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The Role of Hypoxic Inducible Factor-1 Alpha Subunit in Mitochondrial Function in Ischemic Heart Disease

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Declaration

I, Esmaa Bouhamida, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

.....(Esmaa Bouhamida)

I. Abstract

Ischemic heart disease is the most common type of heart disorder and represents a major cause of mortality worldwide. Recently, Hypoxia-inducible factor-1 alpha (HIF-1α) has drawn much attention in many research fields, have outlined its importance as a master transcription factor activated during hypoxia, and acts as a key modulator of diverse target genes in the human body including, apoptosis/ survival, metabolic reprogramming in response to hypoxia [1,2]. Mitochondria are the powerhouse of oxygen consumption, significantly abundant in the heart, and have emerged as an important regulator of cardiovascular health and disease. Several cellular processes are dedicated to maintaining mitochondrial function in cardiovascular homeostasis.

In the present study, we aimed to investigate the role of HIF-1α on mitochondrial homeostasis, especially its contact with the endoplasmic reticulum during hypoxia-induced by deferoxamine in human cardiomyocytes. Further different cellular events in response to hypoxia were studied, such as cell death (apoptosis and necrosis), calcium homeostasis, autophagy/mitophagy. Additionally, we elucidated the effect of HIF-1α in regulating the mitochondrial permeability transition pore (mPTP) in response to hypoxia. Therefore, we aimed to examine the localization of HIF-1α after its stabilization in our cell model and unveil its new possible effective roles during hypoxia. We also compared the effect of HIF-1α on the mitochondrial homeostasis in neonatal cardiomyocytes and cardiac fibroblasts in response to hypoxia.

The first part of the work performed experiments allowed the description of the effects of HIF-1α in response to DFO-mediated hypoxia in human cardiomyocytes. The main finding of this work is that HIF-1α plays a dual role in human cardiomyocytes during DFO induced-hypoxia, affecting the mitochondrial function and MAMs subsequently affecting various fundamental cellular processes, autophagy/mitophagy, cell death, calcium homeostasis in a dose and time-dependent manner. In the second part of our work, we identified for the first time a new localization of HIF-1α in human cardiomyocytes in response to hypoxia. Furthermore, the ongoing study on HIF-1α transcription activity showed for the first time two predicted copies of putative HRE encompassing consensus sequences of ITPR3 gene. Additionally, in other cell model neonatal cardiomyocytes (RNC) and cardiac fibroblasts (RCF), hypoxia-induced by DFO affected both cells differently, in which RCF were more resistant to hypoxia-induced mitochondrial oxidative stress compared with RNC. Indeed, the mitochondrial membrane potential of RNC was depolarized at an increased time of hypoxia induction.

Conclusively, these findings suggest new insights on the role of the hallmark protein HIF-1α in cardiac cells during hypoxia, emphasizing the relationships between HIF-1α /mitochondria and endoplasmic reticulum, focusing on the cardioprotection effect of HIF-1α.

II. Riassunto

La cardiopatia ischemica è il tipo più comune di disturbo cardiaco e rappresenta una delle principali cause di mortalità in tutto il mondo. Recentemente, l'Hypoxia-inducible factor-1 alpha (HIF-1α) ha attirato molta attenzione in molti campi di ricerca; è stata delineata la sua importanza come un fattore di trascrizione principale, attivato durante l'ipossia che agisce da modulatore chiave di diversi geni target nel corpo umano, tra cui apoptosi / sopravvivenza e riprogrammazione metabolica in risposta all'ipossia [1,2]. I mitocondri sono la centrale elettrica del consumo di ossigeno, significativamente abbondanti nel cuore, e sono emersi come un importante regolatore della salute e della malattia cardiovascolare. Diversi processi cellulari sono dedicati al mantenimento della funzione mitocondriale nell'omeostasi cardiovascolare.

Nel presente studio, abbiamo voluto indagare il ruolo di HIF-1α sull'omeostasi mitocondriale, in particolare il suo contatto con il reticolo endoplasmatico durante l'ipossia indotta dalla deferoxamina in cardiomiociti umani. Sono stati studiati altri diversi eventi cellulari in risposta all'ipossia, come la morte cellulare (apoptosi e necrosi), l'omeostasi del calcio, l'autofagia/mitofagia. Inoltre, abbiamo chiarito l'effetto di HIF-1 nella regolazione del poro di transizione della permeabilità mitocondriale (mPTP) in risposta all'ipossia. Pertanto, abbiamo voluto esaminare la localizzazione di HIF-1α, dopo la sua stabilizzazione, nel nostro modello cellulare e svelare i suoi nuovi possibili ruoli efficaci durante l'ipossia. Abbiamo anche confrontato l'effetto di HIF-1α sull'omeostasi mitocondriale nei cardiomiociti neonatali e nei fibroblasti cardiaci in risposta all'ipossia.

La prima parte del lavoro ha permesso di descrivere gli effetti di HIF-1α in risposta all'ipossia mediata da DFO in cardiomiociti umani. La scoperta principale di questo lavoro è che HIF-1α giocaun doppio ruolo nei cardiomiociti umani durante l'ipossia indotta da DFO, influenzando la funzione mitocondriale e le MAMs successivamente influenzando vari processi cellulari fondamentali, l'autofagia/mitofagia, la morte cellulare, l'omeostasi del calcio in modo dose e tempo dipendente. Nella seconda parte del nostro lavoro abbiamo identificato per la prima volta una nuova localizzazione di HIF-1α nei cardiomiociti umani in risposta all'ipossia. Inoltre, lo studio in corso sull'attività di trascrizione di HIF-1α ha mostrato per la prima volta due copie predette di HRE putative che comprendono Sequenze consenso del gene ITPR3. Inoltre, in un altro modello cellulare di cardiomiociti neonatali (RNC) e di fibroblasti cardiaci (RCF), l'ipossia indotta da DFO ha colpito entrambe le cellule in modo diverso, in cui RCF erano più resistenti allo stress ossidativo mitocondriale indotto dall'ipossia rispetto alle RNC. Infatti, il potenziale di membrana mitocondriale le RNC è stato depolarizzato al momento dell'induzione dell'ipossia.

In conclusione, questi risultati suggeriscono nuove Informazioni sul ruolo della proteina hallmark HIF-1α nelle cellule cardiache durante l'ipossia, sottolineando le relazioni tra HIF-1α /mitocondri e reticolo endoplasmatico, concentrandosi sull'effetto di cardioprotezione di HIF-1α.

III. Publications

1. Morciano G, Patergnani S, Bonora M, Pedriali G, Tarocco A, **Bouhamida E**, Marchi S, Ancora G, Anania G, Wieckowski MR, Giorgi C, Pinton P (2020) Mitophagy in cardiovascular diseases. **J Clin Med** 9:892
2. Bonora M, Patergnani S, Ramaccini D, Morciano G, Pedriali G, Kahsay AE, **Bouhamida E**, Giorgi C, Wieckowski MR, Pinton P (2020) Physiopathology of the Permeability Transition Pore: Molecular Mechanisms in Human Pathology. **Biomolecules** 10:998
3. Patergnani S, Danese A, **Bouhamida E**, Aguiari G, Previati M, Pinton P, Giorgi C (2020) Various Aspects of Calcium Signaling in the Regulation of Apoptosis, Autophagy, Cell Proliferation, and Cancer. **Int J Mol Sci** 21(21):E8323
4. Giorgi C, **Bouhamida E**, Danese A, Previati M, Pinton P, Patergnani P (2021) Relevance of Autophagy and Mitophagy Dynamics and Markers in Neurodegenerative Diseases. **Biomedicines** 9:149
5. Patergnani S*, **Bouhamida E***, Leo S, Pinton P, Rimessi A (2021) Mitochondrial Oxidative Stress and "Mito-Inflammation": Actors in the Diseases. **Biomedicines** 9(2):216 *equally contributed.
6. Morciano G, Vitto VAM, **Bouhamida E**, Giorgi C, Pinton P (2021) Mitochondrial bioenergetics and dynamism in the failing heart. **Life** 11:436
7. Patergnani S, Bonora M, **Bouhamida E**, Danese A, Marchi S, Morciano G, Previati M, Pedriali G, Rimessi A, Anania G, Giorgi C, Pinton P (2021) Methods to Monitor Mitophagy and Mitochondrial Quality: Implications in Cancer, Neurodegeneration, and Cardiovascular Diseases. **Methods Mol Biol** 2310:113-159
8. Marracino L, Fortini F, **Bouhamida E**, Camponogara F, Severi P, Mazzoni E, Patergnani S, D'Aniello E, Campana R, Pinton P, Martini F, Tognon M, Campo G, Ferrari R, Vieceli Dalla Sega F, Rizzo P (2021) Adding a "Notch" to Cardiovascular Disease Therapeutics: A Micro-RNA-Bases Approach. **Front Cell Dev Biol** 9:695114
9. Review for Biology, The Interplay of Hypoxia, Mitochondrial dysfunction, and Inflammation in Cardiovascular diseases and Cancer: from molecular mechanisms to therapeutic approaches. "manuscript in preparation"

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care and love, even though we are physically apart.

Special thoughts to all those people who suffer from heart disease

I dedicate this modest work to my father

who inspired me always to chase this path in life science

Table of content

V. Table of figures

I. List of abbreviations

$[Ca^{2+}]$:	Ca^{2+} concentration
$[Ca^{2+}]c$:	Cytosolic Ca^{2+} concentration
$[Ca^{2+}]er$:	Endoplasmic reticulum Ca^{2+} concentration
$[Ca^{2+}]m$:	Mitochondrial Ca^{2+} concentration
3-MA:	3-methyladenine
a.u:	Arbitrary units
BH:	Bcl-2 homology domain
AC16:	Human ventricular cardiomyocytes
AEQ:	Aequorin
AIF:	Apoptosis-inducing factor
ANT:	Adenine nucleotide translocase
ATG:	Autophagy related genes
ATP:	Adenosine triphosphate
bHLH:	Basic helix-loop-helix
BK:	Bradykinin
BM:	CellLight Mitochondria-RFP BacMam 2
BNIP3:	Adenovirus E1B 19-kD-interacting protein 3
Ca^{2+}:	Calcium ions
CABPs:	Intraluminal Ca2+-binding proteins;
Cleaved-PARP:	Cleaved poly (ADP)ribose polymerase
CoCl2:	Cobalt chloride
CsA:	Cyclosporine A
CVD:	Cardiovascular disease
cyt:	Cytosolic
DFO:	Deferoxamine
DMEM:	Dulbecco's Modified Eagle Medium
DMOG:	Dimethyloxalylglycine
EPO:	Erythropoietin
ER:	Endoplasmic reticulum

FBS:	Fetal bovine serum
FIH-1:	Factor inhibiting HIF
GFP:	Green fluorescent protein
GSK3-β:	Glycogen synthase kinase 3-beta
Gtom:	Emerald-TOMM20 probe
HIF-1α:	Hypoxia inducible factor-1 alpha subunit
HIF-1β:	Hypoxia inducible factor-1 beta subunit
HIF-1:	Hypoxia inducible factor-1
His:	Histamine
HRE:	Hypoxic response element
HRP:	Horseradish peroxidise
HS:	Horse serum
IMM:	Inner mitochondrial membrane
IMS:	Intermembrane space
IP3:	Inositol 1,4,5-trisphosphate
IP3R:	Inositol 1,4,5-trisphosphate receptor
IRI:	Ischemia reperfusion injury
LC3:	Microtubule-associated proteins 1 light chain 3A
MAMs:	Mitochondria-associated membranes
MCA:	Plasma membrane Ca^{2+}-ATPase
MOM:	Mitochondrial outer membrane
mTOR:	Mammalian target of rapamycin
$Na_2S_2O_4$:	Sodium dithionite
NaCN:	Sodium cyanide
NaN_3:	Sodium azide
NCX:	Na^+/Ca^{2+} exchanger
ODDD:	Oxygen-dependent degradation domain
OMM:	Outer mitochondrial membrane
OXPHOS:	Oxidative phosphorylation
P2:	Purinergic receptors

PACS-2: Phosphofurin acidic cluster sorting protein 2

PDH: Pyruvate dehydrogenase

PdK: Pyruvate dehydrogenase kinase

PHD: Prolyl hydroxylase domain

PI3K class III: Phosphatidylinositol 3-kinase III

PKCε: Protein kinase C epsilon

PM: Plasma membrane PM

PMCA: Ca^{2+}-ATPases pumps

PTEN: Phosphatase and tensin homolog

PTP: Permeability transition pore

pVHL: Von Hippel–Lindau protein

ROS: Reactive oxygen species

RyR: Ryanodine receptor

SERCA: Sarco-Endoplasmic Reticulum Ca^{2+}-ATPase

Sig-1R: Sigma-1 receptor

siRNA: Small interfering RNA

SOCE: Store-operated Ca^{2+} entry

SR: Sarcoplasmic reticulum

STIM: Stromal interaction molecule

STING: Stimulator of IFN genes

TMRM: Tetramethyl rhodamine, methyl ester

TRPC: Transient receptor potential channels

TSPO: Mitochondrial translocator protein

UCP: Uncoupling protein

UPR: Unfolded protein response

VDAC: Voltage-dependent anion channel

VEGF: Vascular endothelial growth factor

VGCC: Voltage-gated Ca^{2+} channels

VOCs: Voltage operated Ca^{2+} channels

Ψm: Mitochondrial membrane potential

Chapter 1

Introduction

Introduction

The cardiovascular system consists of the heart, blood vessels, and blood and is responsible for the transportation of gases, nutrients, hormones, and blood cells throughout the body. Additional functions of the cardiovascular system are to regulate body temperature and acid-base balance. Chapter 1 contains an overview of the anatomy and the function of the heart and how the heart is dependent on the mitochondria, providing the main functions of the mitochondria in the heart, and a brief description of one of the most common cardiovascular disorders, in which we are interested in Ischemic Heart Disease. Moreover, we discuss the mechanisms of the mitochondrial dysfunction underlying acquired in heart disease under hypoxic conditions, in which multiple processes are involved, including cell death (apoptosis and necrosis), autophagy, mitophagy, and oxidative stress, as well as other processes mentioned in this thesis highlighting the impact of a key factor HIF-1α in response to hypoxia.

1.1 Structure and function of the heart

The heart is a large muscular pump made up of four chambers to provide blood flow to the systemic and pulmonary circulations and prevent backward flow: the left and right atria, and the left and right ventricles. The right receives deoxygenated blood via the venae cavae, which flows through the tricuspid valve to fill the right ventricle, which is the main pumping chamber of the right heart. The right ventricle feeds the left and right pulmonary arteries, which carry blood to the lungs to be re-oxygenated [3]. (figure 1)

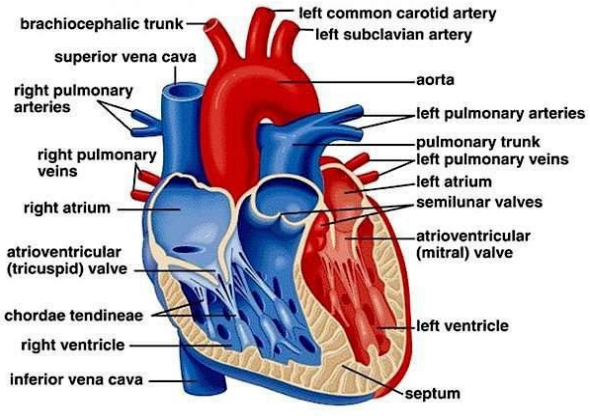

Figure 1: Structure of the heart [4].

As with the right atrium, the left atrium receives oxygenated blood back from the lungs via the pulmonary veins, passing through the mitral valve to the left ventricle. Oxygenated blood is pumped out to the systemic circulation via the aorta. The cycle is then repeated all over again in thenext heartbeat. The wall of the left ventricle is thicker, owing to the pressure required to pumplarger blood volume around the entire body. The heart walls comprise 3 layers: endocardium (the inner), myocardium (the middle), and epicardium (the outer).

The myocardium is a cardiac muscular layer, consisting of involuntary striated muscle tissuesurrender by a complex collagen framework. The myocardium is made up of two types of cells: the cardiomyocytes are highly contractile muscle cells, and smaller pacemaker cells, which have limited contractibility. The pacemaker cells function similarly to neurons, firing synchronized action potentials to stimulate contraction of the entire heart [5]. Due to the incredibly high ATP demands of normal cardiac contractile function, Cardiomyocytes are enriched with mitochondria and account for around 35% of their entire volume [6].

1.2 Mitochondria

Mitochondria play a pivotal role in cardiac myocytes. Their fundamental function is to meet the high energy demand of the beating heart by providing ATP through oxidative phosphorylation (OXPHOS) and tricarboxylic acid (TCA or Krebs cycle). Mitochondria occupy a large portion of each myocyte and are located between the myofibrils and just below the sarcolemma. The abundance of mitochondria ensures a highly efficient localizedATP delivery system to support contraction, metabolism, and ion homeostasis [7].

1.2.1 Mitochondrial structure

Mitochondria are fundamental subcellular organelles in most eukaryotic cells, composed of adouble membrane comprising proteins and phospholipid bilayers. This creates five distinct mitochondrial compartments: the outer membrane, intermembrane space, inner membrane, infoldings of the inner membrane constitute the cristae, while the space created between cristae is termed the matrix, each compartment serves a different function (Figure 2).

The mitochondrial outer membrane (MOM or OMM) is similar to the other eukaryotic cell membranes, has a 1:1 protein: phospholipid ratio, and is largely composed of transmembrane

porins, which allow the inflow and outflow of small molecules between the cytoplasm and the mitochondrial intermembrane space [8].

The intermembrane space (IMS) is a component of a very small volume enclosed by the outer and inner membranes of the mitochondria. Because of their free diffusion via the MOM, ions and other tiny molecules are at the same concentration as in the cytoplasm. However, due to the need for particular signaling sequences to translocate, the protein composition of the intermembrane space differs from that of the cytoplasm. The last enzyme in the respiratory transport chain, cytochromeC, is found here, and its leakage into the cytoplasm is frequently utilized as a marker of cellular stress and/or death [9].

The inner mitochondrial membrane (IMM or MIM) is highly folded, creating cristae, and extremely impermeable with a very high protein: phospholipid ratio, and possesses a membrane potential as a result of the electron transport chain. The enzymatic machinery responsible for OXPHOS, ATP generation, metabolite transport, protein import, and mitochondrial fission and fusion is housed in the MIM. Additionally, the MIM not only wraps around the internal perimeter of the mitochondrion but also invaginates to form numerous cristae, greatly increasing its available surface for ATP production; mitochondria in cells with higher energy demands have greater numbers of cristae [10].

The mitochondrial matrix fills the space between cristae and contains around 60% of all mitochondrial proteins, including enzymes required for the pyruvate oxidation, fatty acids, and the citric acid cycle. Moreover, the matrix contains copies of the mitochondrial DNA genome, and proteins, and ribosomal components and RNAs [11].

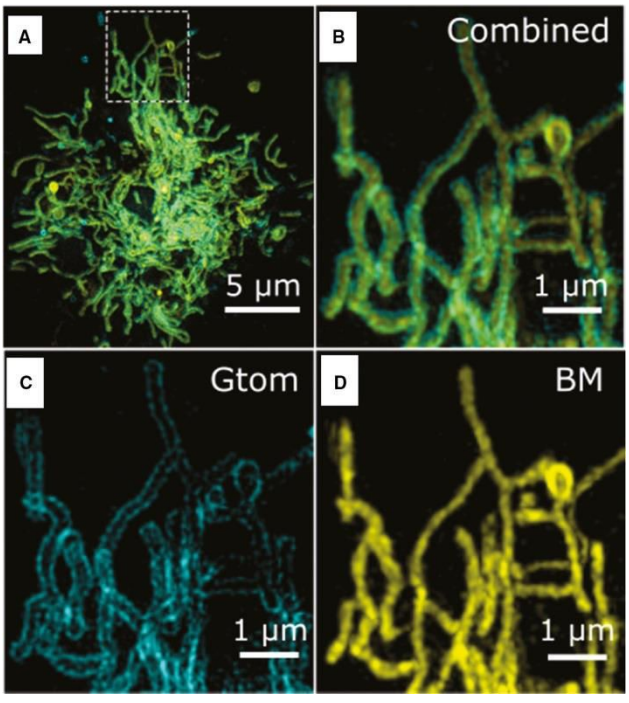

Figure 2: Live imaging of an MCC13 cell duallylabeled with Gtom and BM.

The boxed region in (A) is shown magnified for the combined (B) and individual probes (C and D). Gtom
(C) localizes at the mitochondrial outer membrane, and the BM label (D) localizes in the mitochondrial matrix. Gtom surrounding BM is shown in the merged image (B). Images are maximum intensity projections of a 1.25-µm 3D SIM z-stack. (MCC13 Merkel cell carcinoma). [12]

1.2.2 Mitochondrial functions and energy production

Living organisms require a steady supply of energy to carry out movements like muscle contraction, active transport of molecules and ions, as well as the synthesis of biomolecules from simple precursors. Mitochondria are fundamental organelle that ensure a highly efficient localized ATP delivery system to support contraction, metabolism, and ion homeostasis, playing an important role in intracellular signaling activation of apoptosis and other mechanisms. Indeed, mitochondria are the sites of the OXPHOS and the electron transport chain, and they oxidize hydrogen derived from our TCA cycle and β-oxidation with oxygen to generate heat and ATP [13].

The main energy source for cellular metabolism is glucose, which is catabolized in the three subsequent processes: glycolysis, TCA and finally oxidative phosphorylation to produce ATP. In the first process when glucose is converted into pyruvate the amount of ATP generated is low. Consequently, pyruvate is converted to acetyl- coenzyme A (acetyl-CoA), which enters the Krebs cycle, enable the production of NADH Ultimately NADH is used by the respiratory chain complexes to generate a proton gradient across the inner ATP synthase. Furthermore, cardiac mitochondria are also serving other cellular function including the generation and regulation of reactive oxygen species (ROS), calcium homeostasis, and regulating cellular apoptosis through the mitochondrial permeability transition pore (mPTP) [14].

1.2.3 Mitochondria associated membranes (MAMs)

Mitochondria and the endoplasmic reticulum are tightly associated with highly dynamic platforms termed mitochondria-associated membranes (MAMs), serving as essential hubs for inter-organelle communication in eukaryotic cells [15]. Among the various "close contacts" or "direct interactions" between cellular organelles, MAMs have recently attracted the attention of many researchers, as seen by the expanding number of new publications highlighting their crucial role in various signaling pathways in both physiology and pathophysiology [16].

MAMs consist of regions of the ER involved in direct interactions with the mitochondria. However, proteins from other cellular compartments have also been found in MAMs, suggesting that MAMs

also form close contacts with other intracellular structures in addition to the ER. For instance, plasma membrane (PM) proteins are observed in MAMs, indicating the presence of close contacts between the mitochondria and the PM [17]. Numerous investigations have found that mitochondria-ER interaction sites are dynamic structures. The contacts between these membranes are strong, and they are not disrupted through isolation methods (figure 3).

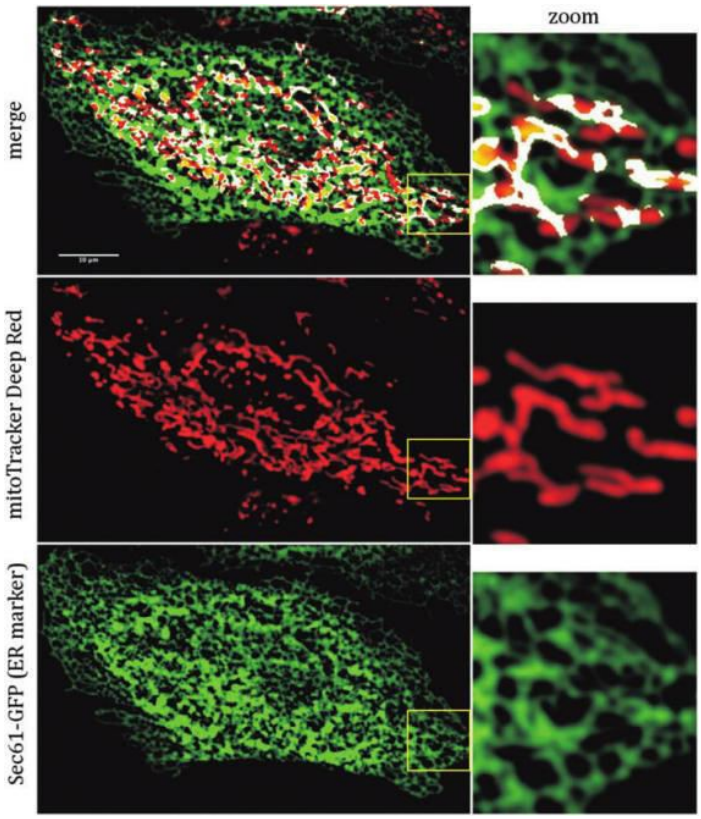

Figure 3: **Determination of MAMs viafluorescence microscopy.**

HeLa cells were transfected with the SEC61-GFP plasmid to label the ER (green) and loaded with Mito- Tracker Deep Red to visualize the mitochondrial compartment (red). In the merged and zoomed images, the white areas correspond to the MAMs. Images were obtained using a confocal laser scanning microscope at 63· magnification. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub .com/ars [16].

1.2.4 Calcium signalling

Calcium (Ca^{2+}) is a highly versatile intracellular signal fundamental for multiple cellular processes, including development, proliferation, and cell death. In resting cells, the [Ca^{2+}]c is maintained at lower levels (100nM) than the extracellular fluid but are stimulated when this level increases to 1000nM. The question that arises is how this Ca^{2+} elevation may control so many functions. The answer lies in the Ca^{2+} signaling mechanism's adaptability in terms of speed, amplitude, and spatiotemporal perspective [18].

There are four functional units in the Ca^{2+}-signaling network:
Signaling is triggered by a stimulus that generates various Ca^{2+}-mobilizing signals; These signals activate the ON mechanisms that promote Ca^{2+} into the cytoplasm; Ca^{2+} acts as a messenger to promote multiple Ca^{2+}-sensitive processes; and the OFF mechanisms, which consist of pumps and exchangers, remove Ca^{2+} from the cytoplasm to restore the resting state [19].

Moreover, in the mitochondria and the nucleus, the concentrations of calcium are similar to those in the cytoplasm. Other intracellular organelles such as the endoplasmic reticulum (ER), the calcium ranges between 100 and 800µM [20]. Cells use both internal and external Ca^{2+} sources to generate Ca^{2+} signals. Internal storage is represented by the endoplasmic reticulum (ER) or corresponding organelles, the sarcoplasmic reticulum (SR) of muscle cells, and the Golgi apparatus, with Ca^{2+} concentrations reaching approximately 500 mM [21]. In all eukaryotic cells, the ER is a vast network of cisternae and microtubules that reaches from the nuclear envelope to the cell surface. Being a dynamic calcium ions reservoir that may be activated by both electrical and chemical cell stimulation, it serves as an essential source for rapid physiological signaling. suppression of ER Ca^{2+}-release and alteration in mitochondrial Ca^{2+} accumulation result in damaged MAM [22]. During cell stimulation, intracellular calcium can increase more than double in the micromolar range and subsequently various channels of plasma membrane stimulate the extracellular calcium influx into the cells. The most important of these channels are transient receptor potential channels (TRPC) [23] store-operated Ca^{2+} entry (SOCE) channels like ORAI and STIM [24], voltage-gated Ca^{2+} channels (VGCC) in excitable cells [25], receptor- operated Ca^{2+} channels like the N-methyl-d-aspartate receptor (NMDA) [26], and purinergic P2 receptors [26,27], the activation of which determines cytosolic Ca^{2+} release from internal reserves, primarily via inositol 1,4,5-triphosphate receptors (IP3Rs) on the ER, may also contribute to intracellular Ca^{2+} increases [28].

IP3Rs are large-conductance cation channels that are activated when cell surface receptors are engaged. The activity of sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA), which pumps Ca^{2+} into the ER with a stoichiometry of 2:1 Ca^{2+}/ATP, and secretory protein calcium ATPase (SPCA), which transports Ca^{2+} into the Golgi apparatus, allows Ca^{2+} absorption into the ER lumen [29]. Therefore, the PMCA (plasma membrane Ca^{2+} transport ATPase) and the Na^{+}/Ca^{2+} exchanger (NCX) are the two processes responsible for Ca^{2+} extrusion on the plasma membrane. PMCA is a Ca^{2+} pump that belongs to the P-type ATPase family and it pumps Ca^{2+} across the plasma membrane (figure 4) [30,31].

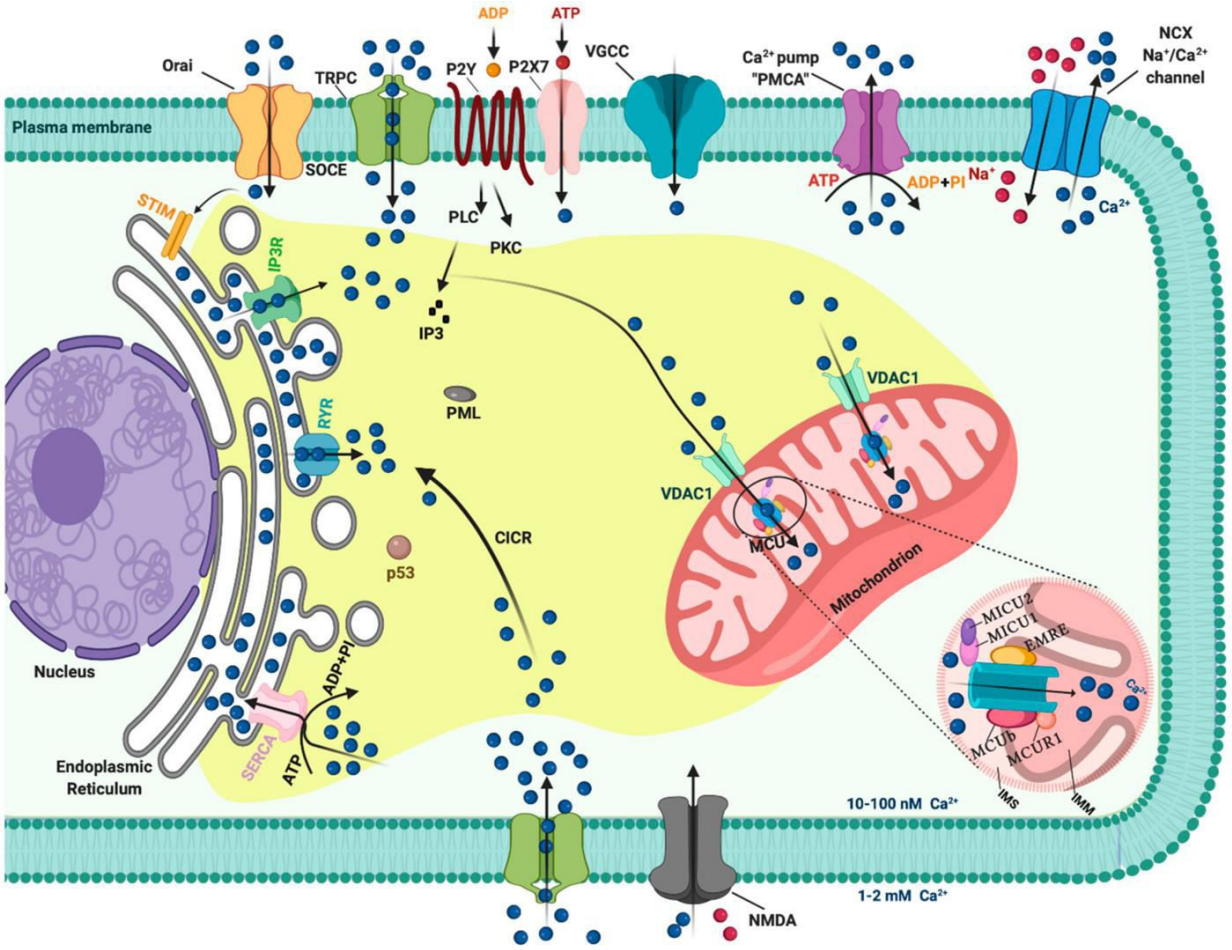

Figure 4: The intracellular Calcium (Ca^{2+}) signaling.

Different Ca^{2+} transporters, channels, exchangers, binding/buffering proteins and pumps mediate the regulation of cytosolic Ca^{2+} concentration. In the plasma membrane (PM), PM Ca^{2+}-ATPases (PMCA)pumps, transient receptor potential channels (TRPC), voltage-gated Ca^{2+} channels (VGCC), Na^{+}/Ca^{2+} exchanger (NCX), and purinergic P2 receptors regulate the transport of Ca^{2+} ions inside and outside cells. Inositol 1,4,5-triphosphate receptors (IP3R), ryanodine receptors (RyR), and sarcoendoplasmic reticulum Ca^{2+}-ATPase (SERCA) pumps control the storage of Ca^{2+} in the endoplasmic reticulum. Finally, voltage-dependent anion channels (VDAC) and members of the mitochondrial Ca^{2+} uniporter family are critical for controlling the mitochondrial Ca^{2+} uptake.

1.2.5 The membrane permeability transition pore

The mitochondrial permeability transition pore (mPTP) is a non-specific large conductance pore residing in the mitochondrial inner membrane that is normally closed [32]. A physiological for of mPTP, which is not firmly demonstrated, is that it may contribute to ion homeostasis when it opens transiently [33].

Despite the extensive research by many laboratories, the exact main molecular identity of mPTP is currently debated and controversial but Cyclophilin D, voltage-dependent anion channel (VDAC), Adenine Nucleotide Translocator (ANT), Bax and Bak, are believed to be involved in the modulation of the mPTP, with the ATP synthase potentially comprising the pore domain, however, it is not fully clear which other proteins are involved in the pore formation [34]. As of now, only CypD remains potentially a crucial component of mPTP. Therefore, it has been documented that mitochondrial ATP synthase is a critical component of the mPTP and /or the c-subunit of mitochondrial ATP synthase serves a pore-forming subunit [35].

This large protein pore forms open and under stress conditions such as mitochondrial matrixcalcium accumulation, depletion of adenine nucleotide, enhanced phosphate concentration or elevated oxidative stress, and optimal pH of above 7, which leads to the release of a huge amount of calcium and proapoptotic proteins from the mitochondria, subsequently leading to cell death [36]. Therefore, Conditions that hinder mPTP opening are low pH, high proton motive force, Cyclosporine A (CsA), which binds to Cyclophilin D, and Bongkrekic Acid (BKA) which inhibits the ANT [32].

Pioneers' studies reported that the solutes with molecular mass up to 1.5 kD can pass through this channel, suggesting that the pore diameter is approximately 3nm. One of the important consequences of the mPTP opening is that the mitochondrial inner membrane no longer maintains abarrier to H^+, which lead to the dissipation of $\Delta\psi m$ and the consequent arrest in all $\Delta\psi m$-dependent mitochondrial activities (figure 5) [14].

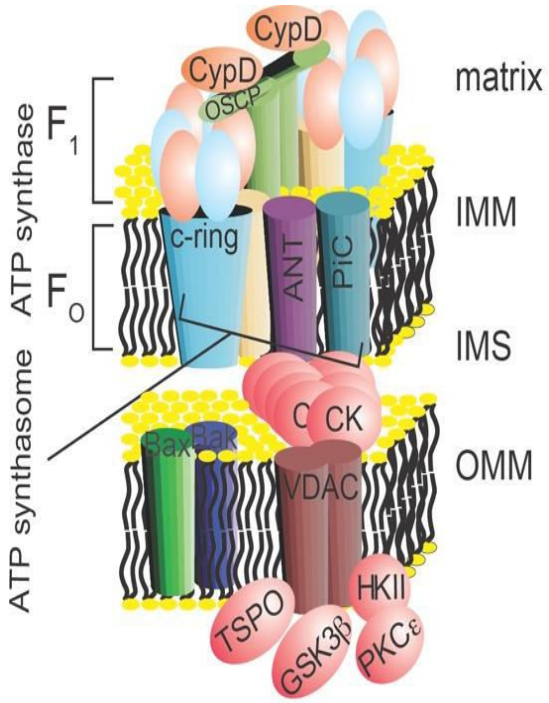

Figure 5: A novel model of mPTP structure

The present model for mPTP is built around F1/FO ATP synthase superstructures (involving the ANT and PiC) that directly interact with the main mPTP regulator CypD. The c-ring of the ATP synthase acts as the pore of the mPTP. The model spans from the inner mitochondrial membrane (IMM) to the outer mitochondrial membrane (OMM) by interactions with the VDAC, Bax, and Bak, and CK oligomers in the intermembrane space (IMS). Finally, the complex is surrounded by regulatory elements, as protein kinase C epsilon (PKCε), glycogen synthase kinase 3-beta (GSK3-β) and mitochondrial translocator protein (TSPO) are involved [37].

1.3 Mitochondria in cardiovascular disorders

1.3.1 Cardiovascular diseases overview

Cardiovascular disease (CVD) refers to all diseases of the heart and circulatory system, this multifactorial disorder encompasses numerous congenital and acquired diseases. CVD represents the leading non-communicable cause of death in Europe (∼50% of all deaths; ∼30% of all deaths worldwide) [38]. CVD is multifactorial with clinical (dyslipoproteinemia and hypertension) and behavioral factors (sedentarily, smoking, diet, and a stressful life). In this chapter, we focus on the role of mitochondria in ischemic heart disease (the most common form of CVD).

1.3.2 Mitochondria and Ischemic heart disease (IHD)

Ischemic heart disease (IHD) is a heterogenous group of pathologic conditions, characterized bylow concentrations of oxygen and insufficient perfusion of cardiac tissue. The current treatment of ischemic heart disease consists only of myocardium reperfusion to restore blood flow, a procedure, known as ischemia-reperfusion injury (IRI). IHD is globally the leading cause of death, primarily by induction of heart attacks according to the World Health Organization, life-years lost and disability worldwide, contributing to over 7.2 million deaths annually [39], (The World Health Organization, The top ten causes of the death fact sheet. http://www.who.int/mediacentre/factsheets/fs310/en/index.html).

Ischemia occurs when blood flow to the tissue is disrupted, resulting in cellular oxygen deprivation (hypoxia) and glucose deprivation. Hypoxia triggers a switch to anaerobic glycolysis, which rapidly acidifies the intracellular milieu. In cardiomyocytes, about 35% of the total cell volume is constituted by the mitochondrial network [40].

Mitochondria are essential dynamic organelles more abundant in energy-demanding cardiac tissue and play a crucial role in both the life and death of cardiac myocytes. In healthy cells, their essential role for survival is to provide energy to the beating heart via oxidative phosphorylation (OXPHOS) to ensure their functional integrity [7,41]. Apart from ATP production, Mitochondria have been recognized as are also the main primary triggers for ischemic heart injury [42].

1.3.3 Pathophysiology of IHD

During ischemia, sudden decrease in oxygen levels and nutrients result in abrupt biochemical and metabolic changes. Insufficient of oxygen causes the cardiomyocyte to rely on anaerobic respiration instead of oxidative phosphorylation, which in turn causes depolarization of the MIM and ATP depletion. subsequently inhibiting contractile function. In parallel, attempts to maintain mitochondrial membrane potential by active pumping of protons (H^+) across the membrane requires ATP hydrolysis, depleting the limited ATP supply. hypoxia triggers a switch in cellular metabolism to anaerobic glycolysis, causing acidification of the cell as H^+ accumulate and inhibiting the opening of the Mitochondrial Permeability Transition Pore (mPTP) [32,43]. This stimulates the Na^+-H^+ ion exchanger, additional mitochondrial Ca^{2+} loading can occur as $\Delta\Psi m$ is restored. Recovery of pH, an increase in ROS, and mCa^{2+} loading can trigger mPTP opening and cell death (Figure 6) [44].

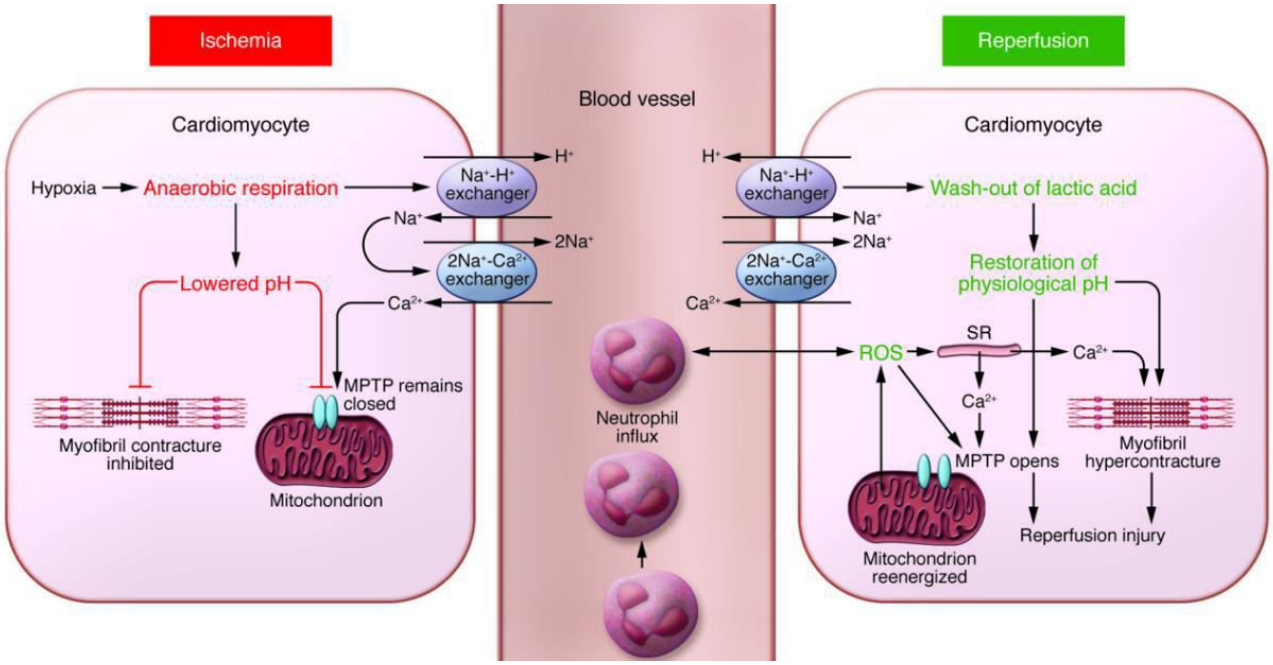

Figure 6: Schematic of main proponents of acute myocardial I/R Injury. The ischaemic pathways are demonstrated in red and the reperfusion in green. Not discussed in the text is the inflammatory response, as it remains unclear whether this contributes to the pathogenesis of lethal myocardial reperfusion injury,or whether it is a reaction to the acute myocardial injury. Figure taken from Hausenloy, D. J. and D. M. Yellon (2013)[45].

1.4 Oxygen balance and the Hypoxia inducible Factors 1 (HIF-1)

Oxygen (O_2) delivery is an effective prerequisite to ensure the normal function of the cell and is fundamental for a wide range of physiological responses including, cell metabolism, and growth. O_2 serves as an important for aerobic respiration that yields the primary cellular energy, the adenosine triphosphate (ATP) [46]. This process normally occurs at the powerhouse of O_2 consumption in the cell, the mitochondria, mainly via OXPHOS and TCA. However, when O_2 supply is insufficient to meet cellular energy demand, cells undergo hypoxia and are forced to use anaerobic respiration, which generates less than a tenth of the amount of aerobic respiration. Subsequently, mitochondria are severely affected by hypoxia, they sense the O_2 levels and initiate cellular responses to hypoxia. Along with those lines, mitochondria are considered the key source of hypoxic damage in the human body [47]. Therefore, hypoxia introduces dysfunctional feedback resulting in mitochondrial damage that exacerbates oxidative stress and inflammatory signaling, correlating to mitochondrial metabolism upon hypoxia [47]. It is well known that hypoxia is a hallmark of various diseases. Indeed, at cellular levels, emerging evidence reported the pathophysiological CVDs including ischemic heart disorder, which involve complicated and coordinated signaling pathways triggered during a decline of tissues or cells O_2 stress (hypoxic milieu).

Insufficient oxygen availability "hypoxia" is a precursor to numerous cardiovascular diseases, including ischemic heart disorder [48], hypoxia launches adaptive mechanisms that converge to hypoxic transcriptional response, this is regulated by hypoxic-inducible factor-1 (HIF-1) [49]. Research of Gregg Semenza's laboratory at Johns Hopkins Medical Institutions led to HIF-1 discovery after discerning hypoxia response element (HRE), putative sequences in the 30 -flanking region of the human EPO gene. Further research found that the transcriptional activation of several regulatory genes is triggered by the binding of a particular protein to the HRE, which is induced by hypoxia. This protein was later identified as HIF-1 [50].

1.4.1 Molecular characteristic and regulation of HIF-1α

In mammalian cells, HIF-1 has been demonstrated to play a fundamental impact in cellular and systemic O_2 homeostasis [50], which mediates adaptation to hypoxia through activation of a multitude of genes encoding proteins needed for improving tissue O_2 homeostasis, energy metabolism, and efficient management of hypoxia-induced toxic stress [51]. HIF-1 is a heterodimeric trans-acting DNA-binding transcription factor that constitutionally comprises expressed subunit HIF-1β (aryl hydrocarbon receptor nuclear translocator, ARNT) and an O_2-sensitive expressed HIF-1α subunit (or its analogues HIF-2α and HIF-3α) a master transcriptional regulator in response to hypoxia and a key modulator for the induction of genes that facilitate adaptation and survival of cells [52]. Both subunits, α and β, exhibit basic helix–loop–helix (bHLH) motifs and belong to the bHLH-Per-ARNT-Sim (PAS) homology protein family. The bHLH domain is a DNA-binding domain that can bind HREs to target specific genes [53,54].

In normal conditions, the HIF-1α subunit is hydroxylated by HIF prolyl-4-hydroxylases at proline 402 and 564 in the O_2-dependent degradation domain (ODDD) of the α-subunits, causing its ubiquitination and proteasomal destruction via the ubiquitin-proteasome (26S) mechanism, which is able to incite constantly the proteasomal degradation. Von Hippel– Lindau (pVHL), which also acts as a tumor suppressor, binds the ubiquitin ligase complex E3, that targets HIF-1α subunit destruction in O2-dependent degradation domain. Because of this, during normoxia HIF-1α protein has a short half-life [55,56] (figure 7).

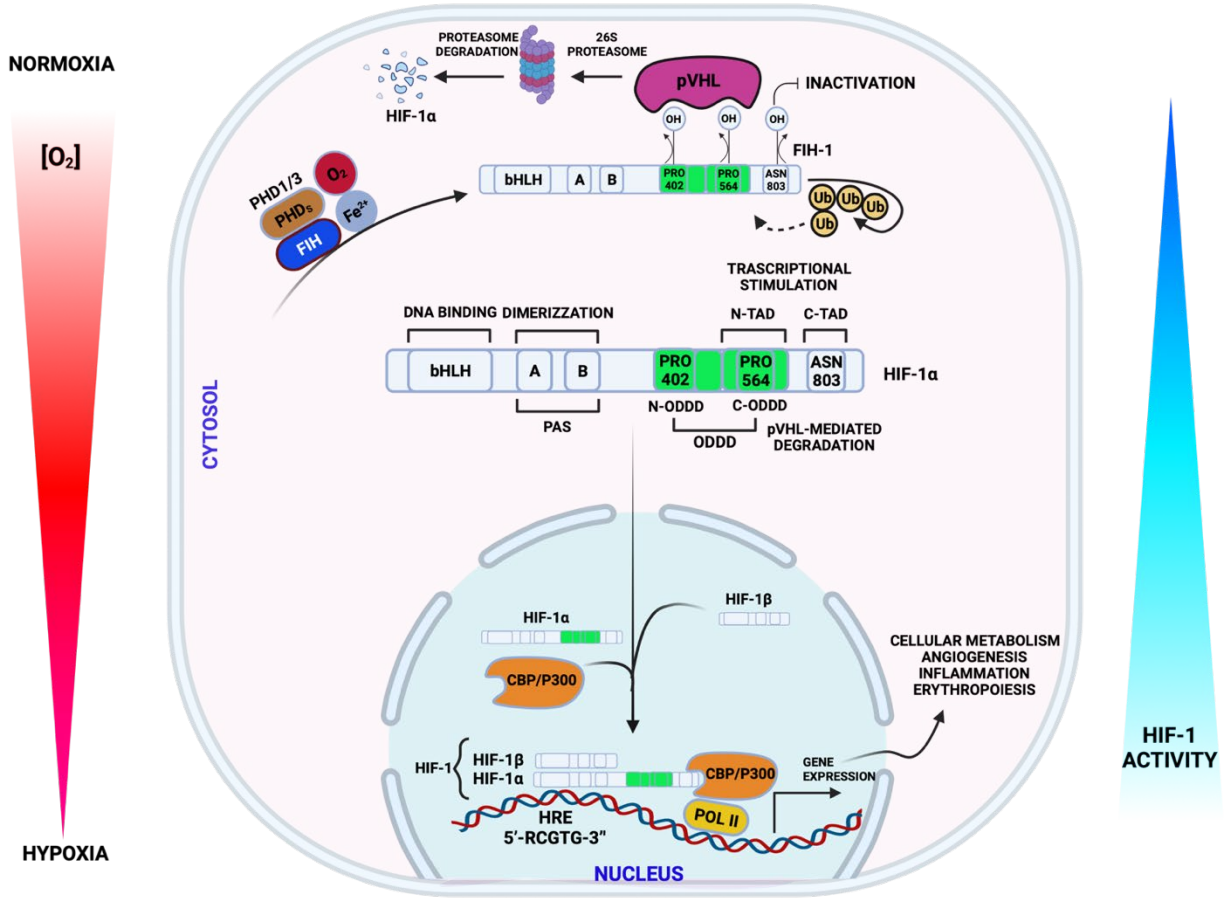

Figure 7: Schematic illustration representing the regulation of HIF-1α protein in response to normoxia and hypoxia. During normoxia, HIF-1α protein is hydroxylated by propyl-hydroxylases (PHDs) and factor inhibiting HIF (FIH). Both oxygen-dependent proteins are stimulated in normal conditions and suppress HIF-1α activity. The hydroxylated prolyl residues permit the binding of HIF-1α by the von Hippel– Lindau protein (pVHL), resulting in ubiquitination and ultimate proteasomal destruction. During hypoxia or PHD inhibition, HIF-1α translocates to the nucleus, heterodimerizes with HIF-1β, and subsequently binds to HREs in the putative region of a target gene to enhance their transcription.

1.4.2 HIF-1α mediated transcriptional activity to hypoxia

Under hypoxia, the repression of O_2-dependent propyl-hydroxylase-1, -2, and -3 enzyme activity (PHD1, -2, -3) results in suppression of HIF-1α hydroxylation. HIF-1α protein is then stabilized, accumulates in the cytosol, and transferred into the nucleus, where it creates a heterodimer complex with HIF-1β and binds to HREs with a consensus sequence (5'-RCGTG-3') in promoter or enhancer of target genes to activate a concerted transcriptional response (Figure 7). The nuclear translocation of HIF-1α is not enough to stimulate the target genes transcription [54]. The HIF-1α/HIF-1β (HIF-1) transcription factor recruits various cofactors that are fundamental for full transcription activity, including CREB-binding protein (CBP)/p300 and transcription intermediary factor 2 steroid-receptor activator that ultimately binds to CTAD domain. Another cofactor recognized is the M2 isoform of pyruvate kinase (PKM2), which enhances the binding of the complex HIF-1 to HRE [57]. Moreover, in response to hypoxia, each cell displays numerous types of signals at the transcriptional and translational levels, consisting of the activation of a group of genes termed as hypoxic-inducible genes, which are involved in different biological processes, including cellular metabolism (lactate dehydrogenase-A (LDH-A) or pyruvate dehydrogenase kinase isoform 1 (PDK)) [58,59], angiogenesis (vascular endothelial growth factor-A (VGFA))[60], erythropoiesis (erythropoietin (EPO)) [61], and inflammation (inducible nitric oxide synthase (iNOS))[62]. Hypoxia stimulates multiple processes to adapt to insufficient levels of O_2 in the environment. Therefore, it has mostly negative consequences for cardiovascular functions, ultimately manifesting in pathology.

1.4.3 Hypoxic mimicking compounds

To generate the hypoxia response at the cellular level, it is fundamental to establish hypoxia models in cell culture. Hypoxia conditions can be assessed by physical hypoxia or chemical hypoxia. Physical hypoxia is the use of hypoxic chamber or a CO_2 incubator with regulated oxygen levels. Alternatively, hypoxia can also be induced by chemical compound. The common chemical hypoxia-inducing reagents include cobalt chloride ($CoCl_2$), sodium azide (NaN_3), and sodium dithionite ($Na_2S_2O_4$), sodium cyanide (NaCN), Dimethyloxalylglycine (DMOG) ($C_6H_9NO_5$) and Deferoxamine (DFO) ($C_{25}H_{48}N_6O_8$). Cobalt in $CoCl_2$ can replace the ferrous ion in hemoglobin and stabilize hypoxia-inducible factors (HIF) 1α and 2α and mimic the hypoxia-induced changes in gene expression, but $CoCl_2$ can damage the cell membrane and fail to keep the degree of hypoxia at a constant state. NaN_3 inhibits cytochrome c oxidase, which restricts OXPHOS of cells, NaN_3 has a significant cytotoxicity, which limits its use [63]. NaCN induced- hypoxia is linked with altered gene expression

in stress-related genes and cellular activity of caspase-3 [64]. $Na_2S_2O_4$, known as sodium hydrosulfite, is one of the most usedcompounds to establish hypoxic models. It reduces oxygen in aqueous solutions rapidly, it depletes the dissolved oxygen in solution, resulting in a hypoxic environment. However, it is a very toxic chemical for living cells [65]. DMOG is ester of N- oxalyglycine and a competitive inhibitor of PHD enzymes that increase the stability of HIF-1 [66]. DFO ($C_{25}H_{48}N_6O_8$) has been commonly used in hypoxia- related research and is an iron chelator; they chelate the iron in the PHD region of HIF-1α that is loosely bound by 2-histidine-l-carboxylate coordination motif. By diminishing the availability of iron, HIF-lα is stabilized because the prolyl-4-hydorxylase requires oxygen, iron and ascorbate as cofactors, recently DFO is the most used chemical to induce hypoxia [52].

1.5 The effects of hypoxia on mitochondrial (dys)function and cellular biological processes in ischemic heart disease (IHD)

1.5.1 Hypoxia signaling and mitochondria in IHD

HIF-1α is an O_2-sensitive transcription factor that regulates adaptive metabolic responses to hypoxia and elicits a crucial impact in various CVDs, such as IHD [67]. Recent insights demonstrated the effect of HIF-1α signaling in the progression of heart disorders [68,69], or its cardioprotective role after I/R in animal model deficient of PHD3 and HL-1 cardiomyocytes [70-72] (Figure 8). Furthermore, HIF-1α overexpression caused mitigation of I/R-enhanced cardiomyocytes loss, suggesting that HIF-1α may drastically influence cardiomyocytes endurance[72]. Recently, studies documented that the HIF signaling pathway not only stimulates disease progression but has a cardioprotective effect as well as the potential for cell recovery from cellular stress in various disorders. This seems to be linked to the duration of hypoxic exposure as well as the stabilization of HIF-1. In fact, chronic exposure to hypoxia is found to increase ischemic ventricular arrhythmias and further cell death [73]. While intermittent exposure to hypoxia reduces arrhythmia during I/R, as also stimulates protective effects against myocardial infarction in rodents [74-77]. The prolonged HIF-1α upregulation was shown to promote dilated cardiomyopathy in transgenic mice with PHD2 depletion [22]. HIF-1α can also influence mitochondrial function and alleviate the severity of ischemic heart. HIF-1 elevates glycolysis by upregulating glycolysis enzyme production, increasing glucose transporters, and repressing the mitochondrial energy metabolism [78]. Moreover, HIF-1α promotes PDK-1 activation, which phosphorylates and inhibits pyruvate dehydrogenase (PDH), from converting pyruvate to acetyl CoA to fuel the mitochondrial TCA cycle and preventing the formation of iron-sulfur (Fe/S) clusters, thereby attenuating complex I activity [79]. It also inhibits the expression of mitochondrial encoded subunits in OXPHOS complexes by blocking the nuclear–mitochondrial interaction [80]. Recent pieces of evidence have also reported HIF-1α in improving mitochondrial function, reducing cellular oxidative stress, and stimulating the cardioprotection [2]. Furthermore, HIF-1α improves mitochondrial respiratory function by triggering various cardioprotective signaling pathways, including the phosphoinositide-3-kinase/Akt (PI3K/AKT) and Janus kinase (JAK) 2/signal transducer and activator of transcription (STAT) 3, to protect the heart during I/R injury [81]. Indeed, Nanayakkara and colleagues reported the transcriptional role of HIF-1α during hypoxia in regulating frataxin expression levels, a highly conserved nuclear-encoded mitochondrial protein, expressed in tissues

such as the heart, neurons, and liver with a high metabolic rate [81], which served as a cardioprotective element against ischemic injury. Ultimately, enhanced frataxin levels can alleviate mitochondrial iron overload, thereby preserving mitochondrial membrane integrity and the cardiomyocyte's viability [82]. HIF-1α stabilization permits cells and tissues to adapt to the hypoxic response in I/R, thus protecting cardiomyocytes against IHD and improving patient prognosis [51].

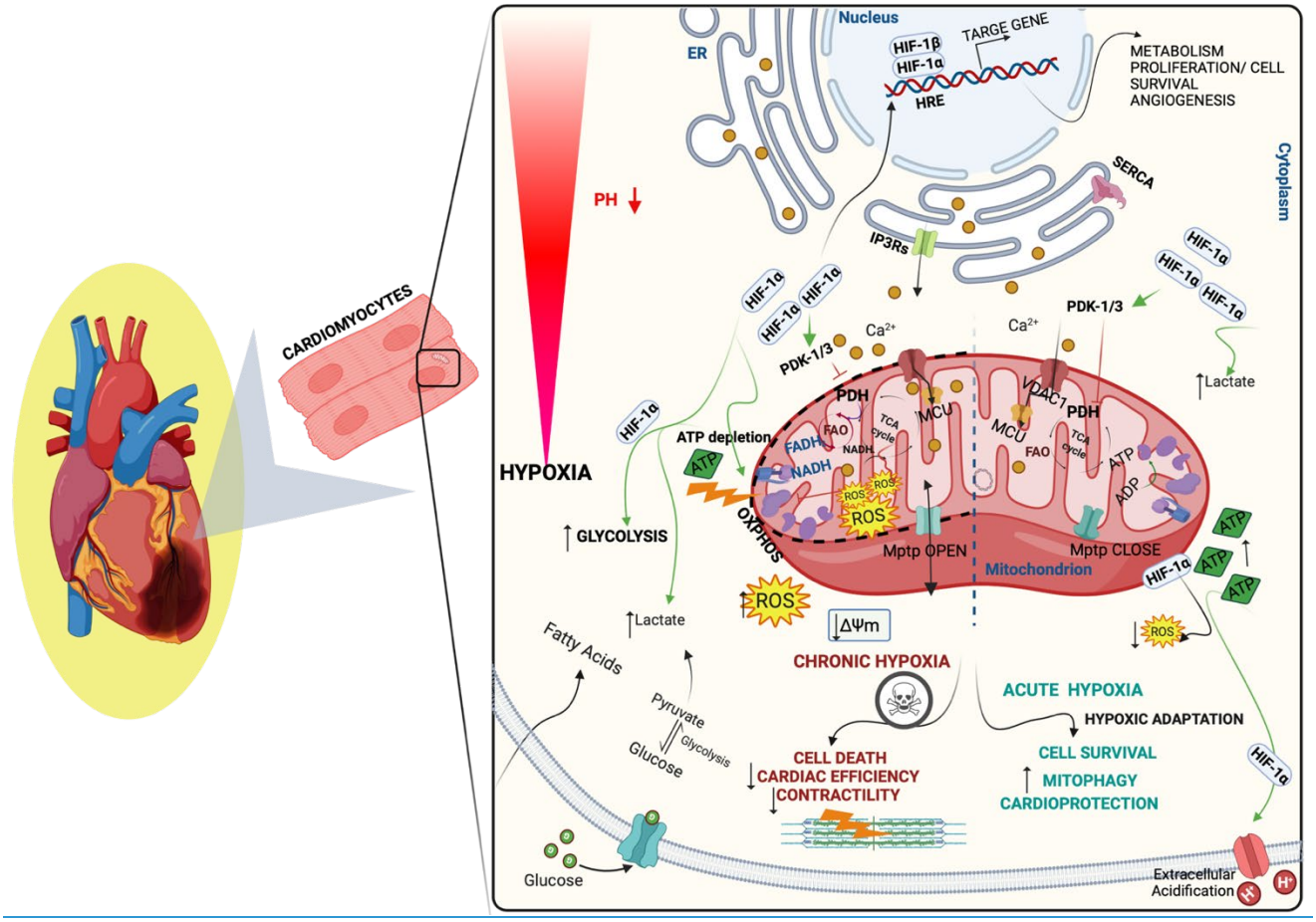

Figure 8: **HIF-1α signaling and mitochondria in CVDs**. Sudden decrease in O_2 levels result in abrupt biochemical and metabolic changes. Hypoxia causes accumulation of HIF-1α that moves to the nucleus to activate genes crucial to a metabolic switch away from OXPHOS to glycolysis, the cardiomyocyte relies on anaerobic respiration instead of oxidative phosphorylation, which in turn causes disruption of the mitochondrial membrane potential (ΔΨm) and ATP depletion, affecting Mitochondrial Permeability Transition Pore (mPTP) opening. Subsequently inhibiting contractile function. Hypoxia triggers a switch in cellular metabolism to anaerobic glycolysis, causing acidification of the cell as H^+ accumulates. Cardiomyocytes damage and mitochondrial deficiency are relatively linked to the degree of hypoxia exposure and due to the dual role of HIF-1α; in acute hypoxia (right), HIF-1α play a protective role against oxidative damage by alleviating ROS generation and stimulating the removal of unwanted mitochondria through mitophagy. While (left) HIF-1α enhances ROS levels and increases cell death, ultimately, reduces cardiac efficiency and contractility.

1.5.2 Autophagy and Mitophagy

The effective functioning of cardiomyocytes requires continual harmonization of mitochondrial function. This is accomplished through mitochondrial adaptation to current energy needs, which necessitates dynamic expansion and contraction of mitochondrial pools, the development of new mitochondria, and the removal of " inefficient and dysfunctional " organelles a process termed mitophagy, mitochondria are degraded under a variety of different conditions, including mitochondrial dysfunction, basal mitochondrial quality control [83]. Recently, it has been reported that mitochondrial autophagy and HIF1-α are involved in myocardial I/R [84]

In IHD, HIF-1α can directly or indirectly regulate numerous signaling pathways [85,86]. In terms of mitochondria, HIF-1α can upregulate mitophagy, mitochondrial autophagy, in cardiac cells through HIF-1α/(BNIP3) BCL2 and adenovirus E1B 19-kD-interacting protein 3 pathways, thereby stimulating their survival following myocardial ischemia-reperfusion. This is only applied to the role of HIF-1α-mediated mitophagy at an early phase of ischemia, which may result in cardiac protection, while prolonged autophagy may activate cell death in H9C2 cardiomyoblasts and Sprague Dawley rat models [2]. In contrast, other studies revealed that HIF-1 activation enhances BNIP3 expression, resulting in (H9C2) cardiomyocyte death, which is a hallmark of ischemia and HF [87,88]. Major interplays have been identified between hypoxia-mediated mitochondrial function and mitophagy in cardiomyocytes. In brief, upregulation of the IMM, Optic atrophy 1 (Opa1) stimulates mitophagy and mitochondrial function in response to hypoxia in mouse cardiomyocytes [89]. FUN14 Domain Containing 1 (FUNDC1) is an OMM protein that accumulates on the MAMs. Several recent studies reported its effective role to mediate mitophagy during ischemic conditions in cardiomyocytes, and it thus conveys cardioprotection [90]. Although the cardioprotective effect of mitophagy in the ischemic heart is widely demonstrated, during the reperfusion stage, mitophagy has a defective impact on cardiac function, and this may be due to the repression of FUNDC1-dependent mitophagy and necrosis upregulation [91]. The contribution of FUNDC1 in response to hypoxia may provide new insight in favor of therapeutic target approaches in CVDs, and further research focused on the FUNDC1-HIF-1 axis may be beneficial. Furthermore, a novel protein, WD Repeat Domain 26 (WDR26), has been detected to localize into the mitochondria, promoting mitophagy in H9C2 cells during hypoxia, suggesting its pivotal effect in hypoxia-enhanced mitophagy [92]. In another line of evidence, HIF-1α accumulation directs mitophagy and promotes the differentiation of H9C2 cells.

These events are useful for counteracting thenegative changes occurring during IHD and in CVD in general, and further comprehension of the molecular players involved can be fundamental for

potential therapies. Autophagy is one of the key cellular pathways and is a highly conserved catabolic process that permits lysosomal-mediated degradation of unwanted or dysfunctional cellular components, and it occurs at low levels under normal conditions in the heart, and alteration in this process cause cardiac dysfunction. This pathway is rapidly enhanced under stress conditions, including ATP depletion, reactive oxygen species, and mPTP. Although autophagy is upregulated in several pathophysiological conditions including ischemia and reperfusion, the functional role of enhanced autophagy is not well elucidated and is currently under intense investigation. Autophagy, as well as HIF-1α has a dual role in cardiovascular diseases: at the initiation, it is protective to support cell survival, but later, it may promote cell death [93]. many studies reported the deleterious role of autophagy due to its stimulation to cell death in heart diseases [94-96]. On the contrary, other studies documented that enhanced autophagy levels are associated with functional recovery of the myocardium after I/R [97]. Similarly, Yan et al. documented that cardiac myocytes with enhanced autophagy were protective against apoptosis [98].

Additionally, ER stress and the unfolded protein response (UPR) have been correlated with the activation of autophagy and mitophagy [99-101]. It has been documented that both cultured neonatal rat and adult mouse ventricular myocytes have shown induction of ER stress and UPR processes during ischemia and reperfusion conditions [102,103].

1.5.3 mPTP and cell death

Cell death are hallmarks for cardiac pathology including ischemic heart disease. Mitochondria are sensitive to alterations in the cellular environment, and are also fundamental regulators of cell death, can switch from a sustainer of cell survival to a promoter of cell death via the necrotic or apoptotic pathways, this can be mediated by opening of the mPTP in the IMM, resulting in a collapse of the membrane potential and swelling of the mitochondria, often culminating in necrotic cell death, or permeabilization of the OMM following the release of proapoptotic proteins, including cytochrome c, and apoptosis-inducing factor (AIF), Smac/Diablo to activate energy- dependent apoptosis (figure 9). Both processes have been involved in myocardial cells death in multiple CVD such as ischemia/reperfusion (I/R), cardiomyopathy, and heart failure [38].

Moreover, mitochondrial dysfunction is correlated with loss of myocytes and the subsequent development of heart failure. Both processes apoptosis and necrosis are regulated by the same biochemical intermediates, including alterations in the levels of high-energy phosphates, intracellular Ca^{2+}, and ROS [104].

The process of necrosis is mostly considered to be initiated by non-cellular mechanisms, such as ischemia, and thrombosis, leading to irreversible cell death, which are characterized by cell swelling, depletion of ATP, and disruption of the cellular membrane, which involves damages in fluid levels. Furthermore, hypoxia causes repression of OXPHOS and switch to glycolytic metabolism, resulting in reduced high-energy phosphates, increased lactic acid production and drop in intracellular PHi, ATP is rapidly depleted, alleviates active Ca^{2+} efflux, and limits the reuptake of Ca^{2+} by the sarcoplasmic, thereby producing intracellular Ca^{2+} overload the process of necrosis will occur because of passive loss of transmembrane ion gradients, following byswelling and loss of membrane integrity. In the heart, these cellular changes are accompanied by theactivation of intracellular proteases that alter myofibrils and enhance hyper contracture. Although necrosis was thought to be the sole cause of death in a myocardial infraction for past years, recent evidence provides growing evidence that the processes of apoptosis play a crucial role in myocytes loss in heart failure [104].

The apoptosis pathway that is activated depends on the nature of the death signal, and it is mediated by two pathways, the intrinsic and extrinsic, both have been described in cardiomyocytes (For instance, loss of the mitochondrial membrane potential loss secondary to mPTP opening triggeredby various pathological conditions, such as calcium overload, ATP depletion, oxidative stress, and high fatty acid). Similarly, to necrosis, apoptosis can be stimulated by the opening of mPTP. However, the stress is often milder in apoptosis. mPTP opening might be transient or maintained in some mitochondria undergoing matrix swelling, unlike necrosis, apoptosis is an active energy- consuming

process that must be executed in the presence of sufficient cellular ATP [106]. It is widely accepted that apoptosis accounts for some of the cell death observed after the myocardial infraction, however, the relative contributions of necrosis and apoptosis is unclear.

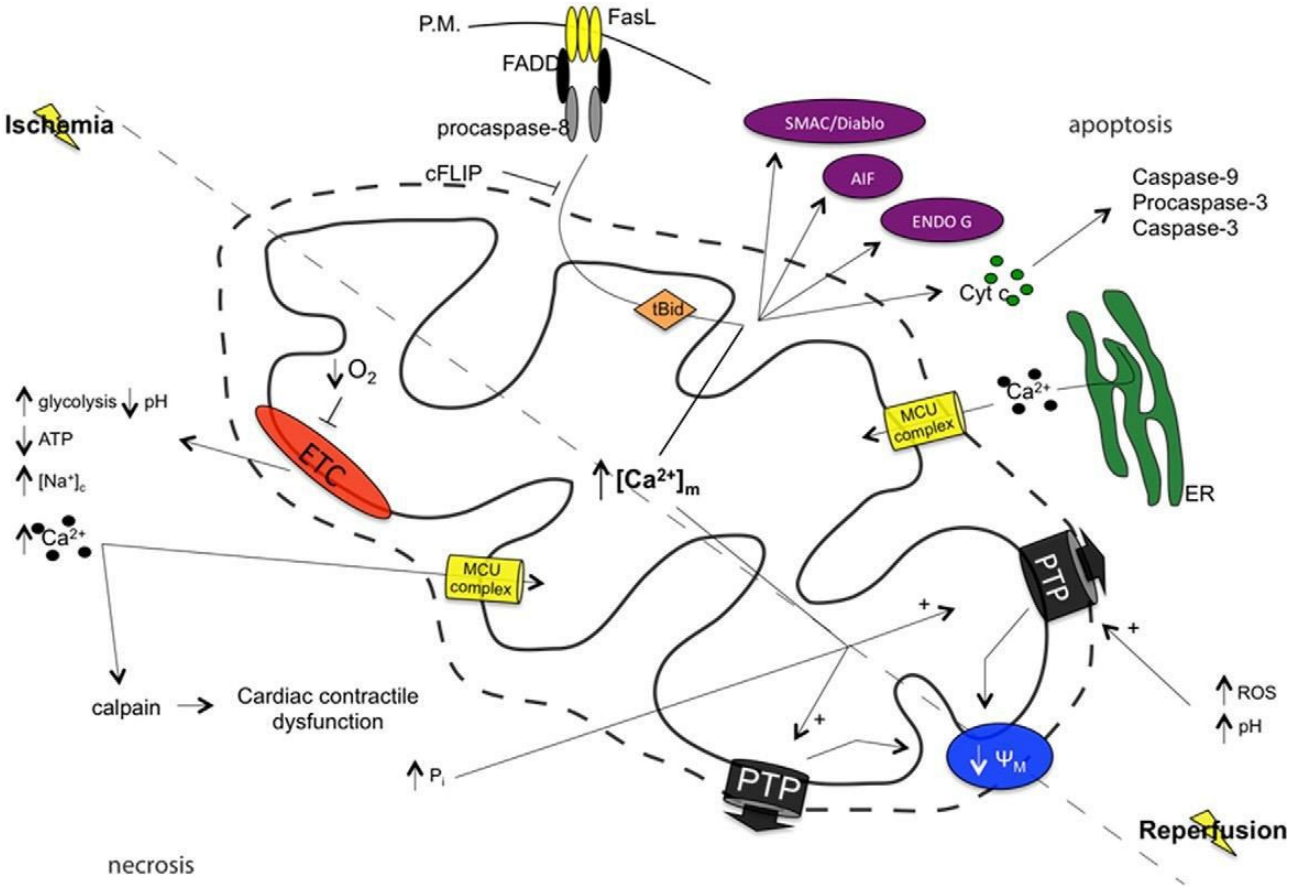

Figure 9: Mitochondrial involvement in cell death during ischemia/reperfusion injury in MI. Ischemia is on the left side; reperfusion is on the right side. The dashed line that divides the mitochondrion reveals that both events share similar pathways that lead to different pathological effects. Ischemia: insufficient blood supply to the heart. Ischemia leads to alterations in the mitochondrial electron transport chain (ETC) complexes, anaerobic metabolism prevails as a consequence of energy failure, lactic acid accumulates, and cellular pH decreases. This accumulation of hydrogen ions causes alterations in intracellular calcium homeostasis that lead to cell death. In the heart, these cellular changes are accompanied by activation of intracellular proteases, which damage myofibrils and result in cardiac contractile dysfunction. Reperfusion: restoring blood flow. Depending on its severity, reperfusion is characterized bythe increased formation of ROS, increased pH, decreased ATP production, and cell death. Some of the main pathways that occur, such as intrinsic and extrinsic apoptosis, permeability transition pore opening and lastly dissipation of the mitochondrial potential and membrane swelling, are represented in the figure [37]

Aims of the study

This present work is the result of my PhD in "Molecular Medicine and pharmacology" attended in "Signal Transduction Lab" leaded by Prof. Paolo Pinton. Under the supervision of Prof. Gianluca Campo and co-supervision of Dr. Simone Patergnani.
Our lab is aimed in studying the signal transduction, a process by which a cell receives and responds to stimuli, which lies at the heart of many interesting and important processes, including cell death, and differentiation process.

The overall aim of this work was to investigate the effects of HIF-1α on mitochondrial functions of cardiac cells in response to hypoxia.

The four specific aims are:

Specific aim 1: To understand the impact of HIF-1α stabilization on the mitochondrial homeostasis and the endoplasmic reticulum as well as, the mitochondrial associated membranes (MAM) that serves as a bridge between both compartments.
Specific aim 2: To demonstrate the effect and the regulation of HIF-1α stabilization in numerous cellular processes, with particular attention to cell death (apoptosis, necrosis) and autophagy/mitophagy (mitochondrial autophagy) during hypoxia.
Specific aim 3: Elucidating the roles of HIF-1α in regulating the mitochondrial permeability transition pore (MPTP) in response to hypoxia.
Specific aim 4: HIF-1α localization in response to hypoxia in human cardiomyocytes.

Chapter 2

Materials and Methods

2.1 Cell culture transient transfection

The AC16 human cardiomyocyte cell line (Merck-Millipore, cod. SCC109) were grown in DMEM/F12 containing 2mM L-glutamine, 10% fetal bovine serum (FBS) and 1x PS solution in 75-cm^2 Corning flasks. Primary rat neonatal cardiomyocytes were grown in DMEM/M199 supplemented with 10% horse serum (HS), mM L-glutamine and 1x PS. Primary cardiac fibroblast were grown in DMEM/F12 supplemented with 10% FBS, 2mM L-glutamine and 1x PS 100mm Petri dishes. All cells were maintained at 37°C under 90% relative humidity in 5% CO_2. Before transfection, cells were seeded onto 24-mm glass coverslips for microscopic analysis. cells were seeded onto 13-mm glass coverslips for other experiments. All experiments were performed 36h after plasmid transfections with Lipofectamine LTX. In experiments involving DFO, cells weretreated with DFO at concentrations 100nM, 500nM, 1μM, 10μM and 50μM in complete medium.

2.2 RNA interference for HIF-1α/IP3R3

The siRNA for the human HIF-1α gene (Hs_HIF1α_5 FlexiTube siRNA, #SI02664053) and the non-silencing siRNA control (Negative Control siRNA, #1027310). The siRNA IP3R3 (Negative Control siRNA, #1027280) (Hs_IP3R3 FlexiTube siRNA, # SI00034585, SI00034587, SI00034594, SI00034601). Both siRNAs were purchased from Qiagen. The siRNAs were transiently transfected into AC16 cells using a Lipofectamine LTX (thermofisher). The transfectants were cultured in complete medium and harvested on days 1–2 after transfection. The transfected cells were cultured in complete medium for 36h.

2.3 Cell viability assay (Crystal violet)

Cells seeded in 12-well plates were treated in the presence or absence of DFO, then the cells were washed with PBS, fixed in 4% paraformaldehyde, and stained with 0.1% crystal violet. Crystal violet was dissolved with 1mol/L acetic acid, and absorbance at 590nm was measured with spectrophotometer.

2.4 Immunofluorescence

Cells were grown on 13-mm coverslips and washed with PBS and fixed in 4% formaldehyde for 10min at 37°C. After washing three times with PBS, cells were permeabilized with 0.1% Triton X-100 in PBS (PBS-T) for 2h at room temperature and thenblocked with PBS-T containing 2% BSA at room temperature for 1h. Cells were then incubated with primary antibodies overnight at 4°C, washed 3 times with PBS-T, and incubated with the appropriate isotype-matched AlexaFluor-conjugated secondary antibodies. Coverslips were

mounted with mounting medium and DAPI reagent at room temperature, and images were acquired with a Zeiss confocal microscope using a 60 3 1.4 NA Plan-Apochromat oil-immersion objective. Acquired images were then analyzed by using open-source Fiji software.

2.5 Annexin-V and Propidium iodide assays

AC16 cells were stained according to the manufacturer's protocols with Annexin V-FITC conjugated (Thermo Fisher Scientific, A13199) and PI (Thermo Fisher Scientific, P3566) in binding buffer (10mM HEPES, 5mM KCl, 15mM NaCl, 1.8mM $CaCl_2$, 1mM $MgCl_2$, pH 7.4) and left in the dark at room temperature for 15min. confocal live imaging were subsequently used to detect the apoptotic and necrotic cells.

2.6 Mitochondrial Ros measurement

Cells were pre-labeled with MitoTracker Red CMXRos. The fluorescence intensity changes among the conditions were subsequently monitored using confocal imaging.

2.7 Mitochondrial membrane potential

Cells were loaded with 20nM tetramethyl rhodamine methyl ester (TMRM) for 30min at 37°C. To obtain and analyze basal levels, cells were stimulated with 10nM carbonyl cyanide p-trifluoro methoxyphenyl hydrazone (FCCP), a strong uncoupler of oxidative phosphorylation.

2.8 Ca^{2+} measurements

AC16 were transfected with either mtAEQwt (mitochondrial calcium measurement) and mtAEQwt or cytAEQwt (cytosolic calcium measurement). The coverslips were incubated with 5mM coelenterazine for 1.5 h in Krebs-Ringer modified buffer (KRB) supplemented with 1mM $CaCl_2$ (KRB: 125mM NaCl, 5mM KCl, 1mM Na_3PO_4, 1mM $MgSO_4$, 5.5mM glucose, and 20mMHEPES, pH 7.4, at 37°C). Aequorin signals were measured in KRB supplemented with 1mM $CaCl^2$ using a purpose-built luminometer. The agonist mix of (500µM Histamin+Bradykinin) for fibroblasts and AC16 was added to the same medium. The experiments were terminated by lysing the cells with Triton X-100 in a hypotonic Ca^{2+} rich solution (10mM $CaCl_2$ in H_2O), thus discharging theremaining aequorin pool. The light signals were collected and calibrated with [Ca^{2+}] values. Further experimental details have been previously described in [107]. For the endoplasmic reticulum (ER) Ca^{2+} measurement we used ER-GCaMP6-210: To test the release of ER Ca^{2+}concentrations with high sensitivity, we used a new Ca^{2+} probe based on the last generation GCaMPtargeted to the endoplasmic reticulum. We chose the GCaMP6 m version because it had the highest

Ca^{2+} affinity. Cells were ER-GCaMP6-210 treated with 50μM DFO at 12h, 24h and 48h. To evaluate its response to agonists, bradykinin (BK) and histamine (His) were added to1mMCa^{2+}/Krebs-Ringer buffer, and the Ca^{2+} ratio was recorded as indicated in the figures. we took advantage of the isosbestic point in the GCaMP6-210 m excitation spectrum; exciting GCaMP6-210 m at 406 nm led to fluorescence emission that was not Ca^{2+} dependent. As a consequence, the ratio between the excitation wavelengths of 494 and 406 nm was proportional to the Ca^{2+} concentration and independent of probe expression levels. Cells were imaged with an IX-81 automated epifluorescence microscope (Olympus) equipped with a 403 oil immersion objective (numerical aperture 1.35; Olympus) and an ORCAR2 charge-coupled device camera (Hamamatsu Photonics).

2.9 Immunoblot analysis

For immunoblotting, cells were lysed in RIPA buffer and then quantified by the Lowry method, and 10mg of protein was loaded on a 4%–20% precast gel. After electrophoretic separation, proteins were transferred onto nitrocellulose membranes that were incubated overnight with the following primary antibodies: HIF-1α (Cell signaling, 14179, 1:1000), GAPDH (Cell signaling, 2118, 1:1000), ATP5A (Abcam, 14748, 1:1000), Caspase 3 (Cell signaling, 9662, 1:1000), cleaved PARP (Cell signaling, 9541, 1:1000), Cleaved RIP (Cell signaling, 77565, 1:1000), PDI (Abcam, ab2729, 1:1000), β -Actin (Merck, A1978, 1:5000), TIM23 (BD Biosciences, 611222, 1:1000), CHOP (Cell signaling, 2895, 1:1000), BiP (cell signaling, 3177, 1:1000), LC3 B (Sigma, L7543, 1:1000), BAP1(Cell signaling, 13271, 1:1000), Hsp90 (cell signaling, 4874, 1:1000), p-mTOR (cell signaling, 2971, 1:1000), mTOR (cell signaling, 2972, 1:1000), PI3K (cell signaling, 4228, 1:1000), Beclin-1 (Cell signaling, 3738, 1:1000). FBXL2 (abcam, ab17018, 1:1000), β-tubulin (cell signaling, 2146, 1:1000), Sigma R1 (Cell signaling, 61994, 1:1000), anti-VDAC1 porin (abcam, ab15895, 1:1000), anti-Cytochrome c (BD Bioscience, 556432, 1:1000), PTEN (Cell signaling, 9559, 1:1000). (The membranes were then treated with specific HRP-labeled secondary antibodies, followed by chemiluminescence detection using a ChemiDoc Touch Gel Imaging System.

3.10 Luciferin-luciferase assay

Experiments were carried out as previously described in Morciano et al [108]. Briefly, AC16 cells expressing a mitochondrially targeted variant of Photinus pyralis luciferase were perfused with Krebs-Ringer modified buffer (KRB) supplemented with 1mM $CaCl_2$ (KRB: 125mM NaCl, 5mM KCl, 1mM Na_3PO_4, 1mM $MgSO_4$, 5.5Mm glucose, and 20mM HEPEs, pH 7.4, at 37°C), and luciferin-dependent luminescence was monitored with a customized luminometer (Elettrofor). The experiments started with background acquisition in which cells were perfused with only KRB solution. Then, KRB supplemented with 25mM luciferin was added to reach the plateau.

3.11 mPTP measurement

AC16 cells were pretreated with DMSO (vehicle), 50μM DFO, and then loaded with 1μM calcein acetoxymethyl ester (Thermo-Fisher) and Co^{2+} as described [107]. Staining solution was added to the cells for 15min at 37°C in a 5% CO_2 atmosphere. Image acquisitions were performed with a motorized Olympus IX81-ZDC inverted microscope with a 40 ×/1.30-N.A. UPlanFLN oil-immersion objective and Cell MT20E xenon lamp. Ionomycin (1μM, Sigma-Aldrich) was administered 30sec after the beginning of the experiment to induce mPTP opening. Finally, images were analyzed, and quenching rates were determined as the slopes of the fluorescence trace over a period of 60sec post-stimulation.

3.12 Protein degradation assay

In order to measure rates of protein degradation in AC16 cells, protein synthesis must be blocked. Protein translation elongation was inhibited by addition of a drug, cycloheximide (CHX). AC16 cells were cultured and protein synthesis was blocked by addition of 50μg/ml cycloheximide (Sigma-Aldrich) for 5h prior to cell lysis. Western blotting was used to quantify rates of degradation.

3.13 Subcellular fractionation

AC16 were seeded in big petries dishes and treated with DFO at 12h, the whole lysates (homogenate), mitochondria, ER, and MAM were isolated by ultracentrifugation as described in the protocol of Wieckowski et al [109].

3.14 Ex Vivo Model

I/R was studied ex vivo using the Langendorff model with minor modifications [110]. In brief, upon euthanasia, the hearts of Wistar rats weighing 270−280g at inclusion into the study were rapidly excised, immediately arrested in ice-cold KHB (pH 7.4; 4°C), cannulated, and retrograde perfused at a fixed-flow rate (11 mL/min) through the aorta with warm KHB (37°C) bubbled with 95% O_2 and 5% CO_2. Upon removal of the left atrial appendage, a latex fluid-filled balloon was inserted into the left ventricular chamber through the atrium to obtain an isovolumetrically beating preparation and connected to a pressure transducer (APT300, Hugo- Sachs, Grünstrasse, Germany) by a fluid-filled polyethylene catheter to monitor performance. An additional transducer above the aortic cannula monitored the CPP. At the start of each experiment, the fluid in the balloon was increased incrementally to achieve a constant EDP of 4 ± 1 mmHg. The LVDP was then measured. The LVDP, EDP, and CPP were continuously recorded using a programmable acquisition system (HSE Isoheart Software for Isolated Heart, Hugo-Sachs, Grünstraße, Germany).

3.15 Isolation and culture of rat neonatal cardiomyocytes

All procedures of animal handling were performed in accordance with animal use guidelines and approved protocols by the Institutional Animal Care and use committee of the University of Ferrara. Neonatal rat ventricular myocytes were isolated from 2/3-day-old Wistar rat hearts by several rounds of digestion with collagenase type II (Sigma- Aldrich, Italy) and pancreatine (Sigma-Aldrich, Italy). Briefly, hearts from neonatal rats were collected, atria excised, and the ventricles minced in ADS buffer (106mM NaCl, 20mM HEPES, 0.8mM NaH_2PO_4, 5.3mM KCl, 5mM glucose, 0.4mM $MgSO_4$, pH 7.4) containing 22.5 mg collagenase type II and 50 mg pancreatine. four rounds of digestion were performed at 37°C for 20min each. Fibroblasts were removed by a round of pre-plating for 1h on plastic tissue culture dishes already coated with laminin 20µg/ml. Cardiomyocytes were seeded on laminin-coated plastic tissue culture plates (TCP) and allowed to adhere for 2 days. Cardiomyocytes were maintained in DMEM/M199 medium, 10% horse serum (Gibco, Italy) and 5% New Calf serum (Gibco, Italy) supplemented with 1% L- glutamine and 1% penicillin streptomycin. During the third and fourth days of culture, then we used DMEM/M199 medium, 10% horse serum and 1% L-glutamine and 1% penicillin streptomycin for seeding and further experiments.

3.16 Statistical analyses

All data were analyzed using Graph Prism Pad 8 (GraphPad software Inc, La Jolla, California). Quantitative analysis was expressed as means ± S.E.M, Anova test and paired t-test was used to generate P values for comparison between groups in each data set $P< 0.05$ is considered assignificant.

3.17 Bioinformatic tool

ConTra v3 was used to verify the predicted binding sites of HIF-1 α transcription factor in the core DNA sequence HRE of the human ITPR3 gene [111]. Results of the predicted hypoxic responsive elements (HRE) in IP3R3 gene, also the homology sequences between species are in this link. http://bioit2.irc.ugent.be/contra/v3/#/results/49710fd636fec056758bae06f1c81fc8

Chapter 3

Results

3.1 Impact of HIF-1α stabilization on the mitochondrial function in Human ventricular cardiomyocytes (AC16) cells

3.1.1 The effect of Deferoxamine (DFO) on cell viabilities of human ventricular cardiomyocytes (AC16 cells)

The starting point of our work is to induce hypoxia in our cell model human ventricular cardiomyocytes (AC16), we used deferoxamine (DFO) that mimics hypoxic conditions by blocking prolyl hydroxylase Domaine-containing enzymes (PHDS), used to assess the molecular mechanisms driven by insufficiency of oxygen (hypoxia) [112].

We first evaluated the nontoxic concentration of iron chelator DFO in AC16 cells. Different concentrations have been tested 100nM, 500nM, 1µM, 10µM and 50µM of DFO, at 3h and 24h. As shown in figure 10.B, at 24h of the pretreatment, DFO decreased significantly the cell viability rate at different concentrations compared with 3h of DFO treatment (figure 10.A). These results have been confirmed with the western blot in the next section.

Further, cells were treated with only 50µM of DFO at different time points 12h, 24h and 48h to check the cell viability of AC16. A 12h DFO exposure did not significantly reduce the cell rate afterthe treatment (according to trypan blue dye exclusion of trypsinized cells, and with crystal violet assay). However, DFO significantly decreased the cell viability rate at 24h and 48h (figure 10.C).

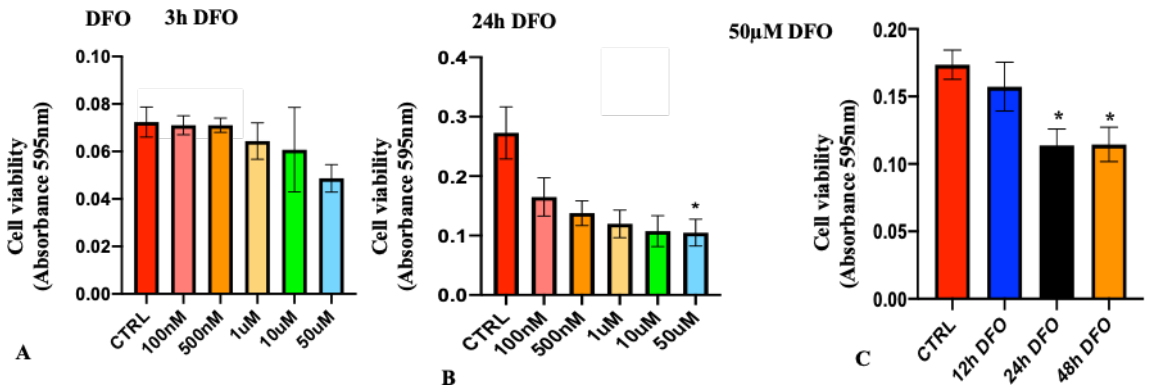

Figure 10: DFO reduced cell viability in AC16 in a dose-dependent manner. **A**. Quantification of cell viability AC16 cells treated with DFO (at 3h), **B**. at 24h, in a dose-dependent manner (100nM, 500nM, 1µM,10µM and 50µM). **C.** AC16 cells were treated with 50µM DFO (CTRL, 12h, 24h and 48h). (staining with crystal violet), The data are presented as means ± SEM.* $p<0.01$

3.1.2. DFO treatment in AC16 induced HIF-1α stabilization

Reductions in oxygen availability (hypoxia) are sensed directly and indirectly by multiple cellular signaling pathways, which elicit a variety of transcriptional, metabolic, and morphological responses to maintain cellular homeostasis. The major transcriptional regulators in response to hypoxia consist of a highly evolutionary conserved oxygen-regulator named hypoxia-inducible factor 1 alpha subunit (HIF-1α) [50].

In human ventricular cardiomyocytes, the hypoxic-mimetic agent DFO induces a rapid accumulation of HIF-1α expression, thus inducing hypoxia at 12h and 24h and a slightly at 48h through its blocking degradation. Immunofluorescence analysis and confirming this elevation by immunoblotting assay (Figure 11. A, B).

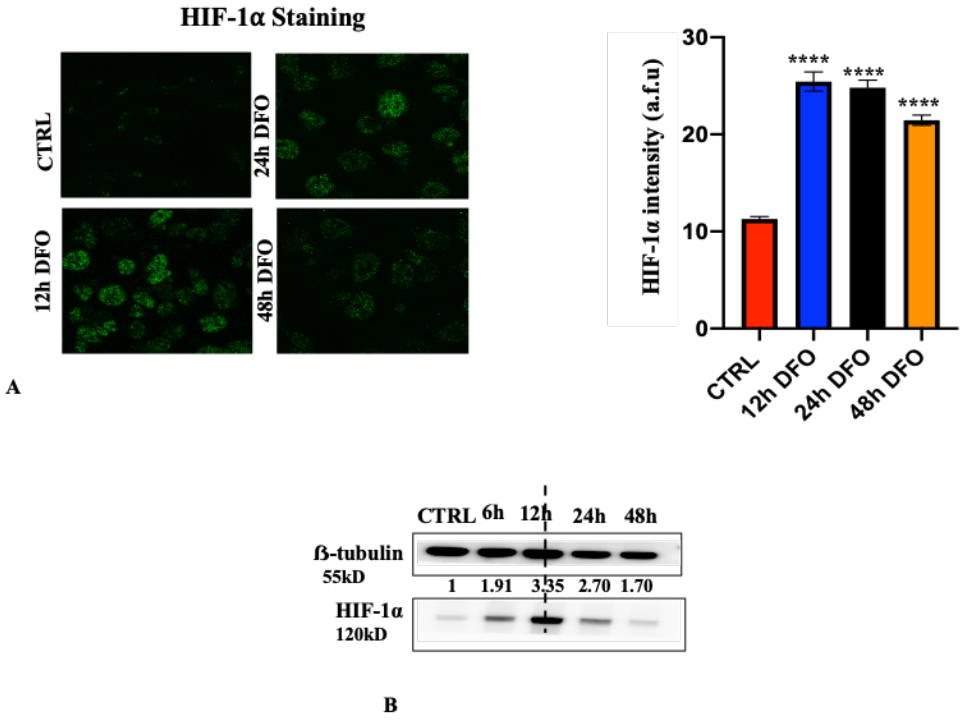

Figure 11: HIF-1α stabilization in AC16. **A**. HIF-1α detection and quantification by immunofluorescence in confocal microscopy in AC16. **B**. Detection of HIF-1α by immunoblot analysis in AC16. The data are presented as means ± SEM. ****p<0.0001.

3.1.3 The effect of DFO-induced hypoxia on the programmed cell death events (apoptosis and necrosis)

Programmed cell death is a crucial event in the life of a multicellular organism and an important finding in multiple forms of cardiovascular diseases. There are two major forms of programmed cell death, appointed, apoptosis and necrosis [113]. Paradoxically, mitochondria also have a central role as a gatekeeper of life and death [114]. Previous studies have documented that HIF-1α could regulate both cell death and cell survival through various pathways [115,116]. However, the role of hypoxia- induced HIF-1α axis in cell death is controversial.

Here we aim to examine whether DFO-induced HIF-1α promotes apoptosis and necrosis at different concentrations of 100nM, 500nM, 1μM, 10μM and 50μM of DFO, at 3h and 24h. To assess apoptotic and necrotic levels on our cellular models, we performed the immunoblotting usingtwo apoptotic markers, including cleaved poly (ADP)ribose polymerase (Cleaved-PARP) and cleaved caspase 3, as well as necrotic marker cleaved receptor-interacting serine-threonine kinase (RIP) as shown in (figure 12.A). Treatment with DFO resulted in stimulation of apoptosis and necrosis only at the concentration 50μM and at 24h, this was correlated with the observation of an increased level of HIF-1α expression, while at 3h no signal of cell death (apoptosis and necrosis) has been detected.

Next, we checked the 50μM at different time points 12h, 24h and 48h (concentration and incubation time were chosen in a manner to not affect drastically cell survival rate; further used in all different experiments shown later). As demonstrated in figure 12, B, C we noticed that DFO inducedapoptosis (figure 12. B) and necrosis (figure 12. C) at 24h and 48h, these results have been confirmed by annexin-V and PI staining (figure 12.D) and western blot.

To further address the possibility that HIF-1α is responsible for hypoxia-induced cell death, we assessed the effects of HIF-1α silencing by RNA interference. As shown in immunoblotting assay (figure 12.E), the apoptotic marker cleaved caspase 3 was drastically decreased at 24h and 48h compared with the scramble 24h and 48h cells, giving an insight that HIF-1α may have a crucial role in inducing the programmed cell death (apoptosis) in human ventricular cardiomyocytes in response to hypoxia.

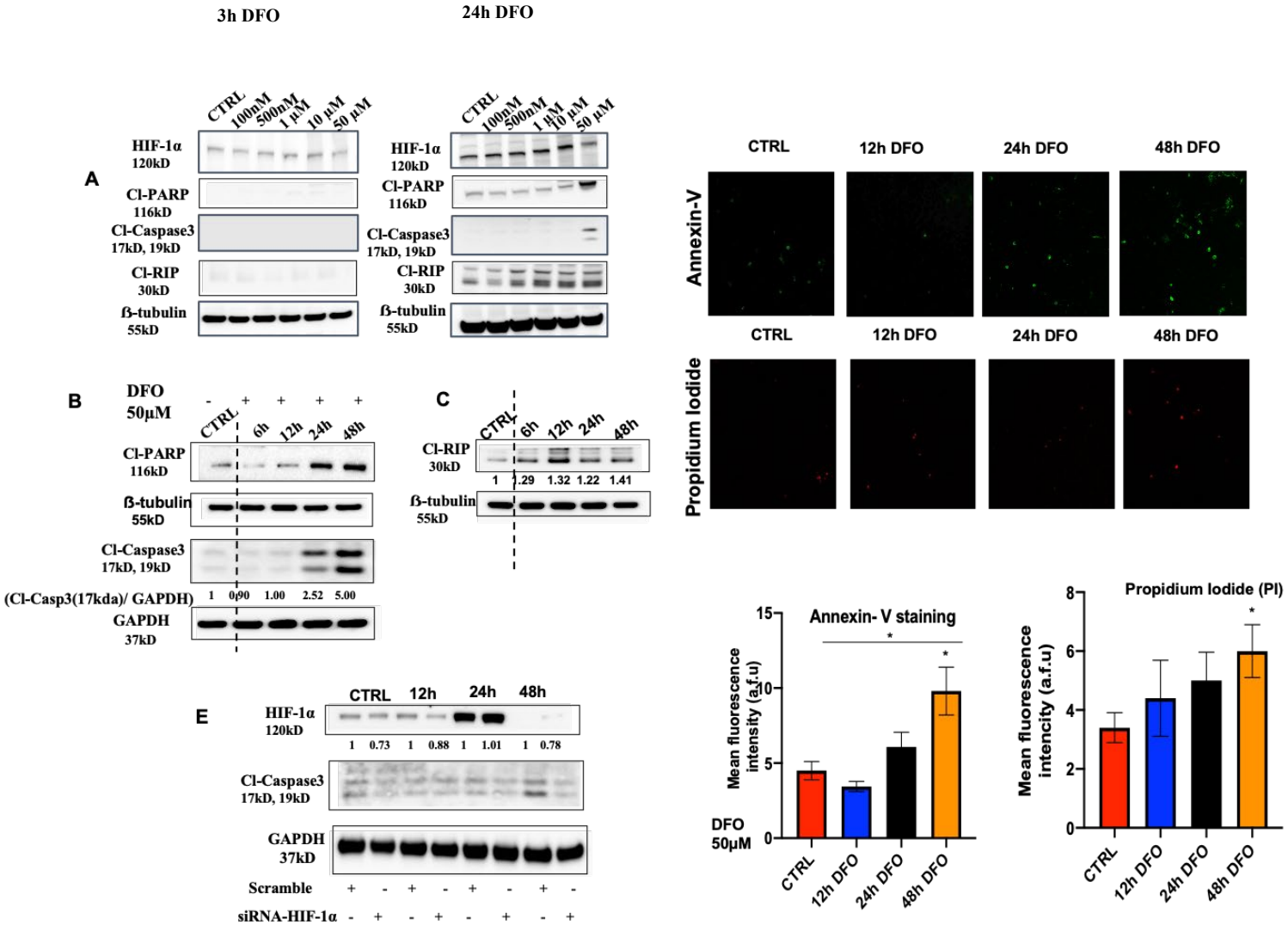

Figure 12: Effect of DFO-induced HIF-1α stabilization on cell death (apoptosis and necrosis) in AC16. **A.** Immunoblot analysis to detect apoptosis (Cl-Caspase3 and Cl-PARP) and necrosis (Cl-RIP) in AC16 treated with DFO 100nM, 500nM, 1µM, 10µM and 50µM at 3h and 24h. **B.** Immunoblot analysis to detectapoptosis in AC16 treated with 50µM DFO at 12h, 24h, and 48h. **C.** Immunoblot analysis to detect necrosis in AC16 treated with 50µM DFO at 12h, 24h, and 48h **D.** Annexin V and PI staining and quantification in AC16 cells treated with 50µM DFO (CTRL, 12h, 24h, and 48h). N=3 **E**. Immunoblot analysis of siHIF-1α detecting apoptosis in AC16 treated with 50µM DFO (CTRL, 12h, 24h, and 48h). The data are presented as means ± SEM. *p<0.01. a.u.f.: arbitrary units' fluorescence.

3.1.4 HIF-1α stabilization increased autophagy and mitophagy

As apoptosis, autophagy has been suggested as an important mechanism in maintaining cellular homeostasis and during the development of multicellular organisms. Therefore, HIF-1α, which is a major transcription factor that responds to cellular oxygen reduction, is low under physiological conditions but increases dramatically in response to hypoxia [61].

To check the effects of HIF-1α on autophagy in our cell model, we quantify the level of autophagy in AC16 transfected with an autophagic marker microtubule-associated proteins 1 light chain 3A (MAP1LC3A, hereafter referred to as LC3) fused to a green fluorescent protein (GFP), LC3 is recruited to autophagosomes forming punctate structures or can be visualized as ring-shaped [99] as showed by green dots (figure 13. A).

Autophagosomes were detected in live-imaging experiments as fluorescent cytoplasmic dots and the number of LC3-positive puncta was significantly more frequent at 12h, 24h, and 48h of DFO treatment compared with the control. Indeed, detection of the LC3-I to LC3-II conversion is monitored through immunoblotting with antibodies against LC3 that recognize the two different forms of LC3 (LC3-I (16kD), LC3-II (14kD), confirmed that autophagy is induced at 12h and 24h of DFO treatment. The level of autophagy was quantified as the ratio of LC3-II to GAPDH as well as and as the ratio of LC3-II/LC3-I (figure 13. B). Furthermore, we evaluated the induction of autophagy in the presence of the lysosomal activity inhibitor NH_4Cl. As a result, after 2h of treatment with NH_4Cl 20mM, in AC16 cells treated with DFO, we observed a marked and comparable accumulation of LC3-II either in control and cells treated with DFO at 12h, 24h, and 48h (figure 13. C).

Therefore, DFO-mediated LC3-II was significantly suppressed by 3-methyladenine (3-MA), an inhibitor of autophagy that targets the class III phosphatidylinositol 3-kinase (PI3K class III), at 24h, in AC16 cells (figure 13. D) Collectively, these results suggested that DFO, which increased higher expression of HIF1α, also induced autophagy in AC16 cells.

In addition, to determine whether HIF-1α highlighted a key role in promoting the process of autophagy in human ventricular cardiomyocytes, we used siRNA-targeted HIF-1α followed by DFO treatment at 12h, 24h, and 48h. As demonstrated in (figure 13. E), the level of autophagy (the ratio of LC3-II to GAPDH) was reduced at 12h, 24h, and 48h of DFO, as well as the autophagic marker Beclin-1. Suggesting that the inhibition of HIF-1α suppresses autophagy.

These findings demonstrate that the autophagic machinery is intrinsically activated dependently on HIF-1αstabilization in AC16.

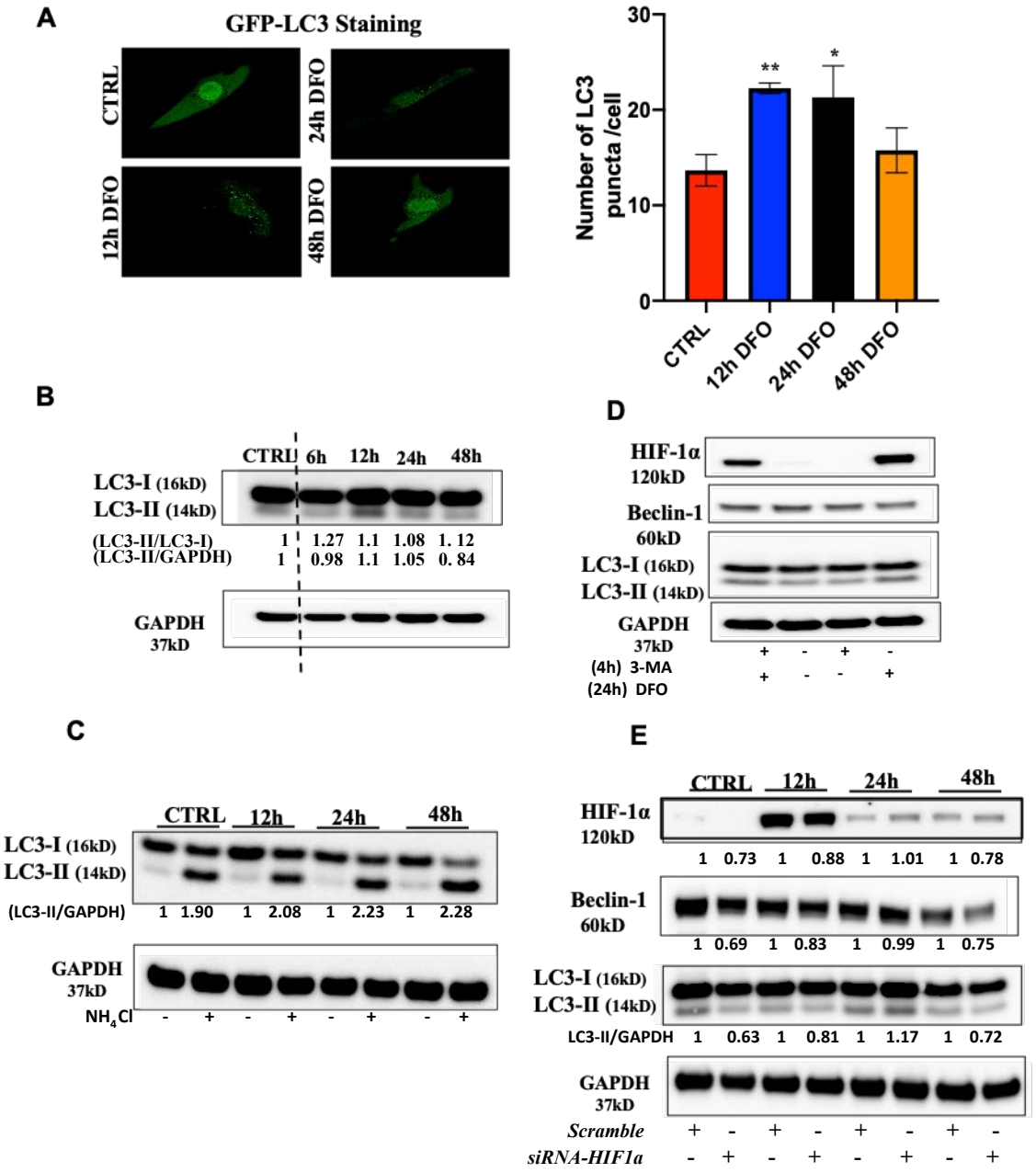

Figure 13: DFO-induced HIF-1α stabilization upregulates autophagy in AC16. **A**. Representative confocal microscopy live-cell imaging of AC16 expressing GFP-tagged LC3 and treated with 50µM DFO (CTRL, 12h, 24h, and 48h) and quantification of colocalization mitochondria-autophagosome. **B.** immunoblot assay to detect autophagy in AC16 treated with 50µM DFO (CTRL, 12h, 24h, and 48h). **C.** Immunoblot analysis of LC3- I/II levels in AC16 cells treated with 50µM DFO (CTRL, 12h, 24h, and 48h), and untreated/treated with NH_4Cl 20 mM for 2h. **D**. Immunoblot of AC16 treated with 50µM DFO (CTRL, 24h), and 3-methyladenine (3-MA) 4h. **E.** Immunoblot analysis detecting autophagy in AC16 with siHIF-1α treated with 50µM DFO (CTRL, 12h, 24h, and 48h). The data are presented as means ± SEM N=3 *P=0.04, **P=0.005.

However, autophagy also exists in selective forms, mitochondrial autophagy, designed as mitophagy, which is necessary for the removal of damaged mitochondria by cellular stress, including hypoxia to preserve a healthy mitochondrial population [117]. We, therefore, investigated whether DFO-induced hypoxia leads to mitophagy in our cell model.

AC16 cells were co-transfected with the autophagic marker fused to a green fluorescent protein (LC3-GFP) and a fluorescent fusion of the localization Tag of a mitochondrial protein (4mt-Cherry)and treated with DFO at different durations at 12h, 24h, and 48h. we analyzed the co-localization of lysosomal and mitochondrial markers in AC16 cells as displayed in (figure 14. A). DFO-induced HIF-1α stabilization at 12h stimulated mitophagy, whereas, at 24h and 48h excessive mitophagy has been observed. Further, we wanted to confirm the mitophagy process in AC16, we co- transfected AC16 with a lysosomal associate protein-1 tagged to green fluorescence protein (LAMP-GFP) with the mitochondrial marker 4mt-Cherry. We observed a significant increase in lysosomal/mitochondrial proteins colocalization at 24h and 48h compared with the 12h and the control cells. Ultimately, this suggests the delayed fusion of the lysosomal to mitochondria (figure 14. B). Along with this, an immunoblotting assay of mitochondrial proteins such as ATP5A and Tim23 blunted out their level in response to hypoxia, thus confirming the previous results (figure 14. C).

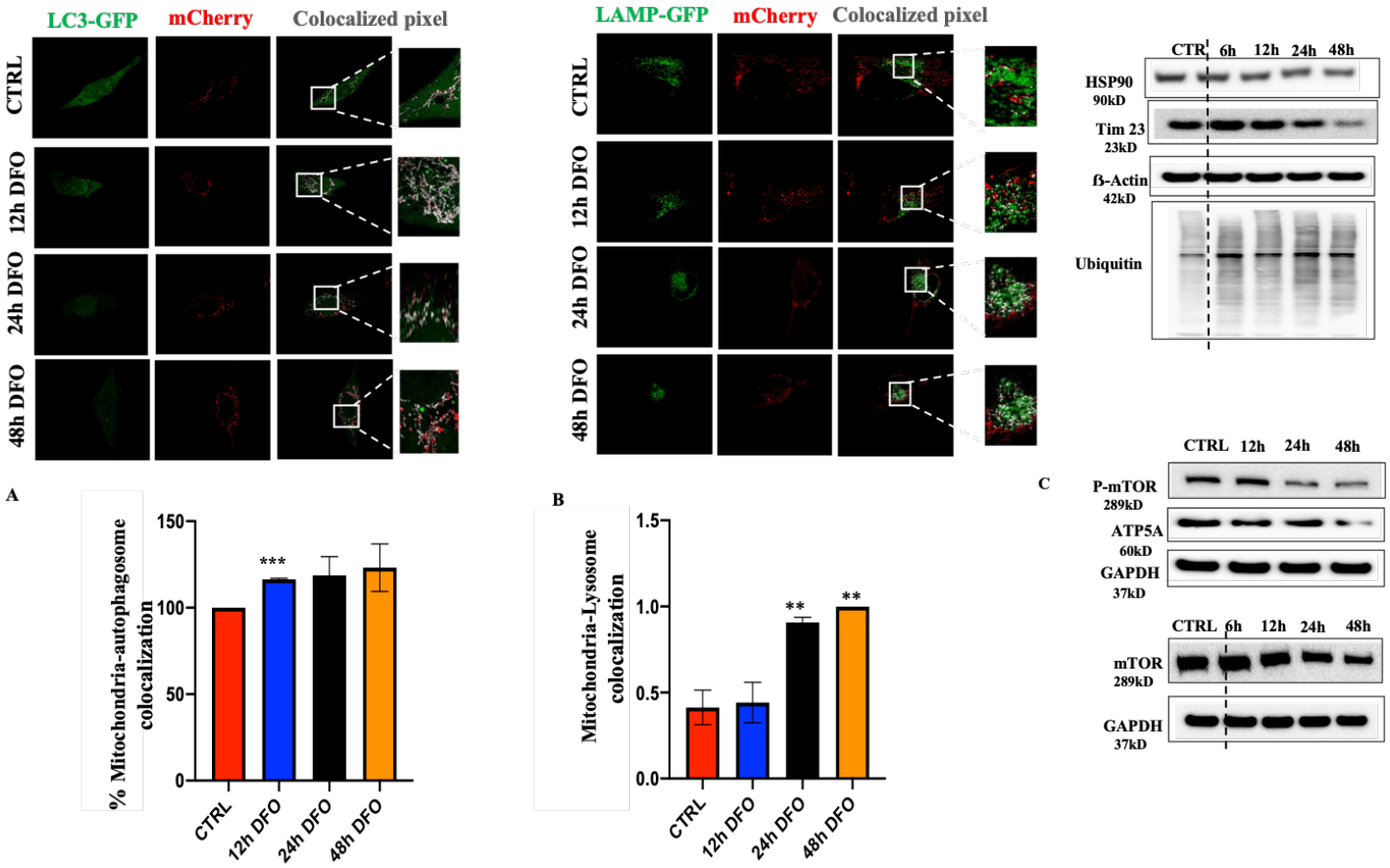

Figure 14: DFO-induced HIF-1α stabilization upregulates mitophagy in AC16. **A.** Representative confocal microscopy live-cell imaging colocalization of AC16 expressing mitochondrial marker (mCherry) and autophagic marker (LC3-GFP) (CTRL, 12h, 24h, and 48h). **B**. Representative confocal microscopy live-cell imaging colocalization of AC16 expressing mitochondrial marker (mCherry) and lysosomal marker (LAMP- GFP) (CTRL, 12h, 24h, and 48h). **C**. Immunoblot analysis of mitochondrial protein change (Tim23, Hsp90, ATP5A), Ubiquitin, mTOR, P-mTOR in AC16 treated with 50µM DFO (CTRL, 12h, 24h, and 48h). The data are presented as means ± SEM N=3. **P=0.007.

3.1.5 HIF-1α effects on the mitochondrial homeostasis

Mitochondria play a potential role in the various cellular processes including energy production, reactive oxygen species (mtROS) generation, and calcium homeostasis during the physiological processes and stress conditions such as hypoxia.

Moreover, in order to understand the effect of DFO mediated HIF-1α stabilization on the mitochondrial homeostasis in response to hypoxia at different times, we assessed the measurement of ATP production level, mitochondrial ROS (mtROS) production, as well as the mitochondrial membrane potential (Ψm) in our cell model AC16.

Given that the mitochondria are the site of most ATP generation, previous works have shown the disruption of the ATP content in response to hypoxic conditions [118]. We prompt to confirm and measure the mitochondrial ATP content level in AC16 using luciferin-luciferase assay as described previously (see materials and methods). As a result, HIF-1α stabilization at12h led to a significant increase in the mitochondrial ATP content level. However, at 24h and 48h, the mitochondrial ATP content was significantly depleted (Figure 15. A).

Next, we measured the mitochondrial reactive oxygen species (mtROS) in our cell model treated with DFO at different time points at 12h, 24h, and 48h. Cells were pre-labeled with MitoTracker Red CMXRos. The fluorescence intensity changes among the conditions were subsequently monitored using confocal imaging. In contrast to the control and the cells treated with DFO at 12h, which showed minimal MitoTracker Red CMXRos fluorescence, whereas treatment with DFO at 24h, we highlighted a significant increase in fluorescence intensity (figure 15. B). Treatment with DFO at 48h resulted in a strong fluorescence originating from the mitochondria. Therefore, we usedsiRNA of HIF-1α, and AC16 cells were treated with DFO 50μM at different times 12h, 24h, and 48h. The repression of HIF-1α contributes to a reversible result in which the generation of mtROSis higher at 12h compared with 24h and 48h of DFO administration (figure 15. C). Furthermore, to better define the impact of hypoxia-induced HIF-1α in the mitochondrial function in AC16, we investigated the effect of DFO-induced hypoxia on the mitochondrial membrane potential (ΔΨm), which plays a vital role in maintaining the physiological function of mitochondria. AC16 were labeled with the membrane-permeant fluorescent dye Tetramethylrhodamine, methyl ester (TMRM), which is readily sequestered in the mitochondria of healthy cells [119]. Then, analysis of the cells by confocal microscopy showed that the ΔΨm of hypoxia-exposed cells by DFO at 12h appeared significantly higher (hyperpolarization) when HIF-1α-is accumulated than

in the control, protecting the cells from cell death (apoptosis). In contrast, the ΔΨm was lower in the cells treated with DFO at 24h and 48h (depolarization) (figure 15. D), indicating, the prevention of the mitochondrial membrane potential loss by HIF-1α accumulation during the acute level of hypoxia.

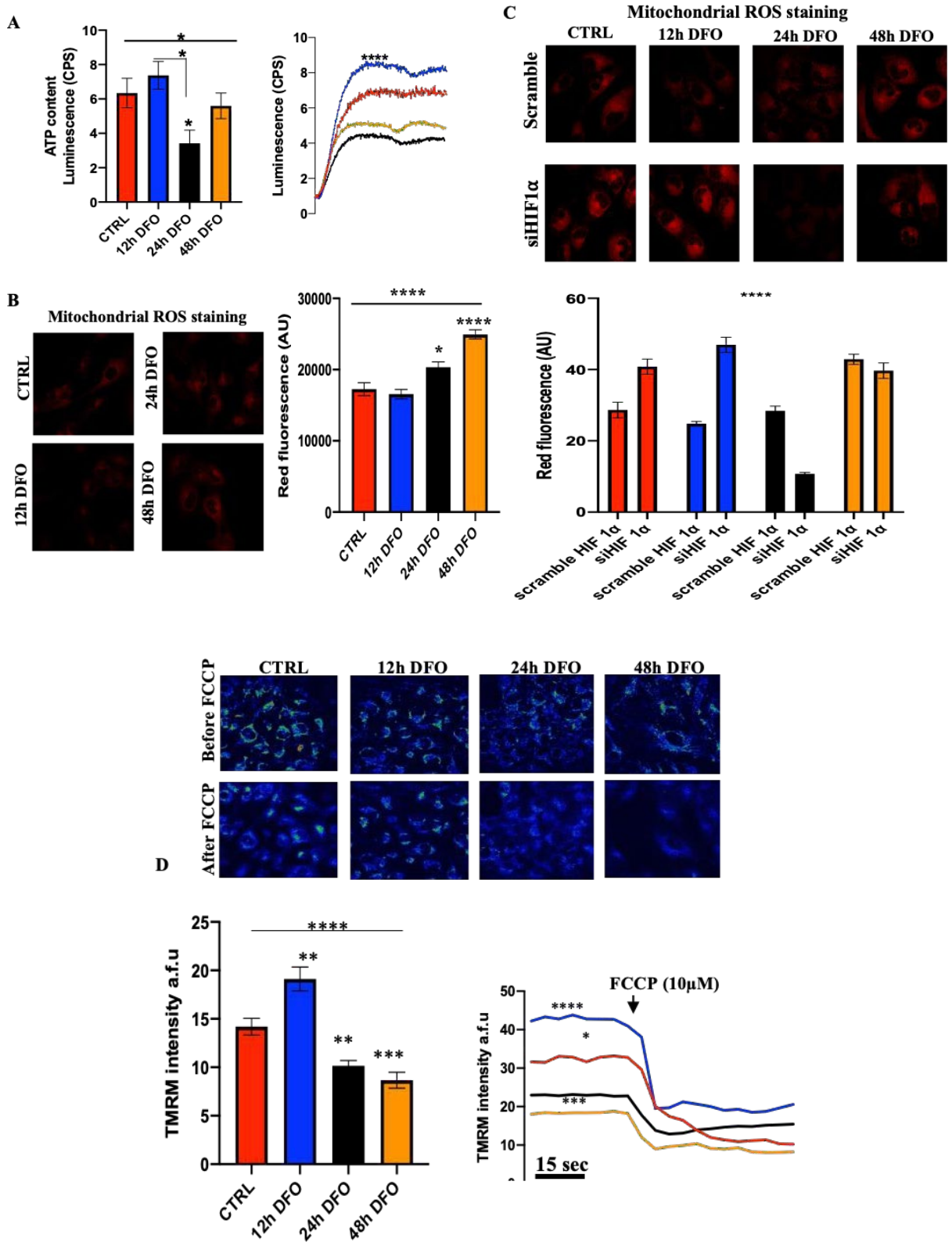

Figure 15: Hypoxia affects mitochondrial homeostasis. **A.** Mitochondrial ATP content measured by luciferin-luciferase assay; cps, counts per second. N=5 **B**. Representative confocal microscopy live-cell imaging of mitochondrial ROS fluorescence in AC16 treated with 50μM DFO (CTRL, 12h, 24h, and 48h). N=4 **C.** Representative confocal microscopy live-cell imaging of mitochondrial ROS fluorescence in AC16 siHIF-1α treated with 50μM DFO (CTRL, 12h, 24h, and 48h). N=2. **D**. Basal mitochondrial membrane potential analyzed by the TMRM probe and fluorescence microscopy. a.u.: arbitrary units. N=3. The data are presented as means ± SEM.

3.1.6 DFO-induced HIF-1α stabilization disrupts the mitochondria-ER contact sites

The interface between mitochondria and the endoplasmic reticulum -termed mitochondrial associated membrane (MAM), as mitochondria-ER contact sites (MERCs), is emerging as a crucial hub for calcium signaling, apoptosis, and auto/mitophagy, with far-reaching implications in cell life and death and the regulation of mitochondrial and endoplasmic reticulum function [120]. Very recently, studies on MAM in the field of cardiovascular diseases have gained more attention due to its role in mediating several processes involved in health and diseases. To date, shreds of evidence enclosed its direct roles in cardiovascular diseases [42]. However, as best in our knowledge no evidence has shown the impact of HIF-1α in MAM.

To demonstrate the effect of DFO-induced HIF-1α stabilization on the mitochondrial associated membrane (MAM), AC16 cells were co-transfected with mitochondrial marker protein (Mito) (mCherry) and endoplasmic reticulum (ER) marker tagged to GFP (PacSec61-GFP), treated with DFO at different time points at 12h, 24h, and 48h. Confocal live cell imaging showed an increase in the colocalized puncta of the mitochondrial marker mCherry and the endoplasmic marker PacSec61-GFP at 12h DFO and followed that 24h and 48h of the treatment (figure 16. A). As it is well documented in the quantification reported in the graph of the ER-Mito contact sites percentage that is increased significantly at 12h compared with cell control. Similarly, at 24h and 48h of DFO, an upregulation of the contact site between the ER and the mitochondria has been shown as well.

We confirmed these results also by immunofluorescence analysis of colocalization between an ER marker and a mitochondrial marker, protein disulfide isomerase (PDI), and ATP synthase F1

subunit alpha subunit (ATP5A) respectively. A high colocalization between the mitochondria and the ER at 12h, followed by an increase at 24h and 48h of hypoxia, as is reported in figure 16. B. we the protein levels of ATP5A and PDI by western blot as shown in figure 16.C.

These findings indicate that DFO-induced hypoxia has also an impact on the mitochondrial associated membrane by increasing high-affinity interaction and contact between the two types of machinery mito-ER, suggesting that HIF-1α may play a crucial role in MAM by activating various processes.

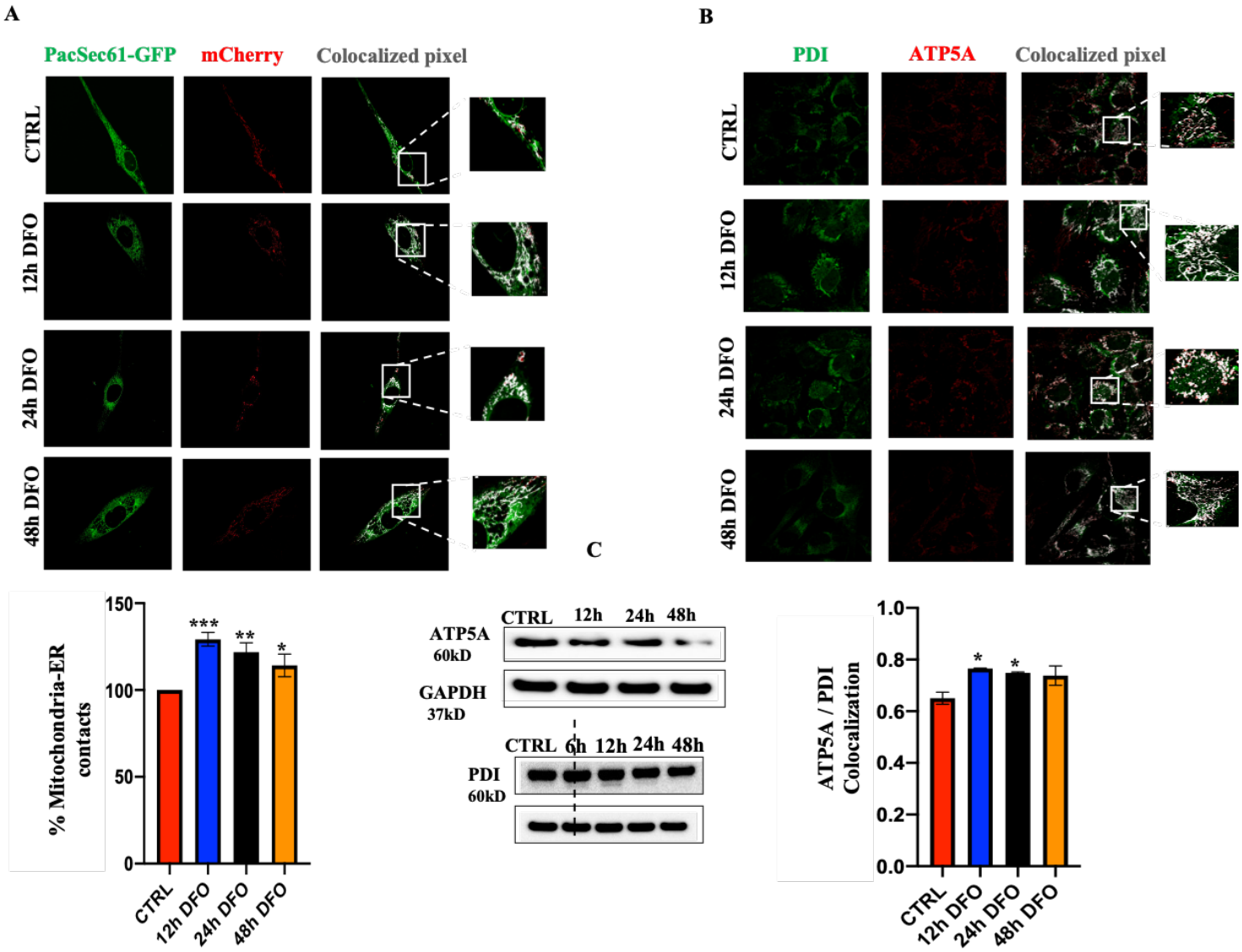

Figure 16: HIF-1α stabilization affects the mitochondria-ER contact sites. **A**. Representative confocalmicroscopy live-cell imaging colocalization of AC16 expressing the mitochondrial marker (mCherry) and endoplasmic reticulum (Pacsec61-GFP) treated with 50µM DFO (CTRL, 12h, 24h, and 48h). **B.** Confocal fluorescence microscopy of AC16 cells incubated and stained with 50µM DFO (CTRL, 12h, 24h, and 48h) antibodies to ATP5A (red, Mitochondrial marker) and PDI (green). **C.**

Immunoblot detecting mitochondrial marker (ATP5A) and ER marker (PDI). The data are presented as means ± SEM. N=4 each experiment. N=3. ***p<0.05. **p<0.01. *p<0.07.

3.1.7 Impact of DFO-induced hypoxia on intracellular calcium (Ca^{2+}) in human ventricular cardiomyocytes

Given that multiple mitochondrial variables have been shown to be altered under the hypoxia-mediated accumulation of HIF-1α at the specific duration of time in our cell model. Here, we verify the modulation of DFO-induced hypoxia to the mitochondrial calcium handling and to analyze the involvement of the mitochondrial Ca^{2+}, AC16 were transfected transiently with aequorin-based mitochondrial calcium probes (mtAEQ) [107], and treated with DFO at the different duration of time 12h, 24h, and 48h. Representative traces of typical experiments and statistical analysis are indicated in (figure 17. A). AC16 were exposed to 500μM histamine (His) and bradykinin (BK), causing the generation of inositol 1,4,5 trisphosphate (InsP3) and the consequent opening of the InsP3 channels of the intracellular stores [107]. DFO-induced HIF-1α accumulation (12h) increased slightly the mitochondrial calcium uptake compared with the control cells, while, at 48h DFO decreased the mitochondrial calcium and significantly at 24h than in cells treated with DFO at 12h (figure 17. A). These results are fully supported by the data showing the level of the mitochondrial ATP content level.

Then measurements of cytosolic Ca^{2+} transients generated by cell stimulation were performed using aequorin probes targeted to the cytosol CytAEQ [107], treated with DFO at the different duration of time 12h, 24h, and 48h. Representative traces of typical experiments and statistical analysis are demonstrated in (figure 17. B). AC16 were exposed to 500μM and histamine (His) and bradykinin (BK), leading to the generation of inositol 1,4,5 trisphosphate (InsP3) and the consequent opening of the InsP3 channels of the intracellular stores [107]. No significant difference has been shown among 12h and 24h compared with the cell control, at 48h cytosolic Ca^{2+} transients in AC16 were significantly increased than those observed in control cells (figure 17. B).

Ca^{2+} is assumed to be first transported from the ER to the cytoplasm, where it is then taken up by mitochondria [19]. To understand whether also DFO-induced HIF-1α accumulation affects the ER calcium release in AC16.

Therefore, Measurement of the endoplasmic reticulum calcium has been assessed using ER-GCAMP6-210, a fluorescent reporter for the ER calcium signaling, followed by DFO treatment at 12h, 24h, and 48h. AC16 cells were stimulated with 500μM histamine (His) and bradykinin (BK), the P2Y receptor agonist that causes the release of Ca^{2+} from the ER. Strikingly, we noticed a

higher decreased level of [Ca^{2+}]ER in AC16 cells treated with DFO 12h in quantitative and kinetic terms, which were faster and larger compared with the control cells and the other conditions. Additionally, a decrease in the [Ca^{2+}]ER has been also observed at 24h and 48h of DFO (figure 17. C).

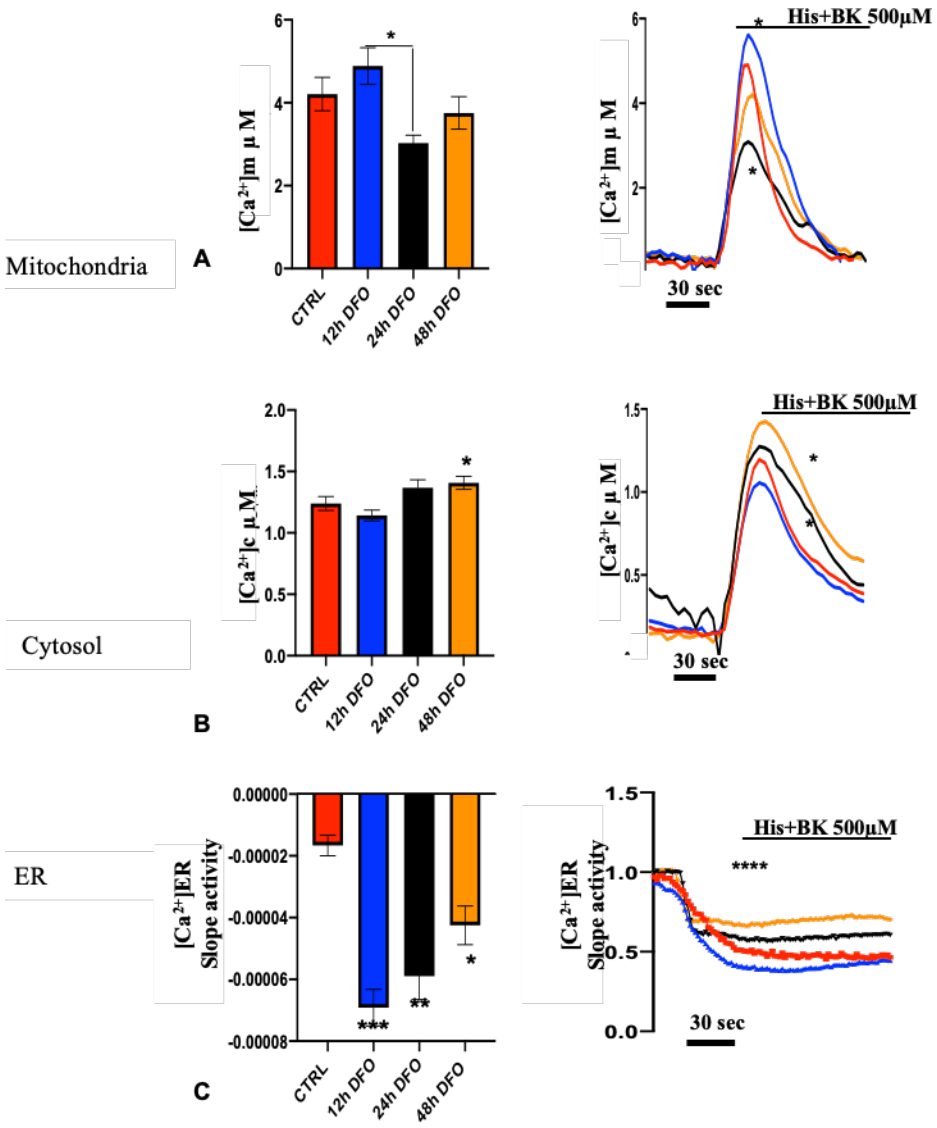

Figure 17: Hypoxia and calcium homeostasis in AC16. **A.** Measurement of [Ca^{2+}] using recombinant aequorin upon agonist (500µM Histamine+Bradykinin) stimulation in mitochondria. **B.** Measurement of [Ca^{2+}] using aequorin upon agonist (500µM Histamine+Bradykinin) stimulation into the cytosol. **C**. ER calcium measurement assessed with ER-GCAMP6-210 reported as slope. AC16 cells treated with 50µM DFO (CTRL, 12h, 24h, and 48h) cells were challenged with (500µM Histamine+Bradykinin). N = 10 [Ca^{2+}]m,c, [Ca^{2+}]ER N=3.The data are presented as means ± SEM ****p<0.001. ***p<0.05. **p<0.01.
*p<0.07.

3.1.8 DFO-mediated hypoxia-induced Endoplasmic reticulum (ER) stress

The ER is a major organelle housing multiple cellular functions and is, therefore, a crucial site for maintaining homeostasis. The ER stress reflects an imbalance between the ER cellular demand for function and ER protein folding ability and has been revealed to be sensitive to the hypoxic environment [121].

To check whether DFO-induced HIF-1α stabilization at different time points (12h, 24h, and 48h) in AC16 may affect the ER machinery and induce stress using immunofluorescence, we examined some ER stress-related proteins including CCAAT-enhancer-binding protein homologous protein (CHOP), which is a pro-apoptotic factor used as a marker for ER stress, and binding immunoglobulin protein (BiP) known as GRP78, which is a central regulator of ER stress due to its role as a major ER chaperone with anti-apoptotic properties [122].

As a result, we observed that CHOP is upregulated significantly at 24h and 48h compared with the control cells and 12h DFO (figure 18. A). Moreover, BiP is significantly more expressed at 12h DFO than at 24h and 48h (figure 18. B), and the level of PDI protein was found to decrease in response to hypoxia initiating at 12h, 24h, and 48h (Figure 18. C). This indicates that DFO-induced cell death is also mediated by the ER stress pathway at 24h and 48h and the ER stress may not activate at a late stage in response to DFO-induced hypoxia in AC16.

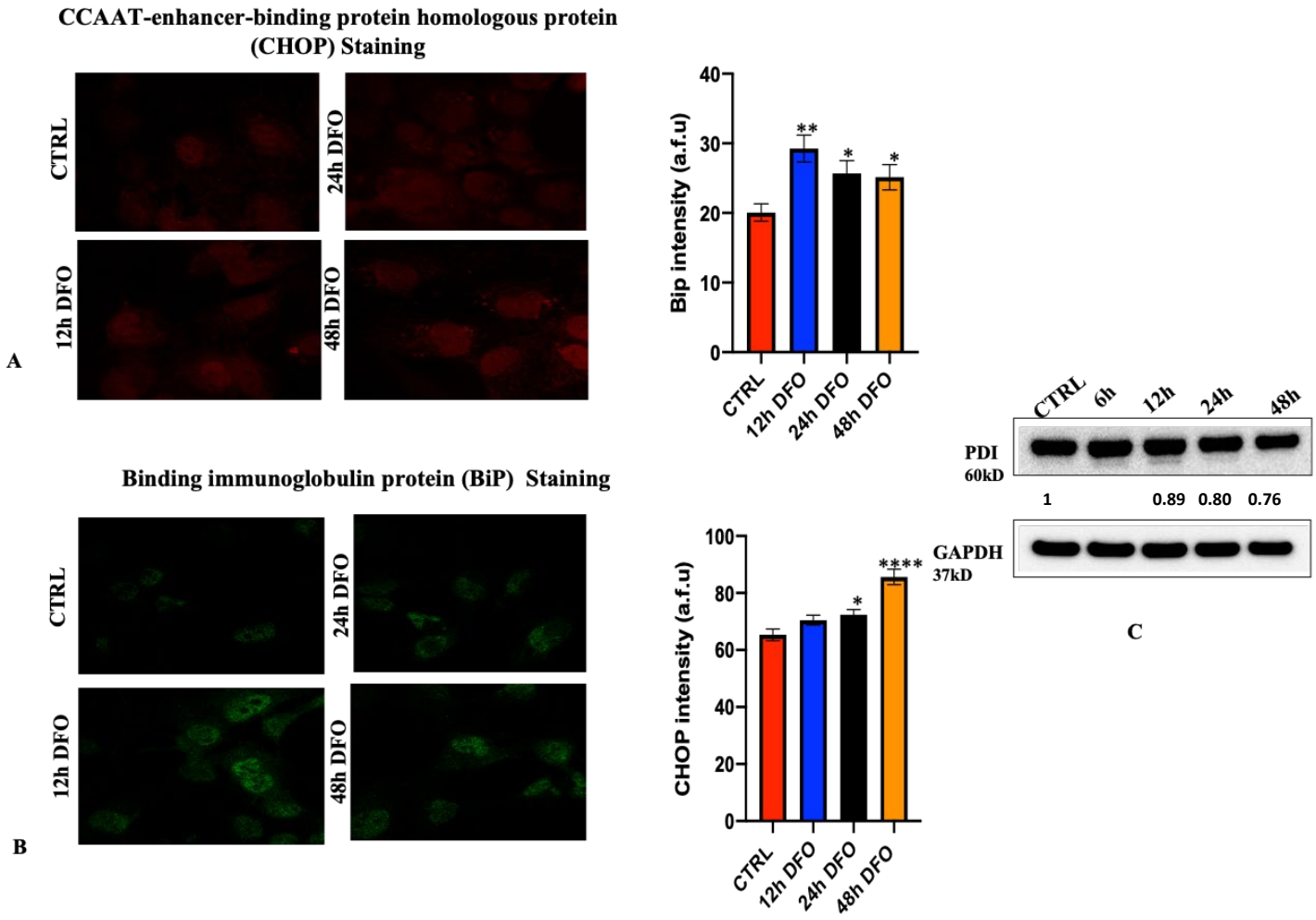

Figure 18: Hypoxia-induced ER stress in AC16 treated with DFO. **A.** CHOP immunofluorescence detection and quantification by confocal microscopy in AC16 cells treated with 50µM DFO (CTRL, 12h, 24h, and 48h). **B**. BiP immunofluorescence detection and quantification by confocal microscopy in AC16 cells treatedwith 50µM DFO (CTRL, 12h, 24h, and 48h). **C.** Immunoblot detecting ER marker (PDI). The data are presented as means ± SEM. ****p<0.001. **p<0.01.

3.1.9 Human ventricular cardiomyocytes show resistance to mPTP opening induced by ionomycin during acute hypoxia

With the aim of understanding whether mPTP activity can change among different timing of inducing hypoxia in human ventricular cardiomyocytes and whether the pore opening is significantly related to HIF-1α stabilization, and further mechanisms underlying the reduction of ΔΨm. We evaluated the opening of mPTP activity, which allows the diffusion of small ions across the mitochondrial inner membrane, and it has been proposed that mPTP opening modulation is a strategic regulator of cell death by apoptosis [123].

By measuring mPTP opening in human ventricular cardiomyocytes (AC16), as evaluated by calcein acetoxymethyl ester (calcein-AM)-cobalt ($CoCl_2$) quenching assay (excitation/emission: 494/517nm) resulting in mitochondrial localization of calcein fluorescence, subjected to DFO at12h, 24h, and 48h, compared with control cells.

Calcein-AM is a membrane-permeable fluorophore that diffuses freely into all subcellular compartments including mitochondria. The acetoxymethyl (AM) group of the fluorophore is cleaved by ubiquitous intracellular esterase. Calcein, which is hydrophilic, is then trapped within all subcellular compartments. The cells are then loaded with the divalent cobalt cation (Co^{2+}), quenches calcein fluorescence in all subcellular compartments except the mitochondrial matrix which is enclosed by a Co^{2+} impermeable inner mitochondrial membrane when mPTP is closed. Thus, the ability of Co^{2+} to quench mitochondrial calcein fluorescence only when mPTP is open allows the determination of open vs closed status of mPTP in the cell, manifesting in a reduction of calcein fluorescence intensity [124].

Variability in the mPTP activity was found in AC16 subject to 12h, 24h, and 48h of DFO. Interestingly, as illustrated in the (figure 19. A) during acute hypoxia at 12h in which HIF-1α is accumulated, we noticed an increase in the calcein fluorescence intensity, the cells exhibit resistance to mPTP opening induced by ionomycin compared with the untreated cells. AC16 at 12h maintained their intensity and hence the mPTP is closed. This result suggests that mPTP is more open in control compared with cells treated with DFO at 12h.

While at 24h and 48h of DFO administration, we observed a significant decrease in calcein fluorescence intensity, indicating a greater quenching of mitochondrial calcein fluorescence after ionomycin stimulation, and subsequently higher increase in mPTP opening compared with the control cells (figure 19. B).

Indeed, we assess a knockdown of HIF-1α followed by a DFO treatment at 24h, in which mPTP initiates the opening, indicating an increase in calcein fluorescence intensity compared with the cells expressing HIF-1α and treated with DFO at 24h. Thus, confirming the fact that HIF-1α accumulation may play a key role in protecting against the opening of mPTP (figure 19. C. D).

Taken together, these findings suggest an interesting relationship between HIF-1α and mPTP activity during acute hypoxia in human ventricular cardiomyocytes under hypoxic stress.

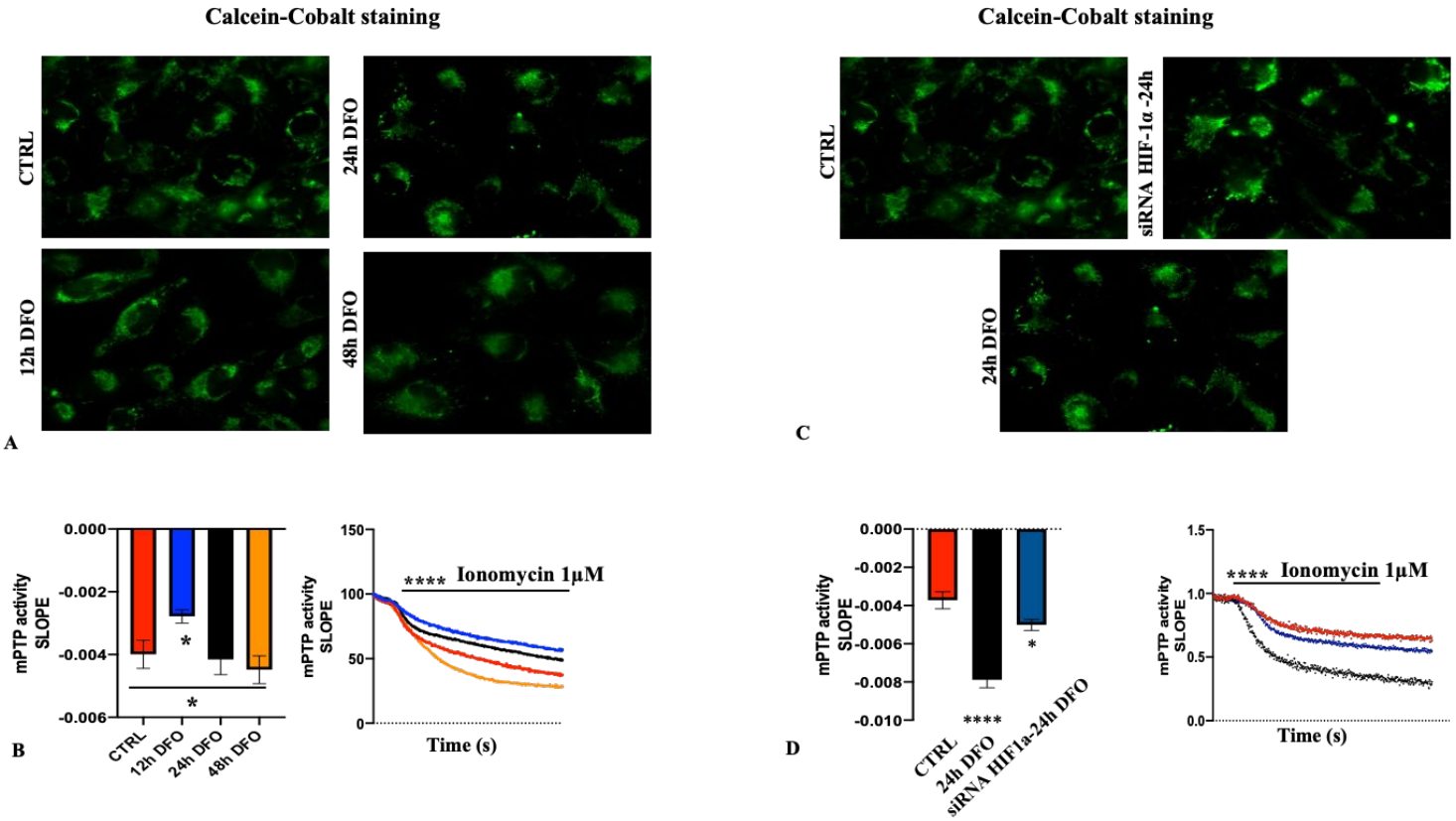

Figure 19: DFO-induced hypoxia affected mPTP activity. **A**. Confocal imaging of Calcein-cobalt quenching assay of AC16 treated with 50µM DFO (CTRL, 12h, 24h, and 48h). **B**. PTPC activity is reported as the slopeof the kinetics following ionomycin (500nM) administration. N=4 **C**. Confocal imaging of Calcein-cobalt quenching assay of AC16 treated with 50µM DFO (CTRL, 24h, HIF-1α -24h DFO), **D.** PTPC activity is reported as the slope of the kinetics following ionomycin (500nM) administration. N=2. The data are presented as means ± SEM ****p<0.0001 ***p<0.05.*p <0.06

3.1.10 Inhibition of autophagy attenuates DFO-mediated mPTP opening

Autophagy is increased under stress conditions, and how autophagic activation signals the mitochondrial permeability transition pore in a particular mitochondrion remains to be investigated and it can promote cell survival or cell death depending on the type of cellular stress such as hypoxia. To understand whether hypoxia-induced autophagy promotes the mPTP activity in human ventricular cardiomyocytes and whether it contributes dependently to survival or cell death. AC16 cells were exposed to an autophagic inhibitor 3-methyladenine (3-MA) followed by DFO treatment at 24h, then we evaluated the subsequent opening of mPTP by using the calcein-cobalt assay. A decrease in mPTP activity was observed in AC16 cells after the inhibition of autophagy and DFO treatment at 24h (24h DFO +3MA) compared with the cells treated only with 24h DFO (figure 20. A. B). This indicates that autophagy at this level may contribute to stimulating the mPTP activity in response to hypoxia at 24h precisely.

To disclose DFO's ability on autophagy induced-apoptosis, Ac16 was administered autophagy inhibitors 3-MA. The cell viability rate of the DFO (24h) + 3-MA group shows no significant reduction compared with the control cells and DFO (24h) (figure 20. C). This effect of DFO's activity and the inhibition of autophagy by 3-MA was observed on mPTP activity, as it is shown to be reduced in the DFO+ 3-MA group compared with DFO (24h). Additionally, as it is documented in the immunoblotting, HIF-1α protein was upregulated in DFO (24h) than DFO (24h) + 3-MA group (figure 20. D). Suggesting, the eventual role of HIF-1α upon autophagy-induced mPTP opening in AC16.

Assessing further experiments in vitro and in vivo to address the possibility that the blocking of autophagy is responsible for cell survival or cell death by affecting mPTP activity in response to DFO-induced HIF-1α at the specific duration of time in human cardiomyocytes are required.

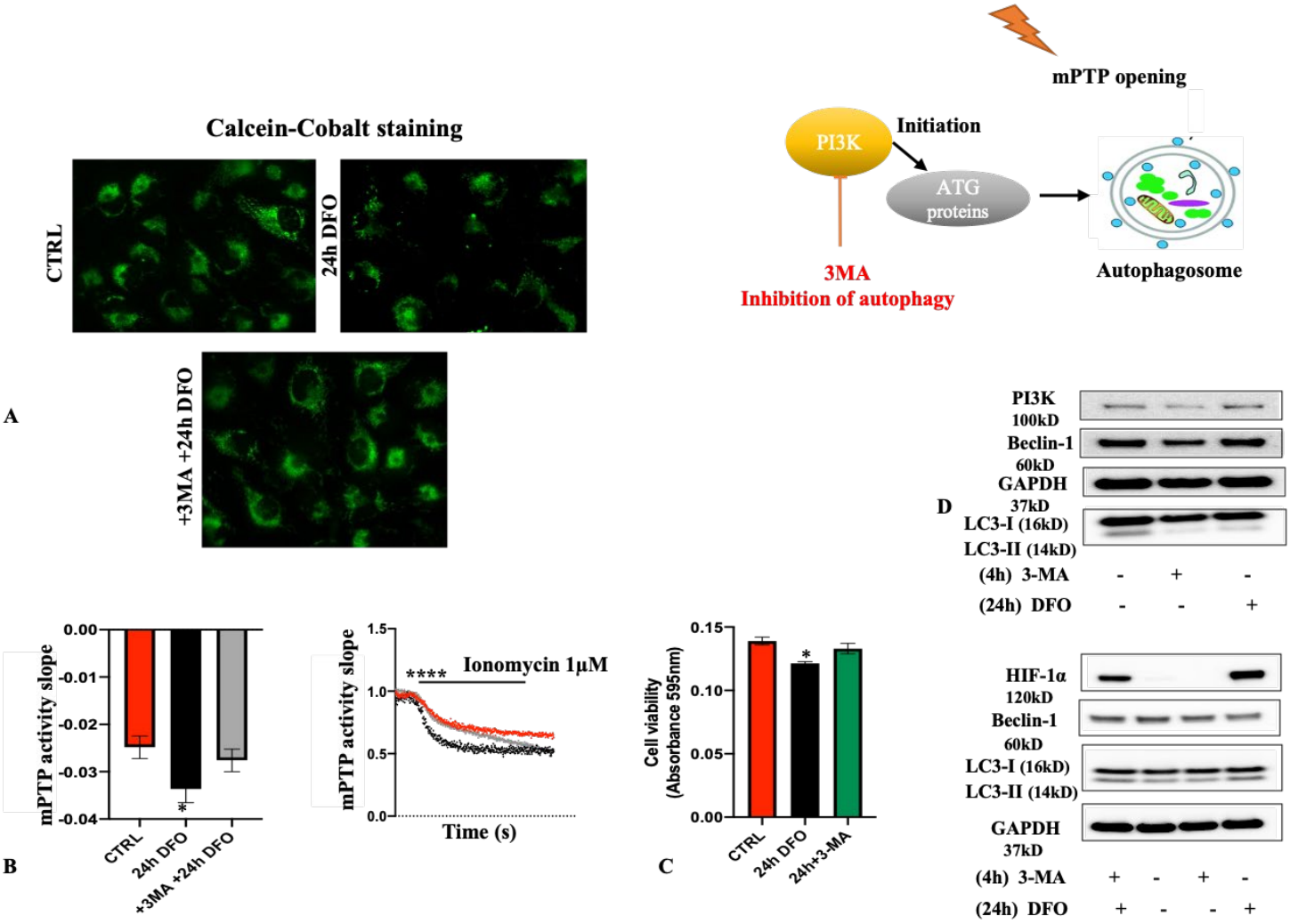

Figure 20: Inhibition of autophagy attenuate mPTP activity in response to DFO-induced hypoxia in AC16. **A**. confocal imaging of Calcein-cobalt quenching assay of AC16 treated with 24h DFO and another group of AC16 treated with (24h DFO +3MA), **B**. PTPC activity is reported as the slope of the kinetics following ionomycin (500nM) administration. **C**. Quantification of cell viability in AC16 treated with or without 24h DFO and (24h DFO +3MA). **D**. Immunoblot analysis for detecting autophagy, and HIF-1α level and PI3K blockade after 3MA.

3.2 New subcellular localization and possible effective roles of HIF-1α in human ventricular cardiomyocytes during acute hypoxia

3.2.1 HIF-1α localization in AC16 during hypoxia

As described previously during hypoxia and as illustrated in (figure 7), the HIF-1α subunit is rapidly ubiquitinated and degraded proteosomally in the cytosol by hydroxylation of prolyl residuesin normal conditions. However, during hypoxia, HIF-1α accumulates in the cytosol and translocasesto the nucleus where it binds and dimerizes the HIF-1ß subunit in hypoxic responsive elements (HRE) of target genes and subsequently activate the transcription of several target genes [49]. Although the HIF-1α activities during hypoxia have been ascribed to its nuclear localization, various processes such as calcium modulation, apoptosis and autophagy are largely regulated at the cytoplasm.

To determine and understand the effect of hypoxia on HIF-1α localization in human ventricular cardiomyocytes cells (AC16). Whole lysates (homogenate), mitochondria, ER, and MAM were isolated by ultracentrifugation [109] from AC16 cells exposed to DFO, which accumulates HIF-1α by blocking prolyl hydroxylases (PHDs) activity, thereby mimicking hypoxic effect at 12h (in which HIF-1α is more stabilized).

Subcellular fractionation of AC16 cells demonstrated for the first time the subcellular localization of HIF-1α in response to DFO-induced hypoxia. Interestingly, we found that HIF-1α was specifically enriched at the ER fraction, and a small fraction of HIF-1α appeared to the pure mitochondria free of ER fraction, as well as at MAM after further exposure to hypoxia at 12h (figure 21. A). Next, we conducted immunofluorescence to detect further interaction between HIF- 1α / ER (PDI) and mitochondria (ATP5A) in response to DFO-induced hypoxia. Colocalization analysis shows a highly significant interaction between HIF-1α and PDI than ATP5A (figure 21. B, C).

Therefore, it has been documented the HIF-1α translocation at the mitochondria in other cell lines [125,126]. However, to the best of our knowledge, no study to date has been documented the translocation of HIF-1α at the ER or MAM during hypoxic conditions, this enrichment of HIF-1α at the ER nicely led us to ask whether HIF-1α plays another effective possible role at this level?

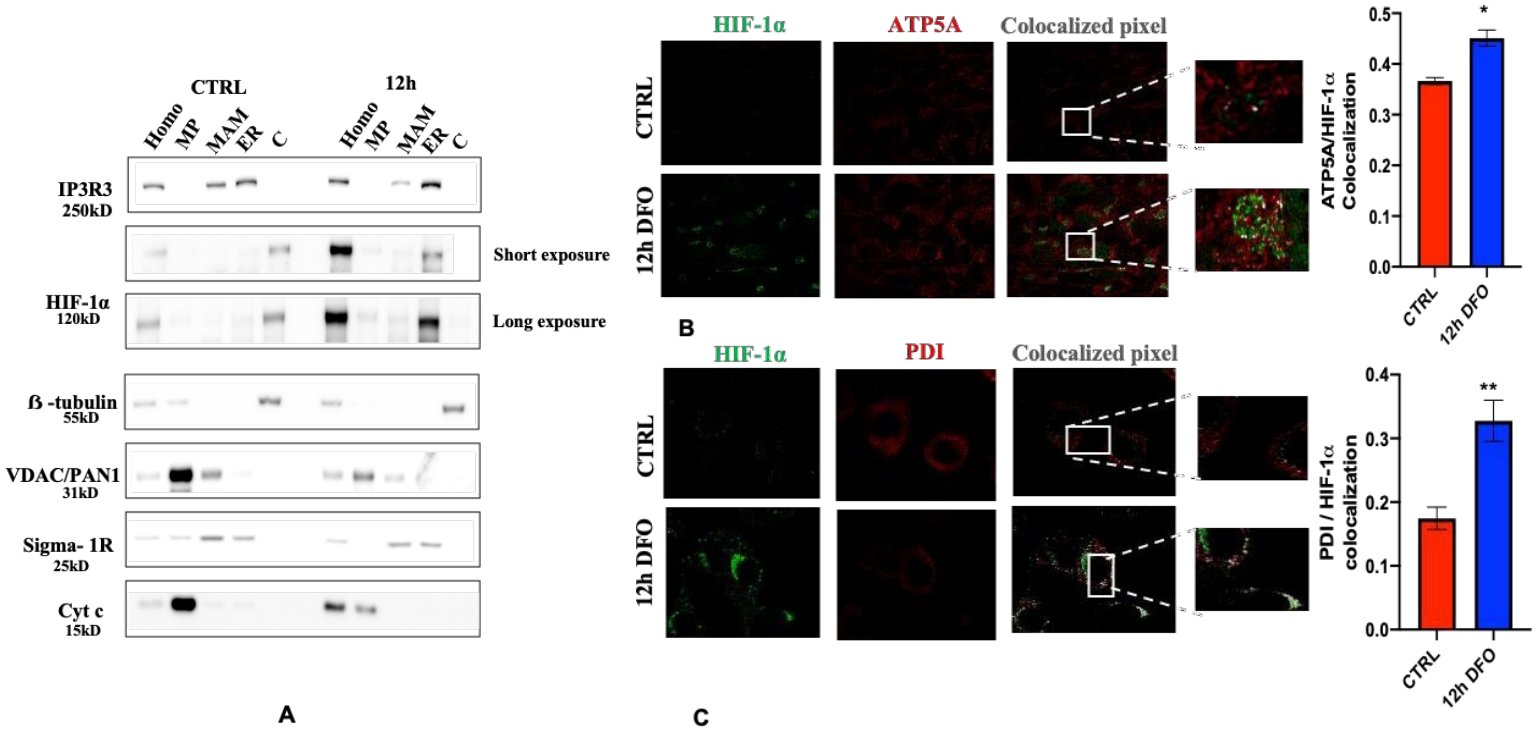

Figure 21: HIF-1α translocation during hypoxic condition. **A.** Subcellular localization of HIF-1α during DFO induced-hypoxia as determined by fine fractionation. IP3R3, β-tubulin, VDAC/PAN1, Cytc, and sigmaR1 were used as markers of ER, cytosol, mitochondria, and MAM, respectively. H: homogenate; MP: pure mitochondria; ER: endoplasmic reticulum; MAM: mitochondria-associated membranes; C: cytosol. **B.** Confocal fluorescence microscopy of AC16 cells incubated with or without DFO for 12h and stained with antibodies to ATP5A (red, Mitochondrial marker) and HIF-1α (green). **C**. Confocal microscopy of AC16 cells incubated with or without DFO for 12h and stained with antibodies to PDI (red, ER marker) and HIF- 1α (green) The merged image shows co-localization of mitochondria and HIF-1α in DFO-treated cells (Colocalized pixel).

3.2.2 Roles of hypoxia-mediated HIF-1α stabilization on inositol 1,4,5-triphosphate (IP3) receptor 3 (IP3R3) in AC16

It has been reported that the ER machinery is the major intracellular calcium store [127]. IP3R3 is an ER channel that regulates and transmits Ca^{2+} from the ERinto the mitochondria through MAM that serves as a bridge for Ca^{2+} transfer between the ER and mitochondria [128].

First, we checked the expression of IP3R3 in AC16 cells treated with DFO at 12h, 24h, and 48h by immunoblotting (Figure 22. A). We found that DFO-induced HIF-1α stabilization affects IP3R3 amounts. Indeed, at 12h DFO the presence of hypoxia results in a decrease in IP3R3 compared with the control and the other conditions (this reduction was rescued by the proteasoamal inhibitor MG132), at 24h and 48h of DFO, IP3R3 levels start to increase at 24h then recovery at 48h is well observed (MG132 treatment abolished this difference) (figure 22. A, B).

To verify this, we transfected AC16 cells with IP3R3 tagged to GFP (IP3R3-GFP) and by using live-cell imaging as reported in (Figure 22. C), IP3R3 intensity was found reduced significantly at 12h compared with the control cells and 48h well, 24h DFO IP3R3 intensity level was reduced compared the control and slightly increased than cells treated at 12h DFO.

Recently, IP3R3 was reported to be regulated by BRCA1-associated protein1 (BAP1), a tumor suppressor in which it binds, deubiquitinates, and stabilizes IP3R3, regulating Ca^{2+} release from the ER into the mitochondria and subsequently promoting apoptosis [128]. Interestingly,similar results of IP3R3 protein level were found for BAP1 as well in response to DFO-induced HIF-1α stabilization, suggesting the involvement of the BAP1-IP3R3 axis (Figure 22. D). We confirmed these findings using immunofluorescence of BAP1 upon DFO treatment at the same concentration and durations pointed out previously, as referred in the (Figure 22. E). Indeed, BAP1 level is increased in rat animals during ischemia and reperfusion (I/R). In addition, autophagy- related proteins were surprisingly found to increase also during I/R (Figure 22. F).

Further, we checked also the phosphatase and tensin homolog (PTEN) that is found to counteract with the F-box protein FBXL2 (the receptor subunit of one of 69 human SCF (SKP1, CUL1, F-box protein) to stimulate IP3R3 and Ca^{2+} mediated apoptosis [129]. Opposite results were found for FBXL2 compared with BAP1, leading to a reduction of PTEN as identified in the immunoblot (Figure 22. G).

In order to understand the effect and the correlation between HIF-1α stabilization and IP3R3 during hypoxia specifically at 12h where HIF-1α is more stabilized, we assessed a knockout mediated by small interferon RNA (siRNA) targeted HIF-1α (siHIF-1α) followed by DFO treatment at 12h in AC16 cells. As referred in the immunoblotting (figure 23. A). IP3R3 amount is reduced when siHIF-1α is used in the untreated cells. Then, IP3R3 levels started to increase very slightly in the cells siHIF-1α treated with DFO at 12h. A further slight reduction in the autophagic related proteins such as LC3-I/II and Beclin-1 has been observed.

Moreover, in siIP3R3 followed by DFO treatment at 12h reduced the level of HIF-1α has been observed compared with the cells treated with DFO only, thus, downregulating the autophagic process (figure 23. B). Overexpressing IP3R3 under hypoxia affects ultimately HIF-1α protein level at 12h DFO, as documented in the immunoblotting, HIF-1α is more accumulated in responseto IP3R3 overexpression resulting in an early autophagy and apoptosis induction (figure 23. C).

To rule out the possibility that DFO impacts HIF-1α proteins synthesis during hypoxia at 12h and may in return affect IP3R3, we used Cycloheximide (CHX), which prevents de novo proteinsynthesis. HIF-1α levels would reflect the process of protein degradation. AC16 cells were exposed to CHX (50µg/ml for 5h) in the presence or absence of hypoxia-induced by DFO at 12h and estimated the expression of HIF-1α levels. HIF-1α was absent in the cells treated only with CHX as well as in CHX+12h DFO, in return IP3R3 was decreased slightly in cells treated only with CHX but a greater downregulation CHX+12h DFO compared with the control cells, as detected by immunoblotting (figure 23. D).

Taken together, overexpression of IP3R3 enhanced HIF-1α expression, and similarly, silencing IP3R3 reduced HIF-1α, this may indicate the interaction between both proteins during elevated hypoxia, and that may regulate IP3R3 gene at the transcriptional level. In the future, it will be necessary to deepen the role of HIF-1α at MAM in the direct/indirect regulation of many processes.

This work proposes for the first-time new localization and roles of HIF-1α in response to hypoxia in human cardiomyocytes. Supplementary and more detailed studies, both in vitro and in vivo, will serve in the future to disclose the possible roles of this hallmark protein in the context of cardiovascular diseases.

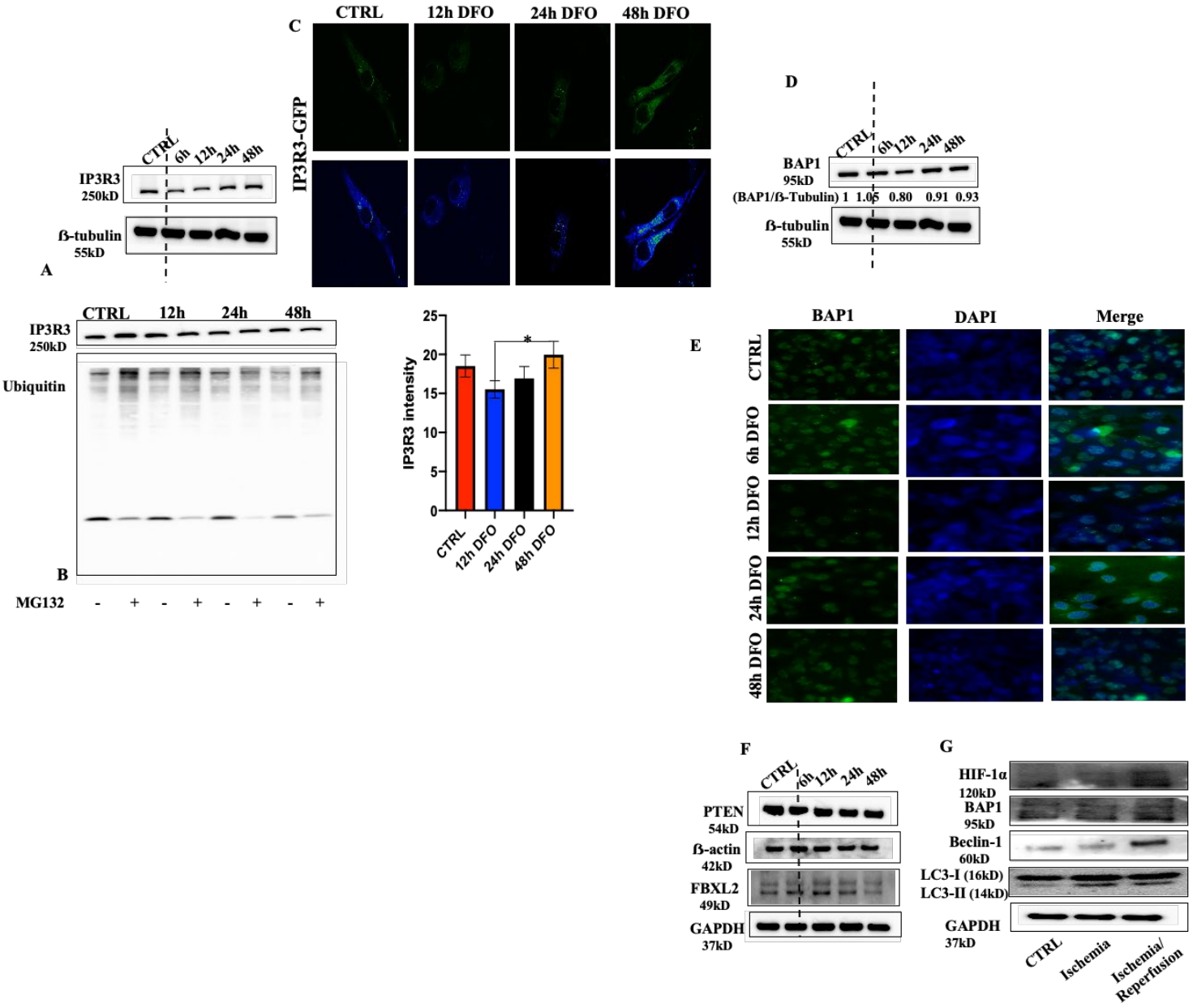

Figure 22: HIF-1α affects IP3R3-BAP1 axis during hypoxic condition. **A**. Immunoblot analysis of IP3R3 protein levels. **B.** AC16 cells treated with DFO then stimulated with or without MG132 (a proteasome inhibitor). **C.** Live cell imaging of IP3R3 tagged GFP in AC16 treated with DFO. **D**. Immunoblot assay of BAP1 protein levels. **E**. Confocal fluorescence microscopy of AC16 cells incubated with or without DFO for12h, 24h and 48h stained with antibody to BAP1 (Green) and DAPI (Blue). **F**. Immunoblot assay of FBXL2, PTEN protein levels.

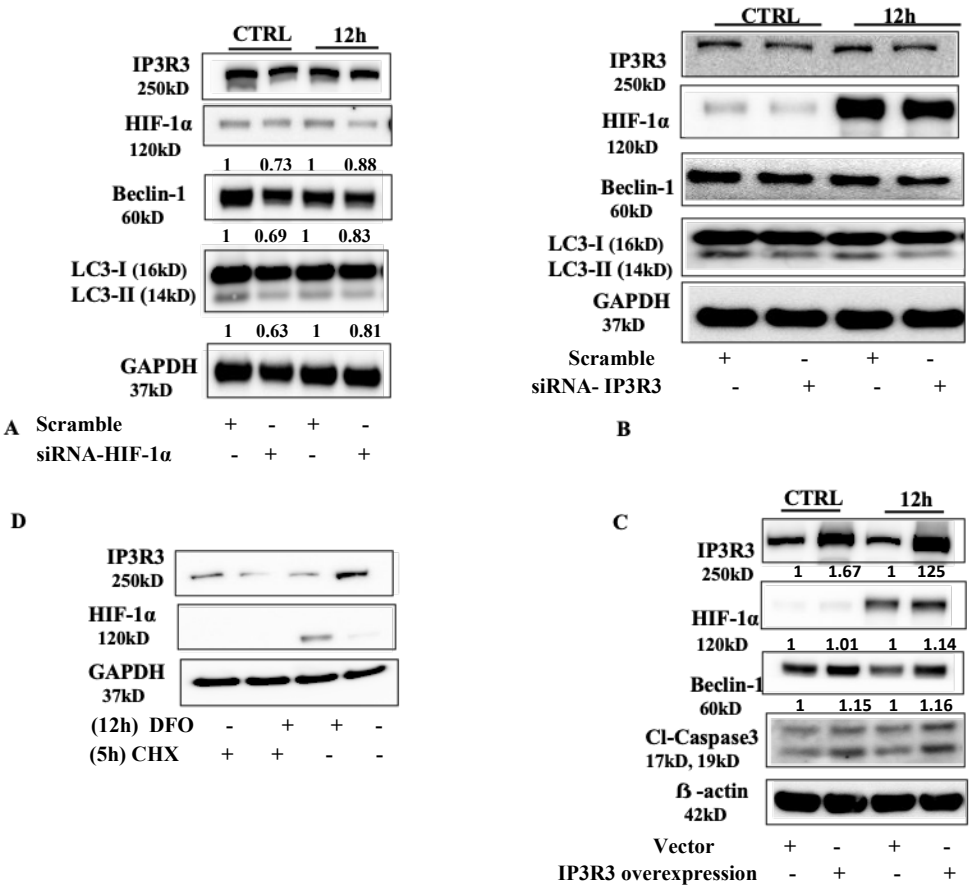

Figure 23: HIF-1α affects IP3R3 during hypoxia-mediated autophagy. **A.** Immunoblot analysis detecting IP3R3 autophagic marker (LC3-I/II, Beclin-1) protein levels after siHIF-1α, and DFO 12h in AC16. **B**. Immunoblot analysis detecting HIF-1α and autophagic marker protein levels after siIP3R3, and DFO 12h in AC16. **C.** Immunoblot analysis detecting HIF-1α, beclin-1 (autophagic marker) and apoptotic marker (Cl- Caspase3). **D**. Immunoblot analysis detecting HIF-1α and IP3R3 after Cycloheximide (CHX) and DFO treatment 12h. N=2

Chapter 4

Discussion

Ischemic heart disease is a major cause of mortality, and currently, there is no effective treatment in clinical practice. One of the most essential activities of the cardiovascular system is the supply of oxygen to respiring tissues. Effective cellular oxygen delivery is a major factor for most eukaryotic species to ensure normal cellular function and survival [78]. Over the past twodecades, biological studies of HIF-1α have notably ameliorated the understanding of oxygen homeostasis. In fact, recently, HIF-1α has drawn much attention in many research fields, have outlined its importance as the master oxygen regulator within the cells and a key transcriptional factor in the cellular response to a hypoxic environment, and playing pivotal roles in regulating several targeting genes involved in diverse processes including, angiogenesis, erythropoiesis, metabolic reprogramming, cell proliferation, and apoptosis/ survival, in response to hypoxia [1]. Disruption in hypoxia-related pathways have been contributed as key for several pathological states including cardiovascular diseases [78]. Mitochondria are major consumers of oxygen in the cell and more abundant in the heart, subsequently, they are impacted byhypoxia.

In this study we attempted to understand the effect of HIF-1α stabilization induced by DFO on the mitochondrial (dys)-function and the mitochondrial associated membrane, which are the mostcritical site of mitochondria and endoplasmic reticulum (ER) and key regulator of several fundamental cellular processes, including, mitochondrial homeostasis, calcium signaling and initiation of autophagy, in human ventricular cardiomyocytes.

We investigated a method of hypoxic culture using a hypoxic mimetic agent DFO under air oxygen culture conditions. After a dose scan of DFO in human ventricular cardiomyocytes (AC16), we selected the right concentration that causes minimal cell count change (50μM) and different duration times (12h, 24h, and 48h). we confirmed the ability of DFO to induce HIF-1α expression in human AC16, and importantly, all the process changes evaluated in this work were correlatedand compared to HIF-1α expression changes. We outlined that the effect of HIF-1α stabilization is highly linked to the incubation time-dependent and not only the doses, in which we observed different cell-responses toward DFO treatment.

During the first stage of DFO-mediated hypoxia 12h, mitochondrial cardiomyocytes exhibit an augmentation in the ATP content level, that may be due to HIF-1α -mediated cardioprotection by altering the balance between glycolytic metabolism and oxidative metabolism as HIF-1α coordinately stimulates the transcription of genes encoding glucose transporters enzymes, thus maintain ATP levels during hypoxia [1].

It is recognized that ROS is considered as a double-edged sword, in low levels of ROS is a key signaling molecule in multiple pathologic processes, while excessive ROS plays a critical role in initiating cell death [81]. HIF-1α stabilization (at 12h) reduced mtROS levels and protected the cells from apoptosis, which is in concordance with the previous studies that reported the role of HIF-1α in inhibiting the mitochondrial oxidative metabolism, thereby a decline in ROS levels in response to hypoxia by various strategies [130]. Reversed results inthis level have been observed on the inhibition of HIF-1α expression by small interfering RNA (siRNA), confirming the effect of HIF-1α in rescuing high ROS levels. This result is in alignment with the previous work of Guo et al, showing that suppressing HIF-1α was found to significantly increase ROS levels and higher cell death after hypoxia [131]. Moreover, at the same duration 12h DFO, variation in ΔΨm is a highly sensitive indicator of the energetic state of mitochondria. In this regard, we found a greater mitochondrial membrane potential (hyperpolarization), promoted the mitochondrial Ca^{2+} accumulation, with no significant changes in the cytosolic calcium and reduced level of ER calcium. It is reported that The Ca^{2+} uptake by the mitochondria determines cell susceptibility to apoptotic stimuli. For example, reduced ER Ca^{2+} flux toward mitochondria results in resistance to apoptosis [107]. Thereby, inhibition of mPTP opening and confirming our previous result the protective effect against apoptosis, which has been also documented to play a crucial role in preventing the heart during acute ischemia and reperfusion [132]. HIF-1α is well established to play a central role in the cellular response to hypoxia; however, much less is known about its cardio specific effects [133].

Additionally, studies reported the disturbance in proper ER function may cause ER stress, as it has been highlighted as an important regulator in cardiovascular disorders [134]. However, our results indicated a non-significant increase in CHOP, which is the most widely ER stress biomarker involved in ER stress-associated apoptosis at 12h DFO, however, BiP protein was found elevated at 12h.

Correspondingly, other previous studies documented the hypoxia-related induction of BiP expression [135-138]. Instead, studies found opposite results in this matter, reported reduced levels of BiP after 12h of hypoxia in HUVEC cells [139], indicating the controversial response of BiP among different cells.

Therefore, MAMs are the most critical site of mitochondria and the endoplasmic reticulum (ER) mediates multiple fundamental cellular processes, including calcium homeostasis regulation, ROS,

mitochondrial dynamics, and initiation of autophagy [140]. MAMs have gained more attention in the field of cardiovascular diseases due to their role in mediating several processes involved in health and diseases. To date, few studies enclosed its direct roles in cardiovascular diseases [15,141].

However, as best in our knowledge no evidence has shown the impact of HIF-1α in MAM. Accumulated HIF-1α at 12h elevated the mitochondria-ER contact sites, indicating the active increasing high-affinity interaction, these events confirm the activation of the autophagic activity and the mitochondrial compartment-specific process (mitophagy) which were expressed at high levels at 12h DFO-induced HIF-1α stabilization.

Furthermore, it has been reported that hypoxia exhibit pro-apoptotic and anti-apoptotic biphasic effects that are dependent upon cell types and the conditions around cells, and multiple studies on hypoxia and hypoxia mimetic agents on cell death displayed that hypoxia mimetic agents significantly upregulated apoptosis [112]. Indeed, in our cell model, DFO-induced accumulation of HIF-1α expression was associated with a significant elevation of cell death (apoptosis and necrosis) in a dose and time-dependent manner (50μM, at 24h and 48h). Conversely to results demonstrated previously, DFO- induced HIF-1α at 24h, and 48h led to ATP depletion, elevated mtROS levels this led to mitochondrial ATP depletion, associated with disruption of the ΔΨm (depolarization) and alteration in the calcium homeostasis, ultimately increased ER stress biomarkers. Enhanced interaction and contact between the mitochondria-ER contact sites has also been identified. These events trigger the mPTP opening through the presence of HIF-1α, as we demonstrated the attenuation of mPTP opening by siRNA- mediated HIF-1α, suggesting the impact of HIF-1α in stimulating the activity of mPTP during hypoxia at 24h. Subsequently triggering autophagy and mitophagy to remove damaged mitochondria. As documented in multiple evidence that autophagy plays a crucial role in removing damaged or excess organelles to ensure intracellular homeostasis and keep the cell healthy, however it is recognized to play a dual role, either beneficial or detrimental in the cell survival, which depends on both the burden of intracellular substance [2].

Following these findings, to better define autophagy in favor of mPTP activity. We intended to expand our understanding of whether hypoxia-induced autophagy promotes the mPTP activity contributing dependently to survival or cell death. To our knowledge, no study demonstrated the direct effect of autophagy on mPTP through HIF-1α activation.

Here, we focused only on 24h DFO as the apoptosis process was initiated in our cells. After treatment with an autophagic inhibitor 3-methyladenine (3-MA) is the most used inhibitor that targets autophagy an early stage [92]. Of great importance, mPTP activity was alleviatedin AC16 cells treated 24h DFO +3MA compared with the cells treated only with 24h DFO, indicating that inhibition of autophagy contributed to attenuate the opening of the mPTP activity in response to hypoxia at 24h precisely, thereby reducing mitochondrial damage and apoptosis. We assessed the cell viability assay of AC16 treated with 24h and 24h +3-MA, in which we confirmed the effect of autophagic inhibitor at 24h to improve cell survival. In our knowledge, no study demonstrated the effect of hypoxia-induced autophagy on the opening of mitochondrialpermeability transition pore (mPTP) through HIF-1α, suggesting a deep study on the molecular pathway in this level, that may contribute to the translational research.

Taken together, the main finding here is that HIF-1α plays a dual role in human cardiomyocytes during DFO induced-hypoxia, affecting the mitochondrial function and MAMs subsequently affecting various fundamental cellular processes in a dose and time-dependent manner.

In the second part of our work, we identified a new subcellular localization of HIF-1α in human cardiomyocytes, and possible explanations on its protective role found at early hypoxia stimulated at 12h DFO.

Firstly, the recognition of HIF's central role in physiology and medicine was very recently investigated by the Nobel Prize award in 2019 to William Kaelin Tr., Sir Peter Ratcliffe, and Gregg Semenza.

HIF-1α is a crucial transcription factor in the cellular response to hypoxia, and abundant studies focused only on its transcriptional activity-dependent functions. Notably, important recent findings to be outlined identified HIF-1α functions outside the nucleus, as reported Li et al. that HIF-1α translocate to the mitochondria in response to oxidative stress to reduce mtROS in human umbilical vein endothelial (HUVEC), L02 liver cells [116]. Mylonis et al. identified the HIF-1α- VDAC1-HK-II complex at the outer membrane of mitochondria which blocks hypoxia-mediated apoptosis [142]. Further, Khan et al. found that HIF-1α colocalized with the peroxisomes and not the nucleus during hypoxia in rat hepatocytes [143]. Clearly, HIF-1α functions may occur at different subcellular localizations also outside the nucleus. We assessed a subcellular fractionation (homogenate, pure mitochondria, MAM, ER, and cytosol) of AC16 to demonstrate the localization of HIF-1α during DFO-mediated hypoxia at 12h. Surprisingly, we found that in response to DFO-mediated hypoxia, HIF-1α enriched mostly at the

ER directly as identified in the subcellular fractionation of AC16 and a small fraction at MAM, as well as at the pure mitochondria, which confirm the previous findings elucidated the translocation of HIF-1α at mitochondria. Moreover, greater colocalization between HIF-1α and ER marker PDI compared with HIF-1α mitochondrial marker ATP5A thus confirming our previous results.

To date, this is the first study demonstrating HIF-1α translocation at the ER and MAM in response to hypoxia and at the mitochondria (using DFO) in human cardiomyocytes. Further continuous study to demonstrate the role and the molecular pathways of HIF-1α translocation to ER and mitochondria and the direct regulation of mitochondria /ER by HIF-1α in response to hypoxia in AC16 cells are highly recommended (experiments ongoing), and confirming these results in vivo may shed light on new therapeutic approaches to reduce the oxidative damage in cardiovascular diseases and other diseases as well.

Additionally, since we showed that HIF-1α stabilization affects the MAM, as well as the calcium signaling, and HIF-1α is enriched at ER in response to hypoxia, this led us to ask whether HIF-1α may affect the ER channel termed IP3R3, which is responsible for calcium release from the ER into the mitochondria. In fact, in our cell model, HIF-1α reduced IP3R3 amount during hypoxia,inducing its degradation and downregulating significantly ER calcium at 12h DFO. In parallel, enhancing the autophagic process, as we speculate that a reduction ER-mitochondrial Ca^{2+} transferis critical for induction of the autophagy process. Consistently, BAP1 a tumor suppressor binds, deubiquitinates IP3R3 have been also shown downregulated in response to DFO-induced HIF-1α stabilization, suggesting further interaction between HIF-1α and IP3R3-BAP1 axis and this may explain the enrichment of HIF-1α during hypoxia at MAM and ER.

Along with this, overexpression of IP3R3 enhanced HIF-1α expression and similarly, silencing IP3R3 reduced HIF-1α, supporting our previous results. Here we hypothesize that HIF-1α as a transcription factor may also regulate the transcription of the IP3R3 gene during enhanced hypoxia. We checked as first whether the IP3R3 gene contains the putative binding sequences (HRE) in which HIF-1α may bind in these sequences and form a complex and subsequently activate the transcription of our gene of interest, during hypoxia. Firstly, we used bioinformatics tools such as ConTra v3 to detect the predicted binding sites of HIF-1 α transcription factor in the core DNA sequence HRE of the human ITPR3 gene. Interestingly, we could identify two predicted copies of putative HRE encompassing consensus sequences in the 5'UTR region "CGTG" and "CACG" near the promoter region of the ITPR3 gene. Therefore, homology of the HRE-ITPR3 predicted copies among different species have been revealed, for example, the mouse, and Chimp. Further experiments are ongoing to confirm this hypothesis are ongoing.

Ongoing and future Directions

4.1 Does IP3R3 play a role as a transcriptional target gene of HIF-1α transcription factor in human ventricular cardiomyocytes (AC16)?

Motivation:

The identification of HRE in the erythropoietin gene was the first discovery toward HIF-1α as a novel transcription factor [144]. Subsequent research later from Dr. Gregg Semenza's team at Johns Hopkins Medical Institutions identified the protein that binds to the HRE during hypoxia as HIF-1 [50,53].

HIF-1α acts as a key regulator of oxygen-regulated gene expression. many putative HIF-1α target genes have been demonstrated [145]. Based on our previous results, we hypothesis that IP3R3 is a novel target gene of HIF-1α and may regulate transcriptionally IP3R3 in response to acute hypoxia. To test this hypothesis, we checked first whether the IP3R3 gene contains the putative binding sequences (HRE) in which HIF-1α may bind in these sequences and form a complex and subsequently activate the transcription of our gene of interest, during hypoxia. Firstly, we used bioinformatics tools such as ConTra v3 to detect the predicted binding sites of HIF-1α transcription factor in the core DNA sequence HRE of the human ITPR3 gene.

Results and discussion

Interestingly, we could identify two predicted copies of putative HRE encompassing consensus sequences in the 5'UTR region "CGTG" and "CACG" near the promoter region of the ITPR3 gene. Additionally, we could detect a homology of the HRE-ITPR3 predicted copies in different species such as the mouse, and Chimp (figure 24). Further experiments are ongoing to confirm this hypothesis are ongoing

Figure 24: HIF-1α stabilization may affect transcriptionally IP3R3 gene. **A1**. Detection of IP3R3 after siRNA HIF-1α by immunoblot analysis in AC16. **B1**. Representative ITPR3 sequences containing twopredicted hypoxic responsive elements (HRE) in 5’ UTR (black) and the promoter region (blue). **C1.** Illustration of the two predicted HRE in ITPR3 gene.

4.2 Does HIF-1α stabilization play an effective role in mitochondrial stability during Myocardial Ischemia and reperfusion in neonatal cardiomyocytes and cardiac fibroblasts?

Motivation:

A comprehensive model of how early environmental changes such as hypoxia may increase pathological responses in cardiac cells such as adult mammalian cardiac fibroblasts, as well as the neonatal mammalian cardiomyocytes that are known as highly resistant to oxygen deprivation [146], and how these changes may affect the mitochondrial function which is far from being fully elucidated, may be partly due to the lack of approaches involving simultaneous environmental stimulation.

Based on our previous results so far, the involvement of reactive oxygen species in the mitochondrial membrane potential instability and further disruption in calcium homeostasis in human ventricular cardiomyocytes (AC16). And given the observation that HIF-1α played a protective role when it is more accumulated (example at 12h of inducing hypoxia). We wondered whether stimulating HIF-1α would prevent the mitochondria from instability not only during ischemia but also reperfusion in RCF and RNC. As reported previously the role of HIF-1α in regulating the transcription of several genes is involved in elevating oxygen supply. Additionally, HIF-1α has been identified to promote efficient use of available oxygen supply via erythropoietin production and angiogenesis to insure tissues protection from ischemic damage [1].

To check our hypothesis, we used adult cardiac fibroblasts (RCF) and neonatal cardiomyocytes (RNC) from rat animals as in vitro models for cardiac pathologies mechanisms research andassessing hypoxia by using DFO. RCF and RNC were isolated and cultured as described previously (see materials and methods).

Results and discussion

To examine the effect of hypoxia-induced by the iron chelator DFO, we screened for cell survival rate in these cells at different time points as the AC16 cells, at 12h, 24h and 48h (using 50µM DFO).

As documented in figure 25. A, DFO treatment slightly reduce the cell viability rate of RCF among the conditions 12h, 24h and 48h than the control cells. Furthermore, compared with RNC, we noticed that DFO alleviate significantly the cell survival rate at 48h, while at 12h and 24h no significant reduction has been observed (figure 26. a). Although it is well documented the resistanceof RNC against oxygen deprivation, here contrary they exhibit nonresistance to hypoxia at 48h compared to RCF.

On the other hand, with the aim to verify DFO-mediated HIF-1α accumulation in both cell models, we assessed an immunofluorescence staining. RCF showed a significant upregulation in HIF-1α expression at 12h and 24h DFO compared to the control cells in, and a non-significative increase of HIF-1α at 48h (figure 25. B). HIF-1α expression in RNC was significantly accumulated at 12h than the other conditions (figure 26. b).

Interestingly, mitochondrial oxidative stress (mtROS) in RCF was documented to be significantly reduced at 12h where HIF-1α is significantly accumulated compared with RNC, indicating further protective roles of HIF-1α against mtROS alteration in response to hypoxia (figure 25.C. Figure 26. c). Of note, at 24h DFO-induced hypoxia mtROS is overloaded in both cells, revealing the instability of the mitochondria, mtROS upregulated only in RNC at 48h DFO than RCF cells.

Next, we wanted to see whether RNC that are the most sensitive to DFO-induced hypoxia may affect the mitochondrial membrane potential as well. To demonstrate this the RNC treated with DFO at the same timing indicated previously, we used TMRM (previously described). The analysis of the cells by confocal microscopy showed that the treatment DFO induced a significant ΔΨm depolarization at 48h (depolarization), a slight decrease in ΔΨm is well observed at 24h, while the cells exposed to DFO at 12h exhibit an increased ΔΨm (hyperpolarization) compared to the control cells as referred in figure 26. d. These results confirm the findings highlighted on the mtROS in RNC.

Assessing experiments to unveil the role of HIF-1α in these cells focusing the mitochondrial associated membrane, which are the fundamental hotspot of various processes. Therefore, performing these experiments on HIF-1α knockout fibroblasts/neonatal cardiomyocytes would uncover protective mechanisms under times of hypoxic stress.

Rat Cardiac

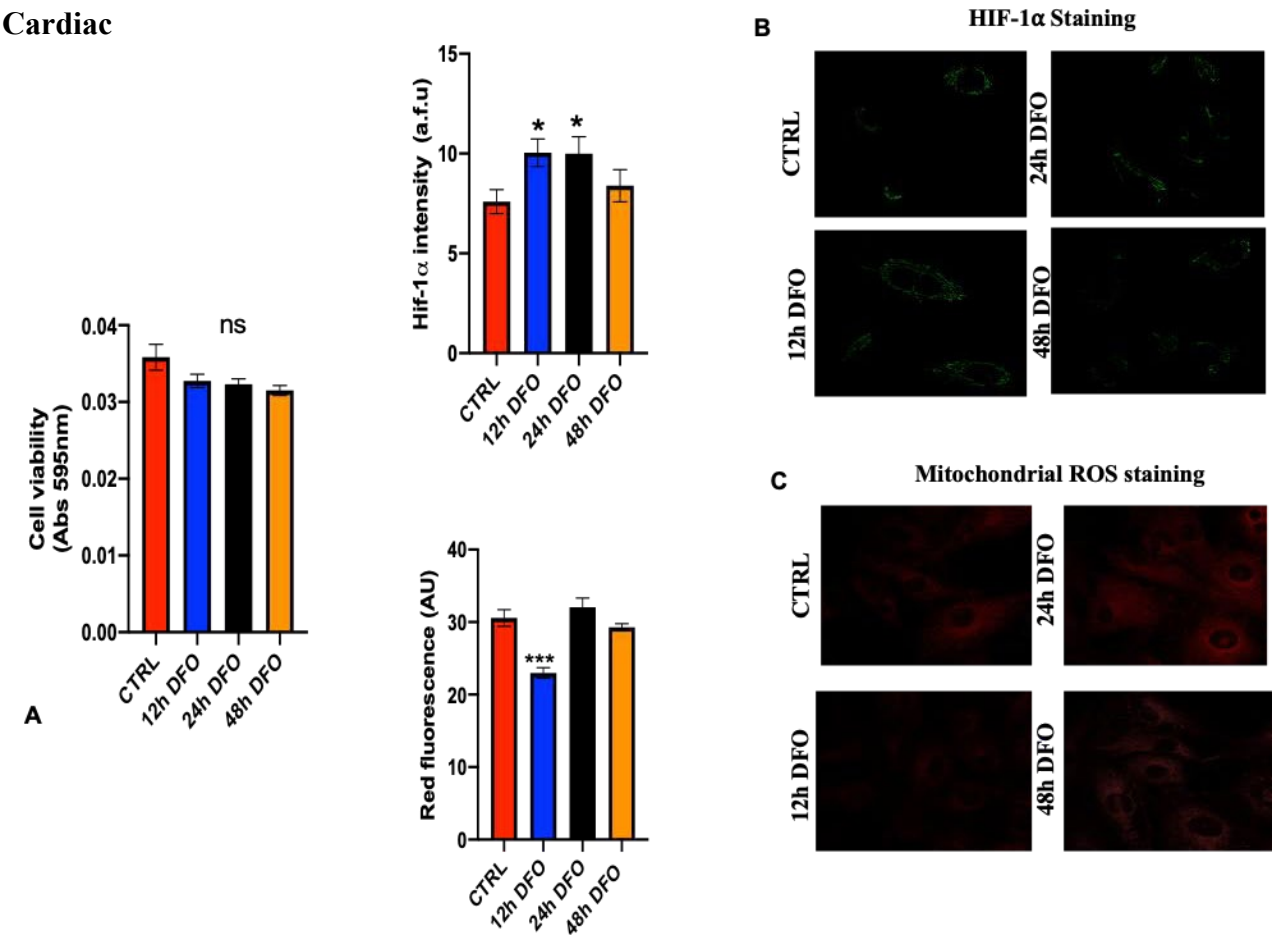

Figure 25: Impact of DFO-induced HIF-1α stabilization on cardiac fibroblasts (RCF). **A.** Cell viability assayin RCF treated with 50μM DFO. **B.** Confocal fluorescence microscopy of RCF cells incubated with or without DFO for 12h, 24h, and 48h stained with HIF-1α antibody (Green). **C.** Live imaging using MitoTracker Red CMXRos to measure mitochondrial ROS in RCF.

Rat neonatal cardiomyocytes

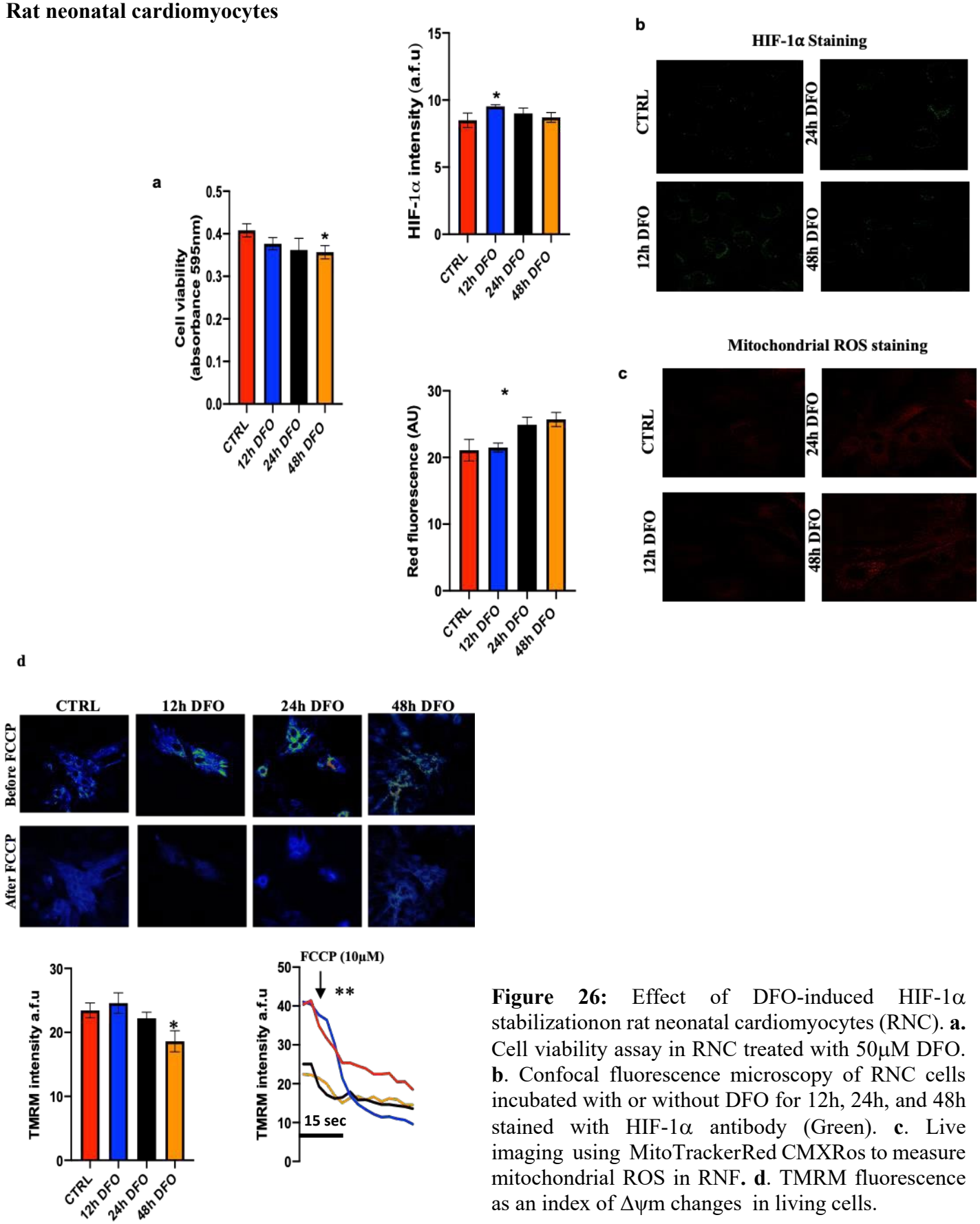

Figure 26: Effect of DFO-induced HIF-1α stabilizationon rat neonatal cardiomyocytes (RNC). **a.** Cell viability assay in RNC treated with 50μM DFO. **b**. Confocal fluorescence microscopy of RNC cells incubated with or without DFO for 12h, 24h, and 48h stained with HIF-1α antibody (Green). **c**. Live imaging using MitoTrackerRed CMXRos to measure mitochondrial ROS in RNF**. d**. TMRM fluorescence as an index of Δψm changes in living cells.

General conclusion

This work has demonstrated the relevance of HIF-1α on cardiac mitochondria as a double agent that affects their function by modulating several processes including calcium homeostasis, autophagy, mitophagy and cell death in response to hypoxia.

Focusing on its cardioprotective side, we believe that HIF-1α is the most promising target to cause a significant change in cardiovascular research.

If there's a will,

there's a way Because life's too short

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