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Metabolic changes underlying caloric restriction and diabetes impact upon intercellular communication activity in the heart

Dissertação para a obtenção do grau de Mestre em Investigação Biomédica
sob orientação científica do Doutor Henrique Girão e co-orientação da Doutora
Maria João Pinho apresentada à Faculdade de Medicina da Universidade de Coimbra.

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METABOLIC CHANGES UNDERLYING CALORIC RESTRICTION AND DIABETES IMPACT UPON INTERCELLULAR COMMUNICATION ACTIVITY IN THE HEART

Dissertação de Mestrado em Investigação Biomédica, na especialidade de Oncobiologia, apresentada à Faculdade de Medicina da Universidade de Coimbra para obtenção do grau de Mestre.

Orientadores: Doutor Henrique Girão e Doutora Maria João Pinho

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Abbreviations

AGEs	Advanced glycation end products
AP-2	Adaptor protein 2
ATG	Autophagy-related gene
Baf	Bafilomycin A1
BSA	Bovine serum albumin
CIP75	Connexin43-interacting protein of 75 kDa
CMA	Chaperone-mediated autophagy
CR	Caloric restriction
CVD	Cardiovascular disease
Cx	Connexin
DAB2	Disabled 2
DMEM	Dulbecco's Modified Eagle Medium
DUB	Deubiquitinating enzyme
Eps15	Epidermal growth factor receptor substrate 15
ER	Endoplasmic reticulum
ERAD	Endoplasmic reticulum-associated degradation
ESCRT	Endosomal sorting complexes required for transport
EV	Extracellular vesicle
E1	Ubiquitin-activating enzyme
E2	Ubiquitin-conjugating enzyme
E3	Ubiquitin-protein ligase
FBS	Fetal bovine serum
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GJ	Gap junction
GJIC	Gap junction-mediated intercellular communication
HIF-1α	Hypoxia-inducible factor 1-alpha
HRP	Horseradish peroxidase
Hsc70	Heat shock cognate proteins of 70 kDa
H9c2	Rat cardiomyoblast cell line
HEK-293	Human Embryonic Kidney 293 cell line

IC	Intercellular communication
ILV	Intraluminal vesicle
IP	Immunoprecipitation
LAMP-2A	Lysosomal-associated membrane protein-2A
LC3	Microtubule-associated protein 1 light chain 3
LIR	LC3-Interacting Region
MGO	Methylglyoxal
mHypoE-N42	Embryonic mouse hypothalamus N42 cell line
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide
MV	Microvesicle
MVB	Multivesicular body
Nedd4	Neural precursor-cell-expressed developmentally down regulated 4
NEM	N-ethylmaleimide
PBS	Phosphate buffered saline
PE	Phosphatidylethanolamine
PFA	Paraformaldehyde
PI3KC3	Class III phosphatidylinositol 3-kinase
PM	Plasma membrane
PMSF	Phenylmethanesulfonyl fluoride
PQC	Protein quality control
PTM	Post-translational modifications
p62/SQSTM1	Sequestosome 1
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SENP	SUMO-specific protease
SUMO	Small ubiquitin-like modifier
TBS-T	Tris-buffered saline-Tween 20
TSG101	Tumor susceptibility gene 101
TEM	Transmission electron microscopy
Ub	Ubiquitin
Ubl	Ubiquitin-like protein
UPS	Ubiquitin-proteasome system
WB	Western blot
ZO-1	Zona occludin protein 1

Resumo

O coração depende de uma rede de comunicação intercelular bem organizada para assegurar a sua correta função. A comunicação intercelular pode ocorrer diretamente, entre células adjacentes, através das *gap junctions*, ou indiretamente, a longas distâncias através de vesículas extracelulares, nomeadamente através de exossomas. A conexina 43 (Cx43) é a principal proteína ventricular encontrada no coração e alterações na sua dinâmica têm um impacto direto na comunicação intercelular cardíaca e consequentemente na função do coração. Resultados anteriores do nosso grupo mostraram que curtos períodos de *starvation* estabilizam a Cx43 na membrana plasmática de cardiomiócitos, sugerindo que o efeito cardioprotetor da *starvation* e restrição calórica no coração pode ser mediada por uma melhoria na comunicação intercelular mediada por *gap junctions*, através do aumento dos níveis de Cx43. Uma vez que os mecanismos que estão subjacentes a estes efeitos benéficos da *starvation* e da restrição calórica no coração são amplamente desconhecidos, o principal objetivo deste trabalho foi averiguar o efeito da *starvation* e da restrição calórica nos níveis de Cx43 no coração. Para além disso, uma vez que o hipotálamo atua como sensor de nutrientes e regula a homeostasia da energia, também foi explorado o efeito de exossomas libertados de células do hipotálamo em *starvation*. Os resultados obtidos neste estudo mostram que a *starvation* e a restrição calórica nos animais leva a uma acumulação dos níveis de Cx43 no coração. Os exosomas libertados por células do hipotálamo foram capazes de aumentar os níveis de Cx43 em células cardíacas, independentemente do estado nutricional da célula de origem e do tempo de exposição a *starvation*. Além disso, reproduzir restrição calórica *in vitro*, através da redução da disponibilidade de glicose (hipoglicemia), também resulta num aumento dos níveis de Cx43 em células cardíacas. No sentido inverso, embora tenha sido mostrado que a hiperglicemia provoca destabilização na Cx43, em células do coração, os mecanismos pelos quais a Cx43 é degradada permanece pouco clara. Por isso, outro objectivo deste estudo foi avaliar o efeito de condições *diabetes-like* nos níveis de Cx43 de células cardíacas. A incubação de células cardíacas, quer na presença de meio com alta concentração de glicose (hiperglicemia)

quer na presença de metilglioxal, um sub-produto da glicólise que se acumula em tecidos em consequência da hiperglicemia, resulta num aumento da ubiquitinação e degradação da Cx43. Além da ubiquitinação, os resultados obtidos neste estudo dão fortes evidências de que a Cx43 sofre *sumoylation*.

Estes resultados suportam a ideia de que os mecanismos base para os efeitos cardioprotetores da *starvation* e da restrição calórica podem, pelo menos em parte, resultar de uma comunicação intercelular mediada por *gap junctions* aumentada, devido ao aumento dos níveis de Cx43.

Abstract

The heart depends on a well-organized intercellular communication (IC) network to ensure its proper function. IC can occur directly, between adjacent cells, through gap junctions (GJs), or indirectly, at long distances via extracellular vesicles (EVs), namely exosomes. Connexin 43 (Cx43) is the major ventricular GJ protein found in the heart and changes in its turnover dynamics have a direct impact on cardiac IC and consequently on heart function. Previous data from our group showed that short-periods of starvation, in cardiomyocyte cells, stabilize Cx43 at the plasma membrane (PM), suggesting that the cardioprotective effect of starvation and caloric restriction (CR) in the heart could be mediated by the improving of gap junction-mediated intercellular communication (GJIC), through the increase of Cx43 protein levels. Since the cellular mechanisms associated to the beneficial effects of starvation and CR in the heart are largely unknown, the main objective of the present work was to investigate the effect of starvation and CR on cardiac Cx43. Additionally, given the role of the hypothalamus in nutrient sensing and energy homeostasis, the effect of starvation-derived exosomes from the hypothalamic cells on cardiac Cx43 was also explored. The results obtained in this study show that starvation and CR in animals leads to an accumulation of cardiac Cx43 levels. Exosomes released by hypothalamic cells were able to increase the levels of Cx43 in cardiac cells, regardless the nutrient state of cell origin and time of exposure to starvation. Moreover, reproducing CR *in vitro*, by reducing the availability of glucose (hypoglycemia), also resulted in increased levels of Cx43 in cardiac cells. In opposite direction, although it has been shown that hyperglycemia results in a destabilization of Cx43, in the heart cells, the mechanisms whereby Cx43 is degraded remains unclear. Therefore, another objective of this study was to evaluate the effect of diabetes-like conditions on Cx43 levels of cardiac cells. Incubation either in the presence of high glucose medium (hyperglycemia) or methylglyoxal (MGO), a by-product of glycolysis that accumulates in tissues in consequence of hyperglycemia, results in increased Cx43 ubiquitination and degradation. Besides ubiquitination, the results obtained in this study provide evidence that Cx43 undergoes sumoylation.

These findings support the notion that the mechanistic basis for the cardioprotective effects of starvation and CR might, at least in part, result from an enhanced GJIC by increasing Cx43.

1. Introduction

1.1. Cardiovascular Diseases

Cardiovascular diseases (CVDs) are a group of heart and blood vessels disorders that include coronary heart disease (heart attack), cerebrovascular disease (stroke), hypertension and heart failure. According to the World Health Organization, CVDs are the leading cause of death and disability worldwide, representing 30% of global deaths [1, 2]. The average lifespan in humans is increasing rapidly and aging is a major risk factor for CVDs. This increase in the average life expectancy combined with the rising exposure of population to other risk factors such as unhealthy diet, overweight and obesity, physical inactivity, tobacco use and stress further promotes the development of heart-related diseases. Although the investment over the last years allowed significant advances in the diagnosis, prognosis, monitoring and treatment of CVDs, these diseases continue to absorb a significant amount of human and economic resources, in part due to fact that the mechanisms underlying these diseases remain elusive [2, 3]. Given its social and economical impact, it is of utmost importance to understand the cellular mechanisms associated with the physiology and pathology of the heart.

1.2. The Heart

The heart is a muscular organ which main function is to pump blood through the blood vessels of the circulatory system, delivering oxygen and nutrients to all the tissues and organs of the body. The human heart is divided into four chambers: upper left and right atria and lower left and right ventricles. The atria act as receiving chambers for blood, so they are connected to the veins that carry blood to the heart, while the ventricles are the larger chambers that pump blood out of the heart through the arteries. The right side of the heart maintains pulmonary circulation to the lungs, while the left side of the heart pumps blood to the extremities of the body through the bloodstream. The heart wall is constituted by three layers. From outer to inner, these are the epicardium, the myocardium and the

endocardium. The middle and thickest layer, the myocardium, is largely made by cardiac muscle cells - cardiomyocytes. To pump blood into the major arteries, the heart depends on the correct and efficient contractility of the myocardium. Cardiomyocytes are the major cell population in the heart, representing approximately one-third of the total number of cells and are responsible for the coordinated and synchronized heart contraction [4, 5]. Besides cardiomyocytes, the heart is formed by other cell types, including immune cells, fibroblasts and endothelial cells [6, 7]. The electrical, biochemical and metabolic communication between the different cardiac cell types is essential for proper structure and function of the heart. Unsurprisingly, impairment of these biological processes has been often implicated in several forms of CVDs, including ventricular hypertrophy, heart failure and ischemic heart disease [7]. In addition to the mechanisms directly associated with heart diseases, there are some other diseases, such as diabetes, that negatively influence the heart function and constitutes a risk factor for CVDs [8-10]. For instance, it was shown by our group that diabetes and hyperglycemia lead to intracellular accumulation of MGO in many different tissues and this leads to hypoxia-inducible factor 1-alpha (HIF-1α) degradation. Consequently, the HIF-1α destabilization causes deleterious effects on the ability of tissues to adapt to low oxygen, suggesting that cell and tissue dysfunction associated with diabetes are related, in part, with loss of cell response to hypoxia [11]. MGO is a highly reactive α-oxoaldehyde formed as a by-product of glycolysis, which leads to the formation of advanced glycation end products (AGEs). These products are related with cell and tissue impairment and, particularly in the heart, can induce cardiac dysfunction [11-13].

1.3. Protein Quality Control

The heart is an organ highly dependent on protein degradation mechanisms to ensure its function. In fact, given the low mitotic activity of cardiomyocytes, these cells largely depend on protein degradation mechanisms to ensure a proper protein quality control (PQC) and, consequently, to maintain proteome homeostasis - proteostasis, and energetic balance of the heart [4, 5].

Cells maintain a state of self-renewal through the continuous synthesis and degradation of proteins and organelles. Thus, PQC and the maintenance of proteostasis assisted by mechanisms that ensure the proper protein folding, trafficking and degradation of proteins, are fundamental for cellular functioning [14, 15]. Not surprisingly, defects in cardiac cellular proteostasis, including the impairment of proteolytic systems affect heart function and have been associated with the initiation and progression of several cardiac disorders, characterized by an inadequate accumulation of unwanted/aberrant proteins [16].

The mechanisms of PQC act at different and consecutive levels of a protein life cycle and require the action of chaperones and proteolytic systems. The first stage of PQC is the correct folding of proteins, provided by chaperones that assist in the folding of *de novo* synthesized proteins, and in the refolding of unfolding proteins. Chaperones identify abnormal and unstable conformations in proteins and help them to regain stability. However, different physiological and pathological conditions can induce alterations on the homeostatic action of chaperones, that can after result in protein aggregation. Under these conditions and for those proteins in which refolding is not possible, cells count on proteolytic systems, not only to recycle amino acids, but also to eliminate the aberrant proteins. These proteolytic systems include the proteolytic pathways ubiquitin-proteasome system (UPS) and autophagy, which are crucial mechanisms in both protein quality and quantity control [14, 17] (Figure 1).

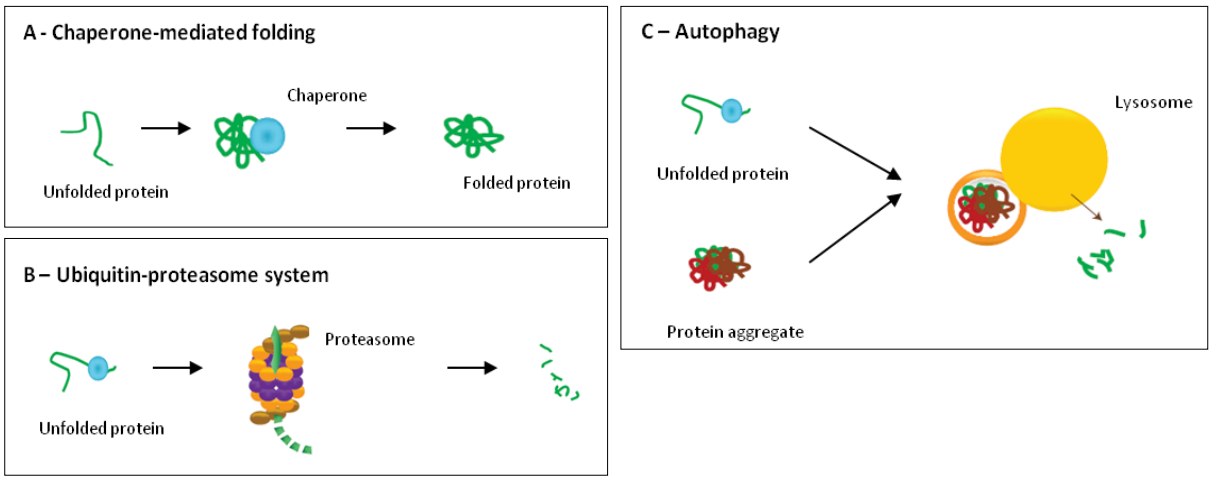


Figure 1. Mechanisms of Protein Quality Control.

A – Chaperone-mediated folding: Chaperones assist in the folding of proteins; Proteins that fail to fold by chaperones can be degraded by two proteolytic systems: **B** – The UPS, where degradation of substrate occurs

in the proteasome; or **C** – Autophagy, where proteins or organelles are engulfed by a double membrane that further fuses with a lysosome, degrading its cargo. (Adapted from Wong E, Cuervo AM. Integration of clearance mechanisms: the proteasome and autophagy. 2010).

The UPS and autophagy are responsible for the degradation of abnormal or unwanted proteins under basal conditions. Indeed, these proteolytic pathways are activated in order to eliminate these aberrant proteins or proteins that have completed their life cycle. In addition to this regulatory role under resting conditions, the activity of these degradative systems can be up-regulated either in response to external stimuli that induce protein or organelle damage, or in the absence of nutrients in order to refill the intracellular reserve of free amino acids that sustains protein synthesis. Failure of the proteolytic systems to maintain basal cellular turnover or to protect cells under stress conditions leads to altered cellular homeostasis, compromises the cellular energetic balance and often promotes intracellular accumulation of damage components that are lethal for cells. Particularly, alterations in cardiac proteasomal and lysosomal degradation are remarkably associated with the majority of heart diseases [14, 17, 18].

1.3.1. The Ubiquitin-Proteasome System

The UPS is the major pathway responsible for the highly regulated extralysosomal degradation of intracellular proteins [19]. Briefly, protein degradation mediated by the UPS involves the covalent attachment of a polyubiquitin chain to a target protein, in a process known as ubiquitination, followed by the recognition and degradation of the ubiquitin (Ub)-tagged protein in the proteasome, with the recycling of Ub [17, 18]. Although ubiquitination was first described as a signal for UPS degradation, more recently, it was shown that Ub also has a role in lysosome protein degradation. Therefore, ubiquitination can be a signal for proteasomal or lysosomal degradation of both cytosolic and membrane proteins [20-22].

Since ubiquitination represents a post-translational modification (PTM), this process will be described in more detail in the “Post-Translational Modifications” section.

1.3.2. Autophagy

Autophagy, also defined as “self-eating”, constitutes an important mechanism for the degradation of cellular components, including proteins and organelles. It is a catabolic pathway through which substrates are engulfed by a double membrane that further fuses with lysosomes [5, 14]. Lysosomes, essential components in this proteolytic system, are single membrane vesicles with acidic pH lumen that contains a large variety of hydrolases with a high enzymatic activity such as proteases, lipases and glycosidases [14].

Despite the fact that all protein degradation by autophagy ends in the lysosomes, until now, three types of autophagy have been described, namely microautophagy, chaperone-mediated autophagy (CMA) and macroautophagy. These pathways differ on the mode of cargo recognition and delivery to the lysosomes occurs, depending on the type of target for degradation and the cellular conditions. In microautophagy, the sequestering membrane is the lysosomal membrane itself, which invaginates substrates into the lysosomal lumen, in a “nonselective” manner. CMA is a direct and selective pathway for transporting targeted soluble cytosolic proteins to the lysosomal limiting membrane and into the lysosome lumen for degradation. In this type of autophagy, cytosolic chaperones, heat shock cognate proteins of 70 kDa (Hsc70) recognizes a KFERQ sequence in substrates and deliver them to lysosome, in a process that involves the lysosomal-associated membrane protein-2A (LAMP-2A). In macroautophagy (hereafter referred to as autophagy), the most well-studied autophagy pathway, double membrane vesicles called autophagosomes sequester proteins, cytosolic components or organelles and then fuse with the lysosome. Following fusion, the cargo is degraded by the lysosomal enzymes and the cell can reuse the resultant degradation products, such as amino acids, that are transported back to the cytosol [5, 14, 17]. The molecular machinery responsible for autophagosome biogenesis and for the fusion of autophagosome with the lysosome involves autophagy-related genes (ATGs) and their respective Atg proteins. The autophagic process can be divided into four steps: the initiation/induction of phagophore formation, the phagophore nucleation, the phagophore elongation/expansion and finally the fusion between the autophagosome and the lysosome for subsequent degradation of its cargo and release of nutrients into the cytosol [23] (Figure 2).

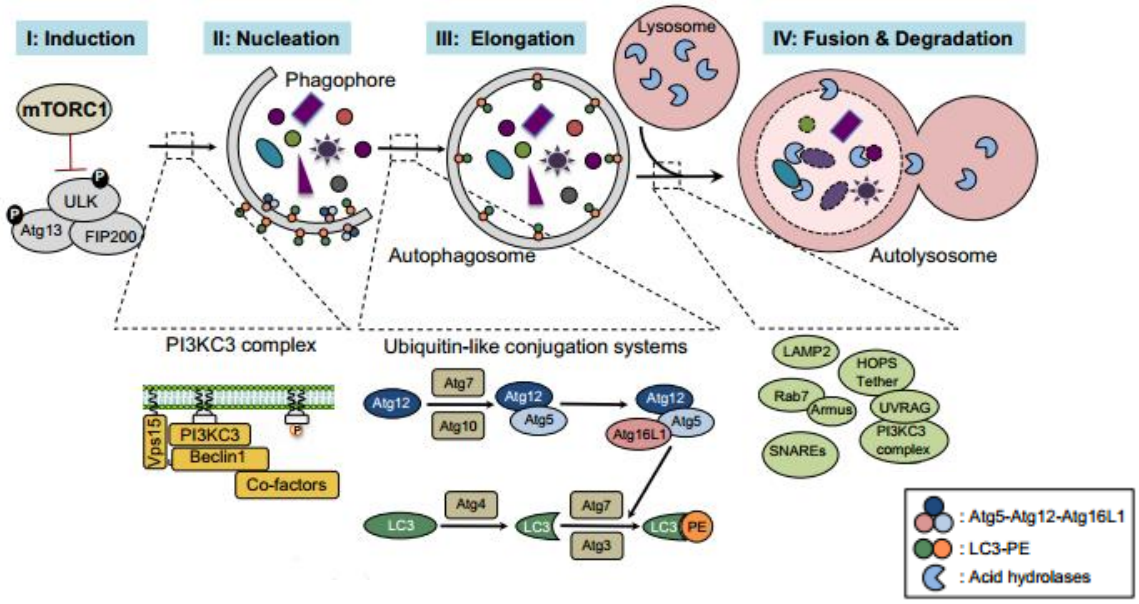


Figure 2. The steps of Autophagy.

The steps of autophagy include induction, vesicle nucleation, membrane elongation, autophagosome formation and subsequent fusion with the lysosome for degradation and recycle of its cargo. **I** – Autophagy induction is tightly regulated by the "nutrient sensor" mTORC1, that, when is inactivated in response to stimuli such as starvation or hypoxia, activates the ULK complex and initiates the autophagy pathway. **II** – Phagophore nucleation is mediated by the Beclin1-associated PI3KC3 complex. **III** – Autophagosome membrane elongation depends on two ubiquitin-like conjugation systems: the Atg12-Atg5 conjugation and the LC3-PE conjugation. **IV** – Fusion of the autophagosome with the lysosome involves membrane fusion factors. Finally, the cargo is degraded into the lysosomal lumen, enriched with acid hydrolases. (Adapted from Liang, et al. MicroRNAs: an emerging player in autophagy. 2014).

Although initially thought to be a non-specific and in bulk degradative pathway, there is now ample evidence that suggests that autophagy can also constitute a highly selective mechanism of PQC. This selective degradation of unwanted cellular components is conferred, in part, by autophagy adaptors, that select and convey the substrates to the autophagic vesicles. Among the several autophagy adaptors already identified, the best studied one is the sequestosome 1 (SQSTM1/p62), an adaptor that recognizes ubiquitinated substrates and directs them to the autophagosome, through the binding of a microtubule-associated protein 1 light chain 3 (LC3)-Interacting Region (LIR) to LC3 present in autophagy vesicles [(4)].

Through these diverse pathways, autophagy occurs at basal levels throughout the organism and is involved in many physiological processes, including quality control of proteins and organelles, source of energy through the generation of amino acids when nutrients are scarce, cell and tissue remodeling, degradation of pathogens and antigen presentation [4, 5, 14].

1.3.2.1. Crosstalk between UPS and Autophagy

Although initially thought as two independent pathways, it is now widely accepted that UPS and autophagy crosstalk [5, 14]. While some studies suggest that UPS and autophagy act as synergetic mechanisms, others show that autophagy can act as a compensatory mechanism when UPS is impaired. In fact, it was demonstrated that if the UPS is impaired, autophagy is activated, with p62 acting as a shuttle, diverting ubiquitinated proteasomal substrates to the autophagy pathway. For this reason, a pivot role has been ascribed to p62 protein in the link between the ubiquitination and the autophagic pathway [24]. Moreover, studies support a broader role of Ub-conjugation in cellular quality control, because the Ub tagging is not limited to targeting substrates for UPS degradation, but it is also involved in selective autophagic degradation [25].

1.3.2.2. Autophagy in the Heart

In the heart, autophagy plays a dual role, being either protective or detrimental, depending on the nature, extent and severity of the stimuli or insult. The precise role of autophagy in cardiac context remains obscure owing to its multifarious actions [26]. The physiological importance of autophagy was already described in the maintenance of heart homeostasis, being today well known that basal levels of autophagy are fundamental to ensure protein and organelle quality control and, consequently, cardiac structure and function [5]. Indeed, this homeostatic role of autophagy is highlighted by the fact that cardiac-directed knockout of key autophagic genes are lethal and, also, cardiomyocyte-specific abrogation of autophagic protein Atg5 leads to changes in cardiac

structure and a decline in performance [26]. On the other hand, autophagic flux is upregulated in several forms of human CVD, including ventricular hypertrophy, heart failure and ischemic heart disease, thus implying an exacerbated activation of autophagy as a causative factor for various cardiac maladies [27-29].

1.4. Post-Translational Modifications

The increase in complexity from the genome to the proteome can be explained by post-translational modifications (PTM) of proteins. PTM are chemical modifications that can occur at any step in the life cycle of a protein and can regulate activity, localization and interaction of proteins with nucleic acids, lipids and other proteins. They constitute key regulatory modifications that facilitate a rapid response to diverse cellular needs, avoiding *de novo* synthesis of proteins. These modifications include phosphorylation, glycosylation, nitrosylation, methylation, acetylation, lipidation, ubiquitination and sumoylation [30].

1.4.1. Ubiquitination

Ubiquitination is a process in which proteins are modified through the covalent attachment of Ub, involving the formation of an isopeptide bond between the carboxy group of C-terminal glycine of Ub and the substrate. Although the first studies described the ε-NH_2 group of a lysine residue on the target substrate as the acceptor of Ub, it is now known that Ub can also be conjugated to the thiol group of a cysteine or to the α-amino group of the N-terminal amino acid [31]. Ub, made up by 76 amino acids, is particularly stable and is ubiquitous in all tissues. The conjugation of Ub to substrates requires the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3). In a first step, Ub is bound in an ATP-dependent reaction by a high-energy thioester bond to E1, thus becoming activated. The activated Ub moiety is then transferred to the active site of E2. In the final step, E3 catalyzes the transfer of Ub to the substrate protein. The E3s are responsible for the specific recognition of the large number of substrates of the Ub system and the specificity of the Ub protein ligase

system relies on numerous E3s that recognize and catalyzes the attachment of Ub moieties to a specific target protein [5, 32]. Because of the presence of internal lysine residues, Ub attached to the substrate can be itself subsequently ubiquitinated through repeated actions of the conjugating enzyme cascade forming polymeric Ub chains. Ub is also a product of the proteasome, since it is released from the substrates before their entrance in the catalytic chamber of the proteasome, thus allowing the recycling of Ub and its use in further cycles of ubiquitination. This is possible because ubiquitination is a reversible process, catalyzed by deubiquitinating enzymes (DUBs) that can remove Ub from substrates [5, 33-35] (Figure 3).

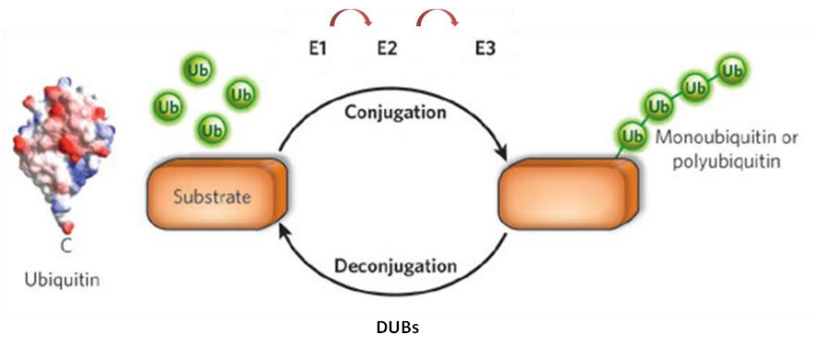


Figure 3. Schematic representation of ubiquitination.

The ubiquitination is a process in which the conjugation of ubiquitin (Ub) to substrates requires the sequential action of three enzymes: the ubiquitin-activating enzyme (E1), the ubiquitin-conjugating enzyme (E2) and the ubiquitin-protein ligase (E3). Substrates can be monoubiquitinated or polyubiquitinated since protein modification can occur by a single Ub or by a chain of covalently linked Ub, respectively. Ub can be deconjugated from the substrates, in a process called as deubiquitination, that is promoted by deubiquitinating enzymes (DUBs). (Adapted from Bergink S, Jentsch S., Principles of ubiquitin and SUMO modifications in DNA repair. 2009).

1.4.2. Sumoylation

Sumoylation is a highly regulated PTM that consists on the attachment of a member of small ubiquitin-like modifier (SUMO) family of proteins to lysine residues in a specific target protein [36-38]. SUMO proteins belong to the ubiquitin-like proteins (Ubls), a small family of protein modifiers, which have almost the same three-dimensional structure as Ub and have similar enzymatic mechanisms for protein modification. Vertebrates have three SUMO

isoforms, SUMO-1, SUMO-2 and SUMO-3 expressed at the protein level. These SUMO proteins can be divided into two families, SUMO1 and SUMO2/3, since SUMO-2 and -3 share 97% sequence identity, whereas SUMO-1 only shares approximately 50% sequence identity with SUMO-2 and -3 [39]. Expression levels, susceptibility to isopeptidases, the ability to form SUMO chains and response to stress are some of the differences between these families. Sumoylation involves a SUMO-specific sequential enzymatic cascade of E1, E2 and E3 enzymes. The SUMO conjugation results in an isopeptide bond formed between the C-terminal glycine residue of SUMO and the ε-amino group of a substrate target lysine. SUMO is first activated by the heterodimeric E1 activating enzyme Aos1/Uba2 (also kwown as SAE1/SAE2), a reaction that involves ATP hydrolysis. This E1 activating enzyme catalyses adenylation of the SUMO C-terminal glycine and subsequently transfers the adenylate to a conserved cysteine, resulting in an E1-SUMO thioester linkage. Subsequently, SUMO is transferred to the catalytic cystein of the E2 conjugating enzyme Ubc9, again forming a thioester bond. In the final step SUMO is conjugated to its substrate, a reaction that is usually performed by a third class of enzymes, the E3 ligases [36, 40]. However, when present in high concentrations, the single E2 conjugating enzyme Ubc9 can directly sumoylate the target protein. Despite the existence of three isoforms, all SUMO proteins are activated and conjugated by the same E1 and E2 enzymes. E3 ligases are a small class of enzymes that determine substrate specificity and increase the SUMO conjugation rates. Sumoylation, similarly to ubiquitination, constitutes a reversible modification, which involves the action of highly active proteases that specifically cleave SUMO from its substrate. All SUMO-specific proteases (SENPs) identified so far belong to the class of cysteine proteases, and these isopeptidases are required for both maturation of SUMO proteins (C-terminal hydrolase activity) and removal of SUMO from targets [35, 40, 41] (Figure 4).

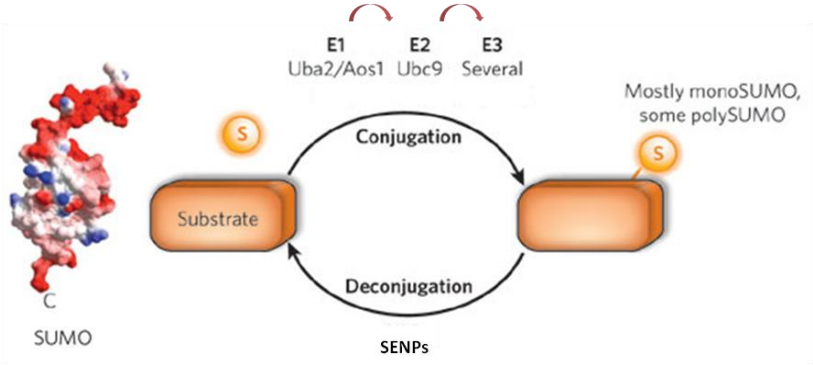


Figure 4. Schematic representation of sumoylation.

The sumoylation is a process in which the conjugation of small ubiquitin-like modifier (SUMO) to substrates requires the sequential action of three enzymes: the E1 activating enzyme Uba2/Aos1, the E2 conjugating enzyme Ubc9 and an E3 ligase. Substrates can be monosumoylated or polysumoylated since protein modification can occur by a single SUMO or by a chain of covalently linked SUMO, respectively. However, monosumoylation occurs more frequently. SUMO can be deconjugated from the substrates, in a process called as desumoylation, that is promoted by SUMO-specific proteases (SENPs). (Adapted from Bergink S, Jentsch S., Principles of ubiquitin and SUMO modifications in DNA repair. 2009).

Protein regulation by reversible attachment of SUMO can modify the activity, stability, interaction, localization and turnover rate of proteins involved in many processes, namely chromatin organization, transcription, DNA repair, macromolecular assembly, protein homeostasis, trafficking and signal transduction, playing a fundamental role in several cellular mechanisms [39, 40].

1.5. Intercellular Communication

In multicellular organisms an efficient communication between cells is essential to ensure the maintenance of organism homeostasis. IC allows cells to exchange information and can occur directly, between adjacent cells, through Connexin(Cx)-containing channels, called gap junctions (GJ), or indirectly, for long distances via extracellular vesicles (EVs), that include microvesicles (Mvs) and exosomes [7, 42, 43].

1.5.1. Gap Junctions

GJ are intercellular channels that provide IC between neighbouring cells. Gap junction-mediated intercellular communication (GJIC) allows the direct transfer of small molecules (with molecular weight typically less than 1.5 kDa) and ions between the cytoplasm of adjacent cells, playing a pivotal role in several biological processes, such as the coordinated contraction of excitable cells, tissue homeostasis, cell growth and differentiation, signaling and cell death [44-47].

1.5.1.1. Structure of Gap Junctions

GJ are formed by the docking of two hemichannels (one from each cell), being each hemichannel composed by the assembly of six subunits of a transmembrane protein called connexin (Cx). Six Cxs oligomerize into a hexameric transmembrane structure with a central hydrophilic pore, called hemichannel or connexon. Two hemichannels, one provided by each of two adjacent cells, dock to form the complete GJ channel that is completely off to the extracellular space, permitting the direct transfer of small molecules and ions between the cytoplasm of adjacent cells. Areas of the PM enriched in hundreds or thousands of Cx-containing channels are called GJ plaques, and constitute the regions where communication takes place. Another feature of Cx is their high turnover, when compared with other membrane proteins, which are usually very stable. For example, Cx43 has an half-life of 1-2 hours, which ascribes to degradation an important role in the regulation of Cx43 levels and, ultimately, in maintenance of GJIC [46, 48].

The human genome encodes 21 different Cx proteins. Despite this number, Cx its structure is highly conserved, being structurally composed of nine domains, namely the N-terminus, two extracellular loops (stabilized by intramolecular disulfide bridges), four α-helical transmembrane domains, a cytoplasmic loop domain and a C-terminus domain. The N-terminus, the cytoplasmic loop and the C-terminus are located in the cytosol. The N-terminus, the two extracellular loops and the four α-helical transmembrane domains are highly conserved among the different Cx isoforms. The major differences between the types of Cxs are due the variability in length and sequence in the cytoplasmic loop and in the

C-terminus domain. These proteins are expressed in most cells and different types of Cxs can be simultaneously expressed in the same cell. This means that hemichannels can be homomeric, if they are formed by the same isoform of Cx, or heteromeric, if they are formed by different types of Cx. However, only some combinations are possible. As consequence, if identical hemichannels dock, homotypic intercellular channels are formed; on the other hand, if the docking occurs between hemichannels containing different Cxs, these channels are called heterotypic [48-50].

The most abundant Cx present in the human body is Cx43, being largely expressed in several tissues, especially in the brain, heart, bone, kidney, eye and skin. Considering its importance in the heart, we will focus on Cx43 [49, 50] (Figure 5).

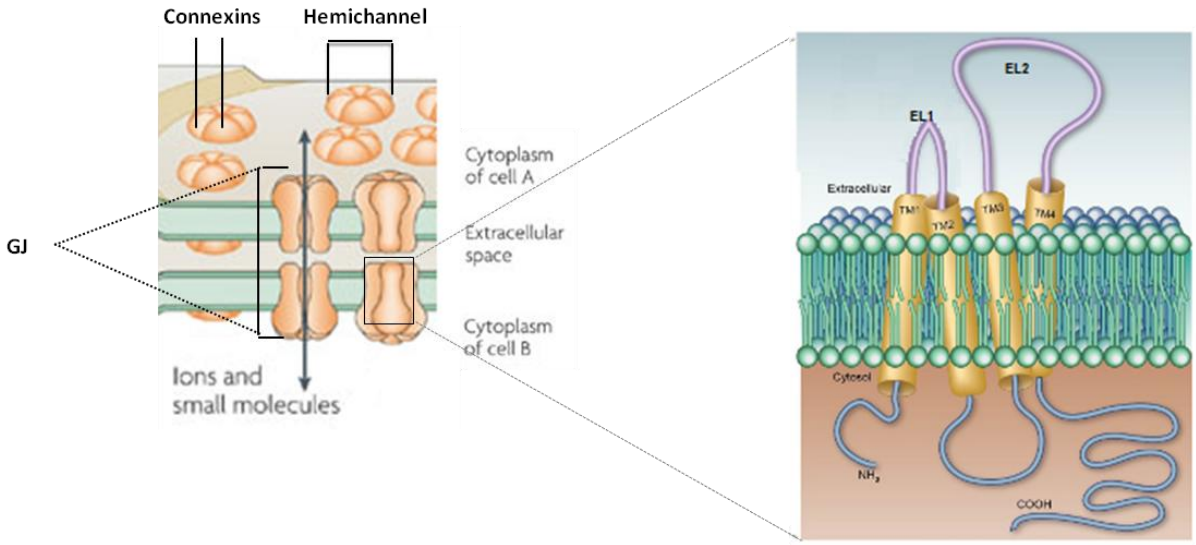


Figure 5. Assembly of GJ and structure of Cx43.

Cx43 is an integral transmembrane protein. Cxs are assembled in groups of six to form hemichannels and two hemichannels can dock to form a GJ. GJ allow the passage of ions and small molecules, mediators of IC, between neighboring cells. Transmembrane domains, extracellular loops (EL1 and EL2) and NH_2 and COOH-terminal tails of Cx43 are illustrated. (Adapted from Bloomfield SA, et al. The diverse functional roles and regulation of neuronal gap junctions in the retina. 2009; and from Chun-hong X, et al. 2012).

1.5.1.2. Cx43 in the Heart

The Cx43-containing channels have a critical role in the heart, since they both provide metabolic communication and mediate electrical coupling between cardiac cells. In the heart, the major ventricular GJ protein found is Cx43, which is mostly localized at the intercalated discs, at the longitudinal termini of cardiomyocytes, where Cx-formed channels allow the anisotropic rapid impulse propagation that is required for a synchronized and coordinated beating of the heart. Therefore, changes in Cx43 turnover dynamics in cardiomyocytes have a direct effect on cardiac IC and consequently the heart function. Unsurprisingly, dysfunction on GJIC in the heart has been associated with several heart diseases, such as heart failure and myocardial ischemia [51-53]. For all that, the mechanisms involved in synthesis, modification, internalization and degradation of Cx43 influence the expression, levels, localization and function of this protein, in a process generally called Cx43 remodeling.

1.5.1.3. Regulation of GJIC

Regulation of GJIC can occur either through the channel gating or the number of functional Cx-containing channels localized at the PM that, in turn, can be regulated at different levels at the Cxs life cycle, including Cx synthesis, trafficking and degradation. Unlike the majority of PM proteins, that are very stable, Cx has a very high turnover. This high turnover of Cx implies a fine-tune regulation of the mechanisms involved in protein degradation, that are very often modulated by PTM of Cx [4, 54]. PTM can induce conformational changes on Cx43 and, consequently, the structural rearrangement of the hexamers can influence the channel gating. An example of PTM that can affect the channel gating is phosphorylation. Phosphorylation consists in the addition of phosphate groups by covalent ligation and can alter protein conformation, hydrophobicity or charge of proteins [55, 56]. Cx43 phosphorylation has been implicated in the regulation of its intracellular traffic, assembly and can induce Cx43 ubiquitination and its further internalization and degradation [57].

1.5.1.4. Post-Translational Modifications of Cx43

Different PTM are involved in Cx43 modifications. PTM can induce alterations in Cx43 by multiple mechanisms, which include hydroxylation, acetylation, disulfide binding, nitrosylation, ubiquitination and, more recently, sumoylation. These PTM can dictate GJIC not only by regulating the level of Cx43 localized at the PM, through the modulation of rate of synthesis, assembly, internalization and degradation of Cx43, but also directly affecting the conductivity and permeability of GJ channels [30, 58].

1.5.1.4.1. Cx43 Ubiquitination

Laing and colleagues provided the first evidence that Ub has a major role in controlling the life cycle of Cx43, suggesting that Ub is a necessary signal for Cx43 degradation [59]. Afterwards, other groups contributed to elucidate in more detail the pathways and molecular players involved in Cx43 ubiquitination. Our group showed that Cx43 ubiquitination signals the degradation of GJ through autophagy and demonstrated that the E3 ligase Nedd4 (neural precursor-cell-expressed developmentally down-regulated 4) mediates Cx43 ubiquitination, constituting a signal for internalization and further degradation of Cx43 [21, 60, 61]. Moreover, it was also demonstrated that interaction of ubiquitinated Cx43 with the autophagic adaptor p62 is an important step in mediating internalization and degradation of Cx43 by autophagy [21, 49]. Therefore, the ubiquitination of Cx43 constitutes a critical point in Cx43 regulation and, consequently, it is pivotal in maintenance of an efficient GJIC between cardiac cells.

1.5.1.4.2. Cx43 Sumoylation

In addition to ubiquitin, Cx43 may also be conjugated with small ubiquitin-like proteins, such as SUMO. Kjenseth et al. demonstrated that all three forms of SUMO proteins can modify Cx43 and identified the lysines at positions 144 and 237, located, respectively, in the intracellular loop and C-terminal tail of Cx43, as SUMO conjugation sites. Moreover, the

authors suggest that the conjugation of SUMO proteins to Cx43, in contrast with Ub conjugation, stabilizes the protein at the PM [39].

1.5.1.5. Internalization of Cx43

The internalization of GJ can be mediated by constitutive endocytic signals present in the sequence of Cx43, such as the Tyr-sorting signal, or by PTM, including phosphorylation and ubiquitination [60]. Results from our group demonstrated that Nedd4-mediated ubiquitination of Cx43 triggers the internalization of GJ [60]. The endocytosis of GJ presents some peculiarities, since the endocytic vesicles that are generated upon GJ internalization, called “annular gap junctions” or connexosomes, are unusual large and formed by a double-membrane vesicle that engulf a significant portion of the PM containing a complete GJ plaque (with hundreds to thousands channels) or a fraction of it. Moreover, GJ internalization occurs into one of the adjacent communicating cells, giving rise to structures containing proteins and PM domains of both of the two connected cells. Besides Nedd4, there are other Ub-binding endocytic adaptor proteins, such as disabled 2 (DAB2), Epsin, epidermal growth factor receptor substrate 15 (Eps15), drebin, adaptor protein 2 (AP2), dynamin and zona occludin protein 1 (ZO-1), that recognize ubiquitinated proteins and promote their internalization and the intracellular sorting of membrane proteins [49].

1.5.1.6. Degradation of Cx43

Since Cx43 has a short half-life, the rate of its degradation is of utmost importance in the maintenance of GJIC. As mentioned above, the level of GJIC largely depends upon the number of Cx-containing channels present at the PM, which in turn can be determined by the internalization and degradation mechanisms. It has been shown that three degradation pathways can be involved in Cx43 degradation: the endosomal, the proteasomal and the autophagosomal [62].

The endosomal pathway includes the internalization of GJ from PM to cytoplasm through endocytosis. After internalization, endocytosed Cx43 can be either recycled back to

the PM or end up in the lysosome, the organelle where complete degradation of Cx43 takes place. Furthermore, studies carried out in our lab established that lysosomal degradation of Cx43 can also occur via the autophagy pathway. Indeed, we demonstrated that GJ are degraded by autophagy, in a process that requires ubiquitination of Cx43, and its further recognition by Ub-adaptors Eps15 and p62 [21, 61]. Therefore, activation of autophagy, as occurs in several pathologies and/ or stress conditions, including, for example, ischemia and starvation, can contribute to modulate GJIC [5]. Strikingly, we have recently shown that ischemia-induced activation of autophagy leads to GJ degradation in cardiac cells [51] (Figure 6).

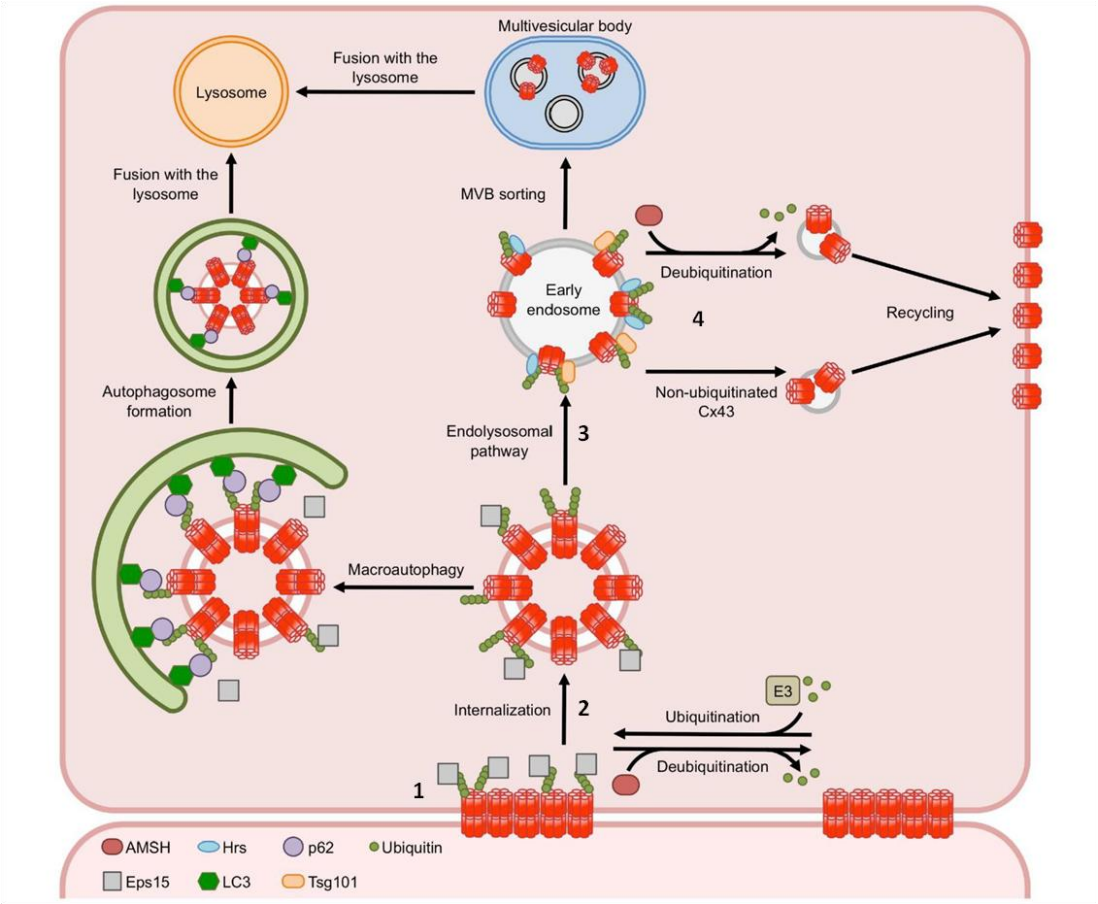


Figure 6. Schematic representation of Cx43 internalization, ubiquitination, sumoylation and degradation.
The ubiquitination of Cx43 at the PM triggers the internalization of Cx43, in a process dependent on the endocytic adaptor Eps15. Once internalized, Cx43 can be recognized by p62 and consequently degraded by autophagy or can be sorted through the endosomal pathway, by the action of Hrs and Tsg101. **1, 2, 3, 4 –** Hypothesis for subcellular locations for sumoylation of Cx43. (Adpated from Ribeiro-Rodrigues T., et al. Connexin 43 ubiquitination determines the fate of gap junctions: restrict to survive. 2015).

Regarding degradation through the proteasomal pathway, it was demonstrated that inhibition of proteasome leads to Cx43 accumulation, suggesting that Cx43 can be degraded by the proteasome [59]. In this case, it is very likely that proteasome degradation of Cx43 makes part of the quality control mechanisms that occur at the endoplasmic reticulum (ER) level – ERAD (endoplasmic reticulum-associated degradation), which recognize misfolded proteins in the ER and target them for ubiquitination with subsequent degradation through proteasome [63, 64]. Moreover, it was shown that both ubiquitinated and non-ubiquitinated Cx43 can be targeted for proteasomal degradation, suggesting that ubiquitination of Cx43 is not required for proteasomal degradation, being, in this case, degradation mediated by connexin43-interacting protein of 75 kDa (CIP75). It was suggested that CIP75 interacts with Cx43 and mediates its transport to the proteasome, through the interaction with 19S subunits, for its subsequent proteasomal degradation [65].

It is generally accepted that PQC occurs at two main levels: folding and degradation. However, it is conceivable that if these two levels of PQC fail, a third level is activated in order to get the cells ride of toxic material that would accumulate as a consequence of proteolysis failure. In this case, the release of these potential toxic products into the extracellular space, in the form of EVs, would constitute an additional level of PQC [66].

1.5.2. Extracellular Vesicles

EVs are vesicles that are physiologically released by most, if not all, cells into the extracellular space. These vesicles were initially thought to be cell debris derivates and a way for cells to remove unwanted material, but, although this hypothesis should not be discarded, emerging evidence suggests that these vesicles may act as intercellular messengers that mediate both local and systemic cell communication. Indeed, EVs play an important role in the transfer of biological information between donor and acceptor cells, since they contain cell-specific cargo proteins, lipids and genetic material that can influence and alter function and behavior of recipient cells [7, 67].

Cells can produce and release different types of vesicles, influencing their direct environment (autocrine effect), or influencing more distant regions from the cell where they

are produced (paracrine effect). In this case, these vesicles constitute long-distance vehicles, conveying the message between different non-adjacent cells of the organism. In agreement, EVs are present in all biological fluids of the body, including blood, urine, saliva, breast milk, amniotic fluid, ascites, cerebrospinal fluid, bile and semen, which make them excellent candidates for biomarkers and promising targets to therapeutic strategies, since they are easy to collect and manipulate [67, 68].

The EVs have a lipid bilayer and can be divided in apoptotic bodies, microvesicles (MVs) and exosomes, depending on their subcellular origin, size and content. Apoptotic bodies have a diameter between 1-5 µm and are released from the PM of cells as blebs when they undergo apoptosis. While MVs derive from direct budding of the PM, exosomes are formed when multivesicular bodies (MVBs) fuse with the PM and release their intraluminal vesicles (ILVs) into the extracellular medium [7, 69] (Figure 7).

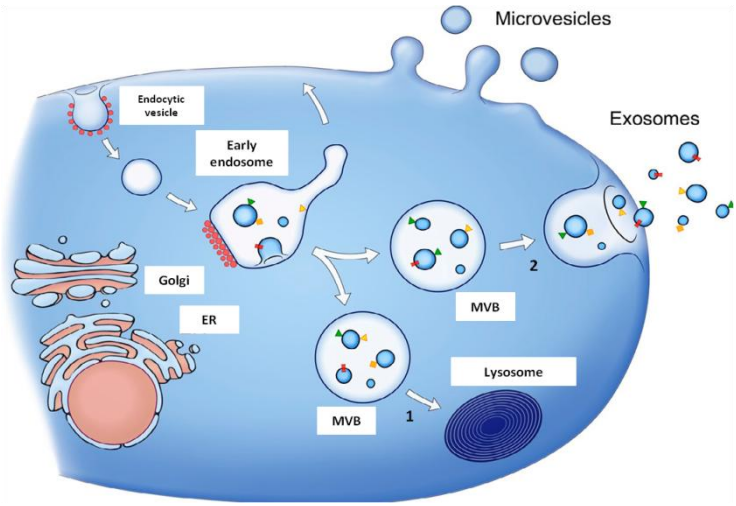


Figure 7. Schematic representation of release of MVs and exosomes.

MVs are released directly from budding of the PM, whereas exosomes are released by fusion of MVBs with the PM. Exosomes derivate from the endosomal pathway. The MVBs formed can fuse with the lysosome **(1)**, or fuse with the PM, releasing exosomes into the extracellular space **(2)**. (Adapted from Raposo G, Stoorvogel W. Extracellular vesicles: exosomes, microvesicles, and friends. 2013).

1.5.2.1. Microvesicles

MVs are a class of vesicles with a diameter between 100 and 1000 nm that are formed from cells by budding or blebbing of the PM.

MVs are released by cells during biological processes, including cellular differentiation or senescence, exposure to high shear stress, or upon pro-inflammatory or pro-thrombotic situations. These vesicles do not have clear markers identified yet, but it is known that they contain a diversity of bioactive molecules, which include cell receptors, cytoplasmic and membrane proteins, cytokines, chemokines, growth factors, functional mRNA and miRNAs, which can trigger an effect on recipient cells. It is generally accepted that the direct cell environment and the nature and extension of determined insults determine MV composition and thereby their action in IC [7, 67, 68].

1.5.2.2. Exosomes

Exosomes are the best characterized class of EVs, with a diameter between 40 and 200 nm, which are secreted upon fusion of MVBs with the cell surface [70]. Besides their size, protein and lipid composition of exosomes are important features that allow their identification. Given their endosomal origin, the typical proteins present in exosomes include tetraspanins (CD63, CD9, CD81 and CD82), luminal proteins (annexin and cytokines), antigen-presentation (MHC-I and MHC-II) and co-presentation molecules (CD86), cell adhesion (integrins), heat shock proteins and chaperones (Hsp20, Hsp60, Hsp70, Hsc70 and Hsp90), metabolic enzymes (glyceraldehyde-3-phosphatate dehydrogenase, fatty acid synthase, peroxidases, pyruvate kinase), proteins involved in exosome biogenesis (the endosomal sorting complexes required for transport (ESCRT) complex: Tumor susceptibility gene 101 (TSG101) and Alix), signaling proteins (kinases, GTPase Hras, RhoA) and proteins involved in trafficking and membrane fusion (Annexins, Flotillin and Rab protein family). Among these proteins, Alix, Flotillin, Hsc70, TSG101 and CD63 can be defined as exosomal marker proteins, because they are particularly enriched in exosomes [7, 67-69, 71, 72].

The exosome biogenesis relies on the endosomal network of proteins, namely the ESCRT complex. The first step of exosome formation involves the inward budding of the

endosome membrane and package the cargo into ILVs, forming the MVB. The MVBs that can either fuse with the lysosome resulting in the degradation of endocytic cargo, or fuse with the PM with the consequent release of ILVs into the extracellular space. Once in the extracellular environment, these ILVs are called exosomes [67-69, 72].

Several studies have attributed different functions to exosomes, including regulation of IC and triggering downstream signaling events, besides the delivery of their content. The uptake of exosomes into a cell is mediated by the direct binding with membrane surface molecules, endocytic internalization or fusion with the PM of the recipient cell, followed by delivery of their cargo. Exosomes can carry different proteins, transcription factors, DNA and coding and noncoding RNA molecules, including microRNAs (miRNAs), being, for this reason, considered intercellular messengers involved in both physiological and pathological conditions. Indeed, the content of exosomes influence the type and amplitude of the response caused by exosomes in acceptor cells and vary according to the cell origin and external stimuli, suggesting that the biological processes underlying exosome biogenesis are complex. Moreover, it is generally accepted that the exosomal content can reflect the cell origin and the physiological state of the origin cell [67, 69, 71, 73, 74].

1.6. Hypothalamus-Heart Communication

The hypothalamus is a brain region which main function is the regulation of the organism homeostasis. This brain region has a pivotal role in feeding behaviour and constitutes the major centre of the organism for energy regulation, since it is responsible for control the food intake and metabolism, among other essential functions in the regulation of many physiological processes such as heart rate and blood pressure, body temperature, fluid and electrolyte balance, thirst, appetite, body weight and circadian rhythms. Regulation of energy balance is a crucial mechanism for the maintenance of life and is centrally regulated by the neuronal network of the hypothalamus [75-77]. In the hypothalamus, the arcuate nucleus neurons have a key role in sense and integrate peripheral signals of nutrition to downstream circuits and consequently produce neuropeptides responsible for the increase or decrease of food intake [77-79]. The hypothalamus receives information from almost all

parts of the nervous system and in accordance to the needs of the organism, the hypothalamic neurons respond through the synthesis and the release of neuropeptides and neurohormones, which in turn stimulate or inhibit the production of hormones throughout the body. Therefore, the hypothalamus is a link between the nervous system and the endocrine system, being this linkage promoted, mainly, by the interaction of the hypothalamus with the pituitary gland (hypophysis) [77, 80]. Indeed, the hypothalamic neurons release neuropeptides, neurohormones and also exosomes, which through the bloodstream can reach and influence the function of many organs, namely, the heart. Due to its high metabolic activity, with a rapid energy turnover and low energy storage capacity, the myocardium strongly depends upon exogenous energy supply. Therefore, the cardiac function is highly influenced by the nutritional status of the organism. Furthermore, several studies have been suggesting a protective role of CR and starvation not only during ageing but also in numerous diseases, such as cancer, neurodegenerative diseases and CVDs. However, how CR and starvation exert their protective effect and the mechanisms underlying that protection have not been clearly defined yet [81, 82]. Therefore, a better understanding of the mechanisms whereby starvation and CR protect the heart can provide new and important insight in the field of cardiovascular biology.

2. Objectives

GJIC is essential for the maintenance of a normal heart function. In the heart, the Cx43-containing channels play a critical role, by providing both metabolic and electrical coupling between cardiac cells. Therefore, changes in Cx43 turnover dynamics have an impact on cardiac IC and consequently may lead to several heart diseases.

Although several studies have shown that CR confers cardioprotection, the cellular and molecular mechanisms underlying this protection remain obscure. Previous data from our group showed that starvation stabilizes Cx43 at the PM in cardiomyocytes. Thus, the present work explores whether the cardioprotective effect of starvation and CR is mediated by an improvement of GJIC through the increase of Cx43 in the heart. Therefore, our first specific aim is to evaluate the effect of starvation and CR on cardic Cx43 protein levels, both *in vivo* (rat and mice) and in cultured cardiac cells.

On the other hand, given the fact that hypothalamus has a central role in energy homeostasis, through the release of hormones, neuropeptides, we further hypothesize that exosomes released by hypothalamic cells in starvation might have a protective effect on the heart, namely by improving GJIC.

Since the effects of CR involve a shift in metabolic rates, we also intend to investigate the mechanisms whereby a metabolic disorder, diabetes, contributes to Cx43 turnover. Therefore, another specific aim is to evaluate the effect of a by-product of glycolysis, MGO, on Cx43 degradation in cardiac cells. It was previously reported that hyperglycemia induces the ubiquitination of Cx43. However, it is conceivable that Cx43 undergoes another PTM, such as sumoylation. For that, we aim to evaluate whether sumoylation is a regulatory signal in GJIC.

With this work we expect to contribute to shed some light on the mechanisms whereby starvation and CR mediate cardioprotection. By understanding the molecular players involved, it might be possible to envision new strategies for the treatment of cardiac diseases.

3. Material and Methods

3.1. Cell culture

The rat cardiomyoblast cell line H9c2 and the embryonic mouse hypothalamus cell line N42 (mHypoE-N42, kindly provided by Dr. Cláudia Cavadas, CNC, Coimbra, Portugal) were cultured in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies, Carlsbad, CA), supplemented with 10% fetal bovine serum (FBS), 1% antibiotics (100 U/ml penicillin, 100 µg/mL streptomycin) and 1% GlutaMAX (Life Technologies, Carlsbad, CA). The cells were maintained at 37^{o}C in a humidified chamber with 5% CO_2.

The human embryonic kidney 293 (HEK-293) cells were cultured in 0.1% fibronectin coated culture vessels and maintained in DMEM (Life Technologies, Carlsbad, CA), supplemented with 10% FBS, 1% antibiotics (100 U/ml penicillin, 100 µg/mL streptomycin) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), at 37ºC under 5% CO_2.

3.2. Cell treatments

When indicated, starvation was induced in H9c2 and mHypoE-N42 cells by culturing cells in DMEM (Life Technologies, Carlsbad, CA), 1% antibiotics (100 U/ml penicillin, 100 µg/mL streptomycin) and 1% GlutaMAX (Life Technologies, Carlsbad, CA), not supplemented with FBS. The cells were maintained at 37^{o}C in a humidified chamber with 5% CO_2.

When indicated, ischemia was induced by a buffer exchange to an ischemia-mimetic solution (118 mM NaCl, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 1.2 mM $CaCl_2$, 25 mM $NaHCO_3$, 5 mM calcium lactate, 20 Mm 2-deoxy-D-glucose, 20 mM Na-HEPES, pH 6.6) and by placing the dishes in hypoxic pouches (GasPakTM EZ, BD Biosciences), equilibrated with 95% N_2/5% CO_2.

When indicated, CR was induced in H9c2 cells by culturing cells in DMEM low glucose (Life Technologies, Carlsbad, CA), supplemented with 10% FBS, 1% antibiotics (100 U/ml penicillin, 100 μg/mL streptomycin) and 1% GlutaMAX (Life Technologies, Carlsbad, CA). The cells were maintained at 37°C in a humidified chamber with 5% CO_2.

When indicated, H9c2 cells were treated with 1, 3 or 5 mM MGO (Sigma-Aldrich, St. Louis, MO, USA).

When indicated, cells were treated with different inducers and/or inhibitors: phosphorylation was induced by the addition of 5 μg/ml PMA (purchased from Sigma-Aldrich, St. Louis, MO, USA); lysosome-dependent degradation was inhibited by the addition of 50 nM Bafilomycin A1 (Baf) (Bioaustralis) and internalyzation was inhibited by the addition of 80 μM Dynasore (Sigma-Aldrich, St. Louis, MO, USA).

3.3. Cell Transfection (Genetic manipulation of cell cultures)

3.3.1. Plasmids

The expression plasmid encoding SUMO-1, SUMO-2 and Ubc9 was a kind gift from Dr. Andrea Pichler (Max Planck Institute of Immunobiology and Epigenetics, Department of Epigenetics, Freiburg, Germany).

3.3.2. DNA isolation

Plasmid DNA was isolated using QIAprep Spin Miniprep Kit (QIAGEN), according to manufacturer's instructions.

3.3.3. Transfection

One day before transfection, HEK-293 cells were seeded in pre-coated 6-well plate, so that the cells were 70% confluent at the time of transfection. For each transfection sample, 0.75 μg plasmid DNA was diluted in 50 μl Opti-MEM I Reduced

Serum Media (Life Technologies, Carlsbad, CA). Subsequently, 9 μl Lipofectamine 2000 (Life Technologies, Carlsbad, CA) transfection reagent were diluted in 50 μl Opti-MEM medium and incubated for 5 minutes at room temperature. After 5 minutes of incubation, diluted DNA and Lipofectamine were combined and incubated together for an additional 20 minutes. After incubation time was completed, DNA/Lipofectamine complexes were added to the medium of the cells. The cells were incubated at 37 ºC, under 5% CO_2, for 24 hours prior to the experiments performance.

3.4. Sample preparation and protein extraction from animal hearts

3.4.1. Animal experiments

Rats and mice were obtained from our local breeding colony (Faculty of Medicine of University of Coimbra, Coimbra, Portugal). All animals received care in accordance with the Portuguese Law on Experimentation with Laboratory Animals (last amendment, 2004), which is based on the principles of laboratory animal care as adopted by the EC Directive 86/609/EEC for animal experiments.

3.4.2. Sample preparation and protein extraction

Hearts were removed from mice euthanized by decapitation, that were submitted to caloric restriction (70% of the amount of food of controls), rats that were submitted to starvation for 24, 48 and 72 hours, mice that were submitted to starvation for 48 and 72 hours and respective controls. The hearts from each condition were excised and a heart slice from each heart was lysed in 600 μl RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7), containing protease inhibitors and phosphatase inhibitors (protease inhibitor cocktail (Roche, Basel, Switzerland), 2 mM phenylmethanesulfonyl fluoride (PMSF), 10 mM iodoacetamide and 2 mM sodium ortovanadate). The samples were homogenized using the Polytron Homogenizer (PT 1200E, Kinematica, Fisher Scientific). Cell lysates were incubated

15 minutes on ice, and the solubilized fraction was recovered in the supernatant after centrifugation at 3200 rpm for 5 minutes. Then, the solubilized fraction was recovered in the supernatant after centrifugation at 13200 rpm for 10 minutes. Protein concentration was determined by the DC Protein Assay (BioRad, Hercules, CA, USA), after which 30 µg of the supernatants (total extract/input) were denatured with 4x loading buffer and then a western blot was performed.

3.5. Isolation of exosomes from cell culture

One day before isolation of exosomes, H9c2 and N42 cells cultured in complete DMEM (see cell culture section) were replaced by DMEM supplemented with 10% FBS depleted of exosomes and maintained at 37^{o}C in a humidified chamber with 5% CO_2, during the indicated time periods. Serum was depleted of exosomes by ultracentrifugation at 154.000 g, for 16 hours in a 1:1 dilution to minimize serum proteins' loss due to its viscosity [83].

Exosomes derived from cultured cells were isolated from conditioned medium after culture in either exosome-depleted medium or starvation medium, for the indicated time periods. Afterwards, the medium was collected and exosomes were isolated by ultracentrifugation [84]. In brief, the harvested supernatants were subjected to differential centrifugation at 4°C, starting with a 300 g centrifugation, for 10 min, followed by a 16.500 g centrifugation for 20 minutes. To thoroughly remove cellular debris and larger particles, the supernatants were filtered with a 0.22-µm filter unit and then ultracentrifuged at 120.000 g, for 70 minutes. The resultant pellet was washed with phosphate buffered saline (PBS) and, after ultracentrifugation, exosomes were resuspended in PBS (sterile when used for biological assays).

3.6. PKH26 exosomes staining and sucrose gradient for exosomes

H9c2 and N42 cells were cultured either in exosome-depleted medium or starvation medium for 4 hours, after which exosomes were obtained by

ultracentrifugation from cell culture supernatants. For dye uptake assays, 5 μg of exosomes per condition were labeled with PKH26 Fluorescent Cell Linker, resuspended in Diluent C, followed by 5 minutes incubation with PKH26. Excess dye was washed by exosomal floatation on a sucrose gradient, where the exosomes were placed at the bottom of the ultracentrifuge tube and filled with a discontinuous gradient of sucrose (from 2.5 M to 0.4 M) and ultracentrifuged overnight, at 160.000 g. Fractions from 4-10 were collected and washed with PBS by ultracentrifugation.

3.7. Immunofluorescence staining

Heart slices obtained from animals subjected to starvation and CR were either embedded in OCT (Tissue-Tek, Histolab, Sweden) for cryosectioning, or snap-frozen in liquid nitrogen for biochemical studies, before storage at -80°C. Tissue samples (5 μm cryosections) were fixed with acetone, at -20ºC for 10 min. The samples were then washed three times with PBS, and blocked with 5% BSA for 1 hour prior to incubation with primary antibodies. Incubation with primary antibodies against Cx43 and N-cadherin proceeded for 1 hour at room temperature. The samples were then washed three times with PBS before incubation with the secondary antibody for 1 hour at room temperature. The specimens were rinsed in PBS and mounted with MOWIOL 4-88 Reagent. Nuclei were stained with DAPI. All antibodies were diluted in 5% BSA. For controls, primary antibodies were omitted. The images were collected by fluorescence microscopy using a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

H9c2 cells grown on 96-well plate, PKH26 stained exosomes were added to these cells and incubated for 6 hours. After, cells were fixed with 4% paraformaldehyde (PFA) in PBS, for 10 minutes, then washed three times with PBS, permeabilized with 0.2% v/v Triton X-100 in PBS, for 10 minutes, and blocked with 2.5% Bovine serum albumin (BSA) for 20 minutes. Nuclei were stained with DAPI. The specimens were rinsed in PBS. All solutions were made in 0.25% w/v BSA containing 0.02% sodium azide in PBS. For controls, primary antibodies were omitted. The images were collected

by fluorescence microscopy using a Zeiss Axio HXP IRE 2 (Carl Zeiss AG, Jena, Germany).

3.8. MTT cell viability assay

After treatments, H9c2 cells seeded onto 96-well plates were washed twice with PBS and incubated with 0.5 mg/ml MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Invitrogen, Carlsbad, CA, USA] in DMEM for 2 hours at 37°C in a cell culture incubator. Subsequently, supernatants were removed and the precipitated dye was dissolved in 300 µL 0.04 M HCl (in isopropanol) and quantified at a wavelength of 570 nm, with wavelength correction at 620 nm, using a Biotek Synergy HT spectrophotometer (Biotek, Winooski, VT, USA).

3.9. Transmission electron microscopy (TEM)

Exosomes were fixed with 2% PFA and deposited on Formvar-carbon coated grids (TAAB Laboratories Equipment, Berks, UK). Samples were washed with PBS and fixed with 1% glutaraldehyde for 5 minutes. Following a total of 7 washes using distilled water, grids were contrasted with an uranyl-oxalate solution, pH 7, for 5 minutes, and transferred to methyl-cellulose-uranyl acetate for 10 minutes on ice, as described by Théry and colleagues [(84)]. The images were collected using a Tecnai G2 Spirit BioTWIN electron microscope (FEI, Oregon, USA) at 80 kV.

3.10. Immunoprecipitation (IP)

After appropriate treatments, HEK-293 cells cultured in pre-coated 6-well plate were washed twice in ice-cold PBS, scrapped off the wells and lysed in 150 µl RIPA buffer pH 7 (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7) with protease and phosphatase inhibitors and 20 mM N-ethylmaleimide (NEM). The lysates were centrifuged at 3200 rpm for 5 minutes, and 10% of the total volume of sample

was denatured (inputs), being the remaining lysates used for IP. Then, 15 µl of protein G-Sepharose (GE Healthcare Biosciences, Uppsala, Sweden) beads were incubated with 0,5 µl of goat polyclonal antibodies directed against Cx43 during 2 hours, at 4ºC, with gentle agitation. Sample pre-cleaning was performed by adding the sample to 15 µl of protein A-Sepharose (GE Healthcare Biosciences, Uppsala, Sweden) beads and incubated during 2 hours, at 4ºC, with gentle agitation. Thereafter, the lysates were added to the protein G-Sepharose sediments washed 3 times in RIPA buffer pH 7, eluted with 15 µl of 2x loading buffer with 40 mM NEM and without β-mercaptoethanol (non-reduction conditions). In reducing conditions the cells were lysed in RIPA buffer (150 mM NaCl, 50 mM Tris-HCl, 1% NP-40 and 0.1% SDS, pH 7.5) without NEM and the loading buffer added does not have NEM neither β-mercaptoethanol.

The electrophoresis was performed at 4^{o}C and the western blot analysis was performed as described in the next section.

3.11. Western blot (WB) analysis

When indicated, H9c2 and N42 cells were washed twice in ice-cold PBS, scraped off the wells and cells were denatured with 2x Laemmli buffer and boiled at 95^{o}C for 5 minutes.

After appropriate treatments, HEK-293 cells cultured in pre-coated 6-well plate were washed twice in ice-cold PBS, scraped off the dishes and lysed in 150 µl RIPA buffer pH 7.5 with protease and phosphatase inhibitors or in 150 µl RIPA buffer pH 7 with protease and phosphatase inhibitors and 20 mM NEM. Cell lysates were incubated 15 minutes on ice, and the solubilized fraction was recovered in the supernatant after centrifugation at 3200 rpm for 5 minutes. Protein concentration was determined by the DC Protein Assay (BioRad, Hercules, CA, USA), after which 30 µg of the supernatants (total extract/input) were denatured with 2x Laemmli buffer and boiled at 95^{o}C for 5 minutes (reducing conditions) or denatured with 2x loading buffer

without β-mercaptoethanol and with 40 mM NEM and boiled at 50^{o}C for 15 minutes (non-reducing conditions).

Total cell lysates were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), and transferred to nitrocellulose membranes. The membranes were blocked with 5% non-fat milk in Tris-buffered saline-Tween 20 (TBS-T) (20mM Tris, 150 mM NaCl, 0.2% Tween 20, pH 7.6), probed with appropriate primary antibodies and horseradish peroxidase (HRP)-conjugated secondary antibodies. All antibodies used in this work are listed in Table 1. The proteins of interest were visualized by chemiluminescence using a VersaDoc system (BioRad). Densitometric quantification was performed in unsaturated images using ImageJ (National Institutes of Health, Bethesda, MD).

Table 1: List of primary and secondary antibodies used for the WB, IP and immunofluorescence microscopy analysis.

Antibody	Host/Clonality	Clone/Cat#	Application	Dilution	Company
anti-Cx43	goat polyclonal	AB0016-500	WB/IP	1:2500/-	SICGEN (Cantanhede, Portugal)
anti-Cx43	mouse monoclonal	610062	IF	1:50	BD Transduction Laboratories (CA, USA)
Anti-Flotillin	rabbit polyclonal	sc-25506	WB	1:500	Santa Cruz Biotechnology (Texas, USA)
Anti-HA	rabbit polyclonal	71550	WB	1:300	Life Technologies (Carlsbad, CA)
Anti-Hsc70	rat monoclonal	ADI-SPA-815	WB	1:500	Enzo Life Sciences
N-Cadherin	rabbit polyclonal	H-63	IF	1:50	Santa Cruz Biotechnology, Santa Cruz, CA
anti-Ubiquitin	mouse monoclonal	P4D1	WB	1:1000	Covance (Princeton, NJ, USA)
anti-SUMO1	mouse monoclonal	21C7-c	WB	1:500	DSHB (University of Iowa)
anti-SUMO2	mouse monoclonal	8A2-c	WB	1:500	DSHB (University of Iowa)
anti-GAPDH	goat polyclonal	AB0049	WB	1:2500	SICGEN (Cantanhede, Portugal)
anti-Calnexin	goat polyclonal	AB0041	WB	1:2500	SICGEN (Cantanhede, Portugal)
anti-Tubulin	mouse monoclonal	T6199	WB	1:2000	Sigma-Aldrich (St. Louis, MO, USA)
anti-goat - HRP	rabbit	61-1620	WB	1:5000	Life Technologies (Carlsbad, CA)
anti-rabbit - HRP	goat	656120#	WB	1:5000	BioRad (Hercules, CA, USA)
anti-mouse - HRP	goat	626520#	WB	1:5000	BioRad (Hercules, CA, USA)
alexa 488 anti-mouse	goat	A-11034	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)
alexa 568 anti-rabbit	goat	A-11011	IF	1:200	Molecular Probes, Life Technologies (Carlsbad, CA)

3.12. Statistical analysis

All data were analysed with GraphPad Prism 6 for Windows, version 6.01 (GraphPad Software, Inc.). Results are expressed as mean ± S.E.M. Student's t-test with Welch correction was applied. Differences were considered significant at $p<0.05$.

4. Results

Caloric restriction (CR), defined as a reduction in calorie intake below the usual *ad libitum* intake without malnutrition, consistently extends lifespan in multiple organisms, from yeast to mammals, and delays the progression of age-related diseases, including CVDs [76, 85]. Indeed, ample of clinical and experimental evidence has demonstrated in numerous species that CR can increase lifespan, reduce the incidence and delay the onset of age-related diseases, improve stress resistance and particularly can exert cardioprotection [86]. However, the cellular mechanisms underlying this kind of protection conferred by CR and starvation remain elusive [87]. Given the crucial role of Cx43 in ensuring IC between cardiomyocytes and its importance in heart function, and based on previous data from our group that showed that short periods of starvation in cardiomyocyte (HL-1) cells stabilizes Cx43 in the PM, we hypothesized that the protective effect of starvation and CR in the heart is mediated by an improvement of GJIC, through the increase of Cx43 levels. Therefore, with the present study we intended to evaluate if animals subjected to starvation and CR have increased cardiac Cx43 levels compared with control animals.

A common approach used to induce CR in animals is to measure the *ad libitum* food intake of an individual animal (control animal) and then reduce the food provided to another animal (animal subjected to CR) by a certain percentage. The magnitude of CR applied in most rodent studies varies between a reduction in calories of 5% (mild restriction) and 50% (severe restriction). In this study it was decided to use a moderate restriction of 30%, so the daily caloric intake to mice subjected to CR has been restricted to 70% of the average food intake provided to mice eating *ad libitum* (control animals). Moreover, to evaluate the effect of starvation on cardiac Cx43 levels, we submitted rats to starvation during 24, 48 and 72 hours and mice to starvation during 48 and 72 hours after which we evaluated the total protein levels of Cx43 in heart lysates by WB.

Results presented in Figure 8A show that heart lysates from Wistar rats subjected to 48 and 72 hours of starvation have increased levels of Cx43 compared to control groups. Furthermore, the heart lysates from C57BL/6 mice subjected to starvation during 48 and

72 hours also show an increase in Cx43 levels compared to control groups, with a statistically significant increase between the group of mice submitted to starvation during 72 hours and control group ($p<0.01$) (Figure 8B). We also evaluated the subcellular distribution of Cx43 in heart sections of Wistar rats subjected to 48 hours of starvation, by fluorescence microscopy. In accordance with WB data, our results show an increased staining of Cx43 in starved animals, compared to control group (Figure 8C).

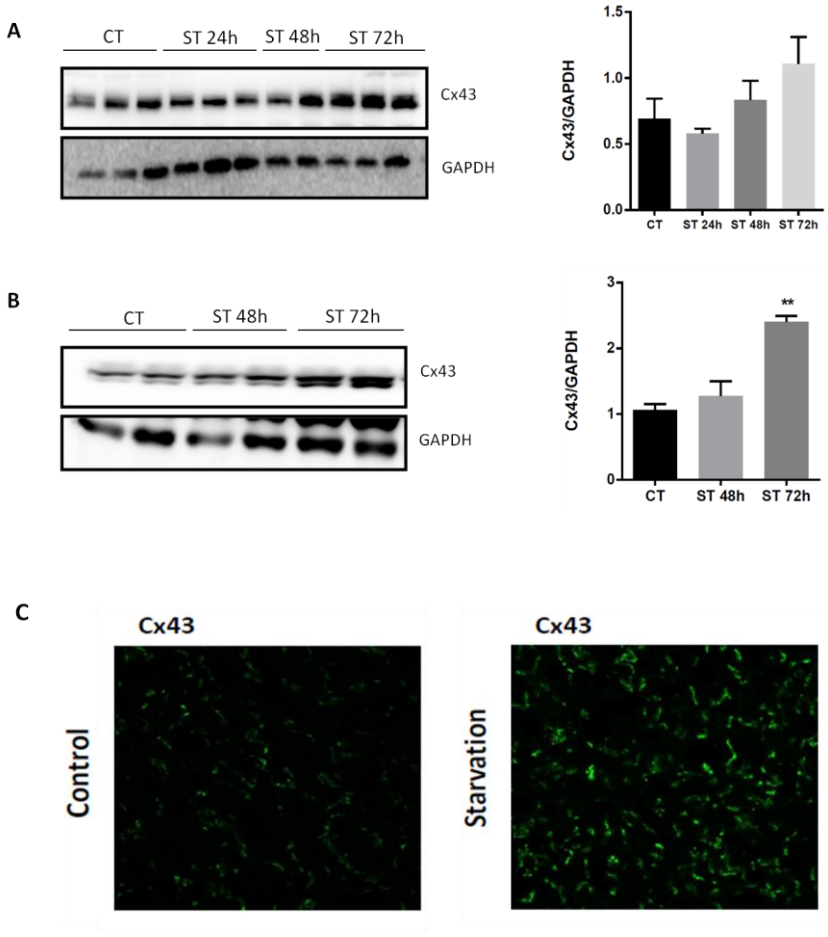
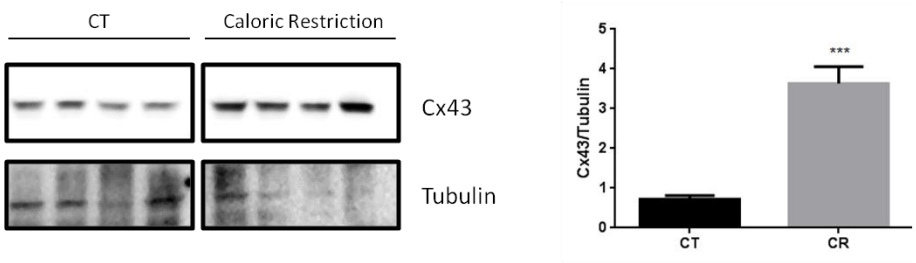


Figure 8. Starvation leads to an accumulation of cardiac total Cx43 levels.

Heart lysates from rats Wistar rats were subjected to 24, 48 and 72 hours of starvation **(A)** and C57BL/6 mice were subjected to 48 and 72 hours of starvation (ST 48h and ST 72h) **(B)** were analyzed by WB using goat polyclonal antibodies anti-Cx43. Starved animals show an increase of Cx43 levels compared to control groups (CT). Representative graphs showing Cx43 protein levels. Values are mean ± SD (n=4). **$p<0.01$ vs CT. **(C)** Fluorescence microscopy analysis of heart sections from control and 48 hour-starved Wistar rats. Starved animals show an increase staining of Cx43.

Likewise, we submitted C57BL/6 mice to CR (70% of total food consumed by control group) during 1 month and we evaluated the total protein levels of Cx43 in heart lysates by WB, and the subcellular distribution of Cx43 by fluorescence microscopy. Our results from WB analysis show that the heart lysates from C57BL/6 mice that were submitted to CR show an increase of total Cx43 levels compared to control group (CT) ($p<0.001$) (Figure 9A). In agreement with biochemical data, the Figure 9B shows an increased staining of Cx43 (in green) in heart sections from animals that were submitted to CR compared to control group ($p<0.01$).

A



B

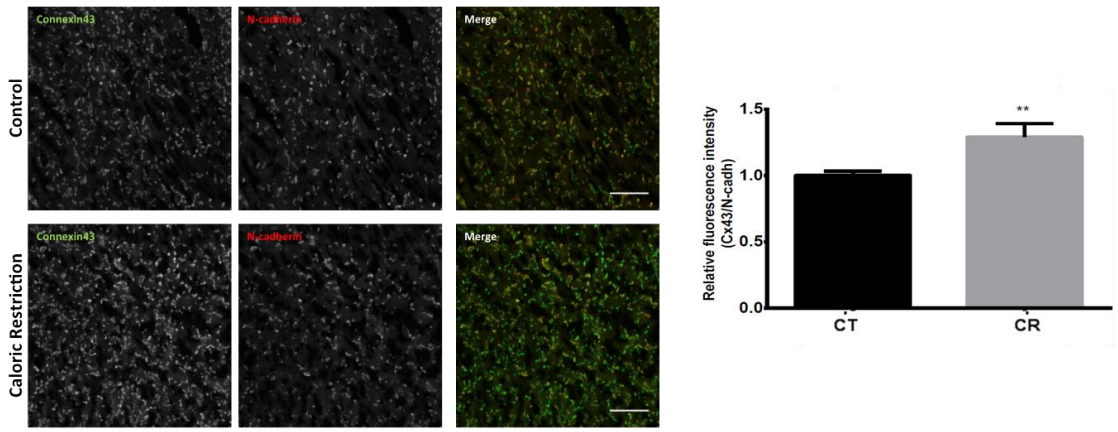


Figure 9. Caloric restriction leads to an accumulation of cardiac Cx43 levels.

C57BL/6 mice were subjected to CR (70% of food intaked by control group) during 1 month. **(A)** Heart lysates from mice were analyzed by WB using goat polyclonal antibodies anti-Cx43. Heart lysates from animals that were submitted to CR show an increase of Cx43 levels compared to control group (CT). Representative graph showing Cx43 levels. Values are mean ± SD (n=4). ***$p<0.001$ vs CT. **(B)** Fluorescence microscopy analysis of heart sections Mice submitted to CR show an increase staining of Cx43 compared to

control group (CT). Representative graph showing relative Cx43 levels. Values are mean ± SD (n=4). **p<0.01 vs CT.

Based on these results, we aimed to explore whether the increase of total cardiac Cx43 levels caused by starvation and CR could be mediated by exosomes. As described above, exosomes are EVs released by the majority of cells and their content varies according to cell origin, environment and stimuli, and as consequence, they can have different effects in recipient cells. In other words, we aimed to investigate if exosomes from cells subjected to starvation have the same effect on cardiomyocytes that starvation have in the heart, focusing on the levels of Cx43. For that, we first evaluated the effect of starvation-derived exosomes from cardiac cells (cardiomyoblast H9c2 cell line) on the total protein levels of Cx43 in recipient H9c2 cells. Then, once the hypothalamus is the first sensor of control of food intake, we evaluated if starvation-derived exosomes from hypothalamus neurons (mHypoE-N42 cell line) could affect the total protein levels of Cx43 in H9c2 cells.

Therefore, in a first stage, we had to test if we could successfully isolate exosomes from cell culture medium, released by cardiomyoblast H9c2 cells and hypothalamus N42 (mHypoE-N42) cells. For that, we evaluated, by WB, the presence of proteins that are consistently found in exosomes, and are, for that reason, considered exosomal markers. We also characterized exosomes based on their size and morphology by TEM. The results presented in Figure 10A and 10B show that vesicle-enriched extract, obtained from both H9c2 cells and N42 cells, present high amounts of proteins usually detected in exosomes, including Hsc70 and Flotillin. We also demonstrate that exosomes released by both H9c2 and N42 cells are within the exosome size range and have the typical exosomal morphology (Figure 10C and 10D, respectively), thus demonstrating that we can obtain an enriched population of exosomes. Furthermore, the Figure 10E shows an enriched population of MVs that was isolated from cell culture medium and visualized by TEM, thus confirming that we can isolate MVs and they are effectively different from exosomes.

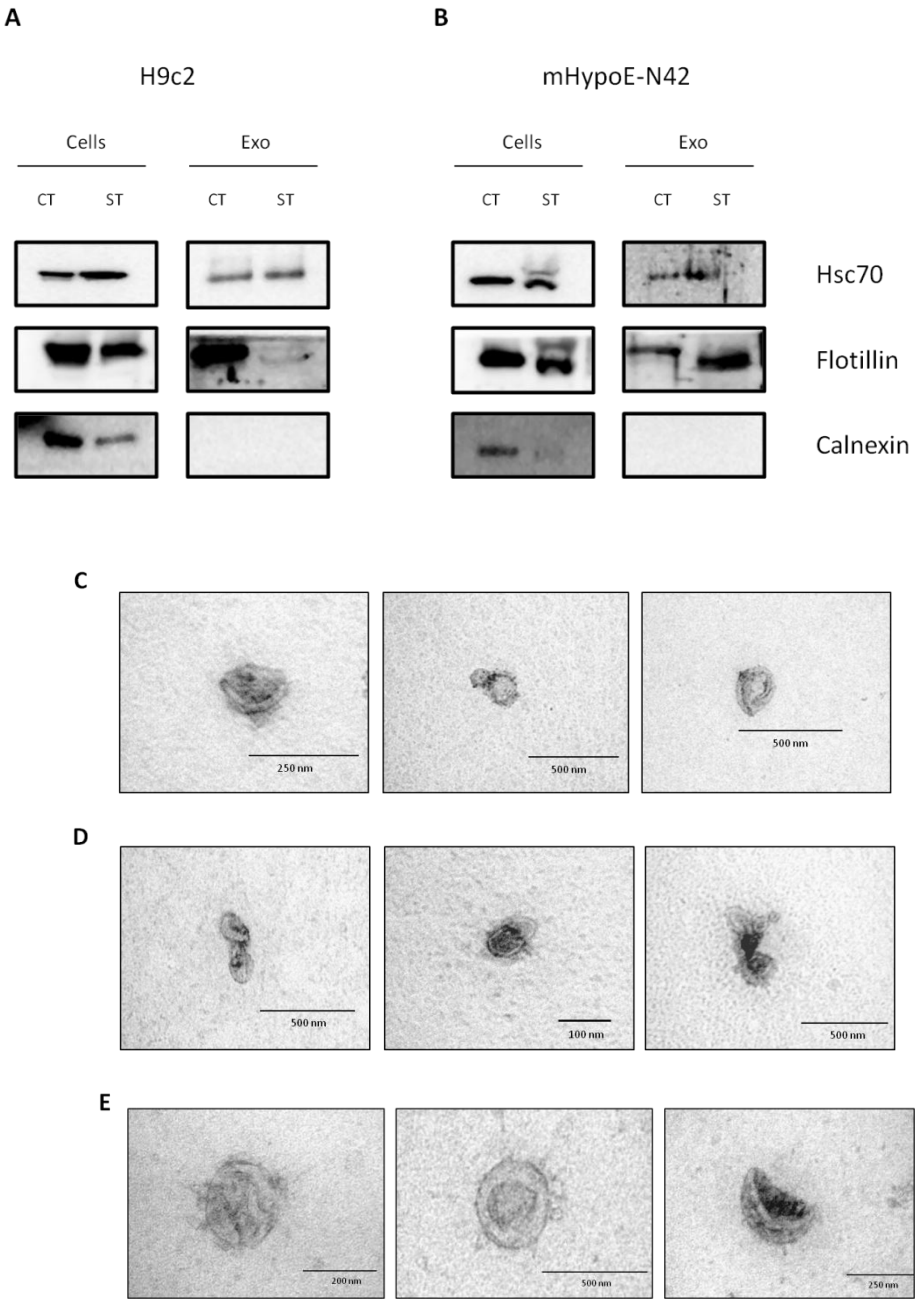


Figure 10. Characterization of exosomes released by H9c2 and N42 cells.

H9c2 and N42 cells were cultured in exosome-depleted medium (CT) or starvation medium (ST) for 4 hours and the released exosomes, during this period of time, were isolated by differential centrifugation. Exosomal (exo) and cellular (cells) extracts from H9c2 **(A)** and N42 **(B)** were evaluated by WB. H9c2 exosomes **(C)** and N42 exosomes **(D)** were observed by TEM. **(E)** H9c2 and N42 cells were cultured in exosome-depleted medium (CT) or starvation medium (ST) for 4 hours and the released MVs, during this period of time, were isolated by centrifugations as already described. MVs were observed by TEM.

One of the main objectives of this part of the work is to establish the exosome-mediated communication between cells. According to our hypothesis, exosomes secreted by cells subjected or not to starvation, can modulate the levels of Cx43 in target cells. For that, exosomes have to be taken up by the acceptor cells. To assess this question, we isolated control and starvation exosomes from H9c2 and N42 cells, incubated either in the presence or absence of serum (starvation), labeled the vesicles with red fluorescent lipidic dye PKH26, and then added them to H9c2 cells. After 4 hours of incubation, cells were fixed with PFA and visualized by fluorescence microscopy. As demonstrated in Figure 11A and 11B, by the intracellular presence of PKH26 stained vesicles, both control and starvation exosomes from either H9c2 or N42 cells were internalized by H9c2 cells.

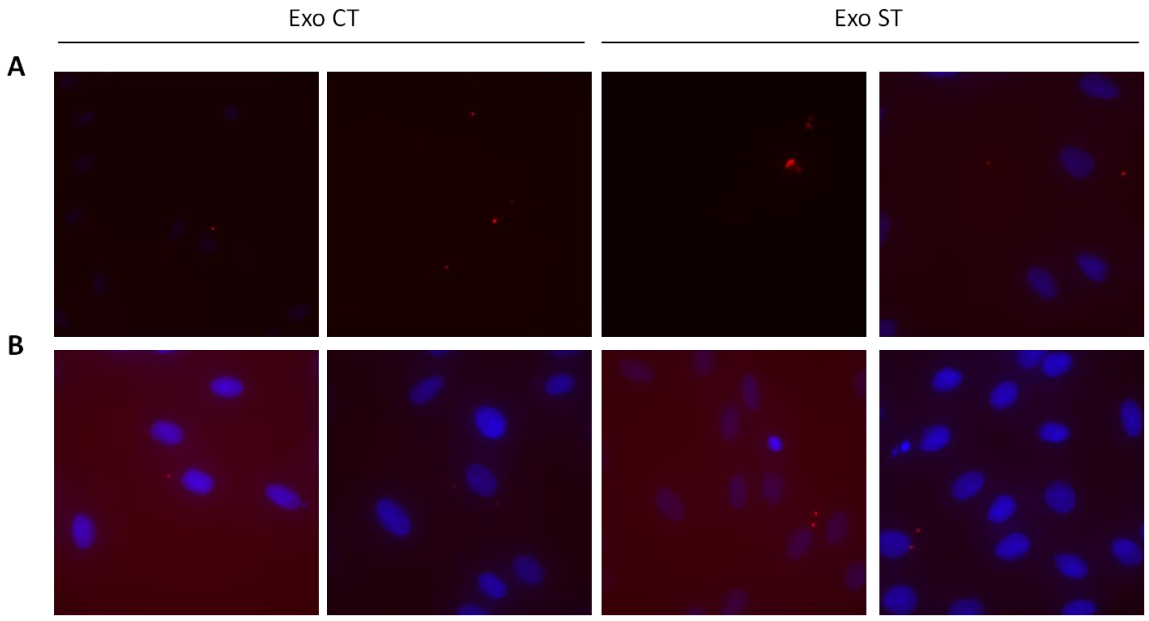


Figure 11. Internalization of both control and starvation-derived exosomes from either H9c2 or N42 cells. H9c2 and N42 cells were cultured in exosome-depleted medium (CT) or starvation medium (ST) for 4 hours and the released exosomes, during this period of time, were isolated by differential centrifugation. Exosomes were stained with PKH26 as described before. Then, H9c2 cells were incubated for 4 hours, in exosome-depleted medium, with exosomes from H9c2 cells **(A)** or exosomes from N42 cells **(B)** stained with PKH26 obtained from either control (Exo CT) or starvation (Exo ST). Cells were fixed and visualized by fluorescence microscopy.

The results previously presented demonstrate that we can successfully isolate a pure population of exosomes released by H9c2 and N42 cells. Moreover, we showed that these exosomes are internalized by H9c2 cells.

We subsequently proceeded to evaluate the effect of starvation-derived exosomes in the total Cx43 protein levels in H9c2 cells, by incubating H9c2 cells either in the presence (control) or absence of serum (starvation) for 30, 60, 120 and 240 minutes conditions. In control conditions, H9c2 cells were incubated in exosome-depleted medium (FBS without exosomes). After these periods of time, the media and the respective cellular extracts were collected. The Figure 12A represents the Cx43 levels present in H9c2 cells subjected to control or starvation. This result shows that in short periods of starvation (60 minutes) there is a slight increase of Cx43 levels whereas for long periods of starvation (120 and 240 minutes) the levels of Cx43 in H9c2 cells decrease. Furthermore, we evaluated the effect of exosomes secreted by control or starvation cells in Cx43 levels of H9c2 cells that receive these exosomes. After 12 hours of incubation of cells with exosomes, H9c2 cells that received exosomes derived from H9c2 cells submitted to control or starvation had increased levels of Cx43 compared to H9c2 cells that did not received exosomes, as showed in Figure 12B. Interestingly, while the levels of Cx43 decrease in cells subjected to increasing periods of starvation, the exosomes secreted by these cells do not induce the same effect regarding the Cx43 levels in target cells.

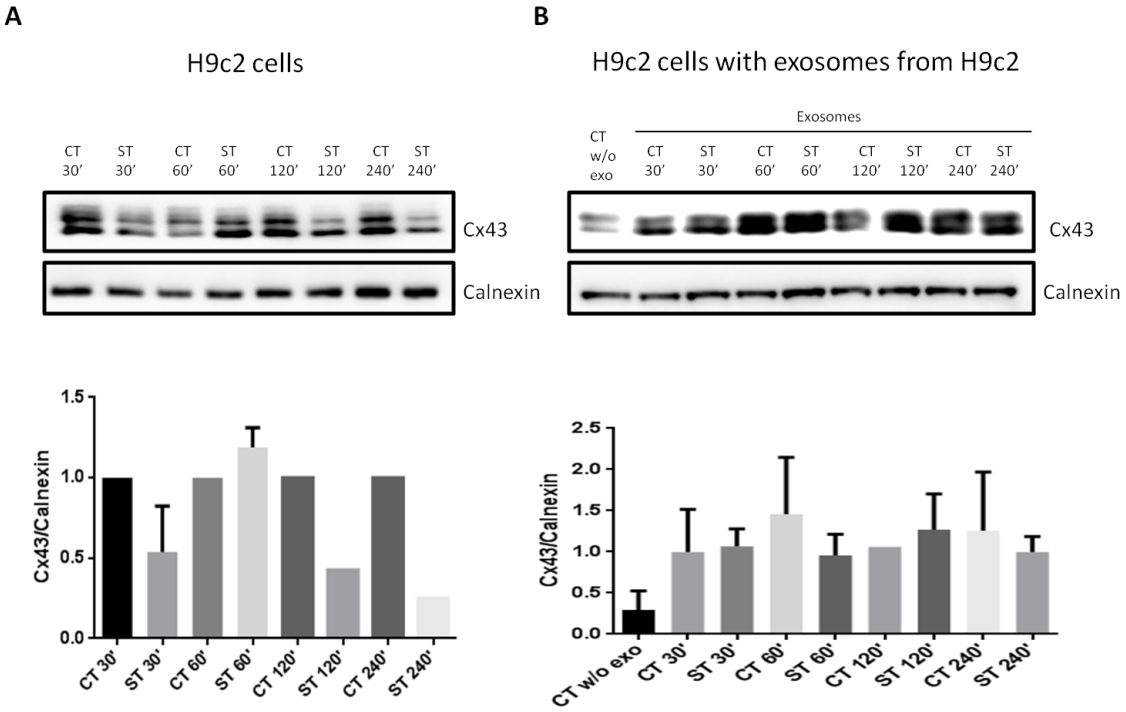


Figure 12. Exosomes from H9c2 cells increase the levels of Cx43 in H9c2 cells.

(A) H9c2 cells were subjected to control (CT) and starvation (ST) during 30, 60, 120 and 240 minutes conditions. Short period of starvation (60 minutes) leads to an increase of Cx43 levels and long periods of starvation (120 and 240 minutes) leads to its decrease. The protein levels were determined by WB. Representative graph showing Cx43 levels. **(B)** Exosomes released by these H9c2 cells that were submitted to control and starvation conditions were isolated by differential ultracentrifugation and then added to H9c2 cells. After 12 hours of incubation, H9c2 cells that received exosomes from H9c2 cells submitted to control and starvation had increased levels of Cx43 compared to H9c2 cells that not received exosomes (CT without exosomes). There are no differences between the levels of Cx43 in H9c2 that were incubated with control-derived exosomes and starvation-derived exosomes. The protein levels were determined by WB. Representative graph showing Cx43 levels.

Next, we evaluated whether these starvation-derived exosomes were protective against ischemic insults, by determining the cellular viability. For that purpose, exosomes isolated from 30, 60, 120 and 240 minutes-H9c2 cells that were submitted to control or starvation were added to H9c2 cells and incubated during 12 hours. After 12 hours of incubation of cells with exosomes, we submitted H9c2 cells to ischemia during 2 hours and then the cellular viability of control and ischemic H9c2 cells was determined by MTT

assay. The representative graph in Figure 13A shows that ischemia decreases cellular viability in H9c2 cells, regardless of the presence or absence of exosomes. The representative graphs in Figure 13B show that only pre-incubation with exosomes released by cells subjected to 30 minutes of starvation seems to promote a tendency to protect cells against ischemic-induced lesion.

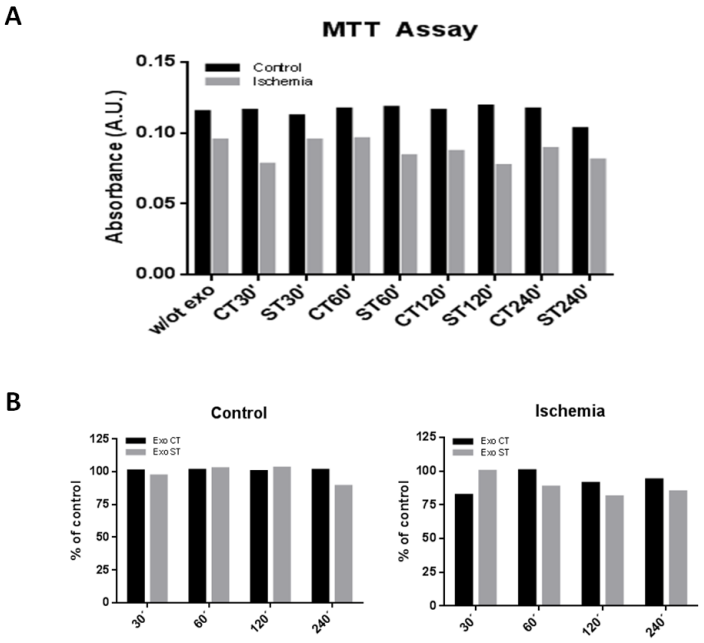


Figure 13. Cellular viability of control and ischemic H9c2 cells is not altered by either control or starvation H9c2-derived exosomes.

H9c2 cells were subjected to control and starvation conditions during 30, 60, 120 and 240 minutes. Exosomes isolated by differential ultracentrifugation were added to H9c2 cells and incubated during 12 hours. After this period, H9c2 recipient cells were subjected to ischemia during 2 hours and then tested for cell viability by MTT assay, with triplicates of each condition. **(A)** Representative graph showing the absorbance in arbitrary units (A.U.) of control and ischemic H9c2 cells. **(B)** Either control or starvation H9c2-derived exosomes do not alter control and ischemic H9c2 cell viability. Representative graphs showing percentage of control and ischemic H9c2 cell viability comparing with cellular viability of H9c2 control cells without exosomes added.

The previous results show that starvation-derived exosomes do not increase the levels of Cx43 in H9c2 cells neither increase the cellular viability in ischemic H9c2 cells. So, in order to explore whether the increase of the Cx43 levels in rats and mice hearts submitted to starvation and CR is a result of exosomes released from hypothalamus, the first sensor of control of food intake responsible for energy homeostasis, we evaluated the effect of starvation-derived exosomes from N42 cells on total protein levels of Cx43 in H9c2 cells. For that purpose, we first subjected N42 cells to control or starvation during 30, 60, 120 and 240 minutes conditions. After that, the media and the respective cellular extracts were collected. First we evaluated the effect of starvation on Cx43 levels, by WB. The Figure 14A shows that the amount of Cx43 levels decrease in N42 cells subjected to starvation. Furthermore, exosomes isolated from these hypothalamic cells subjected to control or starvation conditions were then added to H9c2 cells. After 12 hours of incubation, H9c2 cells that received exosomes from N42 cells submitted to control or starvation had increased levels of Cx43 compared to H9c2 cells that did not received exosomes, as showed in Figure 14B. However, the changes on Cx43 levels in acceptor cells is the same regardless the exosome-producing cells were subject or not to starvation.

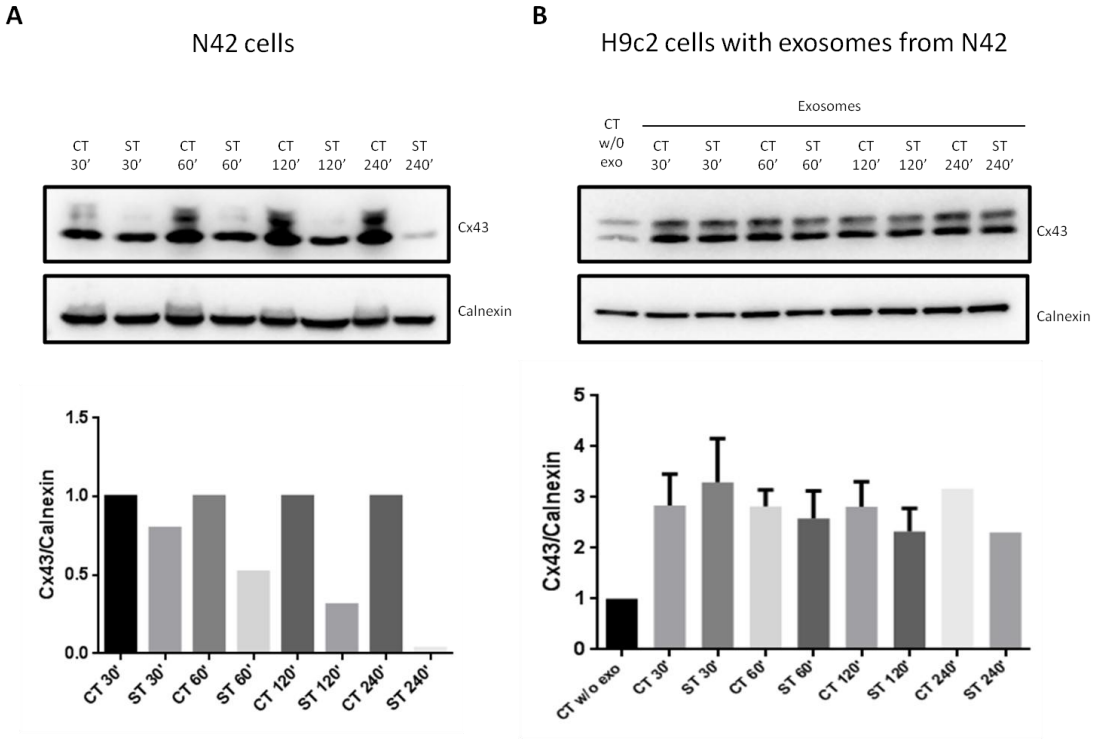


Figure 14. Exosomes from N42 cells increase the levels of Cx43 in H9c2 cells.

(A) N42 cells were subjected to control (CT) and starvation (ST) during 30, 60, 120 and 240 minutes conditions. Starvation leads to a decrease on Cx43 levels in N42 cells. In N42 cells subjected to starvation, the Cx43 levels decrease. The protein levels were determined by WB. Representative graph showing Cx43 levels. **(B)** Exosomes released by these N42 cells were isolated by differential ultracentrifugation and added to H9c2 cells. After 12 hours of incubation, H9c2 cells that received exosomes from N42 cells submitted to control and starvation had increased levels of Cx43 compared to H9c2 cells that did not received exosomes (CT without exosomes). The protein levels were determined by WB. Representative graph showing Cx43 levels.

Next, such as we determined for exosomes from cardiomyoblast H9c2 cells, we evaluated whether the starvation-N42 derived exosomes are protective in the context of H9c2 cellular viability in ischemia. For that, we isolated exosomes from N42 cells that were submitted to control and starvation during 30, 60, 120 and 240 minutes and added them to H9c2 cells. After 12 hours of incubation of cells with exosomes, H9c2 cells were submitted to ischemia during 2 hours and then the cell viability of control and ischemic H9c2 cells was determined by MTT assay. The graph in Figure 15A shows that H9c2 cells in ischemia have decreased cell viability compared with H9c2 control cells. The

representative graphs in Figure 15B show the percentage of control and ischemic H9c2 cell viability comparing with cell viability of H9c2 control cells without exosomes added. Either control or starvation hypothalamus neurons-derived exosomes do not alter control and ischemic H9c2 cell viability.

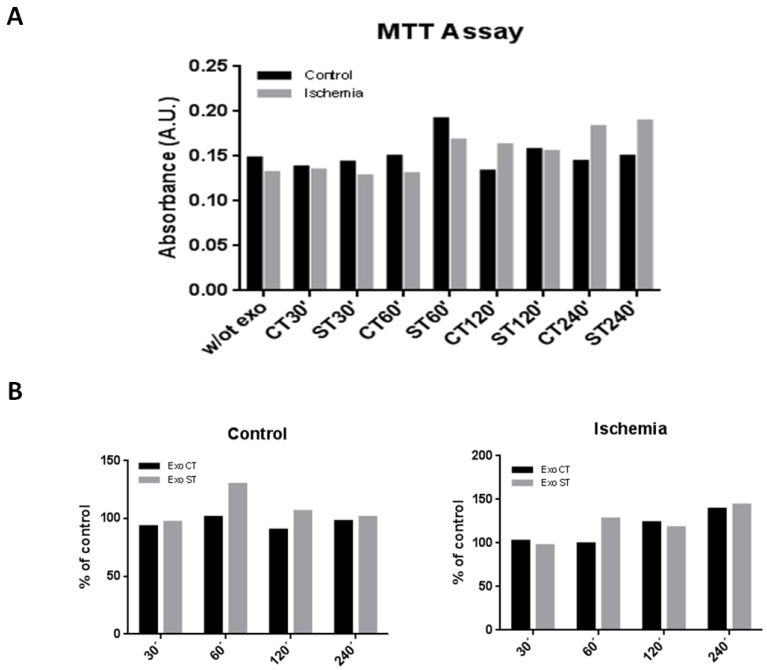


Figure 15. Cellular viability of control and ischemic H9c2 cells is not altered by either control or starvation N42-derived exosomes.

N42 cells were subjected to control and starvation conditions during 30, 60, 120 and 240 minutes. Exosomes isolated by differential ultracentrifugation were added to H9c2 cells and incubated during 12 hours. After this period, H9c2 recipient cells were subjected to ischemia during 2 hours and then tested for cell viability by MTT assay, with triplicates of each condition. **(A)** H9c2 cells in ischemia show a decrease in cell viability, regardless their incubation with control or starvation exosomes from any time. Representative graph showing the absorbance in arbitrary units (A.U.) of control and ischemic H9c2 cells. **(B)** Neither control nor starvation N42-derived exosomes alter cell viability in control and ischemic conditions. Representative graphs showing percentage of control and ischemic H9c2 cell viability comparing with cell viability of H9c2 control cells without exosomes added.

Besides starvation, we also evaluated the effect of CR in H9c2 cells. Like other studies, our strategy to study CR in cells was to reduce the glucose availability, inducing hypoglycemia in H9c2 cells. For that purpose, two sets of H9c2 cells were maintained in high glucose (HG; 1) and in low glucose (LG; 2) media for two weeks and then we tranfered to LG (3) and HG (4) respectively for 24 hours. The results presented in Figure 16 shows that the change of DMEM high glucose to DMEM low glucose (HG-LG) induces an increase on the levels of Cx43 whereas the change of LG to HG (LG-HG) in H9c2 cells leads to a decrease on Cx43 levels in H9c2 cells. These results demonstrate that metabolic changes involving either a decrease (CR) or an increase (hyperglycemia) in glucose results in opposite effects concerning the levels of Cx43. Therefore, and once we established that CR leads to the stabilization of Cx43, in a subsequent satge of this study we proceeded to investigate the mechanisms underlying diabetes-induced degradation of Cx43.

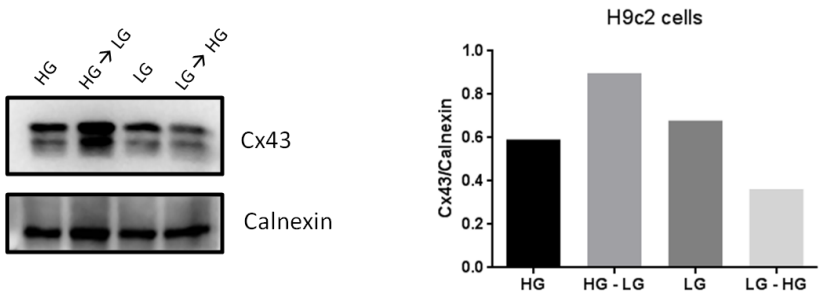
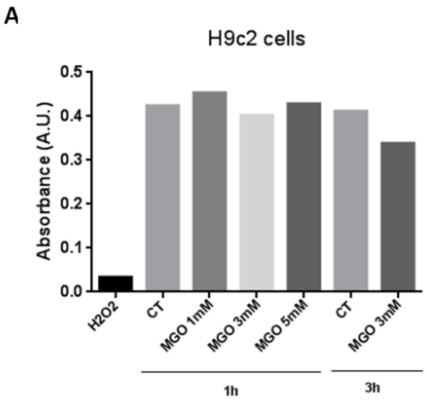
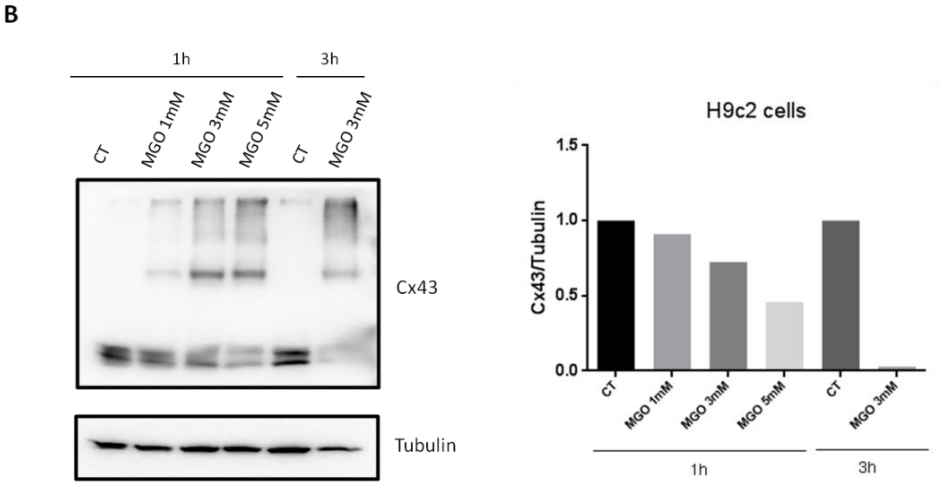


Figure 16. Hypoglycemia leads to an increase on Cx43 levels whereas hyperglycemia induces its decrease. H9c2 cells were maintained in DMEM high glucose (HG) and in DMEM low glucose (LG) during 2 weeks. After this period, the cells that were maintained in DMEM HG were cultured in DMEM LG (HG-LG) and the cells that were maintained in DMEM LG were cultured in DMEM HG (LG-HG) for 24 hours. Protein levels were determined by WB. Representative graph showing Cx43 levels.

It was previously shown by other studies that diabetes and hyperglycemia lead to intracellular accumulation of methylglyoxal (MGO) in many different tissues. MGO is a highly reactive α-oxoaldehyde formed as a by-product of glycolysis, which leads to the formation of advanced glycation end products (AGEs), products that are related with cell and tissue impairment and, and particularly in the heart, can induce cardiac dysfunction [(11-13)]. Since diabetics have an accumulation of MGO in tissues and given our results

showing that hyperglycemia leads to a decrease on Cx43 in H9c2 cells, we aimed to evaluate the effect of MGO (that mimetizes hyperglycemia) in H9c2 cells, in context of diabetic heart disease. For that purpose, H9c2 cells were treated with 1, 3 and 5 mM MGO for 1 hour or with 3 mM MGO for 3 hours. First, we performed a MTT assay to evaluate if MGO alter the cellular viability of H9c2 cells. As show in Figure 17A, MGO does not induce changes in H9c2 cell viability. Next, cells were treated under the same conditions as described above to evaluate H9c2 Cx43 levels by WB. The Figure 17B shows that both increasing concentrations of MGO and a longer incubation cause a decrease on Cx43 levels in H9c2 cells. Finally, we explored if ubiquitination is involved in the MGO-induced Cx43 degradation. So, for that, H9c2 cells were treated with the 3 mM MGO during 3 hours and then cell lysates were immunoprecipitated using antibody against Cx43. The subsequent WB analysis presented in Figure 17C shows that MGO induces an increase of Cx43 ubiquitination.





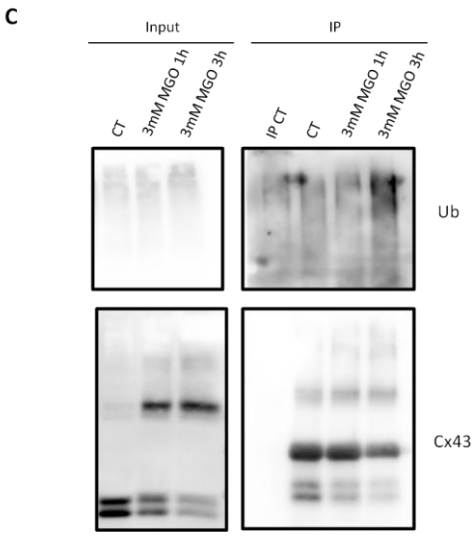


Figure 17. Methylglyoxal induces ubiquitination and degradation of Cx43 in H9c2 cells.

H9c2 cells were treated with 1 mM, 3 mM and 5 mM MGO during 1 hour and with 3 mM MGO during 3 hours. **(A)** MGO does not induce significantly differences in H9c2 cell viability. Representative graph showing the absorbance in arbitrary units (A.U.). Cell viability was determined by MTT assay. **(B)** With the increase of MGO concentration and period of incubation, the levels of Cx43 in H9c2 cells decrease. The protein levels were determined by WB. Representative graph showing Cx43 levels. **(C)** H9c2 cells were treated with 3 mM MGO during 1 hour and 3 hours. Cell lysates were used for IP with goat polyclonal antibody against Cx43. Cx43 and Ubiquitin (Ub) protein levels were determined by WB. MGO induces Cx43 degradation in H9c2 cells, with an increase of Cx43 ubiquitination.

Besides ubiquitination, sumoylation is another PTM modification that can modulate protein turnover. So, in a subsequent stage of this work we aimed to evaluate whether Cx43 undergoes sumoylation.

It has been suggested that sumoylation stabilizes membrane proteins at the cell surface and prevent their degradation. Therefore, we evaluated the endogenous levels of SUMO-1, SUMO-2 and Cx43 in different experimental conditions that were previously shown to affect the turnover of Cx43. To address this question, we first treated HEK-293 cells with either Bafilomycin A1 (Baf), to inhibit the lysosomal degradation, or Dynasore (Dyn), to inhibit endocytosis, after which we evaluated the levels of SUMO-1, SUMO-2 and Cx43 by WB. Furthermore, we used PMA, a PKC activator, known to induce ubiquitination and subsequent internalization and degradation of Cx43. The Figure 18

shows that Dyn increases SUMO-1, SUMO-2 and Cx43 levels in HEK-293 cells. Interestingly, PMA, which induces protein endocytosis endocytosis through phosphorylation, induced a shift in protein migration compatible with Cx43 phosphorylation, but did not affect the total Cx43 levels.

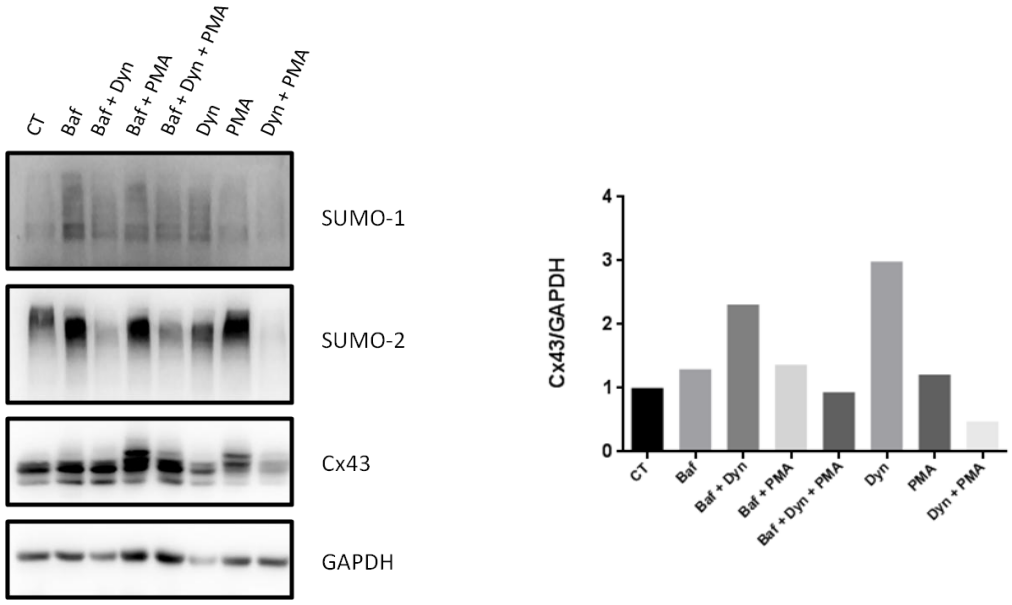


Figure 18. The endogenous levels of SUMO-1, SUMO-2 and Cx43 in different experimental conditions.
HEK-293 cells were treated with Bafilomycin (Baf), Dynasore (Dyn) and PMA. Protein levels were determined by WB. Representative WB showing SUMO-1, SUMO-2 and Cx43 levels. Representative graph showing Cx43 levels.

Since the endogenous levels of SUMO are difficult to detect in basal conditions, we proceeded to evaluate the levels of Cx43 sumoylation in human endothelial kidney 293 (HEK-293) cells transfected with the expression plasmids DNA encoding SUMO-1, SUMO-2 and Ubc9. The WB and graphs presented in the Figure 19 show the transient overexpression of SUMO-1, SUMO-2 and Ubc9, as well as the amount of Cx43 in each experimental condition. Importantly, these results demonstrate that the levels of Cx43 in HEK-293 increase when SUMO-1, SUMO-2 and SUMO-1 with Ubc9 (SUMO-1+Ubc9) and SUMO-2 with Ubc9 (SUMO-2+Ubc9) are overexpressed, comparing to control condition without overexpressions. This increase in Cx43 protein levels is more marked when SUMO-1 is overexpressed.

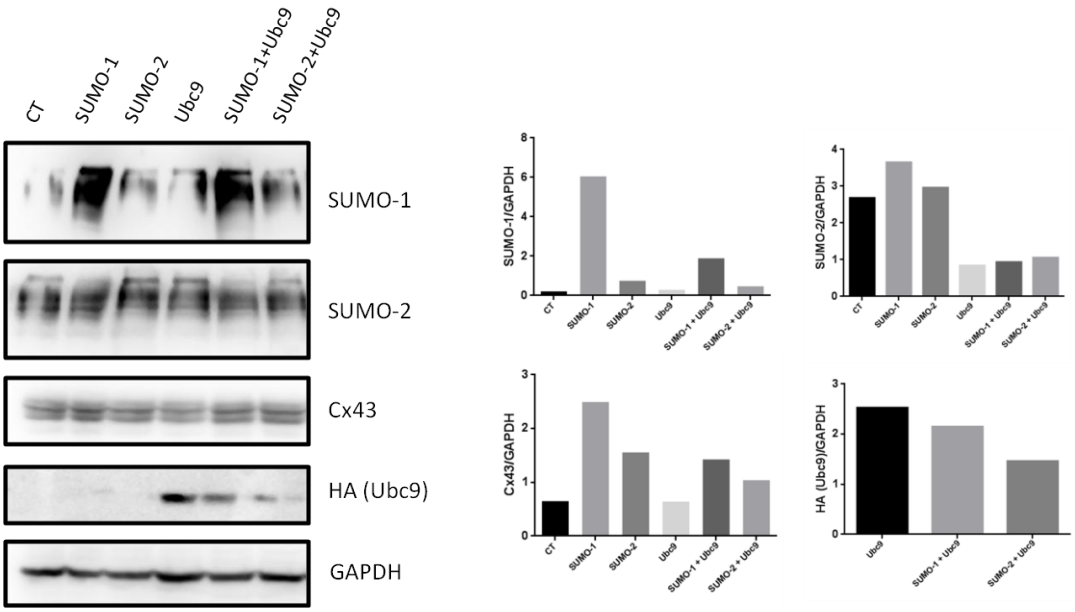


Figure 19. Overexpression of SUMO-1, SUMO-2 and Ubc9 plasmids.

The expression plasmid DNA encoding SUMO-1, SUMO-2 and Ubc9 was isolated and HEK-293 cells were transfected with 0.75 µg of plasmid DNA SUMO-1, SUMO-2, Ubc9, and co-transfected with 0.375 µg of each plasmid DNA SUMO-1 and Ubc9 (SUMO-1+Ubc9), and with SUMO-2 and Ubc9 (SUMO-2+Ubc9). Protein levels were determined by WB. Representative graphs showing SUMO-1, SUMO-2, Cx43 and HA (Ubc9) levels. In the presence of SUMO-1 overexpression, HEK-293 cells show more Cx43 levels comparing to control cells.

Since it is known that, given its labile nature, the detection of protein sumoylation is very difficult, we tested different ways to perform an IP, either in the presence or absence of NEM, a cysteine protease inhibitor that prevents the activity of SENPs and consequently stabilizes the ligation between SUMO and their substrates. The Figure 20 shows that using non-reducing conditions with NEM, it is possible to detect more SUMO-1 levels, comparing with reducing conditions. Moreover, the representative WB shows that Cx43 is sumoylated, demonstrated by the presence of high molecular weight conjugates positive for SUMO-1, following Cx43 IP.

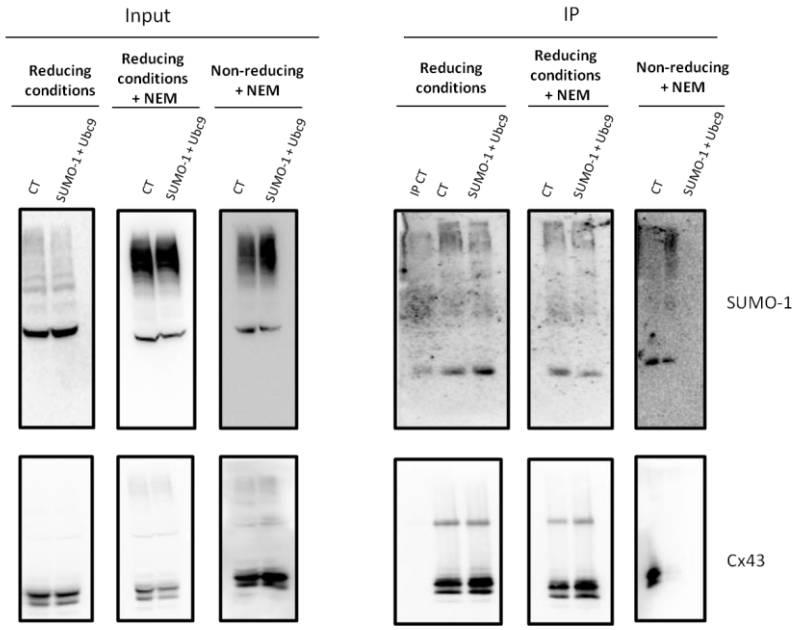


Figure 20. Non-reducing conditions IP with NEM increases the detectable levels of SUMO-1. Cx43 is sumulayted by SUMO-1.

The expression plasmid DNA encoding SUMO-1 and Ubc9 was isolated and HEK-293 cells were co-transfected with 0.375 µg of each plasmid DNA SUMO-1 and Ubc9 (SUMO-1+Ubc9). 10% of the total of cellular extracts was denatured and the remaining lysates were used for IP. In all IP conditions we using goat polyclonal antibodies directed against Cx43. Reduction conditions IP was performed with RIPA buffer and samples were denatured with Laemmli buffer. Reduction conditions with NEM (reduction conditions + NEM) was performed with RIPA buffer with NEM and samples were denatured with Laemmli buffer with NEM. Non-reduction conditions with NEM (non-reduction conditions + NEM) was performed with RIPA pH 7, loading buffer without β-mercaptoethanol with NEM. Protein levels were determined by WB. Representative WB showing SUMO-1 and Cx43 levels.

5. Discussion

Given the robust and widely recognized effect of CR on lifespan extension, there is much interest in elucidating the mechanisms underlying its effects. In fact, the finding that CR can delay the aging process and markedly extend lifespan was one of the most important scientific discoveries of the last century [88]. In 1935, Clive McCay and colleagues reported for the first time that decreasing food intake extends the lifespan of rats. By now, long-term CR is widely recognized to retard the ageing process in a number of organisms from yeast to mammals [82, 87]. Furthermore, it is also well established that CR delays the progression of age-related diseases, including CVDs. Indeed, ample of clinical and experimental evidences in numerous species have demonstrated that CR can exerts protective effects on cardiovascular system [86, 89, 90]. At the myocardium level, Peart et al. demonstrated that CR improves resistance to injurious stressors and restores cytoprotective responses lost with age [85]. Besides CR attenuates cardiac senescence, several studies show that CR can also improve cardiac and vascular function [82, 85, 91]. Focusing on the role of CR specifically in the heart function, some studies demonstrate that CR improves heart function through beneficial effects on blood pressure and inflammatory processes, among others [82, 88, 92]. For example, Han and coworkers showed that long-term CR in mice may preserve cardiac contractile function with consequently improvement of cardiomyocyte function [93]. In addition, Mager and colleagues demonstrated that rats maintained on a CR diet display increased heart rate variability, which is usually associated with improved heart function [94]. Moreover, both systolic and diastolic blood pressures are significantly reduced in rats and monkeys maintained on a CR diet [86, 95, 96]. Other studies also demonstrate that CR reduces the levels of oxidative stress in cardiovascular system, attenuates blood pressure dysfunction and reduces inflammatory processes [88, 97]. Furthermore, it was demonstrated for several studies that both short and long-term CR confers cardioprotection against ichaemia/reperfusion injury and improves ischemic tolerance in both young and aged rodents [82, 86, 98]. However, the cellular mechanisms through which these beneficial effects of CR occur are unclear.

The heart, mainly constituted by cardiomyocytes, strongly depends on a strictly regulated intercellular communication between the cardiac cells to ensuring its proper function. Indeed, it has been consistently shown that communication between the different cardiac cells, either directly, through GJ, or indirectly, for long distances, by exosomes, is vital to maintain the heart homeostasis. Not surprisingly, GJIC impairment has been often implicated in several forms of CVDs, namely ischemic heart disease. Given the crucial role of Cx43 in ensuring the direct IC between cardiomyocytes and its importance in heart function, and based on previous data from our group showing that starvation in cardiomyocyte (HL-1) cells stabilizes Cx43 at the PM, we hypothesized that starvation and CR have a protective effect in the heart by improving GJIC, through the increase of Cx43 protein levels. Therefore, in the present work we further investigate this hypothesis using *in vivo* models of starvation and CR. Our results show that both rats and mice submitted to starvation and CR have increased levels of cardiac Cx43 when compared to their controls, thus confirming our *in vitro* observations. The results present in this work constitute the first evidence that both starvation and CR lead to an accumulation of cardiac Cx43 protein levels.

Interestingly, it has been suggested by some authors that the protective effects of CR derive from the fact that the reduction in calorie intake is a mild stress that provokes a survival response resulting in increased cellular defenses [82, 99-101]. The results obtained in our study provide strong evidence that the stabilization of Cx43, with the improvement of GJIC, can constitute an additional mechanism whereby starvation or CR confers cardioprotection. Future studies are required to elucidate by what cellular mechanisms CR exerts its protective effects, in order to identify novel targets for prevention and treatment of age-related diseases, namely CVDs. Another target of study consists in the development of CR mimetics, i.e. compounds that promote CR-like effects, without a diet [88, 102].

Besides direct communication between adjacent cells, another type of IC has gained particular attention in the two last decades, which involves intercellular transfer of exosomes. Indeed, over the past years, exosomes has emerged as important players for IC that are involved both in physiologic and in pathophysiologic conditions, being

implicated in various different cellular functions and diseases states. Considering that a variety of different cell types within the heart release exosomes, it is reasonable to expect that exosomes play a preponderant role in cardiac cell communication. However, their precise physiological and pathological roles in CVDs are still vague. Interestingly, recent findings demonstrate the role of exosomes in cardiac protection including enhanced myocardial angiogenesis, reduced oxidative stress, limited inflammatory response, decreased cardiomyocyte death and myocardial infarction size [72]. Given the fact that exosomes contain many unique features like surface proteins/receptors, lipids, mRNAs, microRNAs, transcription factors and other proteins, it was suggested that the exosomal contents are highly regulated by various stress and disease conditions, which in turn reflects the parental cell status [7, 69, 72]. Recent studies have also demonstrated that exosomes collected from ischemic preconditioning or various stem cells provide cardioprotection, which may have therapeutic potential in the treatment of CVDs [72]. Moreover, the ability of exosomes to transfer mRNA, miRNA and proteins raises very exciting possibilities for future therapeutic uses. Indeed, the therapeutic potential of exosomes is promising, since they are vehicles that might be used to deliver therapeutics and drugs to cells [7, 68, 69, 72]. Although the promise as a therapeutic approach and a source of novel biomarkers, it is required to understand how exosomes are produced by cells, how their content is sorted and how they affect target cells [7, 68]. To further investigate the mechanism through which starvation and CR induce enhanced levels of Cx43, we explored whether the starvation and CR-induced cardiac Cx43 levels is mediated by exosomes. For that purpose, we determined the effect of exosomes derived from starved cardiac cells on the levels of Cx43 in recipient cardiac cells. Our results show that, in H9c2 cells, short periods of starvation leads to an increase of Cx43 levels, whereas long periods result in a decrease Cx43 amount. A state of nutrient deprivation, including starvation and ischemia, induces autophagy, a crucial degradative mechanism for the preservation of energy status [91]. So, it is plausible that longer periods of starvation lead to protein degradation, namely Cx43 degradation through autophagy. At 60 minutes of starvation there is an increase of Cx43 levels, that may constitutes a survival mechanism consisting in an attempt of cells to increase IC and ensure the passage of nutrients. Moreover, H9c2

cells that received exosomes from H9c2 cells that were submitted to control or starvation conditions had increased levels of Cx43 compared to H9c2 cells that did not received exosomes. Thus, suggesting that exosomes can carry information that can modulate either the transcription or the degradation of Cx43 in recipient cells. To investigate this hypothesis we evaluated the levels of autophagy-related proteins in the recipient cells, however no differences were observed on protein levels of Beclin, p62, Atg7, LC3 (results not shown), thus suggesting that other mechanism is involved, namely activation of Cx43 transcription. Once we demonstrated that starvation increases the levels of cardiac Cx43 levels, we hypothesized that this increase of Cx43 levels on the heart ameliorates the GJIC. Therefore we evaluated whether these starvation-derived exosomes are protective against ischemia insults, by determining cellular viability of the recipient cells. According to our hypothesis, starvation-derived exosomes elicit a response that result in the stabilization of Cx43, even in stress-induced conditions, such as ischemia, where cellular viability would be preserved. Our finding that starvation increases Cx43 in the heart, suggest that the beneficial effects of starvation in the heart are, at least in part, a result of the improved GJIC. So, it was expected that the starvation-derived exosomes would exert a cardioprotection effect against ischemia. Using the evaluation of mitochondrial activity as a mechanism of cell viability (MTT assay) no significant differences were observed between both control and starvation-derived exosomes on either control or ischemic H9c2 cells. Additional methods, such as apoptosis and cell proliferation assays, should be used to confirm this result.

Given the fact that exosomes released by cardiac cells did not present any significant effect on cardiac cells and since hypothalamus act as nutrient sensor and might release exosomes that act on different organs to activate specific responses to starvation and CR, we explored the possible contribution of starvation-derived exosomes from the hypothalamus in this regulation of Cx43 levels in cardiac cells. Thus, we hypothesized that the effect of starvation and CR in the increase of Cx43 levels in H9c2 cells, instead of being derived from starvation-derived exosomes from H9c2 cells, maybe deriving from starvation-derived exosomes from hypothalamic cells. So, based on this theory, the heart could receive “starvation messages” from the hypothalamus and the exosomes from

hypothalamus cells that were submitted to starvation could increase the levels of Cx43 protein levels in H9c2 cells. For that purpose, we explored the effect of exosomes released by hypothalamus N42 cells that were submitted to starvation, on Cx43 protein levels in H9c2 cells. Our results show that the levels of Cx43 in N42 cells subjected to starvation decrease with the increase of incubation period of starvation. Similarly to the result of H9c2 cells submitted to starvation, it is reasonable that longer periods of starvation leads to protein degradation, namely Cx43 degradation and, for that reason the levels of Cx43 in N42 cells that were submitted to starvation decrease. Nevertheless, H9c2 cells that received exosomes from N42 cells submitted to control or starvation had increased levels of Cx43 compared to H9c2 cells that did not receive exosomes, suggesting that exosomes carry mRNAs or miRNAs that can affect the transcription or degradation of Cx43 in recipient cells. Again, no differences in autophagy-related proteins were observed, suggesting that Cx43 transcription might be enhanced, thus need further confirmation. Moreover, as with exosomes released by H9c2 cells, also the exosomes secreted by N42 cells did not present any significant protective effect against ischemia insults. Among the different periods tested, only 60 minutes starvation N42-derived exosomes seems to be protective against ischemia in H9c2 cells. To this point and using the described approaches, we were not able to confirm that exosomes derived from starved hypothalamic cells induce protection against ischemia in the recipient H9c2 cells.

The results presented above show that CR leads to an accumulation of Cx43. The strategy we used to reproduce this metabolic disturbance *in vitro* was the reduction of glucose available to the cells [(103)]. Contrarily, diabetes constitutes another metabolic disorder that involves an increase of glucose levels. Therefore, it is conceivable that opposite to CR/ hypoglycemia, conditions that mimic diabetes/ hyperglycemia leads to Cx43 degradation. Although it was previously shown that Cx43 levels are reduced in both type I and type II diabetic hearts, the mechanisms and signals that regulate Cx43 degradation remain elusive [(104)]. Hence, we aimed to explore the differential effect of CR /hypoglycemia and hyperglycemia (diabetes model) on Cx43 levels of cardiac cells. Interestingly, Inoguchi et al. investigated the underlying mechanisms by which Cx43 is involved in generation of arrhythmias in diabetic rat ventricular cardiomyocytes. The

mechanism proposed was that hyperglycemia causes phosphorylation of Cx43 and impaired ventricular contraction [105]. Although a myriad of mechanisms can be involved, a decrease of Cx43 levels and an impairment of GJIC have been suggested to such defects. Our results show that CR/hypoglycemia in H9c2 cells leads to Cx43 accumulation, whereas hyperglycemia leads to Cx43 degradation. These findings strongly suggest a potential role of diabetes in the regulation of GJIC in the heart. Moreover, previous studies from our group showed that diabetes and hyperglycemia lead to intracellular accumulation of MGO, a by-product of glycolysis, in different tissues throughout the organism. Therefore, it is conceivable that hyperglycemia-induced impairment of GJIC is mediated by MGO-triggered degradation of Cx43. Interestingly, it has been shown that MGO is associated with cardiac dysfunction [106]. Therefore, we investigated the effect of MGO-induced hyperglycemia on Cx43 protein levels in H9c2 cells. Our results show that although MGO did not affect cell viability, it caused a decrease in Cx43 levels and induced Cx43 ubiquitination in H9c2 cells. Altogether, these findings demonstrate for the first time that MGO induces Cx43 degradation and ubiquitination in cardiac cells, thus suggesting that diabetes-associated cardiac dysfunction may be caused by MGO-induced decrease in GJIC.

Besides ubiquitination, sumoylation is another mechanism of PTM that can act as a signal for degradation. PTM by the SUMO moiety is now regarded as one of the key cell regulatory modifications. Since its discovery in the mid-1990s, SUMO was shown to regulate a vast variety of proteins in many pathways and up to now, plenty of sumoylated proteins have been found to be involved in many cellular functions [107]. Indeed, during the last decade, sumoylation has emerged as a central regulatory PTM in the control of the fate and function of many proteins. An increasing number of papers report regulation of sumoylation by stresses and suggests an important role of this modification in cell response. Sumoylation has also been shown to be involved in a number of diseases [40, 41]. Recently, some studies have indicated a potential role for SUMO conjugation in cardiogenesis. SUMO modifies a multitude of transcription factors such as Nkx2.5, GATA4, SRF, myocardin, and prox1, which are essential for normal cardiac development, thus

pointing to a potential role of SUMO in cardiac structural morphogenesis [41]. Another recent studies highlighted SUMO contribution to cardiac development and function, contributing for the identification of more cardiac-associated SUMO targets [41]. Interestingly, into a cardiac context, increased sumoylation seems to be essential for survival, at least under some stress conditions. Furthermore, both hetero- and homozygous SUMO1 knockout mice were found to exhibit congenital heart defects with high mortality rates that could be rescued by the cardiac-specific reexpression of the SUMO1 transgene. Heart failure in humans as well as in mouse and porcine models seems to correlate with reduced SUMO1 levels, and SUMO1 overexpression markedly improved cardiac function in mice [40]. In cerebral ischemia it was found a massive increase in SUMO2/3 conjugates in the hippocampus and the cerebral cortex of mice. Similarly, SUMO2/3 conjugates were strongly increased in a cell-based model of ischemia [108]. Previous studies by Kjenseth et al. have provided the first evidence that Cx43 is modified and regulated by sumoylation, identifying Cx43 as a sumoylation target protein. In this study we show that the overexpression of SUMO-1 results in an increase of Cx43 levels, suggesting that, in line with previous studies by Kjenseth et al., the conjugation of SUMO to Cx43 stabilizes the protein, likely at the PM. The initial observation suggesting modulation of SUMO conjugation by stresses came from Saitoh and Hinchey, who reported that various environmental stresses (osmotic, oxidative stress, heat shock and phosphorylation) increase global sumoylation [38]. To induce stress conditions, we used PMA, known to trigger Cx43 degradation. Our results also show that PMA-induced phosphorylation leads to an increase of the levels of SUMO-1 and SUMO-2. Although further experiments are needed to address this question, it is conceivable that Cx43 phosphorylation act as a prior and precedes sumoylation. This is supported by previous studies that demonstrate that the negative charge introduced by phosphorylation stabilizes the interaction with Ubc9 and thereby enhances sumoylation [109]. As we aimed to evaluate whether Cx43 is post-translationally modified by sumoylation and we were aware from the literature that it was difficult to detect the basal levels of sumoylated proteins, we tested different ways to perform an IP. Among the three types of IP that we tested, our results show that to prevent/inhibit desumoylante enzymes, under non-

reducing conditions with NEM addition we were able to detect more SUMO-1 attached to Cx43, comparing with reducing conditions with or without NEM. In fact, in non-reducing conditions and in the presence of NEM, we can stabilize the SUMO-substrate conjugations. Altogether our results demonstrate that Cx43 is sumoylated. However, the functional consequences of such PTM are still unknown. Indeed, further studies are required to ascertain the role of Cx43 sumoylation as a regulatory signal in GJIC.

In conclusion, our study highlights the fact that the starvation and CR-mediated cardioprotective effects might, at least in part, result from an improvement of GJIC, through the increase of Cx43. Moreover, our findings suggest that diabetes-associated cardiac dysfunction may be caused by a decrease of GJIC, induced by MGO. However, future studies are required to better elucidate the mechanisms whereby ubiquitination and sumoylation regulate the traffic of Cx43 and its turnover, specifically in diabetes, and to clarify their functional implications in the heart.

6. References

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