), Gs (guanine nucleotide-binding protein stimulatory subunit), AC (adenylate cyclase), cAMP (cyclic adenosine monophosphate), Ca^{2+} (calcium), PKA (protein kinase A), p38 (p38 mitogen-activated protein kinases), Bcl-2 (B-cell lymphoma 2), Bcl-xL (B-cell lymphoma-extra-large), Bax (Bcl-2-associated X protein), SOD2 (superoxide dismutase 2), mΨ (mitochondrial membrane potential), mtComplexes (mitochondrial complexes subunits), cyD (cyclophilin F), PARP (cleaved from of poly(ADP-ribose) polymerase-1), LTCC (L-type calcium channel).

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5. Bibliography

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